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Plasma ctDNA enables early detection of temozolomide resistance mutations in glioma

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Abstract

Background. Liquid biopsy based on circulating tumor DNA (ctDNA) is a novel tool in clinical oncology, however, its use has been limited in glioma to date, due to low levels of ctDNA. In this study, we aimed to demonstrate that sequencing techniques optimized for liquid biopsy in glioma patients can detect ctDNA in plasma with high sensitivity and with potential clinical utility.

Methods. We investigated 10 glioma patients with tumor tissue available from at least 2 surgical operations, who had 49 longitudinally collected plasma samples available for analysis. Plasma samples were sequenced with CAPP-seq (AVENIO) and tissue samples with TSO500.

Results. Glioma-derived ctDNA mutations were detected in 93.8% of plasma samples. 25% of all mutations detected were observed in plasma only. Mutations of the mismatch repair (MMR) genes *MSH2* and *MSH6* were the most frequent circulating gene alterations seen after temozolomide treatment and were frequently observed to appear in plasma prior to their appearance in tumor tissue at the time of surgery for recurrence.

Conclusions. This pilot study suggests that plasma ctDNA in glioma is feasible and may provide sensitive and complementary information to tissue biopsy. Furthermore, plasma ctDNA detection of new MMR gene mutations not present in the initial tissue biopsy may provide an early indication of the development of chemotherapy resistance. Additional clinical validation in larger cohorts is needed.

Key Points

- Circulating tumor DNA mutations can be sensitively detected in plasma in glioma patients and 25% of mutations were detected only in plasma.
- Mismatch repair genes indicative of chemoresistance were frequently observed in ctDNA after temozolomide treatment

Analysis of ctDNA has become an important tool in modern clinical oncology for longitudinal assessment of tumor burden and genomic profiling with the advantage of its potential ability to overcome the limitations of temporal and spatial heterogeneity encountered with tissue sampling.[1] Further roles of ctDNA include the identification of eligible patients for clinical trials of targeted therapies, early diagnosis of cancer in asymptomatic patients, and localizing tumors of unknown origin through molecular profiling when tumor tissue is limited.[2–4] Despite the demonstrated utility of liquid biopsy in many cancers, analysis of circulating tumor DNA (ctDNA) in gliomas, the most common primary brain malignancy, has not progressed, due to the challenges with detecting low levels of circulating glioma DNA.[5] Techniques such as fragmentome analysis, which targets DNA sequences between 90 and 150 base pairs (bp) is suggested to improve sensitivity, however, is still in the early stages of investigation.[6] Cerebrospinal fluid (CSF) does contain higher concentrations of ctDNA,[7–11] however obtaining repeated CSF samples is difficult due to the fact that lumbar puncture is not a standard procedure in glioma patients' management. Other strategies including analysis of epigenetic alterations using DNA methylomes or microRNA in

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Importance of the Study

We report vastly improved plasma ctDNA sensitivity using larger plasma volumes and an optimized sequencing method in one of the only studies utilizing longitudinal blood sample series in glioma. Furthermore, we show for the first time that key gene mutations associated with temozolomide chemoresistance (*MSH2, MSH6*) can be readily detected earlier in the blood at the time they develop, before waiting for invasive tissue biopsy or further surgery. This information could potentially be used to decide on the continuation of temozolomide versus switching to another therapy. The results of this study hold importance for the translation of blood-based liquid biopsy for diagnosis and monitoring in glioma.

blood or CSF have shown promise, but these approaches lack the specificity of ctDNA and do not identify prognostic or targetable mutations in the tumor. Blood sampling has major advantages for liquid biopsy as it can be performed non-invasively and at multiple timepoints. The novel next generation sequencing (NGS) technology CAPP-seq[12] has not previously been reported in neuro-oncology, therefore we aimed to assess whether this method, combined with improved blood sampling methods, could allow plasma liquid biopsy to provide valuable genomic information in glioma. In this study, the first longitudinal study of plasma ctDNA in glioma, we show that ctDNA can be successfully detected in the blood of patients with glioma including key genes that could be used to monitor tumor genomic evolution.

Materials and Methods

Patients and Clinical Data

The study was a retrospective analysis of a patient cohort with a diagnosis of glioma who were admitted to the Royal Melbourne Hospital (RMH) or Melbourne Private Hospital (MPH) between November 2018 and January 2021, who had longitudinal blood samples and matched primary and recurrent brain tumor tissue samples available in the RMH neurosurgery biobank. Clinical data, including demographic, surgical, histopathological, and treatment characteristics, as well as follow-up and survival data were obtained from the prospectively collected RMH/MPH Central Nervous System (CNS) Tumor Database, part of the Australian Comprehensive Cancer Outcomes Research Database (ACCORD). Patients were treated as per routine clinical standard of care, including discussion of treatment options at the weekly RMH multidisciplinary neuro-oncology meeting. All patients underwent 2 surgeries, at the time of inclusion into the study and then later at the time of progression. All patients had radiotherapy and adjuvant systemic chemotherapy during or prior to the study period. All patients underwent brain MRIs as indicated by the standard of care (pre-operatively, within 48 h post-op, and then at approximately 3–6 monthly intervals during the monitoring phase). MRI sequences including T1weighted, T2-weighted, FLAIR, and contrast T1-weighted image as well as spectroscopy and perfusion studies were done routinely. Brain MRIs were reviewed at each time-point by an experienced neuroradiologist unaware of any ctDNA results. Standard response assessment in neuro-oncology (RANO) criteria was applied to determine radiographic progression. The project was performed with Human Research Ethics Committee approval from Melbourne Health (HREC 2009.114). All patients gave informed consent for sample collection and inclusion in the study.

Blood Collection

Longitudinal blood sampling was performed with samples taken prior to the initial surgery, within 48 h post-operatively, during the tumor follow-up period (at the time of their MRI), prior to the second surgery at recurrence, and after the second surgery in some cases. A total of 49 plasma samples were taken in the 10 patients (median 4, range 3–7 samples per patient). A minimum of 20mL of whole blood was collected in EDTA Vacutainer tubes (BD, North Ryde New South Wales) in order to recover at least 5 mL of plasma. For plasma isolation, whole blood was centrifuged at 500*g* for 10 min within 6 h of collection to prevent genomic contamination. The supernatant was carefully separated from the red cell pellet and centrifuged at 12 000 rpm for 10 min. 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.

Isolation and Sequencing of cfDNA

Cell free DNA extraction from plasma and targeted sequencing was performed using the AVENIO ctDNA Expanded Kit (Roche Diagnostics), a panel of 77-gene cancer-related biomarkers. Extracted DNA was quantified using fluorimetry (Qubit, Thermo-Fisher Scientific) and stored at –20°C prior to use. Plasma cfDNA was sequenced using the Cancer Personalized Profiling by deep Sequencing (CAPP-Seq) method for quantifying ctDNA as previously described.[12] The method combines optimized library preparations with a multi-phase bioinformatics informatics approach to design cancer specific DNA "selectors," that target recurrently mutated regions in the cancer of interest. Sequencing was performed on libraries generated from approximately 10 ng total cfDNA. AVENIO ctDNA Analysis Software version 2.0.0 was used to align, call, and filter variants against the hg38 human reference genome. Nonsynonymous, non-germline mutations, known to be mutated in cancer were considered significant. Buffy coat DNA was isolated and sequenced using AVENIO Tumor

Tissue Expanded Kit (Roche Diagnostics) that contains the same 77-gene panel, and genes associated with clonal hematopoiesis were removed: *ASXL1, PPM1D, DNMT3A, TET2, GNB1, CBL, JAK2, STAT2, MYD88, SF3B1.* Both filtered and raw data were obtained.

Isolation and Sequencing of Tumor Tissue DNA

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). Glioma tumor tissue underwent Targeted sequencing of 523 cancer genes from DNA and 55 cancer genes from RNA using the TruSight Oncology 500 Assay (TSO500, Illumina, San Diego, CA) on the Illumina NextSeq 550 platform. Illumina Software TSO500 v2.0.0 Local App was used to generate aligned reads and call variants against the hg38 human reference genome. The variants were annotated using the Ensembl's Variant Effect Predictor (VEP) tool[13] and Cancer Genome Interpreter (CGI).

Statistical Analysis

Associations between ctDNA and clinical outcomes were assessed using paired student *t*-test. All statistical tests were two-sided with a <0.05 for statistical significance. Oncoprint diagrams were created in R. Other figures were created in GraphPad Prism version 9. No statistical method was used to predetermine the sample size.

Results

Patient Demographics

From the Royal Melbourne Hospital brain tumor biobank, we included 10 prospectively biobanked patients with glioma who had undergone surgical resection at both diagnosis and at the time of progression. Demographic and tumor details are summarized in Table 1. Eight patients had WHO grade 4 glioblastoma, 1 patient had IDH1-mutant WHO grade 3 astrocytoma and 1 was WHO grade 2 oligodendroglioma. Median time to re-operation was 8 months. In addition to at least 2 surgical operations, all patients underwent radiotherapy and systemic chemotherapy. Most patients were enrolled at the time of their initial diagnosis; 2 patients (nos. 2 and 6) had received surgery and TMZ prior to inclusion in this study. Plasma for ctDNA was collected (median 4 samples per patient) before and after surgery and at the time of each follow-up magnetic resonance imaging (MRI) scan.

Total Cell Free DNA Reflects Tumor Dynamics

Plasma cell free DNA (cfDNA), the total circulating extracellular DNA from all sources, was isolated from at least 5 mL

Table 1. Demographics and Clinical Data

No.	Age	Sex	Tumor Location	Histopathology	Grade	Treatment prior to inclusion	Treatment after inclusion	Time to re-operation (months)	OS (months)
1	24	M	Left Frontal	Oligodendroglioma	2		Radiotherapy TMZ	7	36*
2	31	M	Left Temporal	IDH mutant Astrocytoma	3	TMZ RT	Bevacizumab Lomustine	10	18
3	49	M	Right Temporal	Glioblastoma	4		RT TMZ Bevacizumab	8	10
4	44	M	Right Occipital	Glioblastoma	4		RT TMZ Bevacizumab	7	22*
5	58	M	Left Frontal	Glioblastoma	4		RT TMZ Bevacizumab	16	20
6	61	F	Right Temporal	Glioblastoma	4	TMZ RT	TMZ Bevacizumab	20	33
7	78	F	Left Parietal	Glioblastoma	4		RT TMZ	14	20
8	67	F	Left Temporal	Glioblastoma	4		RT TMZ	8	19
9	50	M	Right Parietal	Glioblastoma	4		RT TMZ	7	16
10	60	M	Right Parietal	Glioblastoma	4		RT TMZ Bevacizumab	6	18

RT: radiotherapy TMZ: temozolomide OS: overall survival.
*Still alive at end of study.

plasma and quantified using fluorimetry (Qubit, ThermoFisher Scientific). Concentrations of plasma cfDNA ranged from 10 to 637 ng/mL and the mean concentration of cfDNA per patient was noted to correlate with tumor grade: 27 ng/mL in grade 2, 60 ng/mL in grade 3, and 116 ng/mL in grade 4 gliomas. The increase in cfDNA concentration observed in higher grade tumors is consistent with findings in other solid cancers.[14] We noted that the concentration of plasma cfDNA was higher in samples taken immediately post-operative compared to the pre-operative sample (mean 167 vs. 77 ng/mL, range pre-op 10–621, post-op 19–638, paired *t*-test $P = .047$), likely due to surgical tissue disruption resulting in direct DNA release from cells into the circulation. In several patients, increasing plasma cfDNA was seen just before or at the time of tumor progression. As previously reported by Bagley et al., tracking plasma cfDNA concentration might be a useful tool in itself for monitoring tumor burden in glioma.[15]

Plasma cfDNA Next Generation Sequencing

We next analyzed plasma cfDNA using the CAPP-seq ("AVENIO," Roche) expanded assay, which includes a targeted panel of 77 genes frequently mutated in cancer (sample metrics and data are available in the Supplementary Material). To compare the spectrum of mutations found in plasma ctDNA with the parental tumor, we analyzed tissue samples with the TruSight Oncology 500 NGS platform (Illumina), which analyses DNA mutations in 523 common cancer genes (results shown in Supplementary Table 1). To exclude germline mutations and mutations associated with clonal hematopoiesis, DNA from peripheral blood mononuclear cells (PBMC) in the buffy coat was sequenced using the AVENIO Tumor Tissue Analysis Kit (Roche Diagnostics) and these mutations were therefore not considered to be ctDNA. These included *mTOR* missense mutations in patients 2, 3, 4, a germline *MET* missense mutation in patient 5, germline *DDR2* mutation in patient 6, *KIT* missense mutation in patient 6, and a *PTCH1* missense mutation in patient 7.

Glioma-derived mutations of ctDNA were detected in at least 1 plasma sample per patient and in 47/49 (93.8%) of all plasma samples (Figure 1A). When considering the 77 genes that were common to both the AVENIO panel and the TSO500 panel, 52.4% of gene alterations were present in tissue only, 22.4% were found in both plasma and tissue, and 25.2% were detected only in plasma (Figure 1B). All mutations found in the 10 patients in both tumor and/or plasma are shown in Supplementary Figure 6. The mutations that were concordant between plasma and tissue for both the primary biopsy and the biopsy at progression are shown in Supplementary Table 2. To analyze the sensitivity of plasma compared to tissue for these mutations we focused on the plasma samples that were taken around the same time as the tissue biopsy, either pre- or post-op within a few weeks of the 2 separate surgical timepoints. For the primary surgery, plasma ctDNA found between 16% and 33% of tissue mutations and for the second surgery at progression, plasma ctDNA found between 0% and 50% of tissue mutations. Plasma-only mutations included those associated with GBM[16] such as *EGFR* amplification and a *PTEN* frameshift mutation and therefore were highly likely to be glioma-derived somatic mutations, despite not appearing in the tissue sequencing (patient 10, Figure 1C). The mean variant allele fraction (VAF) of concordant plasma and tissue gene mutations across all our patients was higher, but not significantly different, to non-concordant mutations (0.32% vs. 0.25%, paired *t*-test $P = .056$), therefore, the detection of mutations in plasma was not simply related to the abundance of ctDNA, underscoring the sensitivity of the AVENIO platform. One patient also had CSF available for AVENIO analysis and this was found to have mutations in *IDH1*, *TP53*, *CCND3*, and *GNAS* concordant to those seen in the plasma, but at higher VAF (patient 2, Supplementary Figure 2). For reference, patients 3, 8, and 9 are also shown in Supplementary Figures 3–5.

Genomic Evolution in Plasma ctDNA

The most frequent mutations detected in plasma included known glioma-associated receptor tyrosine kinase pathway genes, such as *EGFR*, *MET*, *KDR*, *PDGFR-alpha*, *RET*, and *KIT* (Figure 1A). Mutations in cell cycle pathway genes, eg, *CCND1/3*, *TP53*, and *RB1* were also frequently observed, whereas mutations in the PI3-kinase pathway including *PTEN*, *mTOR*, and *PIK3R* were seen less often. We observed *IDH1* mutations in plasma (and CSF) in 1 of the 2 patients with *IDH1*-mutated gliomas. The *IDH1*-mutant patient (patient 1, Supplementary Figure 1) in whom *IDH1mut* was not detected in plasma was a WHO grade 2 oligodendroglioma who had a very low concentration of cfDNA and only a few ctDNA mutations found overall, possibly explaining why *IDH1mut* was not detected in the circulation. Mutation in the *TERT* promoter (*TERTp*) region was found in the plasma of only 1/8 GBMs, despite being present in the tissue in 7/10 tumors. This reflects the known challenges with TERTp sequencing due to the absence of nucleosomes within the promoter region that normally protects the mutation from enzymatic cleavage. This results in shorter DNA fragments (<70 base pairs), which is disadvantageous for detection by sequencing.[6]

Several other ctDNA mutations not frequently reported in glioma were found in the plasma in our cohort. Mutations in the *Discoidin Domain Receptor Tyrosine Kinase 2* (*DDR2*) gene were found in 16%, *Patched 1 gene (PTCH1)* in 14% and *SMAD4* in 10% of plasma samples. Missense mutations in *SMAD4* were observed in 30% (patients 4, 5, and 7) and found almost exclusively at progression following treatment with radiotherapy and TMZ (Figure 2A, B, and D). In all 3 patients, the mutation was not detected until the third follow-up blood sample, 5–14 months after diagnosis. Interestingly, no patients had *SMAD4* mutations identified in tissue sequencing. *SMAD4* is a transcription regulator of the *TGF*β pathway and may be associated with epithelial to mesenchymal transition in glioma progression.[17] Increased levels of TGFβ have been correlated with higher tumor grade and worse prognosis in gliomas and have been suggested as a therapeutic target.[18]

As reported in studies analyzing paired tissue sequencing of glioma patients,[19,20] we observed the evolution of ctDNA mutations over time and in response to treatment. In several patients, new driver mutations

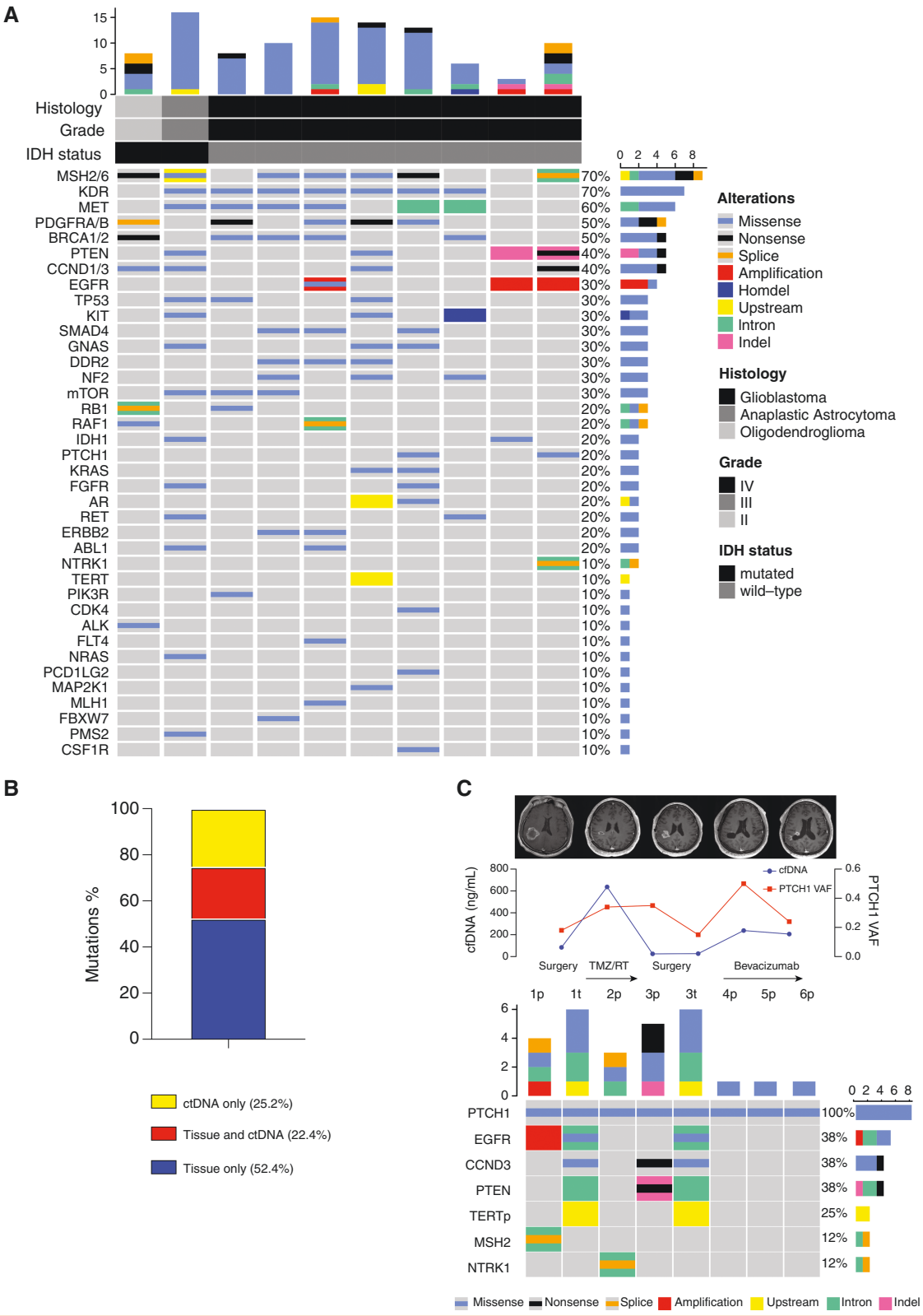

Figure 1. (A) Oncoprint demonstrating mutations identified in at least 1 plasma sample of 10 patients with glioma. Shown are the common genetic alterations (SNVs, single-nucleotide variants; indels, insertions, and deletions; CNAs, copy number alterations; SVs, structural variants). (B) Bar graph showing concordance of mutations between the plasma ctDNA and tumor tissue for the 77-genes included on the AVENIO panel in the 10 patients. (C) Diagram of patient 10 showing representative MR images in the top panel, cfDNA concentration in plasma (blue line), and VAF (red) of key gene mutation over time below. Main panel shows tumor (t) and plasma (p) mutations across longitudinal samples. Timing of surgery and adjuvant therapies listed above the main panel. Mutation types are shown in legend. Detected gene mutations are listed on the left of the diagram.

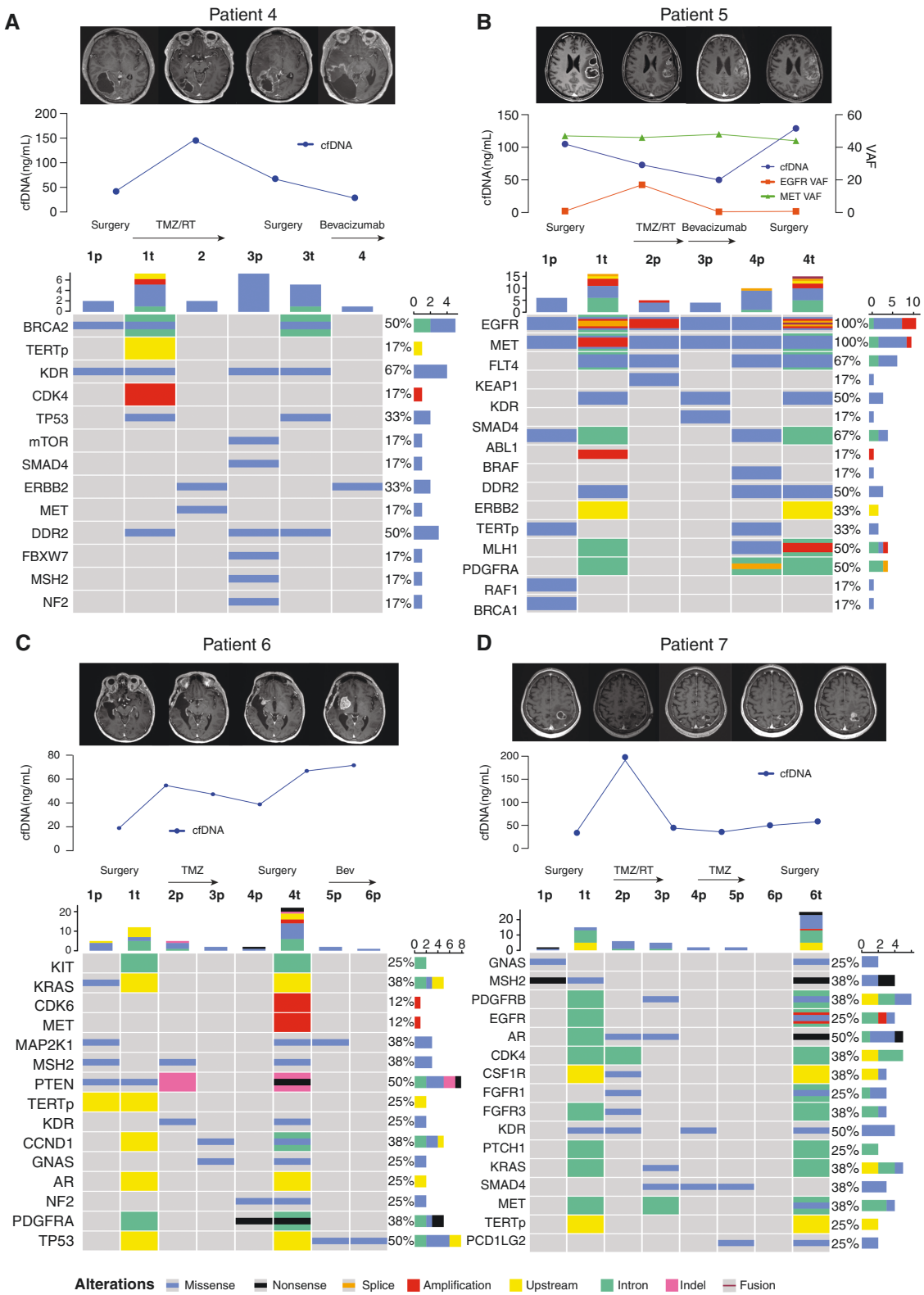

Figure 2. Diagrammatic overview for individual patients: (A) patient 4, (B) patient 5, (C) patient 6, (D) patient 7, showing representative MR images in the top panel, cfDNA concentration in plasma (blue line) and VAF (red) of key gene mutation over time below. Main panel shows tumor (t) and plasma (p) mutations across longitudinal samples. Timing of surgery and adjuvant therapies listed above the main panel. Mutation types are shown in legend. Detected gene mutations are listed on the left of the diagram.

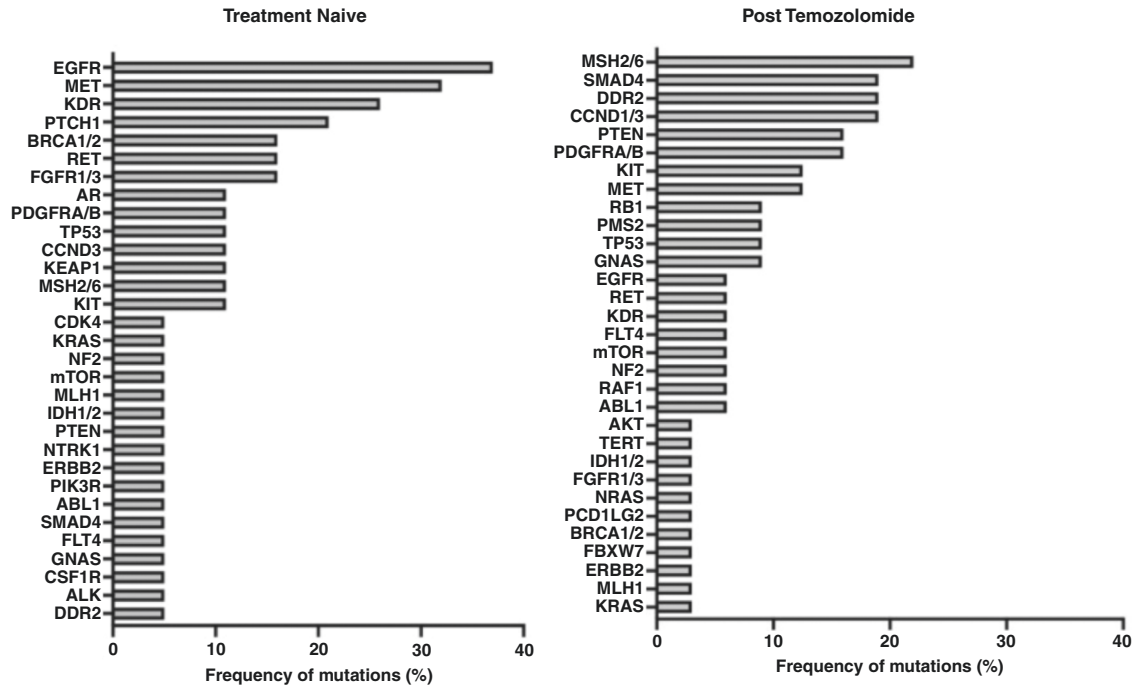

Figure 3. Comparison of mutations detected in plasma in treatment naïve and post-temozolomide groups. Left, bar graph showing frequency of mutations in the treatment naïve and right, patients who had been previously treated with temozolomide.

were detected in plasma prior to detection in tumor tissue at progression. For example, in patient 6, mutations in *CCND1*, *KIT*, and *PDGFRA* were seen in later plasma samples, but were not present in plasma from initial surgery (Figure 2C). Subsequently, all 3 mutations appeared in the tumor tissue at second surgery. Other examples include *FGFR1* and *PDGFRB* missense mutations found in patient 7 (Figure 2D) and a missense mutation in *CCND3* in patient 2 (Supplementary Figure 2).

Mismatch Repair Gene ctDNA Mutations

Following surgery, almost all patients with glioma receive radiation together with alkylator chemotherapy with temozolomide (TMZ). TMZ is more effective in tumors harboring methylation of the *O^6-methylguanine-DNA methyltransferase (MGMT)* gene, which prevents cancer cells from repairing DNA damage.[21] Additionally, mismatch repair deficiency (MMRd) due to acquired mutations in MMR genes such as *MutS protein homologue 2* (*MSH2*), *MutS homologue 6* (*MSH6*), *Mut-L homologue 1* (*MLH1*), and *PMS1 homologue 2 (PMS2),* is another mechanism by which gliomas develop TMZ resistance.[22–24] Even small reductions of *MSH2/6* activity result in markedly reduced TMZ sensitivity in vitro.[25,26] Unlike MGMT methylation, which remains stable throughout glioma progression, mutations in *MSH2* and *MSH6* occur more frequently in recurrent GBM after TMZ treatment.[24,27,28]

To evaluate the impact of TMZ on ctDNA in our cohort we compared plasma samples taken before and after TMZ treatment. *EGFR*, *MET*, and *KDR* mutations were the most common mutations pre-treatment, whereas *MSH2/6*, *SMAD4*, *DDR2*, and *CCND1/3* were significantly enriched following TMZ (Figure 3). We observed mutations in MMR genes including *MSH2* or *MSH6* (20%), *PMS2* (6%), and *MLH1* (4%), in 70% of patients. There were no MMR gene mutations in the PBMC sequencing analysis, suggesting that these MMR mutations identified in circulation were derived directly from the glioma.

The timing of observed MMR mutations with respect to treatment is shown in Supplementary Figure 7. There were 4 patients in whom *MSH2* mutations were detected *after* treatment with TMZ: In Patient 1, a stop gained mutation (p.Arg389*) was identified in the final blood sample 6 months after TMZ was commenced (Supplementary Figure 1), and in patient 4 a missense mutation (p.Ser281Leu) was observed in the third plasma sample, 7 months following TMZ commencement (Figure 2A). Two further patients, who were treated with TMZ before study enrollment, had MMR mutations in their initial plasma samples. A *PMS2* missense mutation (p.Ser457Gly) was observed in 3 out of 4 plasma samples in patient 2, as was a missense and upstream gene variant in *MSH6* (Supplementary Figure 2). Patient 6 had adjuvant TMZ 12 months prior to inclusion in our study after which a missense mutation in the *MSH2* gene (p.Ser284Phe) was detected in the first and second plasma samples but not in the tumor tissue at that time (Figure 2C). The patient was re-challenged with TMZ but subsequently had early progression, consistent with acquired MMRd driving TMZ resistance. Two other patients (patients 7 & 10) were noted to have *MSH2* mutations prior to receiving any TMZ (Figures 2D and 1C), possibly representing sporadic MMR deficiency.[22] Both of these patients

had early tumor progression following completion of combined TMZ and RT, with patient 10 proceeding to further surgery and then change of therapy to bevacizumab. Patient 5 had an MLH1 mutation in both initial and subsequent plasma samples and progressed rapidly on TMZ (Figure 2B).

Hypermutation

In the presence of MMRd, ongoing treatment with TMZ can result in hypermutation with large numbers of G:C > A:T transitions.[19,29] Hypermutation occurs in approximately 2% of newly diagnosed GBMs and 16.6% of progressive tumors and occurs exclusively in those treated with TMZ and those with *MGMT* promoter methylation.[22] Based on tissue sequencing, we observed 2 patients (20%) with hypermutation at progression following TMZ in our cohort, defined as a tumor mutation burden (TMB) greater than 10 mutations/Mb.[22] Patient 7 had an *MSH2* stop gain mutation (p.Gln288*) present in plasma (but not tumor) at the time of initial surgery and was then treated with TMZ and subsequently became hypermutated (TMB 124 at progression vs 3.1 at initial biopsy) (Figure 2D). The same *MSH2* mutation was detected in both the recurrent tumor and plasma. Patient 6 was also noted to be hypermutated (TMB 288.1 vs. 4.7) at progression after 12 months of TMZ therapy and was then treated with bevacizumab (Figure 2B). Both patients 6 and 7 had *MGMT* promoter methylation. Despite having a *PMS2* and *MSH6* mutation, patient 2 did not develop hypermutation at progression, possibly because they received bevacizumab rather than TMZ (Supplementary Figure 2). Interestingly, patient 10 also did not develop hypermutation, likely due to *MGMT* being unmethylated (Figure 1C).

Discussion

Liquid biopsy with ctDNA has emerged as a powerful minimally invasive tool to monitor response, minimal residual disease status, resistance mechanisms and to identify target gene mutations in cancer. The promising utility of CAPP-seq (AVENIO) to analyze ctDNA has previously been reported in colorectal, lung, and ovarian cancer and melanoma but our study is the first to report its use in glioma. Here we demonstrate the sensitivity of plasma ctDNA for diagnosis and monitoring of glioma genomic evolution. Plasma ctDNA mutations were frequently seen prior to their appearance in tissue biopsy, underscoring the advantages of liquid biopsy, which can be performed minimally invasively at multiple timepoints. Previous literature on ctDNA in glioma reported a sensitivity of 50% or less,[7,14,15,30] however, these studies were all based on a single plasma sample, whereas in our study we analyzed multiple samples per patient. Two further factors in our study likely contributed to the improved detection rate: First, we used 2 to 4-fold larger volumes of plasma than previously reported and processed them rapidly, avoiding DNA degradation. Secondly, the AVENIO platform is optimized for liquid biopsy, especially with low levels of input DNA, through the design of cancer specific DNA "selectors," that target recurrently mutated regions in the cancer of interest.[31,32]

The concordance we observed between plasma and tissue mutations was not as high as in other cancer types[2,33,34] and lower than reported between glioma CSF and glioma tissue,[7] however, it is substantially better than has been reported with other assays, where no common mutations were found between plasma and tumor.[15] Furthermore, we found that 25% of all mutations observed, including key glioma drivers such as *IDH1* mutation, *EGFR* amplification, and *PTEN* alterations, were detected only in plasma ctDNA and not in tissue. Similar results were seen in a recent publication where the majority of potentially pathogenic mutations in plasma ctDNA were not found in tumor DNA.[35] The marked intra-tumor heterogeneity and subclonal architecture in glioblastoma,[36] and the necessity for piecemeal surgical biopsies, are all factors that can lead to tissue sampling being under-representative in glioma.[37] In this context, plasma ctDNA analysis could provide valuable additional genomic information for surgical biopsy. Given its specificity, the positive predictive value is high but a negative ctDNA result at any one time-point is weak at ruling out the presence of a tumor mutation, especially given the relative low concentration of plasma ctDNA in glioma, compared to other types of cancer. Data from future clinical trials with larger patient numbers will be necessary to determine whether liquid biopsy could delay or obviate the need for surgical biopsy at either diagnosis or progression.

Importantly, we also showed that plasma ctDNA can detect mutations in mismatch repair genes, a marker of TMZ resistance. Loss of MMR expression in glioma tissue can be diagnosed with a sensitivity of 89% by immunohistochemistry,[38] however, MMRd is rarely present in the initial tumor biopsy and surgery is only performed in ~20% of GBM at progression.[39] Therefore, liquid biopsies during the treatment and monitoring phase to detect MMRd, could facilitate early determination of TMZ resistance and initiate a change of therapy, thereby avoiding the risk of hypermutation with ongoing TMZ exposure. In other cancer types such as colorectal cancer, MMRd and hypermutation are associated with improved prognosis and response to immunotherapies such as PD-1 checkpoint inhibitors.[40] In glioblastoma, however, data suggests that hypermutation indicates a worse prognosis and that there is no or little response to PD-1 inhibitors.[22] Treatment-induced glioma hypermutation has also been associated with progression to higher grade and worse prognosis.[16] If MMRd is detected by liquid biopsy, alternative therapies may be trialed to avoid resistance to TMZ and subsequent hypermutation, such as bevacizumab or lomustine, a drug that has shown promise for *MGMT*-methylated patients who develop resistance to TMZ.[22,41] Another potential strategy to overcome MMRd-mediated TMZ resistance might be the addition of poly (ADP-Ribose) polymerase inhibitors (PARPi), which have demonstrated in vitro activity in *MSH6* deficient glioma cells.[42]

Taken together, our results show that, contrary to previous reports in glioma, ctDNA can be sensitively detected in plasma using multiple longitudinal blood draws, appropriate volumes of plasma, and optimized sequencing

techniques. A significant proportion of mutations may be detected in ctDNA liquid biopsy that are not found in tissue sequencing, a finding that has important translational implications for glioma diagnosis. Finally, mismatch repair gene mutations associated with TMZ resistance can be detected in blood, suggesting that resistance could be identified earlier without the need for repeat surgical biopsy, and in time to guide treatment decisions such as stopping temozolomide therapy and switching to another modality. Accurate assessment of the glioma DNA mutation profile both at diagnosis and during the monitoring phase, might also be able to be used to plan future targeted molecular therapies or immunotherapies. The major limitation of the study is the small sample size, thus validation in larger cohorts is needed before the incorporation of plasma ctDNA liquid biopsy into clinical trials.[33]

Supplementary Material

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

Keywords

biomarkers | ctDNA | glioblastoma | glioma | liquid biopsy

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Conflict of interest statement

The authors declare no potential conflicts of interest.

Authorship statement

J.J.J, A.M., F.G., A.H.K., and K.J.D. conceived and designed the study. J.J.J., A.M., K.J.D., J.T., T.P., and F.G., collected and assembled the data. J.J.J., A.M., K.L.J., S.W., K.J.D., and J.W. analyzed and interpreted the data. S.W., K.L.J, and D.G. were responsible for sequencing and genomic data. A.M., K.J.D., and 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., and T.P. processed the CSF and blood samples. J.J.J., A.M., K.J.D., J.W., and A.H.K. wrote the manuscript. All authors approved the manuscript. Data and code availability statement. All data and code are available on request.
Correspondence and requests for materials.

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