Clinical Cancer Research

Molecular Diagnosis of Diffuse Gliomas through Sequencing of Cell-Free Circulating Tumor DNA from Cerebrospinal Fluid

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Abstract

Purpose: Diffuse gliomas are the most common primary tumor of the brain and include different subtypes with diverse prognosis. The genomic characterization of diffuse gliomas facilitates their molecular diagnosis. The anatomical localization of diffuse gliomas complicates access to tumor specimens for diagnosis, in some cases incurring high-risk surgical procedures and stereotactic biopsies. Recently, cell-free circulating tumor DNA (ctDNA) has been identified in the cerebrospinal fluid (CSF) of patients with brain malignancies.

Experimental Design: We performed an analysis of *IDH1*, *IDH2*, *TP53*, *TERT*, ATRX, *H3F3A*, and *HIST1H3B* gene mutations in two tumor cohorts from The Cancer Genome Atlas (TCGA) including 648 diffuse gliomas. We also performed targeted exome sequencing and droplet digital PCR (ddPCR) analysis of these seven genes in 20 clinical tumor specimens and CSF from glioma patients and performed a histopathologic characterization of the tumors.

Results: Analysis of the mutational status of the *IDH1*, *IDH2*, *TP53*, *TERT*, *ATRX*, *H3F3A*, and *HIST1H3B* genes allowed the classification of 79% of the 648 diffuse gliomas analyzed, into IDH-wild-type glioblastoma, IDH-mutant glioblastoma/diffuse astrocytoma and oligodendroglioma, each subtype exhibiting diverse median overall survival (1.1, 6.7, and 11.2 years, respectively). We developed a sequencing platform to simultaneously and rapidly genotype these seven genes in CSF ctDNA allowing the subclassification of diffuse gliomas.

Conclusions: The genomic analysis of *IDH1*, *IDH2*, *TP53*, *ATRX*, *TERT*, *H3F3A*, and *HIST1H3B* gene mutations in CSF ctDNA facilitates the diagnosis of diffuse gliomas in a timely manner to support the surgical and clinical management of these patients. *Clin Cancer Res; 24(12); 2812–9. ©2018 AACR.*

Introduction

Diffuse gliomas are the most frequent primary malignant tumors of the central nervous system (CNS) and include IDH-wild-type glioblastoma multiforme (GBM), IDH-mutant GBM, diffuse astrocytomas, anaplastic astrocytomas, oligodendrogliomas, anaplastic oligodendroglioma, and diffuse midline gliomas (1, 2). Diffuse gliomas exhibit a wide range of prognosis depending on the grade, from 1 to 15 years median overall survival. Grade IV glioma, GBM, is one of the most aggressive tumors with less than 10% of patients surviving beyond 5 years (3). Magnetic resonance imaging (MRI) is the principal imaging modality for patients with suspected brain lesions but for a definitive diagnosis, tumor tissue (from biopsy or surgical resection) is required (4–6).

Genomic characterization of tumors is crucial for optimal diagnosis and treatment. However, characterization of cancer is challenged by evolving intratumor heterogeneity, which requires thorough and continuous analysis of genomic complexity over time. This is particularly relevant in brain malignancies where the genomic landscape changes in response to treatment or during relapse (7, 8). However, availability of glioma samples for characterization and correct diagnosis can be challenging. The anatomical location of gliomas can complicate tumor access, incurring high-risk surgical procedures and stereotactic biopsies. Moreover, specimens may be small and not representative, hampering correct diagnosis or even necessitating multiple surgical samplings to clarify final pathologic diagnosis. In addition, the surgical intervention strategy and assessment of the surgical risk–benefit balance depend on the glioma subtype (1, 9–11). This implies that an intraoperative histologic diagnosis may be required, possibly delaying the surgical procedure. Repeat surgical interventions may be needed

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Translational Relevance

Diffuse gliomas include different subtypes with diverse prognosis ranging from 1 to 15 years median overall survival. Thus, the classification of this type of tumors is crucial for the correct clinical and surgical managing of patients. Recent advances show that the genomic characterization of diffuse gliomas facilitates their molecular diagnosis. However, the anatomical localization of diffuse gliomas complicates access to tumor specimens for diagnosis, in some cases incurring high-risk surgical procedures. Recently, ctDNA has been identified in the CSF of patients with brain malignancies. We have developed a sequencing platform to simultaneously and rapidly genotype seven genes—*IDH1*, *IDH2*, *TP53*, *ATRX*, *TERT*, *H3F3A*, and *HIST1H3B*—in CSF ctDNA allowing the subclassification of diffuse gliomas. Our results show that CSF ctDNA can be used as a liquid biopsy to diagnose diffuse gliomas, avoiding risky surgical procedures and leading to a better management of CNS malignancies.

to clarify tumor pseudoprogression on treatment or confirm early relapses. Taken together, a relatively noninvasive method for brain tumor characterization to facilitate molecular diagnosis would greatly assist the management of diffuse gliomas, minimizing complex and high-risk surgical procedures.

During the past decade, large-scale DNA sequencing efforts have identified key genomic alterations across glioma subtypes, facilitating classification (12–15). Recently, the 2016 CNS WHO proposed the integration of histology and genetic analyses to define glioma entities. Diffuse gliomas can be subdivided by the presence of *IDH* mutations, and within the subgroup of *IDH*-mutant gliomas the presence of codeletions of the 1p/19q chromosome arms as well as mutations of the *TERT* gene promoter *ATRX* and/or *TP53* distinguish between diffuse astrocytomas and oligodendrogliomas (12, 13, 16). Moreover, diffuse midline gliomas are characterized by mutations in the *H3F3A* and *HIST1H3B* genes (17). Importantly, these tumors are usually located in the brain stem, thalamus, and spinal cord, making surgery to obtain diagnostic tissues extremely difficult.

We and others have reported the presence of cell-free circulating tumor DNA (ctDNA) in the cerebrospinal fluid (CSF) of patients with brain tumors (18–20). While absent or low levels of ctDNA are found in the plasma of these patients, ctDNA is frequently present in the CSF and can be used to characterize brain tumors (18). Here, we postulated that in diffuse glioma patients, CSF ctDNA could complement and facilitate molecular diagnosis. Based on recent knowledge about the repertoire of genomic alterations in diffuse gliomas, we argue that the identification of a set of gene mutations in CSF ctDNA could facilitate classification of this type of tumors. We therefore developed a platform to simultaneously and rapidly genotype mutations in *IDH1*, *IDH2*, *TP53*, *TERT* gene promoter, *ATRX*, *H3F3A*, and *HIST1H3B* by targeted sequencing and droplet digital PCR (ddPCR).

Materials and Methods

Patients

Tumor tissue, CSF (2 mL) were obtained from 20 diffuse glioma patients. Tumors were identified by MRI (Table 1; Supplementary Fig. S1) and diagnosed by histologic analysis. All patients underwent surgical resection, and CSF was collected prior to surgery by lumbar puncture, except for two samples obtained from the cisterna magna during a warm autopsy procedure, and one sample from the brain ventricle through a cerebral shunt. The study was approved by the local ethics committee, and written informed consent was obtained from all patients.

DNA extraction

DNA from fresh tumor tissue samples was extracted using the QIAamp DNA micro kit (Qiagen), and germline DNA from peripheral blood lymphocytes was extracted using the QIAamp DNA blood mini kit. For formalin-fixed paraffin-embedded tumor samples, five 10-μm sections were obtained from the previously selected tumoral area and processed using the QIAamp DNA FFPE tissue kit. CSF-derived ctDNA was extracted using the QIAamp Circulating Nucleic Acids kit and quantified using a Qubit Fluorometer.

Mutational analysis by amplicon sequencing and ddPCR

DNA from matched glioma tumor tissue, peripheral blood, and CSF samples underwent custom amplicon sequencing, targeting all exons of IDH1, IDH2, ATRX, and TP53. In brief, DNA libraries were prepared using the Nebnext Library Prep kit for Illumina, and paired-end 100-bp reads were generated on the Illumina Hiseq2500. Reads were aligned to the reference human genome hg19 using the Burrows-Wheeler Aligner v.07.12. Somatic variants were called using VarScan2, and only mutations with VarScan2 $P < 0.05$, total coverage $\geq$10 reads, variant coverage $\geq$7 reads, and with allelic frequencies >5%, were considered. All candidate mutations were reviewed manually using the Integrative Genomics Viewer.

Genomic DNA (10 ng) from tumor tissues, and germline DNA from peripheral blood lymphocytes, and CSF DNA (2–5 ng) from the same patient were used for digital PCR analysis, using the QX200 Droplet Digital PCR system per the manufacturer's protocols and the literature (21). Custom Taqman SNP genotyping assays for ddPCR were designed to detect IDH1, IDH2, TP53, H3F3A K27M, and HIST1H3B K27M point mutations and the corresponding wild-type alleles. Two ddPCR-specific assays (dHsaEXD72405942 and dHsaEXD46675715, respectively) were used to detect TERT promoter C228T and C250T mutations.

Immunohistochemistry

Immunohistochemistry was performed on 5-μm formalin-fixed paraffin-embedded tissue sections using a BenchMark Ultra immunostainer. Sections were stained with antibodies specific for IDH1-R132H–mutant protein H09 (Master Diagnostica), ATRX HPA001906 (Sigma-Aldrich), and p53 (mouse monoclonal DO-7; Ventana). After deparaffinization, sections were incubated with primary antibody (1:200) for 2 hours. Standard Ventana signal amplification was used. Sections from known mutation-positive and immunoreactive GBM tumors were used as positive controls.

FISH of 1p/19q codeletion

1p/19q deletions were detected by fluorescence in situ hybridization (FISH) on 5-μm paraffin-embedded sections using Vysis 1p36/1q25 and 19q13/19p13 dual-colur probes. Signals were scored with at least 100 nonoverlapping intact

Table 1. Patient and sample characterization

				Tumor characteristics			Tumor analysis				CSF sample		
Patient ID	Sex	Age	Diagnostic	Location	Distance to ventricle (≤5 mm)	Vol. (cm^3)	IDH1/IDH2 AS/ddPCR	ATRX AS	TP53 mutation AS/ddPCR	TERT AS/ddPCR	Source	(ng/mL) CSF	CSF mutations AS/ddPCR
1	M	78	GBM IDH wt	Temporal	Yes	65.9	WT	WT	WT	C228T:WT/43%	Lumbar	103.7	T C228T:100% (AS)
2	M	57	GBM IDH wt	Temporal	Yes	14.3	WT	WT	WT	C228T:79%/43.8%	Cisterna M	560	T C228T:WT/0.23%
3	F	65	GBM IDH wt	Temporal	Yes	47.5	WT	WT	WT	C228T:73%(ddPCR)	Lumbar	64.4	T C228T:75%(ddPCR)
4	F	57	GBM IDH wt	Temporal	Yes	23.47	WT	WT	WT	C250T:WT/9%	Lumbar	35.1	T C250T:WT/3.2%
5	F	81	GBM IDH wt	Parietal	No	2	WT	WT	WT	C250T:WT/3%	Lumbar	17.2	T C250T:WT/1.7%
6	M	66	GBM IDH wt	Occipital	Yes	98	WT			C250T:26%(ddPCR)	Lumbar	13.2	T C250T:4%(ddPCR)
7	M	60	GBM IDH wt	Temporal	Yes	7.4	WT	WT	17.24%/24.5%	C228T:46%(ddPCR)	Lumbar	9.9	T C228T:85%(ddPCR)
8	M	52	GBM IDH wt	Frontal	Yes	31.8	WT	WT	WT	C250T:27.7%/41%	Lumbar	5.4	T C250T:25%(ddPCR)
9	M	68	Gliosarcoma	Fronto-parietal	No	23.5	WT	WT	28.92%/26.7%	C250T:5.66%/17.6%	Lumbar	3.6	T C250T:3%(ddPCR)
10	F	34	GBM IDH mut	Frontal	Yes	70.33	IDH1 R132H: 4.64%/6,6%	7.33%	10.70% (AS)	WT	Lumbar	44.8	IDH1 R132H: 12.4%/20% ATRX:14.9%(AS) TP53:28.2%(AS)
11	F	36	Anaplastic astrocytoma	Insular	Yes	45.02	IDH1 R132S: 43.71%/41%	68.77%	50% (AS)	WT	Lumbar	48.9	IDH1 R132S: 45.1%/46% ATRX:68%(AS) TP53:49%(AS)
12	F	9	Anaplastic astrocytoma	Gliomatosis	Yes	190	IDH2 R172W: 8.87% (AS)	11.30%	11.70%/14.5%	C250T:17%(ddPCR)	Ventricular	4.6	IDH2:8.87%(AS) ATRX:11.3%(AS) TP53:11.7%(AS)
13	M	32	Diffuse astrocytoma	Temporal	Yes	97.27	IDH1 R132H: 25.06%/35.7%	70.84%	27.32%/32.3%	WT	Lumbar	9.0	WT
14	M	34	Oligodendroglioma	Insular	No	58.97	IDH1 R132H: 28.9%/31.2%	WT	WT	C228T:41%/67%	Lumbar	8.7	IDH1 R132H: 6% (ddPCR)
15	F	61	Oligodendroglioma	Parietal	No	4.8	IDH1 R132H: 11.85%(AS)	WT	WT	C250T:15%/25.7%	Cisterna M	152.3	WT
16	F	48	Oligodendroglioma	Frontal	Yes	42.9	IDH2 R172K: 18.5%/21.5%	WT	40.45% (AS)	C250T: 14.7%(ddPCR)	Lumbar	7.25	TP53:3% (AS) IDH2:17% (ddPCR) T C250T:7%(ddPCR)
17	M	44	Oligodendroglioma	Cingulate	Yes	33.1	IDH1 R132H: 37.6%/32%	WT	WT	C250T:12.5%/35%	Lumbar	4.2	WT
18	M	34	Diffuse midline glioma	Bulbo-medular	Yes	14.5					Lumbar	7.25	H3F3A K27M: 7.3% (ddPCR)
19	M	16	Diffuse midline glioma	Thalamus	Yes	20.1					Lumbar	4.785	H3F3A K27M: 17.3% (ddPCR)
20	M	7	Diffuse midline glioma	Bulbo	Yes	17					Lumbar	37.54	H3F3A K27M: 7% (ddPCR)

Abbreviations: AS, amplicon sequencing; ddPCR, droplet digital PCR; Vol., tumor volume.

nuclei. 1p/19q codeletion was defined as the signal ratio of 1p/1q <0.80.

Bioinformatics analysis

In silico genomic analyses were performed using tumor mutational data and RNA-seq of two tumor cohorts from The Cancer Genome Atlas (TCGA), with Firebrowse (01 2016 version; $N = 648$). Tumors were considered as harboring a mutation in the TERT promoter as described previously (12), when expression levels are above a certain threshold, determined through the study of whole genome sequenced tumors. Tumors were considered mutated for IDH1, IDH2, ATRX, or TP53 when any protein affecting mutation (PAM) in the gene's canonical transcript was present, as identified by Variant Effect Predictor (Ensembl release 90). IDH-mutated was defined as a tumor bearing any PAM in either IDH1 or IDH2. *In silico* clinical analyses were performed using TCGA clinical data from the same source as the genomic data. Python lifelines library was used to compute the Kaplan–Meier curve, and its statistical significance between tumor genomic subgroups was assessed through a log-rank test. Survival COX regression was computed by using the same library and was adjusted by age.

Results

Establishment of a gene panel for molecular diagnosis of diffuse glioma

We focused our search for relevant genomic alterations on gene missense or nonsense mutations, which can be detected with a higher sensitivity in ctDNA than genomic deletions or amplifications. Our panel included targeted sequencing of four genes (IDH1, IDH2, TP53, and ATRX) and ddPCR probes for the most common mutations in the TERT gene promoter (C228T and C250T), H3F3A (K27M), and HIST1H3B (K27M). In some cases, we also used ddPCR for mutations in IDH1, IDH2, TP53, and ATRX to increase the sensitivity of the method or validate the results from the targeted sequencing. Importantly, the methodology allowed rapid analysis of genomic alterations, with a 7- to 10-day turnaround time. Based on previous reports and the WHO 2016, the mutational status of these genes can facilitate the molecular diagnosis of diffuse gliomas according to three tumor entities, IDH-wild-type and TERT-mutant tumors suggestive of IDH-wild-type GBM; IDH-mutant and ATRX and/or TP53-mutant tumors suggestive of IDH-mutant GBM or diffuse astrocytomas; and IDH-mutant and TERT-mutant tumors suggestive of oligodendrogliomas. Although we did not analyze 1p/19q codeletions to determine oligodendrogliomas in TERT-mutant tumors, we took advantage of the fact that these codeletions tend not to coincide with TP53 and/or ATRX mutations (12, 13). Analysis of TERT, ATRX, and TP53 thus facilitated the diagnosis of oligodendroglioma.

Mutational analysis in a TCGA cohort

We first determined how our proposed analysis could facilitate the subtyping of diffuse gliomas. To this end, we analyzed the tumor mutational and RNA sequencing data of two tumor cohorts produced by TCGA that contain 648 diffuse gliomas and evaluated how the combination of mutations in the IDH1, IDH2, TP53, TERT, and ATRX genes was distributed (Fig. 1A). We observed that 25% of tumors were IDH-wild-type and TERT-mutant, suggestive of IDH-wild-type GBM; 27% of tumors were IDH-mutant and ATRX and/or TP53-mutant, suggestive of IDH-mutant GBM or diffuse astrocytomas; and 27% of tumors were IDH-mutant and TERT-mutant, suggestive of oligodendrogliomas (Fig. 1A and B). Importantly, analysis of these five genes allowed 79% of gliomas to be attributed to three subgroups (IDH-wild-type GBM, IDH-mutant GBM/diffuse astrocytoma, and oligodendroglioma). As expected, the vast majority of tumors with ATRX mutations and/or TP53-mutations did not exhibit 1p/19q deletions (0.4% showed ATRX mutations and 1p/19q deletions, and 1% showed TP53 mutations and 1p/19q deletions), confirming this tumor group as suggestive of astrocytomas (Fig. 1C). Only one tumor had a K27M mutation in H3F3A and none in HIST1H3B as in this cohort very few pediatric tumors were included.

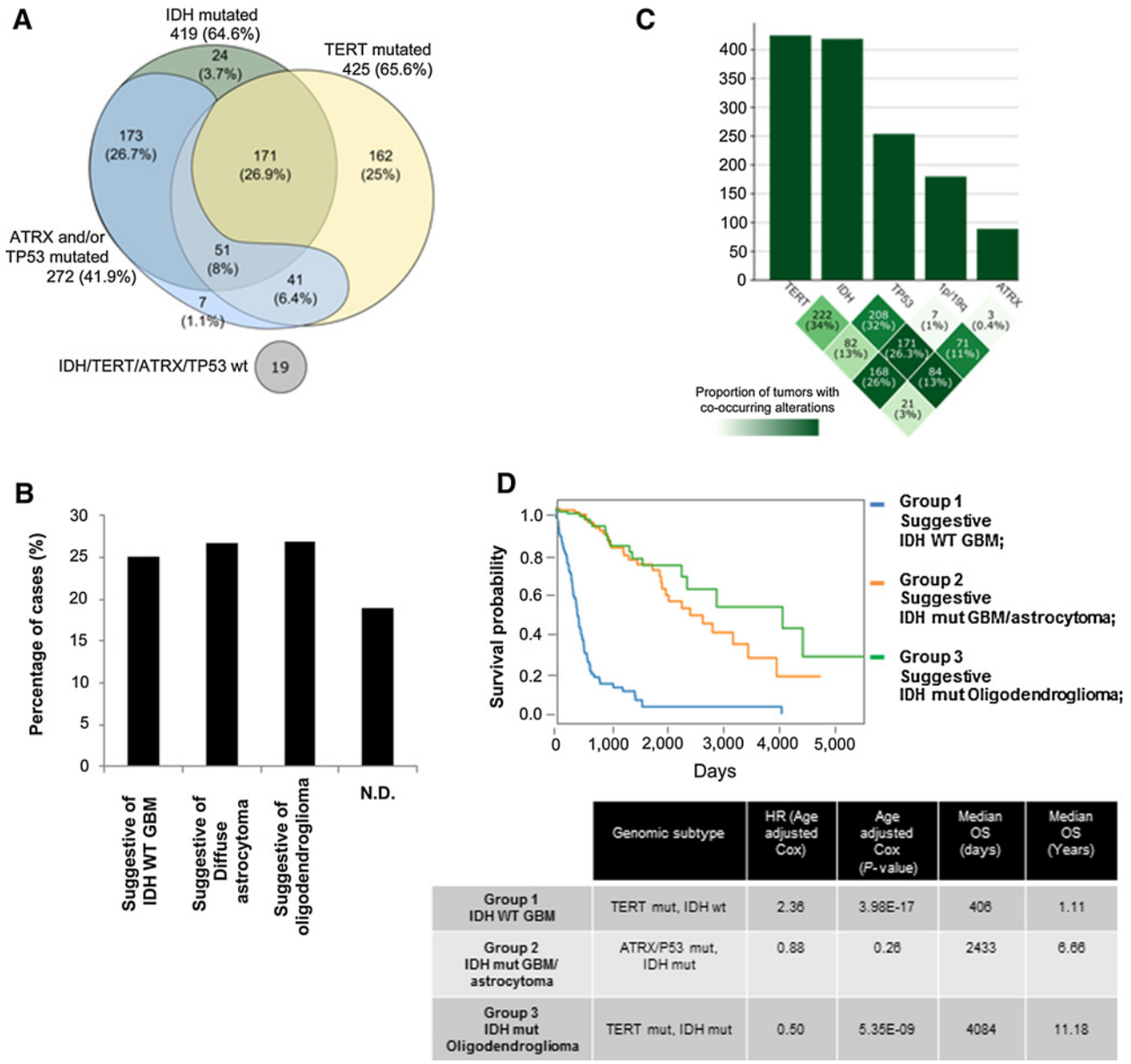
Importantly, the identified subgroups showed quite different prognosis. Median overall survival was 1.1 years for IDH-wild-type and TERT-mutant cases, 6.7 years for IDH-mutant and ATRX and/or TP53-mutant cases and 11.2 years for IDH-mutant and TERT-mutant cases (Fig. 1D). Thus, the molecular classification based on our gene set provided valuable information about prognosis, crucial for the surgical and clinical managing of the patients.

Mutational analysis in diffuse glioma patients

We then tested our sequencing gene panel in a cohort of 20 diffuse glioma cases representing several subtypes, as a proof-of-concept study to compare tumor and CSF ctDNA. CSF ctDNA and tumor DNA were analyzed using the described panel of targeted sequencing and ddPCR probes (Table 1). Gene mutations identified in tumor samples were found in the corresponding CSF ctDNA except for three cases (Fig. 2A). Moreover, the mutational analysis of the CSF ctDNA coincided with the histologic diagnosis. TERT mutations were observed in IDH-wild-type GBM; IDH, TP53, and ATRX mutations were found in IDH-mutant GBM and astrocytomas; and IDH and TERT mutations in the absence of ATRX and/or TP53 mutations were found in oligodendrogliomas where the 1p/19q status was confirmed by FISH (Fig. 2B).

The three cases in which the ctDNA did not recapitulate the results of the tumor analysis, corresponded to a diffuse astrocytoma and two oligodendrogliomas. All were grade II tumor subtypes with diverse tumor volumes, and the distance between the tumors and the brain cortex or ventricles varied from 0 to 1 cm (Fig. 3). Although the number of cases was too small to perform statistical analyses, our results suggest that tumor aggressiveness may be a relevant factor in determining the presence of CSF ctDNA (Fig. 3).

In our cohort, we had three cases of midline gliomas and H3K27 mutations were present in the CSF ctDNA of all of them (Fig. 2A). This is highly relevant because the identification of H3K27 mutations determines the diagnosis of diffuse midline glioma, a type of tumor that is minimally surgically accessible. Our results indicate that sequencing CSF ctDNA can facilitate the diagnosis of this glioma subtype in the absence of a tumor specimen. The three cases had different anatomical locations (thalamus, pons, and bulbous-medullar) and interestingly the allelic frequencies of H3K27 mutations was similar in all three (Table 1; Fig. 4).



	Genomic subtype	HR (Age adjusted Cox)	Age adjusted Cox (*P*-value)	Median OS (days)	Median OS (Years)
Group 1 IDH WT GBM	TERT mut, IDH wt	2.36	3.98E-17	406	1.11
Group 2 IDH mut GBM/ astrocytoma	ATRX/P53 mut, IDH mut	0.88	0.26	2433	6.66
Group 3 IDH mut Oligodendroglioma	TERT mut, IDH mut	0.50	5.35E-09	4084	11.18

Figure 1.

Molecular characterization of diffuse gliomas through the analysis of IDH1, IDH2, ATRX, TP53, and TERT gene mutations. **A,** Incidence of IDH1, IDH2, ATRX, TP53, and TERT gene mutations across 648 diffuse gliomas. **B,** Bar graph plot showing the number of tumors presenting TERT, IDH1/2, TP53, 1p/19q, and ATRX co-occurring genomic alterations. **C,** Bar graph plot representing the percentage of cases of the three glioma subgroups (IDH-wild-type GBM, IDH-mutant GBM/diffuse astrocytoma and oligodendroglioma), and **D,** their corresponding Kaplan–Meier curves. Statistical analyses are summarized in the tables below. ND, not determined.

Our work shows that analysis of mutations in IDH1, IDH2, TP53, TERT, ATRX, H3F3A, and HIST1H3B in CSF ctDNA facilitates the molecular diagnosis of diffuse gliomas.

Discussion

Diagnosis of diffuse gliomas requires access to tumor specimens. Moreover, the evolution of the genomic characteristics of the tumor during progression and relapse, as well as the need to differentiate between pseudoprogressions or true progressions, or clarify early relapse, might imply multiple surgical samplings over time to adequately manage these tumors. In an attempt to overcome the heavy clinical burden associated with the surgical management of diffuse glioma patients, we developed a methodology to allow brain tumor characterization and molecular diagnosis in a relatively non-invasive manner.

The 2016 update of the WHO classification incorporates well-established molecular parameters into the classification of diffuse gliomas, with specific genomic alterations

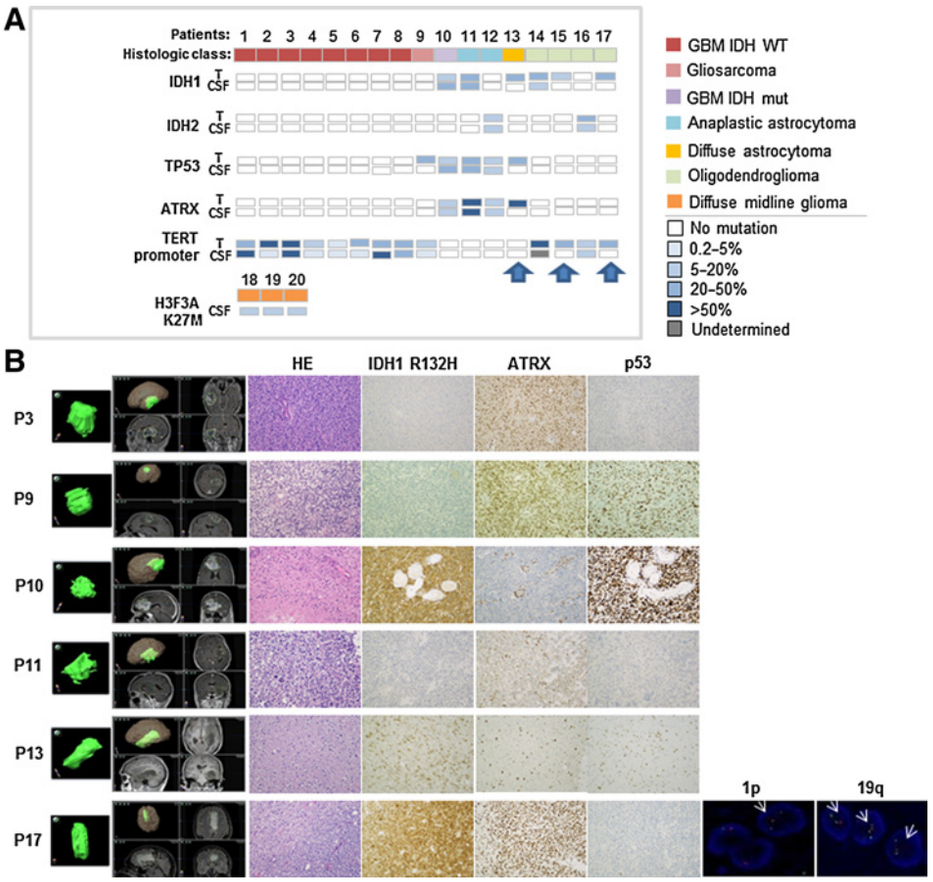


Figure 2.
Gene mutational analysis of tumor and CSF DNA in diffuse gliomas. **A,** Heatmap of the indicated gene mutations. Colored boxes for 20 glioma patients are ordered according to tumor grade, and mutant allelic frequencies (MAF) are depicted following a blue color scale. Blue arrows indicate cases where tumor mutations were not detected in the corresponding CSF sample. T: tumor sample; CSF: cerebral spinal fluid sample. **B,** MRI, histological analysis and FISH of six selected diffuse glioma patients (P3: IDH-WT GBM; P9: gliosarcoma; P10: IDH-MUT GBM; P11: anaplastic astrocytoma; P13: diffuse astroctytoma; P17: oligodendroglioma). Tumor volume measurement images were obtained with iPlan cranial 3.0.5 software from Brainlab. Hematoxylin and eosin (H&E), IDH1 R132H, ATRX, and TP53 staining, and 1p/19q FISH are shown.

guiding diagnosis in combination with histologic analysis. Studies over recent years have shown that the analysis of IDH, ATRX, TP53, TERT, and 1p/19q status facilitates diagnosis of diffuse gliomas (12, 13). Interestingly, almost all TERT-mutant tumors that exhibit 1p/19q codeletion do not show mutations in ATRX and/or TP53, allowing 1p/19q status to be inferred from analysis of TERT, ATRX, and TP53 (12, 13). Thus, identification of IDH1 and IDH2, ATRX, TP53, and TERT status facilitates the classification of diffuse gliomas into IDH-wild-type GBM, IDH-mutant GBM/diffuse astrocytoma, and oligodendroglioma and can have prognostic value. Importantly, our results indicate that the analysis of the mentioned genes will be relevant for the subtyping of astrocytic tumors.

Moreover, mutations in H3F3A and HIST1H3B determine the diagnosis of diffuse midline gliomas (17). This is of great importance because the anatomical location of this type of tumors can prevent the possibility of obtaining surgical specimens.

The *in silico* analysis of a cohort of 648 diffuse gliomas indicated that 79% of them can be subtyped by analyzing IDH, ATRX, TP53, and TERT and thus can be detected by our approach. Furthermore, we could identify ctDNA in a large proportion of patients with diffuse glioma (17 of 20 patients). A lack of ctDNA in the CSF may depend on the aggressiveness of the tumor or the anatomical location (18–20). In our cohort, the three patients without CSF ctDNA (one diffuse astrocytoma and two oligodendrogliomas) were all grade II gliomas. The cohort was not large enough to conclude any tendencies; however, the tumor grade may contribute to predicting the presence of CSF ctDNA. Our results indicate that the analysis of CSF ctDNA in low-grade glioma might have limitations challenging its clinical utility in this context. The inability to detect ctDNA in these three patients in addition to the fact that we could not subtype 21% of diffuse gliomas provides evidence that not all patients will benefit from this type of analysis and that in several cases analysis of CSF ctDNA will not be informative, especially in low-grade gliomas. Nonetheless, for patients in whom this analysis is informative, it could be of crucial relevance in clinical practice, and all the more so as technologic advances improve sequencing sensitivity, thus reducing the number of noninformative cases.

In addition to the possibility of using CSF ctDNA to complement diagnosis and circumvent complex and challenging surgical procedures, CSF ctDNA can play an important role in the context of genomic characterization of tumors, identify potential actionable mutations, and facilitate the monitoring of tumor progression (18). Importantly, serial sampling of CSF after tumor resection might facilitate the characterization of tumor recurrence. Moreover, studies to determine the role of CSF ctDNA in identifying pseudoprogression to treatment as well as the possibility that it could facilitate early diagnosis even before the identification of a relapsed lesion by imaging are still needed.

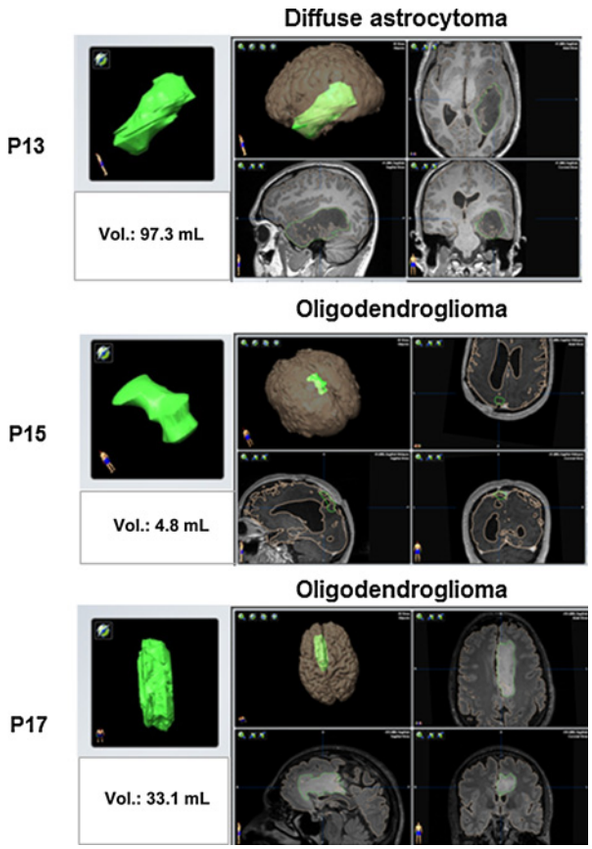


Figure 3.
MRIs of the three glioma patients for whom ctDNA did not recapitulate the tumor gene mutations. Tumor volume is shown.

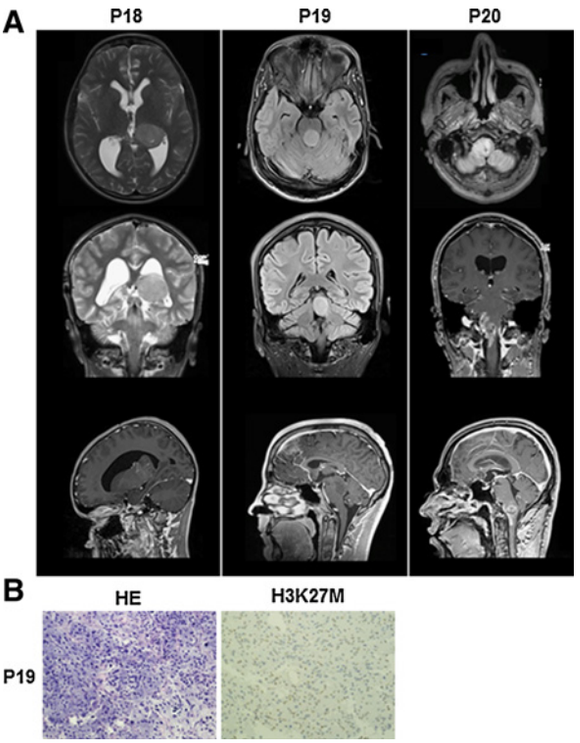


Figure 4.
MRI and immunohistochemistry from three diffuse midline glioma patients. **A,** MRI showing tumor localization. **B,** Hematoxylin and eosin (H&E) and H3K27M staining from patient P19.

Our platform can be improved by assessing more genomic alterations. In this manner, we could improve sensitivity by including gene mutations (such as H3F3A G34A). We could also incorporate targetable gene mutations (such as BRAF mutations) or other genomic alterations to improve prognostic stratification. However, the balance between complexity and speed of the assays and cost should be taken into consideration.

This pivotal study warrants future studies with a larger number of patients, with the demonstration that ctDNA in the CSF can be used as a liquid biopsy to help diagnose brain tumors and avoid risky surgical procedures. This opens a novel avenue in the field of noninvasive methods for molecular diagnosis aimed at better management of CNS malignancies.

Disclosure of Potential Conflicts of Interest

J. Tabernero is a consultant/advisory board member for Bayer, Boehringer Ingelheim, Genentech/Roche, Lilly, MSD, Merck Serono, Merrimack, Novartis, Peptomyc, Roche, Sanofi, Symphogen, and Taiho. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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