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Identification of a novel inactivating mutation in Isocitrate Dehydrogenase 1 (IDH1-R314C) in a high grade astrocytoma

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The majority of low-grade and secondary high-grade gliomas carry heterozygous hotspot mutations in cytosolic isocitrate dehydrogenase 1 (IDH1) or the mitochondrial variant IDH2. These mutations mostly involve Arg132 in IDH1, and Arg172 or Arg140 in IDH2. Whereas IDHs convert isocitrate to alpha-ketoglutarate (α-KG) with simultaneous reduction of NADP+ to NADPH, these IDH mutants reduce α-KG to D-2-hydroxyglutarate (D-2-HG) while oxidizing NADPH. D-2-HG is a proposed oncometabolite, acting via competitive inhibition of α-KG-dependent enzymes that are involved in metabolism and epigenetic regulation. However, much less is known about the implications of the metabolic stress, imposed by decreased α-KG and NADPH production, for tumor biology. We here present a novel heterozygous IDH1 mutation, IDH1R314C, which was identified by targeted next generation sequencing of a high grade glioma from which a mouse xenograft model and a cell line were generated. IDH1R314C lacks isocitrate-to-α-KG conversion activity due to reduced affinity for NADP+, and differs from the IDH1R132 mutants in that it does not produce D-2-HG. Because IDH1R314C is defective in producing α-KG and NADPH, without concomitant production of the D-2-HG, it represents a valuable tool to study the effects of IDH1-dysfunction on cellular metabolism in the absence of this oncometabolite.

Diffuse gliomas are primary tumors of the brain that are thought to develop from (precursors of) astrocytes or oligodendrocytes. Based on histopathology and according to WHO guidelines, these cancers are classified as grade II-IV astrocytomas or grade II-III oligodendrogliomas1,2. The disease is essentially incurable and median overall survival of patients with grade IV astrocytoma (glioblastoma, the most malignant variant) is only 15 months, despite chemo- and radiotherapy1.

In 2008, somatic mutations in the isocitrate dehydrogenase genes IDH1 and IDH2 were identified in the large majority of low grade gliomas and secondary glioblastomas1,2. These mutations are always heterozygous and mostly involve the same amino acid residues; Arg132 in the cytosolic IDH1 and Arg172 or Arg140 in the mitochondrial IDH23,4. In glioma, the majority of IDH mutations are found in IDH1, and about 90% are IDH1R132H. Extensive research has revealed that these mutations are neomorphic gain-of-function mutations, resulting in the conversion of α-ketoglutarate (α-KG), the normal product of IDH-mediated decarboxylation of isocitrate, to D-2-hydroxyglutarate (D-2-HG) which can accumulate to millimolar concentrations6. During the conversion of α-KG to D-2-HG by the mutant enzyme, NADPH is oxidized.

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Because of the molecular similarity between \(\alpha\)-KG and D-2-HG, the latter can competitively inhibit the activity of a range of \(\alpha\)-KG-dependent dioxygenases, of which over 60 have been identified\(^{11,12}\). Most important examples are the Ten-Eleven-Translocation 2 (TET2) DNA demethylases (resulting in the CpG-island hypermethylator phenotype in IDHWT cells) and Jumonji-C (JMJC) histone demethylases (resulting in histone hypermethylation)\(^{11-13}\). The net result is a transcriptional profile that contributes to tumorigenesis via mechanisms that are still poorly understood. Although this has been matter of debate, consensus is now that D-2-HG can promote degradation of the transcription factor hypoxia-inducible factor (HIF-1\(\alpha\)), possibly after non-enzymatic oxidation to \(\alpha\)-KG\(^{14}\). How this exactly contributes to oncogenesis is still an active field of investigation\(^{15-17}\).

The effects of expression of IDH1-mutants and/or D-2-HG on tumor metabolism have started to receive attention now\(^{24}\). It was recently shown that D-2-HG inhibits the tricarboxylic acid (TCA) cycle enzyme succinate dehydrogenase, possibly leading to hypersuccinylation of proteins and anti-apoptotic effects\(^{19}\). Because mutant IDH1 lacks isocitrate-to-\(\alpha\)-KG conversion activity, normally a predominant source of cytosolic NADPH in the brain\(^{25}\), IDH\(^{\text{MUT}}\) cells are predicted to have lower steady state levels of NADPH, an effect that will be augmented by NADPH-oxidation during D-2-HG production. Thus, IDH mutations impact the redox status of glioma cells.

Additionally, IDH mutations may affect anabolic pathways: IDH1/2 can reduce \(\alpha\)-KG back to isocitrate which may serve as carbon source for fatty acid and lipid synthesis via citrate and acetyl-CoA\(^{21,22}\) but IDH1\(^{132\text{H}}\) lacks this reverse activity\(^{23}\). It is conceivable therefore, that IDH\(^{\text{MUT}}\) tumors need metabolic salvage pathways to allow tumor progression and this is supported by the finding of relatively normal \(\alpha\)-KG levels in a patient-derived orthotopic IDH1\(^{R132H}\) glioblastoma model\(^{21}\). Because IDH\(^{\text{MUT}}\) cells display higher sensitivity to glutaminase inhibitors than IDHWT cells\(^{21}\), glutamine has been proposed to feed in into the mitochondrial TCA cycle as an anaplerotic source of \(\alpha\)-KG, via the activities of glutaminase and glutamate dehydrogenase (GDH). We previously postulated that gliomas may resort to direct import of neurotransmitters in brain to allow GDH-mediated \(\alpha\)-KG production\(^{26-28}\). NADH/NADPH, generated during this reaction would simultaneously supplement the NAD(P)H pool. These metabolic changes could all play a role in tumor cell maintenance and therefore be an Achilles heel and target for therapeutic inhibition. However, uncoupling the metabolic alterations that result from NADPH/\(\alpha\)-KG depletion from the pleiotropic effects of D-2-HG is a difficult task.

In this study we describe a novel heterozygous IDH1 mutation which we uncovered by next generation sequencing of a glioblastoma from which a patient-derived xenograft model and corresponding cell line were generated. We show that this IDH1\(^{R314C}\) mutation does not convert isocitrate to \(\alpha\)-KG, unless at non-physiological concentrations of NADP\(^+\), and does not produce D-2-HG. These properties make that IDH1\(^{R314C}\) tumor models are valuable tools to study the relevance of \(\alpha\)-KG/NADPH depletion versus D-2-HG formation in gliomagenesis and tumor progression.

**Results**

**E98 cells contain a rare heterozygous IDH1\(^{R314C}\) mutation located in the NADP\(^+\) binding pocket.** The patient-derived E98 astrocytoma model carries a number of glioma-typical genetic mutations and is phenotypically similar to clinical glioma when grown as orthotopic xenografts\(^{28-32}\). This makes this model of high interest as a prototypical glioblastoma model for testing of targeted therapeutics\(^{31-34}\). Because the design of rational targeted therapies requires a detailed analysis of genetic aberrations, we subjected this cell line to targeted genomic next generation sequencing via Ion Torrent analysis using a primer set that allows parallel deep sequencing of 409 genes with known involvement in cancer-related pathways. As we unfortunately did not have access to blood of the E98 donor, SNPs and variants with mean allelic frequency (MAF) 1% were filtered out using sequence data from pooled blood samples as reference. Due to our general interest in IDH1, our attention was specifically drawn to a heterozygous C to T mutation at position 940 in the IDH1 gene (Fig. 1A) which results in the p.Arg314Cys substitution. Presence of this mutation was verified in the original patient material (Fig. 1B). To check for the incidence of this mutation, 103 DNA samples from glioma (see Supplementary Table 1) were Sanger sequenced. None of these samples contained the p.R314 mutation. Data mining of the Exome Sequencing Project (ESP) database (www.exac.broadinstitute.org/) revealed that this variant has not been identified in 121,410 alleles. Mining of the Cosmic database revealed only one reported IDH1 c.941G>A/p.R314H mutation in a gastric carcinoma (mutation ID COSM4090677). Thus, the R314C mutation is a rare mutation.

Since Arg314 is located in the NADP\(^+\) binding pocket\(^{35}\) we studied the experimentally solved structure of IDH1\(^{\text{WT}}\) and IDH1\(^{R314C}\) in complex with NADP\(^+\) using Yasara. As shown in Fig. 1C, the R314C mutation significantly alters the tertiary structure resulting in an imperfect fit of NADP\(^+\) in the binding pocket, mainly due to the lack of interaction between the negatively charged 2'-phosphate of the \(\alpha\)-co-enzyme NADP\(^+\) and the positively charged amine group of the arginine, and lack of ionic interactions between the Arg314 and glutamate residues on position 247 and 253 in the other subunit.

**IDH1\(^{R314C}\) is defective in isocitrate to \(\alpha\)-KG conversion.** We first examined the activity of purified IDH1\(^{R314C}\)-GST proteins, using IDH1\(^{\text{WT}}\)-GST and IDH1\(^{R132H}\)-GST as positive and negative controls. All enzymes could be readily produced and purified (Fig. 2A). Under the conditions tested, both IDH1\(^{R132H}\)-GST and IDH1\(^{R314C}\)-GST did not show isocitrate-to-\(\alpha\)-KG conversion activity whereas IDH1\(^{\text{WT}}\)-GST did (Fig. 2B). The absence of \(\alpha\)-KG formation by IDH1\(^{R132H}\)-GST and IDH1\(^{R314C}\) was confirmed with LC-MS (Fig. 2C). Importantly, whereas IDH1\(^{R132H}\)-GST was active in converting \(\alpha\)-KG to D-2-HG (as revealed by oxidation of NADPH) the IDH1\(^{R314C}\)-GST mutant was completely devoid of this activity (Fig. 2D). Thus, IDH1\(^{R314C}\) homodimers are inactive in converting isocitrate and NADP\(^+\) to \(\alpha\)-KG and NADPH and, unlike IDH1\(^{R132H}\)-GST, do not consume NADPH, consistent with the lack of D-2-HG production by E98 xenografts that has already been described before\(^{36}\).

The IDH1\(^{R132}\) mutation in human gliomas always occurs in a heterozygous manner, resulting in a theoretical 1:2:1 distribution of IDH1\(^{\text{WT/WT}}\), IDH1\(^{\text{WT/MUT}}\) and IDH1\(^{\text{MUT/MUT}}\) dimers. This stoichiometry may be important for...
balanced IDH activity, because D-2-HG can only be formed via an intimate crosstalk between wild-type and mutant subunits, for which α-KG is a product and substrate, respectively. To analyze the effects of IDH1 R314C overexpression in eukaryotic cells we performed enzymatic assays with cell extracts from HEK293T cells, transiently overexpressing IDH1WT, IDH1R132H or IDH1R314C as well as lentivirally transduced LN229 and U251 glioma cells, stably expressing the different IDH1 mutants (Fig. 3A). To be able to distinguish recombinant from endogenous IDHWT and allow statements on IDH WT/MUT ratios we equipped the mutated reading frames with a BAPHIS (biotin-acceptor peptide-8xHis) tag, increasing molecular weight with ~3 kDa. Intriguingly, irrespective of the method of genetic modification (transfection or lentiviral transduction) expression levels of recombinant enzymes were lower than that of the endogenous enzymes, with IDHR132H consistently being lowest, possibly a result of negative selection for IDH1 R132H-BAPHIS overexpression and associated toxic levels of D-2-HG. Consistent with the absence of activity of purified IDH1R314C homodimers (Fig. 2B), for all cell lines NADP+ dependent oxidative decarboxylation of isocitrate was significantly lower in IDH1R314C-BAPHIS expressing cells than in IDH1 WT-BAPHIS cells (Fig. 3B). Of note, activity of GST-IDH1R314C homodimers at higher concentrations of NADP+ was too low to calculate Km for isocitrate.

Figure 1. Identification of IDH1R314C in a high grade astrocytoma. (A) Sequencing trace showing the heterozygous C to T mutation at position 940 in the IDH1 gene found in the E98 cell line and (B) primary tumor material. This mutation leads to the p.Arg314Cys substitution in the IDH1 protein. (C) Display of the 3D-structure of human IDH1R314C generated with YASARA (PDB-file 3inm). The IDH1WT homodimer is displayed with bound cofactor NADP+ (yellow), and the arginine residue on position 314 (red), both subunits are coloured in a different shade of grey. The inset shows the mutated cysteine (red) superimposed on the original arginine (green).
As expected from the lack of NADPH consumption when incubated with α-KG (Fig. 2D), IDH1R314C-BAPHIS expressing LN229 and U251 glioblastoma cell lines did not produce 2-HG, in contrast to their IDH1R132H-BAPHIS counterparts (Fig. 3D).

Discussion

Since their discovery, hotspot mutations in IDH enzymes have become a major focus of research. The oncogenic potential of IDH1R132H has been attributed to epigenetic alterations, resulting from the production of the alleged oncometabolite D-2-HG9,10,38. Furthermore, it has been shown that proliferation of IDH1R132H cells requires metabolic rescue programs that depend on in vivo tumor-stroma interactions, possibly involving glutamate import26.

We here identify and characterize IDH1R314C, a novel and rare mutation in IDH1 in a high-grade astrocytoma which has not been reported before. We show that IDH1R314C has a greatly reduced affinity for NADP+, in agreement with a previous structure-function relationship study on porcine IDH135 and is consequently defective in oxidizing isocitrate to α-KG. The R314C mutation did not shift the cofactor dependency from NADP+ to NAD+ (Supplementary Figure 2) as was previously reported for mutated IDH1 from the halobacterium Haloferax Volcanii39.

To define an exact role for IDH1R314C in tumor formation and biological behavior of cancer cells is impossible in the context of the many more mutations that are found in E9840. To study this further, isogenic E98 cell lines with a repaired IDH1R314R allele are required and such technically challenging cell lines are not yet available.

The decreased potential of IDH1R314C cells to produce α-KG and NADPH in the cytosol may directly impact on redox potential, lipid synthesis, and membrane production and may explain the slow growth rates of E98 cells in vitro (doubling time of 46 hrs, data not shown). Interestingly, despite these very slow growth rates, in vivo E98 xenografts represent the most aggressive diffuse infiltrative brain tumor model in our lab, killing mice within 4–5 weeks after intracerebral implantation28. This illustrates that the brain micro-environment is crucially involved in allowing growth of E98 cells. Since the redox status of cells may particularly impact on cell migration41 it would be of high interest to investigate how relatively low NADPH/NADP+ ratios in vivo are associated with active cell migration, leading to the diffuse infiltrative phenotype. Further unraveling of the specific in vivo metabolic salvage pathways that are adapted by E98 cells and may be involved in its aggressive behavior may lead to potential novel ways for therapeutic intervention.

In oncology, most NGS protocols aim for detection of actionable hot spot mutations in known genes only, leaving a potentially huge amount of mutations with significance for tumor biology, undetected and this is the reason why we only now recognized that E98 cells carry the IDH1R314C mutation54. Alterations in genes encoding metabolic enzymes may be directly or indirectly involved in tumorigenesis based on subtle distortions in equilibria between metabolic intermediates and/or oxidative milieu.

In conclusion, we identified a novel IDH1R314C mutant that shares with the common IDH1R132H mutant a defect in isocitrate-to-α-KG conversion activity, but lacks the capacity to produce D-2-HG. Future comparative
profiling studies using isogenic cell lines expressing IDH\textSUP{WT}, IDH\textSUP{R132H} or IDH\textSUP{R314C} will allow us to distinguish between epigenetic and metabolic alterations, resulting from NADPH/α-KG depletion or D-2-HG accumulation.

**Methods**

**E98.** Generation of the E98 model which was derived from a grade IV astrocytoma, was described before\textsuperscript{28}. All animal experimental work was performed in accordance with the guidelines of and was approved by the local ethical committee for animal experimentation of Radboud University. An adherent E98 cell line was generated from mouse xenografts via routine procedures\textsuperscript{29,40} and cultured in DMEM (Lonza, Basel, Switzerland) supplemented with 10% FCS (Gibco, ThermoFisher Scientific, Watlham, MA, USA) and 40 μg/ml gentamycin (Centrafarm, Etten-Leur The Netherlands).

**Next generation sequencing.** Genomic DNA from E98 cells and from original tumor material was analyzed by semi-conductor sequencing (IonPGM, Life Technologies, ThermoFisher Scientific, Watlham, MA, USA) using the comprehensive cancer panel (Life Technologies, ThermoFisher Scientific, Watlham, MA, USA) detecting copy number variations and mutations in 409 cancer-related genes. An IonPGM E98 library was generated according to the manufacturer’s protocol. In short, 10 ng of DNA per pool was amplified in 21 cycles by PCR using the Ion AmpliSeq mastermix, followed by barcode and adapter ligation. Amplified products were purified with Agencourt AMPure XP beads (Beckman Coulter Genomics, High Wycombe, UK). The library was diluted to 20 pM and emulsion PCR was performed using the Ion OneTouchTM 200 Template kit following the manufacturer’s protocol. Next, Ion Sphere Particles (ISPs) were recovered and enriched for template positive ISPs using Dynabeads MyOne Streptavidin C1 beads (Life Technologies, ThermoFisher Scientific, Watlham, MA, USA) in the Ion OneTouchTM ES instrument (Life Technologies, ThermoFisher Scientific, Watlham, MA, USA). ISP enrichment was quantified using the Qubit 2.0 fluorometer (Life Technologies, ThermoFisher Scientific, MA,
PCR and cloning of IDH1 constructs. Total RNA was isolated from cell lines using TRIzol reagent (Life Technologies, ThermoFisher Scientific, Waltham, MA, USA) and converted to cDNA using oligo-dT and MMLV reverse transcriptase (New England Biolabs, Ipswich, MA, USA) using standard protocols. Open reading frames for IDH1WT, IDH1R132H, and IDH1R132K were PCR amplified from HT29, E478 and E98 cDNA, respectively, using primers IDH1-EcoRIFw [5′-CAATTCTAGGTAAAGTTTACGGT-3′] and IDH1-AgeIRrv [5′-CCACGTAAGTTGGCCTAGT-3′] and Phusion DNA polymerase (Finzymes, ThermoFisher Scientific, Waltham, MA, USA). PCR products were digested with EcoRI and AgeI (New England Biolabs, Ipswich, MA, USA) and cloned in fusion to a C-terminal biotin-acceptor peptide (BAP) and an 8xhis tag (HIS) in vector pHLeuc-BAPHIS AgeI/EcoRI, derived from pHLeuc-HIS (Addgene, Cambridge, MA, USA) using T4 DNA ligase (New England Biolabs, Ipswich, MA, USA). The BAPHIS tags were added to distinguish recombinant from endogenous IDH1 by molecular weight and to allow Ni-bead purification. Clones containing the respective mutations were selected via Sanger sequencing. For generation of lentiviral constructs, the different ORFs were recloned in vector pENTRY which was recloned with pLENTI using the GATEWAY system via standard procedures. In parallel, IDH ORFs were cloned in fusion with GST in E. coli expression vector pDEST15 as described before.47

GST fusion protein expression and purification. GST fusion proteins were expressed in E. coli strain ER2566 via standard induction protocols. Briefly, 2xTY medium supplemented with 50 μg/ml ampicillin (Roche, Basel, Switzerland) was inoculated with 1% v/v overnight culture. Protein expression was induced with 1 mM IPTG (Serva, Heidelberg, Germany) at OD600 = 0.6. After 3 hours at 30 °C, bacteria were harvested by centrifugation and resuspended in 300 mM NaCl, 50 mM TRIS/HCL pH 8.0 supplemented with Complete protease inhibitor (Roche, Basel, Switzerland). Cells were lysed by 3 freeze-thaw cycles, followed by sonication (4 × 15 seconds, 25% amplitude, Bioruptor, Diagenode) and centrifugation (3500 rpm, 20 min). Proteins were purified using glutathione beads (Pierce, ThermoFisher Scientific, Waltham, MA, USA), eluted with 10 mM glutathione and dialyzed extensively against 150 mM NaCl, 25 mM TRIS/HCL pH 7.5. Expression and purity were checked by SDS-PAGE gel electrophoresis and Coomassie Brilliant Blue staining.
NADP⁺ concentrations (0–500 μM) and 100 μM isocitrate at 22 °C. Absorbance at 340 nm was measured every 20 seconds with a Benchmark Plus Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA). Non-linear regression analysis (Michaelis-Menten) was performed in Graphpad Prism 5.03.

To determine NAD⁺ dependency, 4 μg of IDH1-GST proteins were incubated with 100 μl assay solution containing 2 mM MgCl₂, 500 μM isocitrate and 2, 5 mM NAD⁺ (Sigma-Aldrich, St. Louis, MO, USA) in 100 mM TRIS-HCL pH 7.4 at 22 °C. A sucrose extract made from human liver was taken as a control. Absorbance at 340 nm was monitored as described.

For enzyme measurements in extracts of eukaryotic cells, 50 μg of total cytosolic protein were incubated with assay solution (2 mM MgCl₂, 500 μM isocitrate in 100 mM TRIS/HCL pH 7.4) containing 100 μM NADP⁺ in 96 wells plates (Greiner) at 22 °C and NADPH generation was quantified as described before.

### Table 1. MRM transitions for metabolite detection

<table>
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<tr>
<th>MRM</th>
<th>Compound name</th>
<th>cone V (V)</th>
<th>Coll eV (eV)</th>
<th>Ret. time (min)</th>
</tr>
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<tbody>
<tr>
<td>191 &gt; 111</td>
<td>Isocitrate</td>
<td>22</td>
<td>13</td>
<td>1.28</td>
</tr>
<tr>
<td>145 &gt; 101</td>
<td>α-KG</td>
<td>22</td>
<td>8</td>
<td>1.53</td>
</tr>
<tr>
<td>147 &gt; 129</td>
<td>D-2-HG</td>
<td>19</td>
<td>11</td>
<td>1.54</td>
</tr>
<tr>
<td>152 &gt; 134</td>
<td>13C₂-2-HG</td>
<td>19</td>
<td>11</td>
<td>1.54</td>
</tr>
</tbody>
</table>

D-2-HG, α-KG and isocitrate levels were determined in in vitro IDH1 reaction mixtures. 4 μg of IDH1-GST proteins were incubated in 150 μl freshly prepared assay solution (2 mM MgCl₂ in 100 mM TRIS/HCL pH 7.4) containing 500 μM isocitrate and 100 μM NADP⁺. After 20 min incubation at 22 °C the reaction was stopped using 20 μl 4% HCOOH. 25 μl of this solution was mixed with 25 μl 9 μM 13C₂-2-HG stable isotope (Chiralex, Nijmegen, The Netherlands) in deionized water and 75 μl deionized water before passing it through an Amicon Ultra 30 kDa Centrifugal Filter (Merck Millpore, Billerica, MA, USA) by centrifugation (14000 g, 30 min). 2 μl of the filtrate was injected onto a Atlantis T3 HPLC Column (2.1*100 mM dp 3 μl) and run with 12.5% MeOH in 87.5% 0.3% HCOOH in water. The column was connected to a Waters Quattro Premier tandem mass spectrometry fitted with an ESI probe operated in negative mode, recording the following MRM transitions, as summarized in Table 1.

### Statistics

All experiments were performed in triplicate. Data were processed in Excell (Microsoft, Redmond, WA, USA) and enzyme kinetics were determined by fitting of Michaelis Menten in Graphpad Prism 5.03 (Graphpad Software, La Jolla, CA, USA).

### References


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Author Contributions
S.A.M.v.L., A.C.N. and W.P.J.L. wrote the manuscript and prepared the figures, W.P.J.L., S.A.M.v.L. and K.V. produced recombinant proteins, K.L. and C.J.FvN. were involved in the conceptual design of experiments and manuscript. All authors reviewed the manuscript.

Additional Information
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