Research Square

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Reliable detection of genetic alterations in cyst fluid DNA for the diagnosis of brain tumors

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Abstract

Purpose

Liquid biopsy of cyst fluid in brain tumors has not been extensively studied to date. The present study was performed to see whether diagnostic genetic alterations found in brain tumor tissue DNA could also be detected in cell-free DNA (cfDNA) of cyst fluid in cystic brain tumors.

Methods

Cyst fluid was obtained from 17 patients undergoing surgery for a cystic brain tumor with confirmed genetic alterations in tumor DNA. Pathological diagnoses based on WHO 2021 classification and diagnostic alterations in the tumor DNA, such as *IDH1* R132H and *TERT* promoter mutation for oligodendrogliomas, were detected by Sanger sequencing. The same alterations were analyzed by both droplet digital PCR (ddPCR) and Sanger sequencing in cyst fluid cfDNA.

Results

Twenty genetic alterations were found in 17 tumor samples. All (100%) alterations were detected in cyst fluid cfDNA by ddPCR. Sixteen of the 20 (80%) alterations were also detected by Sanger sequencing of cyst fluid cfDNA. Variant allele frequency (VAF) in cyst fluid cfDNA was comparable to that of tumor DNA (R = 0.67, Pearson's correlation).

Conclusion

Cell-free DNA obtained from cyst fluid in cystic brain tumors is a reliable alternative to tumor DNA when diagnosing brain tumors.

Introduction

Liquid biopsy is used to detect diagnostic markers in body fluids and to detect genetic alterations derived from circulating tumor DNA (ctDNA). The main sources of ctDNA include blood, urine, and cerebrospinal fluid (CSF),[1] and has recently attracted attention as a minimally invasive screening method that can lead to diagnosis and determination of disease status.

In brain tumors, the usefulness of liquid biopsy has been reported in the detection of genetic abnormalities such as *MYD88* L265P in primary central nervous system lymphoma (PCNSL).[2] However, in gliomas and other types of tumors, it is difficult to detect genetic alterations from body fluids except in advanced stages such as those with disseminated foci.[3] Meanwhile, genetic diagnosis is becoming increasingly important, as the World Health Organization Classification of Central Nervous System Tumours 2016[4] and 2021[5] call for the analysis of certain genetic and chromosomal alterations for the diagnosis of gliomas.

To date, there have been no large-scale reports showing the usefulness of liquid biopsy of cyst fluid in brain tumors. Although having a cystic component is not uncommon in brain tumors, it is not known how reliably genetic alterations can be detected from cyst fluid. In the present study, we detected genetic alterations from cell-free DNA of cyst fluid (cyst fluid cfDNA) taken from 12 brain tumor patients and found that in all patients, genetic abnormalities found in tumor tissue DNA (tumor DNA) were also detected in cyst fluid.

Materials and methods

Patients and sample collection

Tumor samples and cyst fluid were collected from 38 patients who underwent surgery at the Department of Neurosurgery, Niigata University, from January 2019 to January 2023 for a cystic brain tumor. In 17 out of 38 (45%) cases, diagnostic point mutations were identified in tumor DNA by routine Sanger sequence analysis. The location of the cyst was confirmed by preoperative imaging. Cysts were defined as having low signal intensity on MRI diffusion-weighted images (DWI) and low signal intensity on contrast-enhanced T1-weighted MRI and uniform signal intensity or fluid-fluid lines on T1-weighted or T2-weighted MRI and more than 1 ml of these areas.[5–7] One to 20 ml of cyst fluid was aspirated manually by inserting a plastic needle or biopsy needle into

the cyst to avoid CSF contamination during surgery with care to avoid blood contamination (Fig. S1A). Cyst fluid was promptly centrifuged at 1,500 G for 10 minutes, and the supernatant was stored at − 80°C (Fig. S1B, C). Tumor DNA and cell-free DNA (cfDNA) of cyst fluid was extracted as previously reported.[2] In brief, tissue DNA was extracted from fresh frozen tissue using the QIAamp Blood & Tissue Kit (Qiagen, Valencia, CA, USA), and ctDNA was extracted using the Maxwell RSC ccfDNA Plasma Kit (RSC; Promega, Leiden, The Netherlands), according to the manufacturer's instructions. For all samples, DNA was stored at − 20°C until further use. The concentration of extracted DNA was measured with a spectrometer (Eppendorf, Tokyo, Japan).

The surgical specimens were fixed with 10% buffered formalin and embedded in paraffin. Histopathological examination was performed on 4-μm-thick sections stained with hematoxylin and eosin. Pathologic diagnoses were made by 3 experienced neuropathologists (A.K., H.S. and M.T.) according to the 2021 World Health Organization classification system.[5] Immunohistochemistry was performed as described previously using primary antibodies against IDH1 R132H (1:100, monoclonal, clone H09, Dianova, Hamburg, Germany) and H3F3A K27M (1:3200, monoclonal, clone RM192, Sigma-Aldrich, STL, USA) .[9] Based on the histological diagnosis, tissues pathologically diagnosed as gliomas were screened for eight mutation hotspots (*IDH1* R132, *IDH2* R172, *HIST 1H3B* K27M, *H3F3A* K27M/G34, *BRAF* V600, and *pTERT* C228/C250) by direct sequencing or ddPCR methods. Similarly, *pTERT* mutations were screened for in a malignant meningioma case. Cases in which alterations were identified in tissue DNA were also searched for in cyst fluid cfDNA, using direct sequencing and ddPCR methods.

This study was approved by the Ethics Committee of Niigata University School of Medicine (Approval #: 2018 − 0353) and written informed consent for liquid biopsy and use of the resected tissues for research purposes was obtained from all patients.

Genetic analysis

Direct sequencing of *IDH1* R132H, *IDH2* R172, *pTERT* C228T/C250T, *H3F3A* K27M/G34, *HIST1H3B* K27M and *KRAS* G12D was performed as reported previously.[2,9–12] A total of 2–20 ng of DNA was used as a template for a single DNA sequencing. The sequences of the primers used in the study are listed in Table S1. The amplified products were fractionated on 2.0% agarose gel at 100 V for 45 minutes. The PCR products were then sequenced on a 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's instructions.

Detailed methods of ddPCR have been previously published.[2,10] Briefly, ddPCR reagents and primer/probe mix for *IDH1* R132H, *pTERT* C228T, *H3F3A* K27M, and *KRAS* G12D were purchased from Bio-Rad (Hercules, CA, USA). Alternatively, primer/probe mix for *pTERT* C228T was purchased from Integrated DNA Technologies Inc (Table S2, Coralville, IA, USA). When detecting *IDH1* R132H, *H3F3A* K27M, and *KRAS* G12D, a 20 μL PCR mix, composed of 10 μL 2× ddPCR Supermix for Probes (no deoxyuridine triphosphate; Bio-Rad), 1 μL ddPCR Mutation Assay (Bio-Rad) and 9 μL DNA, was loaded into sample wells of an eight-channel disposable droplet generator cartridge (Cat. No. 1864007, Bio-Rad). When detecting *pTERT* C228T, a 20 μL PCR mix, composed of 10 μL 2× ddPCR Supermix for Probes (no deoxyuridine triphosphate; Bio-Rad), 1 μL ddPCR Mutation Assay (Bio-Rad), and 6.75 μL DNA, 2 μL 5M Betaine, and 0.25 μL 80mM EDTA was loaded into sample wells, in the same manner. An additional 50 μL of droplet generation oil (Cat. No. 189005 Bio-Rad) was loaded into the oil well for each channel. After droplet generation, the droplets were transferred into a 96-well PCR plate and then thermal cycled using the thermal cycler Dice Gradient (Takara, Shiga, Japan) or MiniAmp™ Plus thermal cycler (Applied Biosystems). Thermal cycling conditions were carried out as follows: 95˚C 10 min, 94˚C 30 sec. 60˚C 30–60 sec. (for 40 cycles) 98˚C 10 min. and hold at 4˚C. After PCR, the 96-well PCR plate was subjected to the QX-200 droplet reader (Bio-Rad), and data were analyzed by QX Manager 1.2 standard edition software (Bio-Rad). Mutation-specific signals were generated in the hexachloro-fluorescein channel. We considered definite mutant cases to have a fractional abundance of 0.1% or more and to have three or more mutant droplets and/or wildtype droplets detected. Variant allele frequency (VAF) was calculated as follows: VAF% = (Nmt/(Nmt + Nwt))×100), where Nmt is number of mutant events and Nwt is number of wildtype events per reaction.

Statistical analysis

The Mann-Whitney *U* test was conducted for comparison of the medians and correlation of VAF between cyst fluid cfDNA and tumor DNA were analyzed using Pearson's correlation coefficients. One way analysis of variance (ANOVA) with Tukey's method

for multiple comparisons was used to compare the mean values of three groups. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using JMP Pro 16 software (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 10 software (GraphPad Software, La Jolla, CA, USA).

Results

Patient characteristics and genetic analysis

In 17 brain tumor cases in which cystic fluid was collected, 20 diagnostic hotspot mutations were detected in the tumor DNA by Sanger sequencing and/or ddPCR (Table 1, Fig. S2). The median age of the 17 patients was 52 years (range 22–83 years), 16 harbored diffuse gliomas (including one diffuse midline glioma) and one had metastatic brain tumor.[12] Six patients had recurrent brain tumors at the time of cyst fluid collection. Importantly, all (100%) 20 hotspot point mutations detected from tumor DNA were also detected in cyst fluid cfDNA by ddPCR.

Table 1
Summary of patient characteristics and genetic analysis

				Tumor DNA		Cyst DNA				
Pt.	Age	Sex	Pathological	Mutation	ddPCR	DNA	Sanger*	ddPCR		
			diagnosis		VAF (%)	concentration (ng/μL)		MT droplets	WT droplets	VAF (%)
1	38	M	Oligodendroglioma, grade 3	*IDH1* R132H	43	21.0	DHP	1414	2509	36
				pTERT C228T	47		DLP	206	778	21
2	61	F	Oligodendroglioma, grade 3	*IDH1* R132H	44	1.7	DLP	31	30	51
				pTERT C228T	32		DHP	13	19	41
3	74	F	Oligodendroglioma, grade 3	*IDH1* R132H	50	27.0	DHP	2093	2275	48
				pTERT C228T	85		DHP	662	820	45
4	22	M	Astrocytoma, grade 4	*IDH1* R132H	31	3.6	DHP	4	11	27
5	37	M	Astrocytoma, grade 4	*IDH1* R132H	33	7.5	DLP	29	452	6
6	37	M	Astrocytoma, grade 4	*IDH1* R132H	11	10.0	DLP	35	533	6
7	45	M	Astrocytoma, grade 4	*IDH1* R132H	39	6.7	DHP	1571	2270	41
8	46	F	Astrocytoma, grade 4	*IDH1* R132H	52	7.4	DLP	52	60	46
9	67	M	Astrocytoma, grade 4	*IDH1* R132H	43	1.8	DLP	48	245	16
10	34	F	Glioblastoma, IDH-wildtype	*pTERT* C228T	21	25.2	ND	213	1946	10
11	44	F	Glioblastoma, IDH-wildtype	*pTERT* C228T	55	34.0	DHP	704	686	51
12	52	F	Glioblastoma, IDH-wildtype	*pTERT* C228T	38	1.0	DHP	8	14	36
13	72	F	Glioblastoma, IDH-wildtype	*pTERT* C228T	12	533.3	ND	217	2335	9
14	83	F	Glioblastoma, IDH-wildtype	*pTERT* C228T	64	264.8	DHP	668	1197	36
15	68	F	Gliosarcoma	*pTERT* C228T	21	1.5	ND	6	9	40

Abbreviations: VAF, valiant allele frequency; MT, mutant; WT, wildtype; DHP, detectable high peak; DLP, detectable low peak; ND, not detected.

* DHP was defined as a mutant peak amplitude half or more than the wildtype peak amplitude. DLP was defined as a mutant peak amplitude that is less than half of the wildtype amplitude signal but is clearly distinguishable from the baseline.

				Tumor DNA		Cyst DNA				
16	55	M	Diffuse midline glioma	*H3F3A* K27M	6	1.2	ND	4	37	10
17	69	F	Enterogenous carcinoma	*KRAS* G12D	22	31.0	DLP	286	2969	9

Abbreviations: VAF, valiant allele frequency; MT, mutant; WT, wildtype; DHP, detectable high peak; DLP, detectable low peak; ND, not detected.

* DHP was defined as a mutant peak amplitude half or more than the wildtype peak amplitude. DLP was defined as a mutant peak amplitude that is less than half of the wildtype amplitude signal but is clearly distinguishable from the baseline.

The median VAF of cyst fluid cfDNA (cVAF) was comparable to that of tumor DNA (tVAF) (36% vs 39%, $P = 0.20$, Mann-Whitney *U* test) (Fig. 1A). Additionally, there was a positive correlation between tVAF and cVAF ($R = 0.67$, $P = 0.0012$) (Fig. 1B). Sixteen of the 20 gene alterations (80%) were confirmed by the Sanger method, although the mutant peaks were small (< 50% amplitude of wildtype peak; detectable low peak (DLP)) in 7 out of 16 (44%) mutations (Table 1). The average VAF (%) determined by ddPCR was significantly higher in cases with high mutant peaks (50–100% amplitude of wildtype peak; detectable high peak (DHP)) compared to those with DLP (40% vs. 22%, $P < 0.05$, one-way ANOVA) and cases with no detectable mutant peak (not detected (ND)) (40% vs. 17%, $P = 0.03$, one-way ANOVA) by Sanger sequencing (Fig. 1C and Fig. S3).

Cases in which liquid biopsy of cyst fluid was especially useful are presented below. Genetic diagnosis with cyst fluid was useful for surgical planning in Case 1 (Patient 1, Fig. 2) and assisted in the pathological diagnosis of Case 2 (Patient 16, Fig. 3).

Case 1

A 38-year-old man presented with increasing headaches. MR images showed a large, cystic mass lesion with mural nodules at the left frontal lobe (Fig. 2A). He was scheduled for surgery to remove the tumor, but due to the rapidly enlarging mass lesion, the patient became drowsy. Therefore, an Ommaya reservoir was placed urgently (Fig. 2B) and cyst fluid was evacuated and collected. *IDH1* R132H and *pTERT* C228T were detected from cfDNA of the fluid (Fig. 2E, F). The tumor was later removed, and the pathological diagnosis was oligodendroglioma, IDH-mutant and 1p/19q-codeleted, WHO grade 3 (Fig. 2C), as *IDH1* R132H and *pTERT* C228T mutations were also detected in tumor DNA.

Case 2

We performed a stereotactic needle biopsy of a 55-year-old man presenting with progressive right hemiplegia with MR images showing a cystic mass lesion located at the cerebral peduncle to midbrain (Fig. 3A, B), but pathological diagnosis was difficult due to contamination of normal tissue (Fig. 3C). Initially, we failed to detect *H3F3A* K27M in DNA extracted from tumor tissue by Sanger sequencing (Fig. 3D). However, *H3F3A* K27M was detected in the cfDNA of the cyst fluid at this time (Fig. 3E). Later, we found *H3F3A* K27M-positive tumor cells by immunohistochemistry (Fig. 3F) of frozen specimens used for rapid diagnosis.

Discussion

In this study, we showed that the same genetic abnormalities found in tumor DNA were detected from cfDNA extracted from cyst fluid. In all cases in which genetic alterations were found in tumor DNA, the same genetic abnormalities were detected in cyst fluid cfDNA by ddPCR. Also, in 12 out of 16 (75%) cases, the Sanger method was sufficient to detect the same genetic abnormalities in cyst fluid.

The frequency of the cystic component in brain tumors depends on the type of tumor.[14] While some tumors, such as hemangioblastoma,[15] are commonly associated with cysts, 8–10% are reported in glioblastoma,[16] 20% in ependymoma,[17] and 2–4% in meningioma[18]. The etiologies of cyst formation can be different from case to case and are generally not well understood. A study on the composition of the fluid content report that cyst fluid is a nutrient source for tumors;[19] a study analyzing cystic components in brain tumors using MR Spectroscopy report that cyst fluid is related to tumor malignancy.[20]

Although the number of cases is small, the present study also analyzed the cyst component biochemically. The electrolyte component of cyst was similar to that of blood, and the sugar component was lower than that of blood, probably because it was consumed (Fig. S1D). Genetic studies on cyst content fluid are scarce. A study of cystic lesions in the pancreas has reported that next generation sequencing (NGS) of cystic content improve the diagnostic accuracy of cytology;[21] in a case series on liquid biopsy of diffuse midline gliomas (DMG), cyst fluid was examined in one of the cases, and in that case, a high VAF of 43% was detected for *H3F3A* K27M mutation.[22] In the present study, we found that VAF of cyst fluid (cVAF) was comparable to that of tumor DNA (tVAF).

CSF is commonly used as a source of liquid biopsy in brain tumors patients.[1,23–26] However, the detection is difficult even by sensitive methods such as ddPCR or NGS, due to low cfDNA concentration in CSF, except for tumors with rapid cell turnover, such as lymphomas,[2] or for advanced stage gliomas that show leptomeningeal dissemination.[3,22,27] Genetic alterations were found in CSF obtained by lumbar puncture in only 42 out of 85 patients (49.4%) harboring gliomas by NGS.[2,3] We previously reported that *H3F3A* K27M mutations are detected in disseminated and advanced stages of DMG.[27] The low cfDNA concentration may be due to the extremely large volume of the CSF cavity. Recent morphological analysis using MR imaging has shown that the volume of CSF in the intracranial region alone is about 300 ml in adults over 60 years of age.[28] In contrast, the cyst cavity is a very small space relative to the CSF cavity and is located close to or inside the tumor, therefore genetic alterations in cyst fluid cfDNA were detected at high VAF and were detected even by the Sanger method. In addition, ctDNA is fragmented DNA and has been shown to be shorter (90–150 bp) than cfDNA, which is approximately 167 bp in size.[29,30] The primers used in the Sanger method in this study all produced PCR products of 160 bp or more (Table S1), suggesting that cyst fluid cfDNA may be longer than ctDNA in CSF or plasma.

Collection of cyst fluid is not minimally invasive because it is usually performed during the surgical removal of tumor. However, in certain situations, a cyst fluid aspiration can be done during minimally invasive surgery. There is a report of navigation-guided puncture of the cyst cavity prior to tumor resection to reduce the cyst volume and safely remove the tumor with minimal damage to the surrounding brain tissue.[31] In another report, patients with metastatic brain tumors who are unable to undergo removal surgery due to poor general condition underwent a cyst fluid aspiration to reduce tumor volume prior to radiation therapy.[32] In these situations, histological diagnosis of the tumor may be difficult, and a genetic search of the cyst fluid may be useful. In addition, as we have shown in this study, a genetic search of cyst fluid may assist the pathological diagnosis even when a small amount of sample is collected in stereotactic brain biopsy.

There are some limitations of this study. First, since our study was focused on specific genetic abnormalities and was not comprehensive, it is unclear whether cyst fluid cfDNA harbors all genetic abnormalities found in tumor DNA. Additionally, it is difficult to determine malignancy (WHO grade 2 and 3) in lower grade gliomas just by analysis of the cyst fluid. However, it is easy to collect and store the liquid, and analyzing it comprehensively by NGS or other methods shows great promise in the future.

We should stress that at the presently, the utility of liquid biopsy of cyst fluid in brain tumors are restricted to cases that have very large cystic components and relatively small solid portion. In this preliminary study, we show the feasibility of liquid biopsy of cyst fluid in cystic brain tumors.

Conclusion

In the present report, we found that certain genetic abnormalities are commonly found in both the cyst fluid and the tumor itself. Moreover, in many cases, the genetic abnormality was also detected in cyst fluid by the Sanger method. Cyst fluid cfDNA in brain tumors may be considered as an alternative source of tumor DNA in brain tumors with cystic components.

Declarations

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Figures

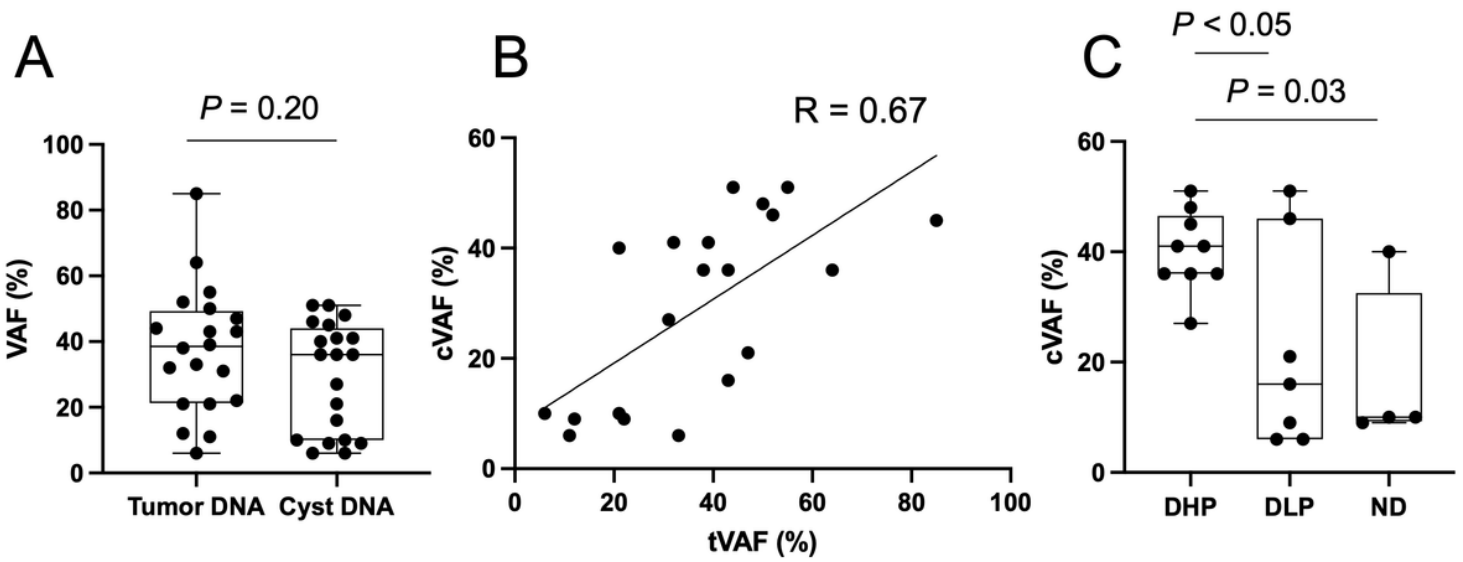

Figure 1

There was no significant difference between VAF in tumor DNA and cyst DNA (P = 0..20)(A). cVAF was strongly related to tVAF (R = 0.67, P = 0.0012) (B). Mean VAF was higher in DHP cases compared to that of DLP (P < 0.05, ANOVA) and ND (P = 0.03, ANOVA) cases (C).

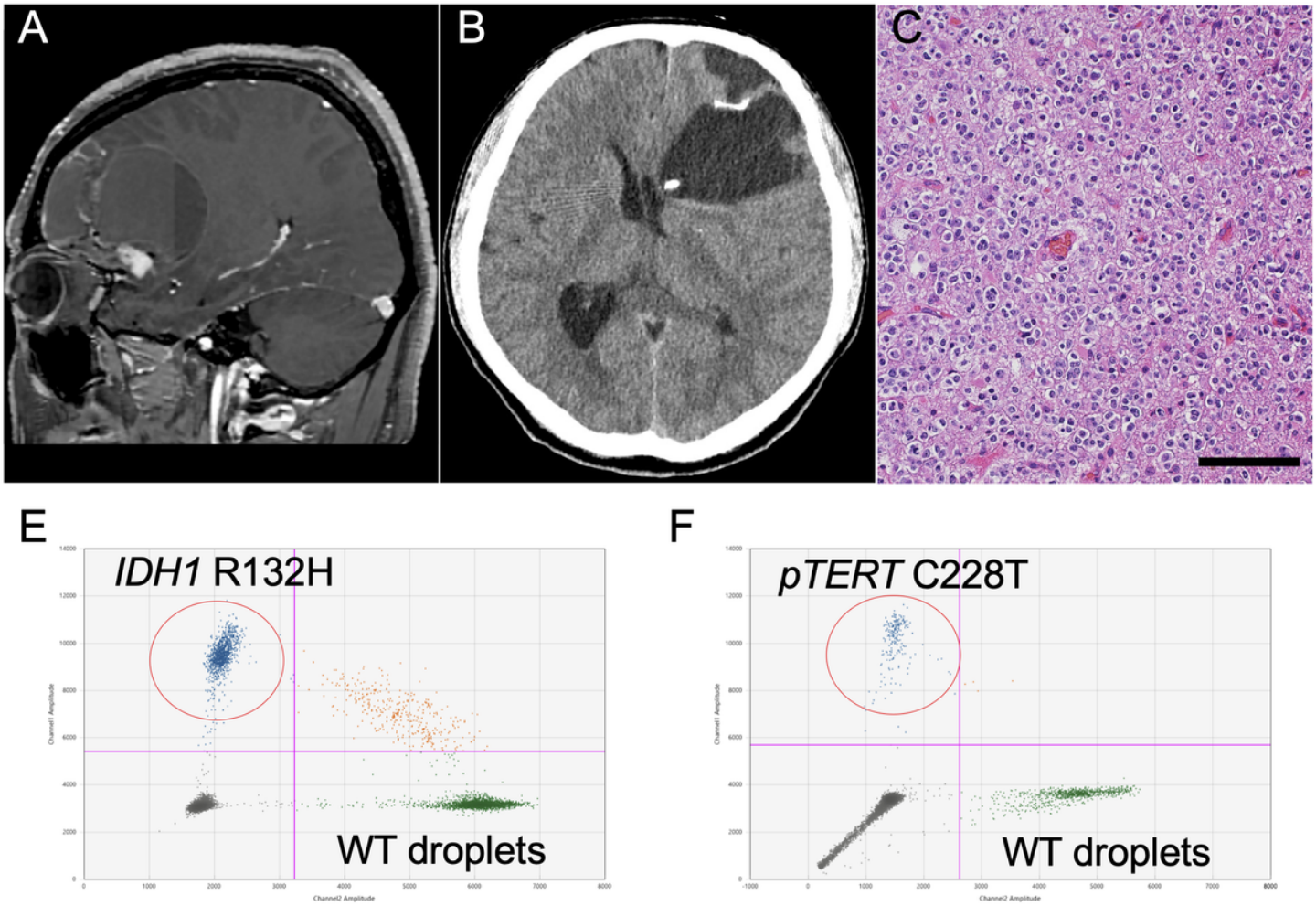

Figure 2

Patient 1. A 38-year-old man with a huge cystic lesion in the left frontal lobe who presented with symptoms of elevated intracranial pressure (A). During the initial surgery, the cyst fluid was aspirated to alleviate intracranial pressure and an Ommaya reservoir was placed in the cyst cavity (B). A total removal was performed in the second surgery, and the histopathological diagnosis was anaplastic oligodendroglioma (C). *pTERT* C228T and *IDH1* R132H mutations were detected in cyst fluid collected at the initial surgery by Sanger (D) and ddPCR (E) methods, respectively. Scale bar C: 100 μm

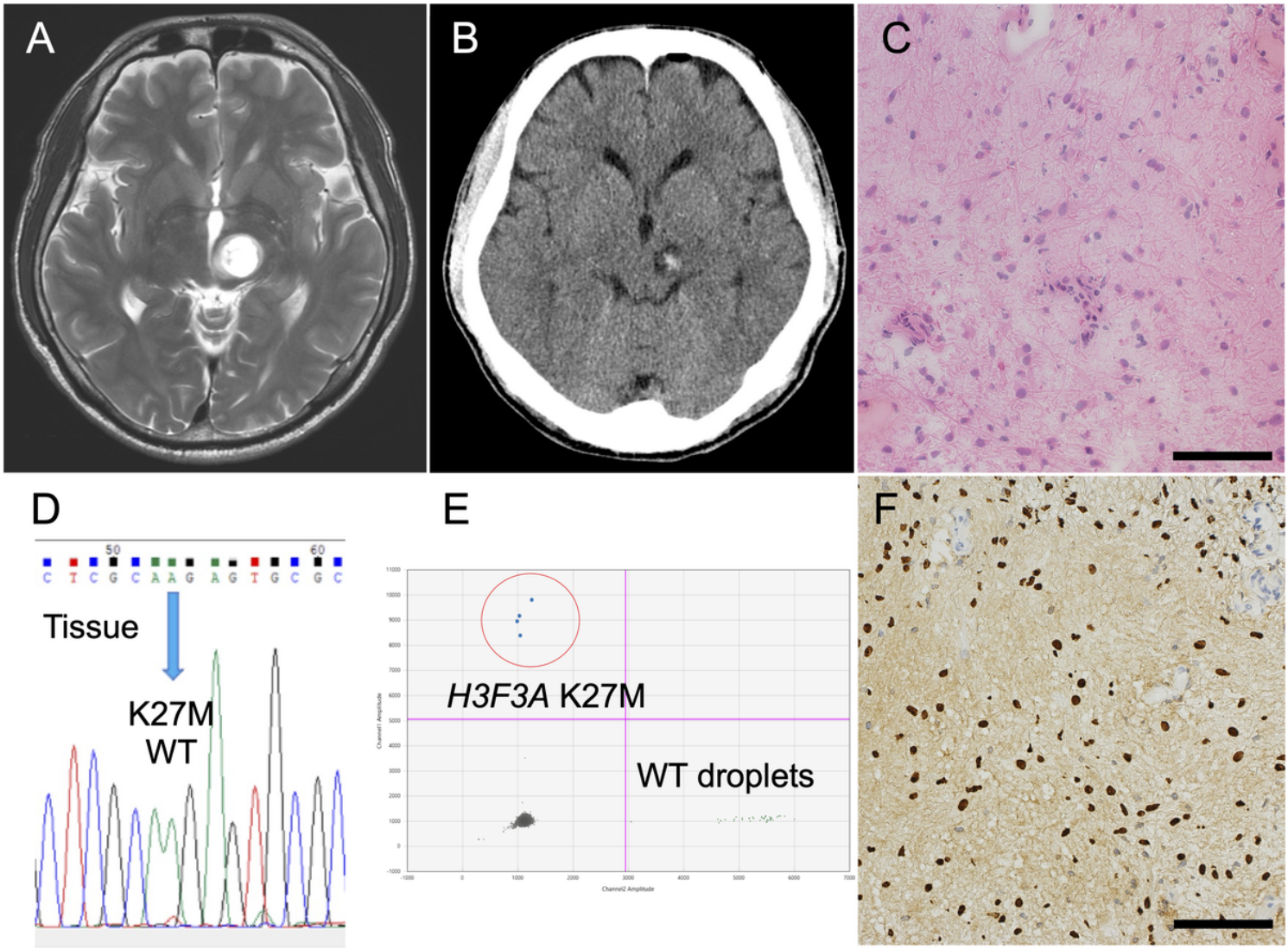

Figure 3

Patient 16. A 55-year-old man with a small cystic lesion in the left midbrain presented with right hemiplegia (A). A stereotactic needle biopsy was performed (B), and cystic fluid and a few tissue fragments were collected. The rapid histology (C) was glioma, but paraffin-embedded tissues failed to reveal H3F3A K27M-positive tumor cells immunohistochemically. Sanger sequencing of preserved tissue failed to detect the *H3F3A* K27M mutation (D). However, *H3F3A* K27M mutation was detected from the cyst fluid (E). The mutation was then confirmed by immunohistochemical staining of the frozen section that had been used intraoperative diagnosis (F). Scale bar C, F: 100 μm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CystbiopsySupplementaryFile.pdf