

Acknowledgment

We thank Susan Archer for technical writing and Stéphanie Reynaud for excellent assistance.

Appendix

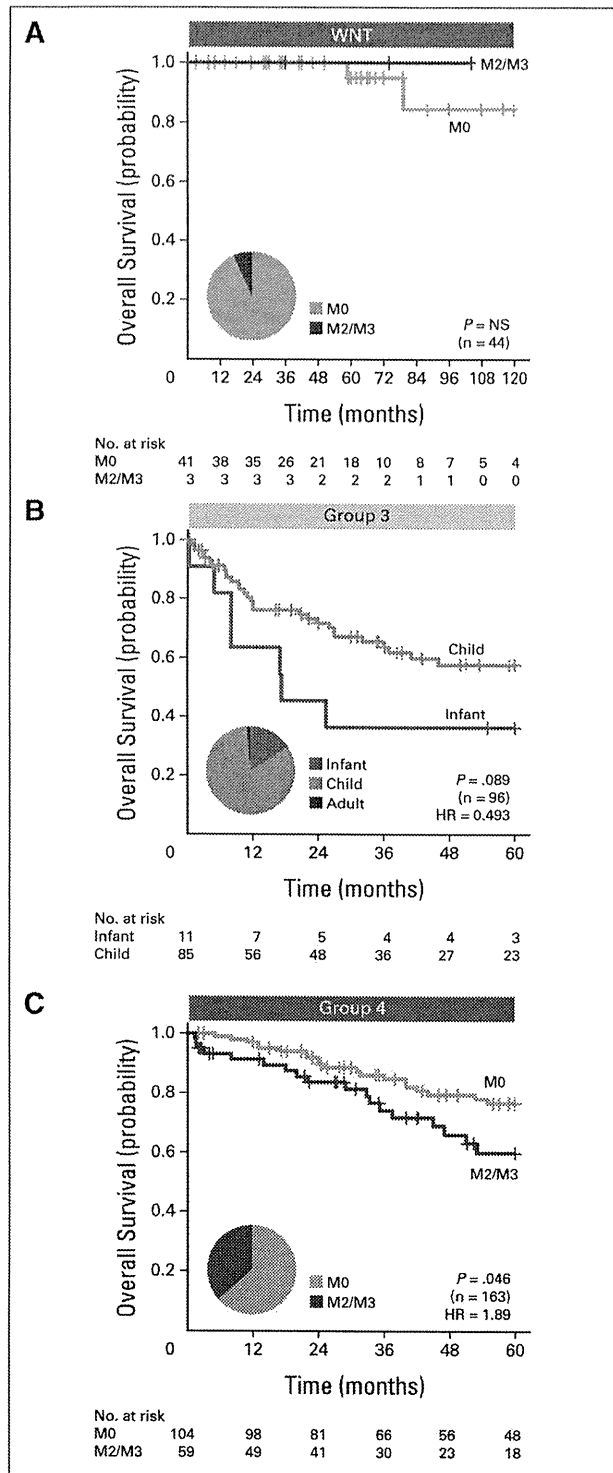


Fig A1. (A) Ten-year overall survival curves for WNT medulloblastoma by metastatic status. (B) Overall survival curves for age groups within Group 3 subgroup (infant, age < 3 years; child, age 3 to < 16 years). (C) Overall survival curves for metastatic status for Group 4 subgroup. Numbers below x-axis represent patients at risk of event; statistical significance evaluated by log-rank tests; hazard ratio (HR) estimates derived from Cox proportional hazards analyses. NS, not significant.

Cytogenetic Prognostication Within Medulloblastoma Subgroups

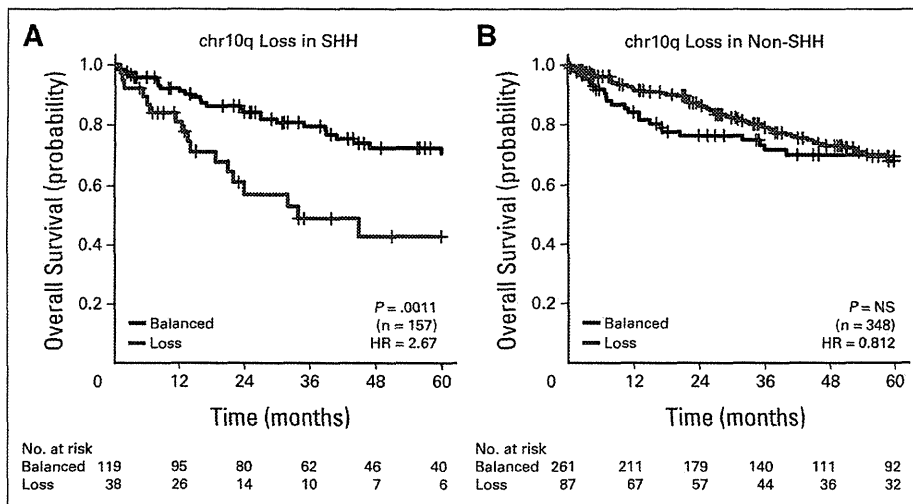


Fig A2. Overall survival curves for chromosome 10q (chr10q) status in (A) SHH and (B) non-SHH medulloblastomas; survival differences evaluated by log-rank tests; hazard ratio (HR) estimates derived from Cox proportional hazards analyses. NS, not significant.

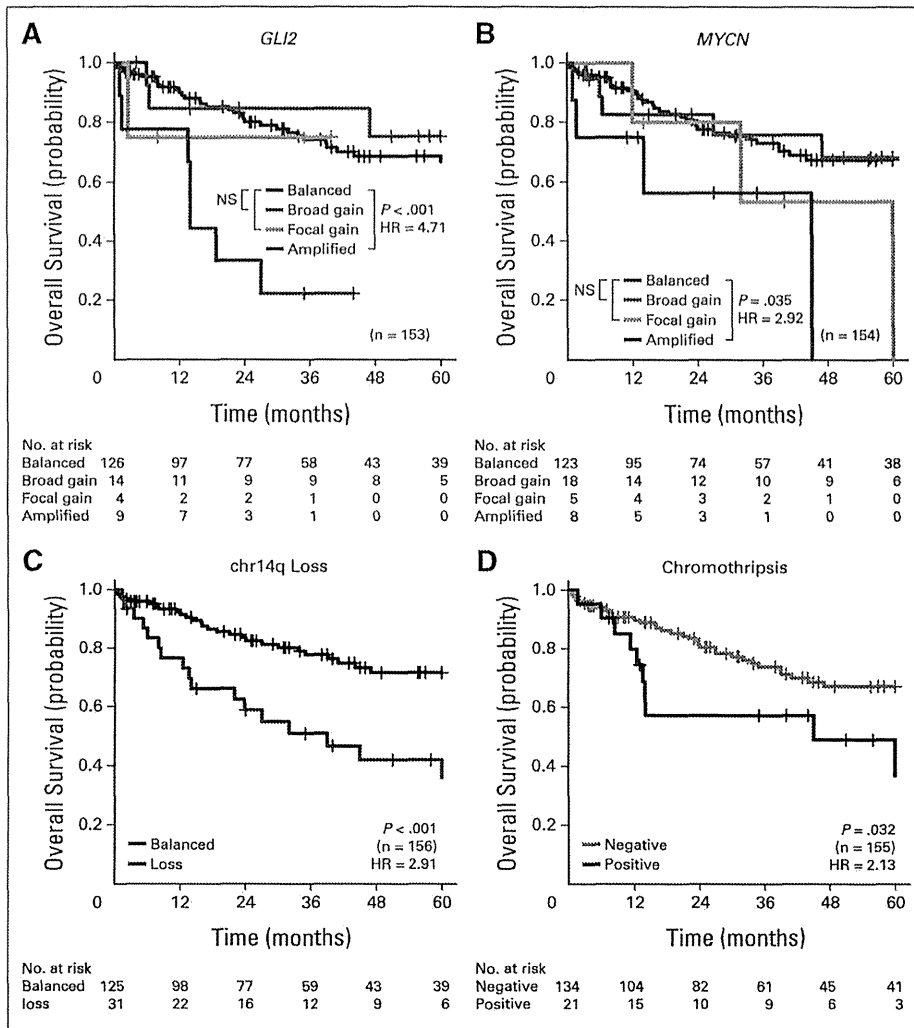


Fig A3. Clinical prognostication of patients with SHH medulloblastoma. Overall survival curves for (A) *GLI2* copy-number status, (B) *MYCN* copy-number status, (C) chromosome 14q (chr14q) status, and (D) chromothripsis status. HR, hazard ratio; NS, not significant.

Cytogenetic Prognostication Within Medulloblastoma Subgroups

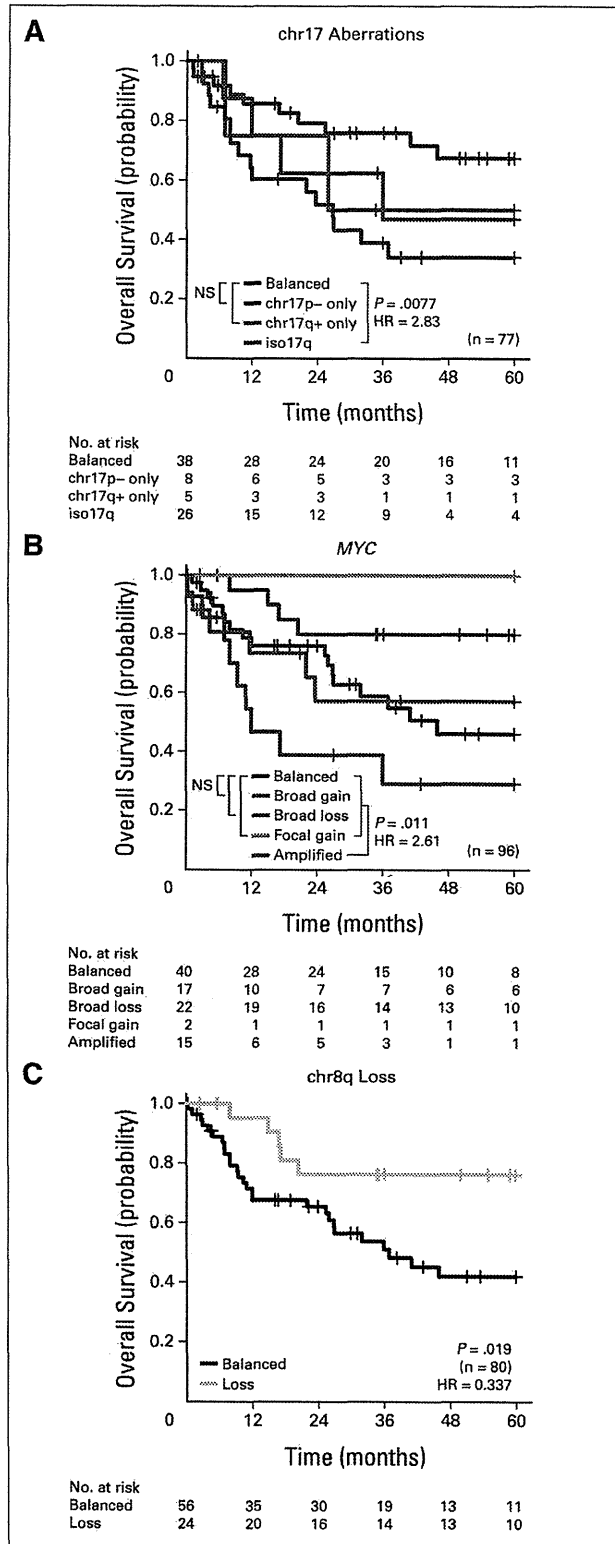


Fig A4. Clinical prognostication of patients with Group 3 medulloblastoma. Overall survival curves for (A) chromosome 17 (chr17) copy-number aberrations, (B) MYC copy-number status, and (C) chr8q status. HR, hazard ratio; iso, isochromosome; NS, not significant.

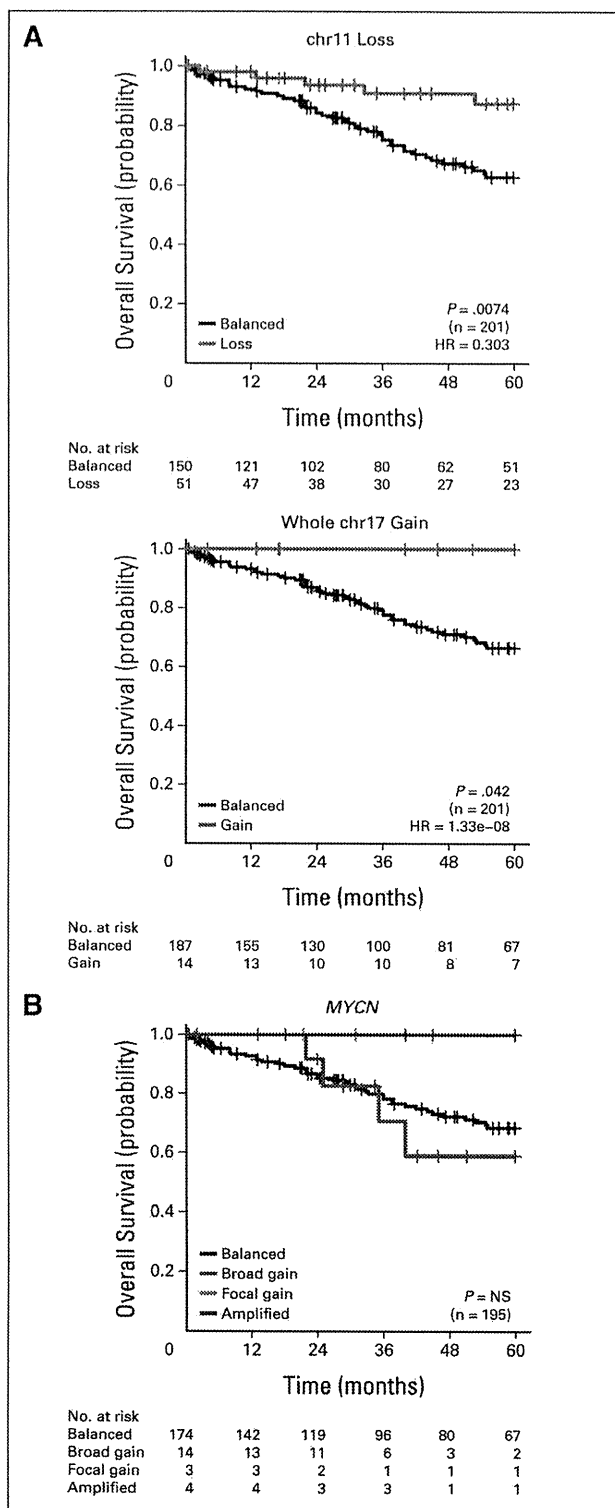


Fig A5. Clinical prognostication of patients with Group 4 medulloblastoma. Overall survival curves for (A) chromosome 11 (chr11) status and whole chr17 status and (B) *MYCN* copy-number status. HR, hazard ratio; NS, not significant.

Rapid and sensitive intraoperative detection of mutations in the *isocitrate dehydrogenase 1* and *2* genes during surgery for glioma

Laboratory investigation

MASAYUKI KANAMORI, M.D., PH.D.,¹ ATSUO KIKUCHI, M.D., PH.D.,²
MIKA WATANABE, M.D., PH.D.,³ ICHIYO SHIBAHARA, M.D., PH.D.,¹
RYUTA SAITO, M.D., PH.D.,¹ YOJI YAMASHITA, M.D., PH.D.,⁴
YUKIHIKO SONODA, M.D., PH.D.,¹ TOSHIHIRO KUMABE, M.D., PH.D.,⁵
SHIGEO KURE, M.D., PH.D.,² AND TEIJI TOMINAGA, M.D., PH.D.¹

Departments of ¹Neurosurgery and ²Pediatrics, Tohoku University Graduate School of Medicine;
³Department of Pathology, Tohoku University Hospital, Sendai; ⁴Department of Neurosurgery, Miyagi Cancer Center, Natori, Miyagi; and ⁵Department of Neurosurgery, Kitasato University School of Medicine, Sagami-hara, Kanagawa, Japan

Object. Intraoperative diagnosis is important in determining the strategies during surgery for glioma. Because the mutations in the *isocitrate dehydrogenase 1* and *2* (*IDH1* and *IDH2*) genes have diagnostic, prognostic, and predictive values, the authors assessed the feasibility and significance of a simplified method for the intraoperative detection of *IDH1* and *IDH2* gene mutations.

Methods. Rapid DNA extraction, amplification with conventional polymerase chain reaction (PCR) or co-amplification at lower denaturation temperature PCR (COLD-PCR), and fluorescence melting curve analysis with adjacent hybridization probes were performed for the intraoperative detection of *IDH1* and *IDH2* mutations in 18 cases of suspected nonneoplastic lesions and low- and high-grade gliomas and in 3 cases of radiation necrosis.

Results. DNA extraction for detection of the mutation took 60–65 minutes. The results of this assay showed complete correlation with that of Sanger sequencing. The sensitivity for detection of mutations in a background of wild-type genes was 12.5% and 2.5% in conventional PCR and COLD-PCR, respectively. The diagnosis of glioma was established in 3 of 5 cases in which definitive diagnosis was not obtained using frozen sections, and information was obtained for the discrimination of glioblastoma or glioblastoma with an oligodendroglioma component from anaplastic glioma or secondary glioblastoma. This assay also detected a small fraction of tumor cells with *IDH1* mutation in radiation necrosis.

Conclusions. These methods provide important information for establishing the differential diagnosis between low-grade glioma and nonneoplastic lesions and the diagnosis for subtypes of high-grade glioma. Although tumor cells in radiation necrosis were detected with a high sensitivity, further investigation is necessary for clinical application in surgery for recurrent glioma.

(<http://thejns.org/doi/abs/10.3171/2014.3.JNS131505>)

KEY WORDS • glioma • intraoperative molecular diagnosis • *IDH1* • *IDH2* • oncology

INTRAOPERATIVE diagnosis is important in determining the strategies to use during surgery for glioma. For example, in the differential diagnosis for low-grade glioma and nonneoplastic lesions, information on the sensitivity to radiation therapy and chemotherapy and the presence of viable tumor cells in recurrent glioma tissue influence the extent of the resection to be performed. Al-

though the rates of diagnostic accuracy of intraoperative frozen-section and cytolytic smear-based examinations are 97.2% and 89.8%, respectively,^{21,22} intraoperative diagnosis sometimes cannot give objective answers to these issues.

Recently, somatic mutations have been identified in the *isocitrate dehydrogenase 1* and *2* (*IDH1* and *IDH2*) genes in adult gliomas.^{1,20,27} These mutations are frequently found in astrocytic and oligodendroglial tu-

Abbreviations used in this paper: COLD = co-amplification at lower denaturation temperature; FMCA = fluorescence melting curve analysis; IDH = isocitrate dehydrogenase; PCR = polymerase chain reaction; Tc = denaturation temperature; Tm = melting temperature.

This article contains some figures that are displayed in color online but in black-and-white in the print edition.

Intraoperative detection of *IDH1/2* mutation

mors at a very early stage of gliomatogenesis, such as in WHO Grade II and III astrocytic and oligodendroglial tumors, and in secondary glioblastomas, but not in primary glioblastoma.^{1,18,20} Thus, these mutations may have diagnostic value for differentiating low-grade glioma from nonneoplastic lesions, non-CNS tumors, or reactive gliosis^{3,8} and for differentiating anaplastic glioma from primary glioblastoma or radiation necrosis.³ Additionally, the mutations have been shown to be prognostic^{1,20,25} in high-grade gliomas and we can expect them to be present after radiation therapy and chemotherapy.^{11,26,29} Based on these findings, we considered that the intraoperative detection of these mutations has potential for clinical application.

The mutations in *IDH1* and *IDH2* are restricted to analogous codons, exon 4 at codon 132 and exon 4 at codon 172, respectively. Various methods have been reported to detect these mutations, including pyrosequencing,⁵ immunohistochemistry with antibody specific to mutant *IDH1* R132H protein,^{3,4} enrichment of low-frequency mutant sequence with co-amplification at lower denaturation temperature polymerase chain reaction (COLD-PCR) combined with high-resolution melting² or with deep sequencing,¹⁵ and conventional PCR, followed by fluorescence melting curve analysis (FMCA) with adjacent hybridization probes (HybProbe, Roche Applied Science),⁸ which provide faster and more sensitive detection than conventional direct sequencing with the Sanger method. These methods allow rapid and sensitive detection of DNA from frozen or formalin-fixed tissues, but application to intraoperative detection of *IDH1* and *IDH2* mutations has not been demonstrated.

The COLD-PCR technique has been reported to be a simple method for identifying low-frequency mutations in the *TP53*, *KRAS*, and *EGFR* genes.¹³ During the PCR process, a critical denaturation temperature (T_c) occurs at a temperature lower than the melting temperature (T_m). Below T_c , the PCR efficiency drops abruptly. The T_c is dependent on the DNA sequence, and DNA amplicons differing by a single nucleotide have different amplification efficiencies, with the PCR denaturation temperature set to T_c . Because most mutations found in the *IDH1* gene are T_c -reducing mutations, COLD-PCR with a denaturation temperature lower than the T_c of the wild-type sequence and higher than that of the mutant sequence should amplify only the mutant sequence,³ thus enriching the low-frequency mutant sequence.

In this study, we developed systems based on conventional PCR and COLD-PCR, followed by FMCA with HybProbe, to achieve rapid, sensitive, and specific intraoperative detection of the *IDH1* and *IDH2* mutations and examined the feasibility and provision of useful information during surgery for glioma.

Methods

Tissues for Validation

We have previously reported the detection of mutations of *IDH1* and *IDH2* genes in glioma, based on Sanger sequencing analysis of DNA extracted with a QIAamp

DNA Mini Kit (Qiagen).²⁷ To confirm concordance between the standard method for sequencing analysis and conventional PCR, followed by FMCA with HybProbe from rapidly extracted DNA, we analyzed 14 tumors previously examined for mutation status: 5 gliomas with *IDH1* mutation, including R132H (CGT→CAT) in 3, R132G (CGT→GGT) in 1, and R132S (CGT→AGT) in 1; 2 gliomas with *IDH2* mutation at R172 K (AGG→AAG); and 7 gliomas without mutations in the *IDH1* or *IDH2* gene. To determine the sensitivity for detection, we examined a dilution series with 50%, 25%, 12.5%, 5%, 2.5%, 1%, 0.25%, and 0% of DNA from the R132H mutation in the *IDH1* gene in a DNA sample from a tumor with the wild-type *IDH1* gene. These DNA samples were amplified with conventional PCR and COLD-PCR, followed by FMCA with HybProbe. Each experiment was performed in triplicate.

Intraoperative Detection of Mutation

Intraoperative detection of *IDH1* and *IDH2* mutations was performed in 18 patients who have undergone cytoreductive surgery or biopsy for intraparenchymal tumors since May 2012. This study was conducted with the approval of the ethics committee of Tohoku University School of Medicine, and written informed consent was obtained from all patients.

Detection of Mutation in Radiation Necrosis

Oligodendroglial tumors carrying the *IDH1* mutation develop recurrent disease with viable tumor cells rather than undergo radiation necrosis.¹² To assess the significance of intraoperative detection of *IDH1* mutation in recurrent cases for differentiation of recurrence from radiation necrosis and reactive astrocytes, we simulated the intraoperative diagnosis in such cases by using DNA rapidly extracted from stored frozen tissues from 3 rare cases diagnosed as radiation necrosis based on small fractions of tumor cells or reactive astrocytes using H & E staining of formalin-fixed, paraffin-embedded sections after treatment of glioma carrying the *IDH1* mutation.

Histological Processing for Intraoperative Detection of Mutation

Tumor specimens (10–20 mg) were cut into two pieces. One piece was immediately frozen in liquid nitrogen, followed by rapid extraction of DNA. The other piece was processed for intraoperative and postoperative histological examination. The intraoperative histological diagnosis was established by the same neuropathologist (M.W.) using current WHO criteria,¹⁴ based on H & E staining.

Rapid DNA Extraction, Amplification, and FMC

DNA from the snap-frozen tissues was extracted with UltraClean Tissue & Cells DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol. The amplification and detection of *IDH1* and *IDH2* mutations was performed using LightCycler 1.5 (Roche Applied Science), as described previously with some modifications.^{3,9} Briefly, the loci flanking codon 132 of the *IDH1* gene and codon 172 of the *IDH2* gene were

amplified with conventional PCR. The forward and reverse primer sequences for the *IDH1* gene were 5'-CGG TCTTCAGAGAAGCCATT-3' and 5'-GCAAAATCACATTATTGCCAAC-3'. For mutation detection, HybProbes complementary to the wild-type allele were designed. The HybProbe sequences for the *IDH1* gene were 5'-CCCCGGCTTGTGAGTGGATGGGTAAAACCTA-Fluorescein-3', and 5'-LC Red 640-CATCATAGGTCGTCATGCTTAT-Phosphate-3'. The forward and reverse primer sequences for the *IDH2* gene were 5'-TTCCGGGAGCCATCAT-3' and 5'-TGCCCAGGTCAGTGGAT-3'. For mutation detection, HybProbes complementary to the wild-type allele were designed. The HybProbe sequences for the *IDH2* gene were 5'-CGTGCCTGCCAATGGTGA-Fluorescein-3', and 5'-LC Red 640-GGCTTGGTCCAGCCAGGGACTAGG-Phosphate-3' (Nippon Gene Research Laboratories). Extracted DNA (20 ng) was amplified with AmpliTaq Gold (Applied Biosystems) in a glass capillary tube in a 20- μ l reaction volume. The conventional PCR conditions for the amplification of *IDH1* and *IDH2* genes were as follows: 95°C for 10 minutes, 40 cycles of PCR consisting of denaturation at 95°C for 15 seconds, and annealing and extension at 58°C for 30 seconds.

Alternatively, the same locus of the *IDH1* gene was amplified with COLD-PCR to achieve more sensitive detection. The same reaction mixture was amplified with COLD-PCR as follows: 95°C for 10 minutes and 10 cycles of PCR consisting of denaturation at 95°C for 15 seconds and annealing and extension at 58°C for 30 seconds; then 30 cycles at 79°C for 15 seconds and annealing and extension at 58°C for 30 seconds.

Postamplification FMCA with HybProbe was performed by gradual heating of samples at 0.1°C/sec from 40°C to 95°C. Fluorescence melting peaks were built by plotting the negative derivative of the fluorescent signal corresponding to the temperature ($-dF/dT$) with LightCycler software 4.1, as described previously.⁸

We confirmed the results of intraoperative detection of the mutations in *IDH1* and *IDH2* genes by Sanger sequence analysis and immunohistochemistry for IDH1 R132H protein as mentioned below.

Immunohistochemical Examination

The primary antibody used in this study was mouse monoclonal antibody for anti-IDH1 R132H protein (Dianova; 1:400). Pathological materials were immediately fixed in 10% buffered formalin (Wako Pure Chemical Industries, Ltd.) at room temperature for several days. Thick paraffin sections (2 μ m) were deparaffinized, rehydrated, and incubated in 0.3% hydrogen peroxide in 100% methanol for 10 minutes at room temperature to block endogenous peroxidase. Antigen retrieval was accomplished by microwaving for 5 minutes in citric acid buffer (2 mmol/L citric acid and 9 mmol/L trisodium citrate dihydrate, pH 6.0). After blocking of nonspecific binding, slides were incubated with primary antibodies overnight at 4°C. Sections were treated with biotinylated anti-mouse immunoglobulin G for 30 minutes at room temperature, followed by peroxidase-conjugated streptavidin for 30 minutes at room temperature, using the Histofine kit (Nichirei Biosciences, Inc.). Sections were de-

veloped with diaminobenzidine solution (0.01 M 3,3'-diaminobenzidine in 0.05 M Tris-HCl buffer, pH 7.6, and 0.006% hydrogen peroxide). Nuclei were counterstained with hematoxylin.

Results

Detection of Wild-Type and Mutant *IDH1* and *IDH2* Genes With Conventional PCR, Followed by FMCA With HybProbe

DNA extraction required 15 minutes, and amplification and detection were completed within 50 minutes. A single peak, representing wild-type allele, was detected in the DNA of 7 tumors with wild-type *IDH1* and *IDH2* genes (Fig. 1A and E). All heterozygous mutations were successfully detected with different T_m from that of wild-type allele in 5 tumors with IDH R132H, 1 tumor with IDH1 R132G, and 1 tumor with R132S mutation (Fig. 1B–D). Similarly, mutation of the *IDH2* gene was detected under the same PCR conditions (Fig. 1F). Dilution analysis detected as low as 12.5% of the *IDH1* mutation (R132H) against a background of the wild-type allele (Fig. 2A).

Detection of Mutant Sequence of *IDH1* Gene With COLD-PCR and FMCA With HybProbe

To enhance the sensitivity of detection for the *IDH1* gene, we applied COLD-PCR in the amplification steps. Amplification with COLD-PCR and detection with FMCA with HybProbe was completed within 45 minutes. Dilution analysis showed this assay detected 2.5% of the mutated allele (R132H) against a background of the wild-type allele (Fig. 2B).

Intraoperative Detection of Mutant Sequence From Surgical Specimen

During cohort periods, 18 cases were enrolled in this study. Mutation of the *IDH1* gene was detected in 10 of 18 cases. The results of intraoperative detection for *IDH1* and *IDH2* are shown in Table 1. These results completely corresponded with those of Sanger sequencing analysis and immunohistochemistry for IDH1 R132H protein. All mutations detected were IDH1 R132H. There were no false-positive or false-negative results.

Detection of *IDH1* and *IDH2* Mutations in Low-Grade Glioma

During the cohort period, the morphological diagnosis based on frozen-section examination was a low-grade glioma in 3 cases and a nonneoplastic lesion difficult to differentiate from low-grade glioma in 5 cases. The diagnoses of low-grade glioma were based on findings such as slightly increased cellularity, nuclear atypia, and uneven distribution of nuclei. A summary of the intraoperative diagnoses using frozen sections; final diagnoses using formalin-fixed, paraffin-embedded sections; and intraoperative detection of *IDH1* and *IDH2* gene mutations is shown in Table 1 (Cases 1–8). In the 5 cases of low-grade glioma and nonneoplastic lesions that were difficult to differentiate, detection of the *IDH1* gene mutation led to an intraoperative definitive diagnosis of low-grade glioma

Intraoperative detection of *IDH1/2* mutation

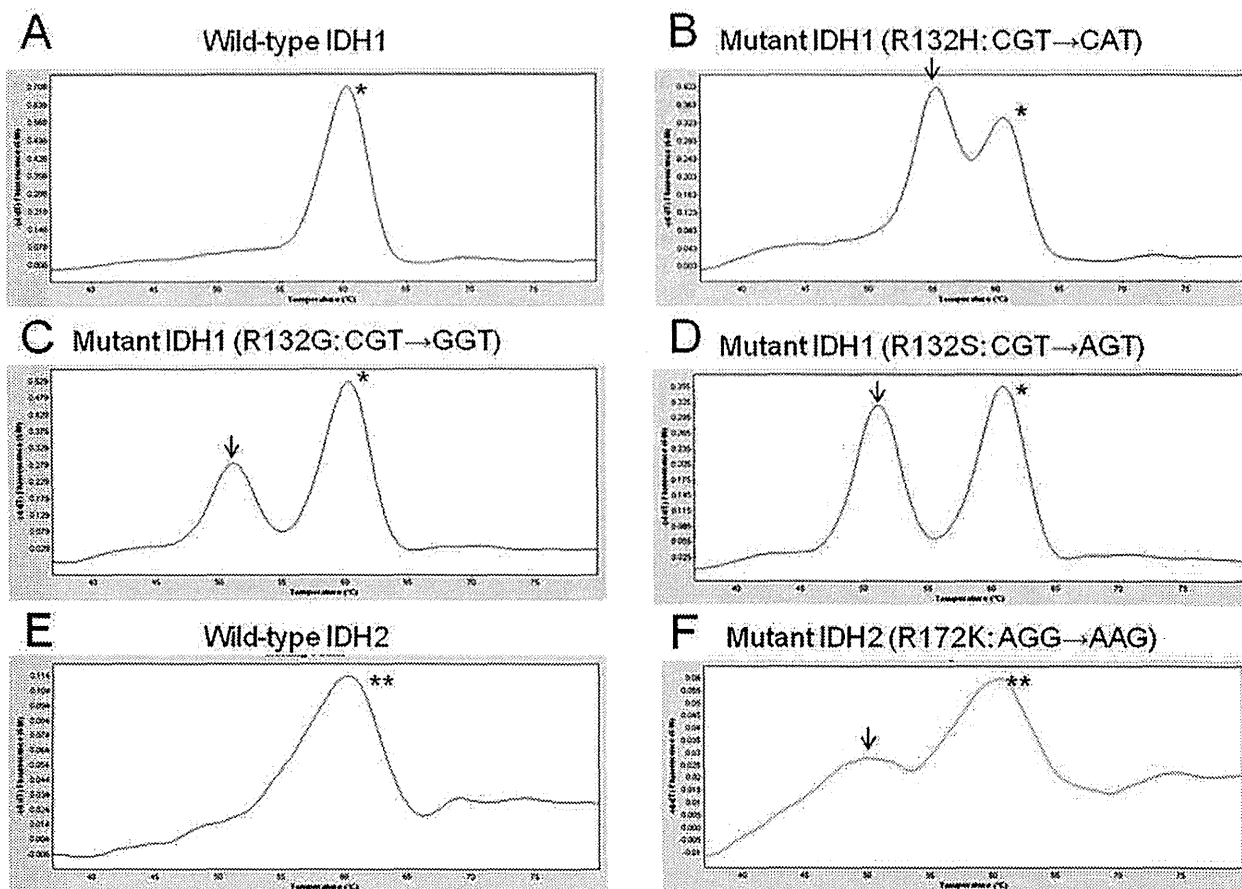


Fig. 1. Fluorescent curve analysis of conventional PCR fragments of *IDH1* and *IDH2* genes from rapidly extracted DNA for intraoperative detection. **A–D:** Fluorescent melting curve analysis of PCR fragments, including the site of *IDH1* gene mutation. All types of mutation (CAT, GGT, and AGT) had a distinct T_m (arrows) from the wild-type sequence (single asterisk). **E and F:** Fluorescent melting curve analysis of PCR fragments, including the site of *IDH2* gene mutations. Mutation of the *IDH2* gene had a distinct T_m (arrow) from the wild-type sequence (double asterisks).

in 3 cases (Cases 2, 3, and 5 in Table 1; Figs. 3 and 4), whereas no *IDH1* and *IDH2* gene mutation was found in 2 cases (Cases 1 and 4). H & E staining of formalin-fixed, paraffin-embedded sections established diagnoses of diffuse astrocytoma in 2, oligodendroglioma in 2, and oligoastrocytoma in 1 case. Case 5 had a diffuse lesion in the left temporal lobe (Fig. 4A). The finding of cells with low-grade atypia distributed unevenly with low cellularity on intraoperative frozen sections is indicative of low-grade glioma (Fig. 4B). Conventional PCR and FMCA with HybProbe detected a slight peak other than that of wild-type allele, and COLD-PCR enriched the mutant sequence (Fig. 4C). Immunohistochemical analysis of the specimen from this lesion revealed that one-third of cells expressed mutant *IDH1* protein (Fig. 4D).

Detection of *IDH1* and *IDH2* Mutations in High-Grade Glioma

During the cohort period, 10 cases of high-grade glioma were suspected based on MRI. The diagnoses based on frozen sections and formalin-fixed, paraffin-embedded

sections, as well as the mutation status, are shown in Table 1 (Cases 9–18). Intraoperative frozen-section examination suggested diagnoses of glioblastoma in 5 cases. Intraoperative detection of the *IDH1* or *IDH2* gene revealed no mutation in 4 of these cases and an *IDH1* gene mutation in 1 of the cases. The final histological diagnoses of the cases without mutation were glioblastoma in 3 and glioblastoma with an oligodendroglial component in 1, and the diagnosis of the case with mutation was glioblastoma. Another 5 cases were diagnosed as high-grade glioma without evidence of glioblastoma. Intraoperative diagnosis based on frozen sections was anaplastic astrocytoma in 2 and anaplastic oligodendroglioma in 3. We detected the *IDH1* gene mutation in 3 cases, and final histological diagnosis of these cases was anaplastic oligodendroglioma.

Differentiation of Recurrence From Treatment-Induced Changes

To examine whether our procedures could detect the presence of tumor cells in radiation necrosis, we evaluated the frozen tissues and formalin-fixed, paraffin-embedded

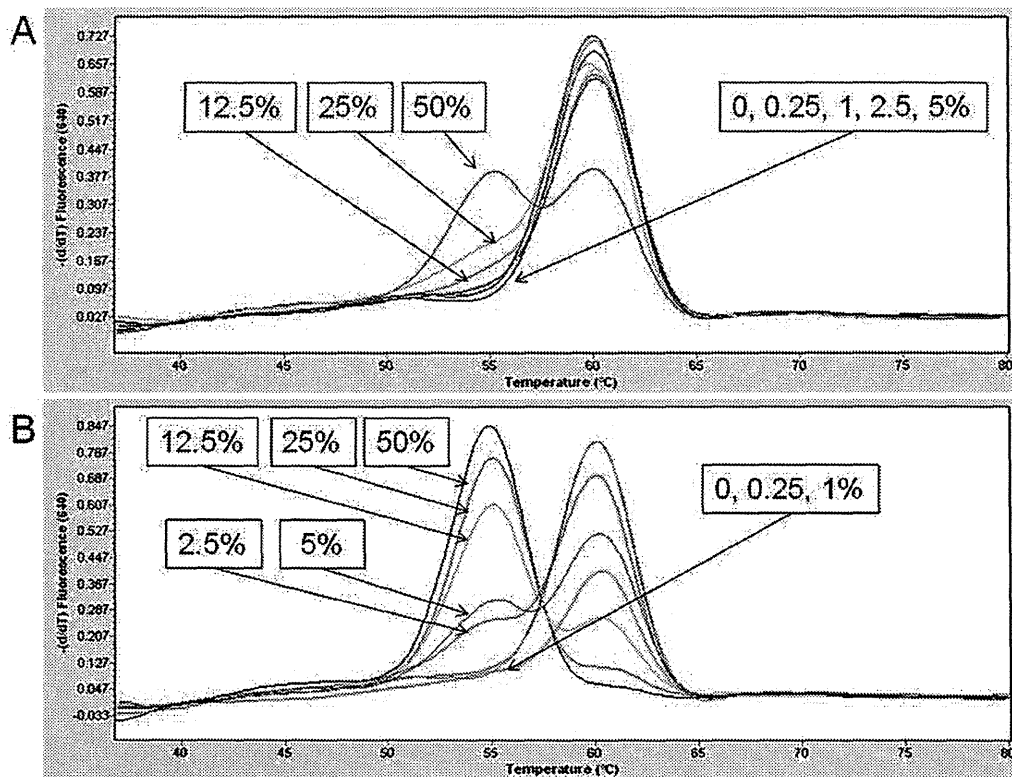


Fig. 2. Fluorescent melting curve analysis after conventional PCR (A) and co-amplification at lower denaturation temperature (COLD-PCR; B) for the detection of *IDH1* R132H mutation. Mutant DNA from tumors with *IDH1* R132H mutation was serially diluted with wild-type DNA from tumor without *IDH1* mutation.

sections from 3 specimens of radiation necrosis containing a small fraction of reactive astrocytes or tumor cells. Conventional PCR and FMCA with HybProbe detected low signals corresponding to the mutated sequence in all 3 cases, and the mutated sequence could be enriched with COLD-PCR in all 3 cases (Fig. 5A). We confirmed this finding with immunohistochemistry for mutated *IDH1* protein, and found a small fraction of cells expressing mutated *IDH1* protein within the necrotic tissue in all 3 cases (Fig. 5B and C).

Discussion

In the present study we investigated intraoperative procedures for the detection of mutations in the *IDH1* and *IDH2* genes. Various methods are available for their detection, with the greatest sensitivity being high as 0.0002% when using COLD-PCR followed by targeted resequencing¹⁵ and the fastest method for amplification and detection being FMCA, which can detect mutation in 80 minutes using extracted DNA.⁸ Our present method detected mutation in 60–65 minutes from sampling of specimens to extraction of DNA and detected *IDH1* mutation as low as 2.5% against a background of the wild-type allele. This study showed that our simple method was feasible for the intraoperative detection of mutation and demonstrated complete concordance with the results

obtained from standard DNA extraction and Sanger sequencing.

The most important application for intraoperative molecular diagnosis is providing definitive, objective, and rapid information to guide the surgical procedures. We found that FMCA with one pair of specific probes for the mutational hotspot⁸ could provide exact information on the mutation status, heterozygosity, and proportions of mutant alleles in the tissue examined. In addition, we employed COLD-PCR as well as conventional PCR. COLD-PCR was highly sensitive for detection of the low abundance of mutated alleles in Case 5 and in cases of radiation necrosis with a small fraction of tumor cells carrying the *IDH1* gene mutation. In addition, this PCR-based detection system requires small samples obtained by 2-mm tumor forceps. Procedures for intraoperative diagnosis require rapidity. We achieved detection of mutation within 60–65 minutes. Double COLD-PCR has been followed by high-resolution melting analysis with an 0.25% sensitivity for detection of mutant allele.² The extraction of DNA using the QIAamp DNA extraction kit, according to the manufacturer's instructions, took 90–270 minutes, and amplification of the DNA fragment and detection of the mutation took 180 minutes.³ Such a method is clearly applicable to postoperative meticulous study, but this method seems to be too sensitive and takes too much time for the intraoperative qualitative diagnosis of glioma. Although

Intraoperative detection of *IDH1/2* mutation

TABLE 1: Results of rapid detection of *IDH1* and *IDH2* mutations and diagnosis based on examination of intraoperative frozen sections and paraffin-embedded sections*

Case No.	Age (yrs), Sex	Diagnosis		<i>IDH1</i> Status		<i>IDH2</i> Status:
		Using Frozen Sections	Using FFPE Sections	PCR	COLD-PCR	PCR
1	18, F	nonneoplastic tissue or LGG	diffuse astrocytoma	wt	wt	wt
2	41, F	nonneoplastic tissue or LGG	oligodendroglioma	mut	ND	ND
3	22, F	nonneoplastic tissue or LGG	diffuse astrocytoma	mut	ND	ND
4	77, M	nonneoplastic tissue or LGG	oligodendroglioma	wt	wt	wt
5	63, F	nonneoplastic tissue or LGG	oligoastrocytoma	mut†	mut	wt
6	31, F	oligoastrocytoma	oligoastrocytoma	mut	ND	ND
7	49, F	oligodendroglioma	oligodendroglioma	mut	ND	ND
8	25, F	ganglioglioma	anaplastic ganglioglioma	mut	ND	wt
9	48, M	anaplastic astrocytoma	GBMO	wt	wt	wt
10	38, F	anaplastic astrocytoma	anaplastic oligodendroglioma	mut	ND	wt
11	52, M	anaplastic oligodendroglioma	anaplastic oligodendroglioma	mut	ND	wt
12	47, F	anaplastic oligodendroglioma	anaplastic oligodendroglioma	mut	ND	wt
13	56, M	anaplastic oligodendroglioma	GBMO	wt	wt	wt
14	52, M	glioblastoma	glioblastoma	wt	wt	wt
15	60, M	glioblastoma	GBMO	wt	wt	wt
16	42, M	glioblastoma	glioblastoma	mut	ND	wt
17	59, M	glioblastoma	glioblastoma	wt	wt	wt
18	62, F	glioblastoma	glioblastoma	wt	wt	wt

* FFPE = formalin-fixed, paraffin-embedded; GBMO = glioblastoma with oligodendroglioma component; LGG = low-grade glioma; mut = mutant; ND = not done; wt = wild-type.

† Low peak of mutated sequence.

we achieved intraoperative detection of the mutation within 60–65 minutes, further shortening of the assay time would increase the applicability of the method. We could shorten the time for DNA extraction (from 90 to 270 minutes to 15 minutes) and for detection of the mutation (from 180 minutes with Sanger sequencing to 5 minutes). The time for DNA amplification took 40–45 minutes, and simpler technology could further reduce the time required. For example, loop-mediated isothermal amplification is a novel nucleic acid amplification method that amplifies DNA with high specificity, efficiency, and rapidity.¹⁷ This method requires a set of 4 specially designed primers and a DNA polymerase with strand displacement activity under isothermal conditions, needs only 15 minutes to amplify the DNA,²⁸ and has been applied to the detection of microbial diseases and metastatic cancer cells.^{10,19}

The quality of any clinical test depends on its validity and predictive value. Validity and predictive value consist of sensitivity and specificity and positive and negative predictive values, respectively. To assess the validity and predictive values, we have to discuss the 2 objectives of this test: 1) intraoperative detection of mutation of *IDH* genes and 2) detection of the “glioma.” If the detection of the *IDH* mutation is the main goal, the sensitivity/specificity and the positive and negative predictive values are all 100%, as no false-positive or -negative results were found in the 14 cases of the validation study and the 18 cases of the intraoperative study. These parameters may be superior to those of Sanger sequencing, in which the detection

threshold was 25% (data not shown) of the *IDH1* mutation against a background of the wild-type allele, and immunohistochemistry for R132H *IDH1* mutation, in which any other type of mutation of *IDH1* or *IDH2* could not be detected. However, we need to be cautious in interpreting this result. The threshold for detection was 2.5% of the *IDH1* mutation against a background of the wild-type allele, whereas an insufficient sampling with a low tumor/normal cell ratio decreased the sensitivity and negative predictive value. If detection of the “glioma” is the main objective, then the sensitivity and specificity are very different. In this series, the sensitivity for the detection of glioma was 44%. Since the mutations of *IDH1* and *IDH2* are high-abundance mutations in gliomas, this result depends on the frequency of the cases with *IDH* mutation established by adequate sampling. Specificity could not be assessed because no cases of nonneoplastic lesions were included in the study. However, high specificity can be expected because previous reports suggested that no nonneoplastic lesions had *IDH1* and *IDH2* mutations.^{3,9}

With regard to predictive value, the intraoperative detection of mutation had a 100% positive predictive value for glioma. This is the most important clinical aspect to apply in intraoperative genetic diagnosis. If intraoperative diagnosis based on morphology cannot establish the definitive diagnosis as glioma, the surgeon has to discontinue the surgery and to await the results of histological diagnosis. However, the detection of an *IDH1* or *IDH2* mutation definitively indicates that the lesion contains

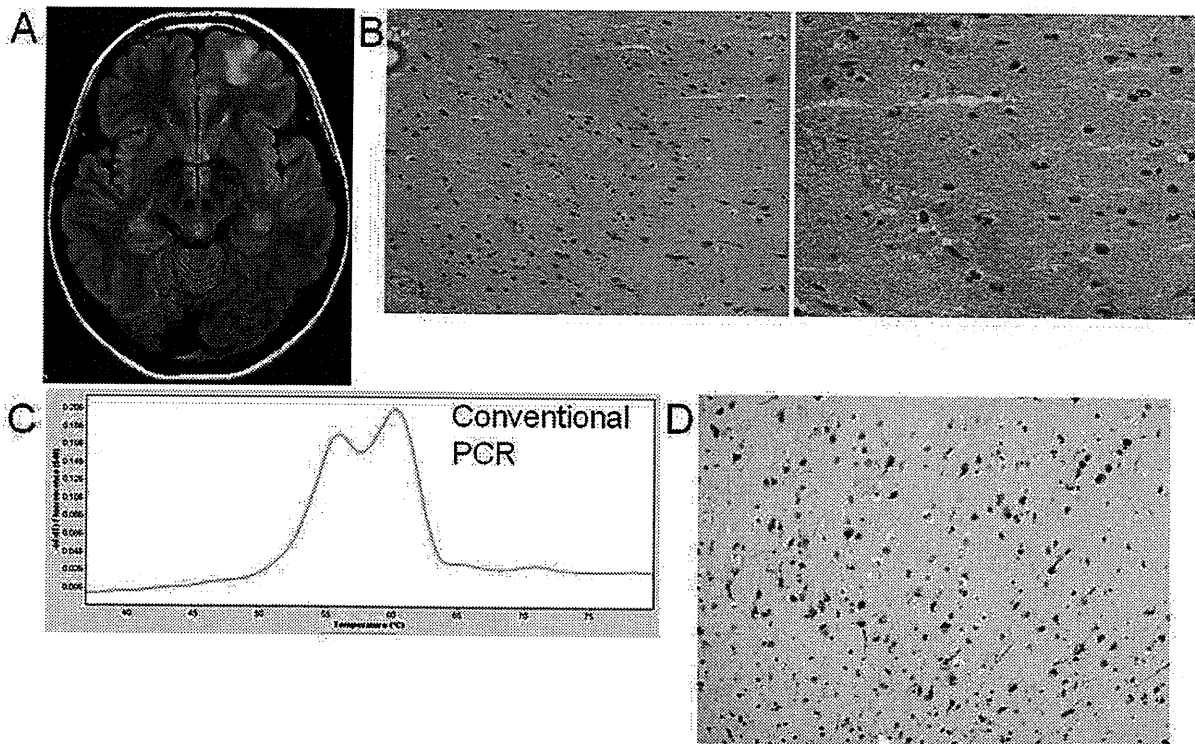


FIG. 3. Case 3. Representative case of a 22-year-old woman with an asymptomatic diffuse lesion in the left frontal lobe. **A:** Preoperative FLAIR MR image demonstrating the diffuse lesion in the left frontal lobe. **B:** H & E staining of the intraoperative frozen section demonstrating cells with slight nuclear atypia distributed unevenly with low cellularity; however, these changes were not conclusive of a diagnosis for low-grade glioma. Original magnification $\times 200$ (left) and $\times 400$ (right). **C:** Fluorescent melting curve analysis after conventional PCR for the detection of the *IDH1* mutation, demonstrating tissues containing the wild-type and mutant *IDH1* gene. **D:** Immunohistochemistry for mutant *IDH1* R132H protein of a formalin-fixed, paraffin-embedded section from the same region, demonstrating that tumor cells express mutant *IDH1* R132H protein. Original magnification $\times 200$.

glioma. Therefore, an intraoperative molecular diagnosis could provide critical information during surgery for tumors suspected to be low-grade glioma. In contrast, 2 of 5 cases in which a nonneoplastic lesion was difficult to differentiate from low-grade glioma had wild-type *IDH1* and *IDH2* genes. Because the absence of an *IDH* mutation does not imply that the lesion is a nonneoplastic lesion, the negative predictive value for glioma was unsatisfactory based only on *IDH1* and *IDH2* gene analysis. Similarly, 6 of 10 cases with high-grade glioma had wild-type *IDH1* and *IDH2* genes. To improve the negative predictive value of intraoperative molecular diagnosis in the future, multiplex analysis with mutation sets for detecting glioma is desirable. To date, the specific mutations such as the *BRAF* V600E mutation in pleomorphic xanthoastrocytoma, ganglioglioma, and pediatric astrocytoma^{23,24} and the histone H3.3 K27 M mutation in diffuse intrinsic pontine glioma³¹ have been reported, and they are potential candidates for intraoperative molecular analysis. Further comprehensive sequence analysis of various types of tumors and nonneoplastic lesions with the next generation of sequencer technology may achieve high negative predictive value for clinical use, and detection of other disease- or tumor-specific mutations may encourage wider use of intraoperative detection systems in the future.

The expected implication of intraoperative diagnosis is that the neurosurgeon can decide the extent of resection based on the mutation status. We found this method useful for the differential diagnosis of anaplastic oligodendroglial tumors, glioblastoma with an oligodendrogloma component, and glioblastoma, as demonstrated in Cases 11, 12, 13, and 15, based on the diverse frequency of the *IDH1* or *IDH2* gene.^{1,7,30} As these entities have quite different prognoses,¹⁶ this method is valuable for establishing the prognosis of the case intraoperatively. However, this intraoperative examination is no more than a diagnostic technique of mutation status and histological subtypes and is not useful for deciding the extent of resection. To this end, the differences in the sensitivity to adjuvant radiation therapy/chemotherapy and in the impact of the extent of the resection between glioma with mutated and wild-type *IDH1* genes should be elucidated. To our knowledge, 3 previously reported studies have demonstrated differences in the sensitivity to adjuvant therapy in tumors with mutated and wild-type *IDH1* genes. A hazards ratio reduction by the addition of chemotherapy (procarbazine, CCNU, and vincristine) was more pronounced in patients with anaplastic oligodendroglial tumors with *IDH1* mutation.²⁹ Similarly, the presence of an *IDH* mutation predicts response to temozolomide in

Intraoperative detection of *IDH1/2* mutation

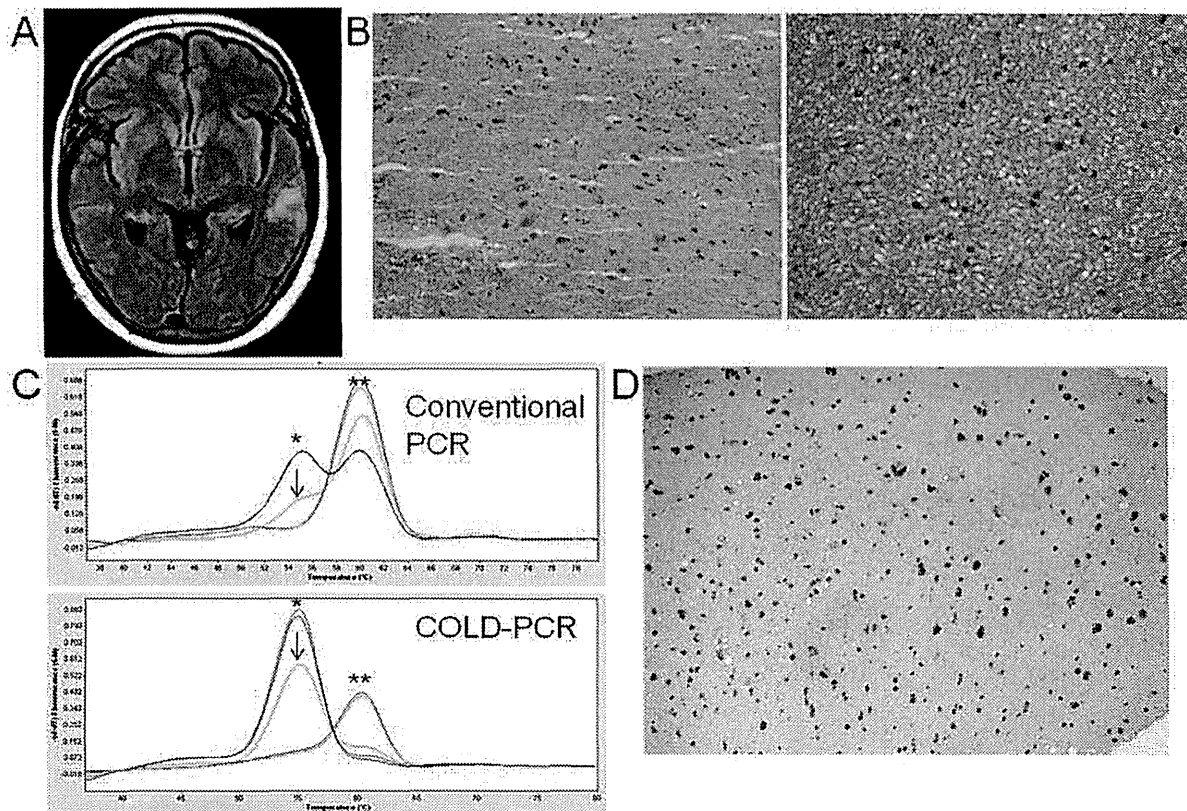


Fig. 4. Case 5. Representative case of a 63-year-old woman with an asymptomatic diffuse lesion in the left temporal lobe. **A:** Preoperative FLAIR MR image demonstrating the diffuse lesion in the left temporal lobe. **B:** H & E staining of the intraoperative frozen section demonstrating uneven distribution of cells with slight nuclear atypia with low cellularity; however, these changes were not conclusive of a diagnosis of low-grade glioma. Original magnification $\times 200$ (left) and $\times 400$ (right). **C:** Fluorescent melting curve analysis after conventional PCR (upper) and co-amplification at lower denaturation temperature (COLD-PCR; lower), demonstrating a low peak of the mutation (arrow) after conventional PCR, and a clearer peak of the mutation (arrow) after COLD-PCR. Same analysis of DNA from the tumor carrying mutated (asterisk) and wild-type (double asterisks) *IDH1* gene was used as the control. **D:** Immunohistochemistry for mutant *IDH1* R132H protein of a formalin-fixed, paraffin-embedded section from the same region, demonstrating that one-third of the cells expressed mutant *IDH1* R132H protein. Original magnification $\times 200$.

low-grade gliomas and secondary glioblastomas.^{11,26} In contrast, little reliable information is available to demonstrate any difference in the impact of the extent of resection in glioma with a mutated and wild-type *IDH1/IDH2* gene. If any difference between glioma with mutated or wild-type *IDH* genes can be demonstrated in the future, an intraoperative molecular diagnosis will provide useful information for designing the surgical strategy.

In the present study, using intraoperative frozen-tissue examination, we detected an *IDH1* mutation in all 3 cases that involved a diagnosis of radiation necrosis with a small fraction of tumor cells. A previous report provided objective evidence for the presence of glioma cells in most cases of radiation necrosis and without definitive tumor demonstrated on H & E-stained photomicrographs.³ Mutant *IDH1* protein was consistently detected in posttherapy glioma with mutation of *IDH1* at first biopsy.³ Considered together with our results, the observation that posttherapy glioma tissues always contain glioma cells indicates that it is meaningless to apply this technology

for only intraoperative detection of tumor cells during the surgery for recurrent glioma. Instead, we can estimate the proportion of tumor cells to normal cells with the PCR and COLD-PCR methods as shown in Fig. 2. For clinical application in the surgery of recurrent glioma, it might be useful to examine the correlation between the proportion of mutated alleles and prognosis, combined with intraoperative assessment of proliferative activity shown using Ki-67 immunostaining.⁶

Conclusions

The combination of rapid extraction of DNA and conventional PCR, followed by FMCA with HybProbe, provides a quick and specific method for intraoperative detection of *IDH1* and *IDH2* gene mutations. COLD-PCR enhanced the sensitivity for detection of the *IDH1* gene mutation and is applicable to intraoperative molecular diagnosis. These methods were useful in the differential diagnosis between low-grade glioma and nonneoplastic

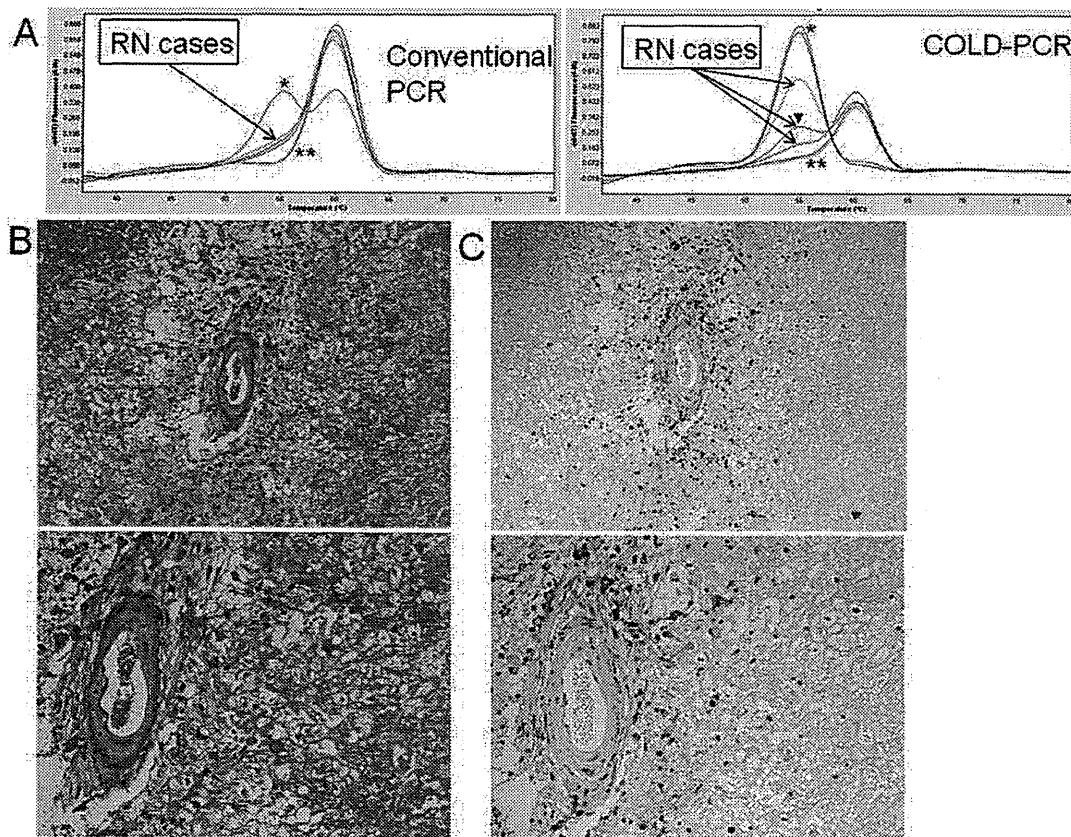


Fig. 5. Simulated detection of mutation from the tissue with radiation necrosis (RN). **A:** Fluorescent melting curve analysis after conventional PCR and co-amplification at lower denaturation temperature (COLD-PCR) for the detection of *IDH1* mutation in the frozen tissues, obtained from 3 cases diagnosed with radiation necrosis on formalin-fixed, paraffin-embedded sections (indicated as RN cases), demonstrating the presence of the mutation in all 3 cases. Note that COLD-PCR enhanced the sensitivity for detection. The same analysis of DNA from the tumor with mutated (*single asterisk*) and wild-type (*double asterisks*) *IDH1* genes was used as the control. **B and C:** H & E staining (**B**) and immunohistochemistry for mutant *IDH1* R132H protein (**C**) of a formalin-fixed, paraffin-embedded section of the representative case with radiation necrosis, demonstrating gemistocytic cells around the vessel in the background of necrotic tissue, and several gemistocytic cells expressing mutant *IDH1* R132H protein around the vessel. Original magnification $\times 100$ (**B** upper and **C** upper) and $\times 400$ (**B** lower and **C** lower).

plastic lesions, the diagnosis for subtypes of high-grade glioma, and the prediction of the prognosis. Although tumor cells in cases of radiation necrosis were detected with high sensitivity, further investigation is necessary to establish clinical application.

Acknowledgment

We thank Ms. Misaki Fue for her technical assistance in the intraoperative assays.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Kanamori, Kikuchi, Kumabe, Tominaga. Acquisition of data: Kanamori, Kikuchi, Watanabe, Shibahara, Saito, Yamashita, Sonoda, Kumabe. Analysis and interpretation of data: Kanamori, Saito, Yamashita, Kure. Draft-

ing the article: Kanamori, Kikuchi, Watanabe, Kure, Tominaga. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Kanamori. Administrative/technical/material support: Kanamori. Study supervision: Kanamori, Tominaga.

References

- Balss J, Meyer J, Mueller W, Korshunov A, Hartmann C, von Deimling A: Analysis of the *IDH1* codon 132 mutation in brain tumors. *Acta Neuropathol* **116**:597–602, 2008
- Boisselier B, Marie Y, Labussière M, Ciccarino P, Desestret V, Wang X, et al: COLD PCR HRM: a highly sensitive detection method for *IDH1* mutations. *Hum Mutat* **31**:1360–1365, 2010
- Capper D, Sahm F, Hartmann C, Meyermann R, von Deimling A, Schittenhelm J: Application of mutant *IDH1* antibody to differentiate diffuse glioma from nonneoplastic central nervous system lesions and therapy-induced changes. *Am J Surg Pathol* **34**:1199–1204, 2010
- Capper D, Zentgraf H, Balss J, Hartmann C, von Deimling A: Monoclonal antibody specific for *IDH1* R132H mutation. *Acta Neuropathol* **118**:599–601, 2009

Intraoperative detection of *IDH1/2* mutation

- Felsberg J, Wolter M, Seul H, Friedensdorf B, Göppert M, Sabel MC, et al: Rapid and sensitive assessment of the IDH1 and IDH2 mutation status in cerebral gliomas based on DNA pyrosequencing. *Acta Neuropathol* **119**:501–507, 2010
- Haapasalo J, Mennander A, Helen P, Haapasalo H, Isola J: Ultrarapid Ki-67 immunostaining in frozen section interpretation of gliomas. *J Clin Pathol* **58**:263–268, 2005
- Hegi ME, Janzer RC, Lambiv WL, Gorlia T, Kouwenhoven MC, Hartmann C, et al: Presence of an oligodendroglioma-like component in newly diagnosed glioblastoma identifies a pathogenetically heterogeneous subgroup and lacks prognostic value: central pathology review of the EORTC_26981/NCIC_CE.3 trial. *Acta Neuropathol* **123**:841–852, 2012
- Horbinski C, Kelly L, Nikiforov YE, Durso MB, Nikiforova MN: Detection of IDH1 and IDH2 mutations by fluorescence melting curve analysis as a diagnostic tool for brain biopsies. *J Mol Diagn* **12**:487–492, 2010
- Horbinski C, Kofler J, Kelly LM, Murdoch GH, Nikiforova MN: Diagnostic use of IDH1/2 mutation analysis in routine clinical testing of formalin-fixed, paraffin-embedded glioma tissues. *J Neuropathol Exp Neurol* **68**:1319–1325, 2009
- Horibe D, Ochiai T, Shimada H, Tomonaga T, Nomura F, Gun M, et al: Rapid detection of metastasis of gastric cancer using reverse transcription loop-mediated isothermal amplification. *Int J Cancer* **120**:1063–1069, 2007
- Houillier C, Wang X, Kaloshi G, Mokhtari K, Guillemin R, Laffaire J, et al: IDH1 or IDH2 mutations predict longer survival and response to temozolomide in low-grade gliomas. *Neurology* **75**:1560–1566, 2010
- Kanamori M, Kumabe T, Shibahara I, Saito R, Yamashita Y, Sonoda Y, et al: Clinical and histological characteristics of recurrent oligodendroglial tumors: comparison between primary and recurrent tumors in 18 cases. *Brain Tumor Pathol* **30**:151–159, 2013
- Li J, Wang L, Mamon H, Kulke MH, Berbeco R, Makrigiorgos GM: Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. *Nat Med* **14**:579–584, 2008
- Louis DN, Ohgaki H, Wiestler OD, Cavenee WK: **WHO Classification of Tumours of the Central Nervous System**. Lyon: IARC Press, 2007
- Milbury CA, Correll M, Quackenbush J, Rubio R, Makrigiorgos GM: COLD-PCR enrichment of rare cancer mutations prior to targeted amplicon resequencing. *Clin Chem* **58**:580–589, 2012
- Miller CR, Dunham CP, Scheithauer BW, Perry A: Significance of necrosis in grading of oligodendroglial neoplasms: a clinicopathologic and genetic study of newly diagnosed high-grade gliomas. *J Clin Oncol* **24**:5419–5426, 2006
- Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al: Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* **28**:E63, 2000
- Ohgaki H, Kleihues P: The definition of primary and secondary glioblastoma. *Clin Cancer Res* **19**:764–772, 2013
- Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K: Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev Med Virol* **18**:407–421, 2008
- Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, et al: An integrated genomic analysis of human glioblastoma multiforme. *Science* **321**:1807–1812, 2008
- Plessec TP, Prayson RA: Frozen section discrepancy in the evaluation of central nervous system tumors. *Arch Pathol Lab Med* **131**:1532–1540, 2007
- Roessler K, Dietrich W, Kitz K: High diagnostic accuracy of cytologic smears of central nervous system tumors. A 15-year experience based on 4,172 patients. *Acta Cytol* **46**:667–674, 2002
- Schiffman JD, Hodgson JG, VandenBerg SR, Flaherty P, Polley MY, Yu M, et al: Oncogenic BRAF mutation with CDKN2A inactivation is characteristic of a subset of pediatric malignant astrocytomas. *Cancer Res* **70**:512–519, 2010
- Schindler G, Capper D, Meyer J, Janzarik W, Omran H, Herold-Mende C, et al: Analysis of BRAF V600E mutation in 1,320 nervous system tumors reveals high mutation frequencies in pleomorphic xanthoastrocytoma, ganglioglioma and extra-cerebellar pilocytic astrocytoma. *Acta Neuropathol* **121**:397–405, 2011
- Shibahara I, Sonoda Y, Kanamori M, Saito R, Yamashita Y, Kumabe T, et al: IDH1/2 gene status defines the prognosis and molecular profiles in patients with grade III gliomas. *Int J Clin Oncol* **17**:551–561, 2012
- SongTao Q, Lei Y, Si G, YanQing D, HuiXia H, XueLin Z, et al: IDH mutations predict longer survival and response to temozolomide in secondary glioblastoma. *Cancer Sci* **103**:269–273, 2012
- Sonoda Y, Kumabe T, Nakamura T, Saito R, Kanamori M, Yamashita Y, et al: Analysis of IDH1 and IDH2 mutations in Japanese glioma patients. *Cancer Sci* **100**:1996–1998, 2009
- Tsujimoto M, Nakabayashi K, Yoshidome K, Kaneko T, Iwase T, Akiyama F, et al: One-step nucleic acid amplification for intraoperative detection of lymph node metastasis in breast cancer patients. *Clin Cancer Res* **13**:4807–4816, 2007
- van den Bent MJ, Brandes AA, Taphoorn MJ, Kros JM, Kouwenhoven MC, Delattre JY, et al: Adjuvant procarbazine, lomustine, and vincristine chemotherapy in newly diagnosed anaplastic oligodendroglioma: long-term follow-up of EORTC brain tumor group study 26951. *J Clin Oncol* **31**:344–350, 2013
- Wang Y, Li S, Chen L, You G, Bao Z, Yan W, et al: Glioblastoma with an oligodendroglioma component: distinct clinical behavior, genetic alterations, and outcome. *Neuro Oncol* **14**:518–525, 2012
- Wu G, Broniscer A, McEachron TA, Lu C, Paugh BS, Beckford J, et al: Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. *Nat Genet* **44**:251–253, 2012

Manuscript submitted July 14, 2013.

Accepted March 13, 2014.

Please include this information when citing this paper: published online April 18, 2014; DOI: 10.3171/2014.3.JNS131505.

Address correspondence to: Masayuki Kanamori, M.D., Ph.D., Department of Neurosurgery, Tohoku University Graduate School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai, Miyagi 980-8574, Japan. email: mkanamori@med.tohoku.ac.jp.

