

Expanding the spectrum of *IDH1* mutations in gliomas

Ruta Gupta^{1,2}, Simon Flanagan^{1,2}, Cheryl CY Li³, Maggie Lee^{1,2}, Brindha Shivalingham⁴, Sanaz Maleki², Helen R Wheeler⁵ and Michael E Buckland^{1,2}

¹Department of Neuropathology, Royal Prince Alfred Hospital, Sydney, NSW, Australia; ²Discipline of Pathology, Sydney Medical School, University of Sydney, Sydney, NSW, Australia; ³Victor Chang Cardiac Research Institute, Sydney, NSW, Australia; ⁴Department of Neurosurgery, Royal Prince Alfred Hospital, Sydney, NSW, Australia and ⁵Department of Medical Oncology, Royal North Shore Hospital, St Leonards, NSW, Australia

Mutations in isocitrate dehydrogenase -1 or -2 (*IDH1* or *IDH2*) are found in the majority of WHO grade II and III diffuse gliomas and secondary glioblastomas. *IDH* mutation screening is rapidly becoming part of the routine pathological work up of human brain tumors, providing both diagnostic and prognostic information. Here, we characterize four rare and novel *IDH1* mutations identified in surgical human glioma samples: two instances of an *IDH1* p.R132S mutation caused by a previously undescribed dinucleotide deletion/insertion mutation, a novel homozygous somatic *IDH1* p.R132L mutation, and an *IDH1* p.R100Q mutation. Characterization of novel and rare *IDH* mutations may provide additional insight into the mechanisms of mutant *IDH* in neoplasia. Furthermore, given the clinical import of *IDH* status, these results highlight the need for comprehensive mutation screening, beyond the targeted identification of common pathogenic variants.

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IDH1 and *IDH2* mutations were first identified during exome-wide sequencing of glioblastomas in 2008,¹ occurring predominantly in those that had arisen through progression of a lower grade glioma (ie, secondary glioblastomas). Subsequently, *IDH* mutations have been described in 70–80% of grade II and III astrocytomas, oligodendroglomas and secondary glioblastomas, but are rare or nonexistent in other brain tumors.² *IDH* mutations tend to occur in younger individuals and carry significant prognostic significance, in that those with *IDH*-mutant tumors have longer disease-free survival and show better response to treatment.^{3–5} For these reasons, assessment of *IDH* mutation status has rapidly become incorporated as a standard component in the neuropathological assessment of brain tumors.⁶

IDH mutations in gliomas are largely known to be heterozygous missense mutations in codon R132 of *IDH1*, or much less commonly, the synonymous codon (R172) of *IDH2*. These mutations result in

substitution of a highly conserved and structurally important arginine residue in the *IDH* substrate binding site.^{2,7} The most common *IDH* mutation is *IDH1* p.R132H, accounting for around 90% of *IDH*-mutant glioma cases; less commonly, the *IDH1* R132 substitution is with serine (p.R132S), leucine (p.R132L), glycine (p.R132G), cysteine (p.R132C) or valine (p.R132V)^{2,8} and, at the molecular level, all but one of the reported cases are due to single-nucleotide substitutions. Mutations of *IDH2* are relatively rare, accounting for ~3–5% of all described *IDH* mutations, but they carry similar prognostic implications.¹

Mutations in *IDH* genes are thought to contribute to neoplasia through a dominant-negative effect on the protein's wild-type function and/or a neomorphic gain of function of the mutant protein.⁶ Downstream effects of mutant *IDH* include decreased cellular NADPH and alpha-ketoglutarate (α KG) levels,⁹ HIF1 α stabilization,⁹ and increased production of 2-hydroxyglutarate (2HG),¹⁰ which competitively inhibits α KG-dependant enzymes such as histone methyltransferases and 5-methylcytosine hydroxylases.¹¹ Homozygous mutations of *IDH1* or *IDH2* in gliomas have not been described, arguing against a simple loss of function mechanism.

Correspondence: Dr ME Buckland, MD, PhD, FRCPA, Department of Neuropathology, University of Sydney and RPA Hospital, Room 502A, D06 Blackburn Building, Sydney, NSW 2006, Australia. E-mail: michael.buckland@sydney.edu.au

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The focused nature of *IDH* mutations has lent itself to a variety of detection techniques. Immunohistochemistry is a well-established component of diagnostic pathology workflows, and antibodies against the common *IDH1* p.R132H mutant protein, as well as against a variant mutant protein, *IDH1* p.R132S, are commercially available.^{12,13} Although the gold standard of clinical mutation detection is unbiased Sanger sequencing, other more focused methods such as pyrosequencing,^{14,15} PCR-based restriction fragment length polymorphism,¹⁶ high-resolution melt (HRM) analysis,^{17,18} and single-base extension¹⁹ methods are commonly used. Most of these alternate mutation detection strategies require prior knowledge of the spectrum of possible mutations, and thus have the potential to miss novel variants.

Here, we report the identification and characterization of very rare and novel *IDH1* mutations, encountered during clinical mutation assessment of human neurosurgical specimens. These findings expand the known spectrum of *IDH* mutations in human gliomas, and have important implications for the design of future mutation detection strategies.

Materials and methods

Tumor Samples

In all, 177 formalin-fixed paraffin-embedded (FFPE) brain tumors referred to the Royal Prince Alfred Hospital, Sydney, Australia for primary or secondary consultation were assessed by immunohistochemistry and bidirectional Sanger sequencing of exon 4 of *IDH1* and *IDH2*; selected mutation cases were also interrogated by single-nucleotide primer extension (SNuPE) and/or pyrosequencing. Demographic and clinical details were obtained during routine diagnostic work up or referral notes. Tumor details are listed in Table 1.

Immunohistochemistry

Monoclonal antibodies against *IDH1* p.R132H (clone H09; Dianova, Germany) and *IDH1* p.R132S; (kind gift from Dr Y Kato, Japan) were used at 1:500 dilution on 5 µm-FFPE tumor sections, with antigen retrieval performed in 10 mM sodium citrate buffer pH 6.0, for 20 min at 125 °C. Sections were incubated in primary antibodies for 1 h at room temperature and antibody detection was performed via Dako EnVision + (Dako, Carpinteria, USA) as per the manufacturer's instructions. Analysis for 1p/19q codeletion was performed via fluorescence *in situ* hybridization (FISH) using commercially available labeled BAC probesets (Abbott-Vysis, USA) as per the manufacturer's recommendations and scored as previously described.²⁰

Table 1 Tumors tested and *IDH* mutation status

Diagnosis	<i>IDH</i> mutation	<i>IDH1</i> (R132H)	<i>IDH1</i> (other)	<i>IDH2</i> (R172K)
Astrocytoma (II), n = 24	19 (79%)	13	2 (R132S) 2 (R132G) 1 (R132C)	1
Oligodendrogloma (II), n = 14	14 (100%)	13	1 (R132S)	0
Oligoastro (III), n = 5	3 (60%)	3	0	0
Anaplastic astro (III), n = 35	14 (40%) ^a	8	2 (R132S) ^b 3 (R132G) 1 (R132L) ^b	0
Anaplastic oligo (III), n = 7	7 (100%)	6	0	1
GBM (IV), n = 77	15 (19%)	13	1 (R132S) ^b 1 (R100Q) ^b	0
Other, n = 15	0	0	0	0

Abbreviation: *IDH*, isocitrate dehydrogenase.

Other tumors tested (bottom row) consisted of gliosarcoma (2), ependymoma (2), anaplastic ependymoma (1), pilocytic astrocytoma (4), central neurocytoma (2), subependymal giant cell astrocytoma (1), ganglioglioma (1), rosette forming glioneuronal tumor (1), and dysembryoplastic neuroepithelial tumor (1).

^aThe anaplastic astrocytoma category included a group of referred H09-immunonegative tumors, biasing the proportion of mutated tumors in this subgroup.

^bDenotes the rare and novel mutations reported in this manuscript.

DNA Extraction and PCR

DNA extraction of each sample was performed on five 10 µm sections of FFPE tissue, which were deparaffinized by washing with xylene twice, followed by a single ethanol wash. Extraction from the deparaffinized tissue was performed using the Qiagen DNEasy blood and tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. All PCRs were performed in 50 mM Tris/HCl, 10 mM KCl, 5 mM (NH₄)₂SO₄ and 2 mM MgCl₂, with 0.2 mM of each dNTP, 2U of FastStart Taq polymerase (Roche, Manheim, Germany), 0.5 µM of each primer and 200 ng DNA in a total volume of 20 µl. PCR cycling conditions consisted of initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation, annealing and extension at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, respectively.

Sequencing

Bidirectional Sanger sequencing of exon 4 of *IDH1* and exon 4 of *IDH2* was performed on PCR products generated using the primer pairs *IDH1e4_1F*/*IDH1e4_2RS* and *IDH2e4F*/*IDH2e4R*, respectively, (see Table 2 for primer sequences). PCR products were sequenced bidirectionally using M13-F and M13-R primers using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Sequencing

reaction products were resolved on a 3730×1 DNA Analyzer (Applied Biosystems).

Pyrosequencing was performed on a 155-bp fragment spanning codon 132 of *IDH1*, amplified using primers IDH1_pyroF and IDH1_pyroR (see Table 2 for primer sequences). Pyrosequencing of PCR products was performed using the Pyromark Q24 pyrosequencing platform (Qiagen, Hilden, Germany) using the internal sequencing primer IDH1_pyroS (Table 2) according to the manufacturer's instructions.

SNuPE and Loss of Heterozygosity (LOH) Analysis

SNuPE was performed on two microlitres of the 155 bp PCR product from the pyrosequencing assay. It was analyzed using the Snapshot Multiplex System (Applied Biosystems) and interrogation primers IDH1_INT1 and IND1_INT2 (Table 2), according to the manufacturer's instructions.

LOH analysis was assessed by PCR amplification of the microsatellite markers D2S325, D2S2321, D2S1276, D2S2178 and D2S2274 using fluorescently labeled primers. Primer sequences for each marker were retrieved from NCBI's UniSTS database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=unists>). PCRs were performed as described above, but with only 30 cycles of denaturing, annealing and extension at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, respectively. Products were resolved on a 3730xl DNA Analyzer (Applied Biosystems).

Results

IDH Mutations in an Australian Glioma Cohort

The 177 brain tumors in this study were from 77 males and 100 females. The mean age was 54 years (median age 55.5 years), and there was no

Table 2 PCR, sequencing and SNuPE primers used in the study

Name	Sequence 5'-3'
M13-F	TGTAAACGACGGCCAGT
M13-R	CAGGAACAGCTATGACC
IDH1e4_1F	TGTAAACGACGGCCAGT catttgtctgaaaaacttgcgtt
IDH1e4_2RS	CAGGAAACAGCTATGACC tcacattattgccaacatgc
IDH1_pyroF	AAATCACCAATGGCACCATA
IDH1_pyroR	TTGCCAACATGACTTACTTGATC
IDH1_pyroS	GACTTACTTGATCCCCATA
IDH1_INT1	GACTGACTGATGGGTAAAACT TATCATCATAGGT
IDH1_INT2	ACTGACTTTGATCCCCATAAGCATGA
IDH2 e4 F	TGTAAAACGACGGCCAGT ggttccaaatctgtggaaagatg
IDH2 e4 R	CAGGAAACAGCTATGACC gctaggcgaggagctccagt

Abbreviation: IDH, isocitrate dehydrogenase; SNuPE, single-nucleotide primer extension.

gender difference in age of onset. The tumor type and mutation status is summarized in Table 1. A number of anaplastic astrocytomas included in this cohort were previously identified as IDH1 (p.R132H)-immunonegative by another laboratory and were referred to us for sequencing; thus, there is likely ascertainment bias accounting for the lower than expected proportion of *IDH*-mutant tumors in this subgroup. Overall, the clinicopathological features of our glioma cohort are similar to those described from other centers across the world. However, four of the 72 mutations we identified were very unusual or completely novel, and are described in detail below.

Two Cases of a Novel Dinucleotide Deletion/Insertion Mutation Leading to IDH1 p.R132S

Two gliomas, an anaplastic astrocytoma and a glioblastoma, were found to contain a heterozygous dinucleotide deletion/insertion at *IDH1* codon 132 (c.394_395CGdelinsTC, p.R132S) on Sanger sequencing (Figure 1). Sequencing was unable to discriminate between involvement of a single *IDH1* allele with the dinucleotide deletion/insertion or a compound heterozygous mutation involving both *IDH1* alleles (Figure 1a). Immunohistochemistry using the IDH1 p.R132S monoclonal antibody SMab-1 demonstrated strong immunoreactivity of tumor cells in the anaplastic astrocytoma, confirming the presence R132S mutant protein expression (Figure 1b) and hence the dinucleotide deletion/insertion event. The glioblastoma was R132S-immunonegative. Multiplex *IDH1* SNuPE assay confirmed the presence of mutations at both c.394 and c.395 positions (Figure 1c), but we cannot exclude a compound heterozygous mutation in this tumor. The R132S-immunopositive anaplastic astrocytoma was removed from a 59-year-old male with a non-enhancing temporoparietal tumor who had previously had a WHO grade II astrocytoma removed 9 years earlier without adjuvant therapy. He is alive and disease free 38 months post resection. His initial resection was unavailable for testing. The immunonegative glioblastoma arose in the splenium of a 67-year-old female, without clinical, radiological or pathological evidence of pre-existing low-grade glioma.

An IDH1 p.R132L Mutation Associated with LOH of *IDH1*

A 5-cm well-circumscribed, solid, non-enhancing lesion in the left parietal lobe was discovered incidentally in a 36-year-old female (Figure 2a). She underwent gross total resection of the tumor, and neuropathological examination indicated an anaplastic astrocytoma, WHO grade III (Figure 2b). There was no 1p36.3 or 19q.13 loss (as determined by FISH), and IDH1 p.R132H immunohistochemistry

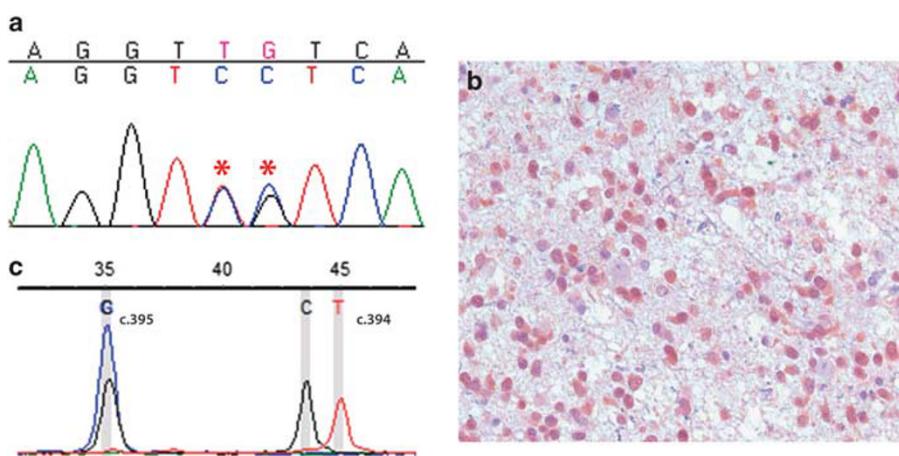


Figure 1 A two base pair insertion/deletion causing *IDH1* p.R132S. (a) Sanger sequencing of DNA extracted from an anaplastic astrocytoma demonstrated heterozygous dinucleotide deletion/insertion of *IDH1* codon 132 (c.394_395CGdelinsTC, p.R132S; asterisks (*)). Sanger sequencing was unable to discriminate between involvement of a single *IDH1* allele with the dinucleotide mutation and a compound heterozygous mutation involving both *IDH1* alleles. (b) Immunohistochemistry with SMab-1 confirmed R132S mutant protein expression restricted to tumor cells. (Original magnification: $\times 400$). (c) Multiplex *IDH1* SNuPE confirmed the Sanger sequencing of an additional case of dinucleotide insertion/deletion mutation in a glioblastoma, which was immunonegative for SMab-1.

was negative. Bidirectional Sanger sequencing demonstrated a seemingly homozygous *IDH1* p.R132L mutation (Figure 2c). Quantitative pyrosequencing of codon 132 of *IDH1* indicated the presence of 76% mutant allele and 24% wild-type allele (Figure 2d). There was no family history of brain tumors or other malignancies, and the patient's peripheral blood was normal (not shown), militating against a germline defect. LOH analysis for the microsatellite markers D2S322, D2S2274, D2S2208, D2S2242 and D2S2321 flanking the *IDH1* locus on 2q was performed on the patient's tumor and peripheral blood. This demonstrated LOH of all three informative markers (D2S2321, D2S2178, D2S2274) restricted to tumor DNA (Figure 2e). D2S2321 is centromeric while D2S2178 and D2S2274 are telomeric to *IDH1*, confirming LOH of at least 2Mb encompassing the entire *IDH1* gene, with retention of the mutant allele in tumor DNA. The patient had postoperative adjuvant therapy (radiation and temozolomide) and is disease-free at 18 months post resection.

IDH1 p. R100Q Mutation

A 54-year-old female patient had an enhancing left insular tumor. Formal histopathology revealed a mixed oligoastrocytic tumor with focal necrosis. In the previous WHO classification (2000), this tumor would be classified as a WHO grade III anaplastic oligoastrocytoma, however, the presence of focal necrosis in the specimen now qualifies it as a glioblastoma with oligodendrogloma component, WHO (2007) grade IV²¹ (Figure 3a). FISH revealed an isolated 19q deletion. *IDH1* p.R132H immunohistochemistry on the tumor was negative. Codon 132 of *IDH1* and codon 172 of *IDH2* were wild-type on bidirectional Sanger sequencing. However, the

IDH1 sequencing amplicon also included codon R100, and identified a heterozygous *IDH1* p. R100Q mutation (c.299G>A, p.R100Q) (Figure 3b). Unfortunately, this patient was from a distant regional center and was lost to follow-up 6 months post-discharge, so long term outcome information was not available.

Discussion

The relative frequency and clinical characteristics of *IDH* mutated gliomas in this Australian cohort are similar to those described by in reports from a variety of centers across the globe including the United States,² India,²² Germany,⁸ France,⁵ China²³ and Japan.²⁴ However, a striking finding is the frequency of rare or previously undescribed mutations in our cohort. This may in part be explained by the fact that many laboratories rely on more focused mutation detection methods such as immunohistochemistry, HRM or single-nucleotide extension assays, which are designed to detect known/characterized *IDH* mutations and may not recognize uncharacterised or rare variants. Australia has a diverse mix of ethnic groups, including a distinct indigenous population as well as numerous residents of South East Asian, Polynesian and Melanesian background, and it is possible that certain racial haplotypes may have a higher frequency of unusual mutations.

Dinucleotide deletion/insertion mutations, or tandem base pair mutations, are uncommon genetic lesions in both human somatic and germline disease.²⁵ Only one dinucleotide deletion/insertion mutation of *IDH1* has been reported previously in gliomas, in that case a (c.394_395CGdelinsGT, p.R132V) was identified in a study of 685 brain

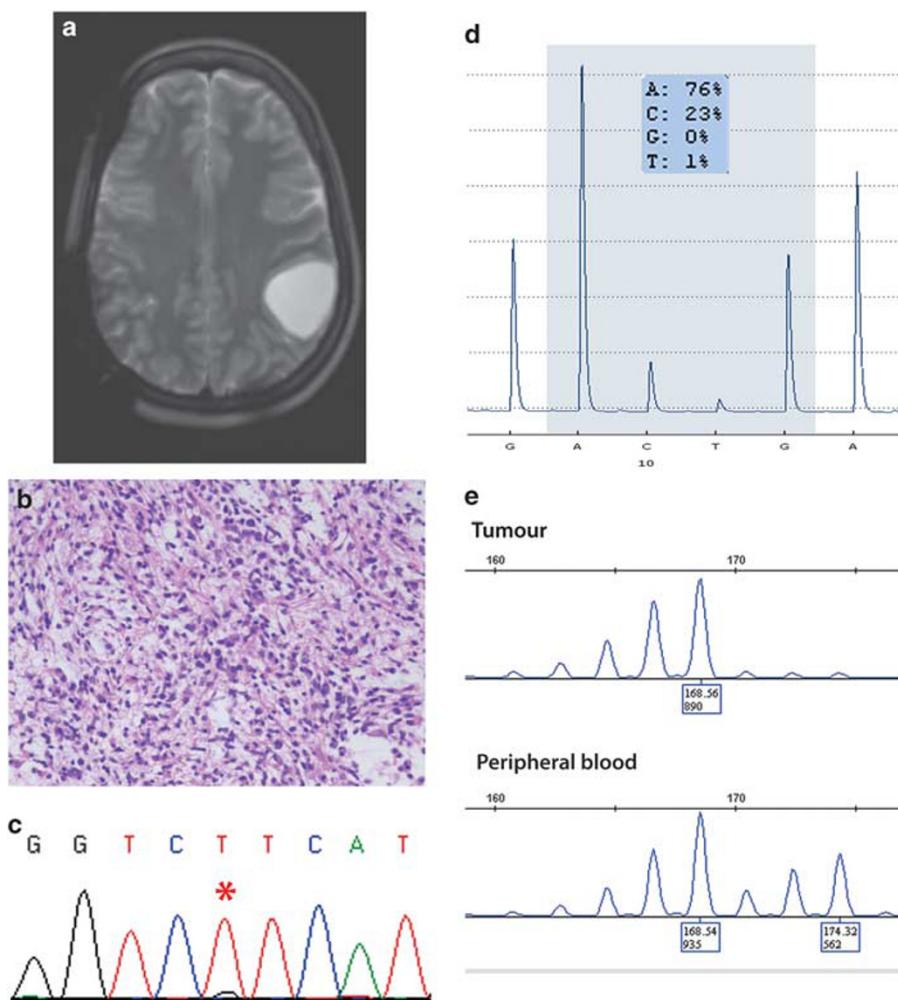


Figure 2 A novel *IDH1* p.R132L mutation with loss of heterozygosity (LOH). (a) Axial T1-weighted MRI of a 5-cm well-circumscribed non-enhancing tumor in the left parietal lobe. (b) Haematoxylin and eosin (H&E) stained sections confirmed an anaplastic astrocytoma WHO grade III (original magnification: $\times 200$). (c) Sanger sequencing of *IDH1* exon 4 demonstrates a seemingly homozygous *IDH1* (c.395G>T, p.R132L) mutation (asterisk). (d) Quantitative pyrosequencing confirms the presence of 76% mutant allele and 24% wild-type allele, consistent with homozygous mutant tumor admixed with some non-neoplastic cells. (e) LOH analysis for the microsatellite markers D2S322 (shown), D2S2274, D2S2208, D2S2242 and D2S2321 flanking the *IDH1* locus on 2q on the patients tumor and peripheral blood demonstrated LOH of all three informative markers in tumor DNA, confirming LOH of at least 2 Mb encompassing the entire *IDH1* gene, with retention of the homozygous mutant allele in tumor DNA.

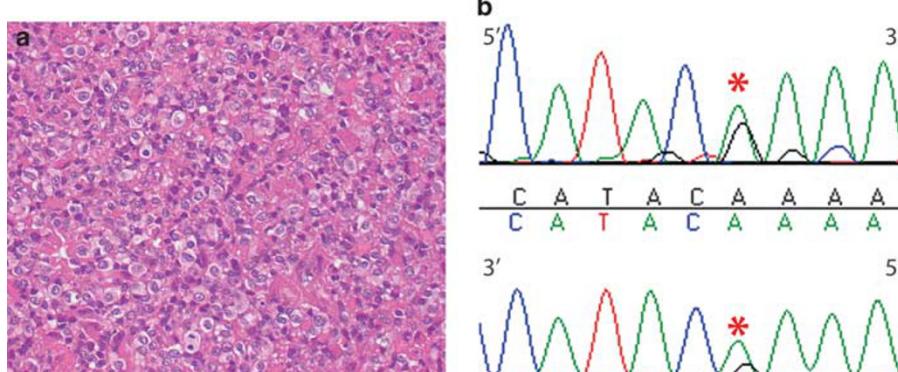


Figure 3 (a) A glioblastoma with oligodendrogloma component, WHO (2007) grade IV. (H&E stain, original magnification: $\times 200$). (b) A heterozygous missense *IDH1* p.R100Q mutation (c.299G>A, p.R100Q) was identified by bidirectional Sanger sequencing. No other *IDH1* or *IDH2* exon 4 mutations were present.

tumors,⁸ the only reported example of the p.R132W mutation. These unusual dinucleotide mutations have also been seen as rare events in AML patients, and four instances of heterozygous dinucleotide deletion/insertion mutation of *IDH1* (c.395_396GTdelinsAC) as an alternate path to the *IDH1* p.R132H mutant protein have been reported in a study of 416 Chinese AML patients.²⁶ Ultraviolet radiation and *cis*-platinum chemotherapy are commonly associated with these dinucleotide deletion/insertion mutations,²⁷ but neither of these factors appear relevant to the two cases described above. The fact that these unusual mutations in both gliomas and AML are missense mutations involving the critical arginine residue at codon 132 is further evidence of the importance of this residue in the effects of mutant *IDH1* in neoplasia.

This cohort also includes an apparently homozygous *IDH1* p.R132L mutation due to LOH at the *IDH1* allele. The pathological features of this case were of a typical anaplastic astrocytoma, although the clinical presentation (an incidental finding) and radiological characteristics were slightly unusual. To our knowledge, only a single case of partial LOH of mutant *IDH1* has been previously characterized. In that case, LOH was identified on progression of a diffuse astrocytoma to secondary glioblastoma with adjuvant radiotherapy.²⁸ However, in contrast to the current case, this previous case demonstrated partial loss of the mutant *IDH1* allele, rather than a loss of the wild-type *IDH1* allele described here. Furthermore, the current case was a primary (ie, untreated) tumor, with homozygous *IDH1* mutation status identified on initial testing. Although LOH in neoplasia is most typically associated with tumor suppressor genes, where it contributes to loss of function of that gene, *in vitro* evidence indicates that mutant *IDH1* is capable of 2HG production, both in the absence and presence of expression of the wild-type *IDH1* allele.¹⁰ In fact, co-expression of wild-type *IDH1* with the mutant protein (as is the case in most *IDH1*-mutated gliomas) may enhance 2HG production, as wild-type *IDH1* activity increases levels of NADPH and α KG, both necessary substrates for neomorphic production of 2HG.¹⁰ LOH of the *IDH1* locus is very uncommon in gliomas,²⁹ while homozygous *IDH1* mutations in AML are very rare,²⁶ and whether the clinical behavior of these tumors is distinct from those with typical *IDH* mutations is not known.

In addition to R132, there are two other arginines present in the *IDH1*-substrate binding site. The *IDH1* R100 residue is synonymous with the *IDH2* R140 residue, which is commonly mutated in myeloid malignancies. *In vitro* assessment of the *IDH1* p.R100Q mutant has demonstrated that it results in overproduction of 2-hydroxy glutarate (2HG)³⁰ in a comparable manner to *IDH1* p.R132 and *IDH2* p.R140 mutations. The case reported here represents the fourth known example of the *IDH1* p.R100Q mutation in gliomas; the three other instances described previously in a single report.²⁸

The prevalence of mutations in the synonymous *IDH2* arginine residue (R140) in myeloid malignancies and the similar functional consequences of both the *IDH1* p.R132H and p.R100Q mutations upon 2HG levels³⁰ suggests that this mutation would have similar clinical implications to the typical *IDH1* R132 mutations.

These results indicate that the spectrum of *IDH1* defects in adult gliomas is more diverse than would be expected from the current literature, and that direct sequencing of exon 4 of *IDH1* and 2 has the potential to identify non-canonical mutations, which may be missed by other methods.

Note added in proof

Between acceptance and publication of this manuscript, Jin and colleagues³¹ have reported two additional cases of LOH at the *IDH1* allele in gliomas. In comparison to our case of *de novo* LOH, these additional cases were identified upon progression of IDH-mutant anaplastic astrocytomas to secondary glioblastomas.

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Disclosure/conflict of interest

Dr Buckland is a Cancer Institute NSW Clinical Research Fellow. The remaining authors declare no conflict of interest.

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