Research article

Cerebrospinal fluid-derived circulating tumor DNA is more comprehensive than plasma in NSCLC patients with leptomeningeal metastases regardless of extracranial evolution

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ABSTRACT

Introduction: Metastases to the central nervous system (CNS) are devastating neurological complications. Circulating cell-free tumor DNA (ctDNA) from cerebrospinal fluid (CSF) better represents genomic alterations in CNS tumors compared to plasma (PLA). However, the clinical value of cerebrospinal fluid (CSF) as a liquid biopsy medium in non-small cell lung cancer patients with leptomeningeal metastases (NSCLC-LM), regardless of extracranial evolution, remains unclear.

Patients and methods: 14/48 NSCLC-BM patients and 34/48 NSCLC-LM patients were enrolled in this study. The genomic mutation profiles in CSF and matched PLA for patients with single CNS progression (cohort one, N = 22) or intracranial progression with extracranial disease progression (cohort two, N = 12) were compared. ctDNA in the CSF and simultaneously collected PLA was subjected to next-generation target sequencing (NGS) of 168 cancer-relevant genes.

Results: CSF is more comprehensive of driver genomic mutation profile than in matched PLA in patients with a single CNS progression. In addition, potential prognostic markers are much higher in CSF samples than related PLA. For example, the detection rate of *EGFR*-amp in CSF was more than twice of the rate in matched PLA. Moreover, *CDKN2A/B*, *PIK3CA/G*, *CDK4/6*, and *MET* were detected uniquely in CSF samples and, all of these genetic mutations were correlated with poor outcomes.

Almost all genetic mutation profiles detected in PLA could be seen in matched CSF samples in cohort two. With the driver genes, such as *EGFR* or *ALK,* have a higher detection rate in CSF compared to PLA. Moreover, the potential survival maker genes *CDK4/6* (6/12, 50%), *CDKN2A/B* (2/12, 17%), *EGFR*-amp (1/12, 8%), *MET* (1/12, 8%), and *PIK3CA* (1/12, 8%) were unique to the CSF samples.

Conclusion: For NSCLC -LM patients, regardless of single intracranial progression or intracranial progression simultaneously with extracranial evolution, CSF is superior to matched PLA.

1. Introduction

Metastases to central nervous system (CNS) is increasing during treating patients with non-small cell lung cancer (NSCLC). The incidence of CNS metastases in NSCLC patients positive for epidermal growth factor receptor (*EGFR)* mutations is 24% at initiation, with the incidence of CNS failure increasing by 40–55% during the disease [1]; 10.4% of patients with NSCLC present with brain metastases (BM), and patients with

adenocarcinoma have a higher frequency of BM [2]. The metastases to leptomeningeal metastases (LM) are 3.4%–3.8% in NSCLC patients, with it being more frequent in *EGFR* mutant NSCLC patients, accounting for 9.4% of NSCLC cases [3].

Multiple studies have been conducted to discuss the dissemination and evolution of the tumor genome from accessible body fluids such as plasma (PLA) and cerebrospinal fluid (CSF). Mok et al. used blood-based circulating tumor DNA (ctDNA) to detect dynamic changes in *EGFR*

mutations and demonstrated that blood-based *EGFR* mutation analysis could predict clinical outcomes [4]. However, for patients with CNS metastases, PLA samples have limited value in establishing the genomic characterization of intracranial lesions. Previous studies identified that CSF ctDNA presents more complete genomic alterations than PLA ctDNA in CNS tumors [5] and can provide the gene profile associated with a worse prognosis. However, the genetic modifications in CSF with single CNS dissemination and evolution or CNS progression simultaneous with systemic disease progression remain incompletely defined.

Here, we performed next-generation target sequencing (NGS) on paired CSF/PLA samples from 48 NSCLC patients with progressive CNS metastases. Our results showed that ctDNA from CSF is better compared to paired PLA, both in single CNS progression and CNS progression simultaneously with systemic disease progression.

2. Materials and methods

2.1. Study population

We retrospectively screened 186 consecutive patients with NSCLC with CNS metastases from June 2019 to May 2021. Patients with no matched CSF or PLA samples were excluded from this study, and 48 patients diagnosed with BM or LM were enrolled. Patients with LM were divided into two subgroups: those with single CNS progression (cohort one) and those with CNS progression simultaneous with systemic disease progression (cohort two). Positive results in CSF cytology or gadolinium-enhanced brain magnetic resonance imaging (MRI) are used to diagnose LM, and BM was diagnosed with clinical signs or symptoms in addition to brain MRI or computed tomography (CT). Approximately 10 ml CSF was obtained by lumbar puncture for clinical use, and blood samples were collected at the same time. According to the standard description, cell-free DNA (cfDNA) from the CSF and blood was isolated using a QIAamp Circulating Nucleic Acid Kit (Qiagen). NGS of 168 cancer-related gene panels (Burning Rock Biotech, Guangzhou, China) was performed using CSF and PLA samples. The preparation of NGS samples was strictly according to the instructions, and compared the sequencing data to the reference human genome (hg19). The enrolled patients in our institute provided written informed consent, and the study protocol was approved and get through by the Research Ethics Committee of Guangdong Sanjiu Brain Hospital.

2.2. Statistical analysis

Paired Wilcoxon tests were used for comparison. P-values were two-sided and deemed significant if p-value was no less than 0.05. SPSS (version 22.0; SPSS) was performed in statistical analyses in this study. GraphPad Prism (version 8) and R statistical software (version 4.04) made graphs.

3. Results

3.1. Clinical characteristics of 48 NSCLC patients with CNS metastasis

We retrospectively screened 186 consecutive NSCLC patients with progressive CNS disease at our institution. Among these patients, forty-eight patients were ultimately included in the present study, all of whom (100%) had matched PLA samples (Figure 1). BM was observed in 14/48 (29 %) patients in the study cohort, and the remaining were LM, accounting for 34/48 (71%) of the cohort. To further analyze the clinical utility of CSF and matched PLA in patients with LM, patients with single CNS progression were defined as cohort one, and cohort two consisted of patients with both intracranial and extracranial evolution. The patients with BM median age were 59 years (range, 36–71 years). For patients with LM, the median ages were 54 years (range, 38–74 years) and 51 years (range, 34–68 years) in cohorts one and two, respectively. *EGFR* mutations were confirmed in 11/14 (79%) of patients with BM, including 4/14 (29%) harboring the exon 19 deletion (Del 19) and 7/14 (50%) who carried the exon 21 mutation (L858R). Besides the *EGFR* mutations,
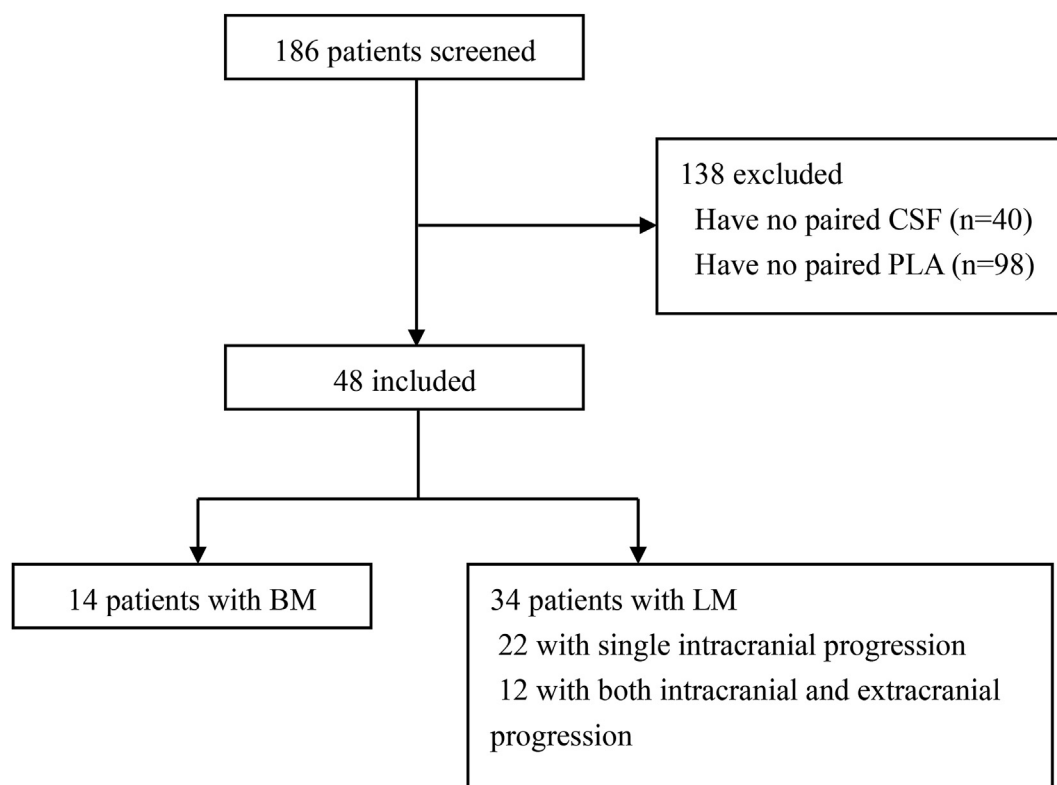
Figure 1. Flowchart of all 48 NSCLC patients with CNS metastases who were included in this study. 14 patients had progressive BM and 34 had progressive LM. NSCLC, non-small-cell lung cancer; BM, brain metastases; LM, leptomeningeal metastases.

two patients had wild-type, and anaplastic lymphoma kinase (*ALK*) was identified in one patient. The *EGFR* mutations, Del 19 and L858R, were detected in 7/22 (32%) and 9/22 (41%) in cohort one, and 3/12 (25%) and 3/12 (25%) in cohort two, respectively; 20 insertions were found in 2/22 (9%) patients in cohort one.

Apart from the *EGFR* mutations, *ALK* and *ROS1* were seen in two patients and a patient in cohort one, respectively; *ALK* was found in two patients in cohort two, with no evidence of *ROS1* in cohort two. Interestingly, of the 2/34 (6%) patients with co-occurring *EGFR, EGFR* 21 L858R and 18 mutations were detected in one patient, and *EGFR* 21 L858R and 25 mutations in another. The characteristics of the 48 NSCLC patients with CNS metastases are summarized in Table 1. Typical gadolinium-enhanced brain MRI diagnosed BM in 14/14 (100%) patients, LM was diagnosed by cytology in 28/34 (82%) patients, and the remaining 6/34 (18%) had typical symptoms and imaging findings. All the patients in this study had matched PLA samples.

3.2. Gene profiling of NSCLC-LM in matched CSF/PLA samples

Prior research has reported that CSF ctDNA is superior to matched plasma in brain lesions [6, 7]. Our study found that ctDNA in CSF is more comprehensive than matched PLA. *EGFR* L858R mutations were shown in 31% of the 34 paired CSF/PLA samples, and *EGFR* 19 deletions were found in 21% of the samples. Rare activating *EGFR* mutations, including exon 18 mutations, 20 insertions, L861Q, and 25 mutations, were found in 10% of patients in the matched CSF/PLA samples. The driver gene *ALK* was found in 7% of matched samples. Previous studies showed that the detection rate of *EGFR* amplification (*EGFR*-amp) was higher in patients with *EGFR*-TKI resistance [8]. *EGFR*-amp was found in 15% of paired CSF/PLA samples, with the detection rate being much higher in CSF

(9/34, 26%) than in the matched PLA samples (1/34, 3%). For patients with *EGFR* variants, the co-existence of *MYC* is associated with poor outcomes [9]. In our findings, *MYC* was observed in 4% of matched samples, with 2/34 (6%) and 1/34 (3%) in matched CSF and PLA, respectively. Genomic characteristics such as *CDK4, CDKN2A, CDKN2B, PIK3CG,* and *MET* were reported to have a poor prognosis, and these genes were more frequently to be identified in CSF samples, detected in 9%, 12%, 4%, 4%, and 4% of the samples, respectively (Figure 2).

The colors for each mutation type are indicated in the legend to the right of the plot. The bar on the right shows the specific mutations, and the left demonstrates the names of the detected genes, while the upper bar plot shows the genetic mutation per patient and MAF in the matched samples. Finally, the bottom bar is annotated according to sex and sample type.

3.3. Comparison of the detection rate of driver and accompanying genes in NSCLC-LM patients with single CNS progression

Paired CSF and plasma samples were obtained serially from 22 patients with single CNS progression. *EGFR, ALK,* or *ROS1* driver mutations were detected in all samples of the matched samples. Modifications of an *EGFR* driver were seen in 18/22 (82%) of the CSF samples, which was much higher than the rate in matched PLA group (10/22, 45%). The *EGFR* mutations included 7/22 Del 19 (32%), 9/22 21 L858R (41%), and 2/22 20 insertion (9%) in CSF, and 2/22 Del 19 (9%), 6/22 21 L858R (27%), and 2/22 20 insertions (9%) in PLA. *ALK* was detected in two patients, *ROS1* in one patient in the CSF sample group, and there was no evidence of driver genes, including *ALK* or *ROS1*, in the matched PLA samples (Figure 3A). In addition, the driver gene mutant allelic frequency (MAF) in the CSF was much taller than in the matched PLA ($P < 0.01$) (Figure 3B).

The most frequent accompanying alteration in the pair-wise samples was *TP53*, which was found in 17/22 (77%) and 4/22 (18%) of paired CSF and PLA samples, respectively. Previous studies have shown that *EGFR*-amp is more frequently detected in patients with exon Del 19 mutations. Patients with *EGFR*-amp treated with erlotinib were associated with a significantly better prognosis. This illustrates the importance of *EGFR*-amp and its potential as a prognostic marker for NSCLC treated with erlotinib [10]. *EGFR*-amp demonstrated a much higher rate in paired CSF samples (8/22, 36%) than in the PLA samples (1/22, 5%). Overexpression of *TBX3* is uniquely associated with tumor size, and inactivation of *PTEN* is notably associated with poor survival in NSCLC patients [11, 12]. The detection rates of *TBX3* and *PTEN* in CSF were almost double those of the paired PLA samples (Figure 3C), making the liquid CSF medium an ideal candidate for categorizing patients treated with *EGFR*-TKI.

Most previous studies reported that the genetic profiles of *CDKN2A/B, PIK3CA/G,* and *CDK4/6* were associated with poor outcomes [9]. In addition, preclinical and clinical evidence suggests that hepatocyte growth factor receptor *(MET)* amplification is a potential pathway for *ALK* target therapy failure, making *MET* a potential therapeutic target in NSCLC (13). Gene signatures, including *CDKN2A/B* (9/22, 41%), *PIK3-CA/G* (4/22, 18%), *CDK4/6* (2/22, 9%), and *MET* (2/22, 9%) (Figure 3D), were exclusively detected in CSF samples, suggesting that CSF delivers a more comprehensive gene alternation profile in patients with single intracranial progression.

A, Bar charts show the detection rates of the driver genes *EGFR, ALK,* and *ROS1* in paired CSF/PLA samples. B, Box charts show the rates of MAF in the matched CSF/PLA samples. C, D, Bar charts show the detection rates of the accompanying genes in paired CSF/PLA samples. *P* values were considered significant if < 0.05.

3.4. ctDNA from CSF is better than plasma in NSCLC-LM patients with both intracranial and extracranial progression

To further compare the ctDNA levels in the CSF and matched plasma samples for NSCLC-LM patients with intracranial progression and
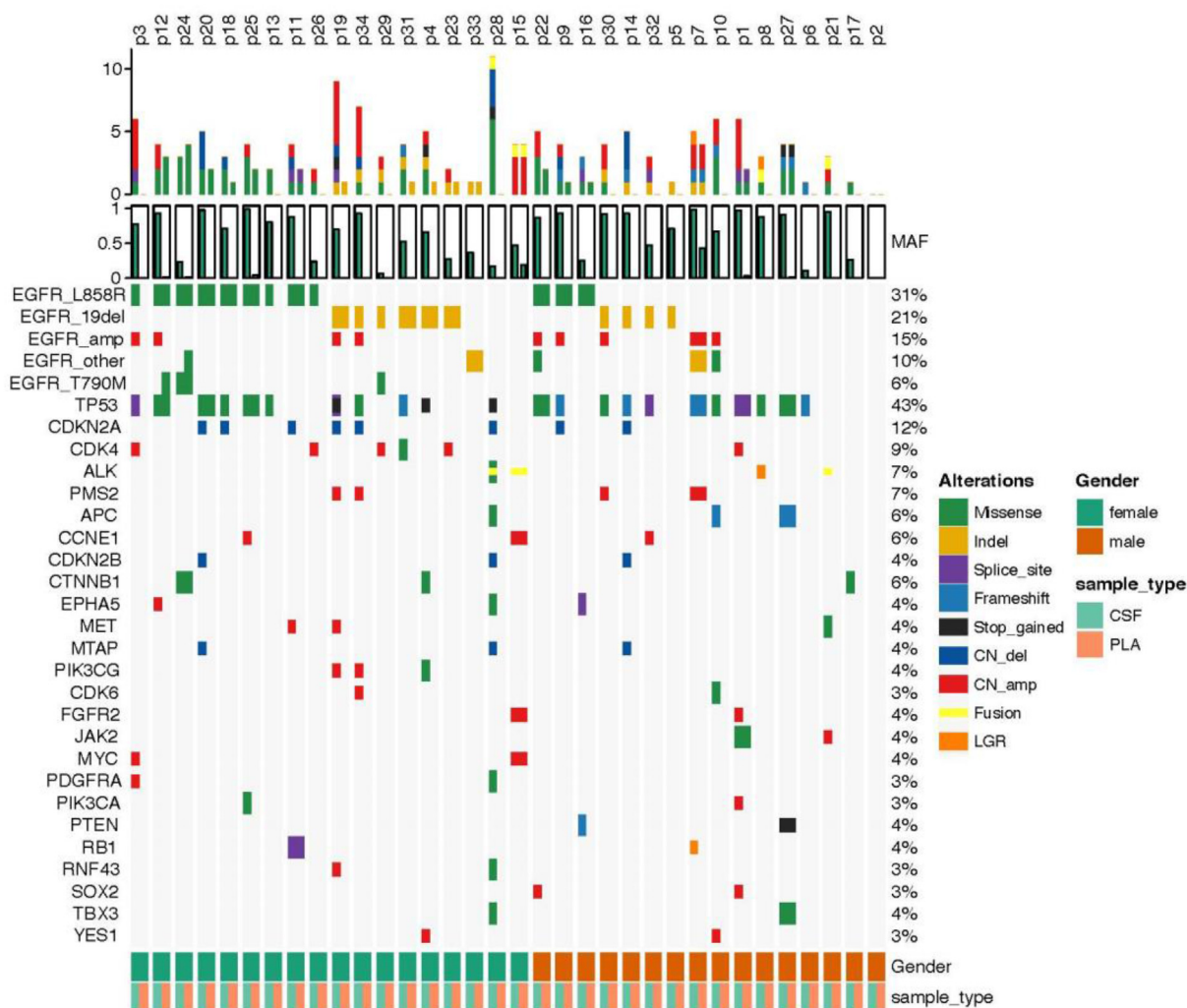
Table 1. Clinical characteristics of 48 NSCLC patients with CNS progression.

Characteristics	NSCLC-BM No. (%)	NSCLC-LM No. (%)	
	14 (29)	34 (71)	
		Cohort one	Cohort two
No. Of patients		22 (65)	12 (35)
Median age year (range)	59 (36–71)	54 (38–74)	51 (34–68)
Gender			
Male	8 (57)	11 (50)	5 (42)
Female	6 (43)	11 (50)	7 (58)
Histology			
Adenocarcinoma	14 (100)	22 (100)	12 (100)
EGFR mutation status			
19 Del	4 (29)	7 (32)	3 (25)
20 insertions		2 (9)	
21 L858R	7 (50)	9 (41)	3 (25)
21 L861Q			1 (8)
others[a,b]		1 (5)	1 (8)
ALK	1 (7)	2 (9)	2 (17)
ROS1		1 (5)	
Negative	2 (14)	1 (5)	3 (25)
Diagnosis of BM			
Typical brain imaging	14 (100)		
Diagnosis of LM			
Positive CSF cytology		17 (77)	11 (92)
Typical brain imaging		5 (23)	1 (8)
Matched PLA			
YES	14 (100)	22 (100)	12 (100)

EGFR, Epidermal Growth Factor Receptor; *ALK*, Anaplastic lymphoma kinase; *ROS1*, Ros oncogene 1.

[a] one patient has co-existing *EGFR* 21 L858R and 25 mutations.
[b] one patient has co-existing *EGFR* 21 L858R and 18 mutations.

Figure 2. Oncoprint of the distribution of gene aberrances in matched CSF/PLA samples of 34 NSCLC-LM patients.

together with extracranial involvement, we performed targeted panel sequencing. The somatic single-nucleotide variants (SNVs) from each patient were classified into CSF unique, PLA unique, or shared. There was one patient with the same negative results in both CSF and PLA samples, we focused on the remaining 11 patients to ensure consistency in this study. PLA unique, CSF unique, and shared SNVs presented in 17%, 33%, and 50% of one patient (P10), respectively. Additionally, all mutations detected in the PLA samples were also identified in matched CSF samples (Figure 4A), and 5/11 (45%) patients had negative results in matched PLA samples and provided no evidence for further analysis.

In addition, driver genes, such as *EGFR* or *ALK*, were much higher in CSF than in matched PLA. *EGFR* mutations included 3/12 L858R (25%), 3/12 exon Del 19 (25%), and 1/12 L861Q (8%) in CSF, and 2/12 L858R (17%) and 2/12 exon Del 19 (17%) in PLA (Figure 4B). The potential survival maker genes *CDK4/6* (6/12, 50%), *CDKN2A/B* (2/12, 17%), *EGFR*-amp (1/12, 8%), *MET* (1/12, 8%), and *PIK3CA* (1/12, 8%) were unique to the CSF samples (Figure 4C). Therefore, we concluded that CSF is superior to matched PLA and demonstrates a more representative gene profile in patients with CNS progression and simultaneous systemic disease progression.

A, The somatic SNVs from each patient were grouped into PLA unique, CSF unique, or shared; B, Bar charts show the detection rates of driver genes in patients with intracranial progression and together with extracranial evolution; C, Bar charts show the detection rates of potential

survival markers in paired CSF/PLA samples in patients with both intracranial and extracranial disease progression.

3.5. The limited value of CSF in NSCLC-BM

We compared them to previous biopsy specimen results to further analyze the genetic alternation in the CSF and paired PLA samples. Overall, all NSCLC-BMs were tested for their *EGFR/ALK* gene status. In addition, 2/11 (18%) had wild-type, 9/11 (82%) patients were confirmed to have *EGFR/ALK* mutations in the primary (metastasis) tumor tissue, 7/14 (50%) patients had *EGFR* mutations in PLA, including 4/14 (29%) patients with an exon 21 mutation (L858R), and 3/14 (21%) patients harbored an *EGFR* Del 19 mutation. The driver gene of the *EGFR/ALK* gene was observed in 5/14 (36%) of the matched CSF, including *EGFR* Del 19, *EGFR* 21, and *ALK,* seen in 2/14 (14%), 2/14 (14%), and 1/14 (7%), respectively. The driver gene of *EGFR* was found in the PLA of three patients, but there was no positive evidence in the paired CSF. Given the lower detection rate of driver gene mutations in CSF, we found a limited utility of CSF in NSCLC-BM. Unfortunately, one patient had an *EGFR*-positive result in pleural effusion but was finally confirmed to have an *ALK*-positive result in the CSF (Table 2). Although our study had a small sample size, we suggest that ctDNA in matched PLA and CSF complements the diagnosis when tissue samples are inadequate.
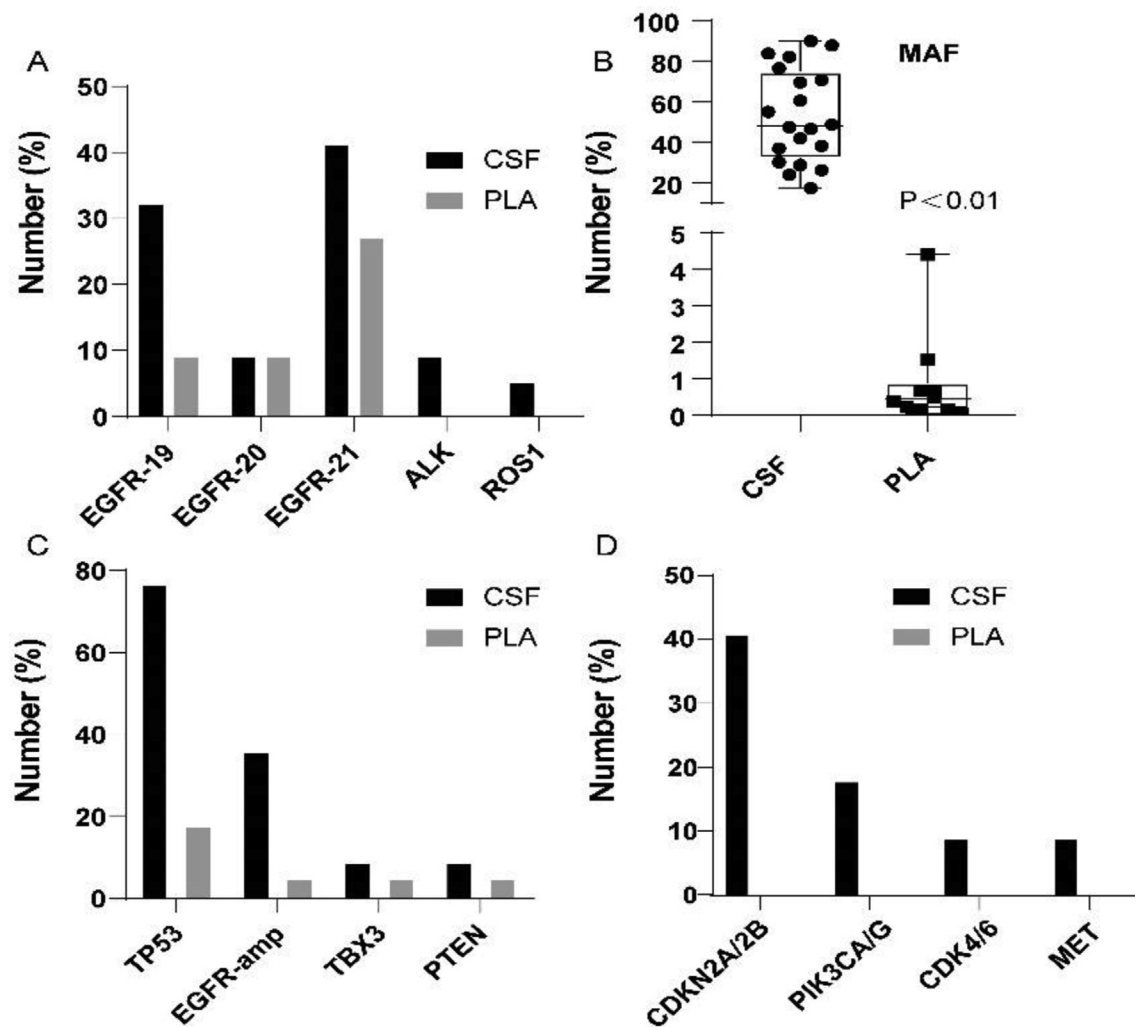
Figure 3. Comparison of the alternation genes in paired CSF and PLA samples in patients with single CNS metastases.

4. Discussion

Dissemination of the CNS is a devastating neurological complication. Owing to improved diagnostics, BM and LM are increasingly common in patients with NSCLC [14]. Previous studies have shown that fluids with a closer site enable more comprehensive detection of relevant mutations than blood and suggest the routine implementation of these 'nonblood' fluids for patients with progressive disease [15]. In addition, the molecular profiles in the CSF from NGS were mainly similar to the tissue biopsy results. Interestingly, CSF has been proven to reveal additional mutations and exhibits higher sensitivity than PLA-based molecular signatures in patients with CNS metastases [7, 16]. However, it remains unclear whether CSF is a better representation of the genomic files than PLA in a single CNS evolution or together with the systematic progression of the disease.

In our findings, CSF demonstrated more comprehensive driver gene profiling in a single CNS progression. The driver gene of *EGFR* was much higher in CSF (82%) than in matched PLA (45%); *ALK* was seen in two patients, *ROS1* was detected in one patient in the CSF sample, and no evidence of the driver genes was seen in matched PLA. In addition, the MAF driver gene was much higher in the CSF than that in the matched PLA ($P < 0.01$). The same trend was observed in patients with simultaneous CNS evolution and systematic progression. Driver genes, such as *EGFR* or *ALK,* were more frequently seen in CSF samples than in matched PLA, with 7/12 (58%) and 4/12 (33%) in CSF, and 2/12 (17%) and 1/12 (8%) in PLA, respectively.

Preclinical and clinical evidence suggests that *MET* amplification is a potential pathway for *ALK* target therapy failure [13]. In our study, *MET* was seen in 2/22 (9%) and 1/12 (8%) patients with single CNS intracranial and extracranial disease progression, respectively. *MET* was found exclusively in CSF samples, which gives CSF a predictive value for target therapy failure. *EGFR* amplification is a known potential pathway for *EGFR*-TKI resistance [8]. In addition, *EGFR*-amp was detected in 8/22 (36%) of CSF samples with only CNS evolution and in 1/12 (8%) of patients with simultaneous progressive intracranial and extracranial disease. In analyses, gene signatures consisting of *PIK3CG*, *CDK4*, *CDK6*, and *CDKN2A* mutations have shown a significant negative association with disease prognosis [9, 17]. Gene alterations, such as *CDKN2A/B*, *CDK4/6*, and *PIK3CA/G,* were seen exclusively in CSF samples, regardless of single intracranial progression or intracranial evolution simultaneous with extracranial development. Consistent with these preclinical predictions, clinical analysis based on ctDNA from CSF can be a potential prognostic biomarker for patients with single intracranial metastases or intracranial progression and extracranial dissemination.

More intriguingly, the predictive value of CSF samples in NSCLC-LM or NSCLC-BM is substantially difference. Although the detection rate of driver genes, such as *EGFR* or *ALK,* was 82% in primary (metastasis) tumor tissues, 50% in PLA, and 36% in matched CSF, the lower detection rate of driver gene mutation in CSF suggested the limited utility of this medium in patients with BM. A detail still needs to improve in our study is that small pair-wise CSF/PLA samples were collected for analysis, and a more extensive sample size study is required to validate our findings.
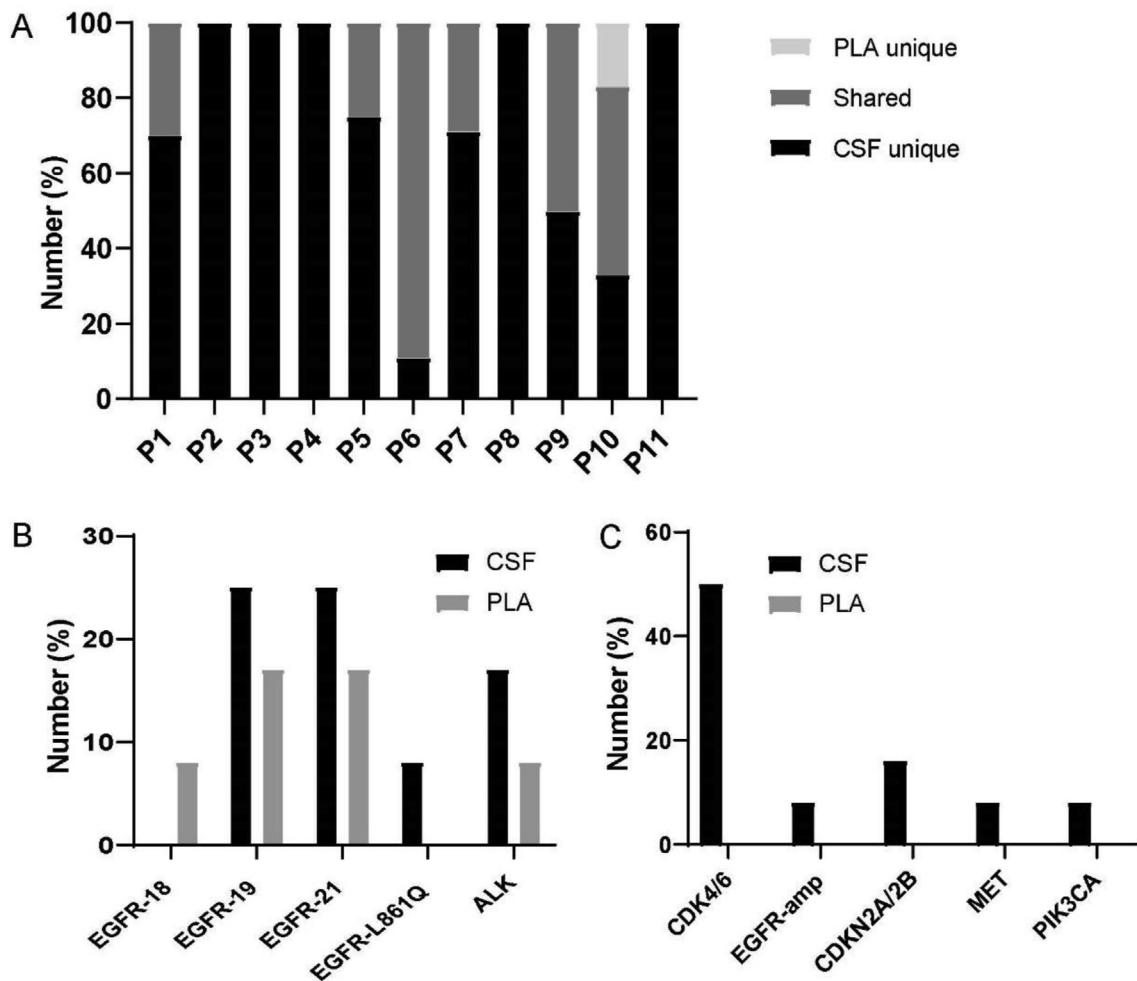
Figure 4. CtDNA from CSF is superior to matched PLA in patients with CNS progression with simultaneous systemic disease progression.

Table 2. *EGFR/ALK* mutations in matched primary (metastasis) tissue/CSF/PLA in patients with NSCLC-BM.

Patient	Tissue/Pleural Effusion	BM-CSF	PLA
P1	*EGFR* 19Del	Negative	*EGFR* 19Del (39.66%)
P2	*EGFR* L858R (36.3%)	Negative	*EGFR* L858R (2%)
P3	-	*EGFR* 19Del (3.37%)	*EGFR* 19Del (7.13%)
P4	*EGFR* L858R	Negative	Negative
P5	-	Negative	*EGFR* L858R (0.70%)
P6	*EGFR* L858R	Negative	Negative
P7	PE *EGFR* L858R	*ALK* EML4 (12.22%)	Negative
P8	-	*EGFR* 19Del (47.48%)	*EGFR* 19Del (6.56%)
P9	*EGFR* L858R	*EGFR* L858R (22.61%)	*EGFR* L858R (9.78%)
P10	Negative	Negative	Negative
P11	*EGFR* L858R	*EGFR* L858R (10.82%)	*EGFR* L858R (0.21%)
P12	*EGFR* L858R	Negative	Negative
P13	*EGFR* 19Del	Negative	Negative
P14	Negative	Negative	Negative

NOTE: mutation status of *EGFR*, and *ALK* in primary (metastasis) tumor, CSF and matched PLA samples from patients P1-14, as detected by panel sequencing.

Our results are consistent with previous evidence. Moreover, in our study, patients with NSCLC-LM were classified into two subgroups: single intracranial progression or intracranial progression and extracranial evolution. Our results indicate that CSF-derived ctDNA is superior to PLA ctDNA, regardless of extracranial development. Our results further confirmed and extended previous evidence in subgroups.

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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