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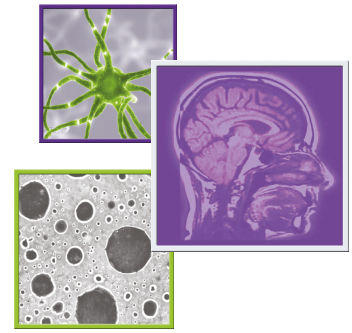

Analysis of cell-free circulating tumor DNA in 419 patients with glioblastoma and other primary brain tumors

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Aim: Genomically matched trials in primary brain tumors (PBTs) require recent tumor sequencing. We evaluated whether circulating tumor DNA (ctDNA) could facilitate genomic interrogation in these patients. **Methods:** Data from 419 PBT patients tested clinically with a ctDNA NGS panel at a CLIA-certified laboratory were analyzed. **Results:** A total of 211 patients (50%) had ≥1 somatic alteration detected. Detection was highest in meningioma (59%) and gliobastoma (55%). Single nucleotide variants were detected in 61 genes, with amplifications detected in *ERBB2, MET, EGFR* and others. **Conclusion:** Contrary to previous studies with very low yields, we found half of PBT patients had detectable ctDNA with genomically targetable off-label or clinical trial options for almost 50%. For those PBT patients with detectable ctDNA, plasma cfDNA genomic analysis is a clinically viable option for identifying genomically driven therapy options.

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Keywords: cell-free DNA • ctDNA • genomic profiling • glioblastoma • Guardant360 • liquid biopsy • personalized medicine • primary brain tumors

Glioblastoma multiforme (GBM), a type of glioma, is the most aggressive type of primary brain tumor (PBT), with limited therapy options and a median survival of 12–15 months [1]. Comprehensive molecular profiling of PBTs can inform more detailed biological classification beyond traditional histopathology [2,3]. Development of therapies directed at molecular targets in gliomas and other PBTs is underway and holds promise as an improvement over current standard therapies [3–5]. However, trials of genomically matched therapies for brain tumors require next-generation sequencing (NGS) of a recent tissue sample, thus limiting progress; tissue requirements also limit the ability to identify and track mutation clonality and clonal evolution of tumors [6–8] and may miss important heterogeneous genomic events [9]. Additionally, recurrent glioblastomas are rapidly growing tumors, and obtaining a biopsy in order to complete molecular profiling is a time-consuming step.

Genomic profiling utilizing tissue samples obtained from invasive biopsy may not always be clinically feasible and is not without risk of morbidity or mortality [10,11]. Additionally, tissue biopsies may be found to have insufficient quantity or quality of material for NGS profiling. Even when tissue sampling is feasible and sufficient for genomic analysis, tissue-based NGS may fail to capture a complete picture of the cancer's genetic profile due to intra- and inter-tumor heterogeneity [8,12–15].

Recently, assays analyzing cell-free DNA (cfDNA) have become commercially available. These tests present an opportunity to genomically profiled patients' tumors through a plasma sample without the need for an invasive tissue biopsy. cfDNA contains fragments of circulating tumor DNA (ctDNA) released into circulation through

apoptosis and/or active DNA release [16,17]. Given the short 2-h half-life of plasma cfDNA fragments in circulation and the ability to capture heterogeneity across multiple areas of a tumor, this technology provides an opportunity to assess cancer genomic signatures in real-time [18–20].

A prior study of plasma ctDNA yield across a variety of solid tumor types identified ctDNA alterations in less than 10% of patients with glioma [21]. The authors hypothesized that the blood–brain barrier is a physical obstacle preventing ctDNA from reaching peripheral circulation, suggesting limited clinical utility of such technology in this cancer type. A recent study utilizing a comprehensive ctDNA analysis yielded a 51% cfDNA detection rate in patients with advanced primary glioblastoma [22] suggesting that ctDNA detection rate in primary brain tumors may vary by assay performance and/or histopathology and grade. We sought to evaluate the ability of a highly sensitive and specific cfDNA NGS assay to identify genomic alterations in patients with GBM and other PBTs, to further characterize ctDNA yield by histopathologic features, and to begin to explore the spectrum of genomic alterations identified in cfDNA in this clinically tested patient population.

Patients & methods

From October 2014 through to December 2017, 665 samples from 419 consecutive patients with PBTs had clinical samples tested in real time with the Guardant360® cfDNA digital sequencing (NGS) assay (Guardant Health, CA, USA); whole blood was collected in Streck tubes, sent to the laboratory and processed as previously described [22–24]. Cases were retrospectively identified via query of the Guardant360 de-identified database of clinical orders for patients with a diagnosis of GBM or other PBTs as indicated on the test request form completed by the ordering provider. 93 patients had more than one cfDNA test result available, as multiple blood draws were performed for tests ordered clinically at multiple timepoints. Analysis was completed under a Quorum Review Institutional Review Board protocol for deidentified and limited datasets which waived the need for individual patient informed consent.

The Guardant360 assay is a laboratory test commercially available for all advanced solid tumors; therefore, the genes interrogated by this assay were not specifically selected with primary brain cancers in mind but rather encompass genomic alterations commonly observed across the spectrum of advanced cancer. The assay composition was expanded over the course of the study. 65 samples were analyzed with the original 54-gene version including comprehensive sequencing analysis of all exons in 18 genes, critical exon (those known to harbor somatic mutations) sequencing analysis of 36 genes and copy number amplification (CNA) analysis of three genes (*EGFR, ERBB2, MET*). An additional 199 samples were evaluated with an expanded 68-gene panel, 219 with a further expanded 70-gene panel and 182 with a 73-gene panel, each including additional exons sequenced, CNAs and select fusion events assessed (Supplementary Figures 1–4). Of note, reported alterations include only those which can be assessed through NGS of fragmented cfDNA; for example, the *EGFR* vIII mutation, large deletions including 1p,19q and epigenetic alterations including *MGMT* methylation, were not detectable alterations in any of these assay versions.

Single nucleotide variants (SNVs), fusions and insertions/deletions (indels) were reported quantitatively as the variant allele fraction (VAF) in cfDNA. CNAs were reported as absolute copy number of the target gene in plasma. The reportable range for SNVs, indels, fusions and CNA on the current 73-gene Guardant360 assay is $\geq$0.04%, $\geq$0.02%, $\geq$0.04% and $\geq$2.12 copies in plasma, respectively [23,24].

Results

The average patient age at the time of first blood collection was 52 years (range 3–88) and 62% were male. Histopathological subtypes included GBM, astrocytoma, oligoastrocytoma, oligodendroglioma, glioma (not otherwise specified [NOS]), medulloblastoma, meningioma and ependymoma (Table 1), with GBM being the most commonly reported diagnosis in the cohort (53%). Tumor types were classified for this study in accordance with the 2016 World Health Organization Classification of Tumors of the Central Nervous System [25].

Overall, somatic alterations were detected in 302 samples (45.4%). When accounting for serial testing, somatic alterations were detected in at least one sample per unique patient in 211 patients (50.4%). Of samples with at least one alteration, the median VAF was 0.33% (range 0.05–41.01%) with an average of 2.14 (range 1–29) alterations identified per sample (Table 2) [22].

Out of the better represented histologic subtypes in this cohort (n $\geq$ 15 samples), cfDNA alterations were detected most frequently in patients with meningiomas (59%), followed by GBM (55%), glioma NOS (49%), anaplastic astrocytoma (43%), oligodendroglioma NOS (33%) and astrocytoma NOS (19%), as shown in Figure 1. As would be expected, cfDNA alteration detection rate increased with increasing grade in patients with astrocytic

Table 1. Patient demographics and histologic subtypes for the 419 unique patients in this cohort.

Histopathology	Number of patients	Percentage of cohort
Astrocytic and oligodendroglial tumors		
– Astrocytoma, anaplastic	21	5.0%
– Astrocytoma, diffuse	2	0.5%
– Astrocytoma, pilocytic	4	1.0%
– Astrocytoma NOS	16	3.8%
– Glioblastoma	222	53.0%
– Oligoastrocytoma NOS	6	1.4%
– Oligodendroglioma, anaplastic	3	0.7%
– Oligodendroglioma NOS	15	3.6%
– Glioma NOS	81	19.3%
Neuronal and mixed neuronal-glial tumors		
– Ganglioglioma	1	0.2%
Ependymal tumors		
– Ependymoma, anaplastic	1	0.2%
– Subependymoma	2	0.5%
– Ependymoma NOS	5	1.2%
Embryonal tumors		
– Medulloblastoma NOS	6	1.4%
Meningiomas		
– Meningioma NOS	34	8.1%
Grade		
Astrocytic and oligodendroglial tumors		
– Grade 1	5	1.4%
– Grade 2	25	6.8%
– Grade 3	35	9.5%
– Grade 4	222	60.0%
– Grade unknown	83	22.4%
Meningiomas		
– Grade 1	4	1.4%
– Grade 2	6	1.4%
– Grade 3	1	5.3%
– Grade unknown	23	5.5%
Gender		
– Males	259	62%
– Females	160	38%
Age: average (range), years	52 (3–88)	

Table 2. Median circulating tumor DNA concentration and average number of alterations detected among samples with cell-free DNA detected obtained from patients with primary brain cancer and among all commercial samples (pan-cancer) previously reported.

	Primary brain series (n = 302 samples)	Guardant360 all cancers (n = 17,628 samples)
Median VAF (range)	0.33% (0.05–41.01%)	0.4% (0.03–97.3%)
Median number of alterations detected per sample (range)	2.14 (1–29)	4.2 (1–166)

Samples from patients with primary brain cancer have lower ctDNA variant allele fraction and fewer alterations than patients with nonbrain solid tumor malignancies.
VAF: Variant allele fraction.

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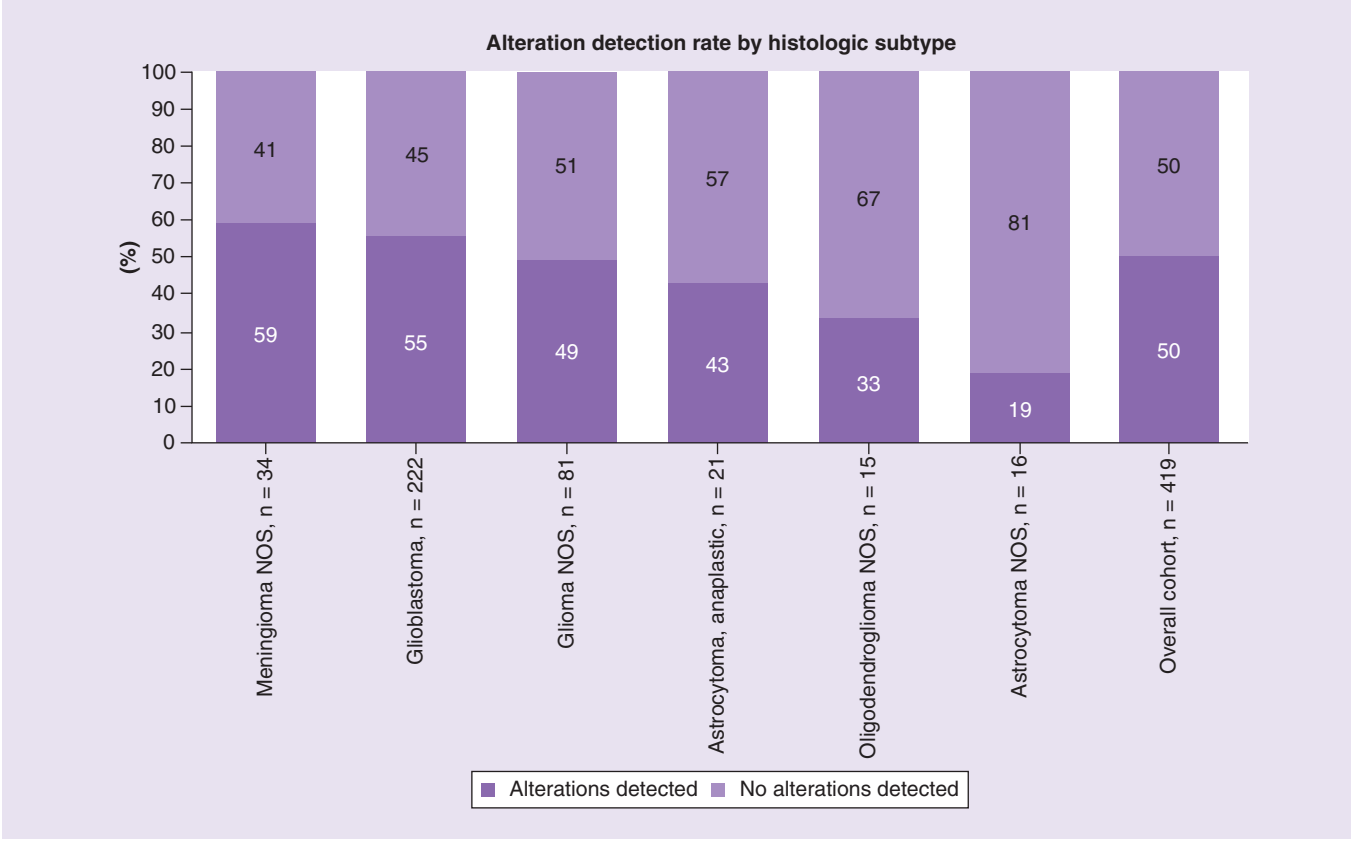

Figure 1. Alteration detection rate by subtype. ctDNA alteration detection rate per patient stratified by histologic subtype. Alteration detection rates for histologic subtype groups with n < 15 not pictured: ganglioglioma, n = 1 (100%); astrocytoma, diffuse, n = 1 (50%); oligoastrocytoma NOS, n = 6 (50%); subependymoma, n = 2 (50%); ependymoma NOS, n = 5 (40%); oligodendroglioma, anaplastic, n = 3 (33%); astrocytoma, pilocytic, n = 4 (25%); medulloblastoma NOS, n = 6 (17%); ependymoma, anaplastic, n = 1 (0%).

and oligodendroglial tumors (AOT): from 20 to 28% to 40 to 55% for AOT Grades 1–4, respectively (Figure 2; p = 0.014). Given the relatively small number of samples with known grades in other histologic groups, this trend was difficult to assess in additional tumor types.

Across all 211 patients with alterations detected at any timepoint, 550 somatic alterations were detected in 61 unique genes (Figure 3A), most frequently in *TP53*. Excluding variants of uncertain significance (VUS, 49% of identified variants) and synonymous alterations (18% of identified variants), characterized point mutations were most commonly seen in *TP53* (n = 79) followed by *JAK2* (n = 10), *NF1* (n = 7), *EGFR* (n = 7), *BRAF* (n = 6), *IDH1* (n = 5), *NRAS* (n = 5), *GNAS* (n = 5) and *ATM* (n = 4). Focusing on GBM, the most commonly altered genes were similar to the overall analysis (Figure 3B), as expected given its strong representation within the total patient cohort.

Multiple alterations potentially relevant to therapeutic targets were identified in several of these commonly altered genes. Recurrent characterized point mutations were detected in *IDH1* (R132H/C/S/G), all identified within the AOT subgroup. The identified characterized *BRAF* point mutations included V600E, an activating mutation common in melanoma and other cancer types (observed in two patients); N581S, an activating mutation in the protein kinase domain (observed in two patients); Q257R, an activating mutation in the cysteine-rich domain of conserved region 1; and R354*, an inactivating mutation predicted to result in loss of the protein kinase domain. All *BRAF* alterations were observed within the AOT subgroup as well.

Characterized *BRCA1* mutations identified were Q380* in a patient with GBM and R1835* in a patient with glioma NOS, expected to result in loss of both BRCT domains and a portion of the C-terminal BRCT domain, respectively. Both of these alterations were observed at VAFs <1%, consistent with somatic, rather than germline, origin. *EGFR* characterized point mutations detected were E142*, S177*, A289V (observed in two patients), R309* and R831H; these mutations occur in multiple domains including extracellular and protein kinase domains,

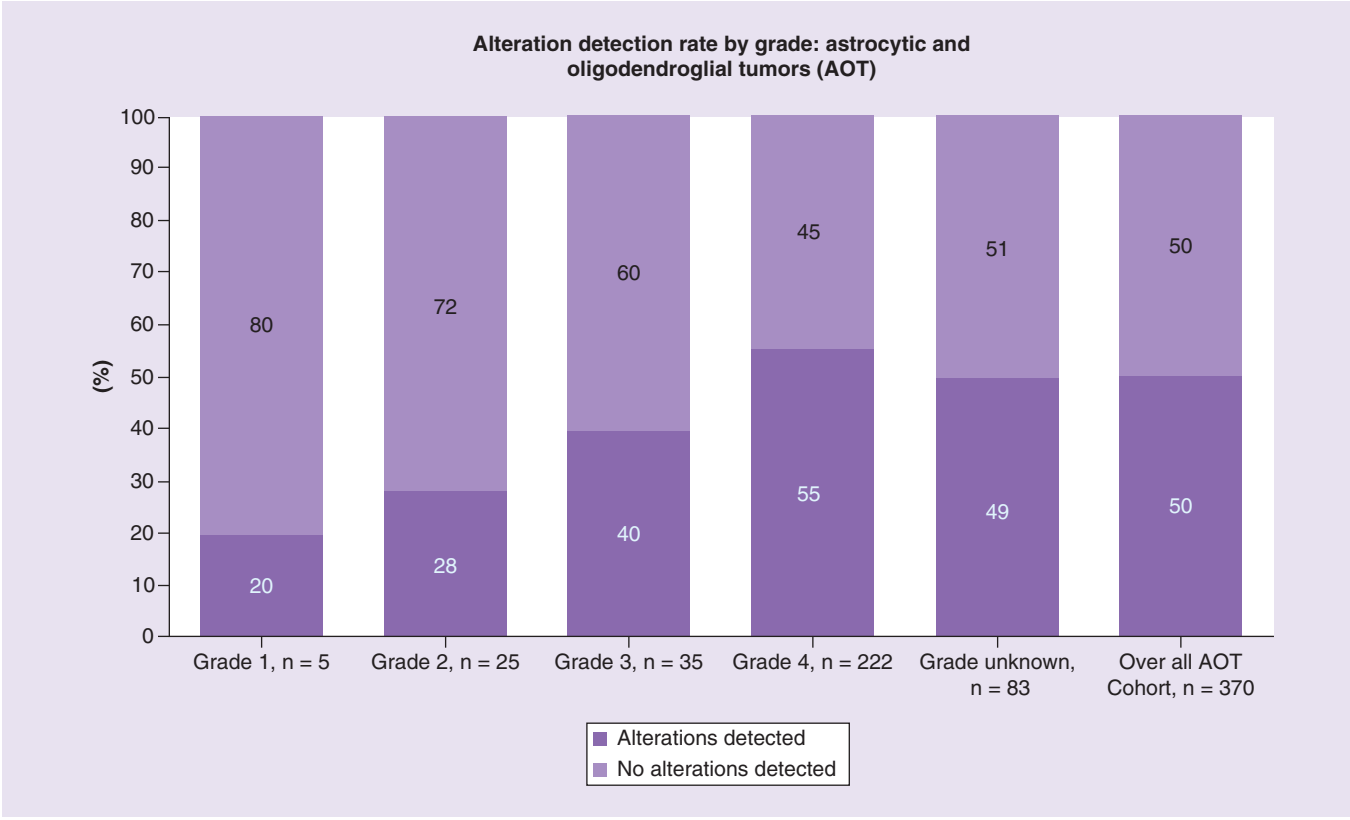

Figure 2. Alteration detection rate by grade: astrocytic and oligodendroglial tumors. ctDNA alteration detection rate per patient stratified by grade, when known. Histologic subtypes included in this cohort include astrocytoma (anaplastic, diffuse, pilocytic and NOS), glioblastoma, oligoastrocytoma NOS, oligodendroglioma (anaplastic and NOS) and glioma NOS.
ctDNA: Circulating tumor DNA; NOS: Not otherwise specified.

and include both activating and inactivating mutations. These characterized *EGFR* mutations were observed primarily, but not exclusively, in the AOT subgroup (one inactivating mutation in a patient with meningioma NOS). Characterized point mutations in *ATM* in this cohort included K342* in a patient with meningioma NOS, R3008H (observed in two patients with GBM), and R3012* in a patient with meningioma NOS, all inactivating mutations.

Common activating mutations in *NRAS* were observed in the AOT subgroup only, including G12D, G13R, Q61K and Q61R. Point mutations were identified throughout the *TP53* gene across the cohort, including patients with AOT, meningioma and medulloblastoma; inactivating mutations were detected in nonrecurrent locations in the *NF1* gene, primarily in AOT but also in meningioma (Figure 4) [26]. Characterized *TP53* mutations were most commonly observed in patients with Grade 4 tumors (n = 56, 5, 2, 2 and 18 in Grades 4, 3, 2, 1 and unknown, respectively); this may be related to inherent biology or the increased detection rate observed in higher grade tumors, or some combination of the two.

CNAs were observed in *ERBB2*/HER2 (4), *MET* (2), *KIT* (2), *BRAF* (1), *EGFR* (1), *CCND1* (1), *CCND2* (1), *CDK6* (1) and *PDGFRA* (1). The majority of these CNAs were identified in patients with GBM, as well as two in patients with glioma NOS and one in a patient with ependymoma NOS. Most CNAs were observed at low levels, consistent with the low overall VAF observed in most cases within the cohort.

Among patients with alterations detected, almost 50% (n = 101) had a potentially therapeutically targetable genomic alteration identified; 53 (25%) had an off-label treatment option identified and 98 (46%) had clinical trial options identified based on the genetic alterations observed, based on annotations in accordance with the published guidelines [27].

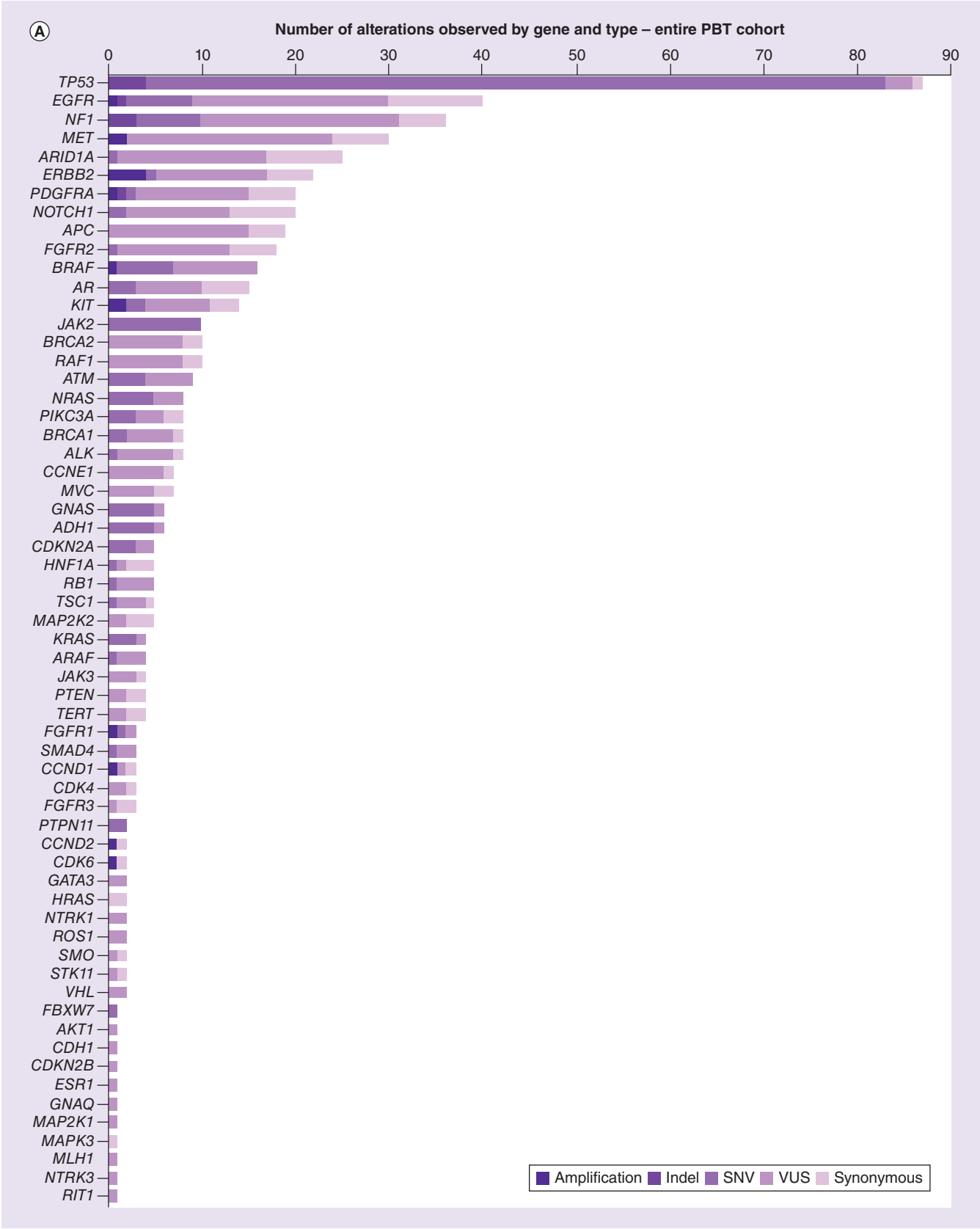

Figure 3. Number of alterations observed by gene and type. Number of alterations in each gene identified stratified by alteration type, including copy number amplifications, characterized indels, characterized point mutations, variants of uncertain significance (VUS) and synonymous (silent) alterations, among **(A)** the 211 patients with alterations detected across all histologic subtypes, and **(B)** the 123 patients with alterations detected with glioblastoma.
SNV: Single nucleotide variant.

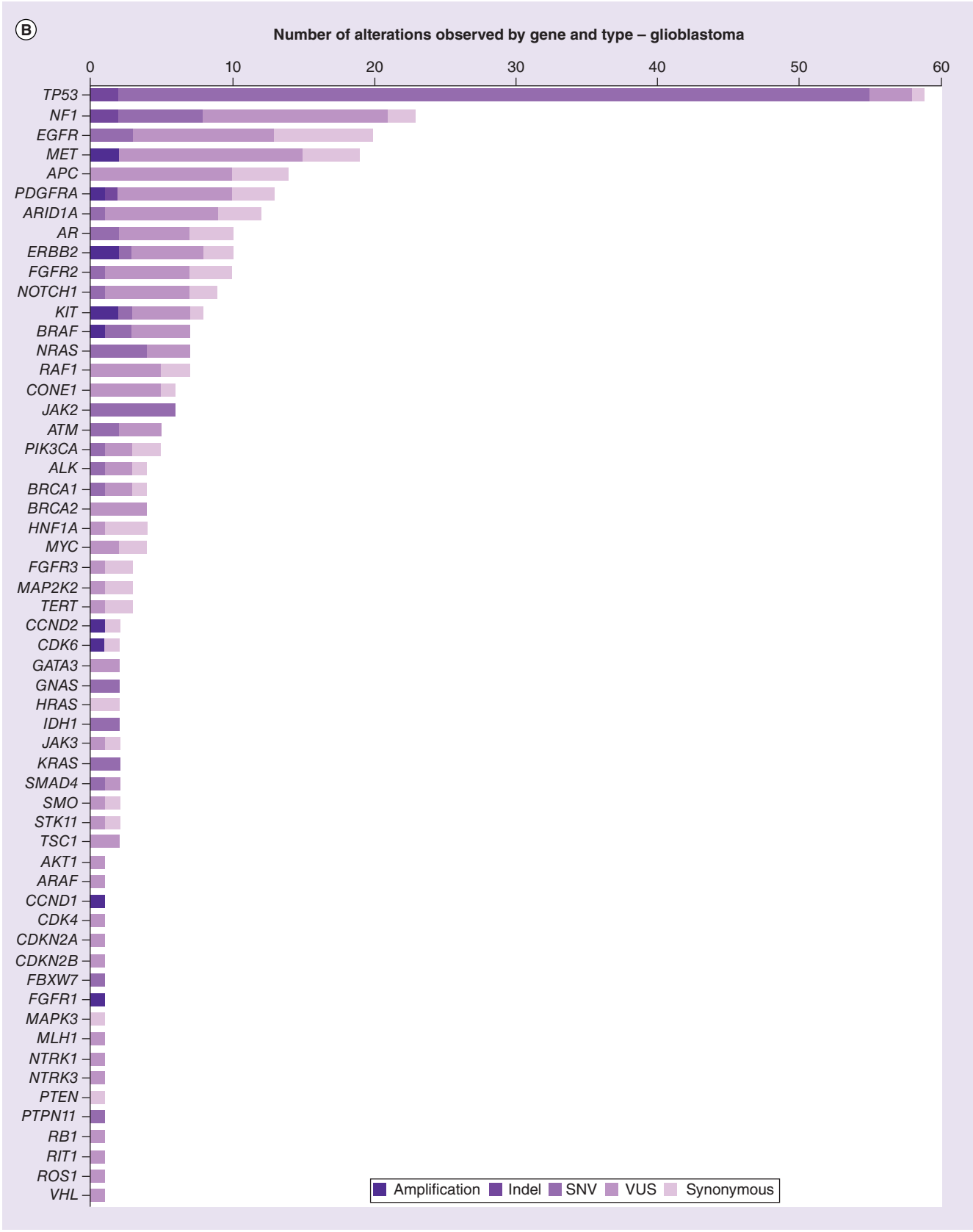

Figure 3. Number of alterations observed by gene and type (cont.). Number of alterations in each gene identified stratified by alteration type, including copy number amplifications, characterized indels, characterized point mutations, variants of uncertain significance (VUS) and synonymous (silent) alterations, among **(A)** the 211 patients with alterations detected across all histologic subtypes, and **(B)** the 123 patients with alterations detected with glioblastoma.
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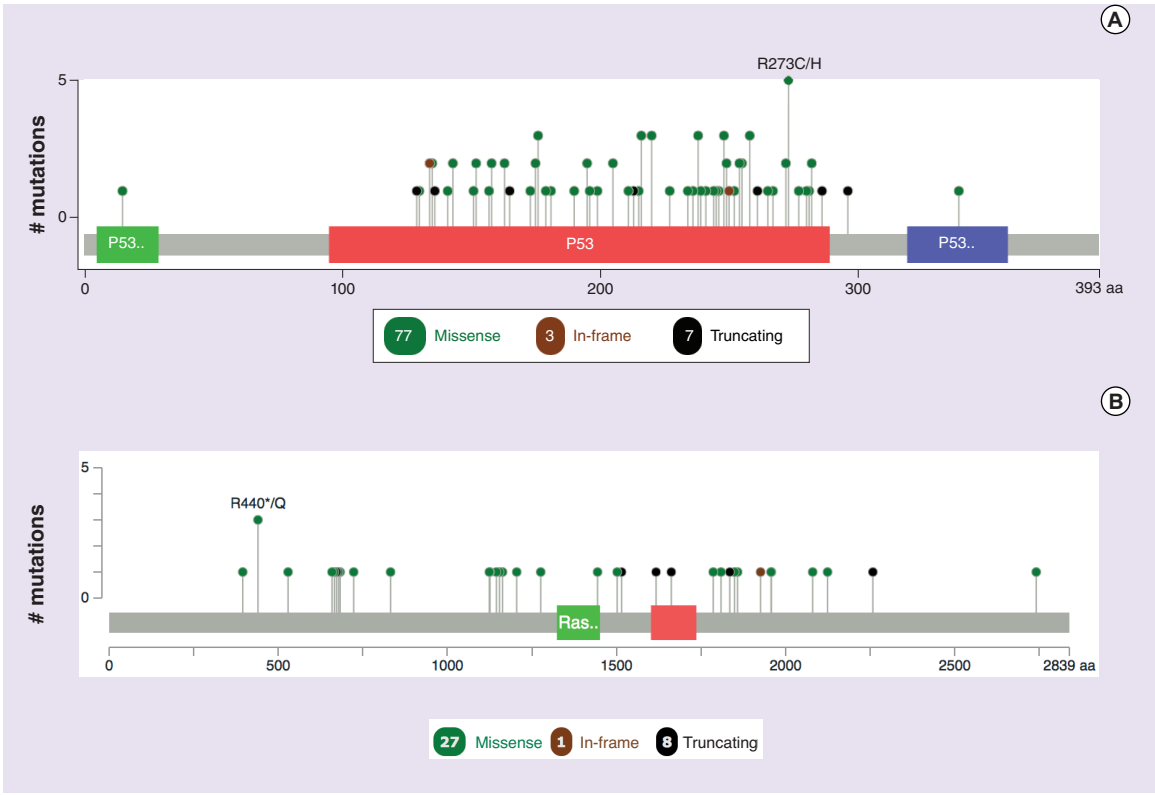

Figure 4. Characterized alterations identified across select genes. Locations of identified characterized alterations across the **(A)** *TP53* and **(B)** *NF1* genes shown in lollipop plots.

Discussion

Contrary to other cfDNA studies which postulated that ctDNA would not cross the blood–brain barrier to reach systemic circulation, we found that half of the patients with primary brain tumors had detectable cfDNA alterations with 48.9% of these having a potentially genomically targetable alteration identified.

Among patients with GBM, who comprised just over half of this cohort, ctDNA alterations were detected 55% of the time. This suggests that cfDNA analysis for GBM genomic profiling may be appropriate to consider prior to an invasive biopsy (performed solely to obtain tissue for genomic testing) and in patients for whom an invasive biopsy is not feasible or who decline. Alterations were detected even more frequently in patients with meningioma, which is consistent with the absence of the blood–brain barrier present in other subtypes of primary brain cancer [28].

With an average VAF of 0.33% and a minimum VAF of 0.05% in this cohort, this study underscores the importance of utilizing a cfDNA assay with high sensitivity for detection of low-level alterations. As seen in Table 2, the number of alterations and cfDNA VAF were both lower in this primary brain tumor cohort compared with a cohort of all solid tumors undergoing this cfDNA assay. The mechanisms that influence the release of tumor DNA into the bloodstream are not entirely understood, and it is possible that the blood–brain barrier may limit the amount of ctDNA able to enter peripheral circulation from a primary brain tumor. The low VAFs observed in this study suggest that technical assay performance is of particular importance when selecting a commercial cfDNA platform for clinical use in this patient population in order to increase the likelihood of identifying these low-level alterations.

This study demonstrates a higher ctDNA alteration yield in patients with primary brain tumors than previously reported. Additionally, one quarter of samples had a ctDNA alteration detected that suggested eligibility for an off-label targeted therapy regimen. Almost half of patients had a ctDNA alteration detected that suggested eligiblity for a targeted therapy clinical trial. This study suggests that the identification of genomic alterations in the cfDNA of patients with primary brain tumors is feasible. This is promising for the continued development and execution of clinical trials of targeted therapies in this patient population, as the ease, convenience and safety of plasma cfDNA

sampling has the potential to make genomic profiling a possibility when tissue is unavailable or unobtainable in the setting of advanced PBT.

Some of the alterations identified in this patient cohort do show potential for molecular targeted therapeutics, including *BRAF*/*IDH1*/*IDH2* mutations, *ERBB2*/*MET*/*EGFR*/*PDGFRA* amplifications and mutations in DNA damage repair genes. For example, at the time of submission, trials using targeted therapies related to genes and pathways described in detail above (e.g., inhibition of RAF/MEK, EGFR and PARP, among others) were available in PBTs. The option to detect these and other genomic alterations through cfDNA analysis may improve access to clinical trials investigating the use of these agents in the setting of primary brain tumors.

As described above, the exploratory analysis presented here utilizes data from an assay commercially available across solid tumor types. Therefore, it is promising that the yield of clinically relevant genomic alterations using a liquid biopsy approach could be even higher from an assay specifically designed with PBTs in mind. However, this may introduce practical challenges, for example, the difficulty of implementing parallel epigenomic and RNA-based methodologies to assess methylation and splice variants, respectively. Additionally, the evolution of personalized medicine has seen multiple pancancer approval for drugs targeting specific biomarkers (e.g., pembrolizumab for MSI-high tumors, larotrectinib for tumors with *NTRK* fusions) and continued success applying targeted therapies from one cancer type to another (e.g., anti-HER2 therapy common in breast cancer showing efficacy in colorectal cancer, BRAF/MEK inhibition common in melanoma showing efficacy in lung adenocarcinoma). Trends such as these may support a broader, less PBT-specific approach to include identification of potential basket or umbrella drug trial targets. There has also been promising work done assessing cfDNA from cerebrospinal fluid [29,30], though this sample collection is still more invasive compared with peripheral blood draw. Future studies investigating ideal liquid biopsy assay composition and sample type may be warranted to further explore these questions [20].

It is important to note an underlying limitation of this study. As the cohort was based on samples submitted to a commercial laboratory, clinical information (including pathologic confirmation of diagnosis, or timing of cfDNA collection in relation to therapy regimen) was not available for all patients. Sample collection may have occurred at various clinical time points (e.g., baseline vs stable disease vs progression) which may have affected ctDNA alteration detection rates and VAF. The likelihood of identifying genomic alterations shed by the tumor in plasma cfDNA is highest prior to treatment and at times of progressive disease, rather than when patients are clinically stable or in active treatment when ctDNA release into the blood is suppressed. However, these clinical details are not available for this cohort from a commercial laboratory, as this information is not required for clinical testing.

This preliminary analysis was intended to focus on overall detection rate of ctDNA in patients with PBTs using an available retrospective dataset, and a breakdown by specific molecular alterations would result in too small of numbers to draw meaningful formal correlative conclusions in this preliminary descriptive analysis. An in-depth exploration of the specific alteration landscape would be best conducted in a cohort with samples collected at consistent and clinically appropriate timepoints (baseline active disease and/or progression) to maximize the likelihood of capturing the tumors' genomic signatures through cfDNA. However, the preliminary spectrum of mutated genes in this cfDNA cohort is similar to that of published data from The Cancer Genome Atlas (TCGA) genomic analysis of tissue, including *TP53, NF1, IDH1* and *EGFR* [31,32].

As this data is from clinical cfDNA analysis performed by a commercial laboratory that does not require detailed clinical data to order testing, genomic profiling results of corresponding tumor tissue for patients who may have had this analysis were not available for comparison in this patient cohort. Any potential discordance may be due to the disease stage, treatment history and clinical status of the patients in the current cfDNA cohort. TCGA recruited patients without any prior therapies, while the current cohort enrolled patients who may have been treatment-naive or previously treated. It is known that the spectrum of mutations observed in treatment-naive versus previously treated tumors differs due to tumor evolution following treatment. Other discrepancies in the results of the two cohorts may be related to sequencing coverage of the cfDNA assay (Supplementary Figures 1–4). For example, the cfDNA assay cannot assess for large deletions, including *EGFR* vIII, and the detection of amplifications in cfDNA analysis is dependent on the level of ctDNA shed being high enough to distinguish CNAs from the vast quantities of germline cfDNA with normal copy number. Additionally, due to the ability of the cfDNA test to capture genomic heterogeneity across disease burden discordance may be due to detection of alterations that were not observed in tumor tissue testing from a single site biopsy.

A future study of tissue plasma alteration concordance in which paired samples are collected contemporaneously at clinically relevant timepoints per published concordance study criteria [33] would be valuable, though perhaps

would be limited by the clinical feasibility of collecting tumor tissue at the time of advanced stage disease when plasma cfDNA analysis is clinically indicated.

The cfDNA assay utilized in this study attempts to report only alterations of somatic origin. However, discrimination between alterations of germline versus somatic origin becomes challenging in cases with high tumor burden and/or chromosomal instability [34]. It is also not possible to rule out hematopoietic origin of alterations through sequencing of cfDNA alone [35], and some alterations, like *JAK2* V617F, occur more frequently in myeloproliferative neoplasms than in solid tumors. Therefore, similar to tissue-only testing [36,37], tumor-derived origin of alterations identified by NGS of cfDNA cannot be confirmed with certainty.

Conclusion

We believe this is the first analysis to interrogate and present plasma ctDNA yield in a cohort of patients with primary brain cancers by histopathologic subtype. Our findings demonstrate a higher ctDNA detection rate than previously reported, particularly among some specific subtypes of primary brain tumors, and will hopefully reinvigorate future clinical research in this area to more deeply explore the role and potential of cfDNA analysis in PBTs. Additionally, cfDNA analysis results identified either a genomically targetable off-label or clinical trial option for almost 50% of samples with cfDNA alterations detected. These results demonstrate that while not all patients with primary brain cancers have detectable alterations by such testing, plasma cfDNA analysis is a viable and safe clinical option to obtain actionable somatic genomic information for some patients with primary brain cancers which may potentially guide clinical therapeutic decision-making.

Summary points

- Glioblastoma and other primary brain tumors (PBTs) can be aggressive with limited therapeutic options.
- They can be difficult to biopsy, limiting the ability to interrogate genomic alterations in the tumor.
- This has challenged the development of and enrollment into genomically matched clinical trials in PBT oncology.
- Cell-free circulating tumor DNA (ctDNA) has shown utility as a biopsy-free alternative for comprehensive genomic profiling in advanced solid tumors, though published small PBT cohorts have suggested low detection rates.
- To investigate ctDNA yield in PBTs, we analyzed the genomic results from over 400 patients with PBTs undergoing ctDNA NGS analysis with a highly sensitive and specific clinical assay.
- Genomic alterations in ctDNA, including single nucleotide variants and gene amplifications, were identified in half of these patients, a much higher yield than previously reported.
- Genomic alterations identified had matched off-label and clinical trial options for almost 50% of patients with detectable ctDNA.
- This study suggests promise in a biopsy-free option to interrogate genomic signatures and evolution in PBTs, which may provide an avenue to further progress in genomically matched clinical trials.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/cns-2018-0015

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Ethical conduct of research

This study has obtained appropriate institutional review board approval for analysis of deidentified and limited datasets which waived the need for individual patient informed consent.

Financial & competing interests disclosure

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Ethical conduct of research

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Acquisition, analysis or interpretation of data: All

Drafting/revision: All

Final version approval: All

Accountable for all aspects of work: All

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Papers of special note have been highlighted as: • of interest; •• of considerable interest

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•• **This thorough review provides background on knowledge gained regarding the molecular biology and genomics of glioblastoma as well as commentary regarding the development of personalized medicine and clinical trials in this patient population.**

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