Neuro-Oncology Advances
6(1), vdae027, 2024 | https://doi.org/10.1093/noajnl/vdae027 | Advance Access date 4 March 2024

Plasma ctDNA liquid biopsy of IDH1, TERTp, and EGFRvIII mutations in glioma

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Abstract

Background. Circulating tumor DNA has emerging clinical applications in several cancers; however, previous studies have shown low sensitivity in glioma. We investigated if 3 key glioma gene mutations *IDH1*, *TERTp*, and *EGFRvIII* could be reliably detected in plasma by droplet digital polymerase chain reaction (ddPCR) thereby demonstrating the potential of this technique for glioma liquid biopsy.

Methods. We analyzed 110 glioma patients from our biobank with a total of 359 plasma samples (median 4 samples per patient). DNA was isolated from plasma and analyzed for *IDH1*, *TERTp*, and *EGFRvIII* mutations using ddPCR.

Results. Total cfDNA was significantly associated with tumor grade, tumor volume, and both overall and progression-free survival for all gliomas as well as the grade 4 glioblastoma subgroup, but was not reliably associated with changes in tumor volume/progression during the patients' postoperative time course. *IDH1* mutation was detected with 84% overall sensitivity across all plasma samples and 77% in the preoperative samples alone; however, *IDH1* mutation plasma levels were not associated with tumor progression or survival. *IDH1m* plasma levels were not associated with pre- or postsurgery progression or survival. The *TERTp C228T* mutation was detected in the plasma ctDNA in 88% but the *C250T* variant in only 49% of samples. The *EGFRvIII* mutation was detected in plasma in 5 out of 7 patients (71%) with tissue *EGFRvIII* mutations in tumor tissue.

Conclusions. Plasma ctDNA mutations detected with ddPCR provide excellent diagnostic sensitivity for *IDH1*, *TERTp-C228T*, and *EGFRvIII* mutations in glioma patients. Total cfDNA may also assist with prognostic information. Further studies are needed to validate these findings and the clinical role of ctDNA in glioma.

Key points

- *IDH1* mutation can be detected in glioma plasma with high sensitivity and specificity.
- *TERT* promoter *C228T* variant was found in 88% of plasma samples.
- Total plasma cfDNA is associated with glioma grade, volume and survival.

Glioma, the most common malignancy of the brain, remains a challenging problem. The genomic landscape of glioma is complex and there is significant inter- and intra-tumor heterogeneity.[1] Improved understanding of the genomic alterations in glioma has allowed more accurate classification and has been incorporated into the WHO system since 2016, with a recent update in 2021.[2] Point mutation in the *isocitrate dehydrogenase 1 (IDH1)* gene is a common origin event and is now a key diagnostic criteria to differentiate low- and high-grade glioma, mutation of the *telomerase reverse transcriptase promoter (TERTp)* is associated with progression to high-grade glioma (WHO grade 4 glioblastoma) and deletion of the extracellular domain of the *epidermal growth factor receptor* (known as *EGFRvIII*) occurs in up to 25% of glioblastomas.[3–5]

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Importance of the Study

Liquid biopsy via detection of circulating tumor DNA has enormous clinical value in several cancers; however, no validated biomarkers exist for glioma. This study demonstrates higher sensitivity using digital droplet PCR than has been previously reported in the glioma literature and shows the ability to detect 3 key glioma mutations, IDH1, TERTp, and EGFRvIII in the blood. Total plasma cfDNA was associated with tumor grade and survival outcomes. None of the plasma mutations had a clear relationship to tumor volume on longitudinal monitoring. The results of this study open the translational possibilities into clinical trials to investigate the role of ctDNA liquid biopsy for noninvasive diagnosis, prognostication, and monitoring for progression in glioma.

Both histopathological and molecular diagnosis of brain tumors currently relies on tissue biopsy which requires invasive neurosurgery either via a burr hole or craniotomy. Liquid biopsy of biofluids such as blood or cerebrospinal fluid (CSF), can be done more frequently and minimally invasively and holds promise in advancing cancer care. Circulating cell-free DNA (cfDNA) is a normal constituent of biofluids and its concentration varies with exercise, infection, trauma, and cancer.[6,7] Circulating tumor (ctDNA) is released from cancer cells by unknown mechanisms and comprises a small fraction (0.1%–5%) of the total cfDNA.[8]

To date, there has been substantial progress in several cancer types utilizing ctDNA for diagnosis, detection of minimal residual disease following therapy, guiding targeted therapies, and monitoring for tumor recurrence.[9–12] For example, detection of the *EGFR T790M* mutation in plasma in NSCLC patients can stratify patients to treatment by specific inhibitors.[13] Mutations associated with acquired drug resistance have also been detected by ctDNA and used to predict targeted treatment failure.[10,14] In glioma, however, analysis of plasma ctDNA has proven challenging with sensitivity <50%,[15–19] because of low levels of glioma-derived DNA in plasma and a lack of adequately sensitive sequencing methods. CSF has been demonstrated to hold higher concentrations of glioma ctDNA,[20–24] but obtaining CSF is more invasive and not as clinically practicable for repeat testing for monitoring compared to blood sampling.

In this study, we aimed to determine whether 3 key glioma gene mutations *IDH1*, *TERTp*, and *EGFRvIII* could be reliably detected in plasma by droplet digital polymerase chain reaction (ddPCR) and thereby investigate the potential of this technique for glioma liquid biopsy.

Materials and Methods

Patients and Clinical Data

The patient cohort was obtained from a prospectively recruited series of patients admitted to the Royal Melbourne Hospital (RMH) or Melbourne Private Hospital with a diagnosis of glioma between November 2018 and January 2021 (Table 1). Clinical data, including demographic, surgical, histopathological, and treatment characteristics, as well as follow-up and survival data were obtained from medical records and the RMH Central Nervous System (CNS) Tumor Database. This project was performed with institutional Human Research Ethics Committee approval (2009.114 and 2020.214) and all patients gave informed consent.

All patients underwent several brain MRIs as part of routine clinical care. MRI tumor volume and longitudinal comparisons were analyzed as in our previous publication.[25] Most patients had radiotherapy and adjuvant systemic chemotherapy during the study period either concurrently (Stupp protocol) or serially.

Blood Collection

Blood was collected from the arterial line or by venepuncture preoperatively, within 48 hours after surgery and then during patient follow-up at the time of each MRI scan, usually every 3–6 months. A minimum of 20 mL of whole blood was collected in EDTA Vacutainer tubes (BD, North Ryde, NSW) to recover at least 5 mL of plasma. For plasma isolation, whole blood was centrifuged at 500 × *g* for 10 minutes within 6 hours of collection to prevent genomic contamination. The supernatant was separated from the red cell pellet and centrifuged at 12 000 g for 10 minutes. The resultant cell-free plasma (at least 5 mL) was separated from the buffy coat residual into an Eppendorf tube and stored at −80° C.

DNA Isolation and Digital Droplet PCR Analysis

Circulating cfDNA was extracted from at least 5 mL of thawed plasma samples using the QIAamp Circulating Nucleic Acid Kit as per manufacturer's instruction (Qiagen, Germantown, MD). DNA was quantified with a microspectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA) and was stored at −20° C prior to use.

Isolation of tumor DNA was performed from fresh tumor specimens snap frozen at −80° C using the AllPrep DNA/RNA Mini Kit as per manufacturer's instructions (Qiagen, Germantown, MD). Extracted DNA was quantified and stored at −20° C prior to use.

Quantification of ctDNA gene mutations was performed using ddPCR. Primers for IDH1 and TERTp were commercially available (Bio-Rad, CA, USA). For *TERTp* ctDNA analysis, an optimized ddPCR protocol was used.[26] A custom-designed *EGFRvIII* primer set was developed due to the lack of a commercially available product. The primer was provided by Applied Biosciences (Thermo Fisher

Table 1. Demographics and Clinical Data

	WHO 2	WHO 3	WHO 4	Healthy Controls	*P*-value
No of patients	26	13	71	26	
Median age (range)	40 (24–66)	42 (26–85)	63 (38–93)	64 (19–83)	
Male	13	10	48	12	
Female	13	3	23	14	
IDH wild type			70		
IDH1 mutation	23	12	1		
IDH2 mutation	3	1			
Astrocytoma	15	7	1		
Oligodendroglioma	11	6			
Status					
Dead	1 (3.8%)	4 (27%)	43 (62%)		
Alive	25	11	26		
Median overall survival (months)	18 (1–34)	18.5 (2–32)	10 (1–33)		<.0001
Median PFS (months)	9 (1–34)	10 (2–32)	5 (1–24)		<.0001
Extent of resection					
GTR	53.8%	30.1%	52.9%		
STR	46.2%	61.5%	30.8%		
Biopsy	0%	7.4%	16.3%		
Tumor volume (cm^3)	25.2	37.1	33.9		

Scientific, Waltham, MA), using mutation sequences described by Koga et al.[27] The number of mutant copies per milliliter of cfDNA and variant allele fraction (VAF) was calculated. Multiple wells were used to confirm results in triplicate.

Confirmation of *IDH1* status in tissue was performed by immunohistochemistry (IHC) using a standard antibody on fixed formalin paraffin sections by the Department of Neuropathology, RMH. Confirmation of *TERTp* mutation status in the tumor tissue was done with the MSK-IMPACT[22] NGS panel by the Royal Prince Alfred Hospital neuropathology service (Sydney, Australia).

Statistical Analysis

The performance of each ctDNA assay was assessed by calculating the sensitivity and specificity compared to the gold standard tissue analysis technique, for both positive and negative tissue controls. The primary clinical endpoint of overall survival was defined as the date of enrollment (first surgery) until the date of death or censored at the date of last follow-up. Progression-free survival (PFS) was defined as the date of enrollment until the date of progression on MRI or clinically, including a second surgery, as determined from medical records including clinic notes and multidisciplinary team meetings. Kaplan–Meier curves were used for survival analysis and compared using the log-rank test. All tests were 2-sided with $P < .05$ considered statistical significance. Statistical analysis was performed with R version 4.2.1 and figures were created in GraphPad Prism version 9.

Results

Patient Characteristics

The patient cohort consisted of 110 patients with glioma comprising 26 WHO grade 2, 13 grade 3, and 71 grade 4 tumors (Table 1). A total of 359 plasma samples were available for ctDNA analysis, with a median of 4 samples (range 1–7) per patient.

Circulating Cell-Free DNA (cfDNA) Is Associated With Tumor Grade, Volume, and Outcome

We found that mean concentrations of cfDNA significantly increased with tumor grade (one-way ANOVA $P = .0067$, 11.94 ng/mL in grade 2, 14.29 ng/mL in grade 3, and 18.55 ng/mL in grade 4 gliomas; Figure 1A). Circulating cfDNA concentration (ng/µl) in preoperative plasma was significantly associated with tumor volume (Pearson coefficient $P = .0002$, $r^2 = 0.127$; Figure 1B). However, the mean concentration of plasma cfDNA was significantly increased 48 hours after surgery compared to preoperatively (mean 7.7 ng/mL in preop, mean 22.4 ng/mL postop, paired *t*-test $P = <.0001$; Figure 1C) possibly due to direct surgical tumor cell or endothelial disruption.

We next compared the relationship between preoperative cfDNA concentrations and survival outcomes in 103 patients. The patients were dichotomized into high- and low-cfDNA groups by the median preoperative cfDNA concentration of 2.2 ng/mL. The 2 cohorts were similar in age

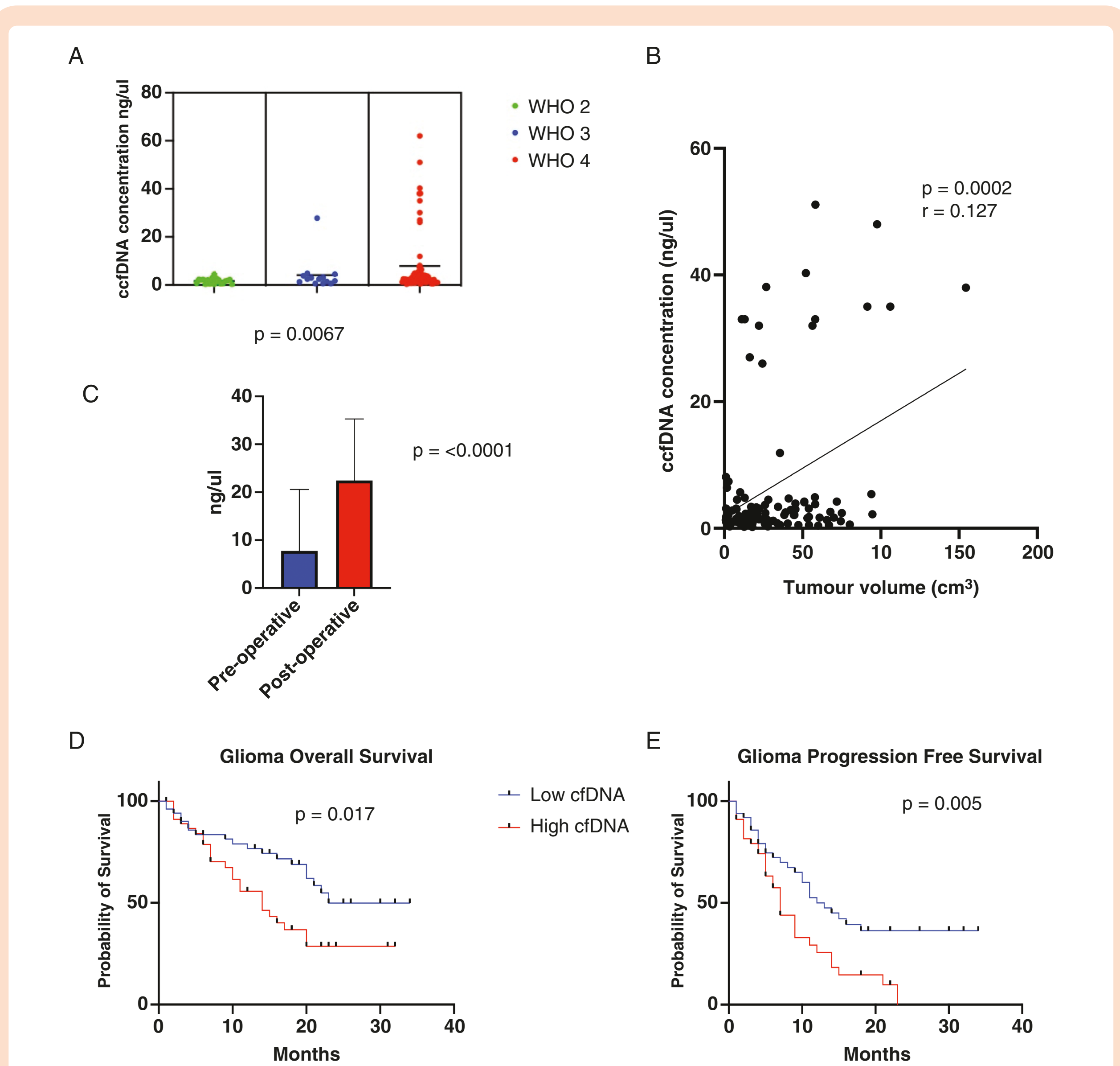

Figure 1. (A) Dot plot graph showing ccfDNA concentration in relation to WHO grade. Black bar shows median value. (B) Scatter plot showing preoperative ccfDNA concentration compared to tumor volume. (C) Bar graph comparing preoperative (blue) and postoperative (red) ccfDNA levels. (D) Kaplan–Meier graph of overall survival comparing low (blue curve) and high (red curve) plasma cfDNA. (E) Kaplan–Meier graph of PFS for low (blue) and high (red) plasma cfDNA.

and extent of resection; however, the low-cfDNA cohort had significantly more grade 2 and *IDH1* mutated tumors. Patients with high cfDNA concentrations had significantly worse overall survival (OS; median 23 vs 14 months, log-rank test $P = .017$, HR 2.13 CI 1.14–3.97) and PFS (median 13 vs 7 months, log-rank test $P = .0054$, HR 2.22 CI 1.27–3.90; Figure 1D and E).

When focusing on WHO grade 4 tumors, the median preoperative cfDNA concentration was 2.9 ng/mL and no significant baseline difference in median age (63 vs 61) or extent of resection was observed between the high (above median) and low (below median) cfDNA concentration cohorts. Again, a high cfDNA concentration was associated with significantly shorter median OS (14 vs 20 months, log-rank test $P = .028$, HR 2.17, CI 1.08–4.34; Supplementary Figure 1A) and PFS (5 vs 7 months, log-rank test $P = .034$, HR 2.02, CI 1.05–3.90; Supplementary Figure 1B). In the 24 WHO grade 2 tumors, cfDNA concentration showed no significant relationship to OS or PFS likely due to the low number of events in this group (Supplementary Figure 1C and D).

To assess the utility of cfDNA as a monitoring tool for glioma progression, we compared plasma cfDNA concentration to tumor volume on MRI in 30 patients who each had more than 3 follow-up plasma samples. We observed that in the majority (25/30) of these patients, increases or decreases in cfDNA did not correlate with a corresponding change in tumor volume. In only 5 patients (eg, patient shown in Supplementary Figure 2), we observed ccfDNA

concentrations reflecting changes in tumor volume (not including the immediate postoperative sample, in which ccfDNA was higher).

The IDH1-R132H Mutation Is Sensitively Detected in the Plasma by ddPCR

We next analyzed 36 patients who were confirmed to have *IDH1-R132H* mutations (*IDH1m*) on IHC of their resected tumor tissue. From this cohort, DNA was extracted from 113 plasma and 35 tissue samples for *IDH1m* quantification with ddPCR. We found that 84.1% (95/113) of all plasma samples had a detectable *IDH1m* (Figure 2A; Table 2). The *IDH1m* was not detected in any of the additional 10 plasma samples from patients with *IDH1 wild type* tumors or in 10 controls without any DNA input. Importantly, in the 36 preoperative blood samples, 77.7% of patients (28/36) had a detectable plasma *IDH1m*. In the immediate postoperative samples, IDH1m was detected in 27/31 (87%). Every patient had at least one *IDH1m* positive sample amongst their collected blood samples.

The mean number of *IDH1m* copies per milliliter was 0.89 (range 0–14.1 copies/mL) and the mean VAF across the cohort was 1.54% (range 0%–10.77%). The mean VAF was higher in the preoperative sample, when compared to the follow-up samples (2.19% vs 1.22%) but unlike total cfDNA concentration, the VAF did not change significantly between preoperative samples and postoperative samples taken 48 hours after surgery (median 2.19% vs 1.49%, unpaired *t*-test $P = .129$; Figure 2B). Similarly, there was no significant difference between the pre- and 48-hour postoperative *IDH1m* concentration (median 0.63 vs 1.01 copies/mL, unpaired *t*-test $P = .19$) or between concentration at

Table 2. Results of ddPCR Analysis With Sensitivity and Specificity % Shown in Parentheses in the Tissue+ and Tissue– Columns, Respectively

Gene/sample type	Tissue+	Tissue–
IDH1mut		
Patients	36	10
ddPCR tissue+	34/35 (97.1%)	–
ddPCR tissue–	1/35	–
Plasma samples	113	10
Plasma+	95 (84.1%)	0
Plasma–	18	10 (100%)
TERTp C228T		
Patients	16	16
Plasma samples	44	16
Plasma+	39 (88.6%)	3
Plasma–	5	13 (81.25%)
TERTp C250T		
Patients	14	16
Plasma samples	39	16
Plasma+	19 (48.7%)	1
Plasma–	20	15 (93.75%)
EGFRvIII		
Patients	7	33
Plasma samples	7	33
Plasma+	5 (71.4%)	2
Plasma–	2	31 (93.9%)

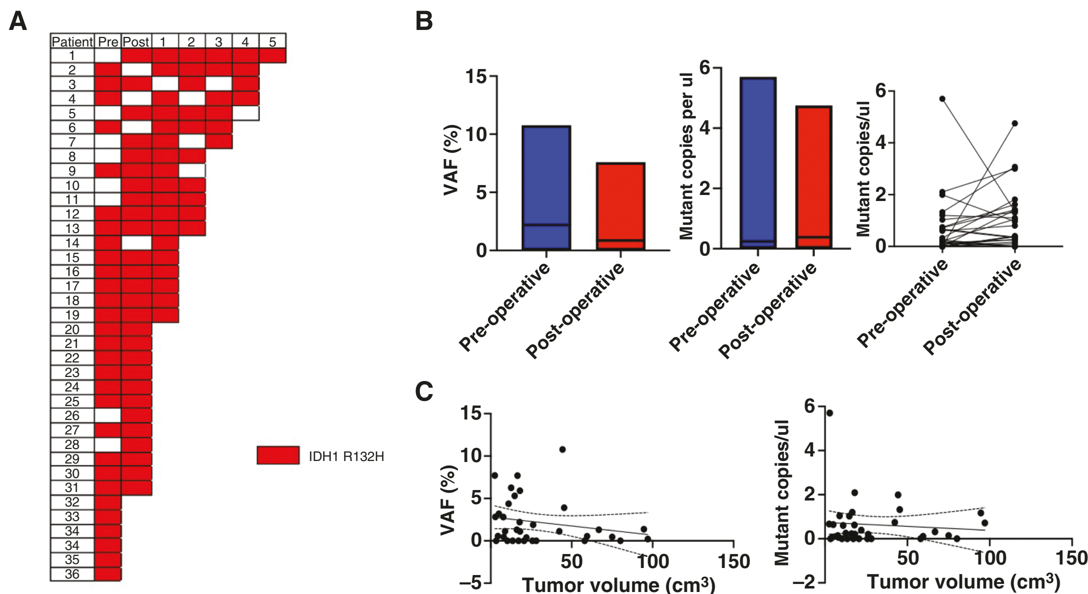

Figure 2. (A) Schematic showing results of IDH1m ddPCR in 36 patients, ranked from greatest to least by number of plasma samples. Red solid square = IDH1m detected, blank square = not detected. (B) Bar graphs showing mean levels of IDH1m preoperatively (blue) and postoperatively (red) with median value shown as black line. VAF% (left panel), mutant copies per milliliter (center panel), and paired difference (right panel). (C) Scatterplot showing correlation between MRI-based tumor volume and IDH1m VAF% (left panel) and mutant copies/mL (right panel). Trend lines with confidence intervals are indicated.

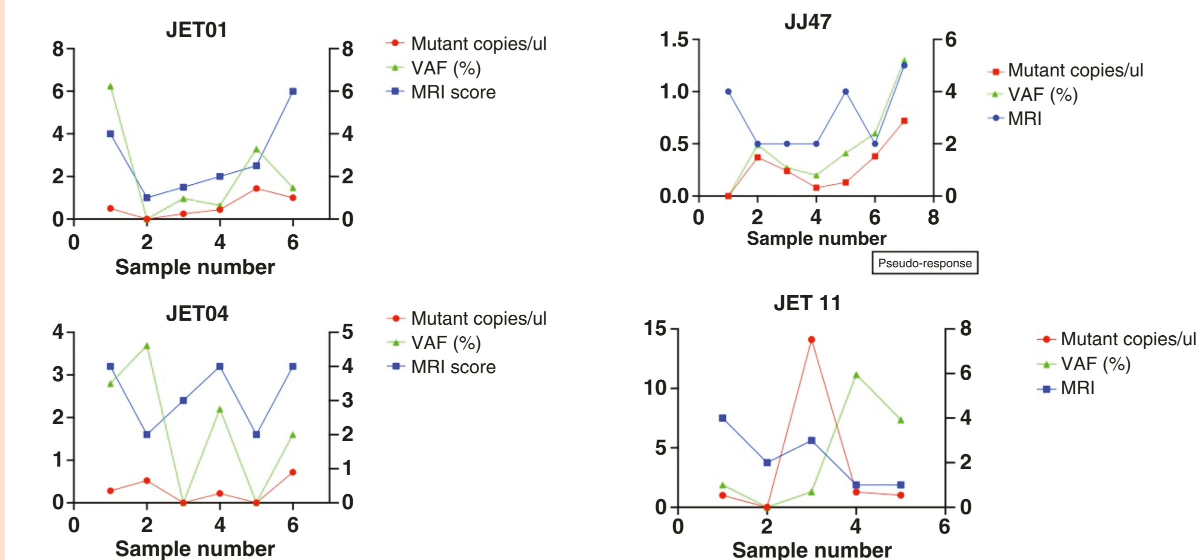

Figure 3. Graphs comparing the relationship of tumor volume as represented by MRI score (blue) to *IDH1 R132H* VAF (green) and number of mutant copies per milliliter (red) in 4 example patients. X-axis numbers indicate longitudinal blood samples. Patient in bottom right panel (JET11) is an example of pseudo-response, with MRI tumor volume reducing after the third follow-up sample following the commencement of bevacizumab.

diagnosis compared to follow-up (0.63 vs 0.79 copies/mL; Figure 2B). We also found no significant difference in the concentration of mutant copies of *IDH1m* for individual patients before and after surgery (mutant copy number paired *t*-test $P = .27$; Figure 2B).

Of 35 patients with *IDH1m* tumors on IHC who had tumor tissue DNA available, 34 were positive on ddPCR, thus the sensitivity (97.1%) of tissue ddPCR was higher than plasma (84.1%) for *IDH1m* detection (Table 2). This finding is not unexpected and likely due to the *IDH1m* concentration (mean 241.21, range 0.32–3930 copies/mL) and VAF (mean 39.4%, range 0.1%–93%) being markedly higher in tissue samples.

Plasma IDH1m Is not Associated With Tumor Grade, Survival, Volume, or Progression

In the 36 patients with *IDH1-R132H* mutated tumors, we did not observe a significant difference in the concentration of *IDH1m* (median 0.36 copies/mL in grade 2, 0.31 copies/mL in grade 3 and 0.33 copies/mL in grade 4, $P = .51$), or VAF concentrations (median 0.86% in grade 2, 0.51% in grade 3, 0.5% in grade 4, $P = .24$) detected between tumor grades. We then compared *IDH1m* concentration and VAF in the plasma prior to surgery with survival outcomes. When the cohort was divided into high and low *IDH1m* copies/mL and VAF by the median value (0.25 copies/mL and 1.1%, respectively), we found no relationship between VAF or mutant copies/mL to either OS or PFS (Supplementary Figure 3A–D). We found no correlation between preoperative plasma *IDH1m* VAF (Pearson's coefficient $r^2 = 0.229$) or concentration ($r^2 = 0.602$) and tumor volume on MRI (Figure 4A and B). In 12 patients who each had 4 or more follow-up plasma samples available for monitoring patient progression, we found that *IDH1m* concentration and VAF were not reliably associated with changes in tumor volume on MRI. In only 4 of 12 patients did either mutant copy concentration and/or VAF reflect corresponding changes in tumor volume (Figure 3). Taking each time point separately, the change (increase/decrease) in mutant copy concentration mirrored the change in MRI tumor volume in only 49% of the 61 MRI/plasma pairs, and similarly the VAF correlated with MRI in only 42% of time points. Therefore, based on our data, plasma *IDH1m* does not appear to be a reliable test to monitor tumor volume or progression.

Plasma Detection of TERT Promoter Mutation

Our cohort included 19 patients with tumors harboring *TERTp* mutations, determined by tissue NGS (Table 2). There were 44 blood samples available from these *TERTp*-positive patients and a further 16 plasma samples were available from *TERTp* mutation-negative tumors. These 60 plasma samples were tested for the *C228T* variant and 55 plasma samples for the *C250T* variant. The majority (39/44) of plasma samples were positive for *C228T* with a sensitivity of 88.6% compared to NGS of tissue, whereas the sensitivity of detection of the *C250T* mutation was lower at 48.7% (19/39; Figure 4A). In contrast, the specificity of a positive *C250T* result was high at 93.7%, with one false positive. However, for *C228T* there were 3 of 16 positive samples in *C228T* tissue negative tumors (specificity 81.2%; Table 2).

We found that, like *IDH1*, although *TERTp* mutation tended to decrease in the postoperative samples, however, the difference was nonsignificant (0.59 vs 0.19 mutant copies/mL, $P = .17$, VAF 1.3% vs 0.23%, $P = .10$; Figure 4B). Neither the *TERTp* mutant concentration nor VAF was significantly associated with survival (Figure 4C) or tumor volume (Pearson coefficient $r^2 = 0.0074$ and $r^2 = 0.0347$,

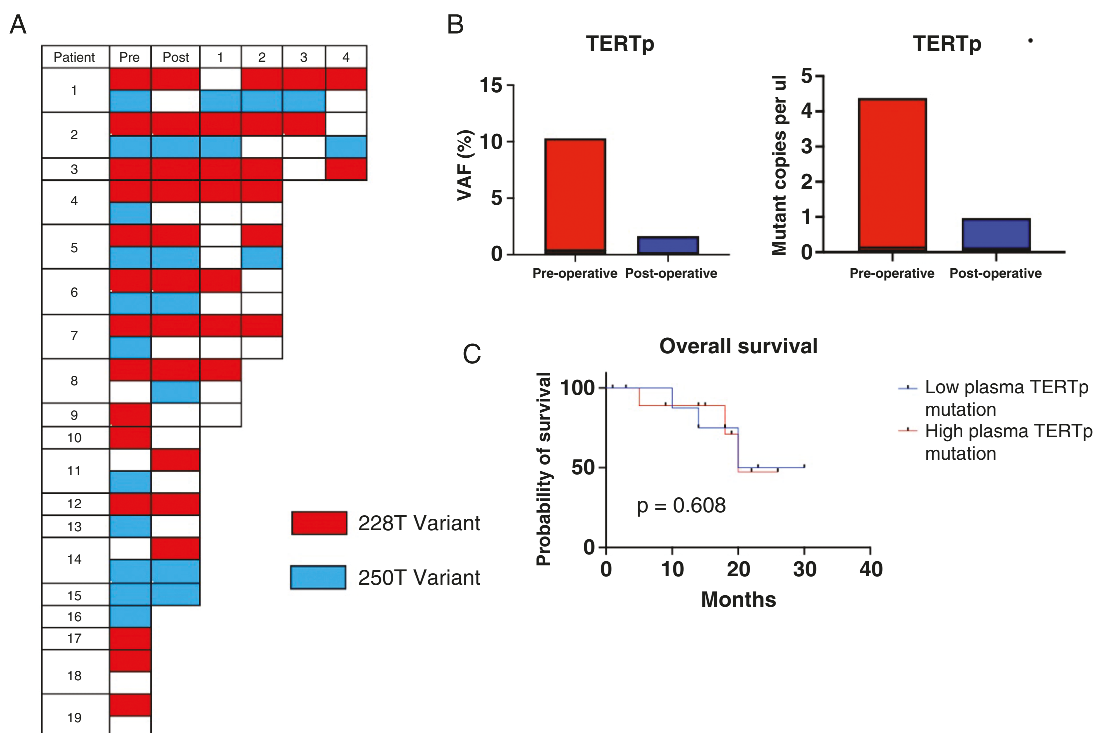

Figure 4. (A) Schematic showing results of 19 patients who were tissue positive for either *TERTp* mutation by ddPCR, ranked from greatest to least by number of plasma samples. Red solid square = *TERTp C228T* mutation detected in plasma, light blue square = *TERTp C250T* mutation detected in plasma, blank square = neither detected in plasma. (B) Bar graphs showing mean levels of *TERTp C228T* mutation preoperatively (red) and postoperatively (blue) for VAF% (left panel) and mutant copies per milliliter (right panel). (C) Kaplan–Meier analysis for overall survival comparing low (blue line) and high (red line) *TERTp C228T* mutation concentration.

data not shown). When we compared plasma levels of *TERTp* mutation in patients with more than 3 follow-up samples, to contrast-enhanced tumor volume based on MRI, we found that changes in plasma *TERTp* mutation level mirrored changes in tumor volume in 19 out of 30 (63%) time points.

EGFRvIII Mutation Is Detected in a Small Number of Patients

To assess the ability of the *EGFRvIII* mutation to be detected by liquid biopsy, we analyzed plasma and tumor DNA from 40 patients with WHO grade 4 tumors. Since there is no commercially available ddPCR primer for the *EGFRvIII* mutation, we designed a custom primer set using mutation sequences described by Koga et al.[27] To confirm the primers, positive control input DNA derived from immortalized *EGFRvIII*-transfected cell lines (U87MG-vIII) was used as positive control and nontemplate controls (lacking any input DNA) were negative. Of the 40 tissue samples, we found 7 patients who were positive for the *EGFRvIII* mutation on ddPCR and of those, 5 had detectable *EGFRvIII* in plasma indicating a sensitivity of 71.4% (Table 2). There were 2 false positives (specificity 93.9%). No wild-type *EGFR* was detected in any of the samples as expected because amplification of wild-type EGFR by the custom EGFRvIII primers should not occur due to its much larger size.[28] Overall the mean concentration of *EGFRvIII* copies in the plasma (0.15 copies/mL) was much lower than that seen with *IDH1-R132H* and *TERTp* mutations.

Discussion

There has been substantial progress over the last decade utilizing ctDNA for genotyping at diagnosis, detection of minimal residual disease, predicting response to targeted therapies, and monitoring for tumor recurrence.[9–12,14] Furthermore, ctDNA has been used to identify mutations associated with acquired drug resistance to predict targeted treatment failure.[10,14] The most clinically advanced ctDNA test detects the *EGFR T790M* mutation in the plasma of patients with NSCLC with sensitivity that is comparable to tumor biopsy.[13,29] Several ctDNA clinical trials are currently underway,[12,30] but there has been minimal progress in liquid biopsies for glioma to date. Therefore, we set out to investigate the utility of ddPCR for plasma ctDNA for 3 key glioma mutations.

In line with previous reports from other cancer types, we found that total cfDNA concentration correlated with glioma volume and higher grade. This may be related to the amount of cellular turnover and apoptosis, as well as increased permeability through the blood–brain barrier.[8] Total cfDNA was also associated with significantly shorter

overall survival in all tumors as well as within grade 4 tumors. Our findings are consistent with 2 previous reports suggesting that cfDNA in itself may be a useful prognostic marker at the time of diagnosis.[18,31] We found that mean cfDNA increased in the immediate postoperative period compared to preoperative levels, likely due to disruption of the tumor cells at surgery, resulting in direct DNA release into the circulation. This suggests that the utility of cfDNA for indicating minimal residual disease is limited. Further work is necessary to determine how and when cfDNA could be used as a prognostic adjunct clinically in glioma patients.

In colorectal, breast and non–small cell lung cancer, total cfDNA concentration increases with tumor recurrence[9–11,14] and similarly in glioma, 2 studies have shown an increase in cfDNA at tumor progression.[18,32] In our present study, however, including 30 patients with more than 3 plasma samples each, we found no correlation between cfDNA concentration and tumor progression. These inconsistent results highlight the difficulties of using nonspecific markers such as cfDNA for tumor monitoring. Total cfDNA concentration is influenced by numerous factors including diet, exercise, surgery, radiotherapy, infection, comorbidities, and medications such as corticosteroids. Since <1% of the total cfDNA is derived from the parental glioma, an increase in concentration due to tumor progression is likely to be greatly diluted.

Circulating tumor DNA has the advantage of very high specificity as a biomarker and ddPCR has become a common method of ctDNA analysis in many cancers, but there are only a few studies to date assessing ddPCR for liquid biopsy in glioma.[23,33–39] In one, *H3K27M* mutation was detected in the plasma of patients with diffuse midline glioma with high sensitivity (90%) comparable to CSF.[34] Two other studies showed that the sensitivity of detecting *IDH1* mutation and *TERTp* mutation in CSF was around 70%.[37,39]

Determination of *IDH1/2* mutation status has become mandatory since the 2021 WHO update that relies on *IDHm* vs *IDHwt* as the primary classifier of glioma grade.[2] Mutations in the *IDH1* or *IDH2* gene are early events in low-grade glioma tumorigenesis and these single base missense mutations make them ideal targets for PCR analysis. The utility of ctDNA *IDH1m* analysis has been reported in several types of cancers. In a phase III study of an *IDH1m* inhibitor in patients with advanced intrahepatic cholangiocarcinoma, a high concordance of *IDH1m* between plasma ctDNA and tissue (92%) was seen,[40] and 10 of 36 patients with disease control also had plasma clearance of *IDH1m*, compared to none of the 40 patients with uncontrolled tumors or those who received placebo. In glioma, the first publication assessing liquid biopsy for *IDH1m* used COLD-PCR and found the mutation in 15/25 (60%) plasma samples.[41] In our present study, using larger blood volumes and ddPCR, we found that the *IDH1m* was detected with 84% overall sensitivity per blood sample, higher than in previous reports in the literature. A further advantage of liquid biopsy, unlike surgical biopsy, is that multiple blood samples can be taken daily or weekly, potentially improving overall sensitivity, however, for routine clinical diagnostic purposes, the sensitivity of the single preoperative blood sample is important; we found that for *IDH1m* this was still very high at 77%. The translational application of this test could provide noninvasive genomic diagnosis in glioma patients who are not suitable for surgical biopsy, or in low-grade gliomas managed with an initial watch-and-wait approach after diagnostic MRI, or in clinical trials of IDH inhibitors. It will be a key question to determine whether a repeat blood draw ctDNA analysis at a later time point will be clinically indicated for those patients who test *IDH1m* negative on their first blood test.

Longitudinal postoperative monitoring of *IDH* status in glioma is also a clinically attractive concept[42] particularly in the context of current trials of *IDH1* inhibitors.[43] Although plasma *IDH1m* was an indicator of tumor volume in other types of cancers,[40] there have been no reports to date comparing circulating *IDH1m* to tumor burden or clinical outcomes by plasma monitoring in glioma. In our cohort, we analyzed multiple longitudinal samples throughout the patients' clinical timeline; however, we did not find that plasma *IDH1m* ctDNA correlated with changes in tumor volume. This could be explained by the fact that plasma ctDNA levels are too low to be a reliable monitoring tool or the fact that *IDH1* mutation is not a driver gene for clonal expansion during glioma progression.[44] Further studies are necessary to determine whether longitudinal monitoring of plasma *IDH1m* will be clinically advantageous and how it compares to the detection of 2-HG levels by MR-spectroscopy.

The *TERTp* mutation is one of the most common genetic alterations in glioma and is found in 70%–84% of GBMs and 74% of oligodendrogliomas.[45] The 2 most common mutations are *C228T* (45% of all *TERTp* mutations) and *C250T* (15%).[4,46] The mutations are heterogeneous and are found in regions with a high CG nucleotide content, making stable, specific, and reproducible PCR challenging. The sensitivity of detecting *TERTp* mutation in CSF has been reported as around 70%,[37,39] whereas *TERTp* mutation detection in plasma by ddPCR showed very low sensitivity.[32] Recently, a ddPCR assay that detects both *TERTp 228T* and *250T* mutations with techniques to reduce secondary structure formation, found moderate sensitivity in plasma of 62.5% (and 90% specificity) in 157 gliomas.[37] In our present study, we used a protocol that reliably detects both the *C228T* and *C250T* mutations down to a VAF of 0.062 and 0.051% respectively in both tissue and plasma samples.[26] We found good sensitivity for plasma *TERTp* mutation detection of 89% for *C228T* but only moderate (49%) for *C250T*. Our results showed that neither *TERTp* mutation concentration nor VAF was significantly associated with tumor volume or survival, but as in previous reports,[36] we observed TERTp tracking tumor size in about two-thirds of patients.

We next focused on *EGFRvIII*, a gain-of-function mutation that arises from genomic deletion of exons 2–7, with ligand-independent constitutively active signaling that promotes proliferation, survival, and angiogenesis.[1] It is mutated in approximately 25% of WHO grade 4 gliomas and is highly oncogenic, making it an attractive therapeutic target. Of the 7 patients with grade 4 tumors whose tumor tissue was positive for the mutation, we found 5 who also had a detectable plasma *EGFRvIII* mutation at diagnosis. Interestingly, we found plasma *EGFRvIII* in 2o patients for whom the mutation was not detected in their tumor tissue on ddPCR. It is unlikely that these *EGFRvIII* plasma copies are from another tissue

source other than glioma, so this finding supports the concept of spatially heterogeneous expression of the mutation in tumor tissue that might have been missed by selective tissue biopsy. Skog et al.[47] also reported 2 patients with *EGFRvIII* negative tumor samples who had serum microvesicles containing the *EGFRvIII* mRNA transcript. Mutations in the *EGFR* gene are amongst the most common reported as ctDNA, particularly the *EGFR T790M* mutation, which has now been incorporated into clinical practice in patients with non–small cell lung cancer. However, ctDNA for the *EGFRvIII* mutation is not well reported, in part due to challenges with reliably detecting the large deletion mutation. Skog et al. observed *EGFRvIII* mRNA in serum microvesicles of 7 of 25 patients with *EGFRvIII* mutated tumors, with no detectable mutation in 5 patients 2 weeks following resection, suggesting a relationship to tumor burden.[47] The same group also reported a higher sensitivity of 61% using CSF in 61 GBMs.[48] In the only prior study based on plasma ctDNA, Salkeni et al.[28] detected *EGFRvIII* in 3 GBM patients using qPCR and found that the 2 patients who had a gross total resection had no detectable levels 3 weeks following surgery. In summary, *EGFRvIII* can be detected in plasma by ddPCR with moderately high sensitivity, in some cases even when it is not found in surgical tissue biopsy. However, the small numbers of patients in our present study and those in the literature means that no strong conclusions can currently be drawn regarding the utility of monitoring plasma *EGFRvIII* levels.

In conclusion, we show that *IDH1-R132H*, *TERTp*, and *EGFRvIII* mutations can be detected in plasma with high sensitivity and specificity. We are designing studies as part of upcoming clinical trials, to definitively determine the performance of these tests with respect to future clinical applications. Potential applications include noninvasive genomic diagnosis, and it remains to be seen if *IDH1*, *TERTp*, or *EGFR* mutations in blood can be used as monitoring biomarkers for tumor response or progression. Total plasma cfDNA is also an attractive test, much simpler to assay and appears to be associated with tumor grade and survival outcome; however, it is not likely to be a reliable monitoring biomarker.

Certainly, ddPCR for ctDNA in glioma could be expanded beyond those genes we have reported here and could be expanded to include any mutation of interest in glioma biology. Alternatively, next-generation sequencing has shown moderate sensitivity and provides widescale profiling information although it remains cost-prohibitive.[16,17] Our results indicate that ddPCR-based plasma ctDNA analysis is a powerful and readily available liquid biopsy technique in glioma that warrants further investigation.

Supplementary material

Supplementary material is available online at *Neuro-Oncology Advances* (https://academic.oup.com/noa).

Key words

ctDNA | glioma | IDH mutation | liquid biopsy | TERTp

Funding

This research was supported by the Cure Brain Cancer Foundation Australia and the Royal Melbourne Hospital Neuroscience Foundation.

Acknowledgements

We acknowledge the patients and families who took part in the study, as well as the staff who contributed to the research at each institution.

Conflict of interest statement.

None declared.

Authorship statement

J.J.J, A.M., F.G., J.W., A.H.K., K.J.D. conceived and designed the study. J.J.J., A.M., K.J.D., J.T., T.P., F.G., collected and assembled the data. J.J.J., A.M., S.W., K.J.D., J.W. analyzed and interpreted the data. A.M., J.J.J., K.J.D., A.H.K. were responsible for the provision of the study materials and the patients. J.J.J., H.N., J.I., S.S., J.T., T.P. processed the blood and CSF samples. J.J.J., A.M., J.W., K.J.D. wrote the manuscript. All authors approved the manuscript.

Data availability

All data and code are available upon request.

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