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Magnetic Resonance of 2-Hydroxyglutarate in *IDH1*-Mutated Low-Grade Gliomas

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Abstract

Recent studies have indicated that a significant survival advantage is conferred to patients with gliomas whose lesions harbor mutations in the genes *isocitrate dehydrogenase 1* and *2* (*IDH1/2*). *IDH1/2* mutations result in aberrant enzymatic production of the potential oncometabolite D-2-hydroxyglutarate (2HG). Here, we report on the ex vivo detection of 2HG in *IDH1*-mutated tissue samples from patients with recurrent low-grade gliomas using the nuclear magnetic resonance technique of proton high-resolution magic angle spinning spectroscopy. Relative 2HG levels from pathologically confirmed mutant *IDH1* tissues correlated with levels of other ex vivo metabolites and histopathology parameters associated with increases in mitotic activity, relative tumor content, and cellularity. Ex vivo spectroscopic measurements of choline-containing species and in vivo magnetic resonance measurements of diffusion parameters were also correlated with 2HG levels. These data provide extensive characterization of mutant *IDH1* lesions while confirming the potential diagnostic value of 2HG as a surrogate marker of patient survival. Such information may augment the ability of clinicians to monitor therapeutic response and provide criteria for stratifying patients to specific treatment regimens.

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Author contributions: G.B. performed the patient consenting, presurgical MR scanning, and tissue sample planning. G.B. and M.S.B. acquired the tissue samples. A.E., L.E.J., and H.A.I.Y. performed the NMR experiments and analyzed the data. J.J.P. performed the *IDH1* immunohistochemistry, genetic sequencing, and pathology analysis. R.P. performed the statistical analyses. A.E. and L.E.J. wrote the paper with assistance from H.A.I.Y., J.J.P., and S.J.N. M.S.B., R.S., S.M.C., S.C., and S.J.N. conceived and designed the experiments and analyzed the data.

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Methods

Fig. S1. J-coupling constants and spectral simulation of 2HG.

Fig. S2. Separation of 2HG resonances at ultrahigh field strength.

Fig. S3. Plots of 2HG correlations with ex vivo metabolites, histopathology, and in vivo MR imaging parameters.

Table S1. Complete patient data for 2HG presence and *IDH1* mutation status.

Table S2. Correlation statistics including an additional statistical method.

References

INTRODUCTION

Infiltrating gliomas are heterogeneous tumors of the central nervous system that include astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas. Overall survival for these diseases can vary significantly depending on the grade of malignancy and histological characteristics, as defined by the World Health Organization (WHO) (1). Although patients diagnosed with infiltrating low-grade (grade II) gliomas generally live much longer than their high-grade counterparts (grades III and IV), there is substantial heterogeneity in outcome, even for patients whose lesions share the same initial diagnosis. Because of the broad range of survival times and limited number of markers that can inform prognosis, there are several challenges for the clinical management of gliomas (1, 2). In 2009, there was a shift in the prognostic paradigm for these lesions, when more than 70% of all patients with low-grade gliomas were discovered to have mutations in the *isocitrate dehydrogenase 1* and *2* (*IDH1/2*) genes, which have been associated with prolonged survival (3–6).

IDH1 and IDH2 are NADP⁺ (oxidized form of nicotinamide adenine dinucleotide phosphate)–dependent oxidoreductases that catalyze the conversion of isocitrate to α -ketoglutarate. IDH1 is found in the cytoplasm and in peroxisomes, where it plays a role in lipid metabolism. IDH2 is localized to the mitochondria and has been implicated in protecting the cell from oxidative damage (7–9). Missense mutations of *IDH1/2* genes were found to result in arginine being substituted by other amino acids at the substrate binding sites of IDH1/2 enzymes. Recent in vitro studies have revealed that mutation of arginine 132 (R132) in IDH1 and of arginine 172 (R172) in IDH2 prevents enzymatic oxidative decarboxylation of isocitrate and confers a new ability to convert α -ketoglutarate to D-2-hydroxyglutarate (2HG) (10). The arginine mutations result in accumulation of 2HG and a vast reduction in normal IDH1/2 enzymatic activity. Although the function of both *IDH*-mutant enzymes and 2HG in gliomagenesis remains unclear, the improved prognosis associated with *IDH1/2* mutations suggests that the presence of 2HG may be of prognostic value as a surrogate for favorable genotypes. Given this potential, developing noninvasive in vivo methods for detecting 2HG in patients and evaluating other descriptive parameters of *IDH*-mutated lesions are of clinical interest.

The objective of this study was to characterize the metabolic profiles of recurrent, infiltrating low-grade gliomas using ex vivo proton high-resolution magic angle spinning (¹H HR-MAS) nuclear magnetic resonance (NMR) spectroscopy of tissue samples acquired by image-guided surgery. Crucial to this aim was determining whether levels of 2HG were sufficient for detection in *IDH*-mutated gliomas and, further, how the concentration of 2HG varied among gliomas that underwent a transition to a higher grade at the time of tumor recurrence. We hypothesized that if 2HG was detected from ¹H HR-MAS spectra, levels of the suspected oncometabolite would be significantly higher in tissue samples that had manifest *IDH1* mutations. As an exploratory analysis, we also sought to evaluate whether histopathological or preoperative in vivo MR imaging (MRI) parameters correlated with quantified levels of 2HG. Such information might be valuable for translational efforts geared toward noninvasively monitoring 2HG and predicting survival in patients with low-grade gliomas.

RESULTS

Characterization of patient population

Our institutionally approved study comprised 60 patients who had previously been diagnosed with WHO grade II glioma and were presenting for surgical resection owing to suspected disease recurrence. Presurgical in vivo MR examinations enabled the planning of

targeted biopsies for sampling tissue from patient lesions. Imaging parameters derived from postprocessed diffusion-weighted imaging and spectroscopic data helped guide the designation of small, putatively defined tumor regions (5-mm-diameter spheres) on surgical navigation software. The criteria for planning image-guided biopsies were based on low apparent diffusion coefficients (ADCs) that represent restricted diffusion associated with tumor cellularity and elevated choline-to-*N*-acetylaspartate (NAA) indices (CNIs) (Fig. 1, A and B), which provide measures of metabolic abnormality. Ex vivo HR-MAS spectra are presented to demonstrate the improved spectral resolution over conventional MR spectroscopy through the separation of individual choline-containing species that contribute to the in vivo total choline (tCHO) peak (Fig. 1C). Histological analysis of tissue samples collected from patients revealed that many had lesions that converted to a higher grade of malignancy at the time of recurrence: 25 patients had converted from WHO grade II to grade III and 6 patients converted to grade IV (Table 1). Eight patients (23 tissue samples) were excluded from the statistical analysis because their samples did not contain tumor, were too small for evaluation, or were contaminated by sterilizing agents used in the operating room. A total of 104 tissue samples from 52 patients remained for evaluation (Table 1). Complete patient data for all 60 subjects, including reasons for exclusion, are provided in table S1.

IDH1 immunohistochemistry

The *IDH1* status of paired tissue samples from each patient ($n = 52$) was assessed to compare wild-type against mutant *IDH1* spectral profiles (11). Antibody staining indicated that 38 patients had gliomas with histidine-mutated (R132H) *IDH1* enzymes. Tissue samples that stained positive for the *IDH1*_{R132H} mutation, as seen colored in brown (Fig. 2A), were shown to visually contrast with those that tested negative for the modified enzymes (Fig. 2B). An electropherogram of sequenced genomic DNA (gDNA) from an *IDH1*_{R132H}-mutant tissue sample illustrates the most common heterozygous mutation of residue R132 (Fig. 2C). Immunohistochemistry results were consistent across all tissue samples from a particular patient in 94.4% of cases (table S1A). Because the *IDH1*_{R132H} antibody is only selective for histidine mutations at residue R132 of *IDH1*, it was expected that samples with other *IDH1* and *IDH2* mutations would not be detected. Genetic sequencing of a grade II sample subset ($n = 21$) revealed that two of the patients testing negative for *IDH1*-mutant enzymes by antibody staining were, in fact, carrying the serine mutation (*IDH1*_{R132S}) and nine carried the histidine mutation (Table 1B), which corroborated the antibody staining (Fig. 2). Overall, 40 patients were classified as being positive for *IDH1* mutations (*IDH1*+) and 12 as *IDH1*-negative (*IDH1*-) (Table 2B). Furthermore, 26 of the 31 (83.9%) patients whose lesions had converted to a higher grade at the time of recurrence were classified as *IDH1*+ compared to 14 of the 21 (66.7%) whose lesions had remained grade II.

Magnetic resonance spectroscopy of a 2HG phantom

The one-dimensional (1D) NMR spectrum of a buffered stock solution of 2HG features four signature multiplets arising from five alkyl hydrogens: one α , two β , and two γ (fig. S1). Although 2HG shares the same AMNPQ spin system as glutamate (Glu), its 2-hydroxyl (2-OH) is more electron-withdrawing than Glu's 2-amino (2-NH₂) substituent, causing the α -proton to resonate further downfield at 4.05 ppm (parts per million) as a doublet of doublets. The chirality of 2HG combined with the distinct chemical environment experienced by each of the resonating protons gives rise to complex vicinal and geminal coupling, which is reflected in the elaborate splitting patterns of the spectra. The β -protons, for example, display highly coupled resonances that are seen to have chemical shifts of 2.01 and 1.85 ppm, respectively. Situated furthest from the chiral center of the second carbon, γ -protons produce overlapping resonances that appear as a single 13-peak multiplet centered at 2.28 ppm. *J*-resolved spectroscopy experiments (Supplementary Methods) allowed for an approximation of the interproton spin-spin coupling constants ($^2,^3J$), which was optimized

using spectral simulation software (fig. S1). Separation of the resonances (Supplementary Methods) was performed at ultrahigh magnetic field strength to demonstrate the γ -proton's mutual coupling with each other and β -protons in greater detail (fig. S2).

Identification of 2HG ex vivo in tissue samples

^1H HR-MAS spectra showed that most of the evaluable tissue samples contained detectable levels of 2HG (Table 2A). Of the 52 patients, 14 had spectra with inadequate resolution and signal-to-noise ratio (SNR) for identifying the signature peaks of 2HG, by either visual inspection or curve-fitting algorithms. This left tissue samples from 38 patients with spectra that could be analyzed. Of these patients, 33 (86.8%) were visually classified as 2HG+ and 5 as 2HG– by referencing discriminating resonances of 2HG from 1D and 2D spectra; additionally, 29 2HG+ patients had levels of 2HG that were quantified using curve-fitting algorithms. At a sample level, this translated to 58 2HG+ and 8 2HG– tissue samples (Table 2A). The fact that there were so few 2HG– samples precluded a statistical analysis between 2HG+ and 2HG– cohorts.

The T2-weighted Carr-Purcell-Meiboom-Gill (CPMG) (12) sequence was chosen for its ability to eliminate broad macromolecular signals, effectively creating a smooth spectral baseline that could resolve subtle metabolite features. CPMG acquisitions provided clear delineation of 2HG from neighboring metabolites γ -aminobutyric acid (GABA), glutamine (Gln), and Glu and were therefore used for all further analyses in this study. CPMG spectra from patients with contrasting mutant and wild-type *IDH1* genotypes show the characteristic presence and absence of 2HG, respectively (Fig. 3, A and B). The blue traces represent the acquired data; red traces represent the spectral curve-fitting performed with the semiparametric high-resolution quantum estimation (HR-QUEST) algorithm. HR-QUEST used an iterative process of approximation to independently fit each of the chemical shifts of 2HG for quantification in the time domain. As shown in Fig. 3A, the 2HG γ -proton multiplet centered at 2.28 ppm offered the best means of visual 2HG recognition on account of its distinct splitting pattern and relative signal intensity, which was comparable to that of neighboring metabolites. Although the β - and γ -proton multiplets are seen as relatively distinct in this particular example (Fig. 3A), they are more likely to be obscured by overlapping resonances in 1D spectra.

The 2D total correlation spectroscopy (TOCSY) (13) spectrum provided further confirmation of the presence of 2HG on the basis of three well-defined cross peaks located along a vertical column at F2/F1, [4.05/1.85 ppm], [4.05/2.01 ppm], and [4.05/2.28 ppm] (Fig. 3C). Each of these resonances corresponds to either the β - or γ -proton's shared magnetization with the α -proton and together constitute a spectral feature that is separable from the resonance of myo-inositol (myo-I) on account of relative chemical shifts. Although the other expected cross peaks of 2HG were detectable between 1.85 and 2.28 ppm, potential interference from the resonances of GABA, Gln, and Glu in this region made the γ -proton cross peaks at 4.05 ppm a more suitable form of identification for 2HG with the TOCSY experiment (Fig. 3C).

Concordance of *IDH1* mutation status and presence of 2HG

There was an 86.4% concordance (51 of 59 tissue samples; 32 of 38 patients) between the presence of 2HG, as detected by ^1H HR-MAS spectroscopy, and *IDH1* mutation status, as determined by antibody staining and genetic sequencing (Fig. 2 and Table 2A). A breakdown of this concordance according to glioma grade is presented in Table 2B. Of the eight discordant tissue samples, one had a level of 2HG that fell below the threshold for visual spectroscopic detection, perhaps as a result of sectioning the tissue sample such that most of the tumor was contained in the portion analyzed for pathology. The remainder of

discordant samples came from biopsies with insufficient tissue (<0.5 mg) for validation by genetic sequencing but may have harbored alternative *IDH1* or *IDH2* mutations (table S1). Two of the samples classified by sequencing as *IDH1*_{R132S} that had not been identified by the *IDH1*_{R132H} antibody staining were also found to contain detectable levels of 2HG. Although this was an expected outcome, because the *IDH1*_{R132H} antibody is only specific for histidine mutations, it proves that MR methodologies are capable of identifying multiple mutations of the *IDH1* gene.

Levels of 2HG among tumor grades

Relative abundance of 2HG in tissue samples suggests varied accumulation in gliomas of different grades. 2HG abundance was reported in relative levels (unitless) because of the T2 relaxation time dependence of the CPMG acquisition and metabolite-tissue interactions, which precluded absolute quantification. Mean 2HG levels were shown to increase across the 29 patients with spectra quantified for 2HG from grade II (0.36 ± 0.22 , $n = 8$) to grade III (0.56 ± 0.38 , $n = 17$) to grade IV (0.77 ± 0.20 , $n = 4$) (Fig. 4A). When analyzed with an exact Wilcoxon rank-sum test, there was a statistically significant difference found between 2HG abundance in grade II and IV samples ($P = 0.016$) (Fig. 4A), whereas the broad range of values shown by tissue from grade III lesions prevented distinction from the other grades. After these levels were normalized by average cellularity (calculated as the number of cells per 200× field), the statistical relationship between grades II and IV no longer existed ($P = 0.38$) (Fig. 4B).

Ex vivo correlations of 2HG

The levels of several metabolites that were quantified from ¹H HR-MAS spectra of biopsy samples were found to correlate with 2HG abundance (table S2 and fig. S3). These were ex vivo metabolites commonly associated with tumor, including all of the choline-containing species: free choline (Cho), phosphocholine (PC), and glycerophosphocholine (GPC), as well as the combined measure of tCHO. As an in vivo marker of cell membrane turnover, tCHO has been used extensively to discern tumor regions with increased cellularity. Its elevation is currently one of the best spectroscopic markers of tumor presence, especially when evaluated alongside attenuated levels of NAA, which indicate diminished neuronal functioning due to neoplastic infiltration. Other brain metabolites related to malignancy, such as aspartate (Asp), GABA, threonine (Thr), hypotaurine (hypo-Tau), creatine and phosphocreatine (Cr, PCr), betaine (Bet), glycine (Gly), lactate (Lac), glutathione (GSH), phosphoethanolamine (PE), Glu, and Gln, were also seen to increase with 2HG levels.

These relationships reflect 2HG's association with several aspects of tumor metabolism: Cr and PCr are commonly used as measures of bioenergetics, Lac is typically a product of anaerobic respiration under hypoxic conditions, GSH is an antioxidant that is thought to combat the extreme oxidative stress caused by reactive oxygen species, and Glu and Gln often signal changes in mitochondrial activity (14). Furthermore, the myo-I-to-tCHO index (MCI) was negatively correlated with levels of 2HG (fig. S3 and table S2). MCI serves as a novel in vivo parameter for evaluating the relative contributions of tumor and treatment-affected tissue, pathologically defined as gliosis (15). Therapeutically treated tissues, although often mimicking tumor on radiographic imaging, express higher levels of myo-I compared to tCHO and thus show a characteristic elevation in this index. The fact that 2HG levels correlate with MCI and the list of tumor-related metabolites helps confirm the association of 2HG with malignant tissue.

Tumor tissue histopathology and 2HG

There was a positive correlation observed between levels of 2HG and mitotic activity, as measured by the MIB-1 index (for evaluating the proportion of cells undergoing mitoses via

immunostaining), relative tumor content [scored by the contribution of tumor to overall cellularity (“tumor score”)], and cellular density (Table 3, fig. S3, and table S2). 2HG levels were negatively correlated with the presence of delicate vasculature found in normal brain tissues using factor VIII staining of vessel lumen. Axonal disruption, as measured by neurofilament antibody staining, was not found to be linked to changes in 2HG concentration in tumor tissue.

In vivo correlations of ADC with 2HG

To test whether MRI parameters were able to provide additional information regarding *IDH*-mutant lesions, we assessed their correlations with levels of 2HG quantified from HR-MAS spectra. By using the coordinates of each targeted biopsy acquired during surgery, it was possible to relate presurgical in vivo patient imaging to sampled tissue. ADCs derived from in vivo diffusion-weighted imaging represent the relative diffusivity of water through tissue. Restricted diffusion in the brain, corresponding to low intensities on ADC maps, is often associated with highly cellular regions of tumor (16). 2HG levels showed a negative correlation with ADC, as well as ADC normalized by normal-appearing white matter in the brain (Table 3 and fig. S3), indicating a regional association with tumor.

Also correlated with 2HG were the eigenvalues describing the magnitude of water diffusion along orthogonally related principal and secondary directions, denoted as λ_1 and λ_2 , respectively (Table 3 and fig. S3). Because ADC is mathematically derived from constituent eigenvalues, this information provides further detail for characterizing how diffusion phenomena in tumor relate to 2HG. These were important parameters to evaluate, because diffusion imaging is routinely used by clinicians to assess treatment response and tumor progression.

A similar analysis was performed on metabolite levels acquired from in vivo spectroscopic measures of Cr, tCHO, NAA, and Lac + lipid; however, owing to the limited coverage volume of our spectroscopy, many of the tissue samples did not have associated metabolite data. Partial voluming with tissue surrounding the primary lesion further limited spectroscopic correlations on the basis of lower in vivo resolution. Despite these limitations, the higher-resolution ex vivo spectroscopy suggests that in vivo tCHO, Cr, and Lac are likely to be elevated in *IDH*-mutant lesions and to correlate with levels of 2HG, along with other metabolites found significant from ex vivo analyses (Table 3).

DISCUSSION

This study has demonstrated the feasibility of detecting 2HG and quantifying its relative abundance in *IDH1*-mutated gliomas using ^1H HR-MAS spectroscopy. There was a strong concordance established between *IDH1*-mutant tissue samples acquired from patients and the presence of 2HG. These data corroborate the previously identified relationship between *IDH1* mutation and the aberrant production of this metabolite that was validated using liquid chromatography–mass spectrometry analysis (10). Because our study design constrained the acquisition of targeted biopsies to relatively small regions with homogeneous in vivo imaging characteristics, the sampled tissue was in some cases not ideal for spectroscopic analysis. Despite this limitation, it was possible to obtain an accurate assessment of the presence and absence of 2HG for most of the patients evaluated. When present, the levels of 2HG were on par with those of the chemically related metabolites Glu and Gln, which are readily detected using ^1H HR-MAS spectroscopy.

We noted several important correlations between 2HG levels in human tissue and parameters that are associated with gliomas. Relationships between levels of 2HG and other ex vivo metabolites may aid in our understanding of the altered metabolic state of *IDH*-

mutated glioma and in determining whether 2HG is a contributing factor. The standing hypothesis that 2HG is a tumor-promoting oncometabolite (3) currently lacks full mechanistic support; however, there are advantages to elucidating the biochemical pathways that are influenced by 2HG and may lead to improved survival for patients with *IDH* mutations. The correlations between histopathology parameters and levels of 2HG that were observed in our study indicated that lesions with *IDH1*-mutant enzymes have increased mitotic activity, with elevated tumor scores and cell density. The strong correlation between these parameters is consistent with there being higher cellularity in *IDH*-mutant gliomas where 2HG is present. This implies that in vivo levels of 2HG may be able to contribute not only to the classification of glioma but also to characterizing the spatial extent of infiltrative lesions.

The negative correlation between 2HG and normal delicate vascularity may assist in designing strategies for treating lesions with mutant *IDH1* enzymes. 2HG has been associated in the literature with the competitive inhibition of oxygen-sensing prolyl hydroxylases (PHDs) and histone demethylases (17, 18). Restraining PHD activity is hypothesized to result in an up-regulation of proangiogenic growth factors, including a common chemotherapy target, vascular endothelial growth factor (VEGF). It is therefore of clinical interest whether patients harboring *IDH* mutations may be ideal candidates for therapies that target 2HG production as well as antiangiogenic agents that promote vascular normalization. With regard to histone demethylases as chromatin-modifying enzymes, their inhibition could alter the regulation of gene expression such that oncogenesis is provoked (18), another potential reason to therapeutically reduce levels of 2HG.

We also found a significant relationship between *IDH1* mutants—as identified via 2HG levels in tissue—and in vivo MR diffusion parameters, which supports the potential role of diffusion imaging in evaluating whether the tissue architecture of mutant gliomas is distinct from that of other neoplasms. The elevation of tCHO that we observed from the ex vivo spectra of *IDH1*-mutant gliomas suggests that in vivo levels of tCHO may also be important for evaluating these lesions. Because MR diffusion and spectroscopy measures are readily obtained on most clinical MR scanners, they may be useful in defining regions of interest for studying variations in levels of 2HG.

The fact that the observed levels of 2HG from tissue samples expressing *IDH1* mutations were similar to those of other routinely detected metabolites, such as Gln and Glu, supports the potential clinical use of 2HG as a noninvasive biomarker for *IDH1*. Given that tissue samples taken from disparate regions of tumor were homogeneous in their mutation status, it is reasonable to assume that 2HG will be present throughout lesions expressing aberrant enzymes. The elevated levels of 2HG in gliomas that had converted to a higher grade at the time of recurrence were attributable to differences in cellularity. This is an important finding because it suggests that the amount of 2HG produced per tumor cell remains unchanged during malignant transformation. From the correlation between levels of 2HG, tumor score, and tissue cellularity, it seems likely that 2HG will contribute to determining the extent of recurrent tumor in an in vivo setting. This will be valuable in differentiating tumor from treatment effects, especially in difficult cases where ambiguous anatomic imaging complicates interpretation by a neuroradiologist.

This study has provided important information that should be considered carefully in the development of technology for translating 2HG detection into an in vivo setting. One of the main challenges is providing a robust in vivo method for resolving 2HG from neighboring metabolites that are of a similar concentration and will have overlapping resonances at clinical field strengths. There have been three preliminary studies that have reported on the detection of in vivo 2HG peaks in patients with *IDH1*-mutated gliomas (19, 20, 21). These

used different acquisition parameters and reported varying accuracies for estimated metabolite levels based on the Cramr-Rao bounds derived using the LCModel algorithm for spectral quantification. Spectral editing and more sophisticated data acquisition methods, including variants of the 2D experiment used in our study, may be useful in separating these components and verifying individual resonances (21). From our data, it appears that focusing on the unobstructed spectral feature created by the α -proton may be of interest for improving the specificity and accuracy of detecting 2HG in vivo.

Considering the survival benefits associated with *IDH1/2* mutations, the presence of 2HG may have significant prognostic value for patients with low-grade gliomas. The characterization of *IDH*-mutated lesions using MR methods could also hold implications for the study of other human cancers that share these genetic abnormalities, including colorectal cancer, prostate cancer, and acute myeloid leukemia, which shows an adverse prognosis for *IDH1/2* mutations, in contradistinction with gliomas (22, 23). Recent evidence suggests that many of these cancers carry chemosensitive properties that could enhance treatment efficacy and inform clinicians on how to stratify patients for different therapies (5). If D-2HG truly bears oncogenic properties (3), then testing for mutations in the *IDH* gene will probably not be sufficient for the clinical management of glioma patients; additional monitoring of 2HG levels would become important, especially for therapies targeting the *IDH* pathway (24, 25). Finally, as a potential marker for tumor in vivo, 2HG may prove especially helpful to clinicians attempting to discern disease recurrence from treatment effects in patients whose radiographic imaging is considered suspect. This information may help clinicians identify candidates for the most appropriate therapies or clinical trials and assist in the monitoring of treatment.

MATERIALS AND METHODS

Patient population

This study was approved by our Institutional Review Board before patient recruitment. Informed consent was obtained from each participating subject. A total of 60 patients whose initial diagnosis was WHO grade II glioma and who were presenting for surgical resection owing to suspected recurrence were enrolled. All participants had previously received standard-of-care treatments that included surgical resection, radiation therapy, and/or chemotherapy. Six patients were excluded because of methanol contamination from the operating room, one patient was excluded because of a lack of tumor cells present in their tissue samples, and one patient was excluded because of insufficient tissue size (<0.5 mg) and degraded cellular integrity. This left 52 patients to be analyzed by this study. A complete list of patients and tissue samples is provided in table S1 along with exclusion criteria.

Preoperative MRI and spectroscopy

Preoperative MR examinations were conducted on a 1.5 T or 3 T EXCITE GE Signa Echospeed scanner (GE Healthcare Technologies) using an eight-channel phased-array headcoil (MRI Devices). Functional imaging included six-directional diffusion-weighted imaging acquired in the axial plane [repetition time (TR)/echo time (TE) = 1000/108 ms, voxel size = $1.7 \times 1.7 \times 3$ mm³, $b = 1000$ s/mm²] and Lac-edited 3D MR spectroscopic imaging (¹H MRSI) using point-resolved spectroscopic selection (PRESS) for volume localization and very selective saturation (VSS) pulses for lipid signal suppression (excited volume = $80 \times 80 \times 40$ mm³, overpress factor = 1.5, TR/TE = 1104/144 ms, field of view = $16 \times 16 \times 16$ cm³, nominal voxel size = $1 \times 1 \times 1$ cm³, flyback echo-planar readout gradient in the SI direction, 712 dwell points and 988 Hz sweep width) (26).

Postprocessing of preoperative MR exam

The in vivo data from the preoperative examination were transferred to a Sun Ultra 10 workstation (Sun Microsystems), and in-house software was applied to derive estimates of in vivo diffusion and spectroscopic parameters. Maps of the ADC were generated on a pixel-by-pixel basis according to a published algorithm (27). MRSI data were processed to quantify tCHO and NAA levels, from which maps of the CNI could be derived. CNI values were generated from a linear regression-based algorithm (28) and represent the changes in choline and NAA levels relative to normal voxels.

Image-guided brain tumor tissue sampling

Regions demonstrating either abnormally decreased ADC and/or elevated CNI values, consistent with viable tumor, were evaluated for the purpose of planning which tissue to biopsy during surgery. One to four tumor tissue samples from each patient were designated as 5-mm-diameter spherical targets on co-registered MR images using BrainLAB surgical navigation software (BrainLAB Inc.). To minimize risks to the patient, no control tissue (normal brain) was acquired. Image-guided navigation was applied to locate tissue corresponding to planned targets and to acquire safely accessible samples. Upon excision, tissue samples were immediately bisected: half were snap-frozen in liquid nitrogen and stored at -80°C for ^1H HR-MAS spectroscopy; the other half was fixed in 10% zinc formalin, dehydrated by graded ethanols, and embedded in Paraplast Plus wax (McCormick Scientific) using standardized techniques for tissue processing and immunohistochemistry.

^1H HR-MAS spectroscopy

Tissue samples weighing between 0.78 and 28.14 mg (mean = 9.56 mg) were loaded into a 35- μl zirconia rotor (custom-designed by Varian) with 3 μl of 99.9% atom-D deuterium oxide containing 0.75 wt % 3-(trimethylsilyl)propionic acid (Sigma-Aldrich) for chemical shift referencing. Data were acquired at 11.7 T, 1°C , 2250 Hz spin rate in a 4-mm gHX nanoprobe with a Varian INOVA 500 MHz multinuclear spectrometer. The nanoprobe gHX is an inverse probe, optimized for the direct detection of protons and the indirect detection of X-nuclei (^{13}C , ^{31}P , ^{15}N) and was equipped with a magic angle gradient coil. A 40 mM phantom solution of 2HG at pH 7 was scanned using the same parameters to provide a basis spectrum for metabolite fitting.

A rotor-synchronized 1D CPMG pulse sequence was run with TR/TE = (4 s)/(144 ms), 512 scans, 40,000 acquired points, 90° pulse, and 20 kHz spectral width for a total time of 35 min. The electronic reference to access in vivo concentrations (ERETIC) method was used to generate an artificial electronic signal that served as an external standard for estimation of metabolite levels in 1D spectra (29). A rotor-synchronized adiabatic 2D TOCSY spectrum was acquired with a 40-ms mixing time, 25 transients, 64 increments, saturation delay of 1 s, 90° pulse, and 20 kHz spectral width for a total time of 62 min (13). TOCSY spectra display a signal intensity map of metabolite cross peaks, based on the transferred magnetization between J -coupled protons. Information derived from this sequence is useful for identifying metabolites by their unique spin systems and separating overlapping chemical signals.

Preprocessing of HR-MAS spectra was done in the time domain using the Java-based magnetic resonance user interface (jMRUI) (30). Quantification of relative 1D metabolite levels was achieved with the semiparametric algorithm HR-QUEST, which fits a customized basis set of metabolites to a given spectrum (31). The HR-QUEST basis set used in this study was composed of spectra from 26 metabolites that are commonly studied in the human brain, as well as spectra acquired from a stock solution of 2HG (Sigma-Aldrich) (14). Metabolite levels with less than 10% Cramer-Rao error estimates were included for statistical analysis. Two experienced spectroscopists also evaluated each spectrum to

qualitatively assess goodness of metabolite fits and whether low resolution or SNR compromised its analysis. Tissue samples found by these spectroscopists to have ambiguous or indeterminate interpretations were eliminated from subsequent analyses, whereas the remainder was classified visually as either 2HG-positive (+) or 2HG-negative (−). This enabled a query of the relative prevalence of aberrant 2HG production among glioma grades and histological subtypes, even in cases where 2HG levels were insufficient for HR-QUEST fitting.

Statistical analysis

All analyses were performed with the statistical software R (version 2.13). To determine differences in 2HG levels by grade, we applied an exact Wilcoxon rank-sum test from the library coin to pairwise compare the distribution of 2HG levels between grades. To identify parameters that were related to levels of 2HG, we performed a correlation analysis. In one method, a single tissue sample was selected at random from each patient and the resulting data set was fit according to a simple regression and Kendall tau model. This process was carried out a total of 100 times, with a correlation being considered statistically significant if the P value for the Kendall tau correlation coefficient was ≤ 0.05 in at least 70% of the individual trials. The second method used a simple regression model, which evaluated all of the tissue samples collectively and correcting for correlated responses from the same patient via bootstrap estimation. All statistical methods are presented in Supplementary Material, whereas the latter simple regression model with bootstrapping is presented as the primary analysis (32, 33). Owing to the exploratory nature of this study, adjustment for multiple comparisons was not made and regression coefficients significant at $P \leq 0.05$ are summarized as predictors of 2HG concentration.

In all cases, 2HG was treated as the outcome. Note that the correlation analysis excluded those patients and tissue samples with an undetermined or negative *IDH1* mutation status and spectra that could not be fit for 2HG, even if it was visually identified. This reduced the data analyzed for correlations to 25 patients and 46 tissue samples. There were two tissue samples from one patient in which the presence of 2HG had been assumed on the basis of histologic confirmation of *IDH1* mutation, but was not corroborated by HR-MAS spectroscopy. 2HG levels in these samples were set to zero for analysis, because their perceived absence was likely owing to concentrations that fell below the level of detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Designating tissue targets from presurgical in vivo imaging. **(A)** ADC map derived from diffusion-weighted imaging (DWI) shows a hypointense region of low diffusion relative to normal-appearing white matter that was designated as a target for tissue sampling in a WHO grade III astrocytoma. Tissue targets were defined as 5-mm-diameter spheres on surgical navigation software. **(B)** In vivo MRSI (extent of coverage defined by yellow box) overlaid on T1-weighted spoiled gradient echo (SPGR) image along with a choline-to-NAA index (CNI) color map. A spectrum from a region of elevated CNI targeted for tissue sampling is displayed in the green box. Cr, creatine; NAA, N-acetylaspartate; PCr, phosphocreatine; tCHO, total choline. **(C)** Sample ex vivo ¹H HR-MAS spectrum from tissue acquired at the site of elevated CNI for comparison with in vivo spectroscopy data in (B). Cho, free choline; GPC, glycerophosphocholine; PC, phosphocholine.

Fig. 2.

IDH1_{R132H} immunostaining and direct sequencing of *IDH1* gDNA. (A) IDH1_{R132H} antibody staining (brown) on a 2HG+ WHO grade III astrocytoma tissue sample, which was representative of 44 tissue samples. The corresponding 1D and 2D spectra for this sample are shown in Fig. 3, A and C, respectively. Scale bar, 100 μ m. (B) Complementary image of a 2HG- sample on which immunostaining for IDH1_{R132H} was also performed and no mutant enzymes were found (representative of seven samples). (C) Electropherogram of polymerase chain reaction (PCR)-amplified gDNA performed on an IDH1_{R132H}-mutant tissue sample. Codon 132 displays signal from the mutant base pair composed of an adenine (mutant) and guanine [wild-type (WT)] nucleobase, each present on complementary strands of DNA. The heterozygous substitution of adenine at R132 results in the production of histidine at the substrate binding site of IDH1 and neomorphic enzymatic activity.

Fig. 3.

Ex vivo spectra from mutant and WT *IDH1* tissue samples. (**A** and **B**) 1D CPMG spectrum from an *IDH1*-mutant WHO grade III astrocytoma (**A**) and WT *IDH1* grade IV astrocytoma (**B**). For both (**A**) and (**B**), the blue trace represents acquired data and the red trace represents the spectral fit used for relative quantification of 2HG levels by HR-QUEST. These spectra are not normalized with respect to each other. (**C**) 2D TOCSY spectrum of *IDH1*-mutant tissue depicted in (**A**). Shared magnetization between nuclei of discrete molecular spin systems is rendered through resonant cross peaks about the F2/F1 diagonal. The correspondence among individual cross peaks of 2HG is highlighted along with the uniquely identifying spectral feature created with respect to the α -proton, labeled as 2HG α . Ace, acetate; Ala, alanine; Gln, glutamine; Glu, glutamate; Gly, glycine; Lac, lactate; myo-I, myo-inositol; scyllo-I, scylloinositol; Tau, taurine.

Fig. 4.

2HG levels according to WHO glioma grade. **(A)** Plot of relative 2HG levels quantified by HR-QUEST in relation to malignancy grades. Mean relative levels per patient ($n = 29$ total: 7, grade II; 18, grade III; 4, grade IV) were evaluated by an exact Wilcoxon rank-sum test. Brackets indicate the statistical comparison of grade II 2HG levels with those of gliomas that had transformed to a higher grade; significance was defined as $P < 0.05$. **(B)** These levels are also shown normalized with respect to cellularity (number of cells per 200× field) for those patients whose tissue samples could be evaluated for this parameter ($n = 22$ total: 6, grade II; 13, grade III; 3, grade IV). NS, not significant.

Table 1

Glioma patient population by tumor grade and histological subtype. Eight patients and 23 tissue samples were excluded owing to a lack of tumor cells observed by histological analysis, an insufficient amount of tissue in a sample for accurate HR-MAS analysis, or contamination of the sample with sterilizing agents. Individual patient information, including reasons for exclusion, is provided in table S1. The remaining tissue samples were classified by grade and histological subtype as astrocytoma, oligodendroglioma, or oligoastrocytoma, using criteria based on WHO II standards, as is the practice at our institution for clinical diagnosis. At the time of recurrence, most patient lesions were found to have converted to a higher grade of malignancy, whereas some remained grade II, as denoted by the arrows.

	Grade	Total patients (tissue samples)	Astrocytoma patients (tissue samples)	Oligodendroglioma patients (tissue samples)	Oligoastrocytoma patients (tissue samples)
Collected	All	60 (127)	—	—	—
Excluded	All	8 (23)	—	—	—
Analyzed	All	52 (104)	21 (38)	22 (44)	9 (22)
	II II	21 (37)	5 (8)	12 (22)	4 (7)
	II III	25 (52)	12 (22)	9 (19)	4 (11)
	II IV	6 (15)	4 (8)	1 (3)	1 (4)

Table 2

Classification of 2HG presence and *IDH1* mutation status. (A) The presence of 2HG in HR-MAS spectra was assessed visually from the 1D spectrum and confirmed by the presence of signature cross peaks in the 2D TOCSY spectrum. *IDH1* mutation analysis was performed with the R132H antibody and further sequencing of gDNA from a subset of patients who had been randomly selected. The concordance between *IDH* mutations and the presence of 2HG was assessed for both tissue samples and patients. This table assumes 2HG presence as positive if at least one tissue sample from a particular patient had detectable 2HG; *IDH* status was assumed positive if at least one tissue sample was confirmed positive by sequencing or antibody staining. Samples that were designated as indeterminate for 2HG presence were excluded from analysis on the basis of insufficient tissue for analysis or inadequate resolution from acquired spectra. Samples were classified as “indeterminate” for *IDH* mutation if there was insufficient tissue for immunohistochemical analysis. (B) Comparison between the assessments of *IDH* mutation and 2HG presence in 52 patients stratified by glioma grade, as determined by clinical pathology.

A. Tissue sample and patient-level concordance between <i>IDH</i> and 2HG							
Tissue-level (n = 104)				Patient-level (n = 52)			
2HG+	2HG–	Indeterminate	Total	2HG+	2HG–	Indeterminate	Total
58	8	38	104	33	5	14	52
<i>IDH</i>+	<i>IDH</i> –	Indeterminate	Total	<i>IDH</i>+	<i>IDH</i> –	Indeterminate	Total
73	24	7	104	40	12	0	52
<i>IDH</i>	2HG–	2HG+	Agree	<i>IDH</i>	2HG–	2HG+	Agree
R132H–	7	7	7/14	R132H–	4	5	4/9
R132H+ *	1	44	44/45	R132H+ *	1	28	28/29
Overall concordance		51/59 (86.4%)		Overall concordance		32/38 (84.2%)	

B. Concordance between <i>IDH</i> and 2HG according to WHO grade						
	Grade II (n = 21 patients)		Grade III (n = 25 patients)		Grade IV (n = 6 patients)	
	<i>IDH</i> + (n = 14)	<i>IDH</i> – (n = 7)	<i>IDH</i> + (n = 22)	<i>IDH</i> – (n = 3)	<i>IDH</i> + (n = 4)	<i>IDH</i> – (n = 2)
2HG+	9	2	15	3	4	0
2HG–	0	2	1	0	0	2
2HG indeterminate	5	3	6	0	0	0
Agreement	11/13		15/19		6/6	
2HG+/ <i>IDH</i> –	2		3		0	
2HG–/ <i>IDH</i> +	0		1		0	

* The *IDH*+ category includes two R132S mutations found from sequencing.

Table 3

2HG correlations with ex vivo metabolites, histopathology parameters, and in vivo diffusion parameters. Each of the study parameters correlated with 2HG is presented. This statistical method used a simple regression model and evaluated all of the tissue samples collectively while correcting for correlated responses via bootstrap estimation. *P* values were obtained by testing the probability of correlation slopes deviating a given value from zero. n_1 and n_2 represent the normalized eigenvalues of the principal and secondary components of diffusion, respectively. Bet, betaine; GSH, glutathione; Hypo-Tau, hypotaurine; MCI, myo-I-to-tCHO index; PE, phosphoethanolamine.

Variables correlated with 2HG	Number of patients	Number of tissue samples	Regression coefficient	<i>P</i>
Ex vivo metabolites				
PC	23	40	0.27	0.003
GPC	25	43	0.15	0.003
tCHO	25	45	0.12	<0.001
Cr, PCr	25	44	0.16	0.03
PE	25	43	0.18	<0.001
GSH	18	28	0.45	0.001
Glu	25	45	0.19	<0.001
Gln	25	43	0.16	0.001
GABA	11	11	6.49	0.02
Ala	12	15	0.92	0.02
Gly	24	39	0.14	0.01
Bet	19	27	12.76	<0.001
Hypo-Tau	10	16	2.68	0.009
Lac	25	45	0.11	<0.001
MCI	25	45	-0.09	0.024
Histopathology parameters				
Average cell density	18	33	<0.001	0.002
Tumor score	22	39	0.25	0.004
MIB-1 index	21	38	0.11	0.006
Delicate vascularity	21	38	-0.21	0.004
In vivo diffusion parameters				
ADC	21	40	<0.001	<0.001
$nADC$	21	40	-0.36	<0.001
n_1	18	31	<0.001	0.007
n_1	18	31	-0.47	0.009
n_2	18	31	-0.32	0.024