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RESEARCH ARTICLE

IDH1 Mutations in Oligodendroglial Tumors: Comparative Analysis of Direct Sequencing, Pyrosequencing, Immunohistochemistry, Nested PCR and PNA-Mediated Clamping PCR

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Keywords
isocitrate dehydrogenase 1, oligodendroglial tumors, PNA-mediated clamping PCR, pyrosequencing.

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Abstract

Mutations in isocitrate dehydrogenase 1 (*IDH1*) are found in a high proportion of glial tumors and have a significant prognostic impact. Although direct sequencing has been considered to be the gold-standard method to detect this mutation, the sensitivity of this technique has been questioned especially because specimens from glial tumors may contain large numbers of non-tumor cells. We screened 141 cases of oligodendroglial tumors for *IDH1* mutations using peptide nucleic acid (PNA)-mediated clamping polymerase chain reaction (PCR) and compared the results with the results of direct sequencing, pyrosequencing, and immunohistochemistry (IHC). Nested PCR was only performed in cases having mutant *IDH1* only discovered by clamping PCR. Using dilution experiments mixing *IDH1* wild-type and mutant DNA samples, clamping PCR detected mutations in samples with a 1% tumor DNA composition. Using PNA clamping PCR, we detected 138 of 141 (97.9%) cases with mutant *IDH1* in our series, which is significantly higher ($P = 0.016$; PNA clamping vs. direct sequencing) than those of direct sequencing (74.5%), pyrosequencing (75.2%) and IHC (75.9%). From our results, almost all oligodendroglial tumors have *IDH1* mutations, and this suggests that *IDH1* mutation is an early and common event especially in the development of oligodendroglial tumors.

INTRODUCTION

Mutations in isocitrate dehydrogenase 1 (*IDH1*) are frequently and selectively found in a high proportion of gliomas (4, 21). They are remarkably specific to a single codon in the highly conserved and functionally important Arg132 residue in *IDH1* gene. Most of these mutations are G395A (Arg132His), while C394G (Arg132Ser), G395T (Arg132Leu), C394G (Arg132Gly) and C394T (Arg132Cys) constitute a minor proportion of *IDH1* mutations (25). Recent studies have demonstrated the prognostic impact of *IDH1* mutations in World Health Organization (WHO) grade II, III and IV gliomas (10, 25), and now *IDH1* mutation has become one of the most significant and clinically relevant issues in the current neuro-oncology field.

Direct sequencing has been considered to be the gold standard for the detection of *IDH1* mutation. However, the sensitivity of this method is influenced by sample quality, requiring at least 50% of cells in a sample to be tumor cells. In addition, the threshold of detection of mutant DNA in a wild-type environment is around 25% (6, 20). Gliomas are innately infiltrative tumors that are often admixed with a normal cell population. Thus, detection of *IDH1* mutations requires an assay that is more sensitive than conventional polymerase chain reaction (PCR)-direct sequencing, and such as assay would be a great asset to the clinical laboratory. Several techniques for the detection of *IDH1* mutations including pyrosequencing (7), single-strand conformation polymorphism (30), PCR- and restriction endonuclease-based detection (15), immunohistochemistry (IHC) (24), melting curve analysis performed on real-time PCR (11) and the PCR-based SNaPshot® assay (22) have been introduced for clinical applications, but these techniques appear not to be significantly better for *IDH1* mutation detection than conventional direct sequencing methods.

Peptide nucleic acid (PNA) oligomers were developed to detect minimal amounts of mutant DNA in clinical samples (19). In PNA-mediated clamping PCR, PNA oligomers suppress the amplification of the complementary sequence by a pair of DNA oligonucleotide primers, as PNA is not a substrate for DNA polymerase (Figure 1). Thus, a PNA-clamped probe assay is more sensitive than direct sequencing, and the sensitivity and validity of this technique have been previously demonstrated in the detection of other genetic mutations (17, 26, 27, 29). In the present study, we describe a simple and highly sensitive PNA-mediated real-time PCR clamping technique for the detection of *IDH1* mutations and discuss the clinical implications of this sensitive technique in the detection of oligodendroglial tumors.

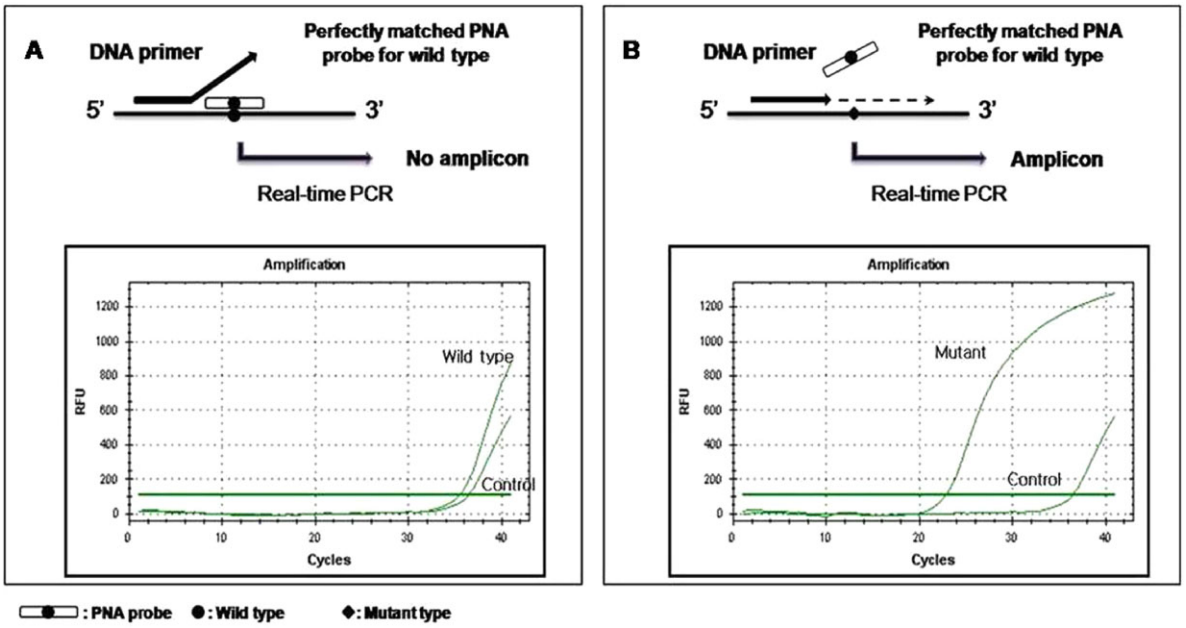

Figure 1. The peptide nucleic acid (PNA) clamping system. The PNA oligomer was designed to bind to the bottom strand of the wild-type sequence, spanning mutational hot spot of the isocytrate dehydrogenase 1 gene. The forward polymerase chain reaction (PCR) primer partially overlapped the PNA binding site. (**A**) A PNA/DNA hybrid with a perfect match prevents annealing of the PCR primer and amplification of wild-type DNA. (**B**) A PNA/DNA hybrid with a single-base pair mismatch does not suppress annealing of the PCR primer or amplification of mutant alleles.

MATERIALS AND METHODS

Patients and tumor samples

A total of 141 oligodendroglial tumor cases of WHO grade II and III were retrieved from the tumor registry of the Samsung Medical Center. Tumors consisted of 55 oligodendrogliomas (OII), 26 oligoastrocytomas (OAII), 37 anaplastic oligodendrogliomas (OIII) and 23 anaplastic oligoastrocytomas (OAIII). Histological diagnoses were made from formalin-fixed, paraffin-embedded tissue following the current WHO classification guidelines (14) by two neuropathologists (DL and Y-LS). The percentage of tumor cells corresponds to the ratio of tumor cells to all cells (tumor and non-tumor cells) on a slide. All samples included in this study were thoroughly selected to contain a tumor cell composition of at least 50%. Twenty nonneoplastic brain tissue samples obtained from epilepsy surgeries were blindly tested by direct sequencing and PNA clamping PCR for negative controls. In addition, to evaluate the *IDH1* mutation statuses in other brain tumors by PNA clamping method, we obtained tumor samples of 20 diffuse astrocytomas (AII), 44 primary glioblastomas (GBMs), 10 medulloblastomas, 15 pilocytic astrocytomas (PAs) and 10 pleomorphic xanthoastrocytomas (PXAs). The DNA we used in this study was all extracted from formalin-fixed paraffin-embedded (FFPE) tissues, and we used the QIAamp DNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. This study was approved by the Institutional Review Board of Samsung Medical Center.

The following clinical data were determined only for the OIII and OAIII patients: age at the time of first operation, gender, type of primary treatment (gross total resection, partial resection, biopsy), type of adjuvant treatment (chemotherapy, radiation therapy), time of the last follow-up and patient status at the last follow-up (alive or deceased). Median follow-ups were 1529 days (range 4–5561) and 1179 days (range 205–4015) for OIII and OAIII patients, respectively. A total of 19 patients (31.7%) died during the follow-up period. The major clinical data of these 60 patients are summarized in Supporting Information Table S1.

PNA-mediated clamping PCR for detection of *IDH1* mutations

The *IDH1* mutation was tested using the PNAClamp *IDH1* Mutation Detection Kit (Panagene, Inc, Daejeon, Korea). All reactions had a total reaction volume of 20 μL and contained template DNA, primer and PNA probe sets and SYBR Green PCR master mix (KapaBiosystems, Woburn, MA, USA). All required reagents were included with the kit. Real-time PCR reactions of PNA-mediated clamping PCR were performed using a CFX96 (Bio-Rad, Hercules, CA, USA). PCR cycling conditions were as follows: 5 minutes at 94°C followed by 40 cycles of 94°C for 30 s, 70°C for 20 s, 63°C for 30 s and 72°C for 30 s. In this assay, PNA probes and DNA primers were used together in the clamping reaction. Positive signals were detected by intercalation of SYBR Green fluorescent dye. The PNA probe, which is complementary to the wild-type sequence, suppresses amplification of the wild-type target. This suppression results in preferential amplification of the mutant sequences by competitively inhibiting the binding of DNA primers to wild-type DNA. PCR efficiency was determined by

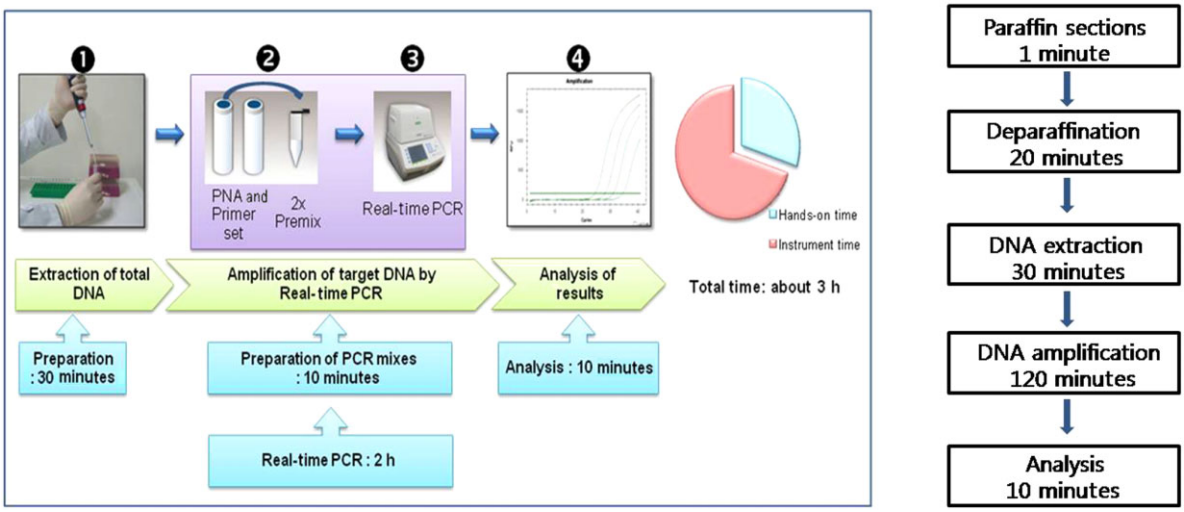

Figure 2. The flowchart and time schedule of the peptide nucleic acid (PNA) clamping method used for the detection of *IDH1* mutations in routinely processed glioma tissue specimens. Note that the total time required from cutting paraffin sections to obtaining sequence results amounts to approximately 3 h.

measuring the threshold cycle (Ct) value. Ct values for control and mutant assays were obtained from SYBR Green amplification plots. Calculations of the delta Ct (ΔCt) value were done as follows: ΔCt1 = [Standard Ct] – [Sample Ct], ΔCt2 = [Sample Ct] – [Non PNA mix Ct]. The gene was considered to be mutated when ΔCt1 values were more than 2.0. When ΔCt1 values were between 0 and 2, a ΔCt2 value was then calculated. The gene was considered to be mutated if the calculated ΔCt2 value was ≤6. PNA clamping PCR for *IDH1* mutations can be applied to any qualified laboratories using FFPE tumor samples, and the entire procedure including DNA extraction can be performed within approximately 3 h (Figure 2)

Pyrosequencing analysis

Pyrosequencing for mutational analysis was performed using a PyroMark Q24 (Qiagen, Germantown, MD, USA). Pyrosequencing primer sets were designed to amplify specific target regions of genes using PSQ Assay Design Software (Biotage, Uppsala, Sweden). Each DNA segment was amplified using 0.5 μmol/L of each primer and a 2X PCR premix (Solgent, Daejeon, Korea) in a TP600-PCR thermal cycler (Takara, Tokyo, Japan). DNA sequences of primers, PNA oligomer and probe sets are summarized in Table 1. Cycling conditions entailed an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation (95°C for 30 s), annealing (58°C for 40 s) and elongation (72°C for 30 s), with a final elongation step at 72°C for 5 minutes. The cycling protocol contained 40 cycles for the purpose of depleting biotin-labeled primers in order to prevent unincorporated primer from binding and competing with the amplicon on the streptavidin-coated beads during pyrosequencing. PCR products were visualized on 2% agarose gel electrophoresis with ethidium bromide staining. Single-stranded products were prepared from 15–20 μL biotinylated PCR products using streptavidin Sepharose® HP beads (Amersham Biosciences, Eubuckingahamshire, UK) following the PSQ 96 sample preparation guide using multichannel pipettes. Fifteen picomoles of the respective sequencing primers were added for analysis. Pyrosequencing was performed on a PyroMark ID system with the Pyro Gold reagents kit (Biotage, Uppsala, Sweden) and was used according to the manufacturer's instructions without further optimization. All experiments included a negative control without a template.

Table 1. DNA sequences of primers, PNA oligomer and probe sets for pyrosequencing and direct/nested PCR sequencing

IDH1 gene	Primer		Size (bp)
Pyrosequencing	Forward	5′- CGGTCTTCAGAGAAGCCATT-3′	131
	Biotinylated-reverse	5′- GCAAAATCACATTATTGCCAAC-3′	131
	Pyrosequencing primer	5′-GGGTAAAACCTATCATCA-3′	—
	Sequence to analyze	TAGGTCGT(GGT, CAT, CTT, AGT, TGT)CA	—
Direct/nested PCR sequencing	PNA probe (clamping)	N′- AGCATGACGACCTAT-C′	131
	Forward	5′- CGGTCTTCAGAGAAGCCATT-3′	131
	Reverse	5′- GCAAAATCACATTATTGCCAAC-3′	131
	Sequencing primer	5′- CGGTCTTCAGAGAAGCCATT-3′	—

Direct/nested PCR sequencing

We used a specially designed IDH1 clamping PNA probe for direct and nested PCR sequencing. PCR was initially performed with 30 ng of clinical sample DNA per reaction, which was amplified using 0.5 µmol/L of each sequencing primer, 5 µmol/L of the *IDH1* clamping probe and 2X PCR premix in a TP600-PCR thermal Cycler (Takara, Tokyo, Japan). The following conditions were used for DNA amplification: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation (94°C for 30 s), PNA annealing (70°C for 20 s), primer annealing (63°C for 30 s) and elongation (72°C for 30 s). After the first round of PCR, consecutive PCR amplification was performed using the PCR products from the initial DNA amplification. Cycling conditions entailed an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of denaturation (95°C for 30 s), annealing (58°C for 40 s) and elongation (72°C for 30 s), with a final elongation at 72°C for 5 minutes. Finally, a direct sequencing reaction was performed using an ABI PRISM 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA) system with Bigdye® Terminator v3.1 Cycle Sequencing Kit (ABI, Carlsbad, CA, USA) according to the manufacturer's instruction.

IHC

IHC was carried out on FFPE, 4-µm thick tissue sections. Anti-*IDH1* R132H was used as the primary antibody (clone H09, Dianova, Hamburg, Germany). Immunostaining was performed using a Ventana BenchMark XT® autoimmunostainer (Ventana Medical Systems, Tucson, AZ, USA) with a cell conditioner 1 for 60 minutes. Slides were then incubated with 1:150 anti-*IDH1* R132H supernatant at 37°C for 32 minutes, followed by standard Ventana signal amplification, counterstaining with hematoxylin for 4 minutes and staining with a bluing reagent for 4 minutes. Slides were then removed from the immunostainer, mounted and examined by light microscopy. Strong cytoplasmic staining in any number of cells was scored as positive. Slides processed without the primary antibodies were used as negative controls.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 18.0, SPSS Inc, Chicago, IL, USA). Fisher's exact test was used to compare the quantitative data. Overall survival (OS) time was defined as the interval between first surgery and death or last follow-up visit. The survival rates were estimated using the Kaplan-Meier method, and the survival curves were compared by the log-rank test. A *P*-value less than 0.05 was considered statistically significant. All the reported *P*-values are two-sided.

RESULTS

We screened 141 oligodendroglial tumors for the presence of *IDH1* mutations by PNA clamping PCR ($n = 141$), direct sequencing ($n = 141$), pyrosequencing ($n = 141$), IHC ($n = 141$) and nested PCR ($n = 27$). Table 2 provides an overview of the results of *IDH1* mutations from the different types of oligodendroglial tumors included in this study. Complete *IDH1* mutation data from all detection methods are presented in the Supporting Information Table S2. The frequencies of detected *IDH1* mutations were similar between direct sequencing (74.5%), pyrosequencing (75.2%) and IHC (75.9%). Only the clamping PCR method showed remarkably high sensitivity and detected 138 of 141 (97.9%) cases with an *IDH1* mutation in our series ($P = 0.016$; PNA clamping vs. direct sequencing). The three cases of wild-type *IDH1* assessed by clamping PCR were also found to have wild-type *IDH1* by direct sequencing, pyrosequencing and IHC. All diagnostic methods for the detection of *IDH1* mutations presented here were tested at least twice.

Detection tool		Number of *IDH1* mutations				
		OII (%)	OAII (%)	OIII (%)	OAIII (%)	Mutant, total (%)
Direct sequencing	Mutant	42 (76.4)	19 (73.1)	28 (75.7)	16 (69.6)	105 (74.5)
	R132H	40	16	28	15	
	R132G	2	2	—	1	
	R132S	—	1	—	—	
	Wild	13	7	9	7	
Pyrosequencing	Mutant	43 (78.2)	19 (73.1)	28 (75.7)	16 (69.6)	106 (75.2)
	R132H	41	16	28	15	
	R132G	2	2	—	1	
	R132S	—	1	—	—	
	Wild	12	7	9	7	
IHC	Positive	47 (85.5)	18 (69.2)	27 (73)	15 (65.2)	107 (75.9)
	Negative	8	8	10	8	
Nested PCR	R132H	4	3	3	5	—
	R132G	—	—	2	—	
	Wild	—	2	1	1	
	Fail	3	—	3	—	
Clamping PCR	Mutant	54 (98.2)	25 (96.2)	37 (100)	22 (95.7)	138 (97.9)
	Wild	1	1	0	1	
Total		43	26	37	23	141

Table 2. Summary of *IDH1* mutation prevalence according to the various techniques used in grade II and III oligodendroglial tumors. Abbreviations: OII = oligodendroglioma; OAII = oligoastrocytoma; OIII = anaplastic oligodendroglioma; OAIII = anaplastic oligoastrocytoma; IHC = immunohistochemistry.

To evaluate the validity and sensitivity of PNA clamping PCR, two additional experiments were performed. First, direct sequencing and clamping PCR were tested in 20 cases of nonneoplastic brain tissue from epilepsy surgery as a negative control. All cases were found to possess wild-type *IDH1* by both methods (Supporting Information Table S3). Second, we performed dilution experiments by mixing wild-type (from U87-MG human glioblastoma-astrocytoma cell line) (5) and mutant *IDH1* (from a mutant clone) DNA samples in different proportions (Figure 3). These results showed that clamping PCR was able to detect *IDH1* mutations with a mutant allele frequency of 1% or more.

Next, we performed quantitative mutational analysis using the results from pyrosequencing and clamping PCR. Pyrosequencing detected a median of 53.3% (range 0–81.4%) of residual wild-type alleles in tumors carrying an *IDH1* mutation. This tumor population had a median percentage of mutant alleles of 46.7% (range 18.6–100%). In tumors carrying a wild-type *IDH1* as assessed by pyrosequencing, the median percentage of tumors with the wild-type allele was 87.5% (range 84.1–98%), while the median percentage of residual mutant alleles was 12.5% (range 2–15.9%). These results indicate that cases which have been presumed to have wild-type *IDH1* still possess a low percentage of mutant alleles, an important finding that may be missed by other conventional detection methods. Alternatively, in tumors carrying mutated *IDH1*, clamping PCR detected mutant alleles of an estimated median of 25% (range 1–80%). The three tumors having wild-type *IDH1* as detected by clamping PCR appeared to possess almost all wild-type *IDH1* alleles only.

We had hypothesized that the three cases without an *IDH1* mutation by clamping PCR in our cohort might have an *IDH2* mutation. Because we did not develop *IDH2* clamping kit yet, we only performed direct sequencing and pyrosequencing for the detection of *IDH2* mutations on these three cases and found only one case with an *IDH2* mutation (R172K).

Using clamping PCR, 27 additional cases of *IDH1* mutation were detected that had been missed by direct sequencing and pyrosequencing (Table 3). All of these cases had a ΔCt1 value of less than 4. It was interesting that the percentages of these cases increased in an ascending order according to the histological diagnoses: OII (7/55, 12.7%), OAII (5/26, 19.2%), OIII (9/37, 24.3%), OAIII (6/23, 26.1%). The estimated median percentage of mutant alleles found by clamping PCR was 1% (range 1–25%) in these cases. To confirm the presence of mutations in these cases, nested PCR was performed. Aside from the six cases in which we failed to amplify DNA, 17 of the remaining 21 cases (81%) demonstrated *IDH1* mutations (R132H 15 cases, R132G 2 cases). In addition, 5 of these 27 cases (18.5%) were tested positive for anti-*IDH1*-R132H, and all of these five cases showed only a few positive cells.

To evaluate the effect of PNA clamping method for the prevalence of *IDH1* mutations in other brain tumors than oligodendroglial tumors, we performed clamping PCR and direct sequencing in a total of 99 cases of brain tumors. *IDH1* mutation was detected in 4 of 20 (25%) cases of AII by both methods. Direct sequencing found only one case of PA with an *IDH1* mutation (1/15, 6.7%), while PNA clamping detected two additional cases having an *IDH1* mutation (3/15, 20%). Of 44 cases of primary GBMs, 2 cases (4.5%) having an *IDH1* mutation was screened by direct sequencing, and the number was doubled by clamping method (4 cases, 9.1%). *IDH1* mutation was not detected even by clamping PCR in all the cases of PXAs and medulloblastomas tested (10 cases each), as previously documented (1).

Then we asked for the clinical usefulness of *IDH1* mutations detected by this sensitive technique. We divided the patients with OIII and OAIII into two groups by ΔCt1 values. The cutoff values for each group were set to best discriminate the patients' survival statistically. The median OS of OIII patients with $\Delta Ct1 > 2.7$ was significantly longer ($P = 0.016$) as compared with OIII patients with $\Delta Ct1 \leq 2.7$ (Figure 4A). In line with this, the median survival of OAIII patients with $\Delta Ct1 > 4$ was significantly longer ($P = 0.002$) as compared with OAIII patients with $\Delta Ct1 \leq 4$ (Figure 4B). Even though PNA clamping method is not a quantitative test, these results suggest that the relative amount of *IDH1*

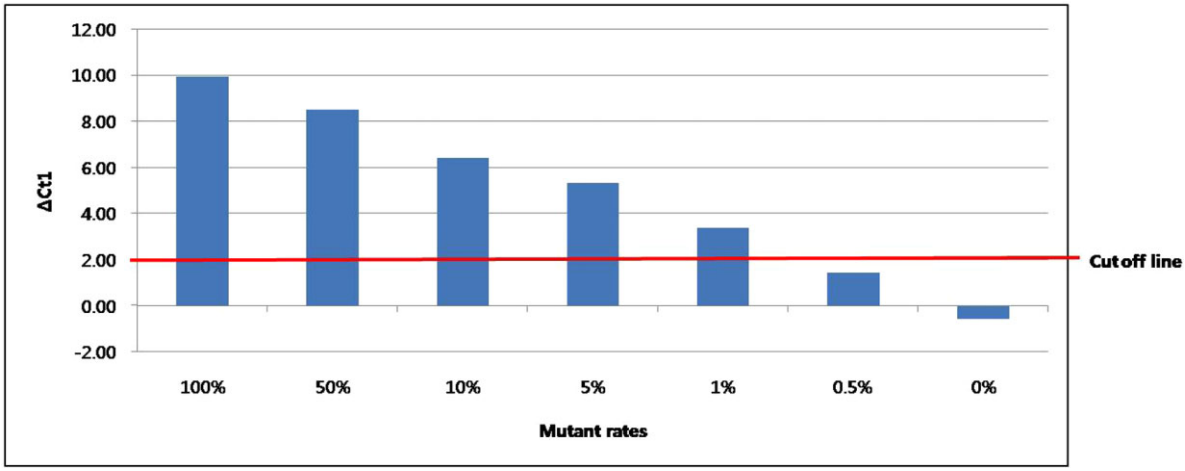

Figure 3. Results of dilution experiments mixing *IDH1* wild-type (from U87-MG human glioblastoma-astrocytoma cell line) and mutant (from a mutant clone) DNA samples in different proportions. These results indicate that the PNA clamping method is able to detect *IDH1* mutations with a mutant allele frequency of 1% or more by our standards (ΔCt1 > 2.0).

Table 3. Results of cases demonstrating a wild-type *IDH1* from the analyses with direct sequencing and pyrosequencing, but showing a mutant *IDH1* when assessed by clamping PCR. Abbreviations: OII = oligodendroglioma; OAII = oligoastrocytoma; OIII = anaplastic oligodendroglioma; OAIII = anaplastic oligoastrocytoma.

Case No.	Diagnosis	Direct sequencing	Pyrosequencing	IHC	Nested PCR	Clamping PCR	ΔCt1	ΔCt2
1	OII	Wild	Wild	–	R132H	Mutant	2.55	6.03
2	OII	Wild	Wild	+	R132H	Mutant	2.44	4.32
3	OII	Wild	Wild	–	R132H	Mutant	2.7	5.27
4	OII	Wild	Wild	+	Fail	Mutant	2.75	2.05
5	OII	Wild	Wild	+	R132H	Mutant	0.87	1.91
6	OII	Wild	Wild	+	Fail	Mutant	0.77	2.61
7	OII	Wild	Wild	–	Fail	Mutant	2.6	7.12
8	OAII	Wild	Wild	–	Wild	Mutant	1.7	5.51
9	OAII	Wild	Wild	–	Wild	Mutant	3.06	6.77
10	OAII	Wild	Wild	+	R132H	Mutant	2.79	5.04
11	OAII	Wild	Wild	–	R132H	Mutant	2.34	7.49
12	OAII	Wild	Wild	–	R132H	Mutant	2.58	5.95
13	OIII	Wild	Wild	–	R132G	Mutant	2.65	5.45
14	OIII	Wild	Wild	–	R132H	Mutant	2.55	6.16
15	OIII	Wild	Wild	–	R132G	Mutant	1.1	5.49
16	OIII	Wild	Wild	–	Fail	Mutant	1.97	5.81
17	OIII	Wild	Wild	–	Wild	Mutant	2.45	7.45
18	OIII	Wild	Wild	–	Fail	Mutant	3.54	6.14
19	OIII	Wild	Wild	–	R132H	Mutant	2.25	5.21
20	OIII	Wild	Wild	–	R132H	Mutant	2.36	5.37
21	OIII	Wild	Wild	–	Fail	Mutant	2.86	6.5
22	OAIII	Wild	Wild	–	R132H	Mutant	3.93	5.1
23	OAIII	Wild	Wild	–	R132H	Mutant	3.42	6.76
24	OAIII	Wild	Wild	–	R132H	Mutant	3	7.78
25	OAIII	Wild	Wild	–	R132H	Mutant	3.23	7.65
26	OAIII	Wild	Wild	–	R132H	Mutant	3.71	6
27	OAIII	Wild	Wild	–	Wild	Mutant	3.73	6.65

mutant alleles influence the clinical outcomes and can stratify the patients.

Direct sequencing and pyrosequencing revealed similar *IDH1* mutation detection results in our cohort. Indeed, results were identical in OAII, OIII and OAIII, while differences were found in only a few cases (three cases) of OII. The overall IHC sensitivity (75.9%) was similar to the sensitivity of other techniques, but there existed some minor differences. For example, five cases found to be positive by IHC tested negative by direct sequencing and pyrosequencing, as described earlier. These five cases carried mutant *IDH1* as determined by clamping PCR: three cases had R132H mutation as confirmed by nested PCR, and in the other two cases, nested PCR failed to amplify the DNA. Mutant *IDH1* was detected by direct sequencing or pyrosequencing in six cases; however, IHC for these cases was negative even after repetitive staining. Furthermore, although the IMab-1 antibody was designed to selectively detect R132H, three cases were tested positive but were found to have R132G mutation. Cases with other types of *IDH1* mutations such as R132G (two cases) and R132S (one case) had negative results by IHC. Samples that had different results by the various detection methods described previously were analyzed in triplicate independent experiments.

DISCUSSION

Technological advances in the life sciences have enabled the development of more sensitive tools than direct sequencing for the detection of DNA mutations. In field of neuro-oncology, various approaches with comparable sensitivity have been developed to detect *IDH1* mutations and have been practically applied (7, 11, 22, 24). PNA clamping PCR is a promising new method for the detection of *IDH1* mutations and has been successfully applied for the detection of other DNA mutations such as epidermal growth factor receptor (EGFR) and K-ras (2, 9). Sensitivity is a critical issue for molecular diagnostic approaches as mutations may only be present in a subset of tumor cells, and wild-type alleles may still be present in tumor cells with mutations in *IDH1*. In addition, biopsy specimens from glial tumors often contain considerable numbers of normal cells. In this study, we showed that clamping PCR allows detection of as little as 1% of *IDH1* mutant alleles in the background of wild-type DNA. The high sensitivity of clamping PCR suggests a potential role for this method in assessing diagnostically ambiguous tissue samples with low tumor cellularity, especially in the infiltrating tumor periphery. In addition, PNA clamping PCR is a fast, more simple and economical method for the *IDH1* mutation detection than direct sequencing, and this test would be a valuable asset in clinical laboratories.

The prevalence of *IDH1* mutations in grades II/III oligodendroglial tumors differs across studies (Table 4). In 2008, Balss *et al* (1) reported that the frequencies of *IDH1* mutations ranged from 67% to 78% in these tumors by direct sequencing. Since then, many investigators have examined *IDH1* mutations in glial tumors using different methods. In addition to direct sequencing (10, 25, 30), other techniques such as pyrosequencing and IHC have been

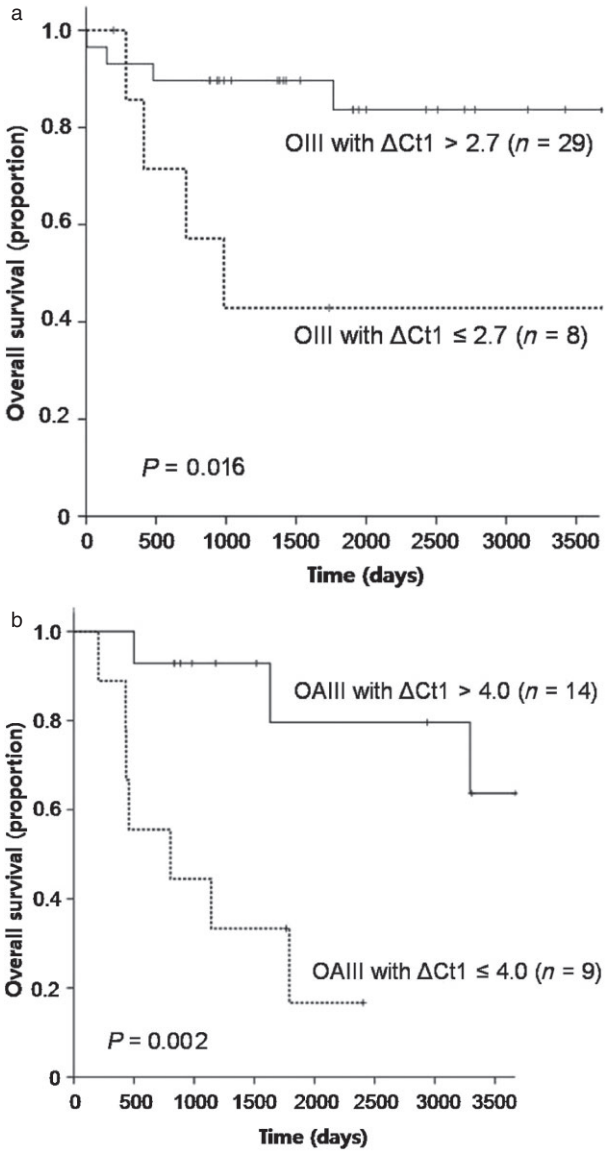

Figure 4. Kaplan-Meier survival curves for overall survival in patients with (i) anaplastic oligodendroglioma (OIII); and (ii) anaplastic oligoastrocytoma (OAIII) according to the ΔCt1 values from clamping PCR.

used (7, 24). The overall frequencies of *IDH1* mutations appear to be somewhat increasing as detection tools become more sensitive. Recently, Capper *et al* (4) reported a frequency of *IDH1* mutation of approximately 90% in grades II/III oligodendroglial tumors when results of IHC and direct sequencing were combined. In the current study, we used PNA clamping PCR and detected higher *IDH1* mutation frequencies ranging from 95.7% to 100% in grades II/III oligodendroglial tumors. Only 3 of 141 cases (2.1%) assessed by clamping PCR contained wild-type *IDH1*. Initially, this unexpected high mutational frequency suggested a false-positive result. However, these results were confirmed by two or more repetitive tests. Furthermore, mutant *IDH1* was not demonstrated in any of 20 cases of nonneoplastic brain tissues using direct sequencing or clamping PCR, indicating the validity of our results.

Recently described next-generation sequencing technology has influenced cancer genomics enormously, and it affords rapid, cost-effective "deep sequencing" of cancer genomes, which enables the genome-wide searches of cancer-associated somatic mutations (16, 23). For the detection of *IDH1* mutations, this new sequencing method appears to be an emerging golden standard. Bettegowda *et al* (3) performed exome sequencing of seven OII, and found the *IDH1* gene was mutated in all the seven tumors. More recently, Yip *et al* (31) also performed exome sequencing of 16 cases of OII, and revealed that all cases had mutations in either *IDH1* (14/16) or *IDH2* (2/16), which was validated by deep sequencing. These previous data strongly support our results and the hypothesis that all oligodendroglial tumors might have mutations in *IDH* genes. To prove this hypothesis, we performed direct sequencing and pyrosequencing for the detection of *IDH2* mutations on three cases having wild-type *IDH1* by clamping PCR, but only found one *IDH2* mutation. However, if we could have used a more sensitive technique, the results might be changed. Regretfully, we did not develop *IDH2* clamp kit yet, we cannot prove this at present and this is our drawback.

Watanabe *et al* (30) analyzed multiple biopsy specimens from the same patients (51 patients) and revealed that there were no instances in which an *IDH1* mutation occurred after the acquisition of either *TP53* mutation or loss of *1p/19q*. This suggests that *IDH1* mutations occur early in gliomagenesis and may affect a common glial precursor cell population. Our results support this idea as almost all oligodendroglial tumors had *IDH1* mutations by clamping PCR. Moreover, this implies that mutations in *IDH1*

Table 4. Previously reported *IDH1* mutation frequencies in oligodendroglial tumors of various histologic subtypes. Abbreviations: OII = oligodendroglioma; OAII = oligoastrocytoma; OIII = anaplastic oligodendroglioma; OAIII = anaplastic oligoastrocytoma; IHC = immunohistochemistry; SSCP = single-strand conformation polymorphism.

	OII (%)	OAII (%)	OIII (%)	OAIII (%)
Balss *et al* (1) (direct sequencing)	74	78	67	78
Sanson *et al* (25) (direct sequencing)	76	76	49	63
Watanabe *et al* (30) (SSCP and direct sequencing)	79	94	75	91
Horbinski *et al* (11) (melting curve analysis)	79.2	50	88	—
Hartmann *et al* (10) (direct sequencing)	82	81.6	69.5	66.1
Felsberg *et al* (7) (pyrosequencing)	62.5	—	—	80
Capper *et al* (4) (IHC and direct sequencing)	91	79	95.7	91
Current study (PNA clamping PCR)	98.2	96.2	100	95.7

are basic, underlying genetic alterations in the development of oligodendroglial tumors. As previously documented (10, 25), *IDH1* mutational status is one of the most powerful prognostic indicators in patients with low and high grade gliomas. However, with this sensitive technique, *IDH1* mutation will no longer have a prognostic value as almost all tumors contained these mutations. We analyzed the newly found *IDH1*-mutated tumors (27 cases, 19.1%) by clamping PCR, and all had a ΔCt1 value of less than 4, suggesting a relatively low percentage of mutant alleles. From the results of pyrosequencing, the median percentage of mutant alleles in these tumors was found to be 3.2% (range 2–15.9%). We confirmed that these tumors had a lower percentage of mutant alleles when compared to those of the other cases carrying *IDH1* mutations (median 44.3%, range 18.6–100%). In addition, the fact that more tumors with higher grade and/or astrocytic component were included in this group of tumors may suggest that not just the presence or absence of *IDH* mutation but low percentage of *IDH1* mutant alleles is associated with disease progression and clinical outcome. Indeed, we proved this that relative quantitation of *IDH1* mutant alleles according to the ΔCt1 value from clamping PCR could stratify the patients' survival. Therefore, quantitative analysis for the *IDH1* mutation appears to be necessary sooner or later from this point of view. In the current study, we applied different cutoff values to OIII and OAIII patients. That is because we set the best cutoff values to stratify patients' survival statistically in two different tumor groups, and they were simply different. This difference may be ascribed to (i) different proportions of *IDH1* mutant alleles in astrocytic and oligodendroglial tumor cells by nature; (ii) different proportions of astrocytic elements even in the OAIII; and (iii) possible interaction between astrocytic and oligodendroglial tumor elements for the *IDH1* mutations.

The most prominent consequence universally accepted in *IDH* mutation is that altered enzyme acquires neomorphic activity to reduce α-ketoglutarate (αKG) into a novel oncometabolite, D-2-hydroxyglutarate (D-2HG) in an nicotinamide adenine dinucleotide phosphate (NADP)-dependent manner (5). Indeed, elevated D-2HG levels were found in acute myeloid leukemia and brain tumor patients with *IDH1* and *IDH2* mutations (5, 8). However, exact mechanisms through which *IDH1/2* mutations contribute to the pathogenesis of gliomas are still not fully understood. There are only several hypotheses regarding the molecular pathogenesis of *IDH* mutations have been proposed. For example, D-2HG may compete with αKG and inhibit prolyl hydroxylase (PHD)-mediated degradation of hypoxia-inducible factor (HIF)-1α. Accumulated HIF-1α may induce expression of vascular endothelial growth factor (VEGF), promoting angiogenesis, which might enhance tumor growth (32). In the second hypothesis, *IDH1/2* mutations promote tumorigenesis by deregulating gene expression from DNA hypermethylation at a number of targeted genes (12, 18). The possibility is also raised that an oxidative DNA damage induced by decreased NADP-dependent *IDH* activity may be involved in tumorigenesis (12, 13). However, there are still limitations to explain the gliomagenesis in *IDH*-mutated tumors only by these hypotheses yet (12). The biologic function of *IDH* and its role in the development and progression of glial tumors still needs to be investigated further.

Unlike oligodendroglial tumors, PNA clamping method failed to find such a high frequency of *IDH1* mutations in other glial tumors including PAs, AIIs and primary GBMs. Of course, clamping PCR increased the mutation frequency almost the double in PAs and primary GBMs, but the general prevalence was still low, measuring only 20% and 9.1% in these tumors, respectively. This result confirms that *IDH1* mutation is highly specific for oligodendroglial tumors, although this mutation also occurs in a small fraction of other glial tumors.

In the current study, different results were derived from direct sequencing, pyrosequencing and IHC in several cases. These differences are likely due to the differing sensitivities of each method or to undiscovered technical problems. Although there is general agreement that PCR is more sensitive than IHC, we found that IHC may be more sensitive in the detection of mutations in *IDH1*, especially in tumors which have just a fraction of tumor cells having mutant *IDH1*. In general, investigators apply different cutoff values when they interpret IHC results. Some consider that 10% of tumor cells should be reactive in order to be rated as positive (28), while others consider a strong cytoplasmic staining in just a few cells to be a positive result (4). We used the latter criteria in this study. As discussed earlier, any number of positive cells identified by IHC should be interpreted meaningfully so as not to miss the presence of *IDH1* mutations in a small number of tumor cells.

CONCLUSIONS

We established a robust method of PNA-mediated clamping PCR to detect *IDH1* mutations with the sensitivity of 1% or more frequency of mutant allele in diagnostic tissue samples. With this technique, we found that 97.5% of oligodendroglial tumors in our cohort had mutant *IDH1* genes, a significantly higher figure than results assessed by other methods. As previously suggested, *IDH1* mutation seems to be an early and common event especially in the development of oligodendroglial tumors. The biologic function of mutated *IDH1* and its role in the development and progression of oligodendroglial tumors needs to be further elucidated in this context.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. The clinical characteristics of the 60 patients with OIII and OAIII.
Table S2. The whole data of results of *IDH1* mutations according to different methods.
Table S3. Results of direct sequencing and PNA clamping PCR for *IDH1* mutations on non-tumor brain tissues.