technique is combined with another method. This method increases the sensitivity to detect a mutant allele contained in a sample [64].

The novelty of COLD-PCR is the use of critical denaturation temperature (Tc), which is lower than the standard denaturation temperature. At the Tc, the mismatched DNA formed by mutant and wild-type sequences is denatured, while the homo-duplex DNA of the mutant or wild-type sequences remains double stranded. Primer annealing and DNA extension by DNA polymerase follow the denaturation at the Tc in this procedure. Since the mismatched DNA formed by the mutant and wild-type sequences is selectively denatured, mutant sequences at low concentration are selectively amplified. This method improves the sensitivity of Sanger sequencing, pyrosequencing, or melting curve analysis [64]. Some studies have reported highly sensitive assays for *IDH1* mutations by combining COLD-PCR with melting curve analysis [60, 61].

Molecular imaging

Another approach to detect *IDH1/2* mutations is molecular imaging. The accumulated (R)-2HG in *IDH1/2* mutated tumors has been considered as a good target for this approach, because this aberrant metabolite is only present at low levels in the normal tissue [65]. Recent studies have shown successful in vivo detection of (R)-2HG using magnetic resonance spectroscopy (MRS) [65–67]. The challenging point of this approach in vivo is that the (R)-2HG spectrum overlaps with that of other metabolites, including glutamate, glutamine, *N*-acetyl-L-aspartate (NAA), or gamma-aminobutyric acid (GABA) [65–67]. These studies have claimed that they overcame this by different MRS sequence optimized in each study (reviewed elsewhere [65]). The signal of (R)-2HG is present in background noises and further improvement will be needed for the robust detection in clinical use. These preliminary reports necessitate further MRS validation and standardization in clinical cases. Nonetheless, this approach potentially poses several advantages over the other *IDH1/2* testing methods. This approach enables noninvasive and quantitative analysis of (R)-2HG, and hence *IDH1/2* status. The concentration of (R)-2HG possibly reflects the tumor status, including cellularity [66]. Identification of (R)-2HG hotspots might also provide information that may help the planning of targeted biopsy [65]. Moreover, the most significant value of this method is the availability for repetitive and temporal measurement during the clinical course. Temporal testing using MRS might enable the dynamic monitoring of the effects of therapeutic agents, including inhibitors of mutant IDH1/2 in the future [68].

Which method should we use for *IDH1/2* testing?

Several studies have compared the sensitivity and robustness of each method. In general, IHC using mutation-specific antibodies, pyrosequencing, or melting curve analysis has a higher sensitivity than Sanger sequencing [46, 49, 57, 62]. The high sensitivity, as reported in each method, is however not always required for all situations, if sufficient tissue specimen is adequately obtained. The optimal method for *IDH1/2* testing varies depending on various factors, including the purpose, sample types (FFPE or frozen tissue), sample number (high throughput analysis is required or not), or laboratory equipment [46].

IHC is sensitive, robust, and accessible. Therefore, this technique is recommended for initial screening [15]. If *IDH1/2* status is critical for a clinical decision, another method should be used in cases showing negative results in IHC, to avoid missing non-R132H mutations. The high throughput techniques, including pyrosequencing and melting curve analysis, are suitable for large-scale studies in which DNA samples are already prepared [46]. Sanger sequencing provides accurate information, if specimens with sufficient tumor cell content are obtained.

Conclusion

As reviewed in this report, *IDH1/2* mutations have distinct relationships with clinical features, pathology, or other genetic/epigenetic alterations. *IDH1/2* status is currently among the most important molecular markers of gliomas. The significance of *IDH1/2* status argues for further improvement and standardization of *IDH1/2* testing methods for practical use. The development of an accurate and robust method for molecular markers is a prerequisite for the establishment of molecular classification of gliomas in the future.

Acknowledgments This work was supported by JSPS KAKENHI Grant Numbers 26861171 (H.A.), 25462283 (K.I.) and by the National Cancer Center Research and Development Fund 23-A-50 (K.I.).

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Brain Tumor Pathol (2015) 32:22–30
DOI 10.1007/s10014-014-0186-0

ORIGINAL ARTICLE

Development of a robust and sensitive pyrosequencing assay for the detection of *IDH1/2* mutations in gliomas

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Received: 17 February 2014 / Accepted: 30 March 2014 / Published online: 19 April 2014

Abstract Assessment of the mutational status of the isocitrate dehydrogenase 1/2 (*IDH1/2*) gene has become an integral part of the standard diagnostic procedure and, therefore, needs to be accurate. This may, however, be compromised by various factors including the method of analysis and a low tumor cell content. We have developed a rapid, sensitive and robust assay to detect all types of mutation in either *IDH1* or *IDH2* using pyrosequencing. The efficacy of detecting mutation was evaluated using a panel of control plasmids representing all the different types of *IDH1/2* mutation and a set of 160 tumor specimens. The sensitivity of the assays was examined by a serial dilution analysis performed on samples containing various ratios of wild-type and mutant alleles. The pyrosequencing assay detected as little as 5 % of mutant alleles for most mutation types, while conventional Sanger sequencing required the presence of at least 20 % of mutant alleles for identifying mutations. The pyrosequencing assay detected *IDH1/2* mutations in three samples which were missed by Sanger sequencing due to their low tumor cell contents. Our assay is particularly useful for the analysis of a large number of specimens as in a retrospective clinical study for example.

Keywords Glioma · *IDH1* · *IDH2* · Pyrosequencing · Mutation detection

Electronic supplementary material The online version of this article (doi:10.1007/s10014-014-0186-0) contains supplementary material, which is available to authorized users.

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Introduction

Isocitrate dehydrogenase 1/2 (*IDH1/2*) mutations are regarded as one of the earliest genetic alterations in gliomagenesis, based on the mutation profiles of various subtypes of gliomas as well as primary and recurrent tumors [13, 30]. *IDH1/2* mutations are predominantly found in World Health Organization (WHO) grade II and III gliomas and secondary glioblastomas [3, 12, 30, 31]. Mutations in *IDH1/2* have been associated with longer survival in every histological type or every WHO grade [25, 31]. It is now clear that the *IDH1/2* mutational status defines two biologically and clinically distinct groups of gliomas. Determining the mutational status of *IDH1/2* has become a part of the standard diagnostic procedure and may be used for stratification in clinical trials as it is one of the major prognostic factors in gliomas [28]. It is, therefore, absolutely essential that the status of *IDH1/2* must be accurately and robustly assessed. Various factors may, however, potentially compromise the authenticity of the results; these include the method of analysis, the type of tumor specimen [frozen or formalin-fixed paraffin-embedded (FFPE) samples] and the tumor cell content.

Almost all reported mutations of *IDH1/2* in gliomas are heterozygous missense affecting either codon 132 in *IDH1* or codon 172 in *IDH2* [11]. About 90 % of all *IDH1/2* mutations are c.395G>A (R132H) in *IDH1* [11]. Other *IDH1* mutations include c.394C>T (R132C), c.394C>A (R132S), c.394C>G (R132G), c.395G>T (R132L), R132P (nucleotide change not reported in the original article) and R132V (c.394C>G and c.395G>T); the latter two mutations reported only in single cases [9, 29]. *IDH2* c.515G>A (R172K) accounts for about 3 % of all *IDH1/2* mutations, and other *IDH2* mutations include c.515G>T (R172M), c.514A>T (R172W), c.516G>C (R172S) and c.514A>G (R172G) [11].

Direct sequencing and immunohistochemistry (IHC) are the most widely used methods for assessing the *IDH1/2* status. Sanger sequencing, however, has the limitation of being unable to detect mutations in tumor samples that contain extensive necrosis or are contaminated with non-neoplastic cells; the accuracy of Sanger sequencing, therefore, largely depends on the quality of the sample [1, 5]. For IHC, two specific antibodies for the mutant R132H, DIA-H09 and IMab-1, are commercially available and well-characterized [4, 15]. The significant advantage that IHC has over Sanger sequencing is that FFPE samples are readily available through routine histopathological examination. IHC, however, can only detect the mutation specific to the antibody used. Antibodies specific for mutations other than R132H have also been developed [14, 16], however, their efficacy needs to be further validated in clinic.

We have developed rapid and robust assays for the detection of *IDH1/2* mutations using pyrosequencing, which is a sequence-by-synthesis technique based on the luciferase–luciferin light release as a signal for nucleotide incorporation into target DNA [24]. Our novel assays enable the detection of all reported mutations in *IDH1* or *IDH2* at a single run for each gene. We describe the details of our original assay and evaluate its potential efficacy in clinical application.

Materials and methods

DNA samples

Frozen tissue samples from a total of 160 glioma cases operated at the National Cancer Center Hospital (Tokyo, Japan) were included in this study; 29 diffuse astrocytomas, 11 oligoastrocytomas, 2 oligodendrogliomas, 28 anaplastic astrocytomas, 21 anaplastic oligoastrocytomas, 8 anaplastic oligodendrogliomas, 55 primary glioblastomas and 6 secondary glioblastomas. Matched FFPE samples were available for analysis in nineteen cases (Supplementary Table 1). Twenty blood samples were also analyzed as a normal control. The study was approved by the local institutional review board. Histological diagnoses were made according to the WHO classification [18]. A DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan) was used to extract DNA.

Control plasmids

All control plasmids that contain every single type of mutation except *IDH1* R132L (see below) were generated by subcloning the mutated sequences from the tumor samples (obtained from the Department of Pathology, University of Cambridge [12]). Briefly, after amplifying the genomic DNA containing the different types of *IDH1/2* mutation, the polymerase chain reaction (PCR) product was subcloned into the pMD20-T vector by a TA cloning procedure using the 10X A-Attachment mix (TOYOBO, Osaka, Japan) and a Mighty Cloning Kit (TAKARA Bio Inc., Tokyo, Japan) according to the manufacturers' recommendations. The control plasmids for R132H, R132C, R132S and R132G in *IDH1*, and R172K, R172M, R172W and R172S in *IDH2* were all generated using the method described above. The plasmid containing the *IDH1* R132L (c.395G>T) mutation was generated by site-directed mutagenesis because no samples in our tumor cohort had this mutation. For this procedure, 50 ng of plasmids with wild-type *IDH1* were unidirectionally amplified using a complementary pair of oligonucleotides containing the mutation, and the non-mutant *dam*-methylated template plasmid DNA was digested using the DpnI restriction enzyme (New England Biolabs Japan Inc., Tokyo, Japan) before the newly synthesized mutated construct is transformed into *E. coli*.

Pyrosequencing

Polymerase chain reaction primers were designed for amplifying relatively small DNA fragments, either 86 bp for *IDH1* or 85 bp for *IDH2* sequences, containing the targeted region so that the assay could potentially be used for DNA extracted from archival tissues. Detailed information about the primers is given in Table 1. Templates for pyrosequencing were prepared by amplifying genomic DNA (10 ng) with primers that were biotinylated for the template strands. The 25 μl PCR mix included 62.5 μM of each dNTP, 0.625 units of Ampli Taq Gold 360 DNA polymerase and 0.5 μM of primers for *IDH1/2* each as per manufacturer's recommendations. The $MgCl_2$ concentration of the PCR mix was optimized for each primer set; 2 mM for *IDH1* and 1.5 mM for *IDH2*. The thermal cycling conditions for amplification were as follows: one cycle of initial denaturation at 95 °C for 10 min, followed

Table 1 Sequences of the primers for PCR for pyrosequencing, Sanger sequencing, and the pyrosequencing assays

Procedure	Sequence
PCR for pyrosequencing	
For *IDH1* (product length 86 bp)	
Forward primer (PC6041)	CAAAAATATCCCCCGGCTTG
Reverse primer (PC6042)	bio-CAACATGACTTACTTGATCCCC
For *IDH2* (product length 85 bp)	
Forward primer (PC6099)	ACATCCCACGCCTAGTCCC
Reverse primer (PC6100)	bio-TCTCCACCCTGGCCTACCTG
Pyrosequencing	
For *IDH1*	
Primer (P0125)	ACCTATCATCATAGGT
Sequence to analyze	CDTCATGCTTAT
Dispensation order	GATCATGTCATG
Assay type	AQ assay
For *IDH2*	
Primer (P0126)	CCCATCACCATTGGC
Sequence to analyze	ANGCAC
Dispensation order	TATGTCACGCAC
Assay type	AQ assay
Sanger sequencing [10]	
For *IDH1* (product length 254 bp)	
Forward primer (IDH1 fc)	ACCAAATGGCACCATACGA
Reverse primer (IDH1 rc)	TTCATACCTTGCTTAATGGGTGT
For *IDH2* (product length 293 bp)	
Forward primer (IDH2 fc)	GCTGCAGTGGGACCACTATT
Reverse primer (IDH2 rc)	TGTGGCCTTGTACTGCAGAG

by 35 cycles 95 °C 30 s, 55 °C 30 s, and 72 °C 30 s. An additional cycle at 72 °C for 5 min was added to complete the elongation step. Amplification of the PCR products was confirmed by running 3 μl of the reaction mix on an agarose gel.

Single-stranded templates for pyrosequencing were prepared as per manufacturer's recommendations using 20 μl of PCR template (Qiagen, Tokyo, Japan). The purified single-stranded PCR products were denatured and annealed to 15 pmol of pyrosequencing primer. Pyrosequencing was performed using the PyroGold Q96 SQA Reagents and the PyroMark Q96 software (version 2.5.7) on a PSQ96 pyrosequencer (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. The data were analyzed using the PyroMark Q96 software. The 3′ end of the pyrosequencing primers was designed immediately upstream of each hotspot. The dispensation orders for pyrosequencing were designed so that all possible mutations at the first two positions of codon 132 of *IDH1* and codon 172 of *IDH2* could be identified in a single assay for each gene (indicated in Table 1). An AQ analysis, which is an analysis mode within the PyroMark Q96 software, was performed so that the percentage of mutant allele could be quantified in this assay.

Sanger sequencing

Templates for Sanger sequencing were prepared by amplifying 10 ng of genomic DNA with a set of primers (Table 1). The 10 μl PCR mix included 2.0 mM of $MgCl_2$, 125 μM of each dNTP, 0.5 units of Ampli Taq Gold 360 DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 0.5 μM of primer pairs (IDH1 fc and rc for *IDH1*, or IDH2 fc and rc for *IDH2* [10]). The same primer pair as the one used for *IDH1* pyrosequencing (PC6041 and PC6042) was used in a single FFPE sample (DA068) which was not sufficiently amplified by the standard primer pairs for Sanger. The thermal cycling for amplification was as follows: one cycle of initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C 30 s, 55 °C 30 s, 72 °C 30 s with an additional cycle of 72 °C for 7 min. Amplification of the 254 bp (*IDH1*) or 293 bp (*IDH2*) product was confirmed by running 3 μl of the reaction mix on an agarose gel. After purification using ExoSAP (Affymetrix Japan KK, Tokyo, Japan) as per manufacturer's recommendations, cycle sequencing was carried out using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and the same forward primer (IDH1 fc, PC6041 or IDH2 fc) as the amplification of genomic DNA.

The thermal cycling for amplification was as follows: 25 cycles of 96 °C 10 s, 50 °C 15 s, and 60 °C 4 min.

Immunohistochemistry

Immunohistochemistry using a mouse monoclonal anti-human IDH1 R132H antibody (H09, Dianova, Hamburg, Germany) was performed by a polymeric method of EnVision FLEX system (Dako Japan Inc, Tokyo, Japan) with an automatic staining machine (Auto-stainer Link 48, Dako Japan Inc, Tokyo, Japan) as previously reported [8]. The presence of positive granular cytoplasmic staining in the tumor cells was judged as being indicative of mutant *IDH1*.

Results

The sensitivity and specificity of the newly developed pyrosequencing assays for *IDH1* and *IDH2* mutation

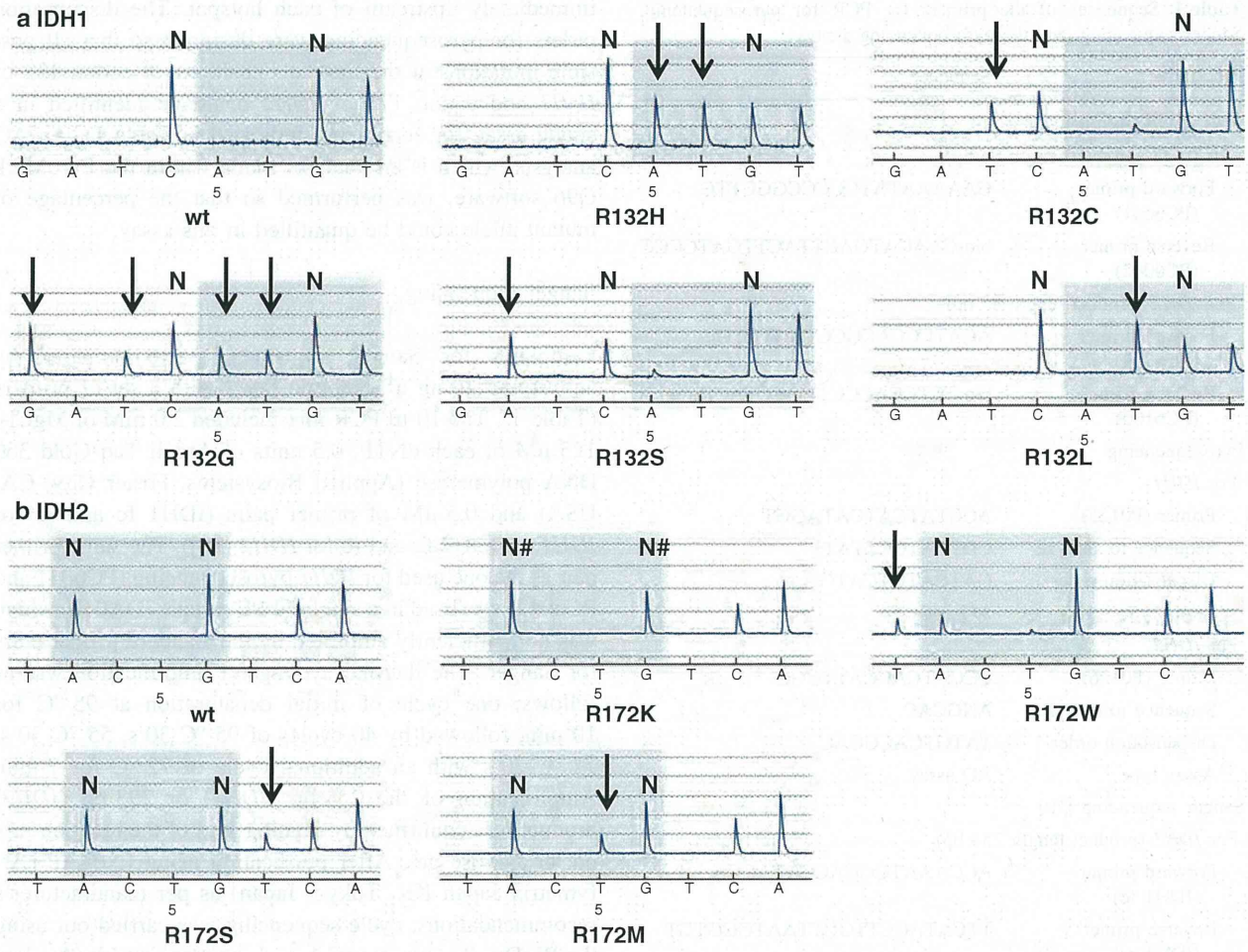

Fig. 1 Pyrograms for each *IDH1/2* mutation. Samples containing equal amounts of wild-type and mutant DNA were subjected to the pyrosequencing assays. The pyrograms show the mutation-specific pattern obtained for each mutation in *IDH1* (**a**) or *IDH2* (**b**) indicated by the *arrows*. "N" denotes the normal peaks. In this assay, all the mutations but *IDH2* R172K have their specific patterns of peaks which are not present in the wild-type samples. The *IDH2* R172K mutation is detected as a higher peak at the 3rd dispensation (*A*) and a lower peak at the 6th dispensation (*G*); those peaks are marked by "N#"

screening were first validated using the control DNA mixture that contains equal amounts of the wild-type and mutated plasmid constructs. All *IDH1* mutations were successfully identified by detecting mutant-specific peaks in a single assay. As shown in Fig. 1a, only one peak at the 4th dispensation (C) was observed among the first 5 dispensations in the pyrogram of the wild-type *IDH1*, while abnormal peaks were observed either at the 1st, 2nd, 3rd, 5th or 6th dispensation only in the mutated DNA, but not in the wild type. As for *IDH2*, two peaks at the 3rd (A) and 6th (G) dispensations were observed in the pyrogram of the wild type, while abnormal peaks unique to each mutation were observed at the 2nd, 5th and 7th dispensations in R172W, R172M and R172S mutants, respectively (Fig. 1b). The *IDH2* R172K mutation could be detected by a peak twice as high as the wild type at the 3rd dispensation and a peak half as high as the wild type at the 6th dispensation. Thus, our assays identify all mutants of *IDH1/2*, except the R172K mutant, by the presence of abnormal peaks which should be absent in the wild-type *IDH2*. The R172K mutation can also be detected by quantifying the mutant allele frequency using an AQ analysis as described below.

Twenty blood samples were then subjected to pyrosequencing to determine the threshold of normal variation. For the c.395 position of *IDH1*, the mean frequency of A, T, G (wild type) and C was 0 %, 0.078 ± 0.065 % (range 0–0.27 %), 99.9 ± 0.1 % (range 99.7–100 %) and 0 %, respectively. For the c.514 position of *IDH2*, the mean frequency of A, T, G (wild type) and C was 7.0 ± 2.6 % (range 3.6–13.9 %), 0 %, 93.0 ± 2.6 % (range 86.1–96.4 %) and 0 %, respectively. Based on the

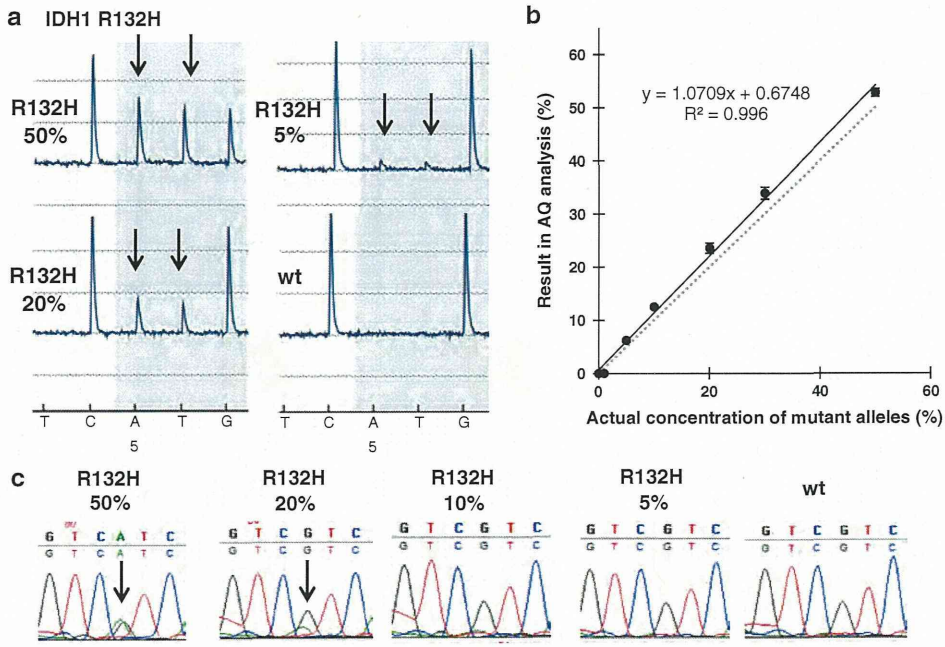

Fig. 2 Serial dilution analysis for the evaluation of the sensitivity in detecting R132H mutation in *IDH1*. **a** A mixture of the control plasmid constructs containing variable ratios of wild-type and R132H mutant alleles of *IDH1* was subjected to pyrosequencing. Even 5 % of mutant allele could be detected as a peak as shown in the pyrogram (the mutated peaks are indicated by *arrows*). **b** The triplicated results of the pyrosequencing assay plotted against the expected concentration showing a very high concordance ($R^2 = 0.996$). **c** The peak of the mutant allele in the Sanger sequencing chromatogram was obscure in samples containing 10 % or less mutant DNA

maximum error ratio in the normal blood controls, the mutant allele frequency of 0.27 % or less for *IDH1* and 13.9 % or less for *IDH2* will be considered as within normal variation.

Next, serial dilution experiments were performed to assess the sensitivity of the pyrosequencing assay to detect *IDH1/2* mutations. Pyrosequencing and Sanger sequencing were performed on either R132H of *IDH1* or R172K of *IDH2* mutant construct serially diluted with the wild-type plasmid to achieve ratios of mutant DNA of 0, 1, 5, 10, 20, 30 or 50 % in triplicate as follows. The presence of the *IDH1* R132H mutant allele was detected as a clearly distinct peak in the pyrogram in samples containing 5 % or more mutant DNA (Fig. 2a) whereas the mutant peak in the chromatograms obtained from direct sequencing was apparent only in samples with 20 % or more mutant DNA (Fig. 2c). The mean measured frequencies of the triplicate experiments showed a strong linear correlation, being almost equal to the actual frequencies of the mutant alleles ($R^2 = 0.996, p < 0.0001$) (Fig. 2b). Based on the threshold defined in the above experiments, the samples showing a mutant allele ratio of 5 % or more were judged to be mutated in this assay. This sensitivity was remarkably comparable with the value found in previously reported assays [7, 26]. As for R172K in *IDH2*, because it was not possible to design an assay to detect the mutation by the presence of a unique peak, this mutation could nevertheless be characterized by quantifying the peak common to wild-type and mutated alleles using the AQ analysis. The mean frequencies of triplicate experiments were slightly higher, nonetheless strongly correlated with the expected frequencies of the mutant alleles ([Percentage in AQ analysis] = 1.07 * [Actual percentage] +4.13, $R^2 = 0.998$, $p < 0.0001$) (Fig. 3b). Based on the results of the blood samples, the samples showing a mutant allele concentration of 10 % or above were considered as mutated in this assay. In Sanger sequencing, the mutant allele could only be detected in samples containing at least 10 % of the mutated DNA (Fig. 3c).

Finally, the pyrosequencing assay and Sanger sequencing were compared in a series of glioma samples to validate the efficacy of detecting mutations on genuine clinical cases. *IDH1* was Sanger sequenced in all 160 cases and *IDH2* in selected cases mainly consisted of those without *IDH1* mutations ($n = 113$). The result of each case is shown in Supplementary Table 1. The pyrosequencing-based analysis for *IDH1* detected mutations in 75 cases (74 cases with R132H and a single case with R132S), while the Sanger sequencing failed to detect three R132H mutant cases (DA068, AA067 and OA 040). The frequencies of mutant alleles measured by pyrosequencing were low in the three discordant cases (10.8–16.2 %), suggesting that those samples contained a low percentage of tumor cells. The results of the pyrosequencing and Sanger sequencing screening for *IDH2*, which identified R172K mutations in 4 tumors (Supplementary Table 1), were identical in all cases analyzed.

Immunohistochemistry was performed on 69 cases including the three discordant cases between Sanger sequencing and pyrosequencing. IHC and pyrosequencing results were concordant in all cases. To ensure that exactly the same specimen was used for comparison, the three

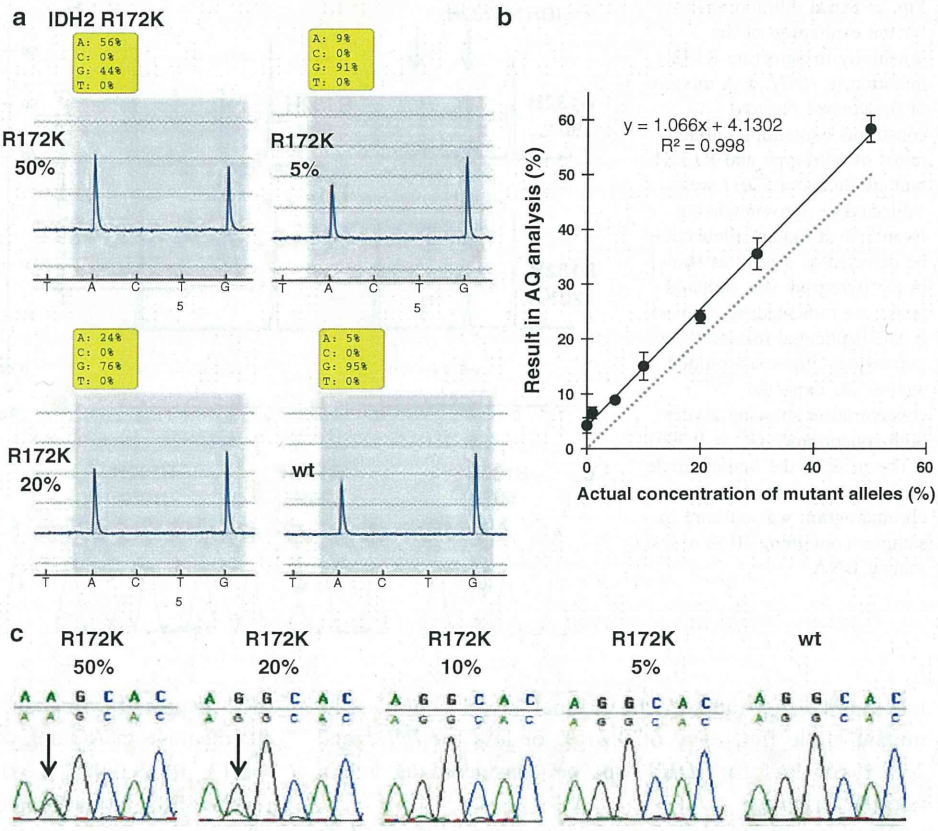

Fig. 3 Serial dilution analysis for the evaluation of the sensitivity in detecting R172K mutation in *IDH2*. **a** Samples containing variable concentrations of wild-type and R172K mutant alleles of *IDH2* were subjected to pyrosequencing. For detection of this mutation, the assessment was based on the allele ratio calculated by the AQ assay as shown in the *insets*. **b** The pyrosequencing assay gives slightly higher values, nonetheless it showed a high concordance with the expected concentration. **c** The peak of mutant allele in the Sanger sequencing (*arrows*) was obscure in samples containing 10 % or less mutant DNA

discordant cases between pyrosequencing and Sanger sequencing (DA068, AA067 and OA 040) were subjected to a new round of Sanger sequencing and pyrosequencing using DNA extracted from sequentially sectioned FFPE samples which were used for IHC. In one of the three previously discordant cases (DA068), the R132H mutation in *IDH1* was detected by pyrosequencing and IHC, but not by Sanger sequencing (Fig. 4). In the other two previously discordant cases (AA067 and OA040), the IHC was found positive and the repeated Sanger sequencing also confirmed the presence of the mutations as a minor allele.

To test whether pyrosequencing is applicable to FFPE, the DNAs extracted from a further 16 matched FFPE specimens were subjected to pyrosequencing for *IDH1*. The results from FFPE and frozen samples were consistent in all cases examined (Supplementary Table 1).

Discussion

The purpose of this paper is to present and discuss in detail a novel pyrosequencing assay for the mutational analysis of *IDH1/2*. This protocol was specifically designed with the clinical setting in mind and should, therefore, be quickly and easily implemented in any laboratories equipped with a pyrosequencer. The assay is fully compatible with Sanger sequencing and shown to be robust, efficient and more sensitive in detecting low-level mutations than the Sanger sequencing method. This assay is particularly useful for the genetic screening of *IDH1/2* mutations in a large tumor cohort as exemplified by its successful application in our recent study of gliomas [2].

Pyrosequencing has been applied to various genetic analyses including mutation detection at hotspots in *KRAS*, or quantitative measurement of methylation levels in the CpG island of the O^6-Methylguanine-DNA methyltransferase (*MGMT*) gene using bisulfite-modified DNA [19, 21, 23]. Several previous studies have also reported the use of pyrosequencing for the detection of *IDH1/2* mutations [6, 7, 17, 20, 26, 27, 32]. These studies mainly focused on the feasibility of detecting *IDH1/2* mutations by pyrosequencing or the clinical significance of these mutations, however, full details of the assay were not given, which prevented independent validation of the assay by others [6, 7, 20, 27, 32]. Our novel pyrosequencing assays for detecting *IDH1/2* mutations are fully validated using engineered controls for almost all known *IDH1* R132 and *IDH2* R172 mutations. We also strived to provide all the technical details to allow an immediate replication of the technique. The robustness of a pyrosequencing-based