



Droplet digital PCR assay for detecting *TERT* promoter mutations in patients with glioma

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Abstract

Two hot spot mutations (C228T, C250T) in the telomerase reverse transcriptase (*TERT*) gene are frequently identified in glioblastoma and oligodendroglioma. *TERT* mutations predicts an aggressive clinical course in isocitrate dehydrogenase (*IDH*) wild-type astrocytic tumors. Therefore, it is important to accurately detect *TERT* promoter mutations in glioma. Sanger DNA sequencing is the currently standard method for analyzing *TERT* mutations. However, PCR amplification in the first step of the sequencing has proven technically difficult because of the high GC content around the *TERT* mutation. In this report, we described a novel droplet digital PCR (ddPCR) assay to evaluate *TERT* hot spot mutations in fresh frozen and formalin-fixed paraffin-embedded (FFPE) specimens of glioma and verified the difference in results from the Sanger DNA sequencing results. We obtained the mutant allele fraction for *TERT* mutations of in a single ddPCR run in all cases, including the micro-dissected FFPE sections. On the contrary, up to twice the DNA sequences were required from fresh frozen tissue to obtain the results, consistent with ddPCR assay. When FFPE specimens were used, more time was required to evaluate *TERT* mutations through DNA sequencing. DdPCR is an effective and sensitive assay compared to the conventional standard Sanger DNA sequencing.

Keywords Glioma · *TERT* promoter mutation · Digital PCR

Introduction

Gliomas are the common malignancies among primary brain tumors, and genetic abnormalities related to their development and progression have been studied [1]. Two-point mutations in the promoter region of the telomerase reverse transcriptase (*TERT*) gene have been identified at high frequencies in adult gliomas [2, 3]. Most commonly, *TERT* mutations of C228T or C250T increases the *TERT* promoter activity by generating binding sites for an activating E26 transformation-specific (ETS) transcription factor in the *TERT* promoter region [4–6]. *TERT* upregulation leads to telomerase activation, and maintains telomere lengthening,

which is a critical step in tumorigenesis [4, 7]. The above-mentioned mutations are frequently found in adult glioblastomas (70–84%) and pure oligodendrogliomas (74%) [2, 8]. This indicates that the presence of *TERT* hot spot mutations is a useful diagnostic biomarker of glioblastoma and oligodendroglioma.

In grade II and III gliomas, Eckel-Passow et al. showed that only *TERT*-mutated gliomas, without isocitrate dehydrogenase (*IDH*) mutation and *chromosome 1p/19q* codeletion, had significantly poor overall survival [9]. The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) update 3 indicates that *IDH*-wild-type diffuse astrocytic gliomas that contain *TERT* promoter mutation correspond to WHO grade IV and should be referred to as diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of glioblastoma, WHO grade IV [10]. From the perspective of prognosis, it is important to establish an assay that can accurately detect *TERT* promoter mutations in patients with glioma.

The *TERT* promoter region around hot spot mutations is characterized by a high GC nucleotide content (more than

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80%), making stable, specific and reproducible PCR amplification difficult [11–14]. In the next-generation sequencing with high DNA analysis accuracy, a bridge PCR is performed with a dedicated enzyme and reagent. Gene amplification is unstable under high GC content in the analysis region or at the site of repeated sequences. In addition, it is impossible to detect *TERT* mutation using immunohistochemistry, because an antibody that specifically recognizes the mutant *TERT* protein has not been developed. Therefore, in many institutions, Sanger DNA sequencing is the standard method for analyzing *TERT* mutations. However, this assay also has a PCR step and sensitivity limits, and positive true gene mutations cannot be revealed if 15–20% or more of the cells do not have the gene mutation [15]. Thus, a novel detection method with high sensitivity and good reproducibility is required to analyze *TERT* promoter mutations, which are an important molecular marker in glioma.

The droplet digital PCR (ddPCR) assay has recently attracted attention as a superior method for the detection and absolute quantification of rare mutated and wild type alleles because of its high sensitivity and robust quantitative performance characteristics [16, 17]. Corless BC et al. showed that the ddPCR assay for *TERT* promoter mutation detected 0.062% and 0.051% mutant allele fractions for the C228T and C250 mutations in *TERT*-mutated cancer cells, respectively [18]. We performed *TERT* mutation analysis using an optimized ddPCR assay for fresh frozen and formalin-fixed paraffin-embedded (FFPE) glioma specimens and verified the difference in results obtained from those obtained using the standard analysis method, the Sanger DNA sequencing.

Materials and methods

Patients and tissue samples

We studied five fresh frozen samples and four FFPE specimens from nine patients with glioma (Table 1). All samples were obtained by surgical resection or biopsy at our institution. Each case was pathologically diagnosed according to 2016 WHO classification [1].

DNA extraction

Genomic DNA was extracted from fresh frozen tissues using DNeasy Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. FFPE slides were prepared using Maxwell® RSC DNA FFPE Kit (Promega, Madison, WI, USA) by the instruction's manual. Human glioblastoma cell line A172-derived DNA and human epidermoid skin carcinoma cell line A431-derived DNA were purchased from BioChain (CA, USA) and were used as positive controls for *TERT* C228T and C250T mutations, respectively [18]. The negative control was genomic DNA obtained from normal human peripheral blood cells (Clontech Takara, Shiga, Japan). Concentrations of extracted DNA were measured using NanoDrop® spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

ddPCR

We used mutation-specific primer/probe combinations (dHsaEXD72405942 and dHsaEXD46675715, Bio-Rad)

Table 1 Glioma samples and results for *TERT* mutation detection by ddPCR and Sanger DNA sequencing

Case No	Sample status	Pathological diagnosis	<i>TERT</i> mutation assay results			
			ddPCR	Sanger DNA sequencing ^a		
				First	Second	Third
1	Fresh frozen	Glioblastoma, <i>IDH</i> -wildtype	C228T (46.1%)	C228T	/	/
2	Fresh frozen	Glioblastoma, <i>IDH</i> -wildtype	C228T (42.4%)	NE	C228T	/
3	Fresh frozen	Oligodendroglioma, <i>IDH</i> -mutant and 1p/19q-codeleted	C228T (55.7%)	C228T	/	/
4	Fresh frozen	Glioblastoma, <i>IDH</i> -wildtype	C250T (59.2%)	C250T	/	/
5	Fresh frozen	Anaplastic astrocytoma, <i>IDH</i> -wildtype	No mutation	NE	NE	No mutation
6	FFPE	Glioblastoma, <i>IDH</i> -wildtype	C228T (51.3%)	C228T	/	/
7	FFPE	Glioblastoma, <i>IDH</i> -wildtype	C250T (37.8%)	NE	NE	C250T
8	FFPE	DMG, H3 K27M-mutant	No mutation	NE	NE	No mutation
9	FFPE	Mixed anaplastic OA, dual-genotype	C228T(31.4%) ^b	NA	NA	NA

FFPE formalin-fixed paraffin-embedded, DMG diffuse midline glioma, OA oligoastrocytoma, NE not evaluable, NA not amplified

^aIn case the result of the first sequencing was different from that of ddPCR, Sanger DNA sequencing was performed up to three times (from First to Third) until the results of the two methods matched

^bThe oligodendroglial but not the astrocytic portion displayed *TERT* C228T mutation

to separately detect C228T and C250T *TERT* promoter mutations, according to the protocol by Corless BC et al. [18]. Mutant and wild-type alleles were labeled with FAM and HEX, respectively. The reaction mixtures were as follows: 1 X ddPCR Supermix for Probes (No dUTP, Bio-Rad), 1 X primer/probe mixture, 0.5 M Betamine, 2.5 U CviQI restriction enzyme, 1 mM EDTA, template tumor DNA of 10 ng, and distilled water to a final volume of 20 μ L.

Droplets were generated using the Automatic Droplet Generator QX200 (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplifications were performed using the following cycle conditions: 1 cycle of at 95 °C (2.5C/s ramp rate) for 10 min, followed by 50 cycles at 96 °C (2.5C/s ramp rate) for 30 s and 62 °C for 1 min, and 1 cycle at 98 °C (2.5C/s ramp rate) for 10 min. The samples were stored at 4 °C until further processing. Droplets were analyzed using a QX200 Droplet Reader (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The QuantaSoft analysis software version 1.7 (Bio-Rad Laboratories, Hercules, CA, USA) was used to acquire and analyze data.

Sanger DNA sequencing

A total of 20–100 ng of DNA was used as a template for a single DNA sequencing. PCR amplification of *TERT* promoter hot spots was performed using forward (5'-TCC CTCGGGTTACCCACAG-3') and reverse (5'-AAAGGAAGGGGAGGGGCTG-3') primers, as reported previously [2]. PCRs were performed in a volume of 25 μ L containing 2 μ L of template DNA, 0.5 μ L of 10 μ M of each primer, 10 μ L of AmpliTaq Gold™ 360 Master-Mix (Thermo Fisher Scientific, Waltham, MA, USA), and 1 μ L of dimethyl sulfoxide, and distilled water. Cycling conditions were 95 °C for 10 min for initial denaturation, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 40 s, and a final elongation at 72 °C for 7 min. Amplification products were purified and cleaned by agarose gel electrophoresis and gel-extracted with the NucleoSpin® gel and PCR clean-up kit (Macherey–Nagel, Dören, Germany) according to the manufacturer's protocol. Purified products were subjected to cycle sequencing using the BigDye® Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA), with the forward PCR primer used as a sequencing primer. Purification was then performed using the BigDye® Xterminator Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the DNA sequences were determined using an Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, CA, USA).

Laser microdissection

For a mixed anaplastic oligoastrocytoma (Case 9), we used laser microdissection to separately obtain DNA from the oligodendroglial and astrocytic portions. FFPE tissue block was sectioned and mounted on slides covered with polyethylene–naphthalate (PEN)-membrane and left to dry overnight at room temperature. The sections were stained with toluidine blue. Visualization and microdissection were performed using LMD-7000 (Leica Microsystems, Germany). DNA from each portion was extracted using the Maxwell® RSC DNA FFPE Kit as described for *DNA extraction*, followed by ddPCR and DNA sequence assays.

Fluorescence in situ hybridization (FISH) for chromosomes 1p and 19q statuses

Five-micrometer-thick paraffin sections of tumors were mounted on positively charged slides and baked at 65 °C overnight. The sections were dewaxed with xylene and ethanol, and treated in 10 mM sodium citrate for 10 min at 80 °C, followed by pepsin digestion (4 mg/mL in saline, pH adjusted to 1.5 with HCl) for 35 min at 37 °C. The sections were denatured for 10 min at 75 °C and hybridized overnight at 75 °C with the two-color FISH probe sets of the differential sites (Vysis LSI 1p36/1q25 and 19q13/19p13 FISH probe kit, Abbott, IL, USA and 1p31/1q25.2 and 19q13.12/19p13.2, GSP Lab, Kobe, Japan). The next day, sections were washed in 50% formamide/1X SSC buffer. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole, and green and red fluorescent signals were enumerated under an Olympus fluorescence microscope BX51 (Olympus Optical, Tokyo, Japan) with appropriate filters. For each hybridization, 100 non-overlapping nuclei were assessed for numbers of green and red signals. An interpretation of deletion was made when more than 50% of the nuclei harbored only one red or one green signal.

Results

ddPCR assay optimization

First, we performed a ddPCR assay for DNA from A172 (*TERT* C228T mutant), A431 (*TERT* C250T mutant) and peripheral blood cell (*TERT* wild-type). To evaluate the least detectable fractions of the *TERT* mutant allele, serial dilutions of mutant *TERT* DNA from cell lines were mixed with normal peripheral blood cell DNA to generate sample series with mutant fractions ranging from 100 to 0% (100%, 10%, 1%, 0.1%, and 0%). The results were shown as two-dimensional ddPCR plots. Wild-type PCR product fluorescence intensity is presented on the X-axis (HEX) and

mutant PCR product fluorescence intensity is presented on the Y-axis (FAM). Next, we set a threshold value of 2000 for the droplet HEX intensity indicating the wild-type and 3000 for the droplet FAM intensity indicating the mutation. This segregated the cluster of droplets with minimal cross-reactivity ($<0.05\%$, data not shown) and excluded false positives. As shown in Fig. 1, the *TERT* C228T mutation was present at 48.1% of DNA only from A172 cells and the *TERT* C250T mutation was present at 33.0% of DNA only from A431 cells, and these mutations were not detected in DNA only from peripheral blood cell. The fraction rate of these mutations obtained through our ddPCR assay was almost equal to that obtained in a previous report [18]. In addition, these control samples were used in all experiments with glioma samples, and the above-mentioned results were reproduced. We could not obtain a detectable mutant fraction from the 0.1% dilution samples of *TERT* C228T and C250T templates. Therefore, we determined that 1.0% or more of the mutant *TERT* DNA was detectable in this assay.

Evaluation for *TERT* promoter mutation using the ddPCR assay in glioma samples

We analyzed *TERT* promoter mutations in nine glioma samples (five fresh frozen and four FFPE specimens) using our optimized ddPCR assay. We obtained convincing data in a single ddPCR run in all cases (Table 1). Representative cases of *TERT* promoter mutation are shown in Fig. 2. Case 9 was a dual-genotype mixed anaplastic oligoastrocytoma (Fig. 3A). Genetic codeletion of chromosomes 1p/19q and positive for IDH1 R132H immunohistochemical staining (data not shown) were demonstrated in the oligodendroglial portion but not in the astrocytic portion. According to our ddPCR assay using DNA from micro-dissected tissue section, 31.4% and 0% mutant allele fractions for *TERT* C228T was observed in the oligodendroglial and astrocytic portions, respectively (Fig. 3B).

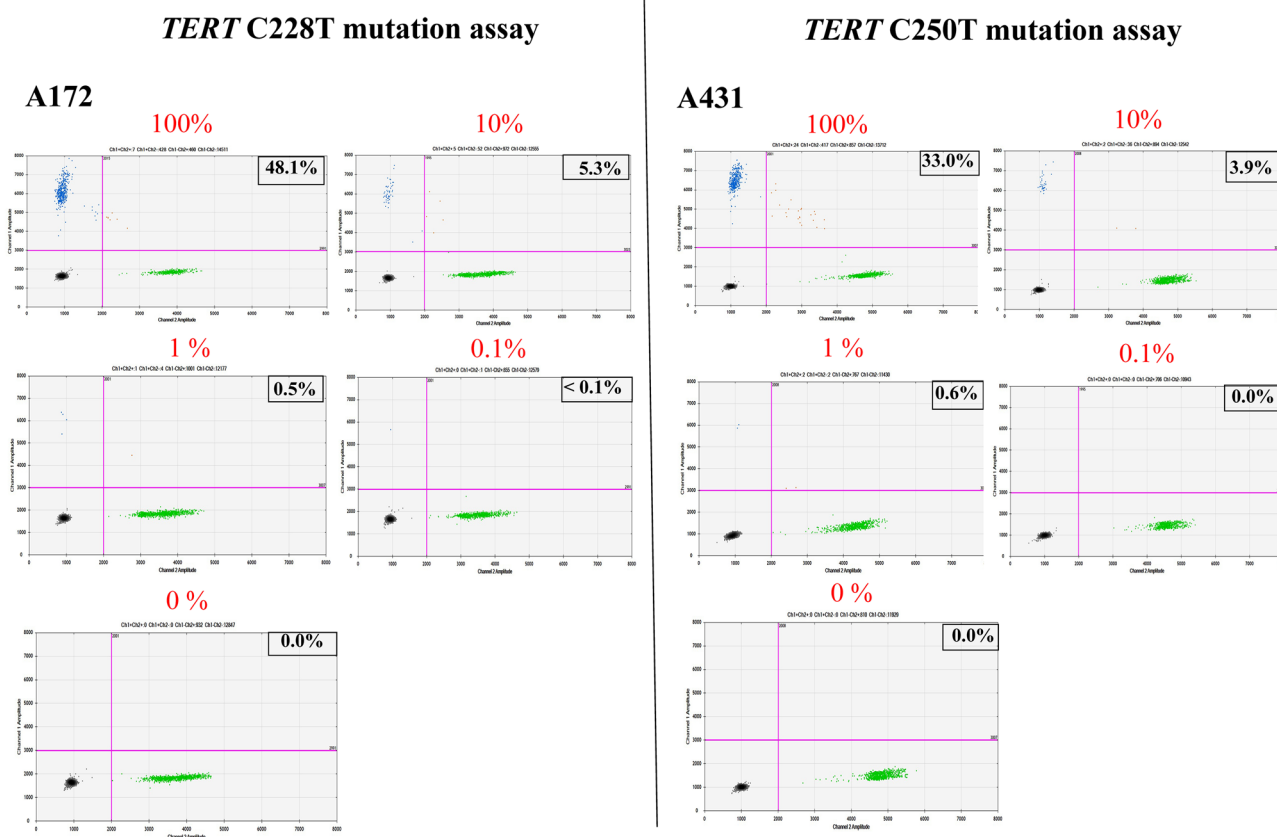


Fig. 1 Two-dimensional droplet digital PCR (ddPCR) plots showing the C228T and C250T *TERT* promoter assays. The Y-axis indicates the intensity of the FAM signal (blue) and represents a *TERT* mutation. The X-axis indicates the intensity of the HEX signal (green) and represents the wild-type. Cross-reactive events for both signals are shown in the upper right corner (brown). The background signals are

shown in the bottom-left corner (black). DNA from A172 cells (left panel) and A431 (right panel) were diluted with normal peripheral blood cell DNA to mutant allele fractions of 100%, 10%, 1%, 0.1%, and 0%. The mutation-positive fraction is indicated as a percentage in the upper right corner

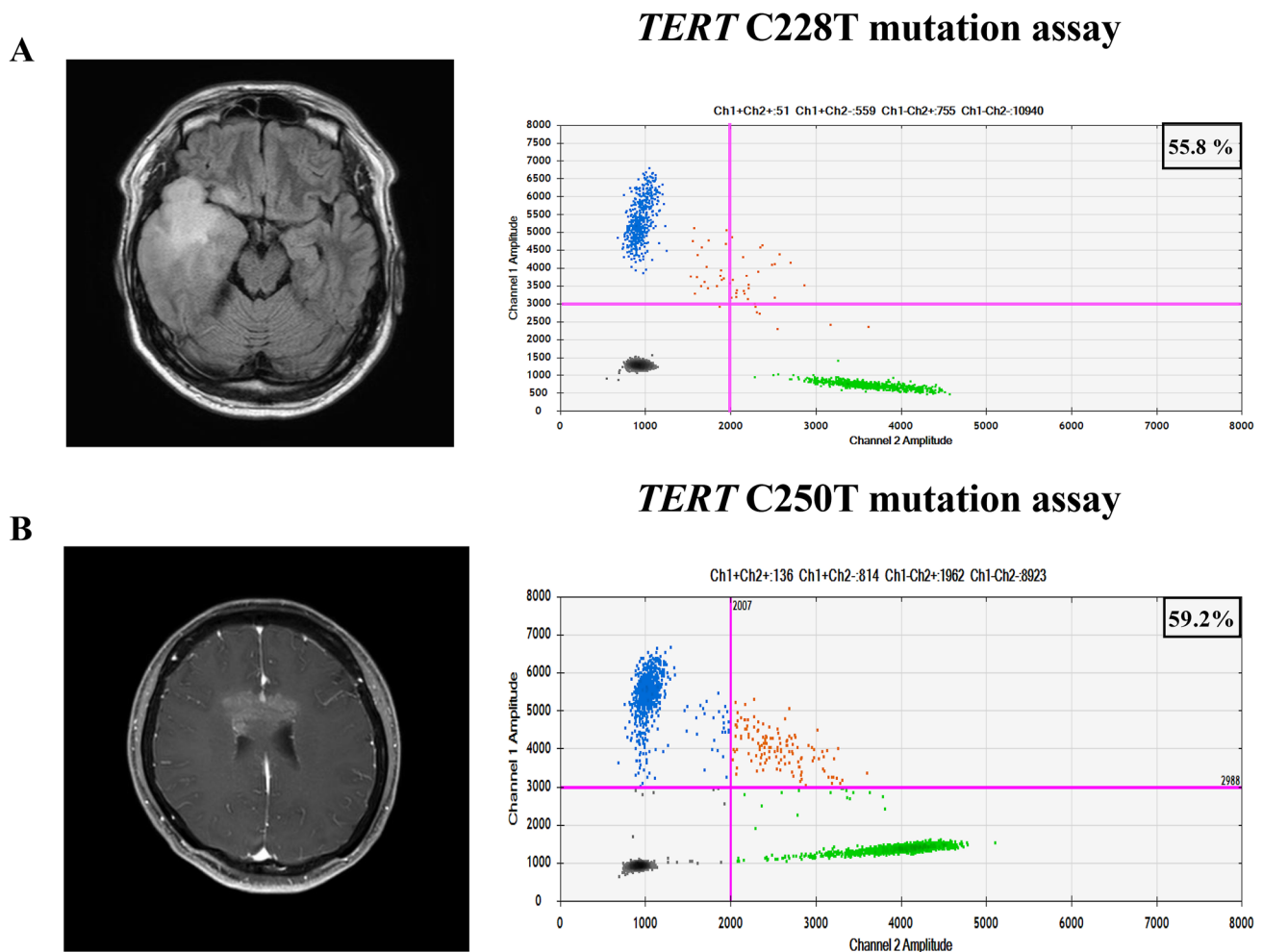


Fig. 2 Representative ddPCR results. **A** Rt. fronto-temporal oligodendroglioma (Case 3). Left panel: a fluid-attenuated inversion-recovery (FLAIR) MRI image; Right panel: ddPCR result of this case shows 55.8% of C228T *TERT* mutation. **B** Glioblastoma in the corpus cal-

losum (Case 4). Left panel: a contrast-enhanced T1-weighted MRI image; Right panel: ddPCR result of this case shows 59.2% of C250T *TERT* mutation

Comparison of the ddPCR assay and Sanger DNA sequencing

Subsequently, we performed Sanger DNA sequencing using the same DNA that had undergone ddPCR and compared the results for *TERT* mutation. As mentioned in *Introduction*, Sanger DNA sequencing is a low-sensitivity method. The sequencing was repeated up to three times if the result of the first sequencing was different from that obtained through the ddPCR assay. When performing the second or third sequencing, we restarted from the PCR step. The comparison results between ddPCR and Sanger DNA sequence are shown on the right side of Table 1. In the case of fresh frozen tissues, DNA sequencing results of the second sequencing were concordant with the results obtained using the ddPCR assay. However, when FFPE specimens were used, more time was required to evaluate *TERT* mutations through Sanger

DNA sequencing. For example, Case 7 was a glioblastoma, *IDH*-wildtype patient, with a FFPE specimen. The ddPCR assay showed a *TERT* C250T mutation of 37.8% for this case (Fig. 4A). In the first and second Sanger DNA sequencing, we could not read the nucleotides peaks because of baseline noise and oddly spaced interstitial peaks. Finally satisfactory results were obtained in the third sequencing (Fig. 4B). In Case 9, DNA extracted from the micro-dissected specimens was very small and could not be amplified by PCR at all three times of sequencing step (Table 1).

Discussion

Here we showed that the ddPCR assay was able to analyze *TERT* promoter mutations in DNA obtained from fresh frozen and FFPE glioma specimens with very high

A

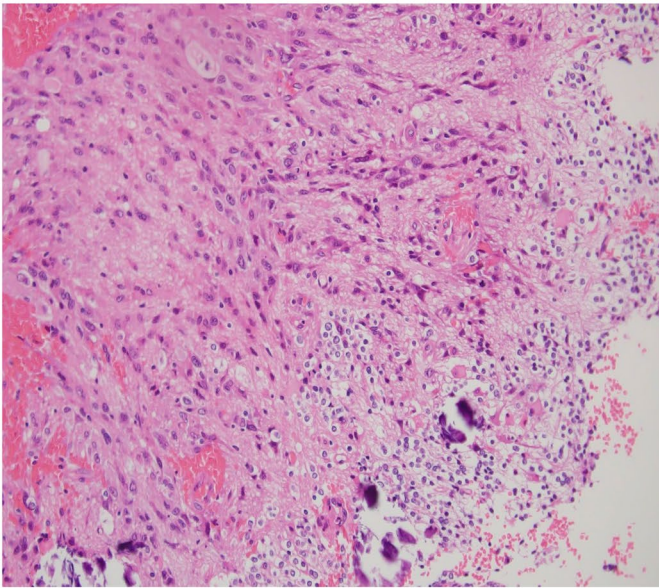
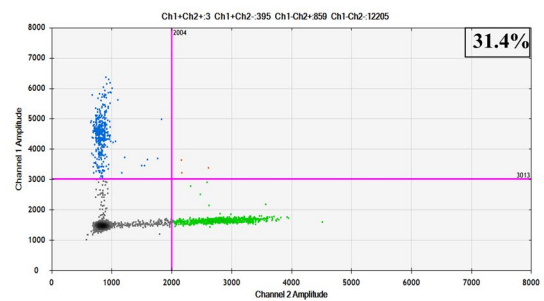


Fig. 3 Mixed anaplastic oligoastrocytoma (Case 9). **A** Hematoxylin & eosin staining of the tumor specimen (X200). The tumor displayed oligodendroglial (lower right) and astrocytic (upper left) differenti-

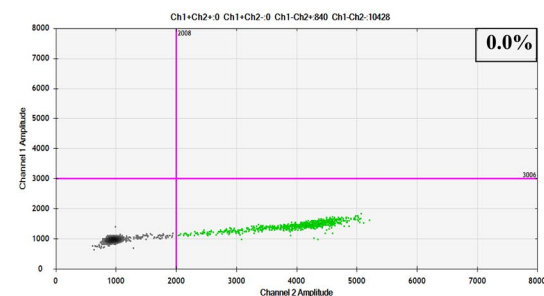
sensitivity compared to the traditional Sanger DNA sequencing method. Our ddPCR assay for *TERT* promoter mutation can detect a mutant allele fraction of 1.0% of mutant *TERT* DNA. This detection limit is significantly lower than those reported for other mutation detection assays such as next generation sequencing (2%), pyrosequencing (5%), and Sanger DNA sequencing (15–20%) [15, 19]. Our results show the preliminary data obtained from nine glioma samples. A validation study of the ddPCR assay for many cases is necessary and is currently in progress. It took a short time of approximately 2.5 h to analyze the nine glioma DNA samples that we prepared. The cost of the ddPCR assay was 1.2 times higher than that of the Sanger sequencing per sample. However, the costs of both were almost the same if 20 or more samples were assayed simultaneously. Although ddPCR misses genetic alterations other than its targeted mutation, it remains an extremely useful assay for the gene analysis of single nucleotide polymorphisms, for which known mutation sites are known, such as *TERT* promoter mutations. This assay may also sensitively detect hot spot mutations, such as *IDH1/2*, *H3F3A* K27M, and *BRAFV600E* mutations, in a subset of glioma [1, 20–22].

B

TERT C228T mutation assay



Oligodendroglial portion

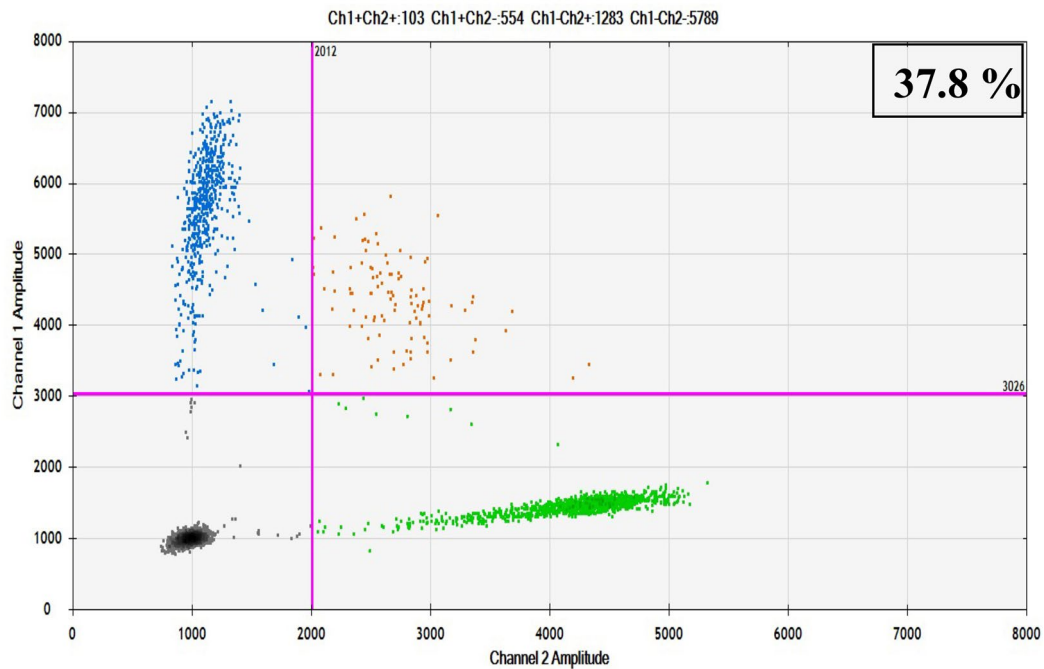


Astrocytic portion

ated portions. **B** DdPCR plots for *TERT* mutations display the results from the micro-dissected tissue of each area

The Sanger DNA sequencing, used as a standard assay in many institutions for *TERT* mutation detection, is problematic as shown in this study. Three of the four FFPE samples did not give clear sequencing results, even in two sequencing assays. This may be because that the DNA quantification method used by us did not correctly assess the concentration of FFPE-derived samples. However, DNA damage and base transitions occur more frequently in FFPE samples than in frozen samples [23]. In addition, the high GC content (more than 80%) within the *TERT* promoter region makes it more difficult for efficient and specific PCR assay to detect *TERT* mutations [11, 14]. Miki S et al. reported that, since the Sanger DNA sequence has low-sensitivity and is non-quantitative, *TERT* mutant cells are possibly masked by *TERT* non-mutant cells, and sequencing results do not reveal positivity for mutation even in fresh frozen samples [24]. However, by increasing the mutation abundance ratio per compartment by compartmentalization with droplets, rare gene mutations can be detected without being buried in the background as long as there is a PCR-amplified gene [16]. As Diplas BH et al. inferred, the true frequency of *TERT* mutations in gliomas, as determined by ddPCR analysis, may actually be higher than previously reported [25].

A *TERT* C250T mutation assay



B

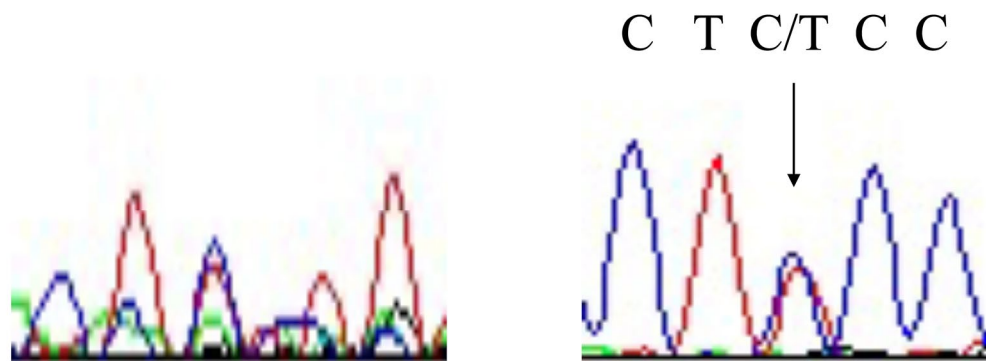


Fig. 4 ddPCR and Sanger DNA sequencing results using FFPE glioblastoma specimens (Case 7). **A** ddPCR plot for *TERT* C250T mutation assay. **B** Sanger DNA sequencing for the same FFPE sample. Left panel: result of the first sequencing was No call; Right

panel: The third-time sequencing showed C250T mutation at 146 bp, upstream of the *TERT* transcriptional start site. Arrow indicates the heterozygous C/T bases at this site

As mentioned above, ddPCR is highly sensitive assay that can detect a small fraction of *TERT*-mutated alleles. *TERT* mutations were successfully verified using 10 ng DNA as a template in this study. It was possible to analyze a very small amount of DNA microdissected from the FFPE section. We found a *TERT* mutation that was detected only in the oligoendroglial portion that was composed of mixed glioma. This ddPCR assay can detect *TERT* mutations with a small amount of tumor DNA template of 1 ng (unpublished data). Therefore, ddPCR can be applied to liquid biopsies

using circulating cell-free DNA (ccfDNA). Muralidharan K et al. reported the feasibility of a ddPCR-based *TERT* promoter mutation assay for ccfDNA from the plasma of glioma patients [26]. According to their results, overall sensitivity and specificity for detecting ccfDNA *TERT* promoter mutations in plasma compared with those for detecting ccfDNA *TERT* promoter mutations in matched tumor tissues were 62.5% and 90%, respectively. Cerebrospinal fluid is better than plasma and a more frequent reservoir of tumor DNA in glioma patients [27]. Using ccfDNA obtained

from cerebrospinal fluid, detection of *TERT* mutation may be more accurate in liquid biopsy of glioma.

It is also attractive that ddPCR is a quantitative method. The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) update 3 reported that the presence of *TERT* mutations predicted an aggressive clinical course in *IDH* wild-type astrocytic tumors with molecular features of glioblastoma, WHO grade IV [10]. Therefore, it would be interesting to determine whether the number of *TERT*-mutant tumor cells is relevant to clinical parameters, such as tumor growth rate, resistance to treatment, and poor survival. The ddPCR assay is a powerful tool for answering these questions.

In summary, we demonstrated here, the validity of a ddPCR assay for specifically detecting C228T and C250T promoter mutations in the *TERT* gene. Especially for FFPE sections, ddPCR has been shown to be far superior to the standard analytical method, Sanger DNA sequencing. The ddPCR assay enables highly sensitive analysis of *TERT* mutations, even using a small amount of micro-dissected DNA, and is expected to be applicable to liquid biopsy in glioma. These results suggest that this assay is a potential tool for diagnosing and monitoring patients with glioma and *TERT* mutations as well as for the development of drugs targeting telomere lengthening.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest related to this work.

Ethical approval All samples were collected and analyzed in accordance with the Declaration of Helsinki (2013). This study was included in Investigation of Brain Tumor Pathogenesis Using Multimodal Neuroimaging, which was approved by the Institutional Review Board of Saitama Medical University International Medical Center.

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