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Journal

Neuro-oncology, 14(12)

ISSN

1522-8517

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Publication Date

2012-12-01

DOI

10.1093/neuonc/nos258

Peer reviewed

Detection of 2-hydroxyglutaric acid in vivo by proton magnetic resonance spectroscopy in U87 glioma cells overexpressing isocitrate dehydrogenase–1 mutation

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The arginine 132 (R132) mutation of isocitrate dehydrogenase –1 (IDH1^{R132}) results in production of 2-hydroxyglutarate (2-HG) and is associated with a better prognosis compared with wild-type (WT) in glioma patients. The majority of lower-grade gliomas express IDH1^{R132}, whereas this mutation is rare in grade IV gliomas. The aim of this study was to noninvasively investigate metabolic and physiologic changes associated with the IDH1 mutation in a mouse glioma model. Using a 7T magnet, we compared MRI and proton magnetic resonance spectroscopy (MRS) in U87 glioma cells overexpressing either the mutated IDH1^{R132} or IDH1 wild-type (IDH1^{WT}) gene in a mouse flank xenograft model. Flank tumors overexpressing IDH1^{R132} showed a resonance at 2.25 ppm corresponding to the 2-HG peak described for human IDH1^{R132} gliomas. WT tumors lacked this peak in all cases. IDH1 mutant tumors demonstrated significantly reduced glutamate by in vivo MRS. There were no significant differences in T₂, apparent diffusion coefficient (ADC), or perfusion values between the mutant and IDH1^{WT} tumors. The IDH1^{R132} mutation results in 2-HG resonance at 2.25 ppm and a reduction of glutamate levels as determined by MRS. Our results establish a model system

Keywords: animal models, glioblastoma, glioma, isocitrate dehydrogenase 1 (IDH1), MRI, MRS.

A comprehensive genomic analysis of human glioblastoma multiforme (GBM) in 2008 revealed a mutation in the active site of isocitrate dehydrogenase–1 (IDH1) (without loss of heterozygosity) in 12% of GBM patients.¹ Over the past 3 years, additional studies have reported the presence of the IDH1 mutation in acute myeloid leukemia,^{2–4} acute lymphoblastic leukemia,⁵ prostate cancer,⁵ colorectal cancer,⁶ and melanoma.⁷ Subsequent investigation confirmed the presence of a point mutation affecting arginine at residue 132 (R132) of the IDH1 protein (IDH1^{R132}) in . 70% of lower-grade gliomas.⁸ The R132 residue is conserved among species, likely representing the substrate binding site.⁹ IDH1^{R132} appears to be an early event in gliomagenesis before the emergence of the p53 mutation,¹⁰ leading to the speculation that it contributes to malignant transformation. Median overall survival among patients with anaplastic astrocytoma harboring mutations in either the IDH1 or IDH2 gene is significantly longer than that among patients without these mutations (65 vs 20 months).⁸ Similarly, glioblastoma

after oxidation to α -ketoglutarate. Reduced levels of α -ketoglutarate were reported as a consequence of IDH1^{R132},¹² suggesting that this mutation may also influence metabolism. Whether oncogenic potential can derive from 2-HG, as previously speculated,^{11,13,14} or from metabolic changes related to IDH1^{R132} is still under investigation.

Based on these data, 2-HG has been proposed as a potential biomarker for patients with glioma. Investigating this possibility, multiple researchers,^{15–17} including our own group,¹⁸ have used proton magnetic resonance spectroscopy (MRS) to noninvasively measure 2-HG levels in human glioma patients. However, gliomas are heterogeneous tumors both genetically and metabolically,¹⁹ so it is difficult to control for tumor differences not related to the IDH1 mutation in human patients (eg, tumor grade or other oncogenic mutations). Conversely, the U87 mouse glioma model presents an opportunity to compare IDH1 mutant and WT tumors in a controlled environment.

Thus, in this study, we used human U87 glioma cells transfected with IDH1^{R132} in order to study the consequences of IDH1^{R132} overexpression on tumor imaging features, physiology, and metabolism. This is the first step in establishing a well-characterized experimental model system in order to test the ability of noninvasive 2-HG measures to act as a biomarker of glioma growth and treatment response.

Methods

Cell Lines and the Generation of Constructs

All cells were cultured at 37°C and 5% CO₂ in a 90% humidified tissue incubator. Culture medium was composed of Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 units/mL of penicillin/streptomycin. Human WT IDH1 coding sequences were amplified from U87 malignant glioma (MG) cells. The cDNA was fused in-frame with a FLAG tag at the C-terminus by the adapter primers (forward primer with the XhoI site underlined: 5'-TACTCGAGATGTCCAAAAAATCAGTGG-3', reverse primer with the EcoRI site underlined and the FLAG tag italicized: 5'-ACGAATTCTTACTTGTCATCGTCATCCTTGTAATCCATAAGTTTGGCCTG-3') and inserted into a XhoI- and EcoRI-linearized pLPCX vector. The IDH1^{R132H} alteration was generated in pLPCX IDH1 WT FLAG using the Quikchange

0.7 mg/mL puromycin. Protein expression was confirmed by immunoblot and immunofluorescence (data not shown).

Animal Model

All animal experiments were approved by the University of California at Los Angeles Institutional Animal Care and Use Committee. Male NOD-*scid* mice (6–8 weeks old) were used for all experiments except 2-HG injection, where a phosphatase and tensin homolog (PTEN)/K-Ras knockout mouse was used (see below). Three groups of NOD-*scid* mice were injected with 10⁶ glioma cells into the right flank as follows: (i) IDH1^{WT}-U87 cells (n¼ 7); (ii) mock-transfected U87 cells (U87-vector control) (n¼ 2); and (iii) U87 cells transfected with the IDH1^{R132} construct (n¼ 8).

Magnetic Resonance Imaging and ¹H Magnetic Resonance Spectroscopy

MRI and MRS were performed on a 7T Bruker system with a custom-built 2.2-cm radiofrequency birdcage coil. Mice were imaged at 14 and 21 days following glioma cell injections. Before imaging, we anesthetized the animals with isoflurane (4% induction, maintenance with 1.5%). During imaging, respiration was monitored and isoflurane levels were adjusted if breathing was < 20 or > 60 breaths per minute. Mice were kept warm with water heated to 37°C circulated using a TP500 water pump (Gaymar Solid State). To quantify T₂ of different tumors, a multislice multi-echo (MSME) spin echo sequence was used (repetition time (TR)/echo time (TE)¼ 2000/7.26–101.64 ms, 14 echoes, 78 mm² in-plane resolution, 1-mm slice thickness, number of experiments [NEX]¼ 2). A diffusion-weighted imaging–echo planar imaging (DWI-EPI) sequence (TR/TE¼ 3800/22.03 ms, with 3 b-values: 0, 500, 1000; 3 diffusion directions; and 156² mm² resolution; 1-mm slice thickness; NEX¼ 2) was used to measure ADC. Nonlinear least-squares regression was used to estimate ADC from multiple b-value DWIs and T₂ from MSME data on a voxel-wise basis. Dynamic susceptibility contrast (DSC) perfusion MRI was collected using an EPI readout with 500 ms temporal resolution (2 slices; TR/TE¼ 500/7.18 ms; flip-angle¼ 45 degrees; 312² mm² in-plane resolution; 0.5-mm slice thickness; NEX¼ 1; 360 repetitions) following tail-vein injection of 0.05 mL of 0.5 mol/L gadolinium-based contrast agent (Magnevist, Berlex). Following DSC MRI, we acquired

steady-state magnetization, calculating the pre-bolus signal intensity on a voxel-wise basis, converting the truncated DSC-MRI time series to a concentration-time curve based on the T_2^* relaxivity of the contrast agent, and estimating relative tumor blood flow (rTBF) on a voxel-wise basis by using the first moment of the delta R2 ($R2 \frac{1}{2} 1/T2$) curves to calculate mean transit time and with leakage correction to calculate tumor blood volume as described in a previous publication.²⁰

MRS was performed using a point-resolved spectroscopic sequence (TR/TE $\frac{1}{4}$ 4000/6.9 ms; NEX $\frac{1}{4}$ 512; 3-mL voxel volume within the tumor) and a variable pulse power and optimized relaxation delay water suppression scheme.²¹ MRS data were quantified using the LCMoDel,²² and 2-HG was modeled based on J-coupling.²³ To optimize detection of 2-HG, MRS was performed on a 2-HG phantom (1-M concentration) using multiple TE times (6.9–30 ms, with 5-ms increments). We found the strongest signal-to-noise ratio for the 2-HG peak in the spectra with the shortest TE (\square 6.9 ms). Importantly, glutamate resonance (at 2.31–2.35 ppm) was distinct from 2-HG resonance (at 2.24–2.26 ppm). Metabolites with standard deviation (Cramer–Rao lower bounds) less than 20% of the estimated metabolite concentration were considered. Relative metabolite concentrations were expressed with respect to choline, since creatine levels were very low. This choice was justified by the lack of significant difference in the absolute choline concentration observed among the flank tumors.

Validation of 2-HG Chemical Shift on MRS in a PTEN / K-Ras Knockout Mouse

Validation of the precise 2-HG chemical shift on the MRS spectrum was determined by injecting exogenous 2-HG (Santa Cruz Biotechnology) into a PTEN /K-Ras knockout mouse²⁴ with a 3-mm optic glioma. This model was chosen to ensure maximum delivery of 2-HG to the tumor, since flank tumors have relatively poor perfusion and areas with limited contrast-agent uptake. Phosphate buffered saline (PBS) was used to prepare 1 M 2-HG solution, and pH was adjusted to 7.4. Serum concentration following a 0.1-mL injection of 1 M 2-HG for a 15-g mouse was \square 10 mM. MRS was performed before and 30 min after 2-HG injection with a similar protocol as previously described, with the exception of NEX $\frac{1}{4}$ 256 and a 2.5-mL voxel volume, which covered the tumor.

from ≥ 2 adjacent sections by 2 experienced neuropathologists (W.Y. and L.M.). The percent of necrosis was calculated as necrotic area within the tumor v the whole area of the tumor and expressed as a fraction of 100 to the nearest decile. In addition, adjacent slices were processed with mouse anti-human antibody specific for IDH1^{R132H} mutation²⁵ (Dianova) for immunohistochemistry. Sections were processed using the Dako EnVision+ system (peroxidase) with blocking steps and secondary antibody dilutions per the manufacturer's instructions (DakoCytomation). NovaRED (Vector Laboratories) was used as a chromogen. Mayer's hematoxylin was used as a counterstain.

Statistical Analysis

MRS metabolites, T_2 , and ADC values within different tumor volumes were analyzed using an analysis of variance (ANOVA) across groups (U87-IDH1^{R132}, U87-IDH1^{WT}, U87-vector). The Holm–Sidak test for multiple pairwise comparisons was used for post hoc comparisons. Sigma Stat 11.0 (Systat Software) was used for ANOVA analysis. $P < .05$ was considered statistically significant. Data are given as means + standard deviation (SD) unless stated otherwise.

Results

Validation of 2-HG Chemical Shift Assignment in MRS Spectra

Validation of the chemical shift assigned to 2-HG on the ¹H-MR spectrum was performed using 0.1 mL of 1 M 2-HG solution injected into a PTEN /K-Ras mouse with an optic glioma. After 30 min following 2-HG injection, a distinct peak appeared at \square 2.25 ppm, the expected location based on J-coupling simulations using the LCMoDel (Fig. 1B). This chemical shift corresponded to the strongest peak of 2-HG (hydrogen attached to C-4 of 2-HG), similar to previously published results for 2-HG using J-coupling modeling and MRS.²³

Measurement of 2-HG in Tumors Overexpressing IDH1^{R132}

Quantitative MRS using the LCMoDel algorithm with J-coupling modeling for 2-HG confirmed the presence of a 2-HG peak in all U87 flank tumors overexpressing IDH1^{R132} whereas 2-HG levels were not detectable in

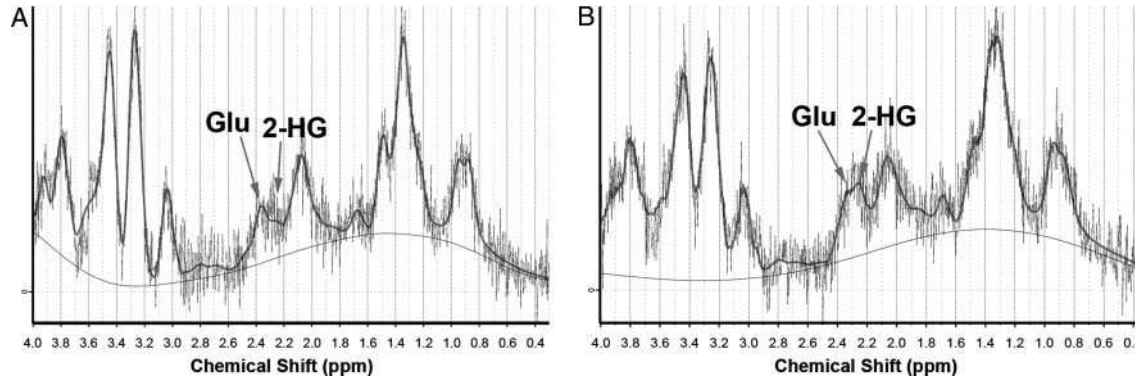
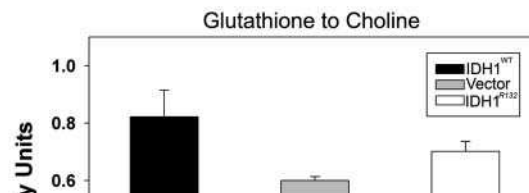
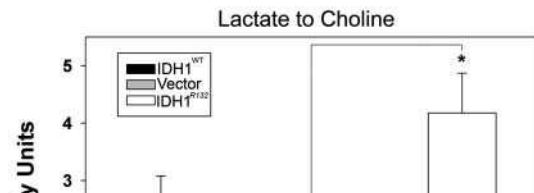
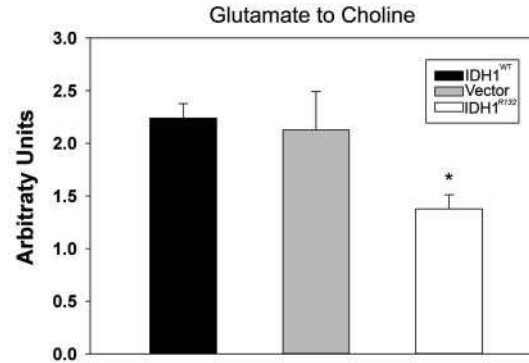
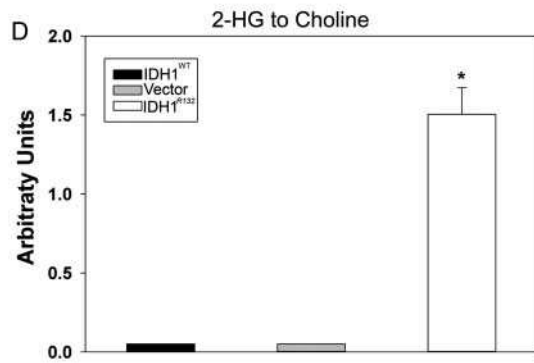
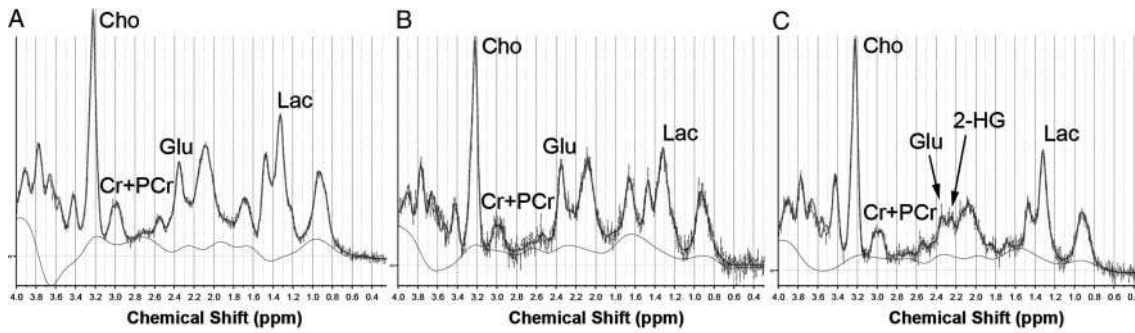


Fig. 1. Verification of 2-HG detection and chemical shift. A PTEN/K-Ras knockout mouse with optic glioma was injected with 0.1 mL of 1 M 2-HG and subjected to MRS before and 30 min after injection. (A) MRS spectrum before injection showing baseline metabolites of the optic glioma. (B) After 30 min following i.v. injection, we noticed the emergence of the additional peak at \square 2.25 ppm that we assigned to 2-HG.



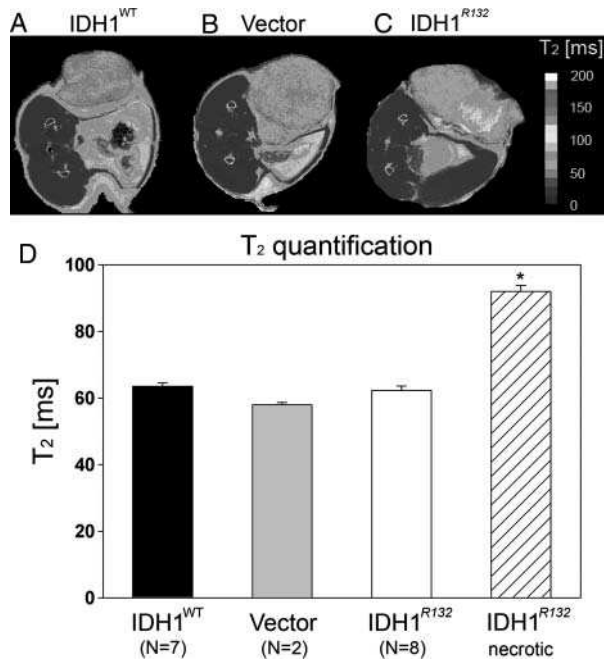


Fig. 3. (A–C) Representative T₂ maps showing T₂ distribution across different flank tumors. Color scale represents different T₂ values. (D) T₂ quantification for all 3 tumor types expressed as mean T₂ values+ SD. Notice similar T₂ distributions except for the necrotic area in IDH1^{R132} tumor (C) with elevated T₂ values, *P, .05 ANOVA with Holm–Sidak correction for multiple pairwise comparison.

(P, .05, ANOVA with Holm–Sidak post hoc test) compared with IDH1^{R132} (Fig. 2D).

Quantification of T₂ and ADC

Increased T₂ values within tumor tissue are typically attributed to the presence of vasogenic edema or macroscopic necrosis. In the absence of vasogenic edema or necrosis, lower T₂ values are expected. No significant differences (1-way ANOVA) in T₂ values were detected between IDH1^{R132} (62.3 + 3.5 ms), IDH1^{WT} (63.5 + 2.8 ms), and U87-vector (57.9 + 1.2 ms) flank tumors (Fig. 3D). However, flank tumors formed from IDH1^{R132}-overexpressing U87 cells had clear areas of macroscopic necrosis with significantly elevated T₂ values (ANOVA with Holm–Sidak post hoc test, P, .05) compared with T₂ values within the nonnecrotic region (91.9* + 3.6 62 ms; Fig. 3D). Additionally, no significant differences in ADC (1-way ANOVA

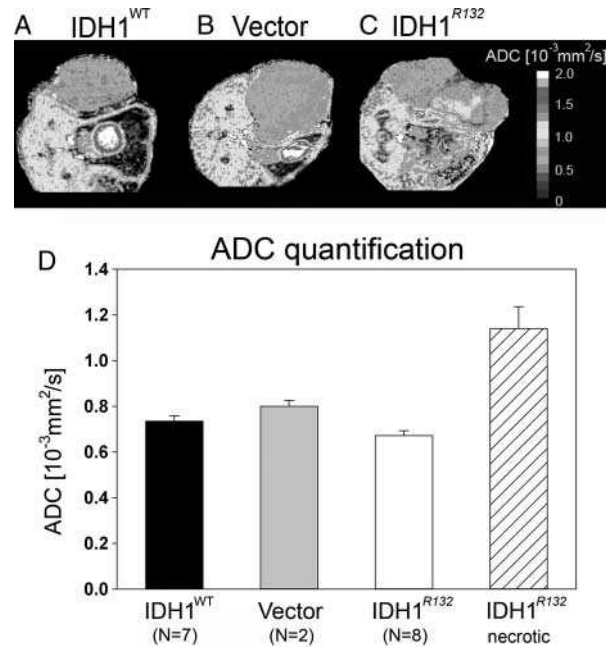


Fig. 4. (A–C) Representative ADC maps showing ADC distribution across different flank tumors. Color scale represents different ADC values. (D) ADC quantification for all 3 tumor types expressed as mean ADC values+ SD. Notice similar ADC distributions except for the necrotic area in IDH1^{R132} tumor (C) with elevated ADC values, *P, .05 ANOVA with Holm–Sidak correction for multiple pairwise comparison.

to decreased cell density and increased extracellular space, as expected for necrotic areas.

MR Perfusion

Independent of IDH1^{R132} or IDH1^{WT} expression, all flank tumors had a similar pattern of contrast enhancement, with an enhancing periphery and non-enhancing T₁ hypointense central region (Fig. 5A–C). The slowest-growing WT tumors had a slightly larger area of contrast enhancement compared with IDH1^{R132} and U87-vector tumors. Central regions exhibiting MR signal intensity consistent with necrosis exhibited very low perfusion, whereas regions with contrast enhancement on postcontrast T₁-weighted images appeared to have elevated perfusion (Fig. 5D–F). Relative TBF was not significantly different among flank tumors of IDH1^{R132} (mean + SEM ¼ 25.01 + 10.328 mL/100 mg tissue/min), IDH1^{WT} (22.29 + 7.748 mL/100 mg tissue/min),

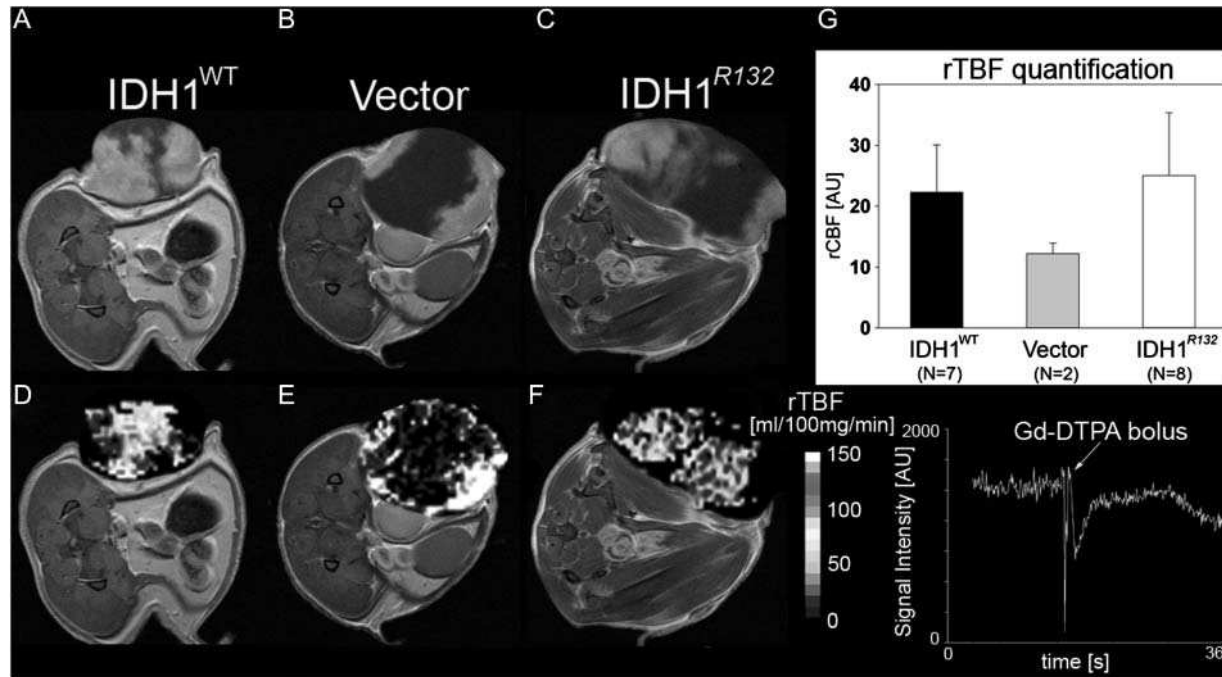


Fig. 5. (A–C) Representative postcontrast T_1 -weighted MRIs of IDH1^{WT}, vector-control, and IDH1^{R132} flank tumors. Notice areas void of contrast agent enhancement in all 3 tumor types. (D–F) Representative perfusion images (color scale represents different perfusion values, mL/100 mg tissue/min) with DSC signal intensity bolus curve. Areas of reduced perfusion were present in all 3 tumor types, usually matched with areas of poor contrast enhancement. Some of the geometric distortion is due to use of echo planar imaging sequence for DSC perfusion. (G) Graph representing relative tumor blood flow (rTBF) measurements expressed as mean blood flow values + SEM.

for IDH1^{R132}, there were occasional cells that did not stain (Fig. 6D). Whether these represented tumor cells that had lost IDH1^{R132} or connective cells from the vasculature or other sources is not clear (Fig. 6D). H&E histology confirmed a necrotic area of \square 40%–50% (Fig. 6H) in flank tumors overexpressing IDH1^{R132} compared with a region of necrosis of \square 10%–20% observed in IDH1^{WT} and vector-control tumors (Fig. 6E–H).

Discussion

The current study is the first controlled experiment validating the link between noninvasive 2-HG detection using MRS and the IDH1^{R132} mutation in glioma cells. IDH1^{R132} overexpression in U87 cells led to in vivo detectable levels of 2-HG using MRS, providing further evidence of a causal relationship between the IDH1 mutation and 2-HG production, as previously

the glutamate and glutamine peaks. As expected, cells overexpressing IDH1^{WT} lacked this metabolic peak. For our experiments, 2-HG levels were estimated to be between 10 mM and 20 mM in IDH1^{R132} flank tumors, somewhat higher than the 2–9 mM range reported for humans.¹⁶ The glutamate resonance (at 2.31–2.35 ppm) was sufficiently distinct from the 2-HG resonance to prevent contamination of the 2-HG peak, which has been a limitation of 2-HG quantification in tumors with MRS performed on clinical (1.5T and 3T) scanners. Given the similarity of the IDH1 mutant spectra in the current study with those previously published from in vivo human data, this use of IDH1^{R132} mutant overexpressing cell lines has provided further evidence that the IDH1^{R132} mutation is responsible for the 2-HG peak and that this resonance is not related to tumor grade or other confounding factors.

Reduced glutamate levels have previously been reported for human oligodendroglioma-derived cell lines

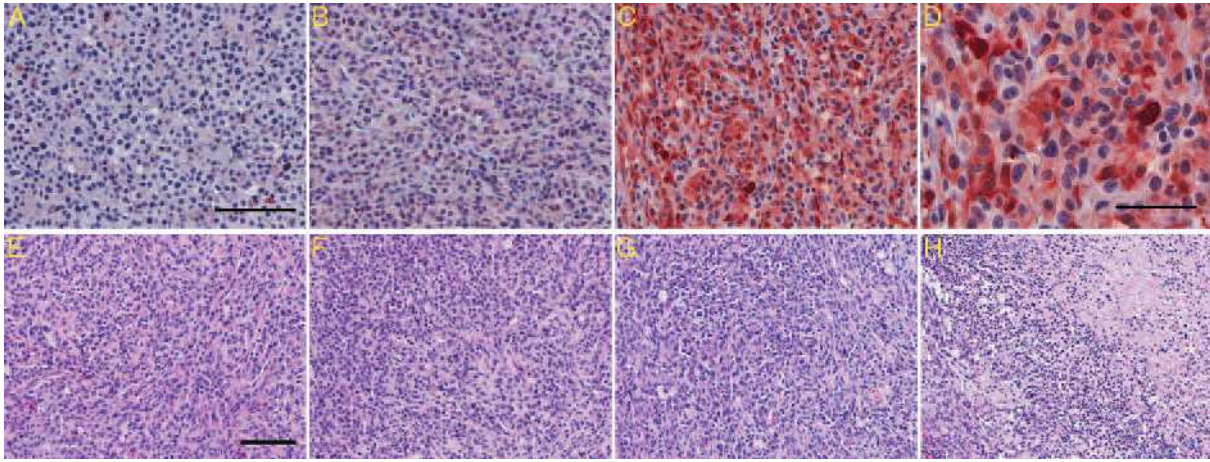


Fig. 6. IDH1 staining and H&E histology of tumors overexpressing IDH1^{WT}, vector control, and IDH1^{R132}. (A–D) Representative IDH1 immunohistochemistry for (A) IDH1^{WT}, (B) vector control, and (C) IDH1^{R132} showing IDH1^{R132}-positive cells for only IDH1^{R132}-transduced gliomas; scale bar (A) represents 100 mm. (D) Higher magnification of IDH1^{R132} immunohistochemistry in IDH1^{R132} flank tumors shows that not all U87 cells are IDH1^{R132} positive; scale bar represents 50 mm. (E–G) Representative H&E staining for (E) IDH1^{WT}, (F) vector control, and (G) IDH1^{R132}; scale bar represents 100 mm. (H) H&E staining shows the necrotic region in the IDH1^{R132} flank tumor.

and therefore the relationship between the IDH1 mutation and glutamate levels could have important clinical implications, meriting further investigation.

Results from the current study suggest that IDH1^{R132} mutant tumors have larger areas of macroscopic necrosis defined by MR compared with IDH1^{WT}. Specifically, extensive areas of necrosis (□40%–50% of the entire tumor volume) were observed in all tumors overexpressing IDH1^{R132} □21 days following injections, compared with □20% macroscopic necrosis observed in IDH1^{WT} or U87-vector flank tumors. It is conceivable that this may be due to slight size differences in the mutant tumors within the context of the current experiment, as more rapid tumor growth could occur quicker than the angiogenesis necessary to maintain a normoxic environment.^{18,30,31} Alternatively, elevated levels of 2-HG could potentially be toxic to the tumor cells, leading to more extensive necrotic area. In humans, 2-HG toxicity is suggested by the observation that inborn errors of metabolism resulting in elevated 2-HG levels result in

encephalopathy.^{32,33} Direct toxicity of 2-HG to rat cerebral cortical slices has been demonstrated (at 5-mM concentration), in similar concentrations to those of 2-HG reported for gliomas,^{11,14,31} and within the range of 2-HG levels calculated for the xenografts used in our study.^{11,14,34} One caveat to this assumption, however, is that IDH1 mutant glioblastomas seem to have less necrosis than their WT counterparts, at least as detected by MRI.³⁵

In summary, we have demonstrated that overexpression of the IDH1 mutation leads to detectable levels of 2-HG in a mouse glioma model that can be measured in vivo and noninvasively by MRS. This model system is of potential utility in establishing the relationships among 2-HG levels and treatment effect, outcomes, and other variables necessary to validate 2-HG as a clinically important biomarker for glioma.

Conflict of interest statement. None declared.

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