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What do we know about IDH1/2 mutations so far, and how do we use it?

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Abstract

Whole genome analyses have facilitated the discovery of clinically relevant genetic alterations in a variety of diseases, most notably cancer. A prominent example of this was the discovery of mutations in *isocitrate dehydrogenases 1* and *2* (*IDH1/2*) in a sizeable proportion of gliomas and some other neoplasms. Herein the normal functions of these enzymes, how the mutations alter their catalytic properties, the effects of their D-2-hydroxyglutarate metabolite, technical considerations in diagnostic neuropathology, implications about prognosis and therapeutic considerations, and practical applications and controversies regarding *IDH1/2* mutation testing are discussed.

Keywords

IDH1/2; 2-hydroxyglutarate; glioma; histone; methylation

Introduction

Mutations in *isocitrate dehydrogenases types 1* and *2* (*IDH1/2*) were first reported in large subsets of gliomas in 2008-2009 [11, 131, 187]. Prior to this discovery there was no reason to think that either gene would be mutated in any tumor, much less gliomas. A 2006 study found an R132C IDH1 mutation in a single colonic adenocarcinoma, but it was understandably not deemed significant at the time [154]. The opinion of the scientific community has clearly changed on the matter, considering that about 500 papers have now been published concerning *IDH1/2* mutations and cancer.

The purpose of this review is to distill what we now know about *IDH1/2* mutations into a thorough yet concise summary that can help readers keep abreast of this rapidly-advancing field. Toward that end, we will discuss the functions of wild-type IDH1 and IDH2, the consequences of mutations on each enzyme's biochemistry, the downstream effects of these mutations on cancer biology, and practical applications of *IDH1/2* mutation testing, specifically in brain cancer.

Wild-type IDH enzyme biochemistry

Three enzymes oxidize isocitrate to alpha-ketoglutarate (α-KG) in human cells: 1. isocitrate dehydrogenase 1 (IDH1); 2. isocitrate dehydrogenase 2 (IDH2); and 3. isocitrate dehydrogenase 3 (IDH3). Despite the similarities of their names, these enzymes differ markedly from each other. IDH1 and IDH2 are single-gene enzymes respectively located on 2q33 and 15q26, each existing as homodimers. IDH3 is a heterotetramer composed of two

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alpha subunits, one beta subunit, and one gamma subunit. These *IDH3A, IDH3B*, and *IDH3G* genes are located on chromosomes 15q25, 20p13, and Xq28. IDH1 and IDH2 utilize nicotinamide adenine dinucleotide phosphate (NADP+) as a cofactor, generating NADPH during catalysis. IDH3 uses NAD+ and produces NADH. IDH2 and 3 are located in mitochondria while IDH1 is in the cytosol and peroxisomes (Figure 1).

Of the three, only IDH3 appears to participate in the Krebs cycle [190]. The exact role of mitochondrial IDH2 is somewhat unclear, though it may act as a source of NADPH for the mitochondria [72]. IDH1 is the primary source of NADPH reducing equivalents in the cytosol and peroxisomes [14, 84]. Both IDH1 and IDH2 are important in the mitigation of cellular oxidative damage induced by intrinsic metabolism and extrinsic factors like radiation [71, 72, 81-83, 100-103]. It is therefore significant that *IDH1* is one of the key genes upregulated in breast cancer stem cells, which have lower levels of reactive oxygen species (ROS) than their progeny and tend to be more radioresistant [38]. IDH1 is also the largest producer of NADPH in the human brain (but not the mouse brain) [9, 14].

IDH3 appears to be a unidirectional enzyme, only capable of oxidizing isocitrate to α-KG. But during hypoxia or mitochondrial dysfunction, IDH1 and IDH2 can reduce α-KG to isocitrate, thereby helping the cell replenish other citric acid cycle intermediates and the fatty acid precursor acetyl-CoA [49, 117, 123, 183].

Mutant IDH enzyme biochemistry

Clinical clues about the function of mutant IDH1/2

IDH1/2 mutations occur in an assortment of seemingly unrelated neoplasms, including gliomas, acute myeloid leukemia (AML), acute lymphocytic leukemia, myelofibrosis, intrahepatic cholangiocarcinoma, melanoma, and chondroid tumors, as well as rare colonic and prostate carcinomas, and even the occasional paraganglioma [6, 17, 50, 78, 108, 153, 164]. Both Ollier disease and Maffucci syndrome—wherein patients develop multiple benign cartilaginous tumors—are caused by somatic mosaic mutations on *IDH1* [7].

In gliomas, several interesting facts about *IDH1/2* are readily apparent. The mutations tend to occur in younger adults, in the 20-60-year range, and are far more common in grades II and III astrocytomas and oligodendrogliomas compared to glioblastomas (GBMs) [15, 131, 187]. *IDH1/2*-mutant gliomas can occur in the pediatric population, but are more likely in adolescents [73, 136]. In fact, about 60-80% of those lower-grade gliomas and GBMs that arose from lower-grade gliomas (so-called “secondary” GBMs) have a mutation, while *de novo* (i.e. “primary”) GBMs only rarely do [167, 178, 187](Figure 2c). Over 90% of the mutations involve *IDH1*, and about 90% of those *IDH1* mutations are CGT>CAT transitions in codon 132, replacing the arginine residue with histidine (R132H IDH1)(Figure 2). Other point mutations also occur at codon 132, resulting in substitutions like R132C or R132S. Analogous mutations occur at R172 in IDH2, but do not show the same preference for histidine as in mutant IDH1 (Figure 2b). Other codons can also rarely be affected, including R49, G97, and R100 on IDH1 and R140 on IDH2 [58, 139, 175]. When an *IDH1/2* mutation is present it is virtually always heterozygous, with the tumor retaining the corresponding wild-type allele. Extremely rare cases of concurrent *IDH1* and *IDH2* mutations have been reported [60], as well as dual R132H and R132C IDH1 mutation in the same tumor [165]. In about 5% of *IDH1/2*-mutant gliomas, the mutation is eventually followed by deletion of the wild-type allele [Hai Yan, M.D., Ph.D., personal communication, 12/12/2012] [67, 69]. Rare cases of mutant allele deletion within an area of a glioma have also been reported [139].

Neoenzymatic activity of mutant IDH1/2

The findings that *IDH1/2* mutations are heterozygous, nearly always target arginine codons involved in the binding of isocitrate, and are missense mutations implied some sort of dominant inhibition or gain-of-function. An early hypothesis was that it acts as a dominant negative inhibitor of wild-type IDH1/2, causing oncogenesis through lower levels of α-KG, leading to inhibition of α-KG-dependent degradation of hypoxia-inducible factor-1α (Hif-1α) [192]. But other studies have shown no difference in either α-KG or Hif1-α between mutant and wild-type gliomas [35, 77, 118, 141, 182]. Furthermore, dominant inhibition of wild-type IDH1 plus consumption of NADPH by the mutant should result in lower cellular NADPH reducing equivalents. Yet *in vitro* overexpression of *IDH1/2* mutants have no effect on NADPH levels [70], and both oxidized and reduced glutathione are lower in mutant-transfected oligodendroglial cells [141]. It is important to remember that a cell is bound to have ways of compensating for deficits of such critical compounds like α-KG and NADPH, like converting more glutamate into α-KG [99, 141]. Therefore, the effects of *IDH1/2* mutations cannot be simply explained by inhibition of their wild-type counterparts, but more likely represent a true gain-of-function.

In 2009 that gain-of-function was discovered by Dang et al., who published a landmark study showing that the R132H point mutation confers neoenzymatic activity on IDH1 [35]. The R132 residue normally helps bind isocitrate substrate, but when it is mutated the enzyme prefers to bind and reduce α-KG to D-2-hydroxyglutarate (hereafter referred to as 2-HG), consuming NADPH in the process (Figure 3). This neoenzyme is so adept at producing 2-HG that mutant gliomas have 10 to 100-fold higher levels of 2-HG than their wild-type counterparts, with tissue concentrations ranging from 10 to 30 millimolar. Similar 2-HG-producing effects have been shown in the other *IDH1* and *IDH2* mutations, both in gliomas and other cancers [7, 17, 57, 151, 175, 177]. Of note, wild-type IDH1/2 are also capable of producing some 2-HG in the reverse reductive reaction under hypoxic conditions, just not nearly as much as the mutants [184].

This neoenzymatic activity begs the question as to how key point mutations of R132 and R172 can so radically alter IDH1/2 functionality. As it turns out, wild-type IDH1 activity can be divided into three distinct phases: a.) initial open isocitrate-binding state; b.) closed pre-transition state; c.) catalysis. A mutation at R132 seems to block the shift to a closed pre-transition state [188]. This impedes isocitrate binding, thereby preventing it from inhibiting the reduction of α-KG to 2-HG—a reaction that, to reiterate, weakly occurs even in wild-type IDH1 [135]. An analogous phenomenon likely holds for R172 and IDH2, as well as for some of the rarer mutations on other arginine-encoding codons like R100 in IDH1 and R140 in IDH2.

Since wild-type IDH1 and IDH2 act as homodimers, and mutations virtually always start out heterozygous (even if the tumor eventually loses its wild-type allele), it raises the question as to how (or if) mutant enzymes interact with their wild-type counterparts. Co-precipitation experiments showed that mutant IDH1 heterodimerizes with wild-type IDH1, but mutant IDH2 does not bind very well to wild-type IDH2, again refuting the hypothesis that these mutated enzymes must bind and inhibit their wild-type counterparts [70]. When substrates are supplied in excess during cell-free *in vitro* experimentation, the R132H:R132H IDH1 homodimer is more efficient at producing 2-HG than a heterodimer [104, 135]. But in a more realistic intracellular environment where substrates are limited, mutant IDH1 produces more 2-HG when it heterodimerizes with wild-type IDH1 [176]. This is consistent with the finding that *IDH1*-mutant gliomas that lose their wild-type allele produce far less 2-HG than they did before the deletion [69]. The wild-type half of an IDH1 heterodimer grabs isocitrate and converts it into α-KG, which is immediately made available to the mutant half for conversion to 2-HG in a process known as substrate channelling [135]. This allows for

efficient 2-HG production across a wider range of local isocitrate concentrations—a feature that could be more important for cytosolic mutant IDH1 than for mutant IDH2, which already resides in the substrate-rich mitochondrion and does not require heterodimerization [176](Figure 3).

Thus it is not surprising that, while virtually all tumor-associated *IDH1/2* mutants produce 2-HG, they are not all perfectly identical in either activity or frequency among tumors. For example, R172K IDH2 produces more 2-HG than R140Q IDH2, and after adjusting for subcellular localization, R132H IDH1 is stronger than R172K IDH2 [176]. But among *IDH1* mutants, R132H IDH1 appears to be the weakest 2-HG producer whereas there is no significant difference among R172 IDH2 variants [70]. In contrast to the aforementioned strong *IDH1* preponderance in gliomas (specifically R132H IDH1), a full one-third of intrahepatic cholangiocarcinomas have *IDH2* mutations; even when IDH1 is the mutated enzyme in these tumors, it is more likely to be R132C than R132H [17, 87], just like cartilaginous tumors [6, 7]. Curiously enough, Li-Fraumeni (germline *TP53* mutant) gliomas also favor R132C mutations [179]. AMLs are more likely to harbor a mutation in *IDH2* than *IDH1*, specifically at R140 rather than R172 [1, 115, 177]. And in gliomas, when R132C IDH1 is present, it tends to be found in astrocytomas, whereas *IDH2* mutations are more frequent in grade III oligodendrogliomas [60] (Figure 4). All this suggests that, from a microevolutionary standpoint, the amount of optimal 2-HG varies depending on tumor site. And within a given site, differing concentrations of 2-HG might nonrandomly affect what kind of tumor is formed.

Parenthetically, to date no cancer-associated *IDH3* mutations have been found [92], suggesting that it is difficult to confer comparable neoenzymatic activity in this heterotrimeric complex via a single point mutation.

Effects of D-2-hydroxyglutarate

2-hydroxyglutaric aciduria

Proving that R132H IDH1 produces D-2-HG [35] was a significant advancement in oncology, not only for solving the question of what the mutation does at a biochemical level, but for spurring a whole new avenue of research aimed at discovering the impact of 2-HG in cancer. Prior to 2009 2-HG studies focused mostly on a very rare inborn metabolic disease called 2-hydroxyglutaric aciduria. The disease was first described in 1980 and was immediately recognized as existing in two forms: L-2-HG and D-2-HG, with each isomer producing its own phenotype [27, 43]. L-2-HG aciduria manifests in early childhood and is slowly progressive, featuring leukodystrophy, psychomotor retardation, cerebellar ataxia, and seizures [156]. In contrast, D-2-HG aciduria can present either as a severe encephalopathy with cardiomyopathy and dysmorphisms affecting the face and other structures, or as a milder variant featuring developmental delay and hypotonia [158]. Remarkably, some siblings of D-2-HG patients also have high serum and urine D-2-HG levels without any symptoms at all. Causative germline inactivating mutations in L-2-hydroxyglutarate dehydrogenase and D-2-hydroxyglutarate dehydrogenase had been known for some time [156, 158], but the genetic defect in a subset of patients with D-2-HG aciduria remained a mystery. In a nice example of symbiotic mutualism between the metabolic and cancer literatures, the discovery of mutant *IDH1/2* in tumors prompted a search for similar germline mutations in that idiopathic subset of D-2-HG aciduria. Sure enough, in 15 of 17 patients with no dehydrogenase mutations, R140Q or R140G IDH2 mutations were detected [91]. Those patients had higher urinary concentrations of D-2-HG compared to those with dehydrogenase mutations, although there does not appear to be a correlation between D-2-HG levels and disease severity [158]. No 2-HG dehydrogenase mutations have yet been

identified in gliomas [20, 92]. Interestingly, 4 patients have been described with both D-2-HG aciduria and chondromatosis; 2 of them had somatic *IDH1* mutations [171].

Because of these organic acidurias, studies on the effects of 2-HG predate the discovery of *IDH1/2* mutations. In slices of rat cortex, 2-HG generates free radicals, causes oxidative stress, inhibits cytochrome c oxidase and ATP synthase, and lowers the rate of aerobic glycolysis [97, 98]. But a great deal of evidence now implicates the epigenome as a critical target of 2-HG in cancer, though this aspect of *IDH1/2* research is particularly fast-moving and requires some background knowledge to appreciate its significance.

IDH1/2 and epigenetics

Histone activity can be modified via attachment of different moieties—such as acetyl or methyl groups—to specific amino acid residues on the histone. These modifications dictate how histones interact with chromatin, thereby affecting things like genomic imprinting, DNA repair, and gene expression [166]. Histone methylation occurs on lysine or arginine residues. The Jumonji C (JmjC) domain family of histone demethylases removes methyl groups from lysine residues. ("Jumonji" is Japanese for cruciform, derived from the abnormal cross-shaped formation of the neural plate and neural groove in mice with the prototypic *JMJ* gene mutation [163].) Each of these histone demethylases contains a Jumonji C DNA-binding domain, requiring Fe^{2+} and α-KG cofactors. Three subfamilies include JHDM1, JHDM2, and JMJD2, each of which contains enzymes that demethylate different histone lysine residues. JHDM1 targets H3K36, JHDM1D is for H3K27, and JHDM2 enzymes demethylate H3K9. JMJD2 is comprised of four enzymes—JMJD2A, 2B, 2C and 2D—all of which demethylate H3K9 or H3K36 [163].

These JmjC domain-containing demethylases and their histone targets had previously been known to regulate processes like cell proliferation and androgen sensitivity, but multiple groups have now shown that the 2-HG product of mutant IDH1/2 inhibits JHDM1A, JMJD2A, and JMJD2C, specifically by competing with α-KG cofactor [30, 88, 112, 186] (Figure 3). As a result of inhibiting these demethylases, methylation of H3K9, H3K27, and H3K36 are higher when *IDH1/2* mutations are present. However, 2-HG does not equally inhibit all JmjC domain-containing demethylases, much less all α-KG-dependent enzymes. This is where 2-HG in cancer becomes very complicated and confusing. For example, one group showed that 2-HG does not inhibit JMJD2D histone demethylase [88]. Another group suggested that 2-HG inhibits prolyl hydroxylase-2, leading to elevated Hif-1α [186], but 2-HG inhibition of prolyl hydroxylase-2 is at least an order of magnitude weaker than for other targets [30]. Surprisingly, 2-HG may actually promote the activity of EGLN1-3 prolyl hydroxylases (derived from "EGg-Laying defective Nine" *C. elegans*), leading to degradation of Hif-1α and facilitation of transformation in astrocytes and hematopoietic cells [88, 109] (Figure 3). Furthermore, the two enantiomers of 2-HG differ in their potency, with L-2-HG generally being a better inhibitor of α-KG-dependent enzymes than D-2-HG [30, 88, 186]. This could account for why L-2-HG aciduria and D-2-HG aciduria have different phenotypes. It could also account for why neoplastic mutations make D-2-HG and not L-2-HG—perhaps a mutation that produced L-2-HG would be too potent for effective oncogenesis in most situations. Though if this is indeed the case, it begs the question as to why tumors have been seen only in L-2-HG aciduria patients, and not D-2-HG aciduria (see "Oncogenesis" section below).

The fact that histone methylation is such an important target of 2-HG dovetails nicely with new research showing the importance of histones in cancer, especially gliomas. Histone H3.3 is encoded by *H3F3A* and is associated with open, active chromatin at telomeres (and probably elsewhere, too) [3, 111]. H3.3 activity is controlled by modifications at key amino acid residues: methylation of K9 and K27 inhibit transcription, whereas K36 methylation

generally promotes transcription. For H3.3 to target and open up telomeric chromatin, a complex including alpha thalassemia/mental retardation syndrome X-linked protein (ATRX) and death-associated protein 6 (DAXX) needs to form with H3.3. This is important for the phenomenon of Alternative Lengthening of Telomeres (ALT), a process by which cancer cells avoid telomere shortening and senescence via homologous recombination of telomeric DNA [42]. ALT is necessary in the minority of tumors that, for whatever reason, either cannot or do not upregulate telomerases. There is now a clear connection between histones, ALT, and gliomagenesis, since a.) ALT is associated with H3.3/ATRX/DAXX mutations in gliomas [111], b.) nearly half of pediatric GBMs have inactivating mutations in the H3.3/ATRX/DAXX complex [149], and c.) the majority of diffuse intrinsic pontine gliomas (DIPGs) have K27M mutations on H3.3 [80, 185]. ALT and H3.3/ATRX/DAXX also have several strong links to 2-HG. First, methylation of K9, K27, and K36 in H3.3 is increased by 2-HG via inhibition of the aforementioned JmjC demethylases [30, 41, 186]. Second, histone mutations appear to be mutually exclusive with *IDH1/2* mutations [159]. Third, ALT is more common in *IDH1/2*-mutant astrocytomas than in wild-type astrocytomas [79, 116, 125, 143]. Fourth, methylation of H3K9—the same histone lysine whose methylation is increased by 2-HG—leads to reduced hTR and hTERT telomerases in cancer cell lines [10]. Finally, *ATRX* mutations are far more common in *IDH1/2*-mutant versus wild-type adult gliomas [79, 107]. While more precise details are still emerging, it thus appears that H3.3 modulation is a critical part of ALT and oncogenesis in a significant proportion of gliomas. In pediatric tumors it happens directly via *H3F3A* mutations, in adults it occurs indirectly via 2-HG, and additional *ATRX/DAXX* mutations contribute to the process in both groups (Figure 3).

In addition to blocking histone demethylases, 2-HG inhibits DNA demethylases, including the α-KG-dependent AlkB homologue 2 DNA demethylase and TET1/2 methylcytosine hydroxylases [30, 48, 186] (Figure 3). Inhibition of DNA demethylation would be expected to promote global hypermethylation, which is exactly what is seen in neoplasms with *IDH1/2* mutations [48, 94, 127, 168]. Consistent with this, TET2 inactivating mutations or TET2 promoter methylation are mutually exclusive with *IDH1/2* mutations, suggesting that both accomplish the same thing [29, 48, 86, 134]. But not all global hypermethylation is equal; profiles will differ depending on which driver gene is altered [5, 134]. It is also interesting (and surprising) to note that levels of the presumed precursor of nucleotide demethylation, 5-hydroxymethylcytosine (5hmC), are not lower in *IDH1*-mutant astrocytomas compared to wild-type [69, 124].

Oncogenesis

Despite these effects of 2-HG on DNA and histone methylation, there is a growing consensus that, while obviously important, *IDH1/2* mutations are insufficient to drive neoplasia. A transgenic mouse model engineered to express mutant *IDH1* in hematopoietic cells showed increased progenitor cells in the bone marrow, but no leukemias [148]. That same group also developed a nestin-R132H IDH1 mouse model, expressing the mutant in neural stem cells [147]. Most mice died *in utero*, and the ones that made it to birth soon died from severe intracranial bleeding. This bleeding was caused by 2-HG inhibiting the α-KG-dependent prolyl hydroxylation of type IV collagen, leading to disorganized basement membrane structures around blood vessels. But not only were no tumors found, no histone methylation was detected. While disappointing to those searching for a robust *IDH1/2*-mutant transgenic tumor model, patient data had already hinted that such a model might be difficult to generate. For although L-2-HG aciduria patients sometimes develop brain tumors [2, 189], no tumors have yet been described in over 85 patients with D-2-HG aciduria [91]. (Remember that clinically relevant *IDH1/2* mutations only produce the D-isomer of 2-HG.) Because 2-HG can slow down cellular differentiation [112], mutant *IDH1/2* might not

directly trigger oncogenesis, but rather extend the opportunity for other tumor-promoting mutations to occur in undifferentiated cells (Figure 3). For example, *IDH1/2* mutations are strongly associated with *TP53* mutations in astrocytomas and 1p/19q codeletion with *CIC* and/or *FUBP1* mutations in oligodendrogliomas [13, 52, 85, 93, 145, 191], although *IDH1/2* mutations appear to precede both *TP53* mutation and 1p/19q codeletion [178].

Application of *IDH1/2* to cancer

Diagnostics

An attractive feature of *IDH1/2* mutations is that, even though our understanding of their full significance is obviously incomplete, there is already considerable utility in screening for their presence in the setting of a brain lesion. These mutations are present in 60-80% of WHO grades II and III astrocytomas and oligodendrogliomas (as well as grade IV secondary GBMs) (Figure 2c), and are never seen in mimickers of glioma like vasculitis, encephalitis, demyelinating disease, or reactive gliosis [21, 64]. Likewise, noninfiltrative gliomas including pilocytic astrocytoma, dysembryoplastic neuroepithelial tumor, and ganglioglioma, do not contain *IDH1/2* mutations [21, 62, 64, 89]. In fact, tumors that were diagnosed as gangliogliomas based on histopathological appearance, but harbored these mutations, ended up behaving like diffusely infiltrative gliomas [65]. Thus, the presence of a mutation, even in an otherwise equivocal biopsy, can be considered solid evidence of an infiltrating glioma, i.e. WHO grade >I. Mutation screening can also help differentiate between anaplastic oligodendrogliomas or glioblastomas with an oligodendroglial component (GBM-O, a.k.a. mixed oligoastrocytoma grade IV) and the more aggressive (and *IDH1/2* wild-type) small cell GBM [74]. Frontal and temporal lobes are the most common locations, whereas infratentorial tumors only rarely have mutations [45, 159]. In fact, the subventricular zone in the supratentorium may be the preferred site for *IDH1/2* mutant gliomas [52]. They can even be found in type I (no discrete enhancing mass lesion) and II (mass lesion present) gliomatosis cerebri [37, 150].

When pooling all *IDH1/2*-mutant infiltrative gliomas together, over 90% will have the R132H IDH1 variant (Figure 2a). This observation makes a R132H mutation-specific antibody effective as a rapid immunohistochemical screen on formalin-fixed, paraffin-embedded tissues [22, 24, 25]. Two such antibodies exist, DIA-H09 and Imab-1; in a head-to-head comparison, the DIA-H09 antibody was slightly more specific, with less background staining, than the Imab-1 antibody [138]. For the immunostain to be interpreted as positive, one should see dark brown cytoplasmic staining that extends out to the tumor processes [62]. Weak staining of neurons or labeling of red blood cells does not count. Sometimes nuclear staining is also seen, but it is not yet clear whether this represents actual abnormal nuclear localization or just antigen diffusion [138]. Another controversy is intratumoral homogeneity of R132H IDH1 expression; some have found 100% homogeneity in all cases [22], whereas others reported that about 15% of mutant gliomas have a subset of tumor cells that are negative [138]. Even so, when interpreted correctly, the degree of concordance between R132H IDH1 immunohistochemistry and sequencing is very high, as the immunostain will match sequencing results 98% of the time [138]. And in biopsies with only a few infiltrating glioma cells amidst mostly nonneoplastic tissue, the antibody has a better shot at detecting mutated cells than does any PCR/sequencing technique [33, 62, 144]. Thus, this antibody has already become a staple of brain tumor workup.

Although the R132H IDH1 immunostain is an excellent first-line screen in brain biopsies, it obviously will not detect less common *IDH1* mutations or *IDH2* mutations. This is a nontrivial issue, since the frequency of those other mutations is uneven between glioma subtypes. In both primary and secondary GBMs, well over 95% of *IDH1/2* mutations will be of the R132H IDH1 variety, but there is variation at the WHO grade II-III level (Figure 4).

For example, about 10% of *IDH1*-mutant grade II and III astrocytomas will have a non-R132H IDH1 mutation, most commonly R132C (Figure 4a & d). Prior work suggested that *IDH2* mutations are more likely to occur in oligodendroglial tumors [60], but combining results from several studies suggests that this is true only at the grade III level (Figure 4e & f), and that WHO grade II oligodendrogliomas have just as strong a predilection for R132H IDH1 as do GBMs (Figure 4b). What all this means regarding gliomagenesis is unclear, but considering we now know that not all *IDH1/2* mutations are identical in their 2-HG production capacity and subcellular localization, such differences cannot be dismissed out of hand. At the very least, it underscores why it is often necessary to do followup sequencing of R132H IDH1 immunonegative cases, especially when dealing with a suspected grade II or III glioma (Figure 2c).

Thus, it is very helpful to supplement R132H IDH1 immunohistochemistry with molecular methods that catch other variants. Multiple assays can do this, with sensitivity well beyond the 20% mutant allele limit of traditional PCR and Sanger sequencing [47, 63, 110, 120, 130, 170]. But regardless of the method used, critical sources of inter-laboratory variability are optimization of DNA extraction and PCR product purification [170]. Of course, the absence of an *IDH1/2* mutation does not exclude the possibility of a glioma, especially if the tissue only contains a few scattered infiltrating neoplastic cells. Follow-up testing of immunonegative cases is probably worthwhile in grades II-III gliomas and secondary GBMs, in patients between 20-60 years, in those whose tumors presented with seizures, and in any GBM with minimal necrosis [95, 126, 157].

In particular, the special association between *IDH1/2* gliomas and seizures deserves special mention. Some have used a 2-HG octyl ester to increase cell permeability [31, 109, 186], but others have shown effects by exogenous 2-HG even without esterification [141]. And recent work showed that exogenous unmodified 2-HG can enter cells via a sodium-dependent dicarboxylate transporter [19]. Since mutant cells secrete 2-HG into culture medium *in vitro* or into the extracellular space *in vivo* [35, 46, 70, 91, 106, 151], its effects may extend to admixed nonneoplastic cells in the tumor. For example, seizures are not only a feature of the 2-HG acidurias, but are also more likely in *IDH1/2*-mutant gliomas than their wild-type counterparts [68, 157]. It is therefore possible that seizures in such patients are not merely sequelae of tumor infiltration or mass effect, but instead represent a direct local activity of 2-HG on nonneoplastic brain cells.

Finally, instead of directly testing for the mutation in biopsied tissue, it is possible to detect elevated 2-HG in *IDH1/2*-mutant brain tumors using proton magnetic resonance spectroscopy [8, 28, 44, 99], though it is not yet clear whether this approach is sensitive and specific enough for routine use. The compound can also be detected in paraffin tissue blocks containing mutant tumor [12]. In *IDH1/2*-mutant leukemias, quantification of 2-HG in serum or even urine is feasible and correlates with disease recurrence [12, 46, 57, 151]. However, a similar approach does not work very well in gliomas, presumably because insufficient 2-HG ends up in the systemic circulation [23]. Likewise, direct detection of mutant *IDH1/2* in the systemic circulation of glioma patients is a relatively insensitive biomarker [16].

Prognosis

Even when a histologic diagnosis of infiltrative glioma is certain, and there is no question about its WHO grade, there is still a very good reason to test for *IDH1/2* mutations—tumors with the mutation tend to be far less aggressive than their WHO grade-matched wild-type counterparts [39, 126, 131, 146, 180, 181, 187]. This is clearly true at the grade III-IV level, where grade III tumors lacking the mutations are just as lethal as wild-type grade IV tumors [59, 187]. In fact, one series of wild-type anaplastic astrocytomas showed progression to

classic ring-enhancing GBMs within just a few months of initial resection, raising the possibility that many such tumors were in fact GBMs at presentation, albeit without necrosis or microvascular proliferation on the initial specimen [33, 129]. And one reason why advanced patient age is an unfavorable prognostic marker is because *IDH1/2* mutant tumors are less likely to occur in older people [55, 59, 187]. *IDH1/2* mutations are not only characteristic of many proneural GBMs, specifically those with global hypermethylation [127, 159, 167], but they comprise the subset of proneural GBMs that actually have a better prognosis [127].

IDH1/2 mutational status is also a very good triaging tool for subsequent 1p/19q testing in suspected oligodendrogliomas, insofar as true whole-arm codeletion hardly ever happens in the absence of a mutation [93, 191]. Thus, a wild-type glioma probably does not need to be tested for codeletion, because even an apparent codeletion (e.g. by FISH) cannot be trusted [34].

As is the case with other aspects of *IDH1/2*, however, there are controversies about its prognostic power in certain contexts. For example, it is not at all clear whether *IDH1/2* status effectively stratifies grade II gliomas, especially astrocytomas; some have suggested a better prognosis [105, 119, 128, 140] while others found no difference [4, 52, 53, 66, 76, 77, 85, 162]. Since grade I gliomas do not have *IDH1/2* mutations, some tumors that appear to be grade II histologically but are *IDH1/2* wild-type might actually represent “overgraded” grade I gliomas. If so, inclusion of such cases would mask the prognostic effects of *IDH1/2* mutations in true diffusely infiltrative grade II gliomas. For example, one study that showed no survival difference in grade II gliomas also reported a 33% higher rate of gross total excision in wild-type tumors, raising the possibility that at least some of their grade II tumors might have been grade I in a biological sense – i.e., essentially noninfiltrative—a point the authors themselves suggested [4]. A different group found that their grade II astrocytomas actually had worse progression-free survival than matched wild-type tumors, but better postrecurrence survival [165]. This incongruity might be explained by accidental inclusion of some wild-type grade I tumors that did not recur, but among tumors that did recur (which were more likely to be real infiltrative gliomas), *IDH1/2*-mutation was favorable.

Another point of debate is whether *IDH1/2* mutations are better prognostic markers than other molecular markers. Several multivariate analyses have shown *IDH1/2* to be more powerful than 1p/19q codeletion and even *O6-Methylguanine-DNA methyltransferase* (*MGMT*) promoter methylation [51, 75, 146, 155, 181], though some have suggested *MGMT* might still be stronger [26, 67, 85, 121]. Given the prominence of *MGMT* in the workup of GBMs, its relationship with *IDH1/2* mutations deserves particular attention.

IDH1/2 and MGMT

MGMT is a DNA repair protein that removes alkyl groups from the O6 position of guanine in DNA, making cells resistant to the alkylating agent temozolomide [161]. When its promoter is methylated, MGMT expression decreases and temozolomide sensitivity increases. As a result of a seminal study by Hegi et al. [61], *MGMT* promoter methylation testing is standard for the workup of GBMs. But that same study also showed that methylated GBMs responded better to a radiation-only regimen, even though MGMT is not known to have a role in repairing the kind of DNA damage induced by radiation. However, considering that *IDH1/2* mutations promote global hypermethylation, including methylation of the *MGMT* promoter [32, 51, 122, 127, 146, 167], it is possible that some of the *MGMT*-methylated GBMs in the Hegi study also had *IDH1/2* mutations, and that it is the latter defect which promotes radiosensitivity. In support of this, some have shown that *IDH1/2*

mutations correlate with radiosensitivity [106, 128, 169], though even this has been disputed [40, 140].

In clinical studies it is difficult to weigh the relative importance of *IDH1/2* versus *MGMT*, because although around half of *IDH1/2* wild-type gliomas will still have *MGMT* promoter methylation [59, 146], the reverse situation—*IDH1/2*-mutant tumors without methylation—is uncommon. Exactly how uncommon seems to depend on the cohort and on how methylation is being tested. For example, one group using methylation-specific PCR reported 15-20% of grade III astrocytomas, and 2% of GBMs, as having *IDH1/2* mutation but not *MGMT* promoter methylation [59]. This is consistent with another study of grade II-IV gliomas using a similar method [146]. In contrast, a different group tested methylation via pyrosequencing of 16 CpG sites in the *MGMT* promoter. They found that, in their cohort of over 400 grade II-IV gliomas, virtually all *IDH1/2*-mutant tumors also had methylation regardless of WHO grade [122]. Others have reported similar degrees of concordance [160, 167, 169].

This does not mean, however, that *MGMT* is now irrelevant. A recent study of GBMs in the elderly (in which *IDH1/2* mutations are uncommon) showed that *MGMT* promoter methylation was associated with better response to temozolomide-containing regimens, but not to radiotherapy alone [114]. It is also unclear whether *IDH1/2* mutations affect temozolomide response independent of *MGMT* [160]. *MGMT* promoter methylation is probably a favorable marker independent of *IDH1/2*, but only in regimens containing temozolomide. *IDH1/2* mutations, on the other hand, may be relevant to a broader spectrum of adjuvant therapies. Thus, having both molecular alterations is likely more favorable than either in isolation. *MGMT* testing is still therefore useful in the workup of GBMs that are wild-type for *IDH1/2*, but in the opinion of this author, if an *IDH1/2* mutation is detected at any grade of glioma, it can safely be assumed that *MGMT* promoter methylation is also present; proving it would therefore be unnecessary.

IDH1/2 variants and outcome

Earlier, differences in 2-HG production among *IDH1/2* variants were discussed; this begs the question as to whether different variants have different outcomes. This is difficult to study in gliomas, where there is a marked preponderance for R132H IDH1 (Figure 2), but in AML there is less enrichment for a specific mutation. In those cancers, mutations on *IDH1* seem to associate with a worse prognosis than *IDH2* [1, 29, 115, 133]. Even variants within the same gene might have differing prognostic significance, as R140 mutations on IDH2 might be somewhat more favorable than R172 [56, 115, 132]. Similarly, R132C IDH1 might be more favorable than R132H [90]. Extremely large cohorts adjusting for other variables like WHO grade, 1p/19q status, and treatment would be needed to reliably determine if similar associations exist in gliomas, although one study has already suggested that there are no survival differences between R132H and non-R132H *IDH1* variants [54].

Yet another layer of complexity regarding *IDH1/2* mutations and prognosis is the phenomenon of monoallelic gene expression (MAE), wherein only one allele of a gene is expressed even though both alleles are present. This of course happens during imprinting and X-inactivation, but it can also occur in otherwise non-imprinted genes. One study found that about 15% of *IDH1*-mutant gliomas had monoallelic gene expression of *IDH1*, that it was usually directed toward the normal allele (i.e. the mutant allele was not expressed), and, as expected, survival was worse in such cases [173]. This could help explain the aforementioned R132H IDH1 immunohistochemical heterogeneity and false-negativity seen in some gliomas [138], and raises the possibility that sequencing might not always be adequate to identify tumors that are functionally *IDH1/2* mutant.

A final word on *IDH1/2* and prognosis is from a report that an rs11554137:C>T single-nucleotide polymorphism (SNP) is present on codon 105 of the *IDH1* gene in about 10% of grades II-IV gliomas [174]. This SNP occurs independently of both *IDH1/2* mutations and WHO grade, and although it does not substitute the glycine residue on codon 105, it nevertheless is associated with worse response to adjuvant therapy in both gliomas and AML [172, 174]. As of yet, there is no satisfying explanation for this remarkable finding.

Future directions

As we have seen, the sheer volume of data that has been generated by multiple laboratories on *IDH1/2* mutations over the last few years is simply incredible. The field has now matured to the stage where rapid, descriptive-type papers on mutation frequencies and outcomes have been done, and mechanistic/experimental papers are becoming more frequent. Predictions as to the next major advances in this subfield are inherently uncertain, but some areas of interest include how *IDH1/2* mutations promote gliomagenesis, what experimental models can be developed, why the mutations affect prognosis, and how to target *IDH1/2* mutations in novel therapies.

Although we do not yet know exactly why *IDH1/2* mutations are found in gliomas, there are a few clues in the literature. For example, if a glioma does not have an *IDH1/2* mutation at its clinical outset, it never acquires one [96]. Likewise, 2-HG levels do not change as a lower-grade glioma progresses to GBM [77]. Mutations predate and are tightly associated with *TP53* mutations in astrocytomas and 1p/19q codeletion in oligodendrogliomas [52, 85, 93, 119, 191]. Even some *IDH1/2*-mutant mixed oligoastrocytomas can have a *TP53* mutation in the astrocytic region and 1p/19q codeletion in the oligodendroglial area [96]. Perhaps the mutation and its 2-HG metabolite are therefore not sufficiently oncogenic on their own, but instead act as selection agents favoring specific additional genetic alterations. And if a glioma manages to arise without a mutation, there is no selection pressure to do so. We also know that certain germline SNPs on 8q24.21 and 11q23 increase the risk of *IDH1/2*-mutant gliomas [68, 142]. What those SNPs are doing to promote *IDH1/2* mutations is unknown, but will certainly be intensely investigated in coming years. Perhaps the SNPs and/or *IDH1/2* mutations are indirectly causing gliomagenesis via congenital disruption of the normal stem cell microenvironment, in turn increasing the odds of neoplasia akin to what was hypothesized for carcinogenesis by James DeGregori [36]. Certainly, the effects of 2-HG on DNA and histone methylation will prove significant in gliomagenesis.

But 2-HG, however beneficial it might be to oncogenesis, clearly can have toxic side effects under certain conditions. Perhaps the favorable prognostic effects of *IDH1/2* mutations are not fully realized unless the cells are abruptly stressed, thereby unmasking metabolic defects for which the tumor had gradually evolved compensatory mechanisms. Recalling the controversy mentioned earlier— whether *IDH1/2* mutations are prognostically important at the grade II level—it is important to remember that most grade II gliomas are not irradiated upfront if it can be avoided, whereas radiotherapy is standard at the III-IV level. And while some have suggested that *IDH1/2* mutations reduce cellular invasiveness and/or proliferation [18, 106], others have found no such correlations [53, 57, 136, 137, 193]. *IDH1/2* prognostic significance may therefore be due more to heightened adjuvant therapy sensitivity as opposed to intrinsic defects in growth or invasiveness. After all, radiotherapy-containing regimens affect not just tumor cells, but also create a hostile microenvironment with necrosis, low pH, low oxygen, low glucose, and increased local concentrations of metabolic wastes. This might help account for the more equivocal prognostic importance of *IDH1/2* in AMLs, as such an unpleasant microenvironment cannot be created in the circulation without killing the patient. Furthermore, glial cells could be particularly sensitive to 2-HG side effects, hence their strong predilection for one of the weakest 2-HG producers,

R132H IDH1. In comparison, AMLs, by virtue of their being in the bloodstream, might have an easier time getting rid of excess 2-HG and thus not be subjected to the same selection pressure for a weak mutation. And recall that these mutations only produce D-2-HG and not L-2-HG, even though the latter is a more potent inhibitor of α-KG-dependent enzymes [30, 88, 186]. Taken together, it seems that the "therapeutic window" for 2-HG to promote oncogenesis is fairly narrow, and what is a beneficial mutation for the untreated tumor can quickly be rendered detrimental during adjuvant therapy.

Regarding its use as a biomarker, while the presence of an *IDH1/2* mutation strongly indicates at least a grade II infiltrative glioma, its absence in an obviously low-grade setting does not automatically equate to a noninfiltrative grade I tumor, especially in children. Other mutations, including those in *H3F3A* and *BRAF V600E*, could be drivers of less common infiltrativesubsets. Nevertheless, the next WHO classification will likely address *IDH1/2* and emphasize its importance in the pathologic workup of gliomas. One group even suggested that a molecular panel including *IDH1/2, MGMT*, 1p/19q, and *TP53* does a better job of prognostic stratification in grade II gliomas than histologic subtyping [105]. And once radiologic measurement of elevated 2-HG is optimized and standardized for routine use, it will have a tremendous impact on the workup and management of brain tumor patients, both in generating a more accurate preoperative differential as well as in discriminating genuine recurrences from therapy-induced changes.

But gliomas apparently have strategies for evolving out of the *IDH1/2* mutations, including monoallelic gene expression of only the wild-type allele [173], deletion of the mutant allele [96, 139], or even deletion of the wild-type allele so as to reduce 2-HG production [67, 69]. This suggests that, for any clinical trials aimed at targeted therapeutics or more accurate prognoses, degrees of mutant expression and functionality are probably more important than just the presence of a mutation [109]. For example, glutaminase inhibition is more effective in *IDH1*-mutant gliomas [152], but only works if the mutation is active. The same would hold for any strategy involving nanotechnology or other small molecules that specifically bind mutant enzyme, such as the R132H IDH1 inhibitor AGX-891 [109].

Generating a robust transgenic mouse model of *IDH1/2*-mutant tumors will be important for the field to really advance. As discussed earlier, Tak Mak's laboratory has nicely demonstrated some of the difficulties in making this happen [147, 148], so for now the emphasis will be on xenografts like the one developed by Luchman et al. [113]. Pairing a conditional knock-in *IDH1* mutation with some other biologically appropriate oncogene or tumor suppressor might eventually work.

Clearly, there is still a great deal to be done on *IDH1/2* in gliomas and other cancers. Our diagnostic and prognostic power has already been strengthened, but realization of the Human Genome Project's ultimate goal—development of personalized, bona fide cures for currently incurable diseases—will require even more hard work and tenacity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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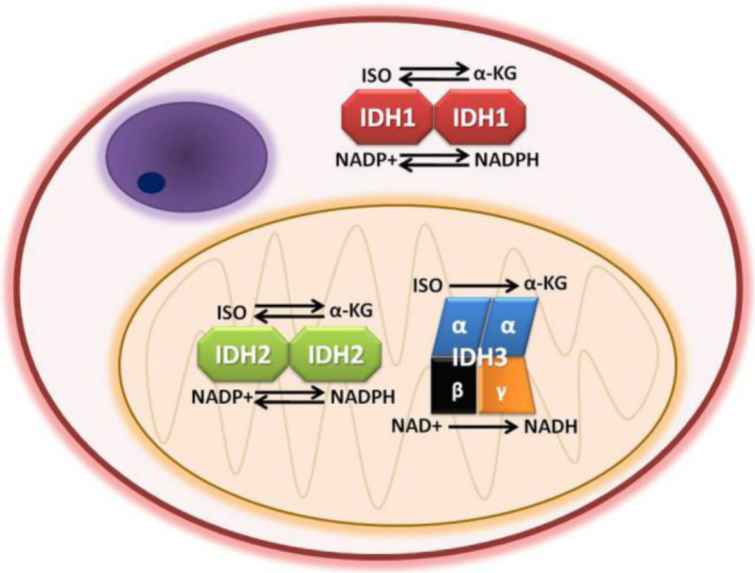

Fig. 1. Normal functions and subcellular locations of IDH1, IDH2, and IDH3

All three enzymes oxidize isocitrate (ISO) to alpha-ketoglutarate (α-KG). IDH1 and IDH2 are homodimers, whereas IDH3 is a heterotetramer. IDH1 and IDH2 utilize nicotinamide adenine dinucleotide phosphate (NADP+) as a cofactor, generating NADPH. IDH3 uses NAD+ and produces NADH. IDH2 and 3 are located in mitochondria while IDH1 is in the cytosol and peroxisomes. In certain circumstances IDH1 and IDH2 can reduce α-KG to isocitrate, whereas IDH3 is unidirectional. (The structure in the upper left of the cell depicts a nucleus.)

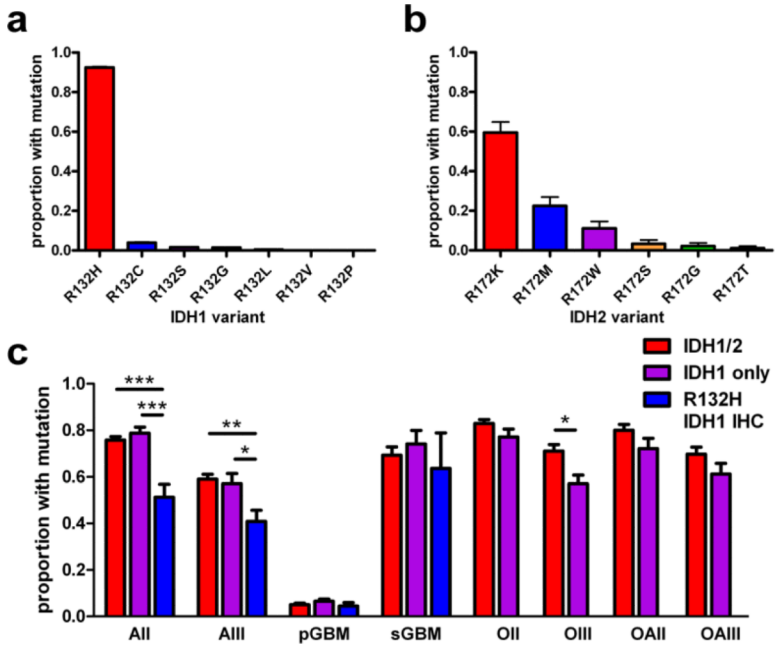

Fig. 2. IDH1/2 mutations by variant, glioma category, and type of screening

(a) In a pooled analysis of over 3400 gliomas from 37 studies in which mutation subtypes were reported, R132H was the most common *IDH1* variant, comprising 92% of all *IDH1* mutations ($P < 0.0001$). The rarest was R132P, occurring in a single case [54]. (b) In contrast, there was less preference for a specific type of *IDH2* mutation, though the R172K variant was present in 60% of *IDH2*-mutant tumors ($P < 0.0001$, N = 89). (c) Inter-study mutation frequencies significantly differ in grades II-III astrocytomas and grade III oligodendrogliomas, depending on whether the studies tested for both *IDH1* and *IDH2* mutations (red bars, N = 4324 gliomas from 26 studies), screened for just *IDH1* mutations (purple bars, N = 2075 gliomas from 12 studies), or only used the R132H IDH1 antibody (blue bars, N = 794 gliomas from 6 studies). Not enough oligodendroglial tumors have been interrogated with R132H IDH1 antibody to be reliably compared with the other columns. Of note, mean mutation frequencies in *IDH1*-only studies sometimes barely exceeded the frequencies reported in *IDH1/2* papers (purple versus red bars in AII and sGBM subgroups). This apparent incongruity can be explained by inter-cohort variations, especially given how rare *IDH2* mutations are in astrocytic tumors (see Figure 4). A list of the studies from which these data are derived is in Supplemental Table 1. All data bars in a-c represent mean ± SEM; statistical analyses were done via Student's t-test or ANOVA with Kruskal-Wallis post hoc test, as appropriate. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. AII = grade II diffuse astrocytoma; AIII = grade III anaplastic astrocytoma; pGBM = primary GBM; sGBM = secondary GBM; OII = grade II oligodendroglioma; OIII = grade III anaplastic oligodendroglioma; OAII = grade II oligoastrocytoma; OAIII = grade III anaplastic oligoastrocytoma.

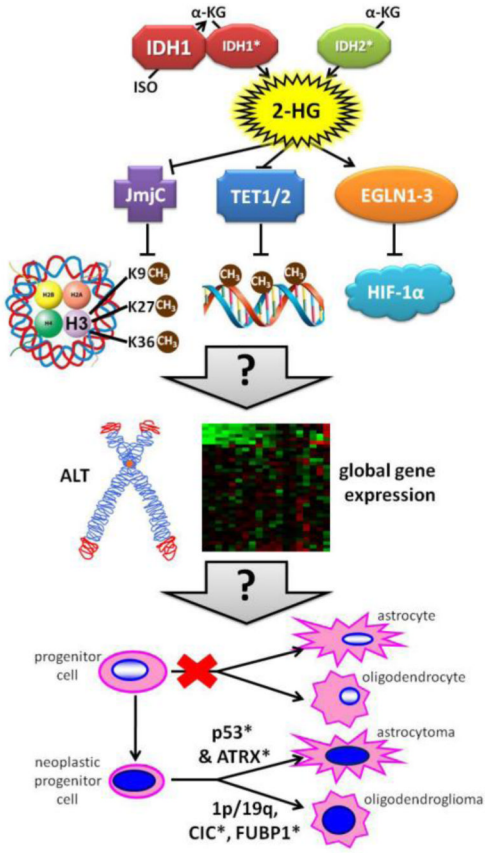

Fig. 3. Postulated effects of mutant IDH1/2 on gliomagenesis

Unlike mutant IDH2, mutant IDH1 is more efficient when it heterodimerizes with a wild-type partner. Both mutations convert α-KG to 2-HG. This 2-HG compound inhibits some enzymes that use α-KG as a cofactor, including JmjC domain-containing histone demethylases and TET DNA demethylases. The result of this inhibition is a net upregulation of histone and DNA methylation, the former occurring at key amino acid residues that are mutated in some non-*IDH1/2*-driven gliomas (involving the *H3F3A* gene encoding histone 3.3). Other proteins involved in chaperoning histone H3.3 include ATRX and DAXX, both of which can be mutated in *IDH1/2*-wt and *IDH1/2*-mutant gliomas (though ATRX is far more likely to be mutated than DAXX)[149]. EGLN1-3 prolyl hydroxylases may actually be activated by 2-HG, thereby increasing degradation of Hif-1α. The exact results of all these alterations are not yet clear, but they definitely cause global modifications of gene expression and may promote Alternative Lengthening of Telomeres (ALT). EGLN activation in particular appears to slow down the differentiation of glial precursors, perhaps providing a greater opportunity for additional mutations to arise. Mutant *TP53* and/or *ATRX* tend to produce astrocytomas, whereas 1p/19q codeletion with *CIC* and/or *FUBP1* mutations produce oligodendrogliomas. Asterisks (*) denote mutated proteins.

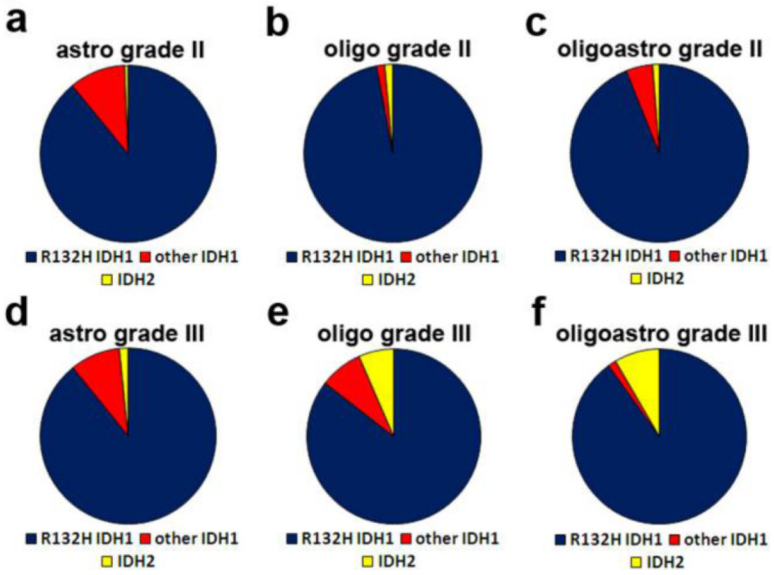

Fig. 4. Frequencies of non-R132H IDH1 mutations differ among subtypes of grade II-III gliomas

Combining data from multiple studies that included details on mutation subtypes by histologic diagnosis (see Figure 2), about 89% of *IDH1/2* mutations in grade II-III astrocytic tumors were R132H IDH1 (a & d), with another 10% being other *IDH1* mutations. Only 0.5-1% of those gliomas had an *IDH2* mutation. In contrast, 7-8% of grade III oligodendrogliomas and oligoastrocytomas were *IDH2*-mutant (e & f). But this increased proportion of *IDH2* mutations was only seen in grade III tumors; 94-97% of grade II oligodendrogliomas and oligoastrocytomas were R132H IDH1, and only 1% were *IDH2*. A list of the studies from which these data are derived is in Supplemental Table 1.