A Novel All-in-one Intraoperative Genotyping System for *IDH1*-mutant Glioma

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[Abstract]

IDH1 gene mutation has been demonstrated to be an oncogenic driver in a majority of lower-grade gliomas (LGGs). In contrast to other central nervous neoplasms and normal brain tissue without *IDH1* mutation, almost 80% of LGGs exhibit *IDH1* mutation. Therefore, expeditious detection of *IDH1* mutation is useful, not only for intraoperative diagnosis of these gliomas but also for determination of the border between the tumor and normal brain tissue. In this study, we established a rapid genotyping assay with a simple DNA extraction method, involving only incubation of the tumor specimen with Tris-EDTA buffer, which can be easily performed in an operating room. In all 11 tested cases, we could identify the *IDH1* status within 90–100 min intraoperatively. In a case of anaplastic astrocytoma, IDH-mutant, we could detect the tumor border by *IDH1* profiling. In addition, with this assay, we could detect *IDH1* mutation using cell-free tumor DNA derived from cerebrospinal fluid in a case of glioblastoma, IDH-mutant.

Considering that clinical trials of mutated IDH1 inhibitors are on-going, less-invasive intraoperative *IDH1* gene profiling might be useful for decision-making of the overall treatment strategy of LGGs. Our assay might be a useful tool for precision medicine and surgery of *IDH1*-mutant gliomas.

[Introduction]

Diffuse glioma, consisting of grade II and III gliomas called lower-grade glioma (LGG) and grade IV glioblastoma (GBM), is the most frequent and malignant neuro-epithelial tumor in the central nervous system. The diffuse infiltrative nature of these gliomas makes a surgical cure unlikely. For diffuse glioma, immediate initiation of optimal adjuvant therapy after removal is required. However, it is frequently difficult to differentiate diffuse glioma from other neuro-epithelial and embryonal tumors radiographically and histologically. Recent comprehensive studies revealed that *IDH1* mutation is an oncogenic driver in the formation of most LGGs and a subset of GBMs [1-3]. Intriguingly, *IDH1* mutation is found specifically in these gliomas, suggesting that this mutation is a powerful biomarker to differentiate LGGs and a subset of GBMs from other neuro-epithelial and embryonal tumors [4]. The highly infiltrative nature of diffuse glioma also makes it difficult to distinguish the tumor from peri-tumoral reactive gliosis tissue intraoperatively. In the normal brain tissue, there are no *IDH1* mutated cells, even in reactive gliosis tissues. Therefore, development of a rapid and convenient *IDH1* genotyping assay that can be performed in an operating room might enable not only intraoperative diagnosis but also detection of the infiltrative front of diffuse glioma during tumor removal.

We established a rapid *IDH1* genotyping assay with tumor-derived DNA, using a new automated genetic typing device (i-densy; Arkray, Kyoto, Japan) [5-7]. This device is an all-in-one system, which performs the pre-treatment of reagents, polymerase chain reaction (PCR), and single nucleotide polymorphism typing within approximately 80 min. In the present study, we established a simple protocol involving 5-min DNA extraction and the all-in-one genotyping system. We successfully obtained sensitive intraoperative diagnoses of the *IDH1* gene mutation status for all 11 tested tumors. In addition, in a newly diagnosed case of anaplastic astrocytoma, IDH-mutant, we could intraoperatively detect the border line for which the *IDH1* mutant status became the wild type. Based on the high sensitivity of our assay, we performed *IDH1* genotyping with cell-free tumor DNA (ctDNA) in the cerebrospinal fluid (CSF). In a case of GBM, IDH-mutant, we could detect the *IDH1* mutation using ctDNA derived from a small amount of the intraoperatively collected CSF.

[Materials and Methods]

Ethics Statement

The study was approved by the institutional review board at Nagoya University Hospital and complied with all provisions of the Declaration of Helsinki. All tumor samples were collected intraoperatively at Nagoya University Hospital upon receiving informed consent from the patients.

Rapid Detection System for IDH1 Gene Mutation with i-densy

The i-densy device uses a fluorescent quenching probe (Q-Probe), in which a fluorescent substance is bound to cytosine on the terminal end of the probe. Q-Probe is quenched upon hybridization with the complementary strand. With increasing temperature, the duplex unravels at a melting temperature that is proportional to the strength of the bond between Q-Probe and the tumor DNA, at which point the fluorescence intensity recovers. Q-Probe is designed to hybridize with the *IDH1* R132H mutant fragment; the temperature for denaturing R132H DNA is 58℃, whereas the wildtype denaturing temperature is 50℃. The sequence of the Q-probe is CATCATAGGTCATCATGCT.

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Sample Preparation of Tumor Samples and ctDNA in CSF for i-densy Analysis

For analysis of tumor samples, we incubated tumor samples with 100 μL of Tris-ethylenediaminetetraacetic acid (TE) buffer at 95°C for 5 min without homogenization and collected the supernatant. We applied 4 μL of the supernatant into the i-densy universal pack with 25 μL Q-probe and primer mix [8]. Intraoperatively, we analyzed 11 tumors whose removal or biopsy was performed at Nagoya University Hospital. The location of each sample was recorded stereotactically in an intraoperative navigation system (Brainlab, Munich, Germany). These tumors consisted of two diffuse astrocytomas, IDH-mutant, one diffuse astrocytoma, IDH-wildtype, two anaplastic astrocytomas, IDH-mutant, one GBM, IDH-mutant, three GBMs, IDH-wildtype and two other tumors harboring the IDH wildtype. For collection of ctDNA from the CSF, we used Maxwell® RSC ccfDNA Plasma Kit according to the manufacturer's instructions. We used 500 μL of CSF and eluted the ctDNA with 50 μL of elution buffer; the DNA concentration was measured with Quantus Fluorometer®. We then performed the genotyping with 4 μL of the ctDNA elution.

Direct Sequencing and Pyrosequencing

DNA was prepared using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amount of DNA obtained from the tumor was deemed to be sufficient for the subsequent genomic analyses. For *IDH1* sequencing, a 129-bp fragment spanning the sequence encoding the catalytic domain of IDH1, including codon 132, was amplified. We applied conventional PCR for 35 cycles with denaturation at 95°C for 30 s, annealing at 56°C for 40 s, and extension at 72°C for 50 s, with a final extension step at 72°C for 7 min, using the forward primer CGGTCTTCAGAGAAGCCATT and reverse primer GCAAAATCACATTATTGCCAAC. Direct sequencing was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The reactions were

carried out using an ABI 3100 Genetic Analyzer (Applied Biosystems). To evaluate the mutant allele frequency of *IDH1* R132H, we performed pyrosequencing for several samples from a given tumor as previously described. In brief, PCR was run for 50 cycles with denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 2 min, using the forward primer GGCTTGTGAGTGGATGGGTA, reverse primer GGGACACCGCTGATCGTTTATGTGTTGAGATGGACGCCTA, and universal primer 5'-Biotin-GGGACACCGCTGATCGTTTA. Detection and calculation of the frequency of the mutant allele was performed with pyrosequencing technology (Pyrosequencing AB, Uppsala, Sweden), using the sequencing primer TGGATGGGTAAAACCTATCATCA, according to the manufacturer's instructions [9].

Immunohistochemistry of Paraffin-embedded Tissue Samples

The HMab-2 antibody, which is commercially available from Wako Pure Chemical Industries Ltd. (Osaka, Japan), was used for immunohistochemistry for the detection of mutant IDH1 [10-12]. The tumor samples were fixed with 10% formalin and embedded with paraffin. Five-micrometer-thick sections were prepared with a microtome (RM2125RT, Leica, Wetzlar, Germany). After deparaffinization and hydration, the sections were incubated in retrieval solution (TE buffer, pH 9.0) for 30 min at 100°C with an electric pot and then blocked with 1.5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in phosphate-buffered saline containing 0.05% Tween-20 at room temperature for 1 h. The samples were then incubated with HMab-2 (diluted to 1 μg/mL) overnight at 4°C. The sections were then incubated with the secondary labeled polymer from the EnVision HRP kit (Dako; Agilent Technologies, Inc., Santa Clara, CA) for 30 min. The substrate-chromogen solution from DAB Substrate Kit (Vector Laboratories) was applied to the samples for 10 min. After washing, the sections were counterstained with hematoxylin and mounted in multi-Mount (Matsunami Glass Ind.) [13].

[Results]

Sensitivity and Specificity of IDH1 Genotyping of Tumor Specimens with i-densy

We evaluated the sensitivity and specificity of our assay for crude tumor specimens using various amount of frozen tumor samples (1 mg, 10 mg, 50 mg). We tested two *IDH1*-mutant tumors (both anaplastic astrocytomas) and two *IDH1*-wildtype tumors (both meningothelial meningiomas). The mutation peak was clearly detected at 58°C, even for the smallest sample amount (1 mg). In addition, for the 50-mg samples, no false-positive peak was detected in the *IDH1*-wildtype tumors (Figure 1). Duplicate analysis using another sample

set showed the same results (data not shown). These data revealed that our rapid and convenient assay, which can be easily performed in an operating room, harbors sufficient sensitivity and specificity for analyses with crude tumor specimens. Based on these experiments, we decided to use 10-mg tumor specimens for the following intraoperative analysis.

Intraoperative Analysis of IDH1 Gene Mutation with i-densy

After obtaining informed consent from the patients, we analyzed the *IDH1* gene status intraoperatively for 11 cases. The entire procedure, from sample collection to mutation diagnosis, took approximately 90–100 min. In all 11 tested cases, we could successfully detect the *IDH1* gene status intraoperatively. To confirm the genotype, we analyzed the *IDH1* status with conventional direct sequencing from tumor-derived DNA using the same samples. Figure 2 shows the collection site of each tumor specimen and the results of direct sequencing followed by quantitative pyrosequencing, and i-densy in two representative *IDH1*-mutant cases. In a previous study, using tumor-derived DNA, we demonstrated that this genotyping system could detect an approximately 10% mutant allele frequency [5]. The present data revealed that our assay could detect *IDH1* mutation from crude tumor specimens with sufficient sensitivity and specificity.

Determination of the Tumor Border from IDH1 Genotyping

We analyzed several specimens derived from various sites of a tumor bulk in a newly diagnosed case of anaplastic astrocytoma, IDH-mutant. We could detect *IDH1* mutation in a specimen from the center of the tumor (Figure 3a). We also collected a specimen from the tumor margin using navigation imaging. This marginal specimen was found to harbor the *IDH1* wildtype, which was confirmed with both immunohistochemistry and direct sequencing (Figure 3b). This case demonstrates that it is possible to intraoperatively detect the borderline at which the *IDH1* mutant gene status becomes intact using our system. This suggests that our assay has sufficient sensitivity to intraoperatively distinguish the peri-tumoral tissue from the tumor tissue in an *IDH1*-mutant glioma case.

Analysis of IDH1 Gene Mutation with ctDNA from the CSF and Blood Plasma

Based on the high sensitivity of our assay for tumor tissue samples, we conducted *IDH1* genotyping from ctDNA of the CSF and blood plasma in the same fashion. In a case of GBM, IDH-mutant, we genotyped the ctDNA derived from the intracranial CSF. This patient developed diffuse astrocytoma, IDH-mutant located on the left insular cortex, and initially underwent partial tumor removal surgery. After a few years, because of regrowth and

malignant transformation of the residual tumor, the second tumor removal surgery was performed, revealing a pathological diagnosis of GBM, IDH-mutant. During this second operation, we collected 500 μL of the CSF and extracted the ctDNA. The concentration of ctDNA was 3.25 ng/μL in 50 μL of elution buffer. Using 4 μL of this eluted ctDNA, we could successfully detect an *IDH1* mutation (Figure 4). We also collected ctDNA from the blood plasma of this patient and performed *IDH1* genotyping in the same fashion. The concentration of ctDNA was 0.300 ng/μL in 1 mL of blood plasma. Using 4 μL of the plasma-derived ctDNA, no mutated peak could be detected. These data suggest that our assay might harbor sufficient sensitivity for genotyping of ctDNA from the CSF.

[Discussion]

IDH1 gene mutation is regarded as a useful biomarker to distinguish the majority of LGGs from other neuro-epithelial tumors and the peri-tumoral area in an individual. In addition, *IDH1* gene mutation is also a favorable prognostic factor in these gliomas [1, 2, 14]. Thus, intraoperative detection of *IDH1* gene mutation allows for immediate initiation of optimal adjuvant therapy after removal and is also useful to determine the tumor border. In this study, our rapid and convenient assay, which can be easily performed in an operating room, enabled establishing a diagnosis of IDH-mutant lower-grade glioma and IDH-mutant GBM, and could help to determine the tumor border intraoperatively. The *IDH* mutation, defined in the 2016 WHO classification, includes various mutation types of *IDH1* and *IDH2* genes, with the *IDH1 R132H* mutation accounting for more than 80% of the mutations. In this study, we used a Q-probe, which hybridizes with an *IDH1 R132H* gene. Now, we are generating new Q-probes, which are designed to hybridize with wildtype *IDH1* and *IDH2* genes. These probes might enable the intraoperative detection of all mutations of *IDH1* and *IDH2* genes. In addition, from a technical point of view, the limitation of our Q-probe, thus far, is that it might not be able to detect *IDH* mutation in tissues containing less than 10% of tumor cells. In order to define the tumor margin of highly infiltrative cases, we need to improve the sensitivity and specificity of the Q-probe. To date, various PCR-based methods for the detection of *IDH* mutations with tumor-derived DNA have been reported as promising tools for clinical use [15, 16]. However, the most DNA extraction methods from crude tumor specimen are labor-consuming, which has been the main obstacle to overcome for achieving a rapid genotyping protocol that is suitable for an operating room. One of the key advantages of our assay is the establishment of a convenient DNA extraction protocol simply involving incubation of the tumor specimen in buffer. This novel DNA extraction protocol takes much less time and effort than conventional methods, which allows for the entire procedure to be performed in an operating room. Another advantage is that we could

analyze the *IDH* gene status of four tumor specimens concurrently within only 90–100 min, as this genotyping device has four chambers for simultaneous analyses. This property might also allow for the real-time determination of the tumor removal extent. Recently, a rapid immunohistochemistry (IHC) assay has been reported to be effective for intraoperative molecular diagnosis [17]. One of the advantages of our assay is that it involves genetic sequencing, which is recommended for use over IHC in the 2016 WHO classification because of its higher specificity [18]. Another advantage is that our assay allows for easier detection of *IDH* mutation than that by IHC, although rapid IHC involves shorter processing time and is also applicable to other molecules such as MIB1.

Various selective inhibitors have emerged for the treatment of several types of cancers. Based on genetic profiling of each tumor, optimal treatment on a case-by-case basis is important, termed precision medicine [19]. Many recent studies on glioma have revealed deep insights into the mechanisms by which mutant *IDH1* induces glioma formation, via production of the oncometabolite 2-hydroxyglutarate [20, 21]. These findings have led to the development of several selective inhibitors for mutant IDH1. A pre-clinical study demonstrated the efficacy of a mutant IDH1 inhibitor [22], and several first-in-human clinical tests of this inhibitor are now in progress. Establishment of an effective mutant IDH1 inhibitor is expected to lead to a drastic paradigm shift in the treatment strategy for cases of LGGs and GBM harboring an *IDH1* mutation. Therefore, in the near future, the pre- and intraoperative detection of *IDH* mutation might become quite important for determination of the overall treatment strategy for these types of gliomas. Recently, liquid biopsy, a novel and less invasive diagnostic tool using ctDNA of body fluid, has been applied for the clinical diagnosis of various cancers. In glioma, a few groups have reported the detection of various genetic alterations using ctDNA from the CSF and blood plasma [23-25]. Such less-invasive detection of *IDH* mutation with liquid biopsy might be quite effective for the pre-operative planning of a tumor removal strategy in an IDH1 inhibitor era. Indeed, in this study, we could detect *IDH* gene mutation with ctDNA derived from a small amount of CSF in a case of GBM, IDH-mutant. Our rapid diagnostic system might be useful for the less-invasive diagnosis of *IDH1* mutation using the CSF. However, in this case, the yield amount of ctDNA was greater than that reported in the few previous cases. This might be because the CSF dynamics were altered after the first tumor removal and/or the recurrent tumor was adjacent to the CSF space. Therefore, to establish a universal pre-operative genotyping assay with CSF, it is important to develop an *IDH1* mutation assay using CSF in a newly diagnosed case.

In summary, to realize the pre- and intraoperative diagnosis of *IDH1* gene mutation, we established a rapid and convenient diagnostic assay system that was successful for both tumor tissue as well as CSF samples. This highly sensitive diagnostic assay for *IDH1*

mutation might be useful as a companion diagnosis method with tissue samples and CSF for achieving precision medicine for patients with IDH-mutant glioma.

[Reference]

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[Figure Legends]

Figure 1. Genotyping of various amounts of frozen crude tumor specimens with i-densy

We performed *IDH1* genotyping of 1-mg, 10-mg and 50-mg tumor specimens with and without *IDH1* mutation. In our assay, a mutation peak and a wildtype peak are detected at 58°C and 50°C, respectively. Typically, a mutated tumor specimen exhibits both peaks, because a glioma tissue contains normal glial cells with the wildtype *IDH1*. In all the amounts of specimens analyzed, we could detect clear peaks, consistent with their *IDH1* gene profiling without false-positive and –negative results.

Figure 2. Illustrative cases with intraoperative detection of *IDH1* mutation

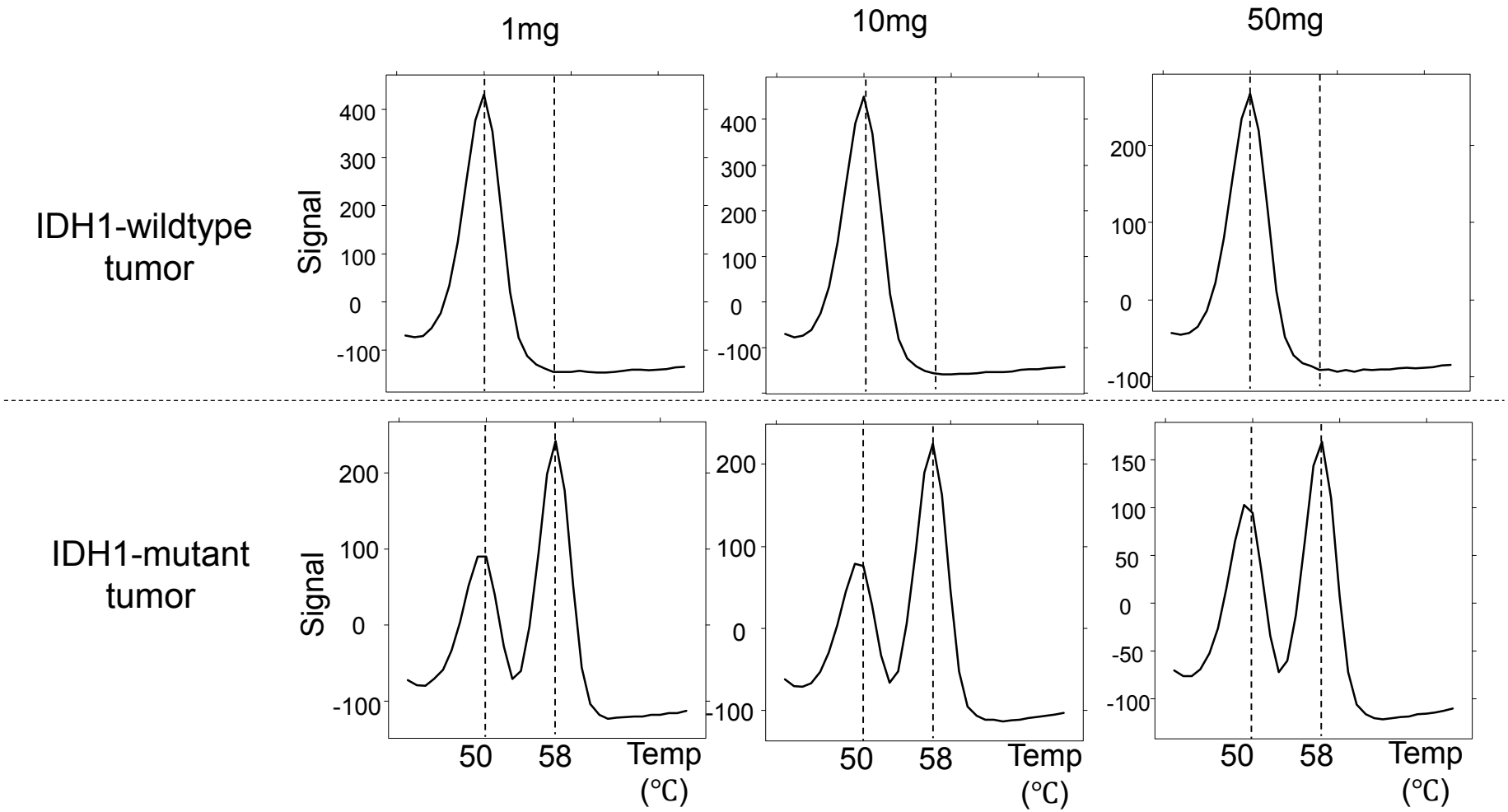
We collected tumor specimen intraoperatively and took screenshots of the sample locations with a navigation system. In cases of *IDH* mutant gliomas, we could detect mutation peaks clearly. We extracted DNA postoperatively from tumor samples, collected from the same site, and analyzed their *IDH1* status by direct sequencing and pyrosequencing. We described

mutation peaks with direct sequencing and calculated the percentage of G to A mutation with pyrosequencing of these cases.

Figure 3. An illustrative case in which an invasive front of tumor is detected intraoperatively
In the case of an anaplastic astrocytoma, IDH-mutant, we performed multi-sampling in an individual tumor. As described in Figure 3a, we could detect *IDH1* mutation in a tumor specimen collected from the center of a tumor. This specimen also had mutated IDH1 as revealed by IHC, direct sequencing, and pyrosequencing. In contrast, a tumor specimen, collected from the border of the tumor as described in the navigation system, revealed wildtype *IDH1* by i-densy, intraoperatively. Consistently, IHC, direct-sequencing, and pyrosequencing revealed no mutation as in Figure 3b.

Figure 4. Genotyping of ctDNA, derived from CSF in a case of glioblastoma, IDH-mutant
We described the T2-weighted image, obtained by MRI, of a case of glioblastoma, IDH-mutant before the 2nd operation. We collected 500μL of CSF from a CSF space (indicated by the arrow) intraoperatively and extracted ctDNA. Using this ctDNA, we could efficiently detect *IDH1* mutation with i-densy.

Figure 1.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IDH1-wildtype tumor
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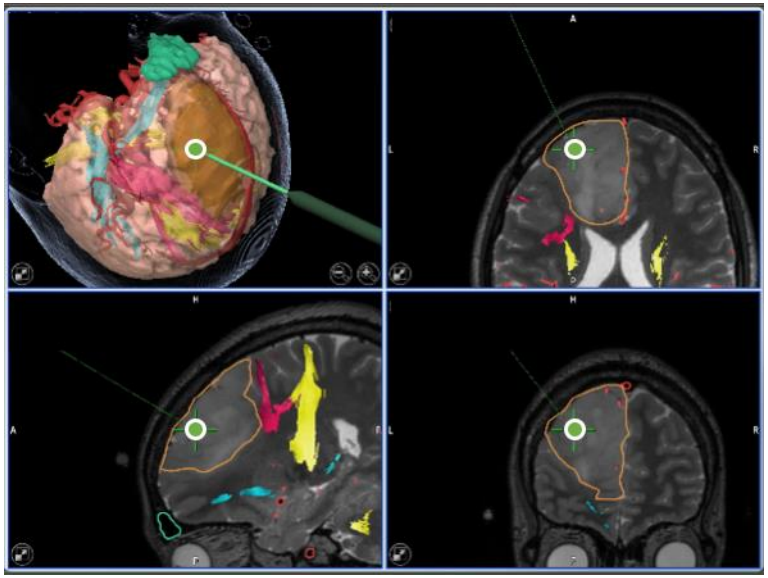
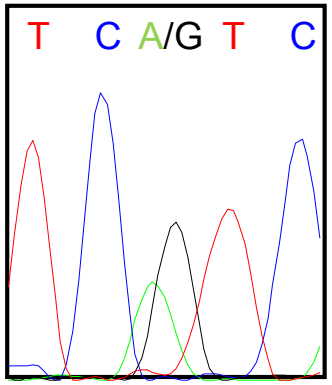
Figure 2.





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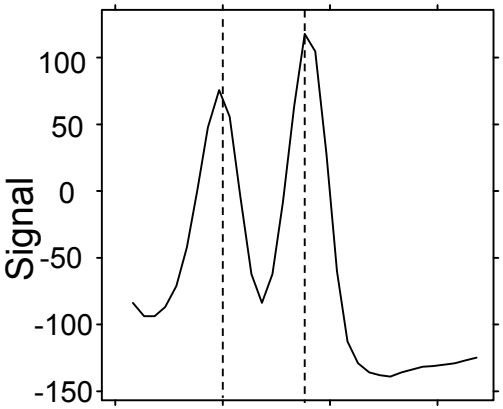

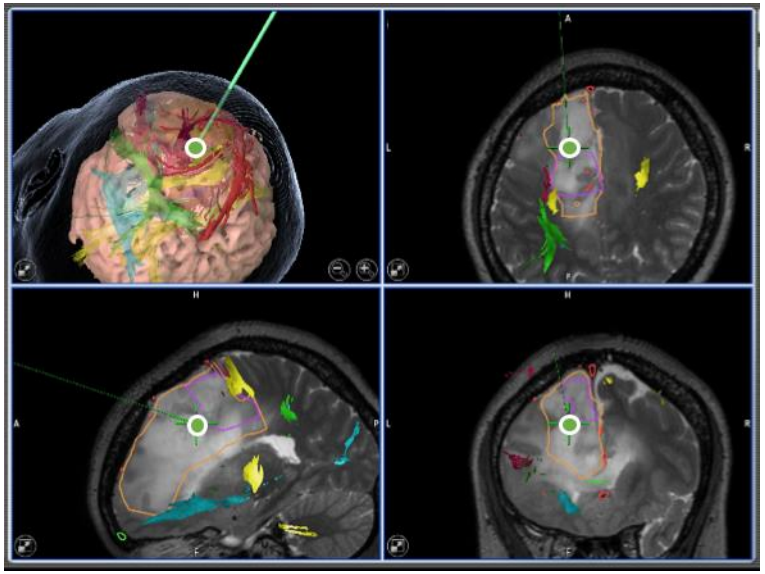

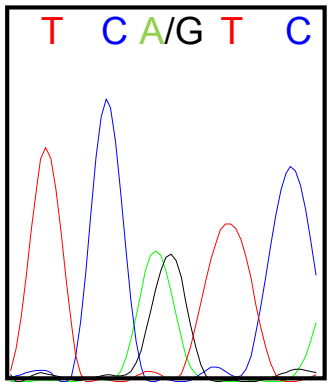

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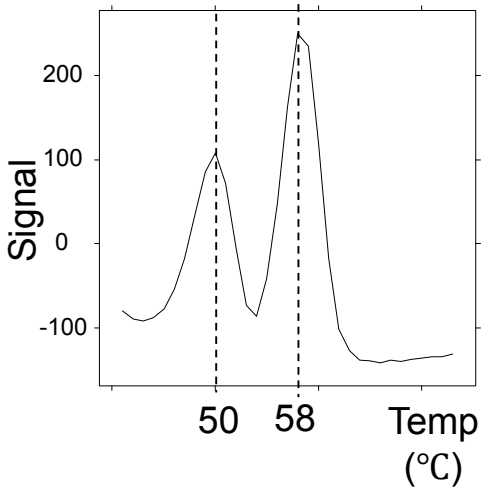
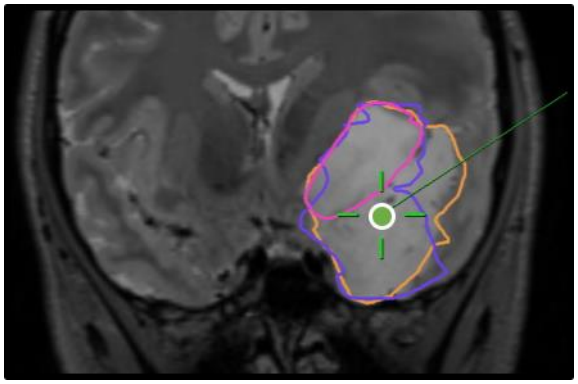
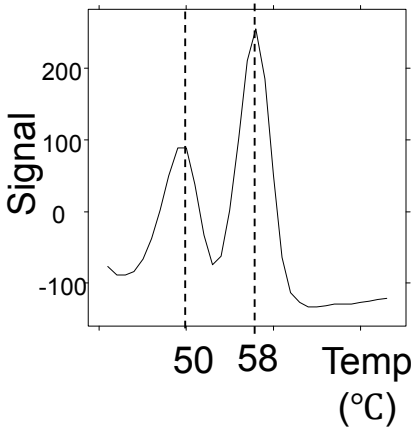
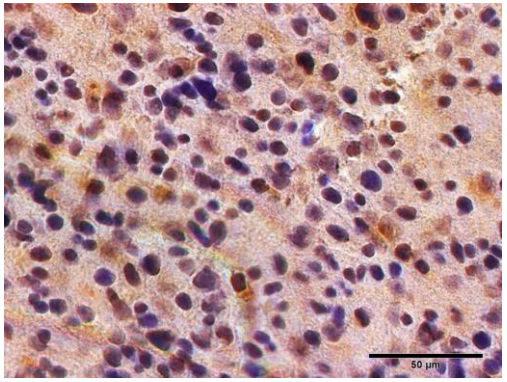


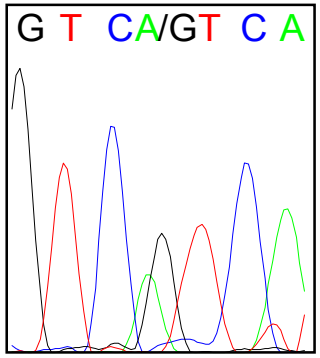
Figure 3.

a)



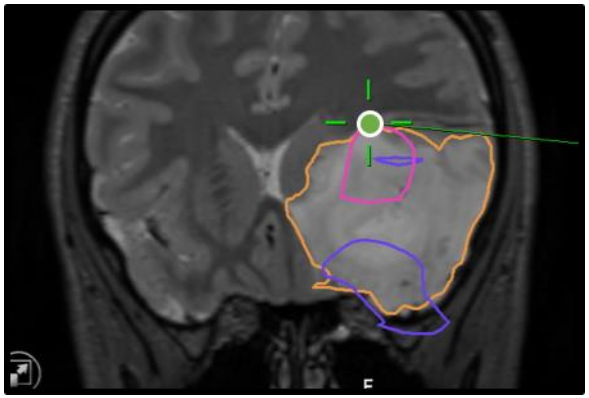


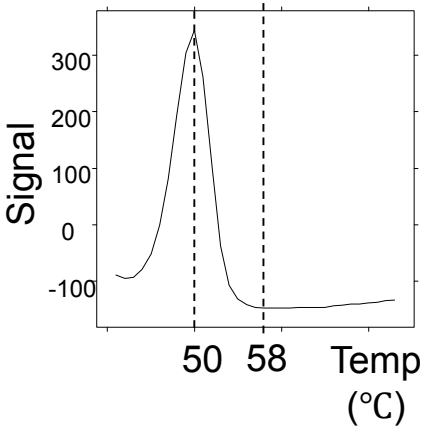


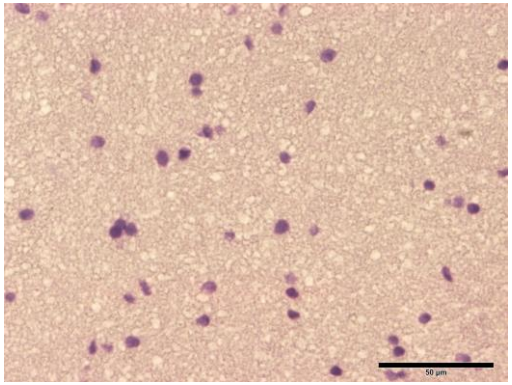


34.7%

b)







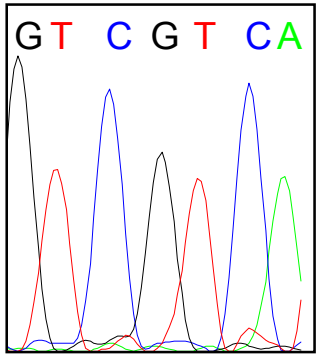


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Figure 4.