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Abstract
Mutations of the isocitrate dehydrogenase 1 (IDH1) gene are among the most prevalent in low-grade glioma and secondary glioblastoma, represent an early pathogenic event, and are associated with epigenetically driven modulations of metabolism. Of particular interest is the recently uncovered relationship between the IDH1 mutation and decreased activity of the branched-chain amino acid transaminase 1 (BCAT1) enzyme. Noninvasive imaging methods that can assess BCAT1 activity could therefore improve detection of mutant IDH1 tumors and aid in developing and monitoring new targeted therapies. BCAT1 catalyzes the transamination of branched-chain amino acids while converting α-ketoglutarate (α-KG) to glutamate. Our goal was to use 13C magnetic resonance spectroscopy to probe the conversion of hyperpolarized [1-13C] α-KG to hyperpolarized [1-13C] glutamate as a readout of BCAT1 activity. We investigated two isogenic glioblastoma lines that differed only in their IDH1 status and performed experiments in live cells and in vivo in rat orthotopic tumors. Following injection of hyperpolarized [1-13C] α-KG, hyperpolarized [1-13C] glutamate production was detected both in cells and in vivo, and the level of hyperpolarized [1-13C] glutamate was significantly lower in mutant IDH1 cells and tumors compared with their IDH1-wild-type counterparts. Importantly however, in our cells the observed drop in hyperpolarized [1-13C] glutamate was likely mediated not only by a drop in BCAT1 activity, but also by reductions in aspartate transaminase and glutamate dehydrogenase activities, suggesting additional metabolic reprogramming at least in our model. Hyperpolarized [1-13C] glutamate could thus inform on multiple mutant IDH1-associated metabolic events that mediate reduced glutamate production. Cancer Res; 74(16); 4247–57. ©2014 AACR.

Introduction
Heterozygous mutations in the gene coding for the isocitrate dehydrogenase 1 (IDH1) enzyme have been reported in several cancer types, most notably in more than 70% of diffuse low-grade gliomas and 80% of secondary glioblastomas (GBM; refs. 1 and 2). The resulting mutant IDH1 enzyme acquires the neomorphic activity of catalyzing the reduction of α-ketoglutarate (α-KG) to the oncometabolite 2-hydroxyglutarate (2-HG), leading to significantly elevated 2-HG levels in tumor tissue (3). The specific mechanisms through which IDH1 mutations and elevated 2-HG drive tumor development are still being uncovered. Nonetheless, it is clear that 2-HG inhibits a range of α-KG-dependent dioxygenases, resulting in a broad range of alterations in the cellular epigenome (4, 5), which ultimately lead to oncogenesis (6–11).

Among other effects, presence of the IDH1 mutation is associated with a range of alterations in the cellular metabolism. These have been reported in mutant IDH1-expressing cell models, ex vivo tumor samples, and in vivo patient studies, and include significant changes in choline-containing metabolites, N-acetyl aspartate, and glutamate (12–15). At the enzymatic level, a recent study showed that expression of the pyruvate carboxylase (PC) enzyme was significantly increased in mutant IDH1 cells and patient samples, suggesting that PC flux could serve as a source of TCA anaplerosis in mutant IDH1 cells that channel glutamine to 2-HG production (16). A separate study recently reported that several glycolytic enzymes were underexpressed in mutant IDH1 glioma patients’ samples, likely because of hypermethylation of their promoter regions. Most notably, the expression of lactate dehydrogenase A was silenced in mutant IDH1 tumors (7). Another enzyme that was recently reported as modulated in mutant IDH1 cells is branched-chain amino acid transaminase 1 (BCAT1; ref. 17). BCAT1 is a cytosolic enzyme that catalyzes the catabolism of branched-chain 1-amino acids (BCAA) to branched-chain α-keto acids (BCKA), while concomitantly converting α-KG to glutamate. The expression of BCAT1 was significantly reduced in mutant IDH1 glioma cells compared with their wild-type counterparts, and this effect was associated with epigenetic silencing likely driven by the IDH1 mutation (17). In addition, studies show that BCAT1 could serve as a...
novel therapeutic target for glioma (18). Innovative methods for noninvasive assessment of BCAT1 activity could therefore help refine the diagnosis and monitoring of tumors harboring the IDH1 mutation, and aid in the development and monitoring of BCAT1-targeting therapies (17, 18).

1H magnetic resonance spectroscopy (MRS) is a noninvasive method that can probe the steady-state levels of several endogenous cellular metabolites (19). It has been widely used in the clinical setting as a diagnostic and prognostic tool for patients with brain tumor (19, 20). More recently, a complementary metabolic neuroimaging approach, hyperpolarized 13C MRS, has been successfully developed and implemented. Through the use of dynamic nuclear polarization (DNP), 13C-labeled compounds can be hyperpolarized, resulting in a 10,000- to 50,000-fold increase in their MR-detectable signal-to-noise ratio (SNR) when compared with thermally polarized compounds (21). Accordingly, hyperpolarized 13C MRS provides a noninvasive method to dynamically image metabolic fluxes. Over the past decade, this method has proven extremely useful in the field of oncology to monitor tumor metabolism, in the absence of ionizing radiation and with convenient integration to standard MR imaging techniques (22). In particular, [1-13C] pyruvate, the most commonly utilized hyperpolarized probe, has been widely used to detect the presence of tumor and response to treatment in several preclinical models of cancer (23, 24), including glioma (25–28). Furthermore, the first clinical trial of this technique was recently completed on patients with prostate cancer, demonstrating the translational value of the hyperpolarized imaging approach (29).

In the context of the IDH1 mutation, both 1H and hyperpolarized 13C MRS have proven useful. 1H MRS methods have been used to monitor the presence of 2-HG in patients with glioma in vivo (15, 30, 31), in preclinical rodent models of GBM (32), and in patient biopsy samples (12, 33). In addition, we recently developed [1-13C] α-KG as a new hyperpolarized probe, and were able to detect the conversion of hyperpolarized [1-13C] α-KG to hyperpolarized [1-13C] 2-HG in real time in mutant IDH1 cells and orthotopic tumors using 13C MRS (34). Considering that BCAT1 requires α-KG as a substrate to generate glutamate while transaminating BCAAs to BCKAs, we sought to expand on the use of hyperpolarized [1-13C] α-KG as an imaging probe, and investigated its conversion to hyperpolarized [1-13C] glutamate as a method for monitoring BCAT1 activity. We studied 2 isogenic cell lines that differ only in their IDH1 status, and show that following injection of hyperpolarized [1-13C] α-KG, the production of hyperpolarized [1-13C] glutamate can be detected, and is reduced in cells and tumors that express the IDH1 mutation. However, in our model, the presence of the IDH1 mutation led not only to a drop in BCAT1 activity, but also to a drop in the activities of two additional enzymes that can catalyze the conversion of α-KG to glutamate, namely aspartate transaminase (AST) and glutamate dehydrogenase (GDH). Our metabolic imaging therefore informs not only on BCAT1 status but also on a broader metabolic reprogramming associated with the IDH1 mutation. As such, hyperpolarized glutamate could serve as an indirect imaging biomarker of IDH1 status that is complementary to 2-HG.

Materials and Methods

Cell models and culture

U87 GBM cells were virally transduced with the wild-type IDH1 gene or the mutant IDH1 gene to produce U87IDHwt and U87IDHmut cells as previously described (34). Unique DNA “fingerprint” identities (i.e., variable number tandem repeat PCR products) established for these cell lines were used to regularly confirm their identities. Both strains were cultured under standard conditions in high glucose Dulbecco’s Modified Eagle Medium (DMEM-H-21, UCSF Cell Culture Facility) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Scientific Hyclone), 2 mmol/L L-glutamine (Invitrogen), 100 units of penicillin per mL, and 100 mg/mL streptomycin (UCSF Cell Culture Facility). Both cell lines were maintained as exponentially growing monolayers at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Spectrophotometric enzyme assays

All spectrophotometric measurements were performed on an Infinite m200 spectrophotometer (Tecan Systems, Inc.) and experiments performed in quadruplicate for each cell line. The BCAT1 activity assay was adapted from methods developed by Cooper and colleagues (35). Briefly, 1 × 106 U87IDHwt and U87IDHmut cells were lysed using lysis buffer (Cell Signaling Technology) in the presence of protease inhibitor (Calbiochem, Merck KGaA). A reaction mix was prepared using 10 mmol/L L-leucine, 5 mmol/L α-KG, 5 mmol/L ammonium sulfate, 50 μmol/L NADH, 0.5 mmol/L GTP, and 1.9 units of leucine dehydrogenase in 100 mmol/L phosphate buffer (in 0.15 M NaCl, pH 7.4; all reagents from Sigma Aldrich). Two hundred microliters of reaction mix and 20 μL of cell lysate were placed in each well of a 96-well plate. The decrease in absorbance at λ = 340 nm was measured every 30 seconds over 10 minutes. BCAT1 reaction rate RBCAT1 was calculated for each cell line and expressed in femtomol of NADH/cell/min.

GDH and AST activities were measured using assay kits (BioVision, Inc.). Note that, for both enzymes, the assays reflect the combined activities of cytoplasmic and mitochondrial isoforms. For both assays, 1 × 106 cells were lysed in 200 μL of kit buffer. After a 1:50 dilution, 50 μL of sample was added to each well and the absorbance at λ = 450 nm was measured every 10 seconds over 70 minutes. The reaction rate of GDH RGDH was expressed in femtomol of NADH/cell/min and the reaction rate of AST RAST in femtomol of glutamate/cell/min.

Alanine aminotransferase (ALT) activity was measured using an Endpoint Assay Kit (Bio Scientific pty. Ltd.). Briefly, 1 × 106 cells were lysed in 400 μL of kit buffer and lysates centrifuged at 14,000 rpm for 30 minutes at 4°C. Ten microliters of lysate was placed in each well and the absorbance at λ = 510 nm was monitored every 10 seconds for 30 minutes. The reaction rate of ALT RALT was calculated by linear regression and reported in femtomol of pyruvate/cell/min.

Western blotting analysis

For each cell line, denatured proteins were electrophoresed on 4% to 15% Bio-Rad Ready gels (Life Science Research) using the SDS-PAGE method and electrotransferred onto polyvinylidene...
fluoride membranes. Blots were blocked and incubated with the primary antibodies anti-BCAT1 (1:1,000 dilution; Cell Signaling Technology), anti-AST1 (cytosolic, 1:100 dilution; Abcam), anti-AST2 (mitochondrial, 1:500 dilution; Abcam), anti-GDH1 (cytosolic, 1:1,000 dilution; Novus USA), anti-GDH2 (mitochondrial, 1:500 dilution; Abcam), anti-ALT1 (cytosolic, 1:500 dilution; Abcam), and anti-ALT2 (mitochondrial, 1:100 dilution; Abcam) overnight in TBS-t with 5% BSA. Blots were then incubated with secondary antibodies goat anti-rabbit horseradish peroxidase–linked (Cell Signaling Technology) or goat anti-mouse horseradish peroxidase–linked (Santa Cruz Biotechnology Inc.) for 1 hour in TBS-t with 5% milk. The immunocomplexes were visualized using ECL Western Blotting Substrate (ThermoFisher Scientific). Bands were quantified using the ImageJ software (NIH) and normalized to β-actin.

Quantitative real-time polymerase chain reaction analysis
mRNA was isolated from 5 × 10^6 U87IDHwt and U87IDHmut cells using RNeasy Kit (Qiagen Inc.). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by the Genome Analysis Core Facility at UCSF (16). Briefly, cDNA was generated from RNA using the qScript cDNA Synthesis Kit (Quanta Biosciences) on a PTC-225 Thermocycler (MJ Research). qRT-PCR was conducted in a 384-well plate with 20 μL Taqman buffer, 5.5 mmol/L MgCl₂, 200 nmol/L of each of the corresponding Taqman probe, 0.2 μmol/L of each deoxynucleotide triphosphate, 0.025 unit/μL AmpliTaq Gold, 5 ng cDNA, and 0.5 mmol/L of each of the following TaqMan primers (all reagents from Life Technologies) for the following genes (n = 9 per gene): BCAT1 coding for the cytosolic BCAT1 isozyme; glutamic–oxaloacetic transaminase 1 and 2 (GOT1 and GOT2) coding for the cytosolic and mitochondrial AST1 and AST2 isozymes, respectively; GLUD1 and GLUD2 coding for the cytosolic and mitochondrial GDH1 and GDH2 isozymes, respectively; glutamic–pyruvate transaminase 1 and 2 (GPT and GPT2) coding for the cytosolic and mitochondrial ALT1 and ALT2 isozymes, respectively. The ABI 7900HT instrument (Life Technologies) was used with 1 cycle of 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds. Analysis was carried out using the SDS software to determine Ct. Expression levels were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript.

Perfusion system
For live cells studies, U87IDHwt and U87IDHmut cells were seeded on Biospin Microcarrier beads (NUNC). After 48 hours, ∼3.5 × 10^6 cells on beads were loaded into a 10-mm NMR tube connected to a perfusion system, which maintained an atmosphere of 5% CO₂/95% air and circulated growth medium through the cells (flow rate 1.5 mL/min; refs. 25, 28, and 36). A port on the inflow line allowed for injection of hyperpolarized material, during which time the perfusion was briefly stopped. All experiments were performed at 37°C.

Hyperpolarized ^13C MR studies of live cells
A volume of 30 μL of [1-13C] α-KG solution (5.9 M; 3:1 water: glycerol, 17.3 mmol/L OX63 radical, 0.4 mmol/L Dotarem) was polarized using a hypersense polarizer (Oxford Instruments) for approximately 1 hour (34). Hyperpolarized [1-13C] α-KG was then rapidly dissolved in isotonic buffer (40 mmol/L Tris, 30 mmol/L NaOH, 3.0 μmol/L Na₂EDTA) and injected into the perfusion medium of U87IDHwt cells (n = 5) and U87IDHmut cells (n = 5) to a final concentration of 15 mmol/L. Dynamic sets of hyperpolarized ^13C spectra were acquired on a 11.7 Tesla INOVA spectrometer (Agilent Technologies, Inc.) starting at the beginning of the injection using a pulse-acquire sequence [13° flip angle (FA), 3 second repetition time (TR), acquisition time 300 seconds; ref. 25].

MR data analysis of perfused cells experiments
All spectral assignments were based on literature reports (e.g., www.hmdb.ca). The integrals of hyperpolarized [1-13C] α-KG and hyperpolarized [1-13C] glutamate were quantified by peak integration of the dynamic ^13C spectra using ACD/Spec Manager 9. The integral of hyperpolarized [1-13C] glutamate was normalized to noise and to cell number. Normalized glutamate kinetics were further analyzed using a gamma-variate analysis (GVA; ref. 37) to derive the following parameters: R value, area under the curve (AUC, expressed in a.u.), time of maximum peak height (peak time, expressed in seconds), maximum peak height (peak height, expressed in a.u.), and full width at half maximum (FMHW, expressed in seconds).

Tumor-bearing animals
All animal research was approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Athymic rats (n = 2 U87IDHmut, n = 2 U87IDHwt, average weight 200 g, male, rnu/rnu homozygous, 5- to 6-week-old; Harlan Laboratories) were used in this study. An hour before the intracranial injection, U87IDHwt or U87IDHmut cells were washed once with PBS solution, collected by trypsinization, counted, and resuspended in serum-free McCoy's medium to a concentration of 3 × 10⁶ cells per 10μL. Rats were then anesthetized by an intraperitoneal injection of a mixture of ketamine/xylazine (100/20 mg/kg, respectively), and 10 μL of cell suspension was slowly injected into the right putamen of the animal brain using the freehand technique (34). Buprenorphine (0.03 mg/kg, V = 600 μL) and bupivacaine (5 mg/kg, V = 300 μL) were injected subcutaneously before injection for optimal pain management.

In vivo hyperpolarized ^13C MR studies
In vivo experiments were performed on a 3 Tesla clinical MR system (GE Healthcare Little Chalfont) equipped with a dual-tuned 1H–13C transmit/receive volume coil (fifth = 40 mm). Rats were anesthetized using isoflurane (1%–2% in O₂, 1.5 L/min) and a 23 gauge catheter was secured in the tail vein. For all in vivo studies, B₀ shimming was performed using the automatic GE shimming routine. Anatomical imaging was performed using a 2D fast spin echo sequence [axial/coronal, echo time (TE) = 20 ms, TR = 1200 ms, field of view = 40 × 40 mm², matrix 256 × 256, 20 slices, thickness = 1 mm, acquisition time 5 minutes 7 seconds, number of transients (NT) = 1]. After 1 hour, hyperpolarized [1-13C] α-KG was rapidly dissolved in...
isoionic buffer (40 mmol/L Tris, 200 mmol/L NaOH, 0.1 mg/L Na2EDTA) to obtain a 100 mmol/L solution. Within 15 seconds after dissolution, a volume of 2 mL of hyperpolarized solution was injected over 12 seconds.

$^{13}$C 2D dynamic CSI was acquired starting 5 seconds after the beginning of hyperpolarized $[1^{-13}$C$]$ $\alpha$-KG injection. The sequence was optimized for glutamate detection using a specialized variable flip-angle (VFA) multiband spectral-spatial RF excitation pulse designed to provide an initial 4° FA for $[1^{-13}$C$]$ $\alpha$-KG (to preserve substrate magnetization) and 25° for $[1^{-13}$C$]$ glutamate. These FAs were progressively increased over time to efficiently use the hyperpolarized magnetization in the presence of metabolic conversion, with both VFA schemes ramping up to 90° FA (38). An echo-planar spectroscopic imaging read-out gradient was used for increased imaging speed. Additional parameters were: adiabatic double spin-echo acquisition, bandwidth 543 Hz, resolution 10.4 Hz, 52 points, TE/TR = 140/215 ms, matrix $8 \times 18.5 \times 5$ mm$^2$ resolution, slice thickness 2 cm, 1.7 seconds per image, images every 5 seconds; refs. 34, 39, and 40).

**MR data analysis of in vivo experiments**

MR datasets were analyzed using the in-house SIVIC software (http://sourceforge.net/apps/trac/sivic/). T2-weighted anatomical images were superimposed to the 2D CSI grid to derive the location of the tumor and normal brain voxels (34). The integral values of the hyperpolarized $[1^{-13}$C$]$ $\alpha$-KG and hyperpolarized $[1^{-13}$C$]$ glutamate peaks were calculated for each voxel type and for each tumor type. The ratios of hyperpolarized $[1^{-13}$C$]$ glutamate to hyperpolarized $[1^{-13}$C$]$ $\alpha$-KG were calculated for normal brain voxels and tumor voxels as the ratio of the integral values. Color heatmaps of hyperpolarized $[1^{-13}$C$]$ $\alpha$-KG and hyperpolarized $[1^{-13}$C$]$ glutamate were generated for the 25 seconds time point using a sinc-based interpolation of the $^{13}$C 2D CSI data to the resolution of the anatomical images using SIVIC.

**Statistical analysis**

All results are expressed as mean ± standard deviation. Two-tailed Student t test was used to determine the statistical significance of the results, with a $P$ value below 0.05 considered significant.

**Results**

**BCAT1 activity and expression are significantly decreased in U87 mutant IDH1 cells**

The goal of this study was to develop a method to noninvasively assess BCAT1 activity as an indirect readout of IDH1 mutational status. First, we therefore had to verify that, in our model, the presence of mutant IDH1 was associated with a drop in BCAT1 activity and expression, as described in other systems (17). As presented in Fig. 1A and Table 1, our results show that BCAT1 activity was significantly decreased by 32% ± 7% in U87IDHmut cells as compared with U87IDHwt: $R_{BCAT1} = 0.17 \pm 0.01$ fmol of NADH/cell/min for U87IDHwt cells versus $R_{BCAT1} = 0.12 \pm 0.004$ fmol of NADH/cell/min for U87IDHmut cells ($P = 0.002, n = 4$ per cell line). BCAT1 Western blot analyses are shown in Fig. 1B. Consistent with the activity assay, BCAT1 protein levels were significantly lower in U87IDHmut cells, down to 38% ± 12% of U87IDHwt ($P = 0.01; n = 3$ per cell line; Table 1), qRT-PCR analysis confirmed this finding: mRNA levels were also significantly lower in U87IDHmut cells, down to 48% ± 19% of U87IDHwt ($P = 0.01; n = 9$ per cell line; Table 1) and within experimental error of the observed drop in protein levels.

**Formation of hyperpolarized $[1^{-13}$C$]$ glutamate from hyperpolarized $[1^{-13}$C$]$ $\alpha$-KG can be observed in live U87IDHwt perfused cells, but not in U87IDHmut cells**

Before performing preclinical animal studies, we assessed the feasibility of monitoring the conversion of hyperpolarized $[1^{-13}$C$]$ $\alpha$-KG to hyperpolarized $[1^{-13}$C$]$ glutamate in live perfused cells using $^{13}$C MRS.

As shown in Fig. 2A, injection of hyperpolarized $[1^{-13}$C$]$ $\alpha$-KG [chemical shift (δ) δ$_{\alpha\text{-KG}} = 172.6$ ppm] into the perfusion medium of U87IDHwt cells resulted in the detectable build-up of a new hyperpolarized metabolite at δ = 177.5 ppm—the chemical shift of $[1^{-13}$C$]$ glutamate. Furthermore, the maximum peak height of this new compound was observed at 12.2 ± 0.1 seconds after the maximum peak height of hyperpolarized $[1^{-13}$C$]$ $\alpha$-KG, in line with an expected delay when metabolism occurs (peak time; Table 2).

Based on chemical shift value and the observed delay in metabolite build-up, we...
attributed this newly detected resonance to hyperpolarized [1-13C] glutamate produced from hyperpolarized [1-13C] α-KG.

In contrast to U87IDHwt cells, hyperpolarized [1-13C] glutamate was detected within 0.2 ± 0.1 seconds after the maximum of hyperpolarized [1-13C] α-KG, and was generally barely above the noise level in U87IDHmut cells, as shown in Fig. 2B. The time courses of hyperpolarized [1-13C] glutamate formation in U87IDHwt and U87IDHmut cells (normalized to noise and cell number) are plotted in Fig. 2C, and show the substantial and delayed build-up of glutamate in U87IDHwt cells only. These kinetics were further analyzed using a GVA, as summarized in Table 2. Importantly, all estimated parameters were significantly different between U87IDHwt and U87IDHmut cells. In particular, the AUC and the peak height were significantly decreased by 84.1% ± 5.1% and 80.0% ± 5.0% in U87IDHmut cells as compared with U87IDHwt, respectively (P < 0.01, n = 5 per cell line), demonstrating that significantly less glutamate is produced from α-KG in mutant IDH1 cells.

Hyperpolarized [1-13C] glutamate can be detected in vivo in U87IDHwt tumors, but not in U87IDHmut tumors

Next, we translated the method developed in the perfused cell experiments, and performed an in vivo proof-of-principle study to confirm the feasibility and potential of using

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>BCAT1</th>
<th>AST1</th>
<th>AST2</th>
<th>GDH1</th>
<th>GDH2</th>
<th>ALT1</th>
<th>ALT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>BCAT1</td>
<td>GOT1</td>
<td>GOT2</td>
<td>GLUD1</td>
<td>GLUD2</td>
<td>GPT</td>
<td>GPT2</td>
</tr>
<tr>
<td>Activity levels (% of U87IDHwt)</td>
<td>68 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>69 ± 15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>b.d.</td>
<td></td>
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<tr>
<td>Protein levels (% of U87IDHwt)</td>
<td>38 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>112 ± 30</td>
<td>b.d.</td>
<td>131 ± 33</td>
<td>103 ± 18</td>
<td>131 ± 32</td>
</tr>
<tr>
<td>mRNA levels (% of U87IDHwt)</td>
<td>48 ± 19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91 ± 18</td>
<td>b.d.</td>
<td>b.d.</td>
<td>132 ± 30</td>
</tr>
</tbody>
</table>

NOTE: Enzymatic activities, protein levels, and mRNA levels were measured by spectrophotometric assays, Western blot and qRT-PCR analysis, respectively (first row, enzyme; second row, corresponding gene). All values are for U87IDHmut cells and expressed in percent of U87IDHwt levels. Significant results are highlighted in bold.

Abbreviation: b.d., below detection level.

<sup>a</sup>P < 0.01.

<sup>b</sup>P < 0.05; n = 3 per protein per cell line; n = 9 per gene per cell line.

Figure 2. Hyperpolarized [1-13C] glutamate formation from hyperpolarized [1-13C] α-KG can be detected in live cells and is decreased in U87IDHmut cells as compared with U87IDHwt. Stack plots of dynamic 13C MRS spectra acquired at 11.7 Tesla following injection of hyperpolarized [1-13C] α-KG in live U87IDHwt (A) and U87IDHmut (B) perfused cells (temporal resolution 9 seconds), showing the formation of hyperpolarized [1-13C] glutamate in U87IDHwt cells. Note the absence of detectable hyperpolarized [1-13C] glutamate in U87IDHmut cells, C, intensities of hyperpolarized [1-13C] glutamate versus the time of maximum hyperpolarized [1-13C] α-KG (vertical dashed line), as expected when metabolism occurs. The fit derived from the GVA is displayed as a continuous line for U87IDHwt and as a dashed line for U87IDHmut perfused cells.
hyperpolarized \([1-\text{^{13}C}]\) \(\alpha\)-KG to monitor hyperpolarized \([1-\text{^{13}C}]\) glutamate production in orthotopic brain tumors at clinical field strength (3 Tesla).

Figure 3A and B illustrates the MR images and spectra obtained from U87IDHwt and U87IDHmut tumor-bearing rats with comparable tumor sizes. The tumors appear as hyper-

Table 2. GVA of the hyperpolarized \([1-\text{^{13}C}]\) glutamate kinetics observed in live cells

<table>
<thead>
<tr>
<th></th>
<th>R-value</th>
<th>Area</th>
<th>Peak time (seconds)</th>
<th>Peak height</th>
<th>FWHM (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87IDHwt ((n = 5))</td>
<td>0.98 ± 0.11</td>
<td>2,444 ± 211</td>
<td>12.2 ± 0.1</td>
<td>119 ± 4</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>U87IDHmut ((n = 5))</td>
<td>0.82 ± 0.21</td>
<td>494 ± 8*</td>
<td>0.2 ± 1.1*</td>
<td>24 ± 0.2*</td>
<td>51 ± 3*</td>
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NOTE: All values are expressed as mean ± SD. Significant results are highlighted in bold.
Abbreviation: FWHM, full width at half-maximum.

*\(P < 0.01\).

\(P < 0.05\); \(n = 5\) per cell line.

hyperpolarized \([1-\text{^{13}C}]\) \(\alpha\)-KG to monitor hyperpolarized \([1-\text{^{13}C}]\) glutamate production in orthotopic brain tumors at clinical field strength (3 Tesla).

Figure 3A and B illustrates the MR images and spectra obtained from U87IDHwt and U87IDHmut tumor-bearing rats with comparable tumor sizes. The tumors appear as hyper-
The activities and expression of other enzymes catalyzing the α-KG-to-glutamate conversion are significantly reduced in mutant IDH1 cells

To determine the specificity of our imaging approach, further biochemical analyses were performed to measure the activity and expression of 3 additional enzymes known to catalyze the α-KG-to-glutamate conversion, namely AST, GDH, and ALT (Fig. 4A).

Results show that total cellular AST and GDH activities were significantly decreased by 17% ± 10% and 31% ± 15%, respectively, in U87IDHmut cells as compared with U87IDHwt (Fig. 4B and C; Table 1): $R_{\text{AST}} = 4.41 \pm 0.45$ fmol of glutamate/cell/min for U87IDHwt cells versus $R_{\text{AST}} = 3.62 \pm 0.15$ fmol of glutamate/cell/min for U87IDHmut cells ($P = 0.03, n = 4$ per cell line and per enzyme).

β-Actin was used as a loading control. (Note: GDH1 was below detection level.)
cell line); \( R_{\text{OHE}} = 1.79 \pm 0.38 \) fmol of NADH/cell/min for U87IDHwt cells versus \( R_{\text{OHE}} = 2.59 \pm 0.53 \) fmol of NADH/cell/min for U87IDHmut cells (\( P = 0.04; n = 4 \) per cell line). ALT activity was below detection level in both cell lines.

When considering protein levels, the Western blot analyses for the 3 enzymes of interest (cytoplasmic and mitochondrial isoforms) are presented in Fig. 4D. The quantified protein and mRNA levels are summarized in Table 1. In mutant IDH1 cells, the protein and mRNA levels of the cytoplasmic isoform AST1 were reduced significantly to 55% ± 11% (\( P = 0.04; n = 3 \) per cell line) and 73% ± 9% (\( P = 0.009; n = 9 \) per cell line) of U87IDHwt, respectively. In contrast, for the mitochondrial isoform AST2, only the mRNA levels were significantly reduced to 79% ± 5% of U87IDHwt (\( P = 0.008; n = 9 \) per cell line) in U87IDHmut, whereas any changes in protein levels were below detection. In the case of GDH and ALT, the protein and mRNA levels were either below detection, or did not change significantly (Table 1).

Discussion

The IDH1 mutation, which is the most common somatic mutation in low-grade gliomas (3), is considered an early oncogenic event (41) and is associated with global modulations in the methylene, transcrptonome, and metabolome (6–11, 14). Consequently, the IDH1 mutation is being used for patient stratification and prognosis, and mutant IDH1 inhibitors are being developed as therapies (42, 43). Noninvasive methods are therefore needed to monitor IDH1 status, and to help develop and monitor new targeted treatments. In this context, and in light of the recently uncovered link between BCAT1 expression and the IDH1 mutation (17), we investigated the potential of hyperpolarized [1-13C] α-KG as an imaging probe to monitor the BCAT1-driven α-KG-to-glutamate conversion and its modulation in the presence of the IDH1 mutation in cells and in vivo.

Decreased BCAT1 activity, protein levels, and mRNA expression have previously been reported in 4 cell models harboring the IDH1 mutation (17). Importantly, our results are in line with this previous report. We show that BCAT1 activity, protein levels, and mRNA expression were significantly decreased in our U87IDHmut cells as compared with their wild-type counterpart, in addition to our previously reported elevation in 2-HG levels in the same model (34).

In an initial cell study, we investigated the potential of hyperpolarized [1-13C] α-KG to provide a noninvasive readout of the confirmed drop in BCAT1 activity in U87IDHmut cells. Injection of hyperpolarized [1-13C] α-KG into the perfusion medium of U87IDHwt cells resulted in a significant build-up of hyperpolarized [1-13C] glutamate at \( \delta_{\text{GLU}} = 177.5 \) ppm, with a maximum of glutamate production at 12.2 ± 0.2 seconds after the maximum of hyperpolarized [1-13C] α-KG. This result is, to our knowledge, the first time the detection of hyperpolarized [1-13C] glutamate from hyperpolarized [1-13C] α-KG is reported in live cells. This result also confirms that enough hyperpolarized [1-13C] α-KG permeates the cells to enable the detection of its conversion to hyperpolarized [1-13C] glutamate within a time frame compatible with a hyperpolarized 13C MRS experiment, as previously observed in our 2-HG study (34). Finally and most importantly, whereas hyperpolarized [1-13C] glutamate was detectable in U87IDHwt cells for up to 80 seconds after injection of hyperpolarized [1-13C] α-KG into their perfusion medium, hyperpolarized [1-13C] glutamate formation was almost undetectable in U87IDHmut cells, reflecting mutant IDH1-driven metabolic alterations in our model.

When injected intravenously in vivo in orthotopic tumor-bearing rats, hyperpolarized [1-13C] α-KG conversion to hyperpolarized [1-13C] glutamate could also be observed in situ. Hyperpolarized [1-13C] glutamate could be detected in the tumor of U87IDHwt animals 25 seconds after injection of the hyperpolarized substrate, or 10 seconds after the maximum of hyperpolarized [1-13C] α-KG, in line with the live cells results. In contrast, hyperpolarized [1-13C] glutamate was barely detected in U87IDHmut tumors, highlighting the translational potential of our approach.

Hyperpolarized [1-13C] glutamate was also detected in normal brain voxels in animals bearing both tumor types. We cannot rule out that a small amount of hyperpolarized [1-13C] glutamate is produced elsewhere in the body and flows to the normal brain through the bloodstream. However, because we did not observe significant levels of glutamate in U87IDHmut tumors, glutamate produced elsewhere is likely below detection in our animals. In contrast, the presence of hyperpolarized [1-13C] glutamate in the normal brain is consistent with our previous work showing that, although hyperpolarized [1-13C] α-KG levels detected in normal brain are lower than in tumor, significant levels are observed in the normal brain (34). Thus, it is likely that hyperpolarized [1-13C] glutamate observed in the normal brains of our rats was produced in situ and reflects normal brain metabolism.

We cannot rule out that the level of hyperpolarized [1-13C] glutamate reflects not only its production, but also its conversion into multiple downstream metabolites such as glutamine, γ-Glu-Cys, etc. However, within the relatively short lifetime of the hyperpolarized species, any downstream metabolites were below detection level in our study. Thus, the level of hyperpolarized [1-13C] glutamate observed in our cells likely reflects primarily glutamate production. Hyperpolarized [1-13C] glutamate can be produced from hyperpolarized [1-13C] α-KG through the activity of BCAT1, but also via 3 other enzymes, namely AST, GDH, and ALT. In addition to the expected drop in BCAT1 activity, we show that, in our mutant IDH1 cells, GDH and AST activities were significantly reduced, suggesting additional metabolic reprogramming. In the case of AST, as in the case of BCAT1, this effect is likely mediated by a drop in expression of the cytoplasmic isoform of AST, AST1. In the case of GDH, its unchanged mRNA expression indicates that our observed drop in cellular activity in mutant IDH1 cells is likely mediated by a posttranslational modification of the enzyme, such as modification of its acetylation (44, 45). Further investigations are therefore needed to fully explore the underlying mechanisms of our findings. Nonetheless, and independent of the mechanisms, our results are in line with the observed modulations of the enzymes catalyzing the α-KG-to-glutamate conversion, as well as previous studies reporting...
that most of the glutamine-derived α-KG pool is channeled toward 2-HG production rather than glutamate production in mutant IDH1 cells (3, 34, 46). Finally, our results are also in line with reports of decreased steady-state levels of glutamate in mutant IDH1 tumors when compared with wild type, including results in our U87-based model (12–14).

From a metabolic imaging perspective, this study is the first report of the detection of hyperpolarized [1-13C] glutamate produced from hyperpolarized [1-13C] α-KG both in cells and in vivo. However, whereas the α-KG-to-glutamate conversion had not been previously reported, other metabolic imaging studies have investigated the reverse metabolic reaction, namely glutamate-to-α-KG conversion. In a recent report, hyperpolarized [1-13C] glutamate was used as the injected hyperpolarized substrate, and detection of hyperpolarized [1-13C] α-KG was reported in hepatoma cells and tumors (47). Interestingly, hyperpolarized [1-13C] α-KG could be detected only when hyperpolarized [1-13C] glutamate was coinjected with pyruvate, the cofactor needed for transamination by the ALT enzyme (Fig. 1). Another study indirectly investigated the glutamate-to-α-KG reaction by monitoring the transamination of hyperpolarized α-keto-[1-13C] isocaproyl (KIC) to leucine by BCAT1, a reaction that concomitantly converts glutamate to α-KG (48). Interestingly, when comparing these 2 studies, hyperpolarized [1-13C] glutamate conversion to hyperpolarized [1-13C] α-KG seems to be driven mainly by the ALT enzyme, whereas hyperpolarized [1-13C] KIC only reflects BCAT1 activity. In our study, we show that hyperpolarized [1-13C] α-KG to hyperpolarized [1-13C] glutamate conversion reflects the activity of several enzymes, namely BCAT1, AST, and GDH. Accordingly, the technique presented in this study is reaction specific (α-KG-to-glutamate conversion) rather than enzyme specific. Future studies combining our approach with other specific metabolic probes, such as hyperpolarized [1-13C] KIC or hyperpolarized [1-13C] glutamate, could prove useful to evaluate the contributions of the different enzymes to the detected hyperpolarized [1-13C] glutamate production.

On a technical level, this study benefits from innovative dedicated pulse sequences that allow preservation of substrate magnetization while enhancing the signal of the detected substrate. Dedicated pulse sequences that allow preservation of substrate magnetization while enhancing the signal of the detected substrate. In contrast, hyperpolarized [1-13C] glutamate informs on mutant IDH1-associated metabolic modulations and, as such, provides an indirect confirmation of mutant IDH1 status. Further development of advanced pulse sequences is expected to lead to simultaneous detection of both hyperpolarized [1-13C] 2-HG and hyperpolarized [1-13C] glutamate following hyperpolarized [1-13C] α-KG injection, providing complementary direct and indirect metabolic information with regard to IDH1 mutational status. The ability to detect both metabolites simultaneously, in a single sequence, would likely enhance the accuracy of mutant IDH1 imaging.

In summary, this study shows that 13C MRS of hyperpolarized [1-13C] α-KG can be used to monitor hyperpolarized [1-13C] glutamate production. Most importantly, the observed level of hyperpolarized [1-13C] glutamate was significantly lower in mutant IDH1 cells and tumors, in line with decreased BCAT1, AST, and GDH activities. Hyperpolarized [1-13C] glutamate could therefore serve as a secondary metabolic biomarker of IDH1 mutational status in gliomas, together with 2-HG.

Disclosure of Potential Conflicts of Interest
P.E.Z. Larson reports receiving a commercial research grant from GE Healthcare. No potential conflicts of interest were disclosed by the other authors.

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