Detection of oncogenic IDH1 mutations using magnetic resonance spectroscopy of 2-hydroxyglutarate

Ovidiu C. Andronesi,[1] Otto Rapalino,[2] Elizabeth Gerstner,[3,4] Andrew Chi,[3,4] Tracy T. Batchelor,[3,4,5]
Dan P. Cahill,[6] A. Gregory Sorensen,[7] and Bruce R. Rosen[1]

[1]Athinoula A. Martinos Center for Biomedical Imaging, [2]Department of Radiology, [3]Department of Neurology, [4]Division of Neuro-Oncology,
[5]Department of Radiation Oncology, and [6]Department of Neurosurgery, Massachusetts General Hospital, Harvard Medical School,
Boston, Massachusetts, USA. [7]Siemens Healthcare USA, Malvern, Pennsylvania, USA.

The investigation of metabolic pathways disturbed in isocitrate dehydrogenase (IDH) mutant tumors revealed that the hallmark metabolic alteration is the production of D-2-hydroxyglutarate (D-2HG). The biological impact of D-2HG strongly suggests that high levels of this metabolite may play a central role in propagating downstream the effects of mutant IDH, leading to malignant transformation of cells. Hence, D-2HG may be an ideal biomarker for both diagnosing and monitoring treatment response targeting IDH mutations. Magnetic resonance spectroscopy (MRS) is well suited to the task of noninvasive D-2HG detection, and there has been much interest in developing such methods. Here, we review recent efforts to translate methodology using MRS to reliably measure in vivo D-2HG into clinical research.

Introduction

Recurrent heterozygous somatic mutations of the isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) genes were recently found by genome-wide sequencing to be highly frequent (50%–80%) in human grade II–IV gliomas (1, 2). IDH mutations are also often observed in several other cancers, including acute myeloid leukemia (3), central/periosteal chondrosarcoma and enchondroma (4), and intrahepatic cholangiocarcinoma (5). The identification of frequent IDH mutations in multiple cancers suggests that this pathway is involved in oncogenesis. Indeed, increasing evidence demonstrates that IDH mutations alter downstream epigenetic and genetic cellular signal transduction pathways in tumors (6, 7). In gliomas, IDH1 mutations appear to define a distinct clinical subset of tumors, as these patients have a 2- to 4-fold longer median survival compared with patients with wild-type IDH1 gliomas (8). IDH1 mutations are especially common in secondary glioblastoma (GBM) arising from lower-grade gliomas, arguing that these mutations are early driver events in this disease (9). Despite aggressive therapy with surgery, radiation, and cytotoxic chemotherapy, average survival of patients with GBM is less than 2 years, and less than 10% of patients survive 5 years or more (10).

The discovery of cancer-related IDH1 mutations has raised hopes that this pathway can be targeted for therapeutic benefit (11, 12). Methods that can rapidly and noninvasively identify patients for clinical trials and determine the pharmacodynamic effect of candidate agents in patients enrolled in trials are particularly important to guide and accelerate the translation of these treatments from bench to bedside. Magnetic resonance spectroscopy (MRS) can play an important role in clinical and translational research because IDH mutated tumor cells have such a distinct molecular phenotype (13, 14).

Biochemistry and metabolic alterations in IDH-mutated tumor cells

The family of IDH enzymes includes three isoforms: IDH1, which localizes in peroxisomes and cytoplasm, and IDH2 and IDH3, which localize in mitochondria as part of the tricarboxylic acid cycle (11). All three wild-type enzymes catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (αKG), using the cofactor NADP$^+$ (IDH1 and IDH2) or NAD$^+$ (IDH3) as the electron acceptor. To date, only mutations of IDH1 and IDH2 have been identified in human cancers (11), and only one allele is mutated. In gliomas, about 90% of IDH mutations involve a substitution in IDH1 in which arginine 132 (R132) from the catalytic site is replaced by a histidine (IDH1 R132H), known as the canonical IDH1 mutation (8). A number of noncanonical mutations such as IDH1 R132C, IDH1 R132S, IDH1 R132L, and IDH1 R132G are less frequently present. Arginine R172 in IDH2 is the corresponding residue to R132 in IDH1, and the most common mutation is IDH2 R172K. In addition to IDH2 R172K, IDH2 R140Q has also been observed in acute myeloid leukemia. Although most IDH1 mutations occur at R132, a small number of mutations producing D-2-hydroxyglutarate (D-2HG) occur at R100, G97, and Y139 (15). However, only a single residue is mutated in either IDH1 or IDH2 in a given tumor.

IDH mutations result in a very high accumulation of the oncometabolite D-2HG in the range of 5- to 35-mM levels, which is 2–3 orders of magnitude higher than D-2HG levels in tumors with wild-type IDH or in healthy tissue (13). All IDH1 G97, R100, R132, and Y139 and IDH2 R140 and R172 mutations confer a neomorphic activity to the IDH1/2 enzymes, switching their activity toward the reduction of αKG to D-2HG, using NADPH as a cofactor (15). The gain of function conferred by these mutations is possible because in each tumor cell a copy of the wild-type allele exists to supply the αKG substrate and NADPH cofactor for the mutated allele.

A cause and effect relationship between IDH mutation and tumorigenesis is probable, and D-2HG appears to play a pivotal role as the relay agent. Evidence is mounting that high levels of D-2HG

Table 1
Summary of in vivo 1H MRS methods used in the literature for detection of D-2HG in patients with mutant IDH glioma

Method	2HG detection optimization	Strength and limitations	Reference
Short-echo MRS (PRESS, TE 30 ms, voxels 1 cm³, acquisition time ~5 min)	None	Widely available for clinical use on all scanners; false positive rate can be large	24
Long-echo MRS (PRESS, TE 97ms, voxels 1–8 cm³, acquisition time ~5 min)	Modulation of 2HG resonances by spectral editing	Small modification of PRESS sequence; relies on spectral fitting to identify D-2HG	30
Difference MRS (MEGA-LASER, MEGA-PRESS, TE 68 ms, voxel 8–27 cm³, acquisition time ~5–10 min)	Removes overlapping resonances from other metabolites	Less available clinically; D-2HG signal can be identified without fitting, no false positive	29, 30
Correlation MRS (COSY-LASER, TE 45 ms, voxel 27 cm³, acquisition time ~10 min)	Separates metabolite crosspeaks in a 2D spectrum	Not standard in clinical examination, longer acquisition, offline processing; unambiguous metabolite assignment, complex quantification	29

alter the biology of tumor cells toward malignancy by influencing the activity of enzymes critical for regulating the metabolic (14) and epigenetic state of cells (6, 7, 16–18). D-2HG may act as an oncometabolite via competitive inhibition of αKG-dependent dioxygenases (16). This includes inhibition of histone demethylases and 5-methlycytosine hydroxylases (e.g., TET2), leading to genome-wide alterations in histone and DNA hypermethylation as well as inhibition of hydroxylases, resulting in upregulation of HIF-1 (19). The effects of D-2HG have been shown to be reversible in leukemic transformation (18), which gives further evidence that treatments that lower D-2HG could be a valid therapeutic approach for IDH-mutant tumors. In addition to increased D-2HG, widespread metabolic disturbances of the cellular metabolome have been measured in cells with IDH mutations, including changes in amino acid concentration (increased levels of glycine, serine, threonine, among others, and decreased levels of aspartate and glutamate), N-acetylated amino acids (N-acetylaspartate, N-acetylserine, N-acetylthreonine), glutathione derivatives, choline metabolites, and TCA cycle intermediates (fumarate, malate) (14). These metabolic changes might be exploited for therapy. For example, IDH mutations cause a depletion of NADPH, which lowers the reductive capabilities of tumor cells (20) and perhaps makes them more susceptible to treatments that create free radicals (e.g., radiation) (21).

In vivo MRS of D-2HG in IDH mutant tumors
D-2HG may be an optimal biomarker for tumors with IDH mutations, as it ideally fulfills several important requirements: (a) there is virtually no normal D-2HG background — in cells without IDH mutations, D-2HG is produced as an error product of normal metabolism and is only present at trace levels; (b) 99% of tumors with IDH mutations have increased levels of D-2HG by several orders of magnitude; (c) the only other known cause of elevated 2HG is hydroxyglutaric aciduria (in this case, high L-2HG caused by a mutation in 2HG dehydrogenase), which is a rare inborn error of metabolism that presents with a different clinical phenotype and marked developmental anomalies in early childhood. Hence, tumors displaying increased levels of D-2HG are unlikely to represent false-positive cases for IDH mutations. Furthermore, this raises the possibility that D-2HG levels could also be used to quantify and predict the efficacy of drugs targeting mutant IDH1
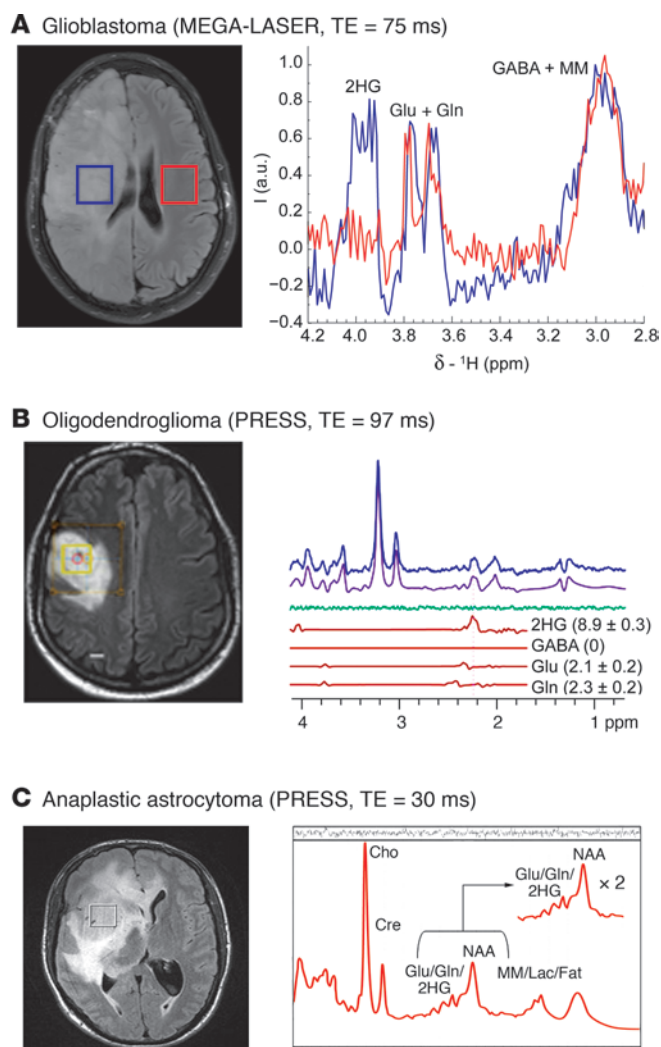
for antitumor therapy (11, 15). In fact, it is hard to find a similar example of another tumor biomarker metabolite that is so well supported by the underlying biology.

The high levels of D-2HG observed in IDH1-mutant gliomas are amenable to detection by in vivo MRS. Given that the detection threshold of in vivo MRS is around 1 mM (1 µmol/g, wet tissue), D-2HG should be measurable only in situations in which it accumulates due to IDH1 mutations. Conversely, D-2HG is not expected to be detectable in tumors in which IDH1 is not mutated or in healthy tissues. In addition, ex vivo MRS measurements of intact biopsies (22) or extracts reach higher sensitivity 0.1–0.01 mM (0.1–0.01 µmol/g) and can be used as a cheaper and faster alternative to mass spectrometry.

However, unambiguous measurement of 2HG with proton MRS, especially in vivo, is challenging because its coupled five-spin system yields a complex spectral pattern (23) that overlaps with other metabolites that are normally present in the brain or may accumulate in cancer. The D-2HG signals from Hβ (1.91 ppm) and Hγ (2.24 ppm) protons are superimposed by glutamate, glutamine, and γ-aminobutyric acid (GABA), while Hα (4.02 ppm) signals are obscured by myoinositol, phosphocreatine, and lactate.

Spectral fitting of conventional 1H magnetic resonance spectra is likely to provide a high rate of false-positive results for D-2HG or may lead to under- or overestimation of D-2HG levels. Indeed, a false-positive rate of approximately 22% was observed by Pope et al. (24) when using conventional MRS and spectral fitting to detect D-2HG in glioma patients. This error rate is a result of the spectral decomposition of the fitting algorithm, which may not be unique, especially when dealing with the limited resolution of in vivo spectra acquired at low fields. This type of error is well documented for GABA, which poses similar problems (25). Indirect proof regarding the difficulty of D-2HG detection is provided by the fact that despite the high frequency of IDH1 mutations in glioma, high levels of D-2HG, and large number of MRS studies of brain tumors performed over the last 20 years (26–28), the existence of D-2HG was not reported in the cancer MRS literature until after it was discovered through genomic and ex vivo metabolomic analysis (1, 2, 13).

Recently, reliable detection of D-2HG using in vivo 1H MRS was demonstrated in glioma patients (29, 30). Andronesi et al. reported the unambiguous detection of D-2HG in mutant IDH1 glioma in vivo using 2D correlation spectroscopy (COSY) and

A Glioblastoma (MEGA-LASER, TE = 75 ms)

B Oligodendroglioma (PRESS, TE = 97 ms)

C Anaplastic astrocytoma (PRESS, TE = 30 ms)

Figure 1
In vivo D-2HG measurements: (**A**) J-difference spectroscopy with MEGA-LASER sequence in a patient with GBM with mutant IDH1. Adapted with permission from Science Translational Medicine (29). (**B**) Spectral editing with PRESS sequence of TE 97 ms (TE1: 32 ms, TE2: 65 ms) in a patient with mutant IDH1 oligodendroglioma. Adapted with permission from *Nature Medicine* (30). (**C**) Spectra acquired with PRESS sequence of TE 30 ms in a patient with mutant IDH1 anaplastic astrocytoma. Adapted with permission from *Journal of Neuro-Oncology* (24). Cho, choline; Cre, creatine; Gln, glutamine; Glu, glutamate; Lac, lactate; MM, macromolecules; NAA, N-acetyl-aspartate.

J-difference spectroscopy (29). In 2D COSY the overlapping signals are resolved along a second orthogonal chemical shift dimension (31, 32), and in the case of D-2HG, the cross-peaks resulting from the scalar coupling of Hα-Hβ protons show up in a region that is free of the contribution of other metabolites in both healthy and wild-type tumors. While 2D COSY retains all the metabolites in the spectrum, J-difference spectroscopy (25, 33) takes the opposite approach instead by focusing on the metabolite of interest, such as D-2HG, and selectively applying a narrow-band radiofrequency pulse to selectively refocus the Hα-Hβ scalar coupling evolution, then removing the contribution of overlapping metabolites. In this case a 1D difference spectrum with the Hα signal of D-2HG is detected at 4.02 ppm. Both methods have strengths and weaknesses: 2D COSY has the highest resolving power to disentangle overlapping metabolites, but has less sensitivity and quantification is more complex; J-difference spectroscopy has increased sensitivity, and quantification is straightforward, but it is susceptible to subtraction errors.

In a study conducted on glioma patients, Choi et al. (30) detected and quantified D-2HG levels using spectral editing at long echo times (TEs) and J-difference spectroscopy. Spectral editing at long TEs takes advantage of the modulation of multiplets and line

shapes due to scalar coupling evolution and achieves some degree of filtering based on this mechanism and difference in transverse relaxation. In the case of D-2HG, a TE of 97 ms maximizes the contribution of Hγ protons (2.24 ppm) against the background of glutamate and glutamine. This approach has the advantage of simplicity because it uses sequences existing on all MR scanners and relies on the fitting to separate D-2HG from glutamate, glutamine, and other metabolites at TE of 97 ms. Good spectral resolution, scanner stability, and absence of motion artifacts is required to reliably disentangle signals at 2.24 ppm. Detection based on spectral difference has been obtained based on the same principle outlined above. In their study, Choi et al were able to correctly identify all patients that have IDH mutations (15 of 30), without false-positive results. They also quantified levels of D-2HG based on the internal water reference signal, and in their patient population in vivo D-2HG was found to range between 2 and 9 mM.

In Table 1, a comparison is made among the published methods for D-2HG detection. Results selected from the literature are shown in Figure 1. Besides the approaches discussed thus far, other methods are available in the in vivo MRS armamentarium that could be perhaps explored for reliable detection of 2D-HG, such as multiple-quantum filtering sequences (34, 35) and a variety of 2D spectroscopic methods (36–39).

Caveats of in vivo MRS
A major determinant of in vivo MRS performance is signal localization. Most in vivo MRS sequences are based on point-resolved spectroscopy (PRESS) (40), employing radiofrequency pulses with limited bandwidth, which results in large chemical shift displacement error, and are affected by B_1 field inhomogeneity, in particular for B_0 fields above 1.5 T. This approach leads to signal loss via inaccurate localization and variability of flip angle, especially in J-difference spectra (41, 42). Improved localization and signal to noise may be obtained for multiplets of coupled spins using localized adiabatic spin echo refocusing (LASER) (43, 44), which minimizes chemical shift artifact and compensates for B_1 inhomogeneity.

J-difference spectroscopy, multiple quantum filtering, and 2D methods require longer acquisition times, which makes them more susceptible to errors due to subject motion and hardware instabilities, causing drifts in the main magnetic field B_0. In order to account for these factors, acquisition can be broken in several shorter time blocks with scanner adjustment in between and retrospectively discarding averages that were corrupted by motion (45). A more precise way for achieving this is to perform prospective real-time motion correction and dynamic shim (46, 47).

Absolute quantification of metabolites is important to assess in vivo enzymatic activity by MRS, but existing methods based on water reference have shortcomings when applied to tumors due to

variations in water content (48). Relative quantification, typically employed in clinical exams, is also affected by confounding effects due to changes in the reference metabolite (49).

Ex vivo MRS of D-2HG in tumors with IDH mutations

The panoply of methods and ability of ex vivo MRS (50) to detect D-2HG in patient samples is far superior to in vivo MRS because the above list of limitations and artifacts is not of concern.

Metabolic profiling of intact tumor biopsies as small as 1 mg can be performed with high-resolution magic angle spinning (HRMAS) (51–53). HRMAS preserves the integrity of the samples that can be further analyzed with immunohistochemistry, genomics, or other metabolic profiling tools such as mass spectrometry. Detection of D-2HG in mutant IDH1 glioma was confirmed by ex vivo HRMAS experiments (29, 54, 55). In addition to D-2HG, ex vivo HRMAS studies can detect quantitative and qualitative changes for a large number of metabolites in IDH mutated tumors (54, 55).

However, as with any ex vivo assay, challenges involved with HRMAS include obtaining a representative tumor specimen, repeatability, and sample degradation. The main limitation of ex vivo measurements is, of course, the need of a biopsy, which in the case of brain tumors incurs risks and might not be repeatedly obtained with ease. In addition, the availability of high-resolution NMR spectrometers compared with MRI scanners is rare in most clinical sites, although tissue samples can be preserved frozen and shipped to a site with such equipment. An alternative to HRMAS of intact biopsies is the measurement of tumor extracts using perchloric acid. However, the extensive sample preparation required with this technique is prone to alter the metabolite content in tumor extracts.

Discussions and future implications

The example of IDH1 mutations is a perfect illustration of the rapid pace of progress brought to the medical sciences by the power and advances of modern technology: genome-wide sequencing, metabolomics, and imaging.

In vivo MRS has the unique ability to noninvasively probe IDH mutations by measuring the endogenously produced oncometabolite D-2HG. As an imaging-based technique, it has the benefit of posing minimal risk to the patients, can be performed repeatedly as many times as necessary, and can probe tumor heterogeneity without disturbing the internal milieu. To date, in vivo MRS is the only imaging method that is specific to IDH mutations — existing PET or SPECT radiotracers are not specific (56, 57), IDH-targeted agents for in vivo molecular imaging do not yet exist, and the prohibitive cost of radiotracers will likely limit their clinical development.

Caution must be exercised, however, when acquiring and analyzing in vivo MRS data. Reliable measurements of D-2HG require customized in vivo MRS sequences to avoid false-positive results. D-2HG has been measured by in vivo MRS in glioma patients, but the potential of in vivo MRS to probe IDH mutations remains to be demonstrated in other cancers. While in most tumors the levels of D-2HG seems to be high enough to grant detection by in vivo MRS, there may be a number of tumors in which levels could be around or below the 1-mM detection threshold, depending on the tumor stage or treatment manipulation. Such a gray zone needs to be further investigated to establish the limits or improve the method.

Other aspects are worth mentioning when comparing in vivo MRS with ex vivo assays. In vivo MRS may be faster and less expensive, as spectra can be read at the time of scan, spectra are available prior to biopsy, and no reagents are involved. In fact, clinicians are starting to include the MRS-derived information regarding the IDH status for neurosurgical planning (58). In particular, glioma IDH mutations are routinely diagnosed based on immunohistochemistry using antibodies targeted against IDH1 R132H (59), which miss around 15% of all IDH mutations. In vivo MRS has also been shown to detect those IDH mutations that are missed by immunohistochemistry (29, 30, 58). Moreover, gene sequencing, which is the ultimate diagnostic for IDH mutation, is not without problems. Sequencing may detect numerous SNPs, uncharacterized artifacts, and/or passenger alterations that have no effect on IDH enzyme activity (15). Also, sequencing is not available in most clinical institutions, and where available, results may be delayed by several weeks.

An alternative to D-2HG detection by in vivo MRS is D-2HG measurement in biofluids (plasma, urine, CSF) using gas/liquid-chromatography tandem mass spectrometry (GC-MS, LC-MS). This approach has been shown in acute myeloid leukemia patients (60), but to date it has failed in glioma patients (61).

Finally, as knowledge about cellular mechanisms continues to accumulate, the possibility of an antitumor treatment targeting IDH mutations may soon become reality (12). D-2HG may serve as a pharmacodynamic biomarker, providing information about whether drugs hit their target and allowing optimization of dosing. Hence, in vivo MRS may help optimize drug design and more quickly translate novel therapies to patients. This strategy is especially important in patients with mutant IDH glioma (which thus far represents the bulk of solid cancers with IDH mutations) and in situations in which nonimaging approaches are problematic (e.g., multiple biopsies in a longitudinal study are not feasible, tumors are heterogeneous, and detection of 2HG in biofluids has not been successful to date) (61).

By a fortunate combination of biological and technical factors that reinforce each other, in vivo MRS has a great potential to noninvasively genotype and monitor IDH mutations in cancer patients. Feasibility studies in glioma patients have shown that D-2HG measurements by in vivo MRS have a very high positive predictive value for IDH mutations. Further validation in other IDH cancers, investigation of reproducibility, repeatability, limits of detection, and improvements for longitudinal studies may be necessary, especially for clinical trials of new drugs targeting mutant IDH enzymes.

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