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Detection of “oncometabolite” 2-hydroxyglutarate by magnetic resonance analysis as a biomarker of *IDH1/2* mutations in glioma

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Abstract

Somatic mutations in isocitrate dehydrogenase (*IDH*)1 and 2 have been identified in a subset of gliomas, rendering these tumors with elevated levels of “oncometabolite,” D-2-hydroxyglutarate (2HG). Herein, we report that 2HG can be precisely detected by magnetic resonance (MR) in human glioma specimens and used as a reliable biomarker to identify this subset of tumors. Specifically, we developed a two-dimensional correlation spectroscopy resonance method to reveal the distinctive cross-peak pattern of 2HG in the complex metabolite nuclear MR spectra of brain tumor tissues. This study demonstrates the feasibility, specificity, and selectivity of using MR detection and quantification of 2HG for the diagnosis and classification of *IDH*1/2 mutation-positive brain tumors. It further opens up the possibility of developing analogous non-invasive MR-based imaging and spectroscopy studies directly in humans in the neuro-oncology clinic.

Keywords

Cancer; 2-Hydroxyglutarate; Isocitrate dehydrogenase; Nuclear magnetic resonance; Biomarker

Introduction

Gliomas are a heterogeneous group of diffusely infiltrating brain tumors that carry overall poor and grade-dependent prognosis [1]. The traditional histopathology classification defines several subtypes of gliomas, including astrocytoma, oligodendroglioma, or mixed oligoastrocytoma (World Health Organization (WHO) grade II), which invariably progress to anaplastic forms (WHO grade III) or the highly malignant secondary glioblastoma (WHO grade IV) [1, 2]. Most of glioblastoma (GBM) cases, however, present de novo (~90% of cases), e.g., without a prior detected lower grade lesion, and represent the most frequent primary brain tumor in adults [1, 3]. Primary and secondary GBM are indistinguishable histologically or radiologically, although there is genetic evidence that they may be separate diseases [4, 5]. Given that glioma progression can be asymptomatic, small grade II and III tumors can go undiagnosed before they reach the final grade IV stage, so the “true” number of GBM, which are secondary, is unknown. Currently, diagnosis and classification of brain tumors is largely based on excised tissue morphology and sometimes “surrogate” radiological markers of tumor physiology [1]. Active research efforts to complement histology with specific genetic and molecular markers are ongoing. Furthermore, the identification of imaging-based biomarkers for specific genetic alterations has not been

achieved and would be very helpful for tumors that cannot be resected, as well as to follow-up the tumor growth post-treatment without the need for invasive biopsies.

Somatic mutations in the gene encoding cytosolic isocitrate dehydrogenase (IDH)1 and its mitochondrial homolog IDH2 have been recently identified in a subset of gliomas and acute myeloid leukemia [6–8]. The mutations alter the evolutionary conserved residues in the active sites of IDH1 (R132) and IDH2 (R172 and R140). These alterations impair the normal ability of *IDH1/2* to convert isocitrate to α -ketoglutarate (α KG) [9] and provide the enzymes with the neofunction of converting α KG to D-2-hydroxyglutarate (2HG) [10]. Consequently, tumors bearing *IDH1/2* mutations accumulate drastically increased levels of 2HG (up to ~100-fold), which, in turn, alter the function of α KG-dependent dioxygenases [11]. The biological consequences of 2HG elevation for tumorigenesis are being actively investigated.

Mutations in *IDH1* or *IDH2* genes are found in about 70% of astrocytomas of WHO grade II and III, and ~12% for GBM (with most mutations present in secondary GBM), while other gliomas such as ependymomas (grade II) or pilocytic astrocytomas (grade I) do not carry *IDH* mutations [6, 7]. Further light was shed by recent molecular genetic analyses, which have defined four subgroups of GBM (pro-neural, classic, mesenchymal, and neural), largely based on their transcriptional profiles [1, 12]. Interestingly, most patients with gliomas harboring *IDH1/2* mutations belong to the “proneural” transcriptional subclass of gliomas [12], suggesting that patients with low- and high-grade gliomas with *IDH* mutations may represent a continuum of a distinct disease. These patients show overall prolonged survival, although they do not appear to respond to the current radio- and chemotherapies, highlighting the importance of identifying this separate prognostic subgroup for the development of patient-tailored therapy to maximize the treatment response and to avoid ineffective treatments, unnecessary toxicity, and side effects.

The *IDH1/2* mutation-specific elevation of 2HG prompted us to examine its potential as a biomarker for noninvasive and, preferably, imaging-based detection of the subset of glioma patients harboring *IDH1/2* mutations. Nuclear magnetic resonance (NMR) spectroscopic analysis is a widely applied analytical method for the detection, characterization, and quantification of bioorganic molecules in vitro and in vivo. Furthermore, MR-based metabolomics has recently become a tool for discovering metabolites as biomarkers for diseases. High resolution magic angle spinning nuclear magnetic resonance (HRMAS NMR) can provide high sensitivity and spectral resolution for characterizing and profiling metabolites in biological samples [13–17], which cannot be achieved by immunohistochemistry. Another advantage of HRMAS NMR analysis is that it uses intact tissue samples. Therefore, it is non-destructive, with no or minimal contamination from the sample preparation, leaving the tissue samples available for concomitant histopathology/immunohistochemistry following the NMR analysis. It can profile and quantify metabolites in great detail, even in small amounts of intact tissue specimens (e.g., <50 mg of a tissue sample). More importantly, the metabolite profiles and concentrations measured by HRMAS NMR provide the critical *a priori* knowledge for the development of analogous magnetic resonance spectroscopy methods to detect 2HG directly in patients, where the sampling

volume for a tumor can be 1,000-fold larger than the 10–50 mg of the sample size used in HRMAS NMR experiments.

The goal of this work was to detect the 2HG “oncometabolite” in human tissues of glioma patients vs. those of non-tumoral controls using NMR and to determine whether elevated 2HG levels could serve as a precise indicator of the presence of *IDH1/2* mutations. Herein, we report that NMR can be used for the reliable detection of mutation-specific metabolite marker 2HG in glioma tissues that harbor *IDH1/2* mutations. The results show that magnetic resonance analysis of 2HG has the potential for translation to the clinic for the typing of a unique subtype of human brain tumors, which has important implications for their management.

Materials and methods

Study design

All human studies have been approved by the Internal Review Board committee. Seventy-five individual brain tissue samples were chosen for the study. These consisted of 65 intact snap-frozen glioma tissues obtained from surgical resections of a representative set of glioma patients with astrocytic, oligoastrocytic, and oligodendrocytic features, as well as 10 non-tumoral control tissues from deceased donors (Table 1). The samples were divided into two study phases. In phase 1 (characterization), the training set, which consisted of 30 samples, 20 tissues from patients diagnosed with gliomas (WHO grade II, III or IV) and 10 samples from non-tumoral control tissues (Table 1), was used for the development and characterization of the NMR methodology for the detection of 2HG. In phase 2 (validation), the test set, which consisted of 45 independent glioma samples (Table 1) was evaluated in a blinded fashion (personnel performing NMR analysis had no prior knowledge of the *IDH1/2* mutational status of the tissue samples) using the developed protocol for NMR analysis.

Sequencing of *IDH1/2* genes in patient samples

Genomic DNA was extracted from all brain tissue samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Open reading frames of exon 4 of *IDH1* (NM_005896.2) and *IDH2* (NM_002168.2) were amplified by PCR using the following primers for *IDH1* R132: sense, 5'-TGAGAAGAGGGTTGAGGAGTTCAAGT-3'; antisense, 5'-AATGTGTTGAGATGGACGCCTATTTGT-3'; for *IDH2* R172: sense, 5'-AGCCCATCATCTGCAAAAAC-3'; anti-sense, 5'-CTAGGCGAGGAGCTCCAGT-3'; and for *IDH2* R140, sense, 5'-CTGCCTCTTTGTGGCCTAAG-3'; antisense, 5'-ATTCTGGTTGAAAGATGGCG-3'. The amplicons' DNA sequence was then analyzed and assessed for the presence/absence of mutations in the hotspot codons R132 (in *IDH1*) and R140 and R172 (in *IDH2*) by MacroGen (www.MacroGenUSA.com). The presence of *IDH1* R132H mutations was confirmed by immunohistochemistry (see below) on the matching tumor tissues, while the other mutations were confirmed in independent PCR products.

Immunohistochemistry

Verification of *IDH1* R132H mutation-bearing tumor tissues was performed using immunohistochemistry (IHC) as described in [18]. Briefly, patient biopsies were embedded

into parafilm cassettes and sectioned onto slides. Deparaffinized sections were subjected to heat-induced epitope retrieval by steaming for 15 min, after which they were incubated with pan-IDH1 (1:125; Sigma, St. Louis, MO, USA) or IDH1 R132H mutation-specific (1:40; Dianova, Hamburg, Germany) antibodies at ambient temperature for 40 min. Detection of an antibody–avidin–biotin peroxidase complex was performed using 3,3'-diaminobenzidine as the chromogen, and the images were taken with a camera-equipped light microscope.

Solid state HRMAS NMR analysis of brain tissues

The sample preparation for ex vivo NMR analysis followed a previously reported procedure [16]. Each of the 75 samples was weighed (range, 12–15 mg) by subtracting the weight of the empty from the loaded sample holder/rotor and then thawed in 99.996% saline deuterium oxide (D₂O, Sigma, St. Louis, MO, USA) in the sample holder/rotor (4 mm ZrO₂). A 50- μ l insert was subsequently placed in the sample holder to stabilize the sample and to provide the balance for the rotor. D₂O (99.996%) containing 0.75% 3-(trimethylsilyl) propionic acid (TSP) was added to obtain a frequency-lock signal for NMR experiments, as well as to serve as an internal reference for chemical shift and concentration measurements. Each tissue sample with added TSP and containing D₂O was reweighed for the metabolite quantification. The preparation of NMR samples was done rapidly on ice to avoid possible sample degradation.

HRMAS NMR experiments were conducted at 4°C using a Bruker AVANCE 600 WB solid state NMR spectrometer (Bruker Instruments, Inc., Billerica, MA, USA) with a dedicated 4 mm HRMAS probe. The probe-head was precooled to 4°C before loading the sample. The sample/probe temperature was maintained throughout the experiment ($\pm 0.1^\circ\text{C}$) via a variable temperature control unit. Sample spinning rates were controlled in the range of 2,800 KHz (± 2 Hz) or at the lower spin rate of 800 Hz if the rotor-synchronized delay alternating with nutation for tailored excitation sequence was used. This sample spin rate was applied to ensure the spin sidebands did not affect the spectrum. The presaturation of water was achieved with a zqpr sequence before acquisition pulses. A rotor-synchronized Carr–Purcell–Meibom–Gill pulse sequence was used to suppress broad signals from macromolecules. The number of transients was 256. The repetition time was 5.0 s, and the spectral width was 10 kHz in all experiments.

For the 2HG resonance identification and assignment, we first examined the NMR features of a 95% pure 2HG compound (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in solution. One-dimensional (1D) NMR spectra of a pure 2HG compound, and a mixture of 2HG and a combination of glutamate (Glu) and glutamine (Gln) compounds (together termed Glx), were obtained at ~10 mM 2HG in D₂O, pH 7.0 and collected at 300 MHz at 25°C. J-coupling correlations and patterns of protons in the pure 2HG were then analyzed by a two-dimensional (2D) J-coupled correlated spectroscopy (COSY) method. This was followed by the analysis of “normal” brain and tumor tissue samples using HRMAS NMR. In the tissue sample analysis, 2D COSY data were collected at 4°C with 6,000 Hz spectral width and 1.5-s relaxation delay. Thirty-two transients in the time domain t₂ were averaged for each of the 512 increments in time domain of t₁ with a total acquisition time of ~3 h.

2HG stability assay

The stability of a commercial 95% pure 2HG compound was assessed by 1D NMR. The 1D spectra of 2HG in D₂O were collected after 12 h and then at 24 h intervals for a period of 7 days while keeping the 2HG samples at 25°C. The peak intensities for the resonances of α , γ , β , and β' protons of 2HG found at δ 03.85, 2.1, 1.82, and 1.65 ppm, respectively, were used to calculate the changes in signal/ noise ratios during the tested period.

To assess the stability of 2HG in the brain tumor tissues, selected samples containing 2HG were tested using both 1D and 2D COSY experiments and retested 24 h after. Signal intensity of the cross-peaks from 2HG was compared as a measure of 2HG stability in the tissue samples.

Data analysis

2D spectral data were analyzed using Spinworks (University of Manitoba, Canada). Data were zero-filled to a 2k×2k matrix and weighted with a shifted square sine bell function followed by Fourier transformation. 2D J-coupling patterns of pure 2HG and a mixture of 2HG and Glx compounds were obtained at ~10 mM 2HG in D₂O, pH07.0 and collected at 300 MHz at 25°C. Utilizing the chemical shifts and the J-coupling correlation pattern obtained from the pure 2HG and samples containing 2HG in solution, the unique cross-peaks arising from 2HG resonances (at δ 04.08, 2.33, 2.05, and 1.88 ppm, at 4°C, corresponding to the α , γ , β , and β' protons of 2HG, respectively) were identified in the complex 2D COSY spectra of glioma tissues. The concentrations of 2HG in the tissue samples were calculated based on the amount of the external standard TSP added into the samples, as well as from a dose-dependent curve obtained from a series of 1D and 2D COSY spectra collected from non-tumoral tissue sample titrated with nine different concentrations of pure 2HG. As HRMAS NMR typically detects metabolite concentrations >0.1 mM, all 2HG levels below this amount were considered to be below the quantification limit (BQL).

Statistical analysis

To individually evaluate the sensitivity, specificity and accuracy of NMR detection of 2HG in the training ($N=30$) and the test sets ($N=45$) as a marker for mutations in *IDH1/2* of the glioma tissues, the following formulas were used [19]:

$$\begin{aligned}\text{Sensitivity}\% &= [N_{\text{true positive}} / (N_{\text{true positive}} + N_{\text{false negative}})] \times 100 \\ \text{Specificity}\% &= [N_{\text{true negative}} / (N_{\text{true negative}} + N_{\text{false positive}})] \times 100 \\ \text{Accuracy}\% &= [(N_{\text{true positive}} + N_{\text{true negative}}) / N_{\text{total}}] \times 100\end{aligned}$$

in which, the true positive indicates tissue samples confirmed for *IDH1/2* mutations and also exhibiting 2HG in their 2D COSY spectra. The false negative corresponds to tissue samples confirmed with *IDH1/2* mutations, but missing 2HG in 2D COSY spectra [i.e., 2HG levels were estimated below quantification limit (BQL) or 0.1 mM]. The true negative is defined as samples confirmed as wild type for *IDH1/2* mutations, and also without the presence of 2HG in 2D COSY spectra. Finally, the false positive is defined as samples showing 2HG spectral features in their 2D COSY spectra, however, confirmed negative for *IDH1/2* mutation in the

genetic analysis. Sensitivity is defined as the ratio of samples found positive for 2HG among all samples from all samples that had confirmed *IDH1/2* mutations by genetic analysis. Specificity is defined as the percent of samples showing absence of 2HG in 2D COSY in all tissue samples confirmed negative for *IDH1/2* mutations. Accuracy was determined from sensitivity and specificity. N_{total} is the total number of samples, including those confirmed as true positive, false negative, true negative, and false positive for *IDH1/2* mutations.

Results

For our analysis, we selected a study set of 75 human brain samples, 65 of which were human glioma specimens representative of the different tissue histologies and grades and the remainder, 10 non-tumoral brain samples, used as controls for baseline of 2HG levels in brain tissue. The brain tumor specimens comprised 41 astrocytomas (five WHO grade II, 10 grade III, and 26 GBM grade IV; 16 primary and 10 secondary), seven oligoastrocytomas (two grade II, five grade III), and 17 oligodendrogliomas (eight grade II, nine grade III). The purpose of including this selection of gliomas was to examine whether 2HG could be used as a reliable MR biomarker for the presence of *IDH1/2* mutations across subtypes and grades. To do that, the samples were divided into two groups: the training set used primarily for methodology development and data collection and the test set for verification and validation purposes.

First, we sequenced the mutational hotspot codons R132 in *IDH1*, and R140 and R172 in *IDH2* in the 75 samples. The summary of genetic alterations identified is shown in Table 1. Somatic mutations in *IDH1* and *IDH2* were detected in 34 (52%) and three (5%) of the 65 tumors analyzed, respectively. Heterozygous G to A substitutions at nucleotide 395 of *IDH1* resulted in the replacement of R132 with predominantly H132 ($N=28$), while alterations with G132 ($N=3$), S132 ($N=2$), and C132 ($N=1$) were less prominent. We confirmed by immunohistochemistry the expression of mutant IDH1 R132H protein in all tumor samples carrying the *IDH1* G395A genetic mutation using a mutation-specific antibody (Fig. 1). No L132 mutations were observed. Three patients with an *IDH2* mutation carried a G to A substitution at nucleotide 515, resulting in the replacement of the R172 residue with K172. No mutations were detected in the codon encoding R140 in *IDH2*. In addition, consistent with the literature [7, 8], the observed mutations varied with grade and histology: *IDH1* or *IDH2* mutations were found in 60, 79, and 80% of patients with grade II and III and secondary GBM, respectively (Table 1). Finally, as expected, no *IDH1/2* mutations were detected in non-tumoral control tissues, and only a single primary GBM sample carried an *IDH1* mutation (Table 1).

Next, we performed MR metabolite analysis for detecting 2HG on the above characterized patient tissue samples using HRMAS NMR [13]. To determine the 2HG specific spin system and identify the proton resonances from 2HG, we analyzed a commercial 2HG standard using 1D and 2D NMR. The regional plot of a spectrum of ^1H 2D COSY of 2HG standard established at 25°C shows unique coupling relationships (cross-peaks) of the α , γ , β , and β' protons of 2HG (Fig. 2), including β' to α at 1.65–3.85 ppm (δ 02.2, red), β to α at 1.82–3.85 ppm (δ 02.03, blue), β' to γ at 1.65–2.1 ppm (δ 00.46, yellow), β to γ at 1.82–2.1 ppm (δ 00.19, green) and β to β' at 1.65–1.82 ppm (δ 00.17, cyan).

Given the identified distinctive cross-peak pattern of 2HG, we examined whether we could resolve the signals of endogenous 2HG from the complex NMR spectra of the 30 tissue samples comprising the training set. In 1D NMR spectra, two sets of cross-peaks, i.e. α to β (between 4.08 and 2.05 ppm; 4°C) and α to β' (between 4.08 and 1.88 ppm), from the J-coupled network of 2HG protons showed significant overlap with those of other metabolites (Fig. 3). However, they became distinguishable in the 2D COSY spectra as shown in a representative glioma sample bearing an *IDH1* R132H mutation (Fig. 4a and b, left panel). In contrast, the 2HG specific cross-peaks were completely absent or unquantifiable (see below) in non-tumoral samples ($N=10$) and mutation-negative *IDH1/2* tumors ($N=8$) (Fig. 4b, middle and right panels). We would like to stress that while the J-coupling constants are independent of the magnetic field strength used, the chemical shift values of protons are temperature-dependent, explaining the slight variations between our measurements in solution carried out at 25°C (Fig. 2) and in tissues at 4°C (Figs. 3 and 4). Using this 2D COSY method, we were able to detect unique 2HG cross-peaks in all of the 11 *IDH1* R132X (X=H, G, and S) and the single *IDH2* R172K mutation-carrying tumor tissue samples, which was highly statistically significant ($p<0.0001$, Fisher's test). In the total of 30 brain tissue samples analyzed by HRMAS NMR in the training set, the sensitivity of detecting 2HG by the 2D COSY method reached 100%; the specificity of detecting 2HG in *IDH1/2* mutation-bearing samples was 94.4%; and the overall accuracy of identification of *IDH1/2* mutations by 2D COSY NMR using 2HG as a marker was 96.7%.

To further validate our methodology, we expanded our analysis in an independent test set of 45 glioma samples in a randomized blinded fashion, i.e., without prior information about the *IDH1/2* mutational status of the samples available to the investigator performing the NMR analysis. The 2D COSY method was successfully used to quantify the unique 2HG cross-peaks in 22 of the 23 *IDH1* R132X (X=H, G, C, and S) and 2 of the 2 *IDH2* R172K mutation-carrying tumor tissue samples ($p<0.0001$, Fisher's test) of the test set with results similar to the training set. The sensitivity of detecting 2HG by the 2D COSY method reached 96.0%; the specificity of detecting 2HG in *IDH1/2* mutation-bearing samples was 95.2%; and the overall accuracy of identification of *IDH1/2* mutations by 2D COSY NMR using 2HG as a marker of IDH mutations was 97.8%.

A tentative quantification in the *IDH1/2* mutation-bearing tissues examined in this study using a calibration curve on spiked tissue (see Suppl. Figure 2 and “Material and methods”) showed 2HG levels in the 0.3–11.2 mM range in 36 of the 37 IDH mutation-positive samples tested, while the levels were below the quantification limit (BQL<0.1 mM) in 37 of the 38 mutation-negative and non-tumoral controls, further confirming the specificity of 2HG detection (Table 1, Fig. 5). A single *IDH1* mutated sample (subject ID no. 0719) showed unquantifiable levels of 2HG in NMR, and one mutation-negative glioma sample (Subject ID no. 0533) showed 2HG levels ~0.1 mM, falling right at the boundary of the quantification limit in this study (Table 1, Fig. 5). Moreover, in comparison to 2HG, the levels of endogenous neuro-transmitters, such as glutamate (Glu) and glutamine (Gln) (together termed Glx), remained uniform (~3–6 mM) in the samples of the three tested groups, suggesting that they showed little or no change in tissue with *IDH1/2* mutations (Fig. 4b).

To examine whether 2HG levels might be affected due to potential sample degradation during NMR analysis, selected tissue samples were reanalyzed by NMR using the same experimental conditions and parameters. The 2HG levels remained constant in the retest of the selected samples (compare Suppl. Figure 1a left and right panels), suggesting that 2HG is stable during the 6–8 h of HRMAS NMR analysis. Stability of 2HG was further assessed by an independent NMR stability assay of a pure 2HG standard, where the signal/noise ratio of 2HG levels in a 1D NMR spectrum was monitored daily for 7 days at ambient temperature. The cross-peak intensities of 2HG show that the signal intensity of 2HG resonances remained constant for the 7 days tested (Suppl. Figure 1b), indicating that 2HG was stable during our analysis (<8 h).

Overall, our findings suggest that the NMR-based detection method for 2HG developed in this study represents a sensitive (overall sensitivity of 96%) and accurate (97.8%) tool for the identification of gliomas with mutated *IDH1/2* genes.

Discussion

Here, we demonstrate that 2HG can be used as a tumor-associated HRMAS NMR biomarker in the brain. Many previous studies and new techniques have demonstrated the feasibility of utilizing MR metabolic imaging/spectroscopy for brain tumor diagnosis and potential monitoring of treatment responses. ^1H , as well as ^{13}C - and ^{31}P -based MRS have been previously used for studying brain tumors in patients in research and clinical applications utilizing brain tumor metabolite markers [20–23]. As an example, both ^1H and ^{31}P NMR studies have been quite successful in particular in characterizing and detecting the aberrant choline phospholipid metabolism in vitro and in vivo. While there are clear benefits, a major limitation is that the metabolites used in conventional MRS analyses are broadly involved in the metabolism of normal and many cancer tissues. We showed that 2HG could serve as an “oncometabolite” NMR biomarker for low- and high-grade gliomas harboring *IDH1/2* mutations, regardless of histological subtype. 2HG is superior to other known imaging and spectroscopic bio-markers as it is indicative of a specific genetic profile of brain tumors and because its levels are >100-fold higher in tumor versus normal brain tissue. Therefore, it can distinguish different tumor types and tumors from non-neoplastic lesions in the brain or therapy-induced alterations. A recent report attempted to utilize 2HG as a potential glioma imaging marker and suggested that it can be identified from a 1D NMR spectrum of glioma tissues [24]; however, the authors recognized that their technique was hampered by overlapping spectra, an observation we made as well (Fig. 3). Our new method overcomes this limitation by resolving 2HG peaks in two dimensions (compare Figs. 3 and 4), which allows for a more accurate estimation of 2HG levels and a 97% accuracy in identifying tumors with mutated *IDH1/2* genes.

The analysis of 2HG levels by NMR on ex vivo tumor samples is not meant to replace the use of tumor DNA sequencing or mutation-specific IHC for the determination of IDH mutation status. Rather, it complements these analyses with a direct (no tissue processing, metabolite or nucleic acid extractions) quantification of 2HG in each tumor sample, which, as shown in our study can vary between individual tumors even if they carry identical mutated enzymes. This provides a quantitative measurement of the activity of the mutant

enzyme in each tumor, which may be important for the understanding of the underlying mechanism and roles of IDH mutations and 2HG levels in glioma progression, and potentially the response to treatment.

Most importantly, our demonstration of the detectability of 2HG in ex vivo tumor samples using an MR approach provides the impetus for the further translation of this technology towards the direct non-invasive detection and quantification of this “oncometabolite” in patients with brain tumors, including those that are inoperable. This raises the possibility of using 2HG as a biomarker in the oncology clinic for routine radiological examinations using MRS methods on clinical MRI scanners in the future.¹

Clearly, in order to translate the methodology and discoveries from ex vivo NMR analysis of glioma specimens to in vivo clinically feasible MRS analysis in patients, a number of steps are still required. A major prerequisite is to establish the feasibility of detecting 2HG in gliomas over a time period tolerable to the patient (typically 20–30 min) in the current clinical MRI scanners, with FDA-approved field strengths of 1.5–3 T, which have limited spectral resolution to resolve the overlapping metabolites. The critical findings of this study, i.e., the 2HG concentrations in *IDH* mutation-bearing tumor tissues and unique J-coupling patterns determined by ex vivo HRMAS NMR analysis, strongly support the feasibility of MRS detection of 2HG in patients with clinically capable MRI scanners. The 0.3–11.2 mM range in 2HG levels detected by NMR in specimens of ~12–15 mg is within the detection limit of 3 T MRI/MRS scanners and is similar to that of Glu and Gln, which are readily detectable in human brain by MRS [25]. In addition, as we demonstrate herein, 2HG is not detectable in normal brain tissues by our NMR approach, vouching for the high specificity and essentially no background signal, which typically reduce the sensitivity of many imaging/spectroscopy-based detection methods. The 2HG specific J-coupling pattern is suitable for selectively detecting the resonance of interest using several J-resolved spectroscopic editing detection methods currently available at clinical 3 T MRI systems [26] to overcome the signal overlap. These selective J-coupling-based resonance detection methods have been applied previously in detecting neurochemicals, such as γ -aminobutyric acid and lactate, whose resonances overlapped with others in the peak-crowd spectral regions, in normal controls [27, 28] or glioma patients [29].

A recent report suggests a number of interesting additional changes in the metabolome of an *IDH1/2* mutation-carrying human oligodendroglioma cell line, including various amino acids, glutathione metabolites, choline derivatives, and tricarboxylic acid cycle intermediates [30]. The variations in the levels of the implicated metabolites observed, however, were modest in comparison to the up to 100-fold difference in the levels of 2HG. It is, therefore, very likely that 2HG may serve as a better and more sensitive marker compared to other metabolites. However, further studies are warranted to address the diagnostic roles of these individual metabolites in the complex tissue pattern by NMR.

¹During our submission the following three related studies were published: Elkhaled A et al. (2012) Magnetic resonance of 2-hydroxyglutarate in IDH1-mutated low-grade gliomas. *Sci Transl Med* 4(116):116ra4; Andronesi OC et al. (2012) Detection of 2-hydroxyglutarate in IDH-mutated glioma patients by in vivo spectral-editing and 2D correlation magnetic resonance spectroscopy. *Sci Transl Med* 4(116):116ra4; and Choi C et al. (2012) 2-hydroxyglutarate detection by magnetic resonance spectroscopy in IDH-mutated patients with gliomas. *Nat Med* doi:10.1038/nm.2682.

In summary, by identifying the NMR spectroscopic fingerprint of 2HG in *IDH1/2* mutation-bearing gliomas we demonstrate the feasibility of using NMR to detect and quantify an “oncometabolite,” 2HG, to type the genetic profile of a tumor *ex vivo*. Furthermore, our study provides the rationale for expanding these studies towards the noninvasive detection and quantification of this “oncometabolite” directly in brain tumor patients, which will accelerate tumor classification and provide a useful new imaging tool for diagnosis, prognosis, and clinical follow-up.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Detection of mutant IDH1 at amino acid R132H in brain tumor samples by immunohistochemistry. Representative images of anaplastic astrocytoma negative (**a–c**; subject ID no. 0047) or positive (**d–f**; subject ID no. 0566) for IDH1 R132H mutation are shown. Tumors were stained with **H&E** in **a** and **d**, with pan-IDH1 antibody in **b** and **e** and with anti-mutant R132H antibody in **c** and **f**. Images were taken at 200× magnification

Fig. 2.

A plot of a two-dimensional (2D) ^1H correlation spectroscopy (COSY) NMR spectrum of the pure 2HG compound (the structure of 2HG is shown) exhibits J-coupling cross-peaks unique to 2HG. The one-dimensional (1D) projection of the 2D COSY spectrum of 2HG shows resonances at δ 3.85, 2.1, 1.82, and 1.65 ppm corresponding to the α , γ , β , and β' protons of 2HG, respectively. The J-coupling relationships of 2HG protons include: β' to α at 1.65–3.85 ppm (δ =2.2, *red*), β to α at 1.82–3.85 ppm (δ =2.03, *blue*), β' to γ at 1.65–2.1 ppm (δ =0.46, *yellow*), β to γ at 1.82–2.1 ppm (δ =0.19, *green*) and β to β' at 1.65–1.82 ppm (δ =0.17, *cyan*). The spectrum was collected with ~10 mM 2HG in D_2O (pH=7.0) at 300 MHz at 25°C

Fig. 3. 1D HRMAS NMR spectrum of a glioma sample with an IDH R132H mutation (oligodendroglioma, grade II; subject ID no. 0655). The resonances from individual 2HG protons show extensive overlap with other metabolites. The α , γ , β , and β' protons of 2HG corresponding to peaks at $\delta=4.08$, 2.33, 2.05, and 1.88 ppm, respectively, are indicated in the expanded regions

Fig. 4.

Detection of 2HG in *IDH1/2* mutation-bearing glioma tissue samples using 2D HRMAS NMR. A 2D COSY spectrum of HRMAS NMR of a representative *IDH1* mutation-positive glioma sample (oligodendroglioma, grade II; subject ID no. 0655) is shown in a. The structure of 2HG and a 1D projection of the 2D COSY spectrum of 2HG is shown atop of the COSY spectrum in a with α , γ , β , and β' protons of 2HG corresponding to peaks at δ =4.08, 2.33, 2.05, and 1.88 ppm, respectively. It should be noted that the chemical shift values of these protons were determined at 4°C and 600 Hz instead of 25°C and 300 Hz for those of 2HG compound in solution (Fig. 2). The expanded section of the boxed region of a with the cross-peaks from the 2HG unique J-coupling pattern (red box) is shown in b (left panel). The 2HG cross-peaks (α - β and α - β' shown in red box) are well resolved as they are separated from the resonances of Glx (a mixture of glutamate and glutamine, see blue box) and other peaks that partially overlap with the 2HG in 1D NMR spectra of the tumor tissues. The 2HG specific cross-peaks therefore can be easily distinguished in the 2D COSY spectra. The 2HG specific cross-peaks are at or below the quantification limit (BQL<0.1 mM) in samples of *IDH1* mutation-negative gliomas (subject ID no. 0551), as shown in b (middle panel) or in non-tumoral human brain (subject ID no. HB01), as shown in b (right panel), respectively. Signal intensity color chart is shown

Fig. 5.

Detection of 2HG in brain tissue samples using NMR. The 2HG levels of 65 glioma samples (*IDH1/2* mutation-positive: $N=37$; *IDH1/2* mutation-negative: $N=28$) analyzed by NMR are shown on a $\log_{10}$ scale in relation to *IDH1/2* mutation presence. Samples marked as BQL by NMR were given a numerical value of 0.01 mM for graphing purpose. Significance level is indicated

Table 1Histology, grade, and *IDH1/2* mutation status of 65 glioma samples and 10 non-tumoral brain tissues analyzed

Subject	ID	Histology	WHO grade	Sex	Age	<i>IDH1</i> status	<i>IDH2</i> status	2HG (mM)
Training set								
1	0582	A	II	M	37	R132H	wt	4.3
2	0153	A	II	M	7	wt	wt	BQL
3	0655	OD	II	F	60	R132H	wt	6.1
4	0373	OD	II	F	4	wt	wt	BQL
5	0566	AA	III	M	29	R132H	wt	6.6
6	0590	AA	III	M	46	R132H	wt	3.5
7	0622	AA	III	M	68	R132H	wt	6.8
8	0501	AA	III	F	35	R132S	wt	6.2
9	0047	AA	III	M	56	wt	wt	BQL
10	0610	AOA	III	M	43	R132H	wt	7.2
11	0634	AOA	III	F	31	R132G	wt	4.3
12	0441	AOD	III	M	41	R132H	wt	5.5
13	0551	AOD	III	M	45	wt	wt	BQL
14	0640	GBM (secondary)	IV	M	35	R132G	wt	3.2
15	0589	GBM (secondary)	IV	M	76	R132H	wt	7.1
16	0513	GBM (secondary)	IV	M	48	wt	R172K	1.8
17	0533	GBM (secondary)	IV	M	32	wt	wt	0.1
18	0893	GBM (secondary)	IV	F	62	wt	wt	BQL
19	0126	GBM (primary)	IV	M	56	wt	wt	BQL
20	0865	GBM (primary)	IV	M	41	wt	wt	BQL
21	HB01	HB	–	F	45	wt	wt	BQL
22	HB02	HB	–	M	46	wt	wt	BQL
23	HB03	HB	–	F	48	wt	wt	BQL
24	HB04	HB	–	M	53	wt	wt	BQL
25	HB05	HB	–	M	57	wt	wt	BQL
26	HB06	HB	–	M	57	wt	wt	BQL
27	HB07	HB	–	M	59	wt	wt	BQL
28	HB08	HB	–	F	61	wt	wt	BQL
29	HB09	HB	–	M	65	wt	wt	BQL
30	HB10	HB	–	F	69	wt	wt	BQL
Test set								
31	0197	A	II	M	57	R132H	wt	1.5
32	0715	A	II	F	40	wt	wt	BQL
33	0731	A	II	M	38	R132H	wt	0.8
34	0588	OA	II	M	26	R132H	wt	0.6
35	0653	OA	II	M	42	wt	wt	BQL
36	99106	OD	II	F	37	R132H	wt	0.26
37	0115	OD	II	M	45	R132H	wt	1.1

Subject	ID	Histology	WHO grade	Sex	Age	IDH1 status	IDH2 status	2HG (mM)
38	0706	OD	II	F	66	wt	wt	BQL
39	0719	OD	II	M	49	R132H	wt	BQL
40	0847	OD	II	F	29	wt	wt	BQL
41	0916	OD	II	M	32	R132H	wt	3.2
42	0735	AA	III	M	44	R132H	wt	2.1
43	0843	AA	III	M	25	R132C	wt	0.26
44	1016	AA	III	F	25	wt	wt	BQL
45	1061	AA	III	M	59	R132H	wt	1.8
46	1090	AA	III	M	35	R132H	wt	0.7
47	0840	AOA	III	M	38	R132H	wt	4.2
48	0948	AOA	III	F	60	R132H	wt	2.2
49	1031	AOA	III	F	53	R132H	wt	1.4
50	0368	AOD	III	M	26	wt	R172K	11.2
51	0757	AOD	III	M	39	wt	R172K	2.4
52	0918	AOD	III	M	31	wt	wt	BQL
53	1036	AOD	III	M	63	R132H	wt	1.3
54	1047	AOD	III	M	51	R132H	wt	0.8
55	1055	AOD	III	M	26	wt	wt	BQL
56	1086	AOD	III	M	32	R132H	wt	1.2
57	0162	GBM (primary)	IV	F	58	wt	wt	BQL
58	0442	GBM (primary)	IV	M	78	wt	wt	BQL
59	0648	GBM (primary)	IV	F	62	wt	wt	BQL
60	0812	GBM (primary)	IV	M	54	R132H	wt	1.6
61	0822	GBM (primary)	IV	M	65	wt	wt	BQL
62	0844	GBM (primary)	IV	F	41	wt	wt	BQL
63	0849	GBM (primary)	IV	F	54	wt	wt	BQL
64	0870	GBM (primary)	IV	M	56	wt	wt	BQL
65	0890	GBM (primary)	IV	M	76	wt	wt	BQL
66	0922	GBM (primary)	IV	F	57	wt	wt	BQL
67	0943	GBM (primary)	IV	F	23	wt	wt	BQL
68	0958	GBM (primary)	IV	M	53	wt	wt	BQL
69	1012	GBM (primary)	IV	F	65	wt	wt	BQL
70	1039	GBM (primary)	IV	M	71	wt	wt	BQL
71	0528	GBM (secondary)	IV	M	44	R132H	wt	0.8
72	0668	GBM (secondary)	IV	M	35	R132H	wt	6.3
73	0701	GBM (secondary)	IV	M	31	R132G	wt	2.6
74	0899	GBM (secondary)	IV	F	28	R132S	wt	3.9
75	08100	GBM (secondary)	IV	M	36	R132H	wt	2.4

The identification of mutations in *IDH1* (R132X; X=H, C, G, or S) and *IDH2* (R172K or R140Q) in the glioma tissues is shown. 2HG levels as calculated by NMR are indicated (BQL<0.1 mM)

A diffusely infiltrating astrocytoma, *OA* oligoastrocytoma, *OD* oligodendroglioma, *AA* anaplastic astrocytoma, *AOA* anaplastic oligoastrocytoma, *AOD* anaplastic oligodendroglioma, *GBM* glioblastoma, *wt* wild type, *HB* human brain, *BQL* below the quantification limit.