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# Applications of cerebrospinal fluid circulating tumor DNA in the diagnosis of gliomas

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## Abstract

**Objective:** The 2016 World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS) was revised to include molecular biomarkers as diagnostic criteria. However, conventional biopsies of gliomas were spatially and temporally limited. This study aimed to determine whether circulating tumor DNA (ctDNA) from cerebrospinal fluid (CSF) could provide more comprehensive diagnostic information to gliomas.

**Methods:** Combined with clinical data, we analyzed gene alterations from CSF and tumor tissues of newly diagnosed patients, and detected mutations of ctDNA in recurrent patients. We simultaneously analyzed mutations of ctDNA in different glioma subtypes, and in lower-grade gliomas (LrGG) versus glioblastoma multiforme (GBM).

**Results:** CSF ctDNA mutations had high concordance rates with tumor DNA (tDNA). CSF ctDNA mutations of *PTEN* and *TP53* were commonly detected in recurrent gliomas patients. *IDH* mutation was detected in most of CSF ctDNA derived from *IDH*-mutant diffuse astrocytomas, while CSF ctDNA mutations of *RB1* and *EGFR* were found in *IDH*-wild-type GBM. *IDH* mutation was detected in LrGG, whereas *Rb1* mutation was more commonly detected in GBM.

**Conclusions:** CSF ctDNA detection can be an alternative method as liquid biopsy in gliomas.

**Key words:** circulating tumor DNA, glioma, diagnosis, liquid biopsy, next generation sequencing

## Introduction

Gliomas are the most common primary malignant brain neoplasms in adults. Grade IV glioma is named as glioblastoma multiforme (GBM), which represents the most malignant subtype. Patients with GBM usually have a median survival of ~14 months [1]. Therefore, early diagnosis of primary gliomas, and monitoring tumor progression or recurrence and prediction of sensitivity to radiochemotherapy might be critical for the outcome of gliomas patients. In 2016, the World Health Organization (WHO) classification of gliomas was updated to introduce an integrated diagnosis that incorporates both tumor morphology and molecular information [2].

The challenge of detecting molecular markers of brain tumors is the difficulty in accessing tumor tissue and the inability to detect circulating tumor DNA (ctDNA) in plasma of patients. Liquid biopsies provide a reliable alternative to conventional biopsies [3,4]. The presence of blood-brain barrier (BBB) means that cerebrospinal fluid

(CSF) reflects the tumor characteristics better than plasma [5] and contains certain biomarkers that can hardly be found in plasma. Thus, the use of CSF as a liquid biopsy for central nervous system (CNS) tumors is promising.

Tumor cells release DNA of ~150–200 bp in length into the surrounding environment, which then further spreads to plasma and CSF, forming ctDNA [6]. CtDNA can reflect well the various types of mutations (e.g. point mutations and amplifications) in glioma cells [7] and seems to be a richer source of tumor biomarkers than circulating tumor cells (CTCs).

In this study, we collected 17 pairs of matched CSF and tumor samples from patients with gliomas. Four CSF samples were collected from patients after intravescular embolization of intracranial aneurysm as normal controls. The next-generation sequencing Ion Personal Genome Machine, used in this study, is capable of screening for mutations in multiple genes simultaneously and can be useful in clinical characterization of plasma ctDNA from cancer patients [8,9]. Through combined analysis of CSF ctDNA with clinical features of patients with gliomas, we showed that CSF ctDNA is a minimally invasive means to dynamically assess the biological processes of tumor and can provide more comprehensive diagnostic information for patients with gliomas.

## Materials and methods

### Ethics statement and patients

The study had been approved by the Ethics Committee of Chinese PLA General Hospital. The methods and protocols were carried out in accordance with the approved guidelines and regulations by the Science and Technology Department and Institutional Review Board of the Chinese PLA General Hospital. All patients provided appropriate written informed consent for the use of CSF and gliomas tissue under approval of the Ethics Committee. All samples and medical data used in this study were irreversibly anonymized. Seventeen patients with gliomas and four normal controls were included in the study. The patients were diagnosed and treated by microscopic resection in the Chinese PLA General Hospital from February to July 2016.

### CSF and tumor tissue sample DNA preparation

Seventeen 5-ml CSF samples were obtained from the Sylvian cistern or interhemispheric fissure cistern before tumor resection (Fig. 1), and seventeen tumor tissue biopsies were collected during surgery. Three of these patients were diagnosed as recurrence. All postoperative tumor tissues underwent histological examination by two independent pathologists. Normal samples of CSF control were collected by lumbar puncture from four patients after aneurysm embolization. All CSF samples and their corresponding tumor samples were subjected to single nucleotide variation (SNV) and Indel analysis using next-generation sequencing data. The EZNA Tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA) was used to extract DNA from fresh tumor tissues. CSF samples in EDTA tubes were centrifuged at 16 000 g for 10 min. The pellet was stored at −20°C, while the supernatant was centrifuged at 16 000 g for another 10 min. The supernatant was transferred aseptically to pre-labeled cryotubes and stored at −80°C. CtDNA was extracted from at least 3 ml of CSF supernatant, using the QIAamp Circulating Nucleic Acid kit (QIAGEN) according to the manufacturer's instructions. Finally, ctDNA was quantified using a Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's protocol.

### Ion proton library preparation and sequencing

Preparation of the ion proton library and DNA sequencing were performed as described in previous publications [10,11]. For each sample type, an adapter-ligated library was generated using Ion AmpliSeq Library Kit 2.0 (Life Technologies), following the manufacturer's protocol. Briefly, pooled amplicons made from 10 to 20 ng of ctDNA were end-repaired and ligated to Ion Adapters X and P1. AMPure beads (Beckman Coulter, Brea, CA, USA) were used to purify the adapter-ligated products, followed by nick-translation and PCR-amplification for five cycles. AMPure beads were used to purify the resulting library. An Agilent 2100 Bioanalyzer and Agilent Bioanalyzer DNA High-Sensitivity LabChip (Agilent Technologies) were used to determine the concentration and size of the library. Sample emulsion PCR and emulsion breaking were performed using Ion OneTouchTM 2 system (Life Technologies) with Ion PI Template OT2 200 Kit v3 (Life Technologies), referring to the manufacturer's instruction. Ion sphere particles (ISPs) were recovered, and template-positive ISPs were enriched with Dynabeads MyOne Streptavidin C1 beads (Life Technologies) on Ion One Touch ES (enrichment system) (Life Technologies). ISPs enrichment was confirmed using Qubit 2.0 Fluorometer (Life Technologies). The Ion Proton System using Ion PI v2 Chips (Life Technologies) was used to sequence barcoded samples for 100 cycles, and the Ion PI Sequencing 200 Kit v3 (Life Technologies) was used for sequencing reactions following the recommended protocol.

### Variant calling

Initial data from the sequencing were processed using the ion proton platform-specific pipeline software, Torrent Suite v5.0, including generating sequencing reads, trimming adapter sequences, and filtering and removing poor signal-profile reads, as described in our previous publications. Initial variant calling from the sequencing data was generated using the Torrent Suite Software with the plug-in 'variant caller v5.0'. Three filtering steps were used to eliminate erroneous base calling and generate final variant calling. In the first-step, the followings were defined for tumor tissue DNA: the average total coverage depth was >1000, the coverage of each variant was >20, the variant frequency of each sample was >2% [12] and the $P$ value was <0.01. The following filtering criteria were defined for the CSF ctDNA: the average total coverage depth was >10 000, the coverage of each variant was >10, the variant frequency of each sample was >0.5% and the $P$ value was <0.01. The second step utilized the Integrative Genomics Viewer (IGV) software (http//www.broadinstitute.org/igv) or the Samtools software (http://samtools.sourceforge.net) to eliminate possible DNA strand-specific errors after visual examination of the called mutations. The final step searched for variants within the 2856 mutational hotspots, following the manufacturer's instruction.

### Statistical analysis

Analyses were performed using SPSS version 19.0 (IBM, Armonk, NY, USA). Comparison between groups was handled by chi-square test or student's $t$ tests, while comparing the difference among multiple groups by variance analysis. Data were thought to be statistically significant when $P < 0.05$.

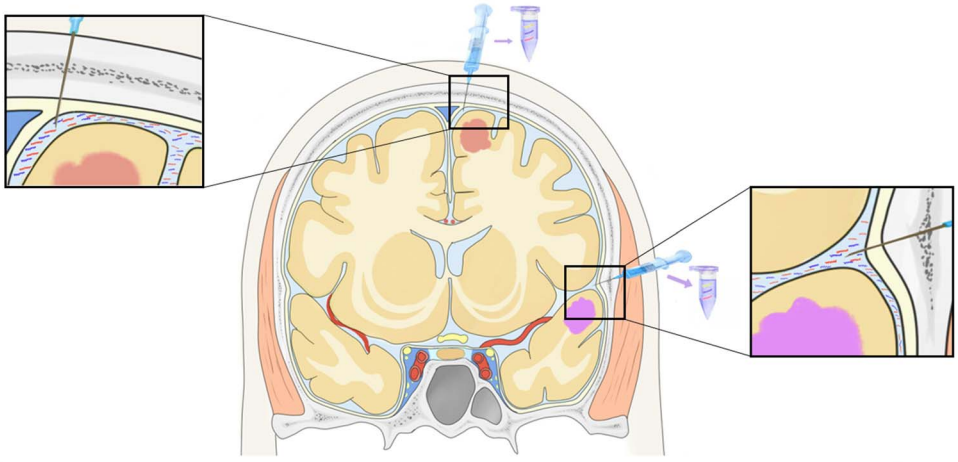

**Figure 1.** Schematic showing the sampling of CSF ctDNA from interhemispheric fissure cistern (top) or Sylvian cistern (bottom).
CSF-derived ctDNA from 17 samples of supratentorial gliomas in our study were collected before tumor resection from interhemispheric fissure cistern or Sylvian cistern. Matched fresh tumor tissues were obtained from surgical resection simultaneously.

## Results

### Patient and tumor characteristics

We collected CSF ctDNA from 17 patients diagnosed with supratentorial gliomas. The patients' characteristics such as age, sex, tumor grade and pathological diagnosis are listed in Table 1. The mean age of the patients was 46.6 ± 11.8 years (range: 28–66 y), including eight male and nine female patients. According to the 2016 WHO classification of CNS tumors, six patients (35.3%) were diagnosed with diffuse astrocytoma (WHO grade II), four patients (23.5%) with anaplastic astrocytoma (WHO grade III), three patients (17.7%) with anaplastic oligodendroglioma (WHO grade III) and four patients (23.5%) with GBM (WHO grade IV). Normal CSF samples were collected from four patients with embolization of aneurysm. Twelve out of 17 patients had a lesion adjacent to the CSF reservoir, including subarachnoid cisterns, ventricles, basal cisterns and other cisterns. The remaining five patients had a lesion >1 cm away from the CSF reservoir. All patients in this study had detectable ctDNA fragments in their CSF.

**Table 1.** Clinical features of 17 gliomas patients

| Characteristic | Parameter value |
|---|---|
| Age, years | |
| Mean (SD) | 47 (11.8) |
| Median (range) | 46 (28–66) |
| Sex | |
| Male | 8 |
| Female | 9 |
| Pathological diagnosis | |
| Diffuse astrocytoma, IDH-mutant | 4 |
| Diffuse astrocytoma, IDH-wild type | 2 |
| Anaplatic astrocytoma, IDH- mutant | 2 |
| Anaplatic astrocytoma, IDH-wild type | 2 |
| Anaplatic oligodendroglioma, IDH-mutant and 1p/19q codeleted | 3 |
| Glioblastoma, IDH-wild type | 4 |
| Tumor grade (WHO) | |
| II | 6 |
| III | 7 |
| IV | 4 |

### Identification of somatic mutations in the CSF ctDNA and tDNA sample pairs

Seventeen CSF ctDNA and genomic DNA (gDNA) sample pairs were identified using the targeted sequencing approach described in 'Materials and Methods' section. Across these paired samples, 404 SNVs and 14 deletions were detected in their CSF. At least one mutation was identified in the CSF from these 17 patients, and the average number of mutations identified per CSF sample was 29.8 (range: 1–192). Among these CSF samples, the most frequently altered genes were *FGFR1* ($n$ = 15, 88.2%), *APC* ($n$ = 10, 58.8%), *EGFR* ($n$ = 10, 58.8%), *RB1* ($n$ = 10, 58.8%), *SMAD4* ($n$ = 9, 52.9%), *ERBB2* ($n$ = 8, 47.1%), *KDR* ($n$ = 8, 47.1%) and *IDH1* ($n$ = 6, 35.3%). In the gliomas tissue, 67 SNVs were detected in the gDNA. The top nine mutations were *RB1* ($n$ = 13, 76.47%), *EGFR* ($n$ = 12, 70.6%), *SMAD4* ($n$ = 12, 70.6%), *TP53* ($n$ = 12, 70.6%), *APC* ($n$ = 11, 64.7%), *GNAQ* ($n$ = 11, 64.7%), *IDH1* ($n$ = 11, 64.7%), *FGFR1* ($n$ = 10, 58.8%) and *RET* ($n$ = 10, 58.8%).

We have found that the ctDNA alterations in the CSF included all the 31 mutations detected in glioma tissue (Fig. 2). The shared mutated genes included some well-known drivers, such as *Rb1*, *TP53*, *EGFR*, *IDH1* and *PTEN* [13]. The concordance of the results of parallel sequencing suggested that these shared mutations identified in the CSF ctDNA might be potential biomarkers for the detection of gliomas. Sixteen gene mutations were detected in the CSF but not in the gliomas tumor specimen (Fig. 3). The discrepancy reflected the spatial heterogeneity of tumor biopsies, while CSF provided more information from mixed tumor cells.

### Relationship between the CSF ctDNA mutations and clinical features

We discovered that *IDH1* CSF mutation was detected more commonly in younger patients ($P$ = 0.049), which was consistent with

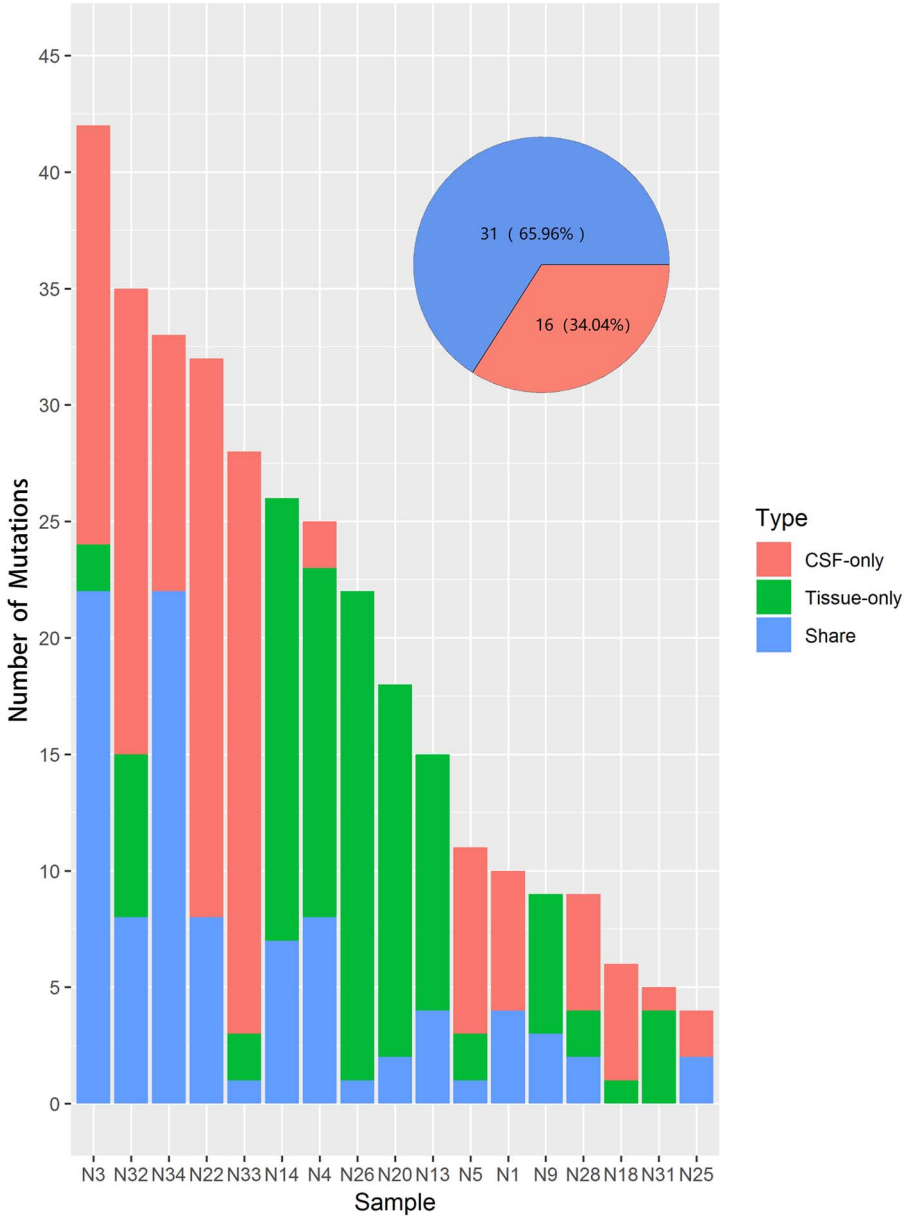


**Figure 2.** Frequency of shared (blue) versus tissue-only (green) or CSF-only (red) mutations in matched tumor tissue–CSF sample pairs.
The number of mutations for each patient. Blue represents the number of mutations detected both in CSF and tumor tissue; red represents the number of mutations detected in CSF only (16, 34.04%); green represents the number of mutations detected in tumor tissue only. At least one mutation could be detected from CSF in the 17 cases. CtDNA alterations in the CSF include all the 31(65.96%) mutations detected in gliomas tissues.

previous studies on young patients with gliomas [14]. We also found the distance between the tumors and brain ventricles or subarachnoid cisterns was associated with CSF-ctDNA levels. Patients with lesions adjacent to a CSF reservoir or subarachnoid cisterns were much more likely to have detectable levels of CSF-tDNA than those with nonadjacent lesions ($P$ = 0.04). CSF-tDNA might be easily released into CSF reservoir, when gliomas are adjacent to these locations [15].

Out of the 17 glioma samples, three samples were recurrent tumor, 14 samples were primary tumor. There was no significant difference in the number of mutations between them. Since *PTEN* and *TP53* are well-known tumor suppressors, mutational status of *PTEN* and *TP53* are associated with the highly invasive behavior and therapy resistance of GBM cells [16]. Mutations of *PTEN* and *TP53* were detected in all three recurrent tumor tissues in our study, and we also found ctDNA mutations of *PTEN* ($n$ = 3, 100.0%) and *TP53* ($n$ = 3, 100.0%) in all three patients with recurrent gliomas. CtDNA mutation frequency of *PTEN* and *TP53* were higher than those in primary gliomas (*PTEN*: $n$ = 3, 21.4%, $P$ = 0.01; *TP53*: $n$ = 4, 28.6%, $P$ = 0.02).

According to the 2016 WHO classification of CNS tumors, CNS tumor grades are still determined based on histological criteria. WHO grade II and III gliomas have been defined as lower-grade gliomas (LrGG) hereafter [17]. We compared CSF ctDNA mutations in 13 LrGGs samples (six WHO grade II and seven WHO grade III) with four GBM samples. Our result showed no significant

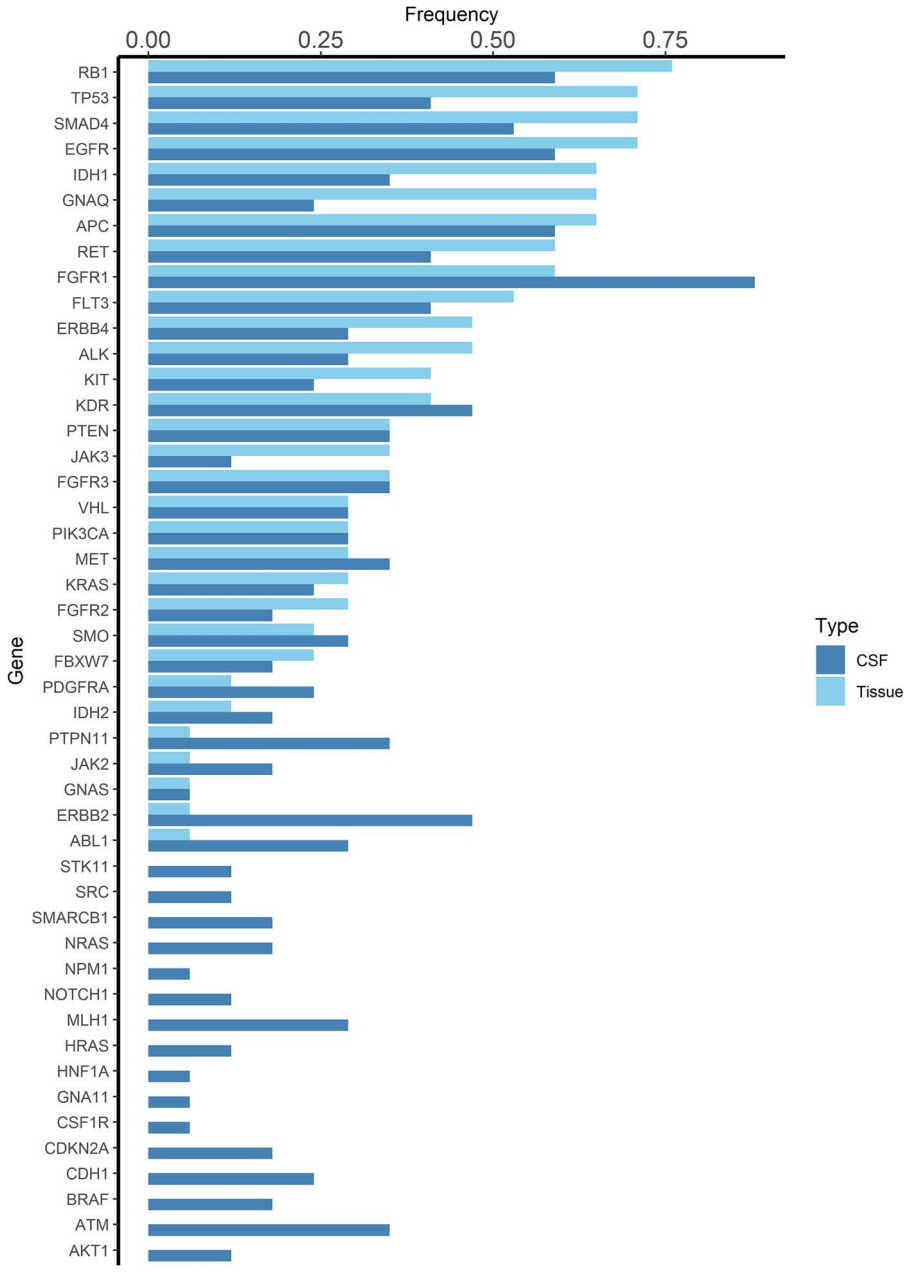


**Figure 3.** Frequency of the most common genetic alterations in tumor tissue (light blue bars) and CSF (dark blue bars).
The shared gene mutations show concordance of the sequencing results of CSF and tumor tissue. Sixteen gene mutations were only detected in the CSF but not in the gliomas tumor specimen. The discrepancy reflects CSF provides more information.

differences in frequency of ctDNA mutations between LrGGs and HGGs ($P = 0.87$). We found that *IDH* ctDNA mutation was detected in about half of the LrGGs ($n = 7$, 53.8%), which was not tested in any GBM samples, while *Rb1* mutation was detected in all GBM samples ($n = 4$, 100.0%) and in less than half of the LrGGs ($n = 6$, 46.2%).

According to the 2016 WHO classification of tumors of CNS, 'integrated' diagnosis is reported based on a combination of phenotypic and genotypic classification [2]. Due to the small cohort size, we did not acquire convincing differences in frequencies of ctDNA mutation among astrocytoma, oligodendroglioma and GBM. We tried to detect several diagnostic gene mutations in the 17 CSF samples (Fig. 4) and obtained similar frequencies with gliomas tissue sequencing. Our study showed that *IDH* ctDNA mutation was detected in most CSF of *IDH*-mutant diffuse astrocytomas ($n = 5$, 83.3%). Highest accuracies were also observed in *IDH*-wildtype primary GBMs, ctDNA mutations of *RB1*and *EGFR* were 100 and 75%, respectively.

## Discussion

In current study, we detected CSF ctDNA to provide pathological information for gliomas according to the latest WHO classification

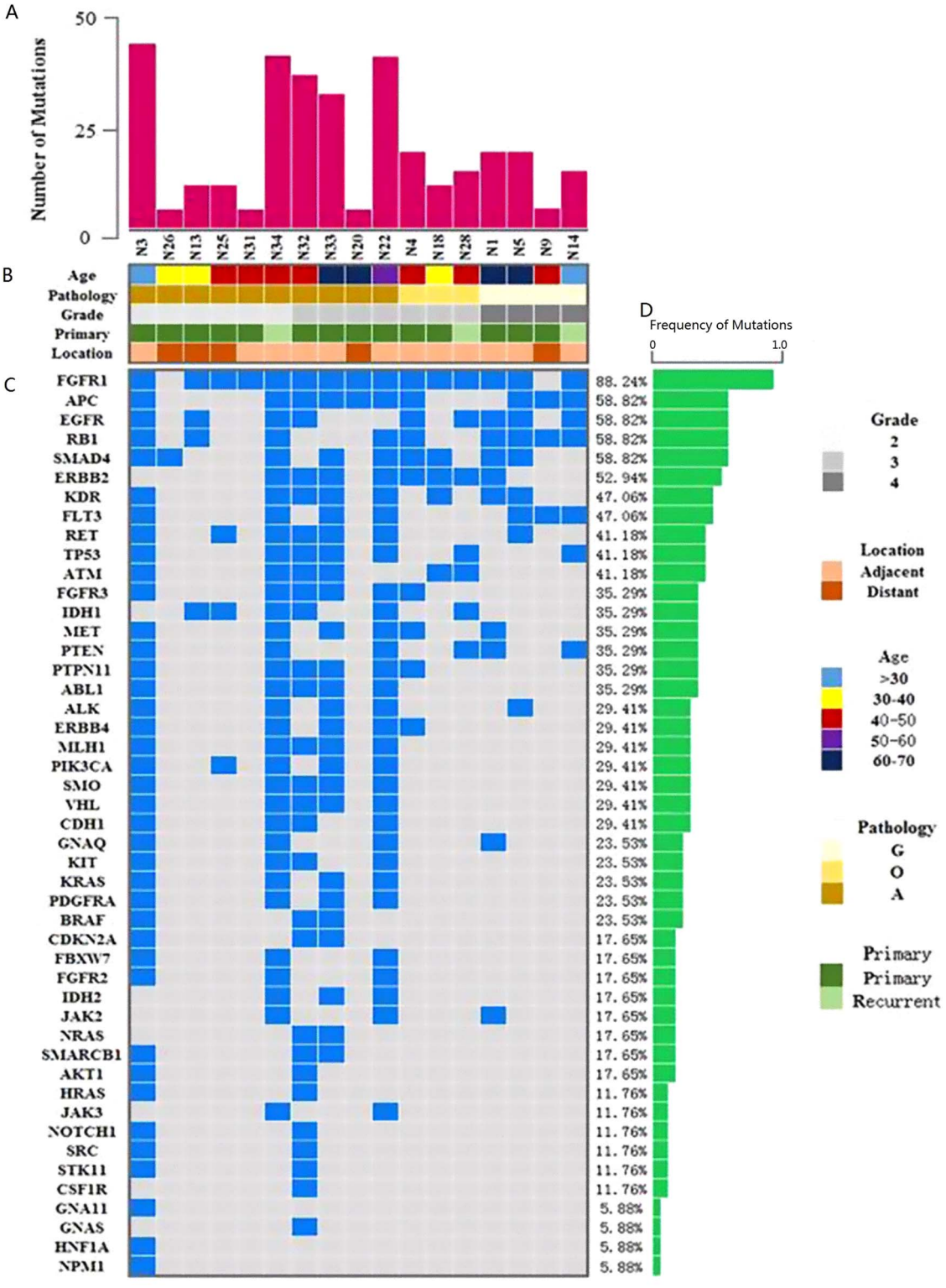


**Figure 4.** Summary of patient characteristics and gene mutations in CSF ctDNA samples.
(A) The number of gene mutations of CSF ctDNA in each sample. The average number of mutations identified per CSF sample was 29.8. (B) Patients were categorized based on age, pathological classification, grade, primary or recurrent and distance to CSF reservoir. (C) Mutational characteristics for mutations detected from the CSF-derived ctDNA. We used a panel-based next generation sequencing approach to identify tumor-specifc mutations in the CSF ctDNA samples. CSF ctDNA was successfully detected in 17 patients with gliomas. Location: light brown represents the location of the tumor is adjacent to the arachnoid cistern; dark brown represents the location of the tumor is nonadjacent. Tumor adjacent to a CSF reservoir or subarachnoid cisterns was much more likely to have detectable levels of CSF-tDNA than those with nonadjacent lesions. The following histologies were included in our study: G, glioblastoma, O, oligodendroglioma, A, astrocytoma. (D) Frequency of gene mutations in CSF samples.

of CNS tumors. Using current technologies, neuroimaging can only identify tumors with a certain volume. Although periodic examination of MRI can be used to dynamically measure tumor progression, genome mutations might occur at an earlier stage, which was hardly can be detected by imaging [18]. In addition, pseudoprogression, which can occur in up to 30% of patients with gliomas after surgery with chemotherapy and radiotherapy is difficult to distinguish from tumor recurrence [19]. Pathological examination is still the gold standard for the diagnosis of primary glioma and its recurrence. However, the craniotomy technique is complex, and biopsy can only reveal tumor characteristics at that single moment and from a small area; thus, it is nearly impossible to provide complete and dynamic information regarding the state of tumor. In addition, pathological examination of the resected specimen often reveals necrosis, scarring or other treatment-related effects rather than recurrent disease [20]. Thus, there is an urgent need to find a convenient method to monitor the progression of gliomas dynamically. CtDNA in patients with lung and breast cancer can be detected in plasma and is representative of the entire tumor genome [21,22]. However, given the existence of the BBB, the frequency of detectable CTC or ctDNA in plasma for gliomas is very low (<10%) [23], whereas level of CSF ctDNA could be detected effectively. Lumbar puncture is an easy and feasible way to obtain CSF samples periodically to make diagnosis and monitor gliomas progression. It is a minimally invasive technique to monitor many diseases of the CNS and has fewer complications, such as intracranial hematoma, compared with surgery. Recently, some studies have demonstrated that tumor mutations are detectable in CSF of patients with various primary and metastatic brain tumors, and ctDNA in CSF recapitulates the genomic landscape of brain tumor better than plasma and precipitates of CSF [24,25].

We obtained CSF samples and matched gliomas tissue samples from the same surgery and observed that they had a high concordance rate. Our results suggested that the concordance of CSF ctDNA closely approximated to the levels found in circulating body fluids adjacent to tumors reported in previous studies [26]. This agreement of CSF ctDNA suggested that it might be a viable means of liquid biopsy in gliomas.

We also found differences between the results of paired CSF sequencing and tumor samples. Some gene mutations could be detected in one patient's tumor tissue, which could not be found in his/her CSF. The negative detection may be caused by low frequency of ctDNA mutations in liquid biopsy. Meanwhile, among all 17 patients, 16 mutations were detected only in CSF ctDNA, but not in any tumor DNA. The heterogeneity of tumor tissues means that mutations of tumor are varied, and biopsies might only represent tumor characteristics of the resected part. Even in the same tissue area, common mutations accounted for only about one-third of the total mutations [27], while CSF ctDNA derived from secretions from different tumor cells may offer a more comprehensive and integrated picture of a tumor's characteristics [28]. Considering tumor space heterogeneity, single-site tissue cannot represent features of gliomas completely. However, it is hard to implement multiple biopsies under the guidance of neuronavigation, especially when the gliomas are close to functional areas or in the brainstem. Patients cannot undergo several surgical biopsies either. Therefore, the detection of CSF ctDNA is superior for allowing continuous sampling to monitor gliomas progression.

Previous studies have reported that ctDNA levels could represent the progression of many somatic tumors after surgery or drug treatment [29]. Increases in ctDNA levels can predict tumor recurrence much earlier than conventional tumor imaging techniques [18,30]. Some tumor-suppressor gene mutations could promote gliomas growth and chemotherapy resistance [16]. In our study, we found that ctDNA of *PTEN* and *TP53* were detected in all recurrent glioams. Thus, ctDNAs might predict sensitivity to adjuvant therapy and represent real-time biomarkers for monitoring the progression or recurrence of gliomas.

In contrast to previous classification principles, the 2016 WHO CNS revision indicated that genotype diagnosis was important for the 'integrated' diagnosis, which might be different from some previous pathological diagnosis of gliomas. Mutation of *IDH* was found in a large percentage of LGG and secondary GBM [14]. Our results showed that *IDH* mutation was detected in most of CSF ctDNA derived from *IDH*-mutant diffuse astrocytomas. *IDH*-wildtype primary GBMs are characterized by *EGFR*, *PTEN*, *TP53* and *RB1* alterations [31,32]. In our study, CSF ctDNA mutations of *RB1* and *EGFR* were found in *IDH*-wildtype GBM, which was similar to published reports about tissue sequencing results. CSF ctDNA parameters could provide a good auxiliary method to estimate the malignancy and subgroup of gliomas preoperatively, but not a complete replacement to tumor tissue biopsy at this stage.

In the initial stage of this study, we thought that lumbar puncture might be unsafe before the resection operation because the mass effect of gliomas elevates intracranial pressure. Therefore, we sampled CSF from cerebral cisterns after craniotomy, and then removed the tumor to take tumor samples. We determined experimentally that 5 ml of CSF was sufficient for ctDNA detection. Some studies showed that 1–2 ml was sufficient [33,34], which suggested that collecting CSF samples preoperatively through a lumbar puncture would be safe, thus making sampling of ctDNA easy. Although CSF ctDNA detection cannot replace tissue pathological diagnosis at present, it would be a good supplement to monitoring gliomas together with radiographical and clinical parameters.

For the small cohort size analyzed in this study, we could not come up with very convincing results relating ctDNA to diagnosis of tumor subtypes and prognosis of gliomas. Following a large sample study in the future, analysis of CSF ctDNA could be very hopeful to provide preoperative information including diagnosis of tumor subtypes and prognosis prediction.

## Conclusions

In summary, we identified that targeted sequencing of CSF ctDNA could be applied to detecting features of gliomas. CSF ctDNA is a potential source to gain diagnostic information, monitor disease progression and response to clinical treatment.

## Conflict of interest statement

General Financial Grant from the China Postdoctoral Science Foundation (Grant No: 2015 M582856); Science Research Foundation of Hunan Provincial Education Department (Grant No: 13B067); National Natural Science Foundation of China (Grant No: 81272804, 81672824); National Key Technology Research and Development Program of the Ministry of Science and Technology of China (Grant No: 2014BAI04B02).

## Ethical approval

All procedures performed in studies involving human participants were in accordance with the Institutional Ethics Committee of the

Chinese PLA General Hospital and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## Informed consent

The study was approved by the Institutional Ethics Committee of the Chinese PLA General Hospital, China.

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## Brief abstract

CtDNA from CSF could provide supplementary diagnostic information on gliomas.