Original Article

Cell-free DNA from cerebrospinal fluid can be used to detect the *EGFR* mutation status of lung adenocarcinoma patients with central nervous system metastasis

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Background: EGFR tyrosine kinase inhibitors (TKIs) have revolutionized the therapeutic approach for *EGFR* mutated patients. However, acquired resistance to EGFR-TKI therapy is unavoidable. Repeat biopsy cannot be used, and peripheral blood detection shows a low positive rate in cases of brain-only disease progression.

Methods: Droplet digital polymerase chain reaction (PCR) (ddPCR) was performed on the plasma and cerebrospinal fluid (CSF) samples of 79 lung adenocarcinoma (LUAD) patients with *EGFR* mutations and central nervous system (CNS) metastasis. The differences in the *EGFR* mutation status between the paired plasma and CSF samples were assessed, and the role of CSF testing as a predictor of overall survival was evaluated.

Results: The CSF of patients with neurological symptoms, EGFR-TKI treatment, or leptomeningeal metastasis (LM) had a significantly higher positive rate of *EGFR* mutation compared to the plasma samples (P=0.001, P=0.035, P=0.019, respectively). Moreover, *EGFR* mutation status in CSF was consistent with neurological symptoms and LM (kappa =0.455, P<0.001; kappa =0.508, P<0.001; respectively). For the patients with brain metastasis, *EGFR* mutation-positive rate in CSF samples was lower than that in plasma samples (28.3% *vs.* 64.2%, P<0.001), while the patients with LM had the opposite result (84.6% *vs.* 38.5%, P=0.004). Moreover, patients with *EGFR* mutation in their CSF experienced worse survival [hazard ratio (HR) =2.93, 95% confidence interval (CI): 1.45–5.92; P=0.003, P_{adjust} <0.0001].

Conclusions: The *EGFR* mutation status of CSF was different from that of plasma and is correlated with patient prognosis. CSF could be helpful in detecting the *EGFR* mutation status of patients, particularly in cases of LM.

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Keywords: Non-small cell lung cancer (NSCLC); leptomeningeal metastasis (LM); liquid biopsy; cerebrospinal fluid (CSF); *EGFR* mutation

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Introduction

Lung cancer, with the highest rate of cancer-related mortality worldwide (1), is among the most common human malignancies. Non-small cell lung cancer (NSCLC) accounts for approximately 90% of all global lung cancer cases (2). Epidermal growth factor receptor (EGFR)-activating mutations, such as L858R and exon 19 deletion (E19Del), have been found in approximately 10–15% of NSCLC patients in Western countries and 40–55% of patients in Asian countries (3). NSCLC patients with *EGFR* mutations have a significantly high risk of developing metastasis of the central nervous system (CNS), including leptomeningeal metastasis (LM) and brain metastasis (BM), which is associated with poor prognosis and compromised quality of life (4). The combined incidence of BM and LM in NSCLC patients is more than 50% (5-7). For NSCLC patients with BM and LM who receive EGFR tyrosine kinase inhibitor (TKI) therapy, the median overall survival (OS) is 10.3–16.2 months (8) and 4.5–11 months (9,10), respectively, which is markedly shorter than that for patients without CNS metastasis. EGFR-TKIs have ushered in a new era of targeted therapy in NSCLC and have dramatically improved the progression-free survival (PFS) of patients who harbor EGFR-activating mutations (11-17). Nevertheless, acquired resistance to EGFR-TKI therapy is inevitable. Therefore, rebiopsy is needed to detect secondary resistance mechanisms in order to develop a new treatment strategy.

With its many advantages, the detection of tumor-specific genetic alterations in body fluids has become a supplement to or even a replacement for established tissue-based tumor diagnostics, and thus has garnered considerable attention in academic research and the medical testing industry (18). Moreover, circulating tumor DNA (ctDNA) has been widely used to detect *EGFR* mutations status in patients who develop resistance to targeted therapy (19). ctDNA derives from tumors or circulating tumor cells (CTCs), and constitutes a fraction of the cell-free DNA (cfDNA) that is extracted from body fluids, which also contain DNA derived from non-transformed cells (20). ctDNA has been shown to be capable of accurately reflecting tumor-specific genomic alterations and can be used to monitor tumor progression, response to treatment, and relapse (21). Therefore, ctDNA testing might be an ideal detection tool in cancer patients. Moreover, cerebrospinal fluid (CSF) has shown utility as a liquid biopsy medium for the gene expression profiling of NSCLC patients with LM, contributing to more sensitive and effective diagnoses (22). However, due to the blood-brain barrier (BBB), the *EGFR* mutation status in CSF is not consistent with that in plasma, and controversy still surrounds which of the two is a better indicator of *EGFR* alterations in patients with CNS metastasis (23,24). Several studies have reported tumor-associated alterations being detectable in the CSF ctDNA of patients with various primary or metastatic brain tumors, while little ctDNA was found in the plasma of these patients (25-28). These findings indicate that CSF has a stronger capability to recapitulate the genomic profile of CNS tumors/metastatic sites than does plasma.

In the present study, we performed droplet digital polymerase chain reaction (ddPCR) on paired plasma and CSF samples from 79 lung adenocarcinoma (LUAD) patients with *EGFR* mutations and CNS metastasis. Through comparing the *EGFR* mutation status of the plasma and CSF samples, we aimed to determine the ability of CSF compared to plasma in detecting *EGFR* mutation in patients with LUAD and CNS metastasis.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/tlcr-21-62).

Methods

Patients

This study included 79 LUAD patients with CNS metastasis harboring *EGFR* mutations in tumor tissue (L858R or E19Del). All patients were diagnosed at Henan Cancer Hospital between 2012 and 2018. The last follow-up took

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Table 1 Characteristics of 79 patients with brain metastases and leptomeningeal metastases

Characteristics	Brain metastases			Leptomeningeal metastases		
	EGFR-TKI naïve	EGFR-TKI treated	Total	EGFR-TKI naïve naivenainaïvenaive	EGFR-TKI treated	Total
Total	24	29	53	5	21	26
Sex						
Male	10	17	27	1	11	12
Female	14	12	26	4	10	14
Age						
<60 years	11	17	28	3	13	16
≥60 years	13	12	25	2	8	10
Smoking status						
No	18	20	38	4	15	19
Yes	6	9	15	1	6	7
Neurological symptoms						
No	11	12	23	0	3	3
Yes	13	17	30	5	18	23
Chemotherapy						
No	19	6	25	4	6	10
Yes	5	23	28	1	15	16
Local CNS RT						
No	23	23	46	5	18	23
Yes	1	6	7	0	3	3
Number of metastases[a]						
Single	8	1	9	1	4	5
Multiple	16	28	44	4	15	19

[a], two patients with leptomeningeal metastases and EGFR-TKI treatment were negative by MRI but positive by ThinPrep cytologic test. TKI, tyrosine kinase inhibitors; CNS, central nervous system; RT, radiotherapy.

place on April 20, 2019, and six patients were lost to follow-up. CNS metastasis was confirmed in all patients by brain magnetic resonance imaging (MRI) and ThinPrep cytologic testing (TCT), which were performed according to standard procedures by experienced radiologists and pathologists. Patient clinical data including sex, age, smoking status, chemotherapy, local CNS radiotherapy, and history of EGFR-TKI treatment were collected from the electronic medical records of Henan Cancer Hospital (*Table 1*).

All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This study was conducted in accordance with the ethical guidelines of the United States' common rule, and the protocol was approved by the Research Ethics Committee of Henan Cancer Hospital. All patients signed informed consent forms.

Sample processing and DNA extraction

After the CNS metastases were diagnosed, CSF (10 mL) and peripheral blood (15 mL) were collected by lumbar puncture and blood sampling respectively (*Figure 1*). The collected samples were preserved in EDTA tubes (SANLI) for the isolation of cfDNA. The DNA isolation procedure

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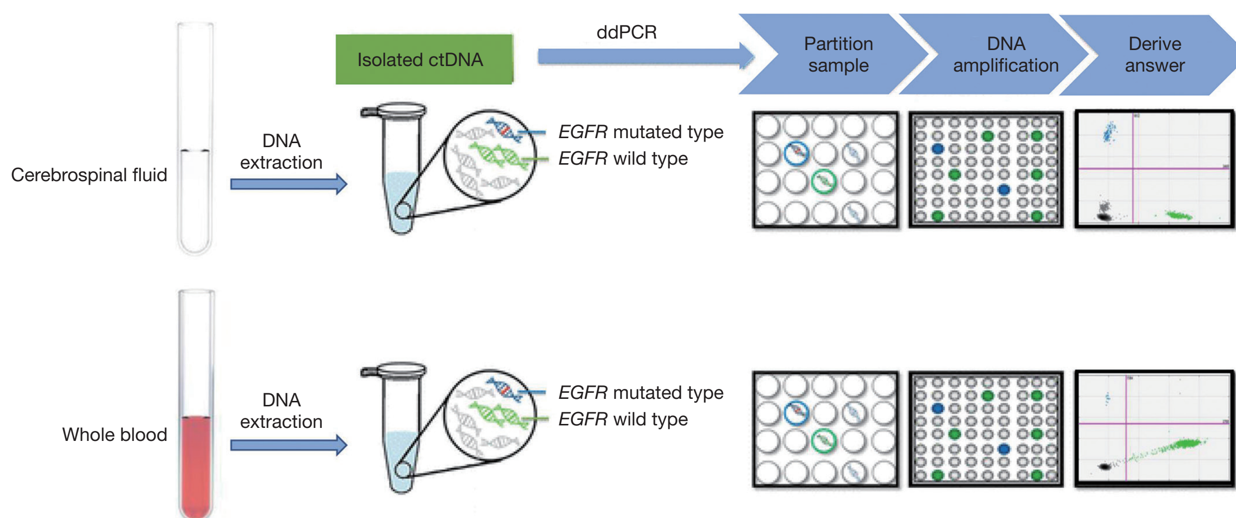

Figure 1 The workflow of the present study. ctDNA, circulating tumor DNA; ddPCR, droplet digital polymerase chain reaction.

was performed within 2 h after sample collection. To collect plasma, the peripheral blood samples were subjected to centrifugation. Then, the plasma and CSF were first centrifuged at 2,000 ×g for 10 min at 4 °C and again at 16,000 ×g for 10 min at 4 °C. The QIAamp Circulating Nucleic Acid Kit (#55114, QIAGEN, Hilden, Germany) was used to capture cfDNA according to the manufacturer's instructions. The quality and concentration of the DNA were assessed using a Qubit dsDNA HS assay (#1204008450, Life Technologies, Carlsbad, CA, USA). DNA was also isolated from the CSF sediment for the ddPCR test. The extracted DNA was stored at −20 °C for later analysis.

ddPCR test

A QX200 digital PCR system (Bio-Rad Laboratories, Hercules, CA, USA) was used to conduct the ddPCR assays (29). For exon 19 deletions, p.L858R and p.T790M were detected using a human-EGFR ddPCR detection kit (#CB240005, YuanQi) according to the manufacturer's instructions. The PCR program was as follows: 95 °C for 10 min; 40 cycles of 94 °C for 15 s and 58 °C for 60 s; 98 °C for 10 min; and 4 °C for 5 min. The reaction volume was set to 40 µL. The reaction temperature was changed at a rate of less than 2 °C/s. After thermal cycling, the amplified samples were loaded into a BioRad reader (#771BR24449, Bio-Rad Laboratories) for quantification. These experiments were performed according to the protocols of the Mutation Detection Best Practices Guidelines supplied by Bio-Rad Laboratories (*Figure 1*).

Statistical analysis

Paired χ^2 (McNemar's test) and kappa tests were utilized to compare the clinical characteristics and *EGFR* mutation status in the plasma and CSF samples from the enrolled patients, and to compare the different subgroups divided according to their clinical data. Wilcoxon signed-rank test was used to evaluate the difference in *EGFR* mutation abundance between paired CSF and plasma samples. The patients' survival probabilities associated with the *EGFR* mutation status of CSF were analyzed by Kaplan-Meier curves and adjusted with clinical variables. Receiver operating characteristic (ROC) curve and time-dependent area under the curve (AUC) analyses were used to evaluate the accuracy of the predictive model by integrating the effects of both clinical variables and the *EGFR* mutation status of CSF on patient survival. All statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA), with the level of statistical significance set at a P value <0.05, unless otherwise indicated.

Results

Patient characteristics

The 79 patients enrolled in this study were diagnosed with advanced LUAD and confirmed to harbor sensitizing *EGFR* mutations, with 25 of these patients being treatment naïve. As shown in *Table 1*, 53 patients were diagnosed with BM and 26 with LM. Furthermore, 50 had a history of EGFR-TKI treatment, 44 patients had received chemotherapy, and

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10 had received local CNS radiotherapy. The median age of the patients was 56 (range, 29–76) years, and 39 patients were male, including 22 who had history of smoking; none of the female patients smoked. Most patients (53 of 79) had neurological symptoms, including dizziness and headache, and most (63 of 77) had more than one metastatic lesion of the CNS.

EGFR gene mutation status in CSF supernatant and sediment

First, to determine whether supernatant or sediment could better represent CSF, McNemar's test and kappa tests were used to compare the *EGFR* mutation status between paired samples of CSF supernatant and sediment from the 79 patients. As shown in Table S1, no statistically significant differences were observed in the positive rate of *EGFR* mutations between the supernatant and sediment of CSF (43.0% *vs.* 34.2%, P=0.092). Moreover, the two samples were generally consistent (kappa =0.656; range, 0.40–0.75; P<0.001). However, a difference was observed in the abundance (mutated alleles/mutated alleles and wild type alleles) of positive supernatant and sediment samples, with the abundance in the supernatant higher than that in the sediment [median (quartile): 33.00% (23.00–49.30%) and 5.30% (2.20–15.00%), respectively].

EGFR gene mutation status and clinical characteristics

To determine if there was any correlation between *EGFR* mutation and clinical features, we compared the *EGFR* mutation status of the patients' plasma and CSF samples with various clinical characteristics. As shown in *Table 2*, a higher frequency of *EGFR* mutation was observed in the plasma samples of never smokers, patients with multiple CNS metastases, patients treated with radiotherapy, and patients without LM (61.4% *vs.* 40.9%, P=0.002; 58.0% *vs.* 40.0%, P<0.001; 58.7% *vs.* 50.0%, P=0.001; and 64.2% *vs.* 38.5%, P=0.015; respectively). No statistically significant differences in *EGFR* mutation status were found for other variables including sex, age, neurological symptoms, chemotherapy treatment history, or EGFT-TKI treatment history (P>0.05). All these characteristics showed poor consistency with *EGFR* mutation status in plasma (kappa <0.4).

For the CSF samples, patients with neurological symptoms (50.9% *vs.* 36.4%, P=0.032), no history of radiotherapy (47.8% *vs.* 40.0%, P<0.001), presence of multiple CNS metastases (including LM) (47.6% *vs.* 35.7%, P<0.001), and an EGFT-TKI treatment history (54.0% *vs.* 34.5%, P=0.035), along with non-smokers (64.2% *vs.* 11.5%, P=0.001) and LM patients (84.6% *vs.* 28.3%, P=0.019), had a higher risk of being *EGFR* mutation-positive. The other variables had no significant correlation with the *EGFR* mutation status of CSF. Moreover, neurological symptoms and LM showed general consistency with *EGFR* mutation status in CSF (kappa =0.455, P<0.001; kappa =0.508, P<0.001; respectively).

EGFR gene mutation status in CSF and plasma samples in different subgroups

To ascertain the difference between CSF and plasma in reflecting the *EGFR* mutations of patients, the *EGFR* mutation status between paired CSF and plasma samples from all patients was compared. As shown in Table S2, there was not a statistically significant difference in the positive rate of *EGFR* mutations between the paired CSF and plasma samples (P=0.360; kappa =–0.081, P=0.466). However, the number of positive CSF samples was significantly higher than that of plasma samples [33.00% (16.80–49.30%) and 2.05% (0.67–14.75%), respectively].

Next, the 79 patients were divided into different subgroups according to their clinical characteristics. The *EGFR* mutation status of the paired CSF and plasma samples from patients in different subgroups was compared. In the BM patient group, the positive rate of *EGFR* mutations was notably lower in the CSF samples than in plasma samples (28.3% *vs.* 64.2%, P<0.001; kappa =0.093, P=0.381), while the opposite result was observed in the LM patient group (84.6% *vs.* 38.5%, P=0.004; kappa =–0.061, P=0.606) (Tables S3,S4). No significant difference between the *EGFR* mutation–positive rate of the CSF and plasma samples was observed in other subgroups, including patients with neurological symptoms (P=0.697), without EGFR-TKI treatment history (P=0.202), with EGFR-TKI treatment history (P=0.777), with *EGFR* E19Del (P=0.821) and *EGFR* 21 exon L858R mutation (P=0.320) (Tables S5-S10).

Different subtypes of EGFR gene mutations in CSF and plasma

Although the positive rates of E19Del and p.L858R mutation were not significantly different between the CSF and plasma samples from any of the patients, as shown in Tables S9,S10, respectively, we questioned whether the

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Table 2 Comparison of *EGFR* mutation status of the patients with different characteristics from plasma and CSF samples of 79 patients

Characteristics	Mutation status in plasma		P	Kappa test		Mutation status in CSF		P	Kappa test	
	Negative (%)	Positive (%)		Value	P	Negative (%)	Positive (%)		Value	P
Total	35 (44.3)	44 (55.7)	–	–	–	42 (53.2)	37 (46.8)	–	–	–
Sex			0.636	–0.014	0.900			0.736	0.115	0.307
Male	17 (43.6)	22 (56.4)				23 (59.0)	16 (41.0)			
Female	18 (45.0)	22 (55.0)				19 (47.5)	21 (52.5)			
Age			0.211	–0.025	0.822			0.880	–0.122	0.278
<60 years	19 (43.2)	25 (56.8)				21 (47.7)	23 (52.3)			
≥60 years	16 (45.7)	19 (54.3)				21 (60.0)	14 (40.0)			
Smoking status			0.002	–0.157	0.100			0.032	–0.120	0.247
No	22 (38.6)	35 (61.4)				28 (49.1)	29 (50.9)			
Yes	13 (59.1)	9 (40.9)				14 (63.6)	8 (36.4)			
Neurological symptoms			0.233	–0.185	0.090			0.001	0.455	<0.001
No	8 (30.8)	18 (69.2)				23 (88.5)	3 (11.5)			
Yes	27 (50.9)	26 (49.1)				19 (35.8)	34 (64.2)			
Chemotherapy			1.000	–0.129	0.253			0.311	0.120	0.278
No	13 (37.1)	22 (62.9)				21 (60.0)	14 (40.0)			
Yes	22 (50.0)	22 (50.0)				21 (47.7)	23 (52.3)			
Local CNS RT			<0.001	–0.073	0.285			<0.001	–0.036	0.643
No	29 (42.0)	40 (58.0)				36 (52.2)	33 (47.8)			
Yes	6 (60.0)	4 (40.0)				6 (60.0)	4 (40.0)			
Number of metastases[a]			0.001	0.057	0.550			<0.001	0.067	0.418
Single	7 (50.0)	7 (50.0)				9 (64.3)	5 (35.7)			
Multiple	26 (41.3)	37 (58.7)				33 (52.4)	30 (47.6)			
EGFR-TKIs			0.451	–0.149	0.181			0.035	0.178	0.094
Naïve	10 (34.5)	19 (65.5)				19 (65.5)	10 (34.5)			
Treated	25 (50.0)	25 (50.0)				23 (46.0)	27 (54.0)			
LM			0.015	–0.218	0.031			0.019	0.508	<0.001
No	19 (35.8)	34 (64.2)				38 (71.7)	15 (28.3)			
Yes	16 (61.5)	10 (38.5)				4 (15.4)	22 (84.6)			

[a], two patients with leptomeningeal metastases and EGFR-TKI treatment were negative by MRI but positive by ThinPrep cytologic test. CSF, cerebrospinal fluid; TKI, tyrosine kinase inhibitors; CNS, central nervous system; RT, radiotherapy; LM, leptomeningeal metastasis.

EGFR mutation status differed between the plasma and CSF samples of patients with BM or LM. Here, three mutations were analyzed: p.L858R in exon 21, E19Dels, and p.T790M in exon 20). As shown in *Table 3*, the positive rates of L858R, E19Dels, and p.T790M mutations in the CSF samples from BM patients were all lower than those in the plasma samples. However, for patients with LM, the positive rates of p.L858R and E19Dels in the CSF samples were both higher than those in the plasma samples, while T790M showed no significant difference.

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Table 3 The *EGFR* mutation types in plasma and cerebrospinal fluid among patients with CNS metastasis

Characteristics	Plasma			Cerebrospinal fluid		
	L858R	19del	T790M	L858R	19del	T790M
Brain metastases						
EGFR-TKI naïve	7/10 (70.0%)	11/14 (78.6%)	1/1 (100.0%)	1/10 (10.0%)	4/14 (28.6%)	0/1 (00.0%)
EGFR-TKI treated[a]	7/15 (46.7%)	10/15 (66.7%)	4/7 (57.1%)	4/15 (26.7%)	7/15 (46.7%)	0/7 (00.0%)
Total	14/25 (56.0%)	21/29 (72.4%)	5/8 (62.5%)	5/25 (20.0%)	11/29 (37.9%)	0/8 (00.0%)
Leptomeningeal metastases						
EGFR-TKI naïve[b]	0/3 (00.0%)	1/3 (33.3%)	0	3/3 (100.0%)	3/3 (100.0%)	0
EGFR-TKI treated	5/14 (35.7.0%)	4/7 (57.1%)	4/9 (44.4%)	11/14 (78.6%)	6/7 (85.7%)	4/9 (44.4%)
Total	5/17 (29.4%)	5/10 (50.0%)	4/9 (44.4%)	14/17 (82.4%)	9/10 (90.0%)	4/9 (44.4%)

[a], 1 patient had both L858R and 19del *EGFR* mutation; [b], 1 patient had both L858R and 19del *EGFR* mutation. TKI, tyrosine kinase inhibitors; CNS, central nervous system.

EGFR gene mutation abundance in CSF and plasma

Since ddPCR allows for the quantitative detection of ctDNA, a Wilcoxon signed-rank test was employed to assess the differences in *EGFR* mutation abundance in paired CSF and plasma samples in positive patients. The median *EGFR* mutation abundances in CSF and plasma samples were 33.50% and 1.75%, respectively, with a median difference of 29.14%. The Wilcoxon signed-rank test results (Z =−3.509 and P<0.001) indicated that the *EGFR* mutation abundance in CSF was significantly higher than that in plasma (Table S11). The comparison of the median *EGFR* mutation abundance in the paired CSF and plasma samples from patients with BM and LM is displayed in *Figure 2*, including three specific mutations: p.L858R in exon 21, E19dels, and exon 20 p.T790M. Overall, in LM patients, the abundance of E19dels, p.L858R, and p.T790M in CSF was higher than that in plasma, while in BM patients, the abundance of p.T790M in CSF was lower than that in plasma.

EGFR gene mutation status and patient survival

Finally, we investigated if there was any correlation between *EGFR* mutation status and OS. Complete follow-up data of 73 of 79 patients were available for analysis. The patients' plasma and CSF samples were separately analyzed using Kaplan-Meier survival analysis, with adjustment for available covariations including sex, age, and smoking status. As shown in *Figure 3A*, the analysis of plasma samples indicated that the difference in median survival time between patients with and without *EGFR* mutations was not significant [19.7 *vs.* 14.4 months, hazard ratio (HR) =1.24, 95%, confidence interval (CI): 0.63–2.45; P=0.531, P_{adjust} =0.819]. However, patients with *EGFR* mutation in their CSF had significantly worse survival than patients without *EGFR* mutation in their CSF (HR =2.93, 95% CI: 1.45–5.92; P=0.003, P_{adjust} <0.0001) (*Figure 3B*).

Then, we assessed the role of the *EGFR* mutation status of CSF in predicting the OS of patients by using time-dependent AUC and ROC curves at the 12th month (or 1-year survival). Compared with the models for age, sex, and smoking status, the time-dependent AUC plotted for additional *EGFR* mutation status in CSF was improved. The AUCs were improved from 70.81–82.40% (P=0.066) for 1-year survival (*Figure 3C,D*).

Discussion

In this study, ddPCR was used to quantificationally detect *EGFR* mutation status in paired plasma and CSF samples from LUAD patients with CNS metastasis. The results showed that for patients with LM, the abundance of *EGFR* mutation in CSF *EGFR* mutation-positive samples was higher than that in plasma, and the *EGFR* mutation status of CSF was significantly different from that of plasma. Further survival analysis indicated that patients with *EGFR* mutation in their CSF had worse survival than patients without *EGFR* mutation in their CSF. These results suggest that for patients with LM, *EGFR* mutations can be detected more effectively in CSF than in plasma samples. This presents a potentially effective alternative or supplement for

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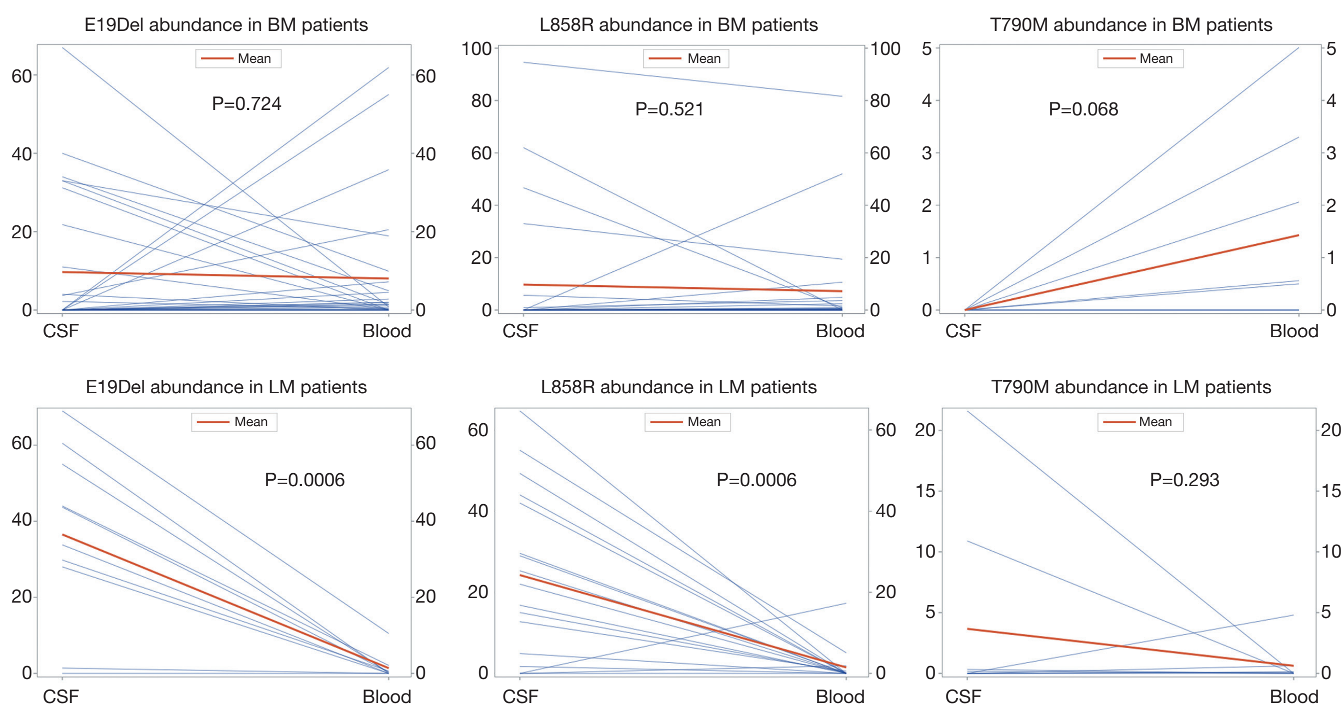

Figure 2 Distribution of different *EGFR* mutation sites in paired plasma and CSF. In leptomeningeal metastasis patients, the abundance of E19del, L858R, and T790M in CSF was higher than that in plasma, while the abundance of T790M in CSF was lower than that in plasma. BM, brain metastasis; LM, leptomeningeal metastasis; CSF, cerebrospinal fluid.

plasma liquid biopsy for the detection of *EGFR* mutations in patients with CNS metastasis. Furthermore, the *EGFR* mutation status of CSF could serve as a biomarker for predicting the OS of patients with CNS metastasis.

Next-generation sequencing (NGS) can be used to monitor LM development and guide precision medicine (4). However, NGS has not been widely used in clinical practice due to its high cost and technological complexity. ddPCR was used as our liquid biopsy method over SuperARMS and NGS due to its higher sensitivity and specificity (the sensitivity is lower than 0.1%), which suggests that more patients would benefit from this method.

Most patients harboring *EGFR* mutations develop CNS metastasis (30); however, treatment with EGFR-TKIs eventually results in acquired resistance. The acquired resistance mechanisms can be either *EGFR*-dependent or EGFR-independent, such as is in T790M or *MET* amplification, respectively. The latter mechanism seems to be the most frequent, appearing in nearly 50% of LM cases (31). Therefore, the treatment strategy selected depends on the patient's *EGFR* mutation status at a particular time. Liquid biopsy facilitates the detection of the *EGFR* mutation status of patients who are unwilling to undergo tissue aspiration biopsy or from whom a tumor tissue sample cannot be obtained (32). cfDNA is a short fragment (usually 130–180 base pairs) double-stranded DNA that is present in plasma and other body fluids (33-36). It is thought to originate mainly from apoptotic or necrotic cell death, although active release mechanisms have also been investigated (37,38). In cancer patients, cfDNA is derived from both non-malignant and malignant cells, with the percentage of ctDNA originating from cancer cells ranging from 3% to 93% (38). Moreover, cancer patients are frequently observed to have increased levels of cfDNA in their plasma, and under certain circumstances, increased cfDNA is considered to be an adverse prognostic factor (39). Increased levels of cfDNA might also be attributable to impaired renal clearance function or production of white plasma cells (WBC) (40,41). In contrast, ctDNA in CSF is quite stable, meaning it has the potential to facilitate and supplement the diagnosis of LM due to the low sensitivity of CSF cytology, especially in cases that cannot be detected by traditional cytopathological analysis (26).

We included both EGFR-TKI-naïve and EGFR-TKI-treated patients in this study. In the EGFR-TKI-naïve patients, the *EGFR* mutation-positive rate in plasma was

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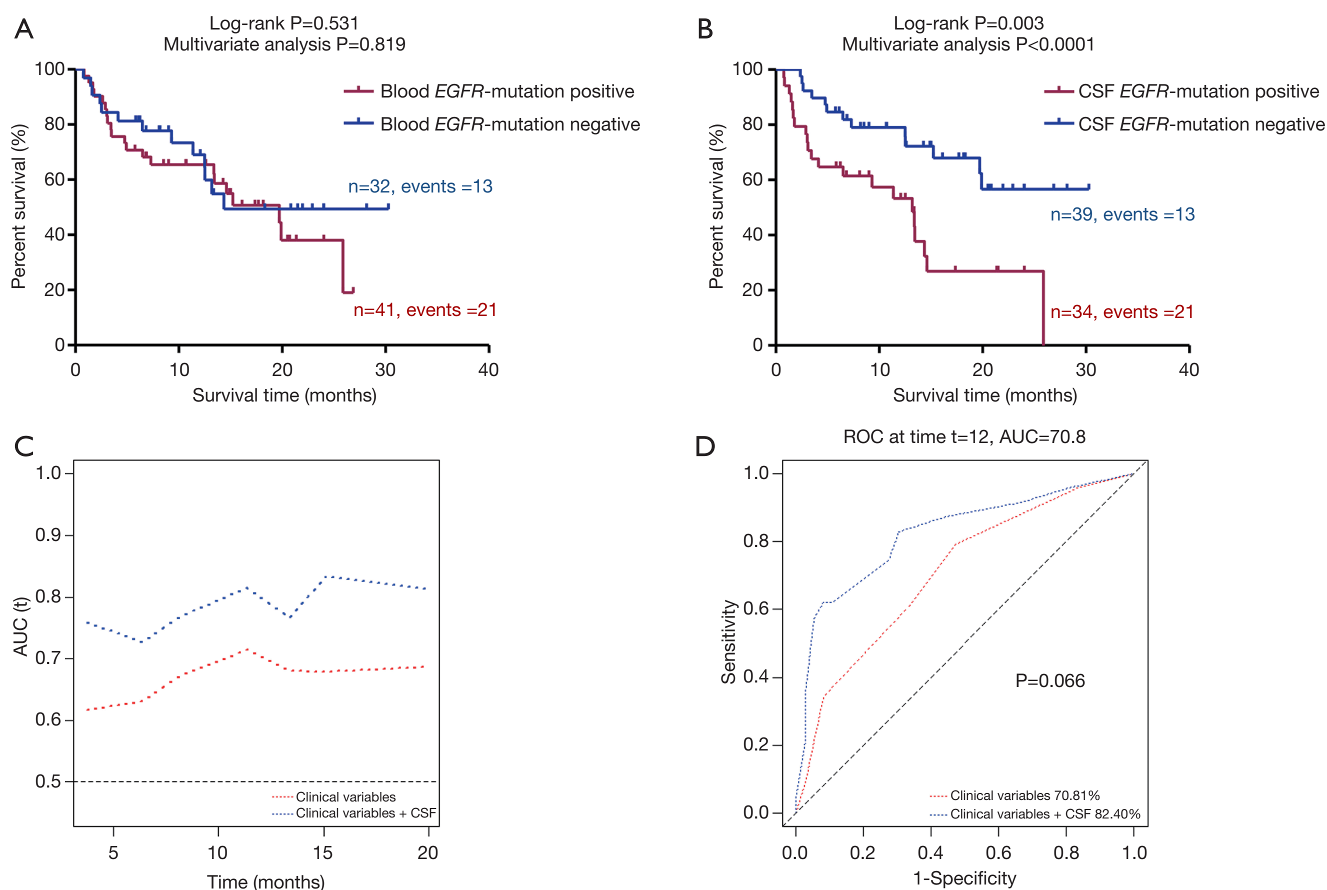

Figure 3 Kaplan-Meier survival curve according to *EGFR* mutation status in plasma and CSF. The difference of median survival time between patients with and without *EGFR* mutations was not significant (A). The patients with *EGFR* mutations in their CSF had significantly worse survival than patients without *EGFR* mutation in their CSF (B). Compared with the model for age, sex, and smoking status, the model with addition of *EGFR* mutation status in CSF showed improvement of time-dependent AUC plot (C,D). CSF, cerebrospinal fluid; AUC, area under the curve.

65.5%, which is generally consistent with previous reports. However, a downward trend in the *EGFR* mutation-positive rate was observed in the plasma of patients with a history of EGFR-TKI therapy. This can be explained by the fact that few *EGFR* mutation-positive NSCLC cells survive in extracranial circulations after EGFR-TKI treatment. Nevertheless, the *EGFR* mutation-positive rate in the CSF samples displayed an opposite trend. The reason for this might be that leptomeninges and CSF could become a safe harbor for protecting *EGFR* mutation-positive NSCLC cells during EGFR-TKI therapy because of the BBB.

In this study, although no statistically significant difference or consistency was observed in the positive rate of *EGFR* mutations in CSF and plasma samples when the entire cohort was analyzed, for patients with neurological symptoms, LM, or those with an EGFR-TKI treatment history, *EGFR* mutation-positive rates in CSF were higher than those in plasma. Moreover, in the patients with LM, the mutation abundance in CSF was significantly higher than that in the plasma samples. This might be due to ctDNA only constituting a small fraction of the cfDNA in plasma arising from the large amount of DNA produced by normal cells, while few normal cells exist in CSF, which results in a notably higher proportion of ctDNA. These results suggest that for patients with EGFR-TKI resistance and CNS metastasis, testing CSF-derived cfDNA might be more effective than testing plasma. Furthermore, our analysis indicated that patients with *EGFR* mutations in their CSF had worse survival, meaning that meningeal metastases might have occurred in these patients, which

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would dramatically increase the risk of death, although this was not diagnosed by TCT or MRI.

The mechanisms of cfDNA release are poorly understood, and their predictive role and relationship to tumor burden are controversial and still being investigated (42,43). We assessed the correlation between the OS of patients and the cfDNA status in both plasma and CSF samples, and found that the cfDNA status in CSF was negatively correlated with patient survival. This result suggests that *EGFR* mutation status or cfDNA in the CSF might be a promising predictor for OS in LUAD patients with CNS metastasis. Previous studies demonstrated a correlation between plasma levels of ctDNA and survival (21,22). The lack of correlation found in this study could be due to the fact that plasma is not adequate to predict patients' survival for patients with CNS metastases.

As far as we know, the present study is the largest to explore *EGFR* mutation status in the CSF of LUAD patients with CNS metastasis, and has yielded new insight into the comparison of *EGFR* mutation status in paired CSF and plasma samples with ddPCR. Nevertheless, this study has some limitations. First, the sample size of 79 patients was not large enough, which might have resulted in the introduction of biases, including low statistical power, higher false discovery rate, and low reproducibility. Secondly, only L858R, E19del, and T790M mutations can be detected by ddPCR, which limited the exploration of the possible molecular mechanism of BM. Finally, this study was performed in a Chinese population and, therefore, may not be representative of other ethnic groups.

In conclusion, we found that the *EGFR* mutation status in CSF was different from that in plasma, and CSF could more effectively reflect the *EGFR* mutation status of LUAD patients with CNS metastasis. Our results indicate that the detection of *EGFR* mutation status in CSF could serve as an efficient alternative or supplement to plasma testing. Furthermore, for patients with acquired resistance to EGFR-TKIs, liquid biopsy of CSF would be an excellent tool for detecting *EGFR* mutation status to inform future treatment decision-making.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised

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in 2013). This study was conducted in accordance with the ethical guidelines of the United States' common rule, and the protocol was approved by the Research Ethics Committee of Henan Cancer Hospital. All the patients in the present study have signed informed consent.

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