Towards personalised medicine for cancer

*From initial therapy to follow-up*

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Chapter 5: IDH1-mutated gliomas rely on glutamate to compensate for defective isocitrate processing: consequences for studies on IDH1 function


Abstract
Eighty to ninety percent of diffuse gliomas carry hotspot mutations in isocitrate dehydrogenase 1 (IDH1). Mutant IDH1/2 enzymes produce D-2-hydroxyglutarate (D2HG) at the expense of α-ketoglutarate (αKG) and NADPH, resulting in metabolic stress. In vitro studies have suggested that cancer cells expressing mutant IDH1 depend on glutaminolysis to generate αKG via glutamate in a two-step reaction. Because glutamate is abundantly secreted by neurons we previously postulated that IDH1-mutated gliomas may directly use this neurotransmitter to escape from metabolic stress. To test this hypothesis, we applied a novel technique of targeted next-generation sequencing of RNAs encoding for metabolic enzymes, on a cohort of 75 clinical gliomas. Metabolic pathway analysis suggests that IDH1-mutated gliomas depend heavily on glutamate and not on glucose for tricarboxylic acid cycle (TCA) anaplerosis. This was functionally confirmed by in situ enzyme activity mapping experiments in a second cohort of clinical gliomas with known IDH1/2 mutational status. We functionally validated these findings further in E478, one of the few existing stable patient-derived glioma xenograft models carrying an endogenous IDH1R132H mutation. In line with the findings in clinical gliomas, targeted RNA and whole RNA next-generation sequencing revealed very low expression levels of enzymes and transporters involved in glycolysis, and high transcript levels of glutamate dehydrogenase (GLUD1/2) and glutamate importer EAAT2 (SLC1A2). These results were validated at the protein level by immunohistochemical staining, enzyme activity mapping studies and in vivo magnetic resonance spectroscopic imaging. We conclude that upon acquisition of the IDH1 mutation, glioma cells adapt to the metabolic stress via epigenetic manipulation of metabolic genes. This process cannot be mimicked by artificial overexpression of the mutant IDH1 protein in IDH1 wild-type cell lines in vitro.

Introduction
Heterozygous hotspot mutations in cytosolic IDH1 and, less frequently, mitochondrial IDH2 occur in 80% of WHO grade II-III and secondary grade IV gliomas and are considered ancestral events in the formation of these and other neoplasms. IDH1 is an NADP+-dependent homodimeric enzyme that reversibly oxidises isocitrate to αKG with concomitant NADPH production. This enzyme reaction is responsible for 65% of total NADPH production capacity in the cytoplasm of glioblastoma cells.

Oncogenic driver IDH1 mutations mainly involve Arg132 in the isocitrate binding pocket. The mutation introduces a neomorphic activity of reducing α-KG to D2HG, while oxidizing NADPH. Because it is metabolised slowly, D2HG accumulates up to millimolar concentrations at which it competitively inhibits a range of αKG-dependent enzymes, ultimately resulting in epigenetic alterations that are associated with malignant transformation.

Mutations in IDH1/2 are oncogenic, but they are also associated with better prognosis. This may be related to metabolic stress, resulting from excessive consumption of αKG and oxidation of NADPH, which directly impacts on fatty acid synthesis. Furthermore, diminished NADPH production capacity results in decreased production of reduced glutathione. Reduced glutathione is an important scavenger of reactive oxygen species (ROS) in stress conditions such as irradiation, providing an explanation for increased radiotherapy sensitivity of IDH1-mutated gliomas.
Progression of IDH1-mutated gliomas can therefore only occur after metabolic rewiring that reduces metabolic stress, by supplying cancer cells with sufficient amounts of αKG and NADPH for anaplerosis. In a previous study, we reported that cells of the patient-derived IDH1-mutated oligodendroglioma xenograft model E478 contain an extremely high density of mitochondria suggesting that IDH1-mutated gliomas revert to mitochondrial metabolism as a rescue pathway.\textsuperscript{129,159} In previous studies it has been proposed that IDH1-mutated cells use glutamine as a source for anaplerosis of the TCA cycle.\textsuperscript{134,144,188} In this setting, IDH1-mutated cells are suggested to produce αKG from glutamine through the sequential activities of glutaminase (GLS) and glutamate dehydrogenases (GLUD1/2) or glutamic acid oxaloaminotransferases (GOT1/2), whereas IDH1 wild-type cells utilise aerobic glycolysis (Warburg effect; \textbf{Figure} 1).\textsuperscript{159} Since grade II and III gliomas express the glutamate importer EAAT2,\textsuperscript{137} we previously postulated that IDH1-mutated cells directly utilise glutamate instead of glutamine as a source for αKG and NADPH production.\textsuperscript{16} Mining of glioma gene expression datasets in The Cancer Genome Atlas (TCGA) showed significant differences between metabolic transcriptomes of IDH1-mutated and IDH1 wild-type gliomas.\textsuperscript{159}

Here, we present experimental data obtained with human glioma tissues that strongly suggest that IDH1-mutated gliomas are more dependent on glutamolysis than on glutaminolysis, and have very low levels of glycolysis. We also present evidence that the study of metabolism in cell systems with artificial overexpression of IDH1 mutant protein need to be interpreted with caution.

\textbf{Figure 1. Overview of the metabolic pathways of investigation in this chapter.}

\textit{Indicated are the pathways of glycolysis (A), glutaminolysis (B), tricarboxylic acid (TCA) cycle (C), oxidative phosphorylation (D) and the pentose phosphate pathway (E), responsible for nucleotide and NADPH synthesis. Enzymes and transporters in the different pathways are indicated in the figure. 6PGL 6-phosphogluconolactonase; Ac-CoA Acetyl-CoA; αKG Alpha-ketoglutarate; ACLY ATP citrate lyase; ACo Aconitase; CS Citrate synthase; CT Citrate; F6P Fructose-6-phosphate; G6P Glucose-6-phosphate; Glu Glutamine; Glu Glutamate; HK2 Hexokinase 2; ICT Isocitrate; IDH Isocitrate dehydrogenase; LDH Lactate dehydrogenase; MCT Monocarboxylate transporter; OAA Oxaloacetate; OXPHOS Oxidative phosphorylation; PEP Phosphoenolpyruvate; RSP Ribose-5-phosphate; TCA Tricarboxylic acid.}
Materials and methods

Patient tissues. All experiments involving human tissues were performed according to institutional guidelines of Radboud UMC and Academic Medical Centre and involved informed consent. All research was performed on ‘waste’ material that was stored in a coded fashion. From Radboud UMC, biobanked frozen glioma samples \((n = 75)\), collected between 2013 and 2017, were used for targeted RNA sequencing (see further) and immunohistochemical analysis. For enzymatic mapping experiments, human glioma samples \((IDH1^{WT/R132H}, n = 7; IDH1^{WT/WT}, n = 5, \text{ all astrocytomas})\) were obtained from the tumour archive maintained by the Departments of Neurosurgery and Neuropathology of the Academic Medical Centre (Amsterdam, The Netherlands). Written consent for tissue storage in the tumour bank for research purposes was obtained and documented in the patients’ medical records and waived by the Medical Ethics Review Committee of the Academic Medical Centre at the University of Amsterdam (reference number W14_224 #14.17.0286). All tumour samples in this study contained >80% cancer cells, as was verified by experienced pathologists using H&E-stained sections.

Targeted RNA next generation sequencing. Enzymes were from New England Biolabs (NEB) unless indicated otherwise. RNA was isolated from glioma cryosections using the standard TRIzol method and treated with DNase. One µg of total RNA was reverse transcribed using Superscript II according to routine protocols in a volume of 20 µL. Fifty ng of cDNA was used for targeted next-generation sequencing (NGS) according to a recently described method using single molecule molecular inversion probes (smMIPs). In short, smMIP-based NGS is based on the hybridization of an extension and ligation probe, joined by a backbone sequence, in an inverted manner to a cDNA of interest, followed by gap-filling/ligation and PCR. SmMIPs against the antisense strand of 104 predicted transcripts (UCSC human genome assembly hg19) encoding enzymes involved in lipid metabolism, glycolysis, oxidative phosphorylation (OXPHOS), tricarboxylic acid cycle, pentose phosphate pathway (PPP), glutaminolysis and control of reductive potential were designed based on the MIPgen algorithm as described by Boyle et al. For each transcript, a minimum of 5 smMIPs targeting different regions were included in the panel to compensate for inter-smMIP variations in efficiency. In case mutations in a transcript were expected, extension and ligation probes in smMIPs were selected to flank the area with the potentially variant nucleotide. When possible, smMIPs were included with ligation and extension probes located on adjacent exons to ensure reaction on fully matured mRNAs. The smMIP set also contained probes for detection of β-actin and β-tubulin as housekeeping genes. Generation of libraries was performed with a procedure adapted from O’Roak et al. In short, a total of 642 smMIPs (Integrated DNA Technologies) were pooled at 100 µM/smMIP. The smMIP pool was phosphorylated using T4 Polynucleotide Kinase in T4 DNA ligase buffer at 37°C for 45 min, followed by inactivation for 20 minutes at 65°C. The capture reaction was performed with 50 ng of cDNA and an estimated 8000-fold molar excess of the phosphorylated smMIP pool in a 25 µL reaction mixture containing Ampligase buffer, dNTPs, HemoKlenTaq enzyme and thermostable DNA ligase (all from Epicentre). The capture mix was incubated for 10 minutes at 95°C (denaturation), followed by incubation for 18 hours at 60°C, during which hybridization and concomitant primer extension and ligation occurred. Directly after this step, non-circularised smMIPs, RNA and cDNA were removed by treatment with 10 U Exonuclease I and 50 U of Exonuclease III for 45 minutes at 37°C, followed by heat inactivation (95°C, 2 minutes). The circularised smMIP library was subjected to standard PCR with 2x iProof High-Fidelity DNA Polymerase master Mix (Bio-Rad) with a backbone-specific primer set containing a unique barcoded reverse primer for each sample. Generation of PCR products of correct size (266 bp) was validated on agarose gel electrophoresis, and PCR libraries of different samples were pooled based on relative band intensity. The pool was then purified using AMPureXP beads (Beckman Coulter Genomics) according to manufacturers’ instructions. The purified library was run on a TapeStation 2200 (Agilent Technologies) and quantified via Qubit (Life Technologies, ThermoFisher Scientific) to assess quality of the library.
**Sequencing and annotation.** Libraries were sequenced on the Illumina NextSeq platform (Illumina) at the RadboudUMC sequencing facility to produce 2x 150 bp paired-end reads. Reads were mapped to the reference transcriptome (hg19) using the SeqNext module of JSI SequencePilot version 4.2.2 build 502 (JSI Medical Systems). The random 8 nt sequence flanking the ligation probe was used to reduce PCR amplicates to one hybridised smMIP (unique reads), making the assay quantitative. Transcript levels were expressed as fragments per million (FPM), calculated by \( \frac{\text{mean \# unique smMIPs per transcript} \times 10^6}{\text{total \# unique smMIPs in sample}} \). Nucleotide calling was considered reliable when a coverage >10 was achieved. Heatmaps of FPM values for each transcript were generated with the open source matrix visualization and analysis platform GENE-E.\(^\text{218}\)

**RNAseq.** Whole-transcriptome RNAseq of E478 (derived from oligodendroglioma; \( \text{IDH1 WT/R132H} \)), E434 (derived from oligodendroglioma, \( \text{IDH1WT/WT} \)) and E98 xenografts (derived from astrocytoma) and E98FM and E98FM-IDH1R132H cell lines was performed at ServiceXS. Samples were prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB #7420S/L). Ribosomal RNA was removed using the NEBNext rRNA removal kit. After fragmentation of the rRNA-depleted RNA, cDNA synthesis was performed, after which sequencing adapters were ligated and PCR was performed. The quality and yield of the product was measured with a fragment analyser. Size of the resulting products was consistent with the expected size distribution of 300-500 bp. Sequencing was performed on an Illumina NextSeq500 and yielded 40-60 million reads per sample (single-end sequencing protocol). The dataset was analysed using the ‘Tuxedo’ protocol;\(^\text{219}\) reads were mapped against the RefSeq human genome (hg19) with TopHat and final transcript assembly was performed with the Cufflinks package.\(^\text{219}\) For xenografts, mouse reads were filtered from the data sets using Xenome\(^\text{220}\) prior to the ‘Tuxedo’ protocol. Transcript values were expressed as FPKM (fragment per kilobase per million human reads). Since all sequencing data were obtained during the same Illumina run, comparison of FPKM values between samples was allowed.

**Metabolic mapping of GLUD and GLS activity and immunohistochemical stainings.** Maximum production capacities of glutaminolysis enzymes were investigated by metabolic mapping in clinical glioma tissue and glioma xenografts.\(^\text{209,221}\) The protocol for the determination of GLUD activity is described in Chapter 4 of this thesis. GLS activity in cryostat sections was measured indirectly as described before.\(^\text{209}\) In short, sections were positioned on glass slides coated with a film containing >40 units bovine GLUD (Serva) and incubated with medium containing 37.5 mM glutamine. NADH production was quantified indirectly by measuring formazan production from NBT as described above. This method enables zero-order GDH kinetics and therefore NADH and α-KG are generated stoichiometrically by GLS via GLUD activity. Negative control reactions were performed in the absence of substrate (glutamate and glutamine for GLUD and GLS activity, respectively). Immunohistochemistry (IHC) was performed on 4 µm-thick sections of formalin-fixed paraffin-embedded tissue. After epitope retrieval by boiling in Tris-EDTA buffer pH 9.0 (Klinipath), sections were stained with rabbit-anti-GLUD1/2 (Cell Signaling Technologies, #12793), rabbit anti-GLS (Abcam, ab156876) or rabbit anti-human vimentin (Thermo Scientific, MA5-16409). Primary antibodies were detected using BrightVision polyHRP-anti-rabbit IgG (Immunologic). All IHC-stained sections were counterstained with haematoxylin and mounted in Quick-D mounting medium (Klinipath). For all tissue samples, control stainings with secondary antibody-only were performed.

**Cell lines and xenograft models.** All animal experiments were approved by the local committee for animal welfare of the Radboud University. Patient-derived E98FM astrocytoma cells, expressing luciferase and mCHERRY, have been described before.\(^\text{222,223}\) E98FM cells were cultured in DMEM medium (Lonza) supplemented with 10% foetal bovine serum (Gibco) and 40 µg/ml gentamycin (Centrafarm) in 5% CO\(_2\) at 37°C. E98FM-IDH1R132H cells were generated by transducing E98FM cells with lentiviruses encoding IDH1R132H as described\(^\text{224}\) and maintained in the same medium, supplemented with blasticidin. Since IDH1R132H expression tends to decrease in overexpressing cells at later
passages, cell lines were always used between passage 5 and 25. E98FM and E98FM/IDH1R132H cells were grown as spheroids for at least 2 passages in serum-free Neurobasal/B27 medium (Gibco, Invitrogen), supplemented with EGF, basic FGF (both PromoCell), heparin, L-glutamine and 40 µg/ml gentamicin (Centrafarm) prior to xenografting. Mice were checked weekly for tumour growth using bioluminescence imaging on the IVIS imager (Perkin-Elmer) and killed when showing signs of discomfort or weight loss (>15% within 2 days). Brains were fixed in formaldehyde, embedded in paraffin, sectioned and examined after H&E and anti-human vimentin immunostaining. The patient-derived orthotopic IDH1WT/R132H and IDH1WT/WT xenograft models E478 and E434 have been described before and were maintained by direct serial transplantation in BALB/c nude mice (Janvier Labs). Expression of IDH1R132H in the different models was validated by western blotting of cell extracts and by D2HG measurements via liquid chromatography mass spectroscopy (LC-MS, Quattro LC, Micromass).129

**Proliferation assays.** E98FM and E98FM-IDH1R132H cells were seeded in 96-well plates at 2,000 cells/well and grown in standard serum-containing DMEM. At days 2, 4, 6 and 8 after seeding, total cell protein was measured using sulforhodamine B (SRB) assays (Sigma-Aldrich) as described.

**In vivo MRSI.** Healthy mice (n = 3) and mice carrying E478 and E434 xenografts (n = 5 for each model) were subjected to in vivo magnetic resonance spectroscopic imaging (MRSI). Animals were anesthetised using an isoflurane/N2O/O2 mixture and investigated on a 7T MR system (ClinScan, Bruker) with a dedicated mouse brain transmit-receive-coil. Breathing of the mice was monitored throughout the MR experiment, and body temperature was maintained at 37.5°C using a continuous flow of warm air (SA Instruments). The presence of tumour was verified in retrospect by comparing T2-weighted MR images to haematoxylin-eosin staining of corresponding brain sections. Guided by these MR images, voxels of interest (VOI) were selected for further analysis while preventing partial volume artifacts. 3D MRSI was performed with a semi-LASER short echo time (TE = 24 ms, n = 5) and a long echo time (TE = 144 ms, n = 3) sequence using a 12x12x16 elliptical weighted phase encoding scheme, interpolated to 16x16x16. The 11x11x16 mm FOV contained 0.85 mm³ nominal voxels. For all measurements, the repetition time (TR) was set at 1500 ms. Water signal was suppressed using WET over a bandwidth of 120 Hz and residual signals from surrounding fat and bone tissue were suppressed with 1 mm thick saturation slabs. The RF saturation pulse was positioned at -3.40 ppm relative to the water resonance. Four averages were acquired; total acquisition time was 28 minutes. A non-water suppressed short TE dataset was acquired (1 average) for calibration of the metabolites to the water signal. For absolute quantification we used known T1 and T2 relaxation times available from literature. Water content was assumed to be 79%. As an anatomical reference, multi-slice T2-weighted images were made in 3 directions parallel to the MRSI grid. Unfiltered spectra in the chemical shift range of 0.5 – 4.2 ppm were fitted with LCModel software using simulated spectral basis sets of 23 metabolites for 7T with TE = 24 ms (for detection of glutamate and glutamine) or TE = 144 ms (for detection of lactate and N-acetylaspartate). Cramér Rao Lower Bound (CRLB) values were reported for all integrated peak values. In case peak intensities could not be assessed independently (metabolite correlation coefficient (MCC) < -0.3), values were excluded from analysis.

**In vivo bioluminescence imaging.** Mice carrying E98FM-IDH1R132H xenografts received an intraperitoneal injection of 200 µl filter-sterile PBS containing 15 mg/ml D-luciferin. After 5 min, mice were anesthetised and positioned in an IVIS Lumina system. Images were acquired at 10 minutes after D-luciferin administration using the Living Image 3.0 software (all from Caliper Life Sciences) and analysed by drawing 2-dimensional regions of interest (ROIs) around the skull. Luciferase activity (photons emitted per second per cm²) was expressed as fold increase, relative to luciferase activity on day 15 after xenografting.
**Statistical analysis.** Statistical analyses were performed in Prism v5.03 (GraphPad Software). For statistical testing of differential expression, IDH1WT/R132H tumours were compared with IDH1WT/WT tumours. Using a Mann Whitney U test, $P$ values are marked as follows: $<0.05$ (**); $<0.01$ (**); $<0.001$ (***); $<0.0001$ (****). For MRSI data analysis, a two-sided unpaired T-test was performed, assuming normal distribution.

**Results**

**GLUD activity is increased and GLS activity decreased in clinical IDH1-mutated glioma.** In vitro observations in cell lines overexpressing IDH1 R132H suggested that metabolic rewiring in IDH1-mutated cancers involves anaplerotic feeding of glutamine into the TCA cycle.\(^134\) We previously showed, using datasets from The Cancer Genome Atlas, that metabolic differences between IDH1 wild-type and IDH1-mutated gliomas are reflected at the level of the metabolic transcriptome,\(^159\) corroborating reports that the transcriptome is directly correlated with the proteome.\(^233\) To investigate metabolic gene activity in clinical glioma samples, we applied a novel method of targeted RNA sequencing, allowing simultaneous quantitative detection of levels of 104 transcripts of interest, and possible mutations therein. Blind metabolic profiling of 75 biobanked clinical gliomas, followed by hierachical unsupervised clustering of the dataset\(^218\) separated the cohort into 2 major groups (Figure 2). Variant calling of the IDH1 transcripts revealed that the 2 groups differed in their IDH1 mutational status. Of note, we also identified other variants than IDH1 R132H (1 case of heterozygous IDH1\(^{V183C}\) of unknown significance and 6 cases of IDH1\(^{V78I}\), a known polymorphism, see annotations in Figure 2). Three cases with IDH2 mutations (IDH2\(^{R172K}\), IDH2\(^{R172W}\), IDH2\(^{R172M}\)) grouped with IDH1 mutations. In all cases, the IDH1\(^{R132H}\) and IDH2 mutations were in retrospect confirmed by standard genetic analysis (data not shown). In IDH1/2-mutated gliomas, levels of transcripts encoding glucose-processing transporters or enzymes were strongly reduced as compared to IDH1 wild-type gliomas, including those encoding glucose transporter (GLUT3/SLC2A3; $P < 0.0001$) and hexokinase 2 (HK2; $P < 0.0001$) and lactate dehydrogenase A (LDHA; $P < 0.0001$) (Figure 3a and 3b). SLC16A3, the gene encoding monocarboxylate transporter 4 (MCT4), an exporter of lactate that is expressed on hypoxic/glycolytic cells,\(^129\) was also significantly lower in IDH1/2-mutated glioma ($P < 0.0001$), whereas SLC16A7, the gene encoding the lactate/pyruvate importer MCT2, was expressed at significantly higher levels ($P = 0.004$). Expression of glutamate dehydrogenases (GLUD1, GLUD2) were significantly elevated in IDH1/2-mutated gliomas (both $P < 0.0001$), whereas glutaminase (GLS) did not differ between groups ($P = 0.85$; Figure 3b). SLC1A2, the gene encoding glutamate importer EAAT2, was expressed at significantly higher levels in IDH1/2-mutated gliomas ($P = 0.002$), in line with previous observations of increased expression in low-grade gliomas.\(^137\) The glutamine importer SLC7A1 was also expressed at higher levels in IDH1/2-mutated gliomas ($P < 0.0001$). Together, these data suggest a more important role for glutamine and glutamate metabolism than for glucose metabolism in IDH1/2-mutated glioma cells in line with a previous report.\(^234\) Levels of transcripts encoding BCAT1, the enzyme converting α-KG to glutamate, were close to zero in IDH1-mutated gliomas in contrast to IDH1/2 wild-type gliomas (Figure 3b; $P < 0.0001$) in line with reports that the BCAT1 promoter is hypermethylated in IDH1/2-mutated glioma.\(^159,175\) Expression levels of the majority of mitochondrial enzymes involved in the TCA cycle (CS, $P < 0.0001$; IDH2, $P = 0.0007$; IDH3A, $P = 0.0002$; SDHD, $P = 0.03$; MDH1 $P = 0.0005$) were significantly higher in IDH1/2-mutated gliomas (Figure 3b). We next investigated expression levels of genes encoding enzymes that are responsible for pyruvate entry into mitochondria. Whereas pyruvate dehydrogenase (PDHA1) expression was significantly higher ($P < 0.0001$), expression of its inhibitor enzyme pyruvate dehydrogenase kinase (PDK1) was significantly lower in IDH1/2-mutated compared to IDH1/2 wild-type ($P < 0.0001$). Pyruvate carboxylase (PC), the enzyme responsible for production of oxaloacetate that can shortcut the TCA cycle, was significantly higher in IDH1/2-mutated gliomas ($P < 0.0001$) (Figure 3b). To validate transcript levels at the functional level, we mapped in situ enzymatic activities of GLS1/2 and GLUD1/2 in glioblastoma with known IDH1/2 mutational status. As shown in Figure 4a and 4b, GLUD1/2 activities were significantly higher in IDH1-mutated gliomas as compared to IDH1/2 wild-type counterparts whereas, conversely, GLS enzymatic activities were significantly
lower. The high glutamate dehydrogenase enzymatic activity, corresponding protein levels and transcript levels, combined with low levels of glucose processing transporters and enzymes, strongly suggest that \textit{IDH1/2}-mutated gliomas depend on glutamate, rather than glucose, as primary fuel.

\textbf{Figure 2. Targeted RNAseq of clinical glioma samples.}

Heatmap and hierarchical clustering of metabolic genes in 75 sequenced clinical gliomas. Gene expression levels were expressed as fragments per million (FPM). Each row represents relative expression of a specific gene across the samples. The dendrogram at the top visualises the Kendall’s Tau correlation between the samples. This clustering separates all samples based on IDH1/2 status.
Figure 3. Overview of gene expression levels of enzymes in the metabolic pathways based on IDH1/2 mutational status.

Gene expression levels of genes involved in glycolysis (A), glutaminolysis (B) and the TCA cycle (C). IDH1WT/IDH1R132H ratios were calculated on the basis of gene expression levels to indicate the relative contribution to a particular pathway. Genes in blue indicate higher gene expression in IDH1 wild-type gliomas, whereas yellow indicates higher gene expression in IDH1R132H-mutated gliomas. Boxplots of expression of genes involved in glycolysis, glutaminolysis, TCA and pyruvate processing enzymes, as indicated. Note that IDH1R132H-mutated gliomas express significantly higher levels of genes involved in glutaminolysis, whereas in IDH1 wild-type gliomas a glycolytic metabolotype is more prominent. IDH1 wild-type glioma cells display the typical Warburg effect by upregulating transporters and enzymes involved in glycolysis, among others GLUT3 and HK2, to ultimately form pyruvate. Pyruvate can either be metabolised to lactate by LDHA and exported out of the cell, or can feed into the TCA via Ac-CoA through the activity of pyruvate dehydrogenase. IDH1-mutated cells express high levels of glutamate import EAAT2 (SLC1A2) suggesting glutamate import and GLUD-dependent production of α-KG that may enter the TCA (B,C in panel 3a). Increased levels of lactate importer MCT2, LDHB and PC suggest that alternatively, lactate from the tumour environment is used to produce pyruvate by pyruvate carboxylase as a TCA shortcut. Abbreviations: 6PGL 6-phosphogluconolactonase; Ac-CoA Acetyl-CoA; αKG Alpha-ketoglutarate; CS Citrate synthase; CT Citrate; D-2HG D-2-hydroxyglutarate; F6P Fructose-6-phosphate; G6P Glucose-6-phosphate; Glu Glutamate; Gln Glutamine; HK2 Hexokinase 2; ICT Isocitrate; IDH Isocitrate dehydrogenase; LDH Lactate dehydrogenase; MCT Monocarboxylate transporter; OAA Oxaloacetate; OXPHOS Oxidative phosphorylation; PDH pyruvate dehydrogenase; PEP Phosphoenolpyruvate; RSP Ribose-5-phosphate; TCA Tricarboxylic acid cycle.
Glutamine-glutamate metabolism in orthotopic IDH 1-mutated glioma model E478 resembles that of patient-derived IDH1-mutated gliomas. Glutamine- and glutamate-processing enzymes are part of the glutamine-glutamate cycle and are therefore highly expressed in normal brain astrocytes and neurons (see also Figure 4a and 4b). Because diffuse gliomas are interspersed with non-neoplastic cells, these are an unavoidable source of contamination that may obscure cancer cell-derived transcripts. This may result in underestimation of IDH1 mutation-associated metabolic differences. To be absolutely sure that the metabolic differences observed between IDH1-mutated gliomas and IDH1 wild-type gliomas are attributable to cancer cells and not tumour stroma, we took advantage of the xenograft setting of our patient-derived glioma xenograft models. Whereas it may be difficult to discriminate mouse and human enzymes by immunohistochemistry or enzymatic mapping (due to the highly conserved nature of these fundamentally important enzymes), on the nucleotide level the inter-species sequence differences allow reliable distinction between mouse and human-derived transcripts after processing by the Xenome routine. The E478 model, developed by us, is a stable, non-engineered xenograft model of IDH1 WT/R132H glioma whereas E434 is a counterpart IDH1 wild-type model. Both models lack chromosome arms 1p and 19q, a characteristic of oligodendrogliomas. Xenome-based whole RNAseq analysis of the human transcripts in E478 and E434 xenografts confirmed almost all metabolic differences that we found in our cohort of clinical gliomas. GLUD1 expression was higher in E478 cancer cells relative to E434 cells (Figure 5a), whereas BCAT1 expression levels were 28-fold lower. In line with the high density of mitochondria in E478 cancer cells and the elevated levels of mitochondrial enzymes in clinical IDH1-mutated glioma samples, TCA cycle enzymes citrate synthase (CS), ketoglutarate dehydrogenase (OGDH), succinate dehydrogenases (SDHD), fumarate hydratase (FH), malate dehydrogenase (MDH2) and all subunits of the NAD+-dependent IDH3 (all enzymes of the TCA cycle) were expressed at higher levels in E478 than in E434 cells (Figure 5a). We validated some of the RNAseq data at the protein level using western blotting with a human-specific antibody against BCAT1 (Figure 5b), confirming low expression levels of the BCAT1 enzyme in E478 xenografts relative to E434. Expression of the IDH1R132H mutant protein was also confirmed on western blot (Figure 5b). Increased GLUD expression and activity in E478 cells was verified on the protein and the enzymatic level with the use of immunohistochemistry and in situ metabolic mapping (Figure 5c). To investigate the relative contributions of glucose and glutamate to the TCA cycle, we also investigated gene expression levels of enzymes that determine the fate of glucose. In line with the clinical dataset, the rate-limiting glycolysis enzyme HK2 was barely expressed in E478 cells (FPKM values of 2 versus 327 in E478 and E434 respectively; Figure 5a). Also with respect to lactate production, E478 closely resembled clinical IDH1-mutated glioma. LDHA levels were lower whereas LDHB expression levels were higher in E478 cells as compared to E434 cells. Expression of PC was 4-fold higher in E478 than in E434 cells (FPKM values of 436 versus 114).

In vivo MRSI. Despite highly significant differences in the metabolic transcriptome of IDH1-mutated and IDH1 wild-type gliomas, metabolic activity is a net result of a combination of factors, among which concentrations of substrate and product, availability of cofactors such as NAD(P)(H) and enzyme concentrations. Enzymatic activities are further determined by enzyme stability at the protein level and the transcript level. Thus, metabolic transcript levels are not necessarily synonymous with levels of the corresponding metabolic activities. Therefore, to test whether the metabolic transcriptome provides relevant information on metabolic pathways in cancer, we performed in vivo MRSI on mice carrying E434 and E478 xenografts. The tumour location in the brain of mice as observed on T2-weighted anatomical MR images matched with H&E-stained histopathological sections (Figure 6a). Analysis of spectra of selected voxels in the brain showed that the neuronal signals of N-acetylaspartate (NAA) and N-acetylaspartate-glutamate (NAAG), together tNAA, were almost absent in tumour tissue of both xenograft models. In normal appearing brain tissue of E478, tNAA was somewhat lower as compared to normally-appearing brain tissue in E434 mice and in healthy control brains (Figure 6). The signal of lactate is inverted at TE = 144 ms and could be detected properly in all animals. We observed distinctly lower lactate concentrations in
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E478 than in E434 xenografts in both tumour tissue and normally-appearing tissue, which is in line with the very low LDHA:LDHB ratios on the RNA level in E478 xenografts (Figure 6a, see also Figure 5a). The levels of glutamine and glutamate were assessed from short echo time spectra (TE = 24 ms). Even though these resonances partially overlapped with other metabolites between 2.0 and 2.4 ppm, we successfully detected both metabolites separately in all selected E478 tumour voxels. We noticed minor dependencies between the levels of glutamate and glutamine and/or NAA(G) in a few cases, but none exceeded the MCC limit and therefore, we included all values for further analysis. CRLB values mostly ranged between 5 and 10%, with the exception of CRLB values for tNAA fits in E478 tumour voxels, indicating almost complete absence of tNAA.

Glutamine levels in normal appearing tissue of E434 and E478 did not differ from glutamine levels in healthy mouse brain. In tumour tissue of E478, the levels of glutamine were increased, whereas E434 tumour tissue contained levels that were significantly decreased relative to healthy brain. Glutamate levels were decreased compared to levels in healthy brain in both tumour and normally-appearing tissue of E478, whereas E434 only showed a drop in glutamate levels in tumour tissue (Figure 6b).

**IDH1R132H overexpression does not induce IDH1 mutation-specific metabolism.** The proposed importance of the glutamine-glutamate cycle in brain for survival and progression of IDH1-mutated glioma cells may explain the difficulty to establish stable in vitro cell cultures. Most research is therefore performed with IDH1R132H-overexpression models with an IDH1 wild-type background. To test whether metabolism in such cell models reflects that of gliomas with endogenous an IDH1 mutation, we generated an IDH1R132H-overexpressing variant of the E98FM cell model and confirmed IDH1R132H expression and D2HG production (Figure 7a and 7b). IDH1R132H expression reduced cell proliferation rates (Figure 7c). Comparative RNAseq analysis showed that expression profiles of glutamate-processing enzymes in E98FM/IDH1R132H cells did not resemble those of preclinical and clinical IDH1R132H glioma (eFigure 1). Note that values obtained with E434 and E478, included here

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**Figure 4. In situ metabolic mapping of glutamate dehydrogenase (GLUD) and glutaminase (GLS) activity in IDHwt and IDH1MUT glioma.**

(A) Representative examples of cryostat sections of normal brain, IDH1 wild-type or IDH1-mutated gliomas after in situ metabolic mapping of activity of GLUD1/2 and GLS. Production of reduced insoluble formazan salt (blue dye) is used as a readout to visualise NADH and NADPH production capacity. (B) Quantification of GLUD and GLS activity levels derived from metabolic mapping in IDH1wt (n = 5) and IDH1mut (n = 7) gliomas.
Figure 5. Transcript levels of genes of metabolic enzymes in E434 and E478 xenografts.

Human reads in whole transcriptome RNAseq were mined from the xenografts. For each transcript, levels are expressed as fragments per kilobase per million reads (FPKM). Genes are shown that are involved in glutaminolysis, TCA, glycolysis and pyruvate fate (lactate formation versus TCA entry), respectively. (B) Western blot of E434 and E478 protein extracts, stained with an antibody specific for human BCAT1 and IDH1R132H, showing low BCAT1 transcript levels in E478IDH1-R132H xenografts. γ-Tubulin served as a loading control. (C) IHC analysis and in situ metabolic mapping (ISMM) of the glioma xenograft models E434 and E478 for GLUD1/2. Note that GLUD expression levels as determined with IHC (upper panels), and activity as determined with metabolic mapping (lower panels), are considerably higher in E478 as compared to GLS. Inserts represent control metabolic mapping staining patterns in the absence of substrate.
IDH1-mutated gliomas rely on glutamate metabolism

Figure 6. In vivo multivoxel MRSI of E434 and E478 xenografts.

(A) Local concentrations of lactate, as acquired with MRSI, mapped to corresponding T2-weighted MR images. Left column: E434, right column: E478. The middle panel shows an example of selected voxels for analysis of tumour tissue (yellow) and normal appearing tissue (green). The bottom panel shows corresponding H&E-stained sections. (B) Quantification of lactate, tNAA, glutamate and glutamine levels in both xenografts and healthy mice, based on MRSI data. Values represent the mean concentrations (± standard error) of metabolites in tumour-related quantifiable voxels in 3 or 5 mice per model, and in 3 healthy animals. Asterisk (*) without a bar indicates significantly different levels of the metabolite relative to healty brain (E000), whereas asterisks with a bar indicate significantly different metabolite levels of E434 relative to E478.

to allow a direct comparison to both E98 cell lines, are the ones presented in Figure 5 as well. To test whether a relevant IDH1-mutated metabolic phenotype can be induced by introducing the cells in the brain microenvironment, we injected E98FM-IDH1R132H cells intracerebrally in nude mice. In contrast to control E98FM-EV cells that developed into invasive tumours in all mice within 5 weeks, E98FM-IDH1R132H cells gave rise to very small tumours in 3 out of 5 mice in the same time span (Figure 8a). Only in one of these mice, a tumour was detectable via luciferase imaging (Figure 8b). These experiments indicate that IDH1R132H-overexpressing in vitro models do not resemble gliomas with the endogenous mutation.

Discussion

Since the discovery of frequent IDH1 mutations in diffuse gliomas, research has focused on unraveling the effects of D2HG. Recognizing the important role of this oncometabolite in glioma development, specific inhibitors of mutant IDH1/2 function have been developed. These inhibitors prevent D2HG production and, supposedly, tumour initiation. However, during tumour progression these inhibitors also prevent NADPH oxidation, resulting in normalised redox-status and reduced sensitivity to radiotherapy.

IDH1-mutated gliomas consume α-KG and NADPH, important intermediates and cofactors for fatty acid and membrane synthesis. This may explain slower tumour progression and better prognosis for patients with IDH1-mutated glioma. However, the metabolic stress in IDH1-mutated cancer cells
requires metabolic rewiring for anaplerosis of αKG and NADPH. Here, we present evidence using \textit{in situ} mapping of enzymatic activities and analysis of corresponding transcripts, that clinical and preclinical IDH1-mutated gliomas use glutamate as a prime carbon source for αKG that may partly feed the TCA cycle, but is also used as substrate for D2HG production. We propose that cells adapt to this stress situation by increasing mitochondrial metabolism, and increasing expression of PC to shuttle as much as possible pyruvate to the TCA via oxaloacetate, simultaneously preventing the energy-inefficient flux from pyruvate to lactate. The highly increased levels of PC in our study confirm similar findings in previous reports. Figure 9 depicts these proposed metabolic fluxes in a model of IDH1 wild-type glioma versus IDH1-mutated glioma metabolism. This is in agreement with our recently published \textit{in silico} gene expression data, as described in Chapter 4.

By means of \textit{in vivo} MRSI, we compared metabolite levels in an IDH1-mutated oligodendroglioma xenograft model and an IDH1 wild-type oligodendroglioma xenograft model. The very low levels of tNAA, a neuronal marker, confirmed a high tumour burden in the selected voxels of both models. A decreased level of tNAA in the normal appearing tissue of E478 reflects the infiltrative nature of this model. The decreased level of tNAA is accompanied by decreased glutamate levels which is often observed in MRS of brain tumours which at least partly can be attributed to replacement or displacement of neurons harboring the major depot of glutamate in the brain. In our study the steady state glutamate levels within tumour tissue was similar for both E478 and E434 xenografts, but lower in the normal appearing brain of E478 xenografted mice. Based solely on these data it is difficult to assign this to an effect of the mutation as the tNAA level is also lower in this area. However, combined with other reports on lower glutamate levels in IDH1-mutated gliomas, it is tempting to speculate that lower levels of glutamate in IDH1-mutated brain result from increased consumption.

We found relatively high glutamine levels in E478, which is in line with a decreased GLS activity, in line with other MRS studies of low grade oligoastrocytomas and oligodendrogliomas. Thus, our MRS data indicate significantly less glutamine consumption by E478 cells as compared to E434 cells.

\textbf{Figure 7. IDH1\textsuperscript{R132H} overexpression in E98\textsuperscript{FM} cells does not simulate endogenous IDH1\textsuperscript{R132H}-mutated metabolism.}

\begin{itemize}
  \item \textbf{a} Y-Tubulin, BCAT1, IDH1\textsuperscript{R132H} Western blot showing expression of IDH1\textsuperscript{R132H} in E98\textsuperscript{FM} cells and empty vector (EV) controls. Expression of the mutated IDH1 does not associate with loss of BCAT1 expression in E98\textsuperscript{FM} cells, as is observed in gliomas and xenografts carrying the endogenous IDH1\textsuperscript{R132H} mutation.
  \item \textbf{b} LC-MS showing that IDH1\textsuperscript{R132H} expression in E98\textsuperscript{FM} cells results in high levels of D-2-HG.
  \item \textbf{c} Sulforhodamine B (SRB) cell proliferation assay showing that IDH1\textsuperscript{R132H} expression significantly reduces cell proliferation rates.
\end{itemize}
Figure 8. Overexpression of IDH1^{R132H} in E98FM does not reflect biology of endogenous IDH1^{R132H} models.

(A) Representative examples of H&E staining and IHC staining for human vimentin (used as a tumour marker) of mouse brains with an E98FM and an E98FM-IDH1-R132H xenograft. Only one out of 5 mice showed tumour take, based on luciferase imaging (B,C) whereas control mice developed large tumours in five weeks (B,C).
which can be compensated for by increased uptake and consumption of glutamate, which is in line with a previous in vitro study.\textsuperscript{234} A model of glutamate dependency is further supported by relatively high expression levels of the glutamate importer EAAT2, a finding that was previously reported for low-grade gliomas,\textsuperscript{137} and assigns a role to glutamate in the brain as a chemotactic factor for IDH1-mutated glioma cells, possibly explaining the diffuse invasive character of these cancers.\textsuperscript{106,187

According to this model, the crucial importance of tumour-stroma interactions in IDH1-mutated gliomas may explain the difficulty to establish stable in vitro glioma cell models with the endogenous mutation. It seems reasonable to assume that culture of IDH1-mutated cells in glutamine-containing media results in a selection of cells that have been able to resharlue metabolic pathways in the direction of the use of glutamine instead of glutamate. Conversely, IDH1\textsuperscript{R132H} overexpression in cells with an IDH\textsuperscript{wt} background may directly interfere with cell proliferation because it disrupts metabolic equilibria via excessive αKG and NADPH consumption. As an example, we show here that overexpression of IDH1\textsuperscript{R132H} in E98\textsuperscript{PM} cells does not lead to silencing of BCAT1 and LDHA (eFigure 1), unlike in IDH1-mutated gliomas.\textsuperscript{159,175} Co-expression of BCAT1, GLUT and IDH1\textsuperscript{R132H} leads to a situation in which BCAT1 converts αKG back to glutamate, resulting in a futile glutamate-αKG-glutamate cycle.

In IDH1-mutated gliomas, the prolyl hydroxylase EGLN is hyperactive, resulting in low steady-state levels of hypoxia-inducible factors (HIFs).\textsuperscript{31} In good agreement, expression of HIF target genes HK2, LDHA and carbonic anhydrase 12 are low in IDH1-mutated gliomas, and this was corroborated by low levels of lactate found in E478 xenografts (Figure 6). These data confirm results from 1-\textsuperscript{13}C-pyruvate flux-based MRSI studies showing lack of lactate production in mice carrying orthotopic patient-derived IDH1-mutated glioma xenografts.\textsuperscript{243} Thus, transcriptome analyses allow valuable insight in the metabolic pathways that are active in a tissue, despite the fact that metabolic activity is a result of a complex network of multiple factors. Therefore, targeted RNA next-generation sequencing with smMIPs to elucidate the most prominent metabolic pathways in an individual cancer may in the end aid in the development of novel, rational personalised therapy concepts that include metabolic inhibitors.

In conclusion, our combined data, showing higher activity of GLUD than GLS, and near absence of enzymes involved in glycolysis, suggest that IDH1-mutated gliomas use glutamate to process it as a metabolic rescue pathway for defective isocitrate processing. Glutaminolysis has been suggested as a promising target in IDH1-mutated cancers.\textsuperscript{134} However, our data pinpoint glutamolysis by GLUD as the preferred therapeutic target of choice. Inhibition of GLUD in IDH1 mutant-cells is expected to prevent formation of αKG for TCA cycle anaplerosis and to reduce levels of NADPH, sensitizing cells to chemotherapy and radiation therapy. A second important conclusion from this work is that artificial overexpression of IDH1\textsuperscript{R132H} in IDH1 wild-type glioma cells does not mimic patient-derived IDH1-mutated gliomas, and results from such studies must therefore be interpreted with caution.\textsuperscript{177}

\begin{figure}
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\includegraphics[width=\textwidth]{figure9}
\caption{Summary of metabolic fluxes in IDH\textsuperscript{wt} and IDH\textsuperscript{mut} gliomas.}

IDH1 wild-type gliomas (blue arrows) are predominantly glycolytic, metabolizing glucose to pyruvate (A) and subsequently lactate (C), instead of shuttling pyruvate into the TCA (B). IDH1-mutated gliomas (red arrows) use significantly less glucose than IDH1 wild-type gliomas, and shuttle available pyruvate, possibly lactate-derived, into the TCA as much as possible. Furthermore, IDH1-mutated gliomas primarily utilise glutamolysis (D) as a source of αKG anaplerosis.
\end{figure}