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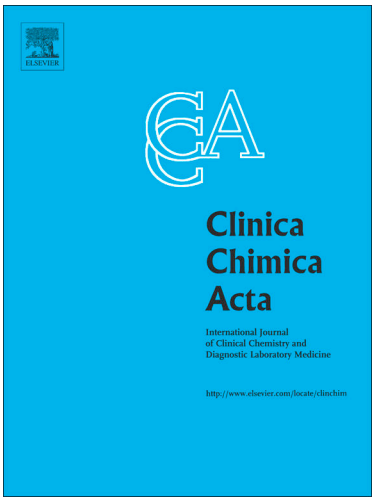

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Detection of mutation profiles and tumor mutation burden of cerebrospinal fluid circulating DNA by a cancer genomic panel sequencing in glioma patients

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Abstract

Background and aims Circulating tumor DNA (ctDNA) has been recognized as a reliable source to reflect the molecular and genetic landscape of corresponding tumors in recent years. In this study, we tested the application of a cancer genomic panel sequencing on the cerebrospinal fluid (CSF)-derived ctDNA for the less invasive detection and diagnosis of glioma.

Materials and methods CtDNA was extracted from 26 CSF samples and subject to a cancer genomic panel sequencing of 520 genes to analyze the mutation profiles and tumor mutation burden (TMB), which were compared with their corresponding tumor DNA samples. Associations between mutations or TMB and clinical characteristics were also evaluated.

Results A high detection rate of ctDNA (24/26, 92.3%) was observed in CSF. CtDNA mutations had high concordance rates with tumor DNA, especially in non-copy number variations and in glioblastoma. CSF ctDNA TMB also exhibited a strong correlation with tumor DNA TMB ($R^2 = 0.879$, $P < 0.001$), particularly in glioblastoma ($R^2 = 0.992$, $P < 0.001$). Age was significantly associated with CSF ctDNA TMB in glioma patients.

Conclusion We established a less invasive but effective molecular diagnostic approach using a cancer genomic panel sequencing system targeting CSF ctDNA for glioma, especially in glioblastoma.

Keywords: Glioma, circulating tumor DNA, cerebrospinal fluid, copy number variation, tumor mutation burden

1. Introduction

Glioma is the most frequent malignant primary tumor in the central nervous system (CNS) of adults, with glioblastoma multiforme (GBM) bearing the highest degree of malignancy[1-4]. Current gold standard treatment strategy remains to be the Stupp regimen, which is maximal safe surgical resection followed by adjuvant focal ionizing radiation plus concomitant and adjuvant chemotherapy (temozolomide)[1-3]. Although great efforts have been made in the past decade in the development of novel treatment

modalities such as immunotherapy, molecularly targeted therapies and tumor treating fields (TTF), alone or in a combination, the median survival of glioma patients remains largely unchanged, with that of GBM patients being less than 17 months[2, 3]. In 2016, the World Health Organization (WHO) revised the classification system of glioma by incorporating both histopathological characteristics and molecular and genetic parameters, which offers a more comprehensive and precise understanding of diagnosis, prognosis prediction and guidance for treatment options[5]. Therefore, technical development for early-stage diagnosis, tumor progression monitoring and prediction of sensitivity to therapies holds the promise for better outcome of glioma patients.

Conventional molecular diagnosis of glioma is established using tumor specimens obtained during biopsy or maximal surgical resection, which however represents a high-risk intervention. Repeated tissue sampling for continuous monitoring of the disease course may also be challenging. Liquid biopsy, referred to the analysis of bodily fluids such as blood, urine and cerebrospinal fluid (CSF) to inform on the presence or on the molecular evolution of neoplastic diseases, has emerged as a less invasive and promising alternative for molecular-diagnostics applications in recent years, specifically in cancers[6]. Due to the presence of blood-brain barrier, CSF has been considered as a better source for liquid biopsy to reflect the molecular and genetic landscape of brain tumors compared to other body fluids such as blood and urine[4, 7-9]. Circulating tumor DNA (ctDNA), around 150-200 bp in length, is one of the important substances released by tumor cells into CSF and plasma and is more stable

($t_{1/2}$: ~2h) compared to other molecules including mRNA and microRNA[7]. CtDNA could reflect various mutations such as copy number variation (CNV), point mutations and tumor mutation burden (TMB)[7]. Of note, TMB has shown promise as a biomarker for immune checkpoint blockade therapy in several types of cancer[10, 11], indicating that detection of TMB using ctDNA may guide the choice of immunotherapy for the treatment of tumors including glioma.

In this study, 26 pairs of matched CSF and tumor samples were collected from glioma patients and 2 CSF samples were collected from patients with subarachnoid hemorrhage as normal controls. A cancer genomic panel sequencing of 520 genes were used to detect the molecular profiles of glioma from CSF-derived ctDNA and tumor tissue-derived DNA (tisDNA). Through combined analysis of CSF ctDNA with clinico-pathological features of glioma patients, we showed that CSF ctDNA may represent a minimally invasive approach for early detection and diagnosis of glioma, and guidance of the choice for treatment such as immunotherapy based on TMB.

2. Materials & Methods

2.1 Patient information and ethics statement

Twenty-six glioma patients treated by microscopic resection in the Guangdong Provincial People's Hospital from July 2018 to December 2019 and two normal controls were included in this study. All patients had a confirmed diagnosis and histological classification by two independent specialists in pathology according to the

criteria of 2016 WHO classification system of CNS tumors. Detailed clinico-pathological information of all the subjects was given in Table A.1. Written informed consent for research purpose was obtained from all subjects or legal guardians. This study was reviewed and approved by the Institutional Review Boards (IRB) and Research Ethics Committees of Southern Medical University and Guangdong Provincial People's Hospital (Guangzhou, China) and carried out in accordance with *The Code of Ethics of the World Medical Association* (*Declaration of Helsinki*).

2.2 Categorization of the tumor attachment

Emerging studies have demonstrated that tumors in contact with the circulating system are more likely to release circulating tumor DNA and we hypothesized that gliomas abutting a CSF space were able to release the circulating tumor DNA into the surrounding CSF whereas those enwrapped by brain tissues would fail so (Figure 1A). In this study, contrast-enhanced magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) were implemented for each patient before surgery and glioma position and attachment were accordingly categorized into two types: abutting a CSF space or enwrapped by brain tissues, which was further confirmed on MRI images by two independent specialists in neurosurgery and neuroradiology. Examples of representative MRI images of glioma patients were shown in Fig. A.1.

2.3 Preparation and quantification of CSF and tumor tissue DNA (tisDNA)

Matched CSF and fresh glioma tissue samples were obtained from each patient.

Twenty-four 8-mL CSF samples were collected through lumbar puncture before tumor resection while 2 were obtained from brain ventricle or cistern during surgery (Figure 1B). Normal samples of CSF (8mL) were collected through lumbar puncture from 2 patients with subarachnoid hemorrhage. CSF samples in EDTA tubes were centrifuged at 1,900 g for 10 minutes at 4°C. The pellet was discarded while the supernatant was transferred to a new tube and recentrifuged at 16,000 g for another 10 min at 4°C. The supernatant was collected and stored at -80°C for later use. CtDNA was extracted from the supernatant (at least 5mL) using the QIAamp Circulating Nucleic Acid kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Fresh CSF-matched tumor tissues were obtained from each patient during surgery. Genomic DNA (gDNA) was extracted from fresh tissue using the QIAamp DNA FFPE tissue kit (Qiagen), in accordance with the manufacturer's instructions. Quantification of ctDNA and tisDNA was performed using the Qubit 2.0 Fluorometer with the dsDNA HS assay kit (Life Technologies, Carlsbad, CA, USA).

2.4 Next-generation sequencing (NGS) library preparation and sequencing

A minimum of 50 ng ctDNA or tisDNA was required for NGS library construction. TisDNA was fragmented using the M220 Focused-ultrasonicator (Covaris, Woburn, MA, USA). Both fragmented DNA and CSF ctDNA were subjected to end repair, phosphorylation, and adaptor ligation. Fragments between 200–400 bp in size were selected by AMPure beads (Agencourt AMPure XP kit; Beckman Coulter, Brea, CA, USA) followed by hybridization with capture probe baits using a 520-gene panel

(Burning Rock Biotech, Guangzhou, China), hybrid selection with magnetic beads, and polymerase chain reaction amplification. Finally, a high-sensitivity DNA assay was performed to assess the quality and size of all fragments using Bioanalyzer 2100 (Aligent Technologies, CA, USA). Indexed samples were sequenced using the Nextseq500 sequencer (Illumina, Inc., Hayward, CA, USA) with pair-end reads and average sequencing depth of 1000x for tisDNA and 4000x for CSF ctDNA.

2.5 Variant calling, CNV detection and TMB calculation

Initial sequencing reads were cleaned with Trimmomatic (v0.36) and then mapped to the human genome (hg19) using Burrows-Wheeler aligner 0.7.10. Local alignment optimization, variant calling, and annotation were performed using the GATK 3.2 and MuTect (both from Broad Institute, Cambridge, MA, USA) and VarScan (Genome Institute, Washington University, USA) software. Variants were filtered using the VarScan filter pipeline. Loci with a depth of less than 100 were filtered out. At least two and five supporting reads were needed for insertions/deletions in CSF and tissue samples, respectively, while eight supporting reads were needed for single number variations (SNVs) to be called in both CSF and tissue samples, and selected exons and introns of 520 genes were captured. According to the Exome Aggregation Consortium, 1000 Genomes Project, dbSNP, and ESP6500SI-V2 databases, variants with a population frequency of over 0.1% were grouped as single-nucleotide polymorphisms (SNP) and were excluded from further analysis. Remaining variants were annotated with the ANNOVAR and SnpEff v3.6 software. DNA translocation analysis was

performed using Tophat2 (Center for Computational Biology, Johns Hopkins University and the Genome Sciences Department, University of Washington, USA) and Factera 1.4.3.

The copy number variation was detected by our in-house analysis scripts based on depth of coverage data of capture intervals. Coverage data was firstly corrected of sequencing bias resulting from GC content and probe design. The average coverage of all capture regions was utilized to normalize the coverage of different samples to comparable scales. Copy number was calculated based on the ratio between depth of coverage in tumor samples and average coverage of an adequate number (n>50) of samples without copy number variation as references as to each capture interval. Gene copy number variation would be reported if the coverage data of the gene region was quantitatively and statistically significantly different from its reference control. Three criteria should be met for copy number variation: 1) the coverage of more than 60% capture intervals of the genes should be significantly different from the reference. The significance was evaluated by z-test comparing coverage of each capture interval to the mean coverage of the interval in all control samples (p<5e-3 for hotspot genes and p<1e-3 for others); 2) the coverage of the total region of the gene in CSF samples was significantly different from the mean coverage of the reference controls, where the significance was evaluated by student t test (p<5e-3 for hotspot genes and p<1e-3 for others); 3) copy number should reach the minimum threshold as to gain and loss (as to copy number gain, CN>2.25 for hotspot genes and CN>2.5 for others; as to copy number loss, CN<1.75

for hotspot genes and CN<1.5 for others).

TMB was computed for each patient as the ratio between the total number of nonsynonymous mutations detected with the total size of the coding region of the panel using the formula below. Mutations occurring on the kinase domain of epidermal growth factor receptor (*EGFR*) or anaplastic lymphoma kinase (*ALK*) were excluded from the mutation count.

TMB = mutation count (excluding copy number variations and fusion)/total size of coding region counted

2.6 Statistical analysis

Analyses of all data were processed using SPSS version 19.0 software package. Comparison between groups was performed by chi-square test and comparison of means among multiple groups was handled by variance single-factor test. Correlations were analyzed by using the Pearson correlation. Data were determined to be statistically significant when $P < 0.05$.

3. Results

3.1 Clinical features of the patients and tumors

Twenty-six glioma patients treated by surgery in the Guangdong Provincial People's Hospital from July 2018 to December 2019 were included in this study. The patients' characteristics such as age, sex, tumor grade (WHO), histological subtype and primary or recurrent status were listed in **Table 1**. The average age of the patients was 40 ± 17.6

years (Range: 5-70 y). Nine of the patients were female while 17 were male. According to the 2016 WHO classification of CNS tumors, 11 patients (39.2%) were diagnosed with GBM (WHO grade IV), 4 patients (14.3%) with anaplastic oligodendroglioma (WHO grade III), 2 patients (7.1%) with oligodendroglioma (WHO grade II), 2 patients (7.1%) with anaplastic astrocytoma (WHO grade III), 1 patient (3.6%) with diffuse astrocytoma (WHO grade II), 2 patients (7.1%) with diffuse midline glioma (WHO grade IV), 3 patients (10.7%) with medulloblastoma (WHO grade IV) and 1 patient (3.6%) with epithelioid glioblastoma (WHO grade IV). Four of them were recurrent while the remaining 22 were primary. Matched tumor tissue and CSF sample were obtained from these glioma patients. Twenty-four CSF samples, whose matched tumor lesions were abutting a CSF space including ventricles, basal cistern, Sylvian cisterns and other cisterns, were collected through lumbar puncture before surgery while the remaining 2 CSF samples whose matched tumor lesions were enwrapped by brain tissues were obtained through cisternal or ventricular puncture during surgery. Accordingly, twenty-four of the CSF samples from glioma patients had detectable ctDNA fragments while the remaining 2 CSF samples from glioma patients and the 2 CSF samples from normal controls showed undetectable levels of ctDNA. The median concentration of ctDNA was 25 ng/μL.

3.2 Comparison of the mutation profiles between CSF ctDNA and tisDNA

Twenty-four paired patient CSF ctDNA and tisDNA samples were detected while tisDNA but not CSF ctDNA was detected in the remaining 2 patients (**Figure 2; Figure**

3). CSF ctDNA was also undetectable in the two normal control CSF samples. Therefore, the detection rate of CSF ctDNA was 92.3% (24/26) (**Figure 2**). Across the 26 tisDNA samples and the 24 matched CSF ctDNA samples, at least one tumor-specific mutation could be detected (**Figure 2**; **Figure 3**). There were 255 and 157 mutations in total in tisDNA and CSF ctDNA, respectively. Among them, there were 132 CNVs and 141 non-CNVs detected in total (**Figure 4A and B**). Thirty-seven CNVs (28%) while 102 (72.3%) non-CNVs were shared in CSF ctDNAs and tisDNAs (**Figure 4A and B**). Moreover, the percentages of cases that showed at least one, at least 50% and all of shared non-CNVs were 92%, 80% and 56% respectively, compared to those in CNVs (50%, 30% and 15%) and all somatic mutations (92%, 69.2% and 19.2%), indicating a much higher consistency in non-CNV profile between paired CSF ctDNAs and tisDNAs (**Figure 4C-E**). We further divided our cohort into GBM and non-GBM subgroups and revealed that the GBM subgroup may account more for the high accordance in non-CNV profile. Nineteen CNVs (35.8%) while 91 (80.9%) non-CNVs were shared in CSF ctDNAs and tisDNAs (Figure 4F). Furthermore, the percentages of cases that showed at least one, at least 50% and all of shared non-CNVs were 91.7%, 83.3% and 66.7% respectively, compared to those in CNVs (63.6%, 45.5% and 18.2%) and all somatic mutations (91.7%, 83.3% and 25%).

The most frequently altered genes in the CSF ctDNA and tisDNA samples were illustrated in **Figure 5**. Notably, the top 4 altered genes between the CSF ctDNA and tisDNA samples were identical: *TERT* (both *n*= 13, 50.0%), *TP53* (both *n*= 9, 34.6%),

PTEN (8 vs 9, 30.8% vs 34.6%) and EGFR (both $n = 6$, 23.1%). There were another 37 identically altered genes between the paired CSF ctDNA and tisDNA samples such as IDH1, ATRX and so on. Many of the shared genes have been recognized as important drivers of glioma and the high concordance of parallel sequencing results between CSF ctDNA and tisDNA samples suggest that CSF ctDNA may serve as a reliable source for the detection of molecular characteristics of glioma.

3.3 Associations of TMB between CSF ctDNA and tisDNA

Although multiple studies have demonstrated the immunosuppressive nature of glioma, immunotherapy, a revolution therapy in cancer, has become a promising strategy with the ability to penetrate the blood brain barrier[12]. Emerging studies have also highlighted the significance of TMB in the prediction of sensitivity to immune checkpoint blockade therapy in several types of cancer including glioma[10, 13, 14]. In this study, we also calculated TMB in both CSF ctDNA and tisDNA samples with the formula described in the "Methods and Materials" section and The TMB profiles were presented in **Figure 6A-B**. Median TMBs in tisDNA and CSF ctDNA were 4 (ranging from 0.8-11.1) and 4 (ranging from 0.8-8.7), respectively. Since one pair of tisDNA and CSF ctDNA had only CNV detected, they were excluded for further correlation analysis, which demonstrated a strong positive correlation between the TMB values in the remaining 23 paired CSF ctDNA and tisDNA samples ($R^2 = 0.879$, $P < 0.001$) (Figure 6C). Notably, a more significantly positive correlation between the CSF ctDNA and tisDNA TMBs was observed in the GBM cohort alone ($R^2 = 0.992$, $P < 0.001$).

These data suggest that CSF ctDNA may provide a less invasive alternative for the prediction of sensitivity to immune checkpoint blockade therapy for glioma treatment.

3.4 Associations between CSF ctDNA mutation and clinical characteristics

In our cohort, we found that the detection rate of CSF ctDNA was negatively associated with the distance between the tumor and brain ventricles or cisterns (**Table 2**). CSF ctDNA was detected in all 24 patients with gliomas abutting a CSF space, but not in those 2 patients with gliomas enwrapped by brain tissue. CSF ctDNA mutation was also compared in different WHO grades of gliomas and our results showed no significant difference in frequency of CSF ctDNA mutation between low-grade gliomas (3 WHO grade II and 6 WHO grade III gliomas) and high-grade gliomas (17 WHO grade IV gliomas) ($P = 0.2084$). Out of the 24 CSF ctDNA-positive gliomas, 21 were primary while 3 were recurrent. No significant difference in frequency of CSF ctDNA mutation between these primary and recurrent gliomas ($P = 0.9818$). IDH1 and IDH2 are two essential enzymes involved in a variety of metabolic processes[15-17] and mutation of these two genes are frequent in patients with low-grade gliomas and secondary GBMs and are associated with a better prognosis[18, 19]. Consistently, our results showed that IDH mutation was more frequent in WHO II and III gliomas (7/9, 77.8%) than in WHO IV ones (0/15, 0%). Conversely, we also found that GBM more frequently harbored TERT, EGFR and RB1 mutations than other gliomas (GBM vs non-GBMs: TERT, 9/11, 81.8% vs 4/15, 26.7%; EGFR, 5/11, 45.5% vs 2/15, 13.3%; RB1, 3/11, 27.3% vs 0/15, 0%), which were also in line with previous reports[20-22].

TP53 is an important tumor suppressor and mutation of TP53 is associated with high invasiveness and recurrence of glioma[23]. We found that mutation of TP53 was detected in paired CSF ctDNA and tisDNA samples of all three recurrent gliomas. Moreover, CSF ctDNA frequency of TP53 in recurrent gliomas was much higher than that in primary ones (n = 6, 28.6%, $P = 0.042$).

The relationships between the level of TMB in CSF ctDNA samples and clinical features were also investigated. As shown in Table 3, only age was significantly associated with a higher level of TMB ($P = 0.049$).

4. Discussion

Although pathological examination is the gold standard for the diagnosis of tumors, its application in intracranial tumors is quite often limited due to the lack of easy access to the tumor tissues. In the present study, we detected the mutation profiles and tumor mutation burden of paired CSF ctDNA and tumor tissue DNA using a cancer genomic panel sequencing system of 520 genes and showed that CSF ctDNA could serve as a less invasive but reliable alternative for the molecular diagnosis of glioma according to the 2016 update of the WHO classification of CNS tumors.

In the clinical setting, neuroimaging and pathological examination are still the main two diagnostic methods for glioma. Although great advances have been achieved in

these techniques, neuroimaging can only identify tumors with a certain diameter (> 1 mm)[24, 25]. Pathological examination is still the gold standard for the diagnosis of glioma[3]. However, acquisition of tumor specimens by biopsy or tumor resection requires complex and high-risk craniotomy, which would be a hard decision for glioma patients to make[26]. Moreover, tumor biopsy can only disclose the features of glioma from a mall area at that moment due to the high heterogeneity of glioma. Thus, precise molecular diagnosis of glioma by biopsy may also be temporally and spatially limited[27]. CtDNA is one of the important substances released by tumor cells and has been reported to be representative of the entire tumor genome in several types of cancers including breast[28] and lung cancers[29]. Recent studies have also demonstrated that ctDNA could be detected in CSF and that mutation profiling of ctDNA exhibited high concordance rate with their matched tumor tissue DNA in both primary and metastatic tumors in the brain[30-33]. Consistent with these studies, we showed a high detection rate of ctDNA in CSF (92.3%, 24/26) and most of the important mutations in glioma could be well detected from CSF ctDNA such as IDH1/2, TERT, PTEN, EGFR, TP53, RB1, H3F3A, ARTX, PIK3A, BRAF, PDGFA and so on. We also showed that high concordance rates were observed between many of frequent mutations detected from tumor tissue and CSF ctDNA such as TERT, EGFR, RB1, TP53 and IDH1. Together with other similar studies, our results suggest that CSF ctDNA could serve as a stable and reliable alternative for the detection and diagnosis of glioma.

Studies over the past decades have demonstrated that molecular biomarkers play critical roles in the guidance of treatment for patients with glioma[5]. For example, patients with oligodendroglioma harboring IDH1/2 mutation and 1p/9q codeletion are highly sensitive to chemotherapy[34]. Molecularly targeted therapies have also been put into clinical trials or clinical use. Generations of EGFR inhibitors or vaccines have been developed and evaluated in the clinical setting[21]. Bevacizumab (a VEGFA-targeted angiogenesis inhibitor) together with concomitant temozolomide therapy have been reported to prolong progression-free survival but not overall survival in recurrent GBMs and approved by Food and Drug Administration for clinical use[35]. Our study showed that IDH mutations were more frequently observed in CSF ctDNA from patients with low-grade gliomas while EGFR mutations were more likely to be detected in CSF ctDNA from GBMs. Moreover, TP53 is an important tumor suppressor and mutations in TP53 could lead to gliomagenesis or chemotherapy resistance in glioma[23]. Concertedly, our results demonstrated that TP53 mutation was present in both CSF ctDNA and tisDNA samples of all recurrent gliomas (n = 3, 100%). In line with previous studies, our results suggest that analysis of CSF ctDNA could also provide a less invasive but reliable basis for the treatment options.

One thing to note is that CSF ctDNA was not detected in two patients in our study. In one case (ID15), the tumor tissue was enwrapped by brain tissue and had no contact with a CSF reservoir. In the other case (ID26), though part of the tumor was extremely close to the surface of cortex and seemed to be abutted to the CSF, detailed reviewing

of the MRI images and intraoperative findings suggested that the tumor may be capsulated by its cystic part, leading to no contact between solid tumor tissue and the CSF circulation system. These observations were consistent with the reports by Wang et al. that ctDNA was not detected in CSF samples whose matched tumors were not abutting a CSF space (n = 5, 100%)[31]. However, a recent study in glioma by Zhao et al. demonstrated that all CSF samples had detectable ctDNA even when the tumor lesions were far way (> 1 cm) from a CSF reservoir (n = 5, 100%)[27]. These conflicting findings suggest that tumor position may not be a significant variable affecting the detectability of CSF ctDNA. Sensitivities of the detection method or platforms might be a possible reason for the discrepancy, which needs further investigation.

Some differences in the mutation profiles were also observed between CSF ctDNA and tisDNA in our study. On the one hand, there were 116 gene mutations detected only in tisDNA but not CSF ctDNA. The negative detection may be caused by the low frequency of ctDNA mutations. On the other hand, 18 gene mutations were present in CSF ctDNA but not tisDNA. This discrepancy may result from tumor heterogeneity, where mutations of different tumor cells in the same mass vary. Mutations detected by biopsies may only reflect the tumor characteristics of the resected part[27]. In addition, a multiregional sequencing in intra-tumoral heterogeneity study have also revealed that common mutations in the same region accounts for only one-third of the total mutations[36]. In comparison, ctDNA is a compilation of DNA substance released from

different tumors cells and may offer a more comprehensive profile of the characteristics of the corresponding tumor[28, 29]. To further analyze the concordance rate in different mutation types, the mutations were divided into copy number variations and non-copy number variation, and our results showed a significantly higher concordance rate in non-CNV (72.3%) than that in CNV (28.0%) between paired CSF ctDNA and tisDNA samples from glioma patients. Notably, a higher concordance rate in non-CNV (80.8%) was observed in the GBM cohort alone. To our knowledge, our study for the first time reported the difference in the CNV and non-CNV mutation profiles of CSF ctDNA in glioma.

In recent years, immunotherapy such as immune checkpoint blockade (ICB) has shown encouraging therapeutic response in a wide spectrum of tumors, such as melanoma and lung cancer[37, 38]. Several clinical studies of immunotherapy have also been launched in glioma[12]. However, efficacies of immunotherapy vary in patients even with the same type of cancer, demonstrating the significance of identifying effective predictive biomarkers. TMB, which is often proportional to the neoantigen burden, has been adopted as an independent predictor of immune response in melanoma and lung cancer[38], but not consistently in gliomas[10, 14]. Notably, a just recently published study reveals that TMB inversely correlates with the immune score in IDH-wildtype gliomas but shows no correlation in IDH-mutant gliomas although there is a positive correlation between TMB and expressed neoantigens[10]. Our results showed that TMB values could be calculated from CSF ctDNA by using the sequencing technology

with a panel of 520 target genes and were highly correlated with those detected from tumor tissues, suggesting that CSF ctDNA may serve as an easily accessible and reliable source for TMB detection to predict the response to immunotherapy in IDH-wildtype gliomas. In addition, the levels of TMB have also been found to be associated with some other characteristics of glioma patients such as patient's age, tumor grade and subtypes of glioma[39]. Consistently, our results revealed that TMB levels detected from CSF ctDNA were positively correlated with the age of glioma patients.

One major limitation of this study must be mentioned here. Due to the small cohort size, no convincing conclusions could be drawn in terms of the associations between CSF ctDNA mutations and pathological subtypes or prognosis of glioma patients. Further investigations with a larger sample size of glioma patients would be preferred to confirm our findings and provide more comprehensive information.

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Conflict of Interest. The authors have no conflict of interest.

Author contributions. W.L.G.: Conceptualization, Data curation, Formal analysis, investigation, Methodology, Resources. L.J.: Data duration and Writing – original draft. J.H.L., G.W.L., J.T.Z., D.Z. and S.Q.Z.: Resources, Formal analysis, Validation and Visualization. H.T.S. and X.D.J.: Conceptualization, Project administration, Funding acquisition and Supervision.

Data statement

All datasets generated/analyzed during this study are available from the corresponding author upon reasonable request.

Appendices

Table A.1 Patient demographics.

Fig. A.1 Representative T2 MRI images from glioma patients with tumors abutting a CSF space or not.

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Figure legends

Figure 1. Schematics showing the release of ctDNA from tumors into ventricles or cisterns and sampling of CSF ctDNA in the brain.

A. Brain tumors attached to a CSF reservoir releases the circulating tumor DNA into the surrounding CSF (purple arrows) whereas those enwrapped by brain tissues would fail so. B. CSF samples from 24 glioma patients and two patients with subarachnoid hemorrhage was collected through lumbar puncture before tumor resection while the remaining 2 CSF samples from glioma patients were obtained through ventricular or

cisternal puncture in the brain during surgery.

Figure 2. Summary of the somatic mutation profiles of the 26 CSF ctDNA samples collected from corresponding glioma patients.

A. The number of gene mutations in each CSF ctDNA sample. The average number of gene mutations identified per CSF sample was 6.0. B. Mutational characteristics of mutations detected from the CSF ctDNA. C. The frequency of each gene mutation in the 26 CSF samples. D. Post-operative pathological diagnosis of the 26 glioma samples. GBM: glioblastoma. AO: anaplastic oligodendrocytoma. AA: anaplastic astrocytoma. DA: diffuse astrocytoma. DIPG: diffuse intrinsic pontine glioma. O: oligodendrocytoma. M: medulloblastoma. EGBM: epithelioid glioblastoma.

Figure 3. Summary of the somatic mutation profiles of the 26 tumor DNA samples collected from corresponding glioma patients.

A. The number of gene mutations in each tisDNA sample. The average number of gene mutations identified per tumor DNA sample was 9.8. B. Mutational characteristics of mutations detected from the tisDNA. C. The frequency of each gene mutation in the 26 CSF ctDNA samples. D. Post-operative pathological diagnosis of the 26 glioma samples.

Figure 4. Summary of the ctDNA-specific, tisDNA specific and matched mutations in each paired ctDNA and tisDNA samples collected from corresponding glioma patients.

A. The number of gene mutations in each paired CSF ctDNA and tisDNA sample. B. Mutational characteristics of mutations detected from the paired CSF ctDNA and

tisDNA samples. Dark green represents the matched gene mutations. Red represents CSF-specific gene mutations. Dark blue represents tisDNA-specific gne mutations. C. The frequency of each gene mutation in the 26 paired CSF ctDNA and tisDNA samples. D. Post-operative pathological diagnosis of the 26 glioma samples.

Figure 5. Summary of the ctDNA-specific, tisDNA specific and matched mutations in each paired ctDNA and tisDNA samples collected from corresponding glioma patients.

A. The number of gene mutations in each paired CSF ctDNA and tisDNA sample. B. Mutational characteristics of mutations detected from the paired CSF ctDNA and tisDNA samples. Dark green represents the matched gene mutations. Red represents CSF-specific gene mutations. Dark blue represents tisDNA-specific gne mutations. C. The frequency of each gene mutation in the 26 paired CSF ctDNA and tisDNA samples. D. Post-operative pathological diagnosis of the 26 glioma samples.

Figure 6. Correlation of TMB between CSF ctDNA and tisDNA in glioma patients.

A-B. TMB levels in each CSF ctDNA (A) and tisDNA (B) samples were calculated and presented as histograms. C. Association between TMB levels in paired CSF ctDNA and tisDNA samples was evaluated using Pearson correlation analysis.

Figure(s)

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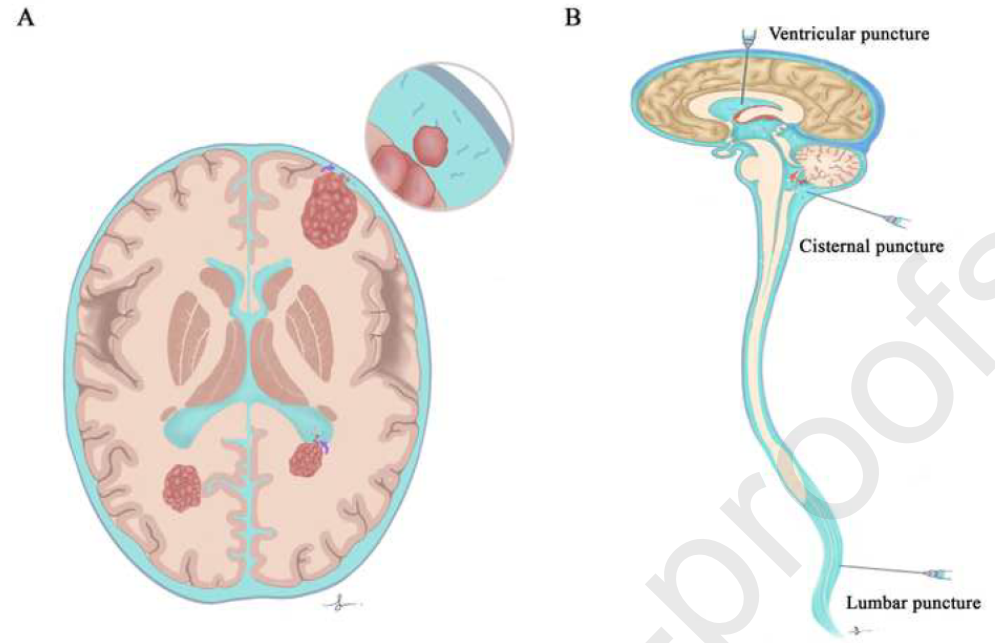
Figure 1



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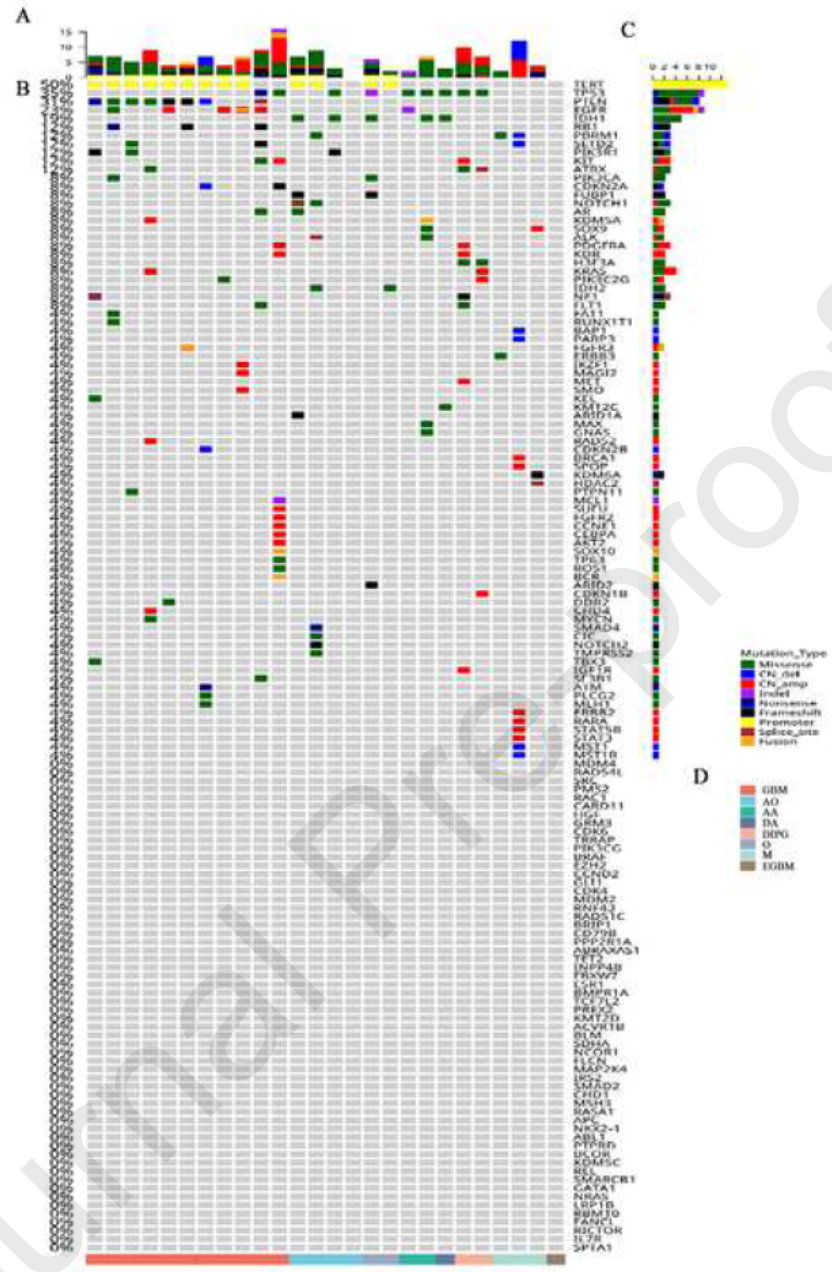

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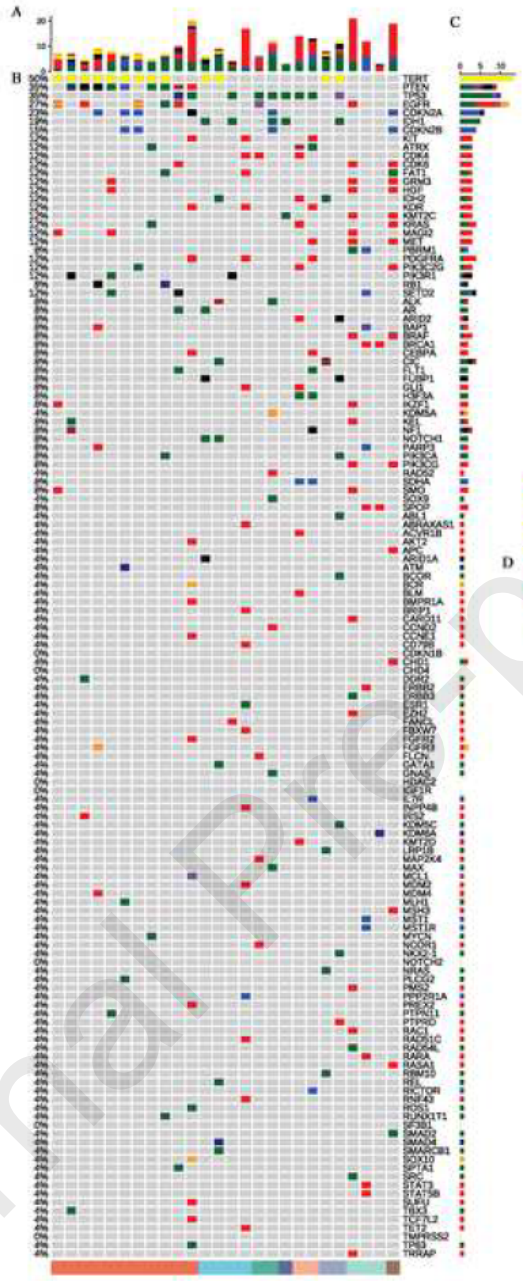

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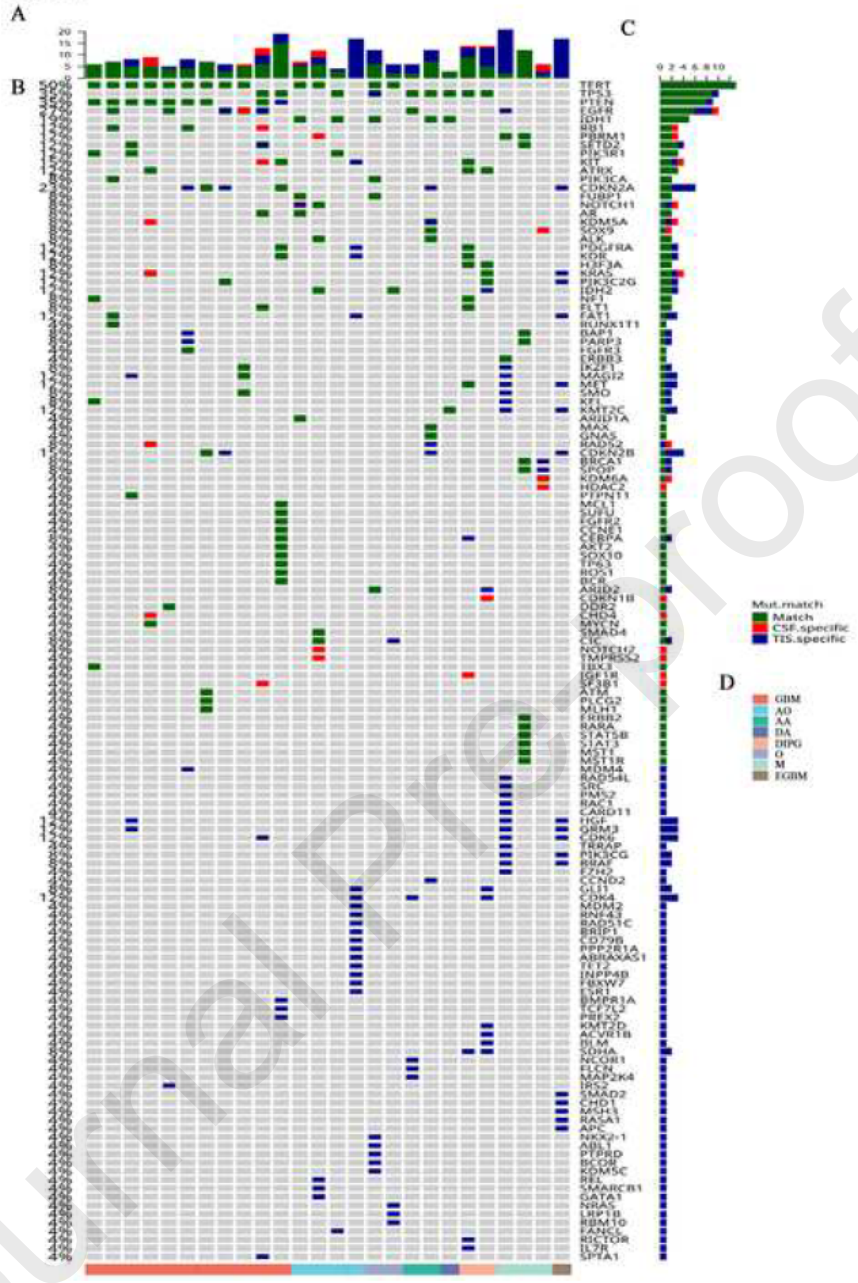

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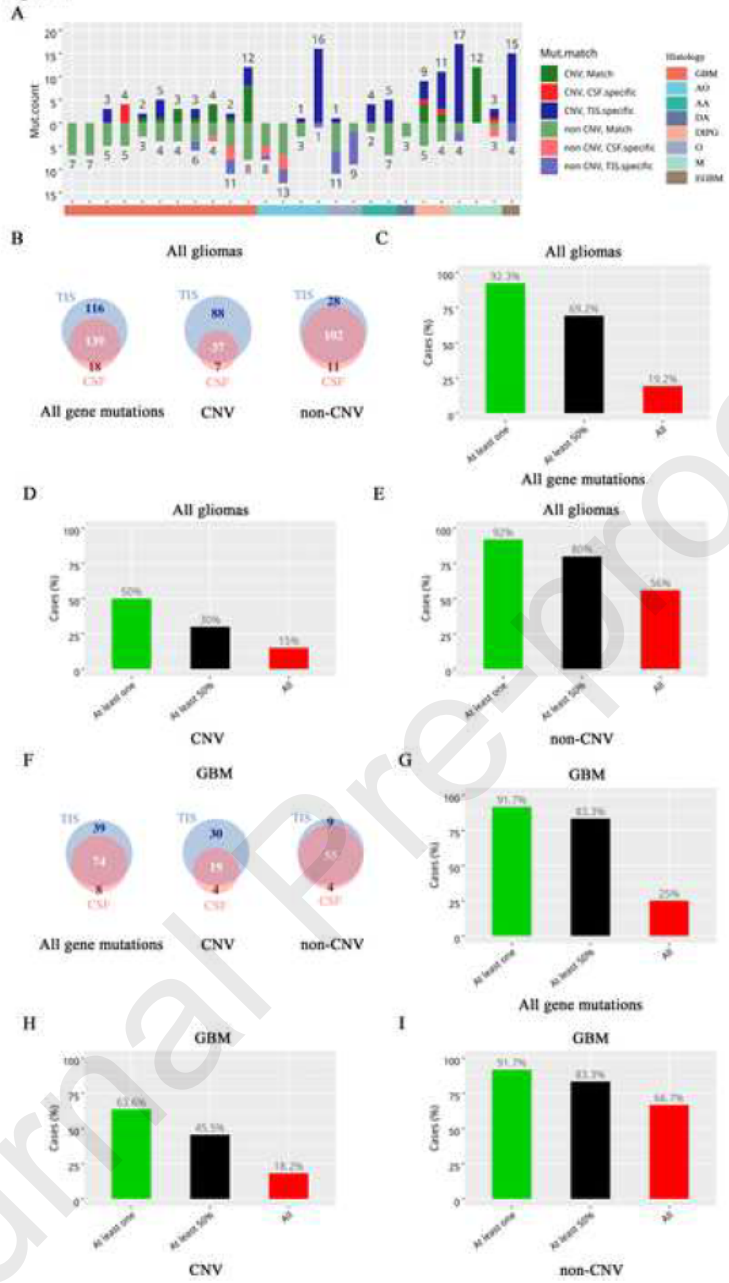

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Figure 6

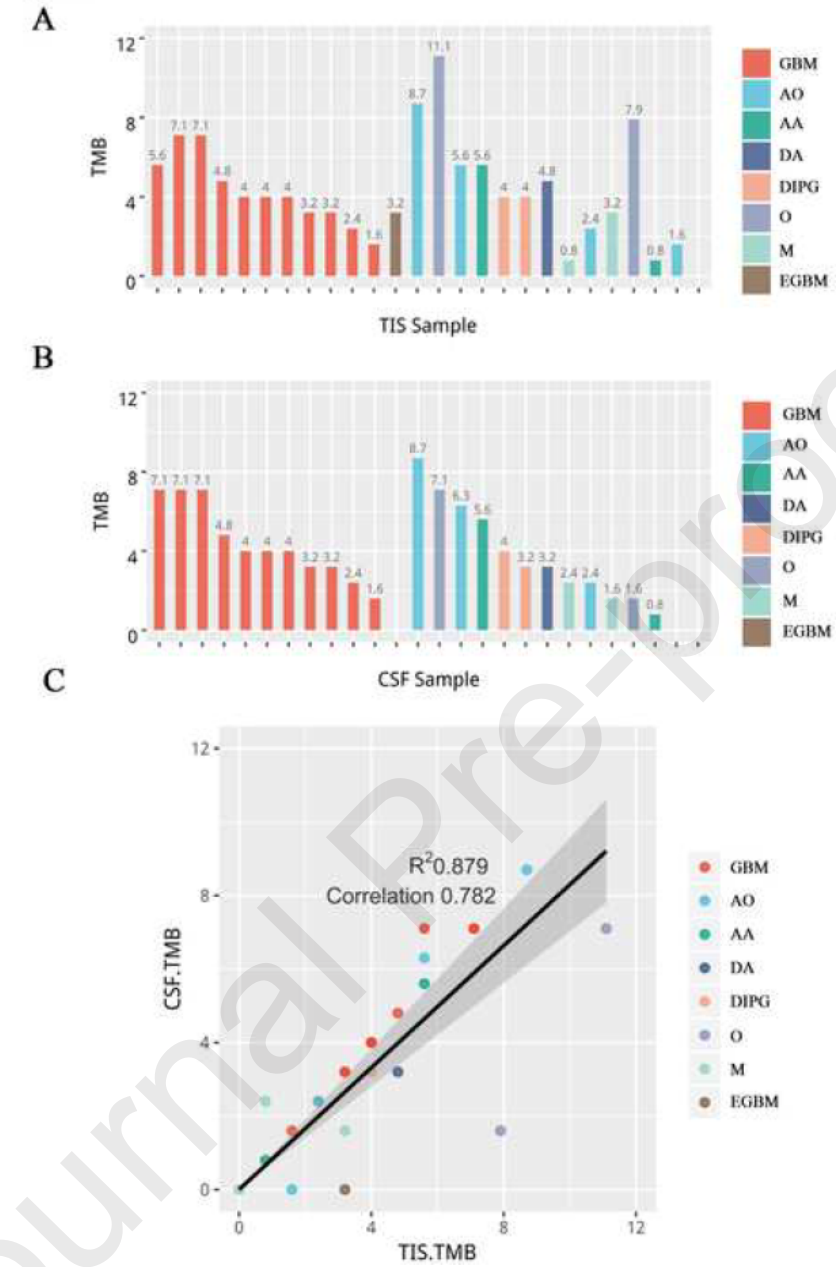

Table 1. Clinico-pathological characteristics of the 26 glioma patients in our study

Characteristics	Parameter value
Age	

Mean (SD)	40 (17.6)
Median (Range)	44.5 (5-70)
Gender	
Male	17
Female	9
Tumor grade (WHO)	
II	3
III	6
IV	17
Histology	
Diffuse astrocytoma	1
Diffuse midline astrocytoma	2
Oligodendrocytoma	2
Anaplastic astrocytoma	2
Anaplastic oligodendrocytoma	4
Medulloblastoma	3
Glioblastoma	12
Adjacent to ventricles or cisterns	
Yes	24
No	2
Primary or recurrent status	
Primary	22

Recurrent		4		

Table 2. Associations between CSF ctDNA detection and clinical characteristics of the 26 glioma patients

Characteristics	*n*	CSF ctDNA(+)	CSF ctDNA(-)	*P* value
Age				> 0.05
< 44	13	11	2	
> 44	13	13	0	
Gender				> 0.05
Female	9	9	0	
Male	17	15	2	
IDH1/2 status				> 0.05
Wild-type	16	14	2	
Mutant	7	7	0	
WHO grade				> 0.05
II and III	9	8	1	
IV	17	16	1	
PRS_type				> 0.05
Primary	22	21	1	
Recurrent	4	3	1	
H3K27M				> 0.05
Wild-type	18	16	2	

Mutant	5	5	0	
Adjacent to ventricles or cisterns				< 0.05
Yes	24	24	0	
No	2	0	2	

Table 3. Clinicopathological characteristics of 26 glioma patients in association with CSF TMB.

	TMB value		
Variables	Low (n=13)	High (n=13)	P-value
Gender			> 0.9999
Male	9	8	
Female	4	5	
No. of tumor loci			> 0.9999
Single	10	11	
Multiple	3	2	
IDH mutation status			0.6584
Wild-type	10	9	
Mutant	3	4	
Age			0.0499*
<45	9	4	
≥45	4	9	
Disease status			> 0.9999
Primary	11	11	
Recurrent	2	2	
WHO grade			0.6802
II-III	5	4	
IV	8	9	
TERT mutation status			0.2393
Wild-type	8	5	
Mutant	5	8	
TP53 mutation status			0.2162
Wild-type	10	7	
Mutant	3	6	
PTEN mutation status			0.0892
Wild-type	11	7	
Mutant	2	6	
H3K27M mutation			0.1807

Wild-type	8	10	
Mutant	2	3	
NA	3	0	
Abutting a CSF space			0.1410
Yes	11	13	
No	2	0	
Histology			0.4314
GBM	5	7	
Non-GBM	8	6	

CSF, cerebrospinal fluid; TMB, tumor mutation burden; IDH, isocitrate dehydrogenase; TERT, telomerase reverse transcriptase; TP53, tumor protein 53; WHO, world health organization; PTEN, phosphatase and tensin homolog; GBM, glioblastoma.

Highlights

1. A high detection rate of ctDNA (24/26, 92.3%) in CSF of glioma patients was observed.

2. CSF ctDNA exhibited high concordance rates with tumor DNA in CNV mutations.

3. CSF ctDNA TMB displayed a strong correlation with that of tumor DNA in glioma patients.