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Original Research

Clinical utility and predictive value of cerebrospinal fluid cell-free DNA profiling in non-small cell lung cancer patients with leptomeningeal metastasis

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ABSTRACT

Leptomeningeal metastasis (LM) is a challenging complication of non-small cell lung cancer (NSCLC). Cerebrospinal fluid (CSF) cell-free DNA (cfDNA) analysis using next-generation sequencing (NGS) offers insights into resistance mechanisms and potential treatment strategies. We conducted a study from February 2022 to April 2023 involving patients from five hospitals in Taiwan who had recurrent or advanced NSCLC with LM. These patients underwent CSF cfDNA analysis using a 118-gene targeted panel for NGS, with comprehensive clinical data collected. Among 25 enrolled patients, 22 (88.0 %) had *EGFR* mutations, while three (12.0 %) had *EML4-ALK* fusion, *KIF5B-RET* fusion, and *ERBB2* A775_G776insSVMA. CSF cfDNA sequencing of 27 samples (from 25 patients) all confirmed their original driver mutations. Of total cohort, 18 patients (72.0 %) underwent intrathecal pemetrexed (ITP), with a median survival time of 7.4 months (95.0 % confidence interval, 3.3–11.6) from the initiation of ITP to death. Among them, ten individuals (55.6 %) survived beyond 6 months. Notably, *MET* copy number gain (CNG) correlated significantly with survival time exceeding 6 months after ITP ($p = 0.007$). The coexistence of *EGFR* T790M and EGFR-independent resistance alterations was associated with shorter survival times after ITP, with a median survival time of 1.9 months compared to 9.9 months for those without *EGFR* T790M ($p = 0.010$). Our results highlight CSF cfDNA NGS's potential in LM resistance understanding and ITP efficacy prediction. *MET* CNG positively impacts survival for ITP recipients, whereas the coexistence *of EGFR* T790M and EGFR-independent resistance mechanisms leads to poor outcomes.

Introduction

Leptomeningeal metastasis (LM) is a dismal complication occurring in 3–5 % of advanced non-small cell lung cancer (NSCLC) cases [1]. Its incidence among NSCLC patients with oncogenic drivers is rising due to prolonged survival from targeted therapies, particularly in the *EGFR*-mutant (9.4 %) and *ALK*-rearranged (10.3 %) subgroups [2,3]. While median overall survival (OS) post-LM diagnosis has improved from 1–3 months to 3–11 months with the advent of drugs that penetrate cerebral and spinal subarachnoid spaces[1], like second- (brigatinib and alectinib) and third-generation (lorlatinib) ALK inhibitors for *ALK*-rearranged NSCLC [4–6], and osimertinib for *EGFR* mutated

Abbreviations: 19DEL, exon 19 deletions; cfDNA, cell free DNA; CI, confidence interval;CNG, copy number gain; CNV, copy number variations; CSF, cerebrospinal fluid; EGFR, epidermal growth factor receptor-tyrosine; ITC, intrathecal chemotherapy; ITP, intrathecal pemetrexed; LM, leptomeningeal metastasis; MRI, magnetic resonance imaging; MSI, microsatellite instability; NGS, next-generation sequencing; NSCLC, non-small-cell lung cancer; OS, overall survival; SNV, single nucleotide variants; TMB, tumor mutation burden; TTD, time-to-treatment discontinuation; VAF, variant allele frequency.

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patients [7], acquired resistance inevitably occurs, and subsequent treatment options remain limited [8].

Comprehensive molecular profiling of tumor tissue or plasma cell-free DNA (cfDNA) after the progression of targeted therapies might unveil resistance mechanisms and inform further druggable molecular targets to overcome resistance [9]. However, discordance in genomic alterations has been reported between cerebrospinal fluid (CSF) cfDNA and plasma cfDNA or primary tissue in patients with LM from NSCLC [10]. Owing to the challenges in obtaining biopsy samples from LM and limited cfDNA detection in plasma due to the blood-brain barrier, CSF derived cfDNA is ideal for detecting and monitoring genomic alterations in LM patients [11]. Genomic profiling of CSF cfDNA reveals more unique alterations and outperforms plasma in detecting copy number variations that accurately reflect resistance mechanisms for LM therapy matching [12]. In patients without matched targeted therapy based on CSF cfDNA genomic profiling, subsequent systemic chemotherapy could be a treatment option; however, its efficacy is limited.

In addition to systemic chemotherapy, intrathecal chemotherapy (ITC) allows drugs to bypass the brain barrier for LM patients treatment [13]. In a pooled analysis of ITC in NSCLC, response rates based on cytological, clinical, and radiographic criteria were 55 %, 64 %, and 53 %, respectively. Furthermore, the median survival time (from the initiation of treatment) was determined to be 6.0 months [14]. In a phase I/II clinical trial, a dose of 50 mg of intrathecal pemetrexed (ITP) demonstrated a clinical response rate of 84.6 % and a median OS of 9 months, which exceeded those observed in patients receiving conventional intrathecal injection drugs [14]. Most enrolled patients developed progressive LM on osimertinib treatment, and ITP proved effective for NSCLC patients with LM who had progressed on osimertinib [14].

Our study primarily focused on heavily treated NSCLC patients with driver mutations who developed LM after targeted therapies exposure. We retrospectively analyzed CSF cfDNA genomic profiles to investigate their capability in identifying resistance mechanisms to targeted therapies, predicting ITP efficacy, and influencing survival outcomes.

Materials and methods

Patients and data collection

We conducted a retrospective study of recurrent or advanced NSCLC patients diagnosed with LM using magnetic resonance imaging (MRI), who subsequently underwent lumbar puncture to obtain CSF samples for next-generation sequencing (NGS) analysis. Comprehensive clinical Data were collected at the National Taiwan University Hospital (NTUH), National Taiwan University Cancer Center, National Taiwan University Hsinchu Hospital, National Taiwan University BioMedical Park Hospital, and NTUH Yunlin Branch from February 2022 to April 2023. The study was approved by the Research Ethics Committee of NTUH (No.202304110RINA) and conducted in accordance with the principles of the Declaration of Helsinki and International Conference on Harmonization Good Clinical Practice Guidelines.

Clinically, our patients underwent molecular testing to identify driver oncogenes using various methods, including the cobas® EGFR Mutation Test v2, the VENTANA ALK (D5F3) CDx Assay, cDNA Sanger sequencing [15], as well as NGS analysis for both liquid biopsy and tissue specimens.

To evaluate the treatment response, clinicians assessed patients' neurological symptoms, and two neuroradiologists interpreted brain MRI responses following the principles set by the Response Assessment in Neuro-Oncology (RANO) LM working group [16].

ITP administration followed the protocol described previously [17]. As part of induction therapy, patients initially received ITP once or twice during the first week of treatment. Subsequently, for consolidative therapy, the patients underwent ITP once every 4 weeks. The pemetrexed used in the treatment was Alimta® 100 mg/vial, manufactured by Eli Lilly and Company. Clinicians prescribed a fixed dose of pemetrexed ranging from 25 to 50 mg, which was then reconstituted in 0.9 % sodium chloride solution. Pemetrexed was administered through lumbar puncture.

Time-to-treatment discontinuation (TTD) for ITP was defined as the time from ITP initiation to ITP cessation or death. The interval between NSCLC and LM diagnosis was defined as the period from the diagnosis of advanced or recurrent NSCLC to the confirmation of LM diagnosis through brain MRI assessment. The survival time after ITP was defined as the time interval from the initiation of ITP to death. The duration from LM to death was calculated as the time from LM diagnosed by brain MRI to death. OS was calculated as the time from the confirmation of recurrence or advanced stage (stage IIIB–IVB) NSCLC to death.

CSF collection, cfDNA extraction and targeted panel sequencing

Each individual sample required 20 ml of CSF for this study. All CSF samples were collected and transferred to Cell-Free DNA BCT tubes (Streck, USA). Each CSF sample was centrifuged at 2000 × g for 10 min. cfDNA was then extracted from supernatant of CSF using the Maxwell® RSC cfDNA Plasma Kit (Promega, USA) following the instructions provided by the manufacturer. The cfDNA was quantified using a 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA).

The DNA NGS library was constructed using the IMBdx NGS DNA Library Prep Kit. At IMBdx, Inc. (Seoul, South Korea), and solution-based target enrichment was performed using the AlphaLiquid® 100 target capture panel (118 cancer related genes) (Supplementary Table). Subsequently, the captured DNA libraries were subjected to sequencing on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) in the 2 × 150 bp paired-end mode [18]. Variant calling was conducted in the same manner as in previous studies [18–20].

Specific thresholds were set for cfDNA mutations to minimize the impact of noise, contamination, and sequencing errors. These thresholds required a variant allele frequency (VAF) of at least 0.1 % and a minimum of four altered duplex consensus sequence counts. Additionally, a manual review was performed and unexpected false positives were curated by visually examining the longitudinal cfDNA mutation profiles. This step was allowed to identify and exclude any potential false positives that may have arisen because of technical artifacts or other confounding factors, thus ensuring the accuracy and reliability of the final mutation calls.

Gains were defined as having a copy number of 4 or greater, or when the copy number ranged from 2.2 to 4, applying statistical criteria with a *p*-value below 0.001. Regarding gene fusion, computational time was optimized by selecting paired-end reads that overlapped with the target regions. Candidate fusion genes were identified using the dual-fusion caller approach with two software tools, Genefuse [21] and SViCT [22]. To ensure high-confidence fusion gene detection, only the fusions supported by two or more reads with a mapping quality of 60 were considered. In addition, the predicted transcript resulting from the fusion event must be considered functional.

The tumor mutation burden (TMB) score was determined by dividing the number of somatic synonymous and non-synonymous variants, which were not driver mutations, by the size of the coding region, which was approximately 321 kilobases (kb).

Statistical analysis

Categorical and continuous variables are summarized as percentages and medians, respectively. Categorical variables were analyzed using the chi-squared test. One-way analysis of variance (ANOVA) or Student's t-test was used to analyze continuous variables. Kaplan-Meier method was used to assess clinical and survival outcomes, including TTD, interval between NSCLC diagnosis and LM diagnosis, survival time after ITP, duration from LM to death, and OS. The median values for these survival outcomes are presented along with their corresponding 95.0 % confidence intervals (CIs). Log-rank tests were performed to

compare the differences in clinical and survival outcomes. Statistical significance was defined as a two-sided p value <0.05. Statistical analyses were performed using the Statistical Package for the Social Sciences (version 22.0; SPSS Inc., Chicago, IL, USA). To plot survival curves, we used Stata for Windows software (version 14.0; StataCorp, College Station, TX, USA). The fragment size calculation was performed using Python on the BAM file, and the plot was created using the Python package matplotlib. An oncoplot was generated using the R complex heat map.

Results

Study population and patient characteristics

Initially, 28 patients with recurrent or advanced NSCLC who underwent lumbar puncture and CSF collection for NGS were identified. However, three patients were excluded from the study because LM were not detected on the MRI scans. Consequently, 25 patients were enrolled (Supplementary Fig. S1). The data cutoff date was June 30, 2023.

The median age of the 25 enrolled patients was 59. Among them, 15 (60.0 %) were women, and 21 (84.0 %) had never smoked (Table 1). Five patients (20.0 %) received treatment for recurrent NSCLC, and 11 (44.0 %) had brain metastasis at the time of initiating systemic treatment. The histological type in all 25 enrolled patients was adenocarcinoma.

Regarding the driver oncogenes, 11 patients (44.0 %) had the *EGFR* L858R mutation, 8 (32.0 %) had an *EGFR* exon 19 deletion (19DEL), 3 (12.0 %) had uncommon or compound *EGFR* mutations, and 3 (12.0 %) had other oncogenic drivers, including one with *EML4-ALK* fusion, one with *KIF5B-RET* fusion, and one with *ERBB2* A775_G776insSVMA (Table 1).

All 25 LM patients exhibited neurological symptoms such as visual field changes, dizziness, altered consciousness, unsteady gait, etc. (Tables 1 & 2). Sixteen patients (64.0 %) had brain metastases confirmed using brain MRI at the time of LM diagnosis. However, of the 22 patients who underwent cytological examinations of CSF study, only 10 (45.5 %) were found to have malignant cells (Table 1).

Of the cohort, the median interval between the diagnosis of recurrent or advanced NSCLC to the confirmation of LM was found to be 19.8 months (95.0 % CI, 6.2–33.5) (Supplementary Fig. S2A). When analyzing 22 patients with *EGFR* mutations, the median interval between NSCLC diagnosis and LM diagnosis for patients with *EGFR* mutations was 18.6 months (95.0 % CI, 7.4–29.7). Furthermore, 11 patients with *EGFR* L858R and 8 *EGFR* 19DEL had median intervals of 15.8 months (95.0 % CI, 0.0–31.6) and 24.4 months (95.0 % CI, 19.2–29.5), respectively (p = 0.400), from NSCLC diagnosis to LM diagnosis (Supplementary Fig. S2B).

For the number of lines of systemic treatment received by these 25 patients following LM diagnosis, the median was 3 (range, 1–9) (Table 2).

Table 1
Characteristics of 25 enrolled non-small cell lung cancer patients diagnosed with leptomeningeal metastasis (LM).

	Overall patients	Patients received intrathecal pemetrexed	
	(N = 25)	Yes (N = 18)	No (N = 7)
Age, median (range)	59 (43-73)	59 (43-72)	67 (54-73)
Female sex, *n* (%)	15 (60.0)	11 (61.1)	4 (57.1)
Never smoker, *n* (%)	21 (84.0)	17 (94.4)	4 (57.1)
Histological type, *n* (%)			
Adenocarcinoma	25 (100)	18 (100)	7 (100)
Cancer stage, *n* (%)			
Recurrence	5 (20.0)	2 (11.1)	3 (42.9)
IVA and IVB	20 (80.0)	16 (88.9)	4 (57.1)
Brain metastasis when initiating systemic treatment, *n* (%)	11 (44.0)	7 (38.9)	4 (57.1)
Driver mutations, *n* (%)			
EGFR L858R	11 (44.0)	8 (44.4)	3 (42.9)
EGFR 19DEL	8 (32.0)	6 (33.3)	2 (28.6)
Uncommon or compound *EGFR* mutations	3 (12.0)	2 (11.1)	1 (14.3)
EML4-ALK Fusion	1 (4.0)	1 (5.6)	0
KIF5B-RET Fusion	1 (4.0)	1 (5.6)	0
ERBB2 A775_G776insSVMA	1 (4.0)	0	1 (14.3)
Diagnosis of LM, *n* (%)			
MRI confirmed	25 (100)	18 (100)	7 (100)
Brain metastasis when LM diagnosis	16 (64)	13 (72.2)	3 (42.9)
Neurological symptoms	25 (100)	18 (100)	7 (100)
Positive CSF cytology	10 (45.5 % of 22 patients)	7 (43.8 % of 16 patients)	3 (50.0 % of 6 patients)

Abbreviations: ALK, anaplastic lymphoma kinase; CSF, cerebrospinal fluid; EGFR, epidermal growth factor receptor; LM, leptomeningeal carcinomatosis; MRI, Magnetic Resonance Imaging.

Quality control (QC) for NGS analysis of CSF cfDNA

Totally, we analyzed 27 CSF cfDNA sequencing results derived from 25 patients, two of whom provided two samples each (CSF-03 and 08). These results were subsequently compared to the patients' prior molecular testing outcomes (Table 3). All 27 samples tested positive for original driver mutations. A cumulative count of 167 variants was identified in 27 samples, with variant counts ranging from 1 to 13.

The median interval between LM diagnosis and CSF sample collection was 18 days (range, 1–453 days). The median nucleic acid input for cfDNA was 9 ng (with a range of 0.7 to 30.0 ng), while the median molecular sequencing depth reached 3669 (spanning from 195 to 13874) (Table 3). Instances of low-input DNA were correlated with lower molecular depths. The mean insert sizes were approximately 160 bp (main peak) and 320 bp (minor peak), consistent with previously reported results for CSF cfDNA specimens (Supplementary Fig. S3A). The majority of cfDNA concentrations measured below 1 ng/μl, with only two samples exceeding 3 ng/μl (Supplementary Fig. S3B), and the largest input DNA amounted to 30 ng. Remarkably, despite modest cfDNA input, this study consistently yielded positive results.

Of the 27 CSF samples, 11 tested positive for malignant cells, seven showed suspicious/atypia results, six were negative, and the results of cytologic assessment were unavailable for three samples (Table 2). Among the six samples with negative cytology results (CSF-09, 12, 16, 18, 21, and 24), all except CSF-24 had input cfDNA below 6 ng. Samples with positive cytology and atypia tended to have higher cfDNA inputs than those with negative results (Supplementary Fig. S3C&D). Even for the six negative cytology samples, the sensitivity of driver mutation identification from CSF cfDNA was 100 %.

Analysis of 27 CSF specimens (n = 27) from 25 enrolled patients (N = 25) showed that the most frequent co-occurring alterations were *EGFR* copy number gain (CNG) (n = 19, 70.4 %), followed by *TP53* alterations (n = 14, 51.9 %), *CDK6* CNG (n = 11, 40.7 %), *MET* CNG (n = 10, 37.0 %), *MYC* CNG (n = 10, 37.0 %), *CDK4* CNG (n = 9, 33.3 %), and *MDM2* CNG (n = 8, 30.0 %) (Fig. 1A). The median TMB was 9.44 mutations per megabase (Muts/Mb) and ranged from 0 to 25.16 (Fig. 1A).

ITP in NSCLC patients with LM

Of total 25 patients, 18 (72.0 %) underwent ITP as indicated in Supplementary Fig. S1 and Tables 1 & 2. Among the 18 patients with ITP, 16 harbored *EGFR* mutations, one harbored *EML4-ALK* fusion, and the remaining patient had *KIF5B-RET* fusion. Fourteen of the 16 patients with *EGFR* mutations received ITP after experiencing disease progression while receiving osimertinib. Two patients, one with *EML4-ALK* fusion and the other with *KIF5B-RET* fusion, received ITP after encountering disease progression on lorlatinib and pralsetinib, respectively. The median TTD for ITP was 3.1 months (95.0 % CI, 0.4–5.7

Table 2
The detailed clinical information of 25 enrolled patients with leptomeningeal metastasis.

Case No.	Age/ Sex	Driver mutations (clinical tests)	LM diagnosis		Time to LM Dx (month)	Prior lines of Tx	Intrathecal pemetrexed (ITP)							Interval between LM Dx to death (month)	OS (month)	Death event
			Brain metastasis	CSF cytology			Neurological symptom evaluation	Response evaluation by MRI	TTD of ITP (month)	TTD event	ITP dose (mg)	Neutro-penia ≥ Gr.2	Survival time (month)			
CSF-03	44/M	EGFR 19DEL	Yes	Positive	27.9	3	Response	SD	12.5	Yes	50	No	13.4	21.2	49.1	Yes
CSF-06	54/F	EGFR L858R	Yes	Atypia	0.5	1	Response	SD	10.8	No	50->40	Yes	10.8	25.6	26.2	No
CSF-24	60/F	EGFR L858R	No	Negative	18.6	3	Stabilization	SD	2.3	Yes	25->40	No	10.2	10.4	29.0	Yes
CSF-01	68/F	EGFR 19DEL	No	Atypia	24.4	3	Response	NA	8.9	Yes	30	No	9.9	9.9	34.3	Yes
CSF-12	58/F	EGFR L858R	Yes	Negative	34.4	4	Response	SD	9.2	No	50	No	9.2	9.3	43.7	No
CSF-26	46/M	EGFR G719A	Yes	Atypia	14.2	4	Response	PR	4.2	Yes	30	No	7.8	8.6	22.8	No
CSF-19	43/F	EML4-ALK Fusion	Yes	Positive	122.1	5	Response	SD	7.5	No	50	No	7.5	7.6	129.6	No
CSF-14	44/M	EGFR L858R	No	Atypia	3.0	3	Response	SD	6.2	Yes	25->50	No	7.4	10.9	13.8	Yes
CSF-23	60/F	EGFR L858R	No	Positive	28.6	1	Response	SD	7.2	No	50	No	7.2	7.2	35.8	No
CSF-08	59/F	EGFR G719X+T790M	No	Positive	19.8	3	Response	SD	4.2	Yes	40->30	Yes	6.5	6.9	26.7	Yes
CSF-27	72/F	EGFR L858R	Yes	Positive	48.3	2	Progression	SD	1.5	Yes	40	No	5.6	6.0	54.3	Yes
CSF-13	59/F	EGFR L858R	Yes	NA	55.6	9	Progression	NE	0.9	Yes	40	No	5.1	14.3	69.9	Yes
CSF-28	60/M	KIF5B-RET Fusion	Yes	Atypia	53.4	1	Response	SD	3.1	Yes	50	Yes	4.3	4.6	58.0	No
CSF-02	55/M	EGFR 19DEL	Yes	Atypia	108.1	9	Stabilization	NE	2.0	Yes	50	No	2.8	5.0	113.0	Yes
CSF-21	67/F	EGFR 19DEL	Yes	Negative	24.2	7	Stabilization	NE	1.4	Yes	30	No	1.9	2.7	26.9	Yes
CSF-04	65/M	EGFR 19DEL	Yes	Positive	31.9	3	Stabilization	NE	0.2	Yes	40	No	1.5	10.8	42.7	No
CSF-18	57/F	EGFR L858R	Yes	Negative	3.9	1	Progression	NE	0.9	Yes	50	Yes	1.4	2.3	6.3	Yes
CSF-25	55/M	EGFR 19DEL	Yes	NA	9.6	8	Progression	NE	0.2	Yes	25	No	0.4	0.6	10.2	Yes
CSF-15	73/F	EGFR L858R	No	Positive	14.5	1								8.7	23.2	No
CSF-16	58/F	EGFR L858R	No	Negative	15.8	1								8.0	23.9	No
CSF-09	72/F	EGFR 19DEL	No	Negative	38.1	3								7.0	45.2	Yes
CSF-05	75/F	EGFR L858R	Yes	Positive	2.2	3								7.0	9.2	Yes
CSF-10	59/M	ERBB2 A775_G776insSVMA	Yes	Positive	15.1	3								4.8	19.9	Yes
CSF-30	54/M	EGFR 19DEL	Yes	NA	14.5	1								4.4	18.9	No
CSF-29	67/M	EGFR G719X	No	Positive	11.1	2								1.5	12.6	No

Abbreviations: ALK, anaplastic lymphoma kinase; CSF, cerebrospinal fluid; Dx, diagnosis; EGFR, epidermal growth factor receptor; F, female sex; ITP, intrathecal pemetrexed; LM, leptomeningeal metastasis; M, male sex; MRI, Magnetic resonance imaging; NA, not available; NE, not evaluable; NGS, next-generation sequencing; OS, overall survival; PD, progressive disease; PR, partial response; SD, stable disease; TTD, time-to-treatment discontinuation; Tx, treatment.

Table 3
The comprehensive clinical information and CSF cfDNA data of 25 enrolled patients with leptomeningeal metastasis.

Case No.	LM Dx to CSF sampling (day)	Treatment before ITP	Status of CSF sampling	Survival time after ITP	NGS QC of CSF cfDNA			NGS results of CSF cfDNA		Strategy of systemic treatment		
					Input DNA (ug)	Sequencing depth	Driver mutations (Clinical tests)	Driver mutations (AlphaLiquid® 100)	Co-occurring genomic alterations (AlphaLiquid® 100)	Treatment when CSF sampling	Treatment after CSF sampling	Responses after treatment changed
CSF-03	239	Osimertinib	Pre ITP	> 6 months	3.4	703.2	*EGFR* 19DEL	*EGFR* T751_I759delinsD	*EGFR* CNG *SMAD4* D537Kfs*14	Osimertinib	Osimertinib/ Cisplatin/ Pemetrexed	SD
CSF-06	453	Osimertinib	Pre ITP	> 6 months	9.0	8784.0	*EGFR* L858R	*EGFR* L858R	*CTNNB1* S37Y *SMAD4* D404Lfs*21 *TP53* I254F	Osimertinib	Osimertinib	SD
CSF-24	287	Osimertinib/ Pemetrexed/ Bevacizumab	Post ITP	> 6 months	28.2	7516.0	*EGFR* L858R	*EGFR* L858R	*BRAF/CDK4/CDK6/ EGFR/KRAS/MDM2/ MET* CNG	Osimertinib/ Pemetrexed/ Bevacizumab	Docetaxel/ Osimertinib/ Ramucirumab	NE
CSF-01	1	Osimertinib	Pre ITP	> 6 months	5.8	1418.4	*EGFR* 19DEL	*EGFR* E746_A750del	*TP53* K132M	Osimertinib	Erlotinib/ Ramucirumab	SD
CSF-12	2	Osimertinib/ Paclitaxel	Pre ITP	> 6 months	4.0	2307.0	*EGFR* L858R	*EGFR* L858R	*CDK6/EGFR/MET/ MYC* CNG *TP53* L137Q	Osimertinib/ Paclitaxel	Osimertinib/ Paclitaxel	SD
CSF-26	201	Pemetrexed/ Carboplatin/ Bevacizumab/ Atezolizumab	Post ITP	> 6 months	11.4	4623.0	*EGFR* G719X	*EGFR* G719A *EGFR* I759M	*AKT/EGFR/MET* CNG *TP53* K120E	Osimertinib/ Dacomitinib	Osimertinib/ Capmatinib	NE
CSF-19	3	Lorlatinib/ Bevacizumab	Pre ITP	> 6 months	30.0	11465.0	*EML4-ALK*	EML4-*ALK*	*EGFR* CNG *TP53* c.375+2T>G	Lorlatinib/ Bevacizumab	Lorlatinib/ Erlotinib	SD
CSF-14	308	Osimertinib	Post ITP	> 6 months	14.9	7210.0	*EGFR* L858R	*EGFR* L858R	*CDK6/CCND1/ CCNE1/KRAS/ MAPK1/MET/MYC/ PIK3CA* CNG *TP53* P278R	Osimertinib	Afatinib/ Bevacizumab	PD
CSF-23	1	Osimertinib	Pre ITP	> 6 months	5.4	3639.0	*EGFR* L858R	*EGFR* L858R	*EGFR/CCND2/CDK4/ CDK6/ERBB2/KRAS/ MDM2/MET* CNG	Osimertinib/ Pemetrexed	Erlotinib	SD
CSF-08	11	Osimertinib	Pre ITP	> 6 months	3.1	2722.0	*EGFR* G719X *EGFR* T790M	*EGFR* G719A *EGFR* T790M	*CCNE1/CDK4/CDK6/ EGFR/MET/MYC* CNG *ATM* F2558Lfs*5*BRAF* V600E	Osimertinib	Osimertinib	PD
CSF-27	85	Osimertinib	Post ITP	< 6 months	30.0	5627.0	*EGFR* L858R	*EGFR* L858R	*EGFR/MYC* CNG *TP53* R175H	Osimertinib	Osimertinib/ Cetuximab/ Capmatinib/ Amivantamab	PD
CSF-13	278	Osimertinib/ Paclitaxel	Pre ITP	< 6 months	14.6	10068.0	*EGFR* L858R	*EGFR* L858R	*CCND1/CCND2/ CCNE1/CDK4/ CDK12/EGFR/ERBB2/ KRAS/MDM2/MYC/ MYCN* CNG	Osimertinib/ Paclitaxel	Osimertinib	PD
CSF-28	76	Pralsetinib	Post ITP	< 6 months	3.1	2774.0	*KIF5B-RET*	*KIF5B-RET*	*ARID1A* W2050*	Pemetrexed/ Cisplatin	Pemetrexed/ Cisplatin	NE
CSF-02	67	Osimertinib/ TS-1/ Bevacizumab	Pre ITP	< 6 months	20.0	3669.5	*EGFR* 19DEL	*EGFR* E746_A750del *EGFR* T790M	*APC* H286Lfs*7 *NF2* Y144*	Osimertinib/ TS-1/ Bevacizumab	Osimertinib/ Brigatinib/ Bevacizumab	SD
CSF-21	1	Osimertinib	Pre ITP	< 6 months	1.4	2157.0	*EGFR* 19DEL	*EGFR* E746_A750del *EGFR* T790M	*EGFR/FGFR1* CNG *PTEN* T319* *TP53* c.672+1G>T	Atezolizumab/ Paclitaxel/ Bevacizumab	Osimertinib	PD

(*continued on next page*)

Table 3 (*continued*)

Case No.	LM Dx to CSF sampling (day)	Treatment before ITP	Status of CSF sampling	Survival time after ITP	NGS QC of CSF cfDNA			NGS results of CSF cfDNA		Strategy of systemic treatment		
					Input DNA (ug)	Sequencing depth	Driver mutations (Clinical tests)	Driver mutations (AlphaLiquid® 100)	Co-occurring genomic alterations (AlphaLiquid® 100)	Treatment when CSF sampling	Treatment after CSF sampling	Responses after treatment changed
CSF-04	1	Osimertinib/ Gemcitabine	Pre ITP	< 6 months	0.8	3426.7	*EGFR* 19DEL	*EGFR* L747_T751delinsQ *EGFR* T790M	*ATK1* CNG *PIK3CA* H1047R *TP53* H179L	Erlotinib	Osimertinib/ Ramucirumab	PD
CSF-18	13	Osimertinib	Pre ITP	< 6 months	0.9	195.0	*EGFR* L858R	*EGFR* L858R	Nil	Erlotinib	Osimertinib	NE
CSF-25	5	Amivantamab	Pre ITP	< 6 months	21.4	7773.0	EGFR 19DEL	*EGFR* E746_A750del	*CDK6/EGFR/MYC/ MYCN/PIK3CA* CNG	Amivantamab	Nil	NE
CSF-15	7				3.6	2189.0	*EGFR* L858R	*EGFR* L858R	*BRAF/CDK6/EGFR/ MET/MYC* CNG *CDKN2A* Y44* *PIK3CA* Q546K *TP53* E221*	Gefitinib/ Bevacizumab	Osimertinib	SD
CSF-16	3				5.5	3273.0	*EGFR* L858R	*EGFR* L858R	*BRAF/CCND2/CDK4/ CDK6/EGFR/KRAS/ MDM2/MET* CNG *TP53* N311Tfs*34	Erlotinib	Erlotinib/ Bevacizumab	SD
CSF-09	18				0.7	1517.0	*EGFR* 19DEL	*EGFR* E746_A750del	*CDK4/MDM2* CNG	Erlotinib	Osimertinib/ Vinorelbine	SD
CSF-05	103				20.0	10219.0	*EGFR* L858R	*EGFR* L858R	*EGFR/MDM2* CNG	Erlotinib/ Gemcitabine	Erlotinib	NE
CSF-10	18				20.0	9686.0	*ERBB2* A775_G776insSVMA	*ERBB2* A775_G776insSVMA *ERBB2* R487W	*CCDN2/CDK4/ CDK12/ERBB2/ KRAS/MDM2/MYC/ PIK3CA* CNG *TP53* V157F	Trastuzumab deruxtecan	Afatinib	PD
CSF-30	48				11.0	5121.0	*EGFR* 19DEL	*EGFR* E746_A750del	*BRAF/CCND2/CDK4/ CDK6/EGFR/KRAS/ MDM2/MET/MYC* CNG *BARD1* Y736* *TP53* V197Q	Osimertinib	Osimertinib/ Capmatinib	PD
CSF-29	2				3.0	2456.0	*EGFR* G719X	*EGFR* G719A	*EGFR/CCNE1/CDK6/ MYC* CNG *TP53* H214L	Afatinib	Osimertinib	PD

Abbreviations: ALK, anaplastic lymphoma kinase; CNG, copy number gain; CSF, cerebrospinal fluid; Dx, diagnosis; EGFR, epidermal growth factor receptor; ITP, intrathecal pemetrexed; LM, leptomeningeal metastasis; NE, not evaluable; NGS, next-generation sequencing; PD, progressive disease; PR, partial response; SD, stable disease; Tx, treatment; QC, quality control.

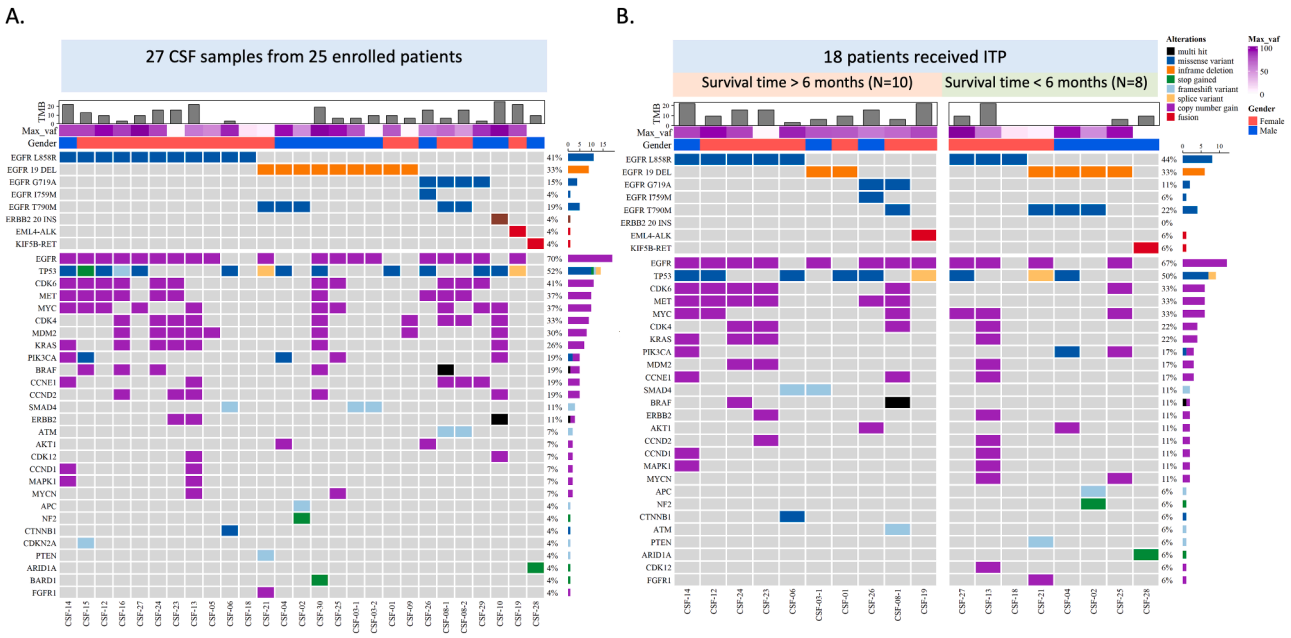

Fig. 1. Oncoprints depicting the prevalence of genomic alterations in CSF cfDNA NGS results from 25 NSCLC patients with LM. (A) Illustrated are 27 CSF cfDNA specimens derived from 25 patients. (B) This subFig. showcases patients with an interval between ITP initiation and death surpassing 6 months (N = 10) and those with an interval <6 months (N = 8). ITP, intrathecal pemetrexed; LM, leptomeningeal metastasis; TMB, tumor mutation burden.

months) (Table 2).

The responses to ITP were evaluated based on changes in neurological symptoms and brain MRI images. Among the 18 patients who underwent ITP, 10 (55.6 %) exhibited positive neurological responses, four (22.2 %) experienced stable symptoms, and four (22.2 %) showed progressive symptoms, as presented in Table 2. However, the improvement observed in the follow-up brain MRI images of patients with LM who received ITP was not significant, with only one patient (5.6 %) showing a partial response on brain MRI (Table 2).

A fixed dose of ITP ranging from 25 to 50 mg was administered. Among the 18 patients, four (22.2 %) experienced grade 2 or higher neutropenia despite receiving folic acid and vitamin B_{12} supplementation. Remarkably, the occurrence of neutropenia was not significantly correlated with the administered dose or TTD of ITP. The median survival time of LM patients from the initiation of ITP to death was 7.4 months (95.0 % CI, 3.3–11.6). Among the 18 LM patients who

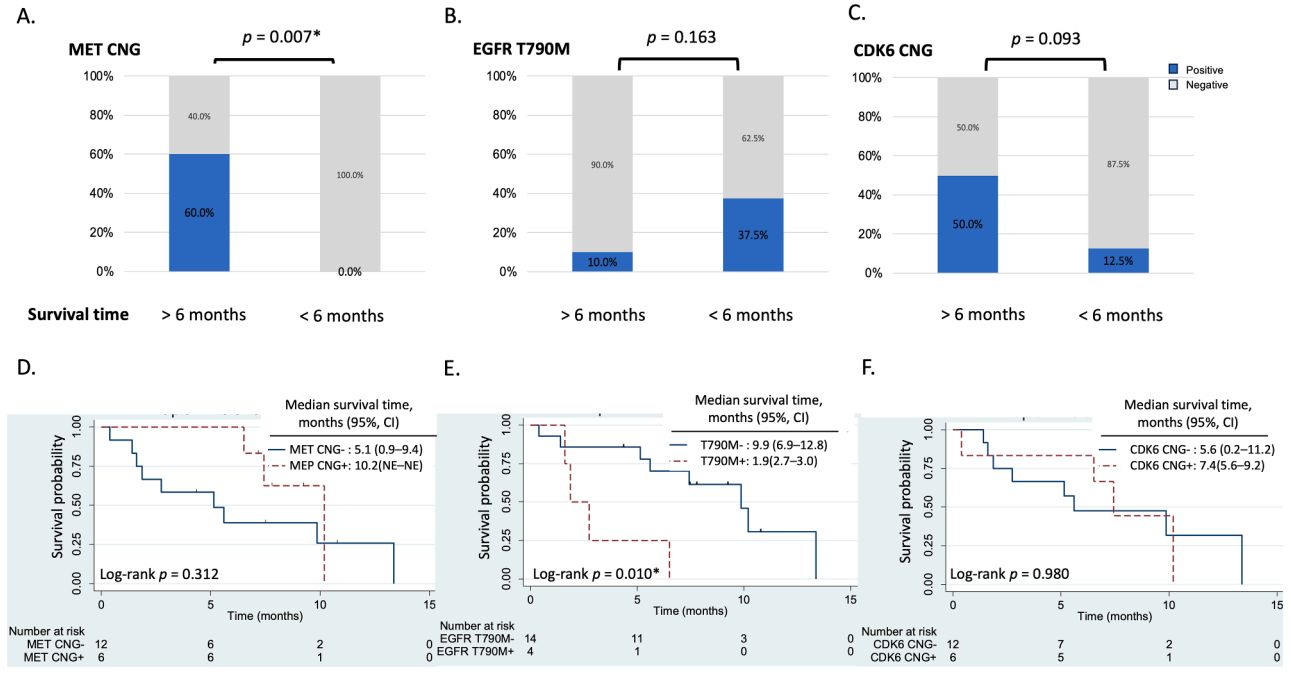

Fig. 2. Percentage of co-occurring genomic alterations in patients with intervals between ITP initiation and death. This subFig. illustrates patients with an interval exceeding 6 months (N = 10) and those with an interval below 6 months (N = 8). (A) *MET* copy number gain (CNG), (B) *EGFR* T790M, and (C) *CDK6* CNG. Kaplan–Meier curves depicting the interval between ITP initiation and death. (D) *MET* CNG, (E) *EGFR* T790M, and (F) *CDK6* CNG. ITP, intrathecal pemetrexed; TMB, tumor mutation burden.

underwent ITP, ten individuals (55.6 %) achieved a survival time exceeding 6 months (Table 2).

Exploring the correlation between clinical factors/co-occurring genomic alterations and the survival outcomes of ITP

To identify predictive factors associated with survival time after ITP > 6 months, we explored various clinical factors, including the coexistence of brain metastasis when LM was diagnosed, prior brain radiotherapy, prior anti-angiogenesis therapy, positive CSF cytology study, and the number of prior lines of systemic treatment before ITP. However, these clinical factors lacked a significant correlation with a survival time > 6 months in patients with LM.

Furthermore, we analyzed co-occurring genomic alterations present in the CSF cfDNA profiles, categorized based on patient survival times exceeding 6 months and those below 6 months (Fig. 1B). We investigated the frequent co-occurring genomic alterations in our cohort, including *EGFR* T790M, *TP53* mutations, copy number gain (CNG) of *EGFR, MET, MYC, MDM2, CDK4/CDK6/CCND1/CCND2, BRAF* mutations, *SMAD4* mutations, and TMB. Notably, patients harboring *MET* CNG who underwent ITP demonstrated a significant association with survival times exceeding 6 months ($p = 0.007$) (Fig. 2A). Out of six patients with *MET* CNG detected in CSF cfDNA, five patients exhibited concurrent CNG in *CDK6/BRAF*. Notably, these three genes (*CDK6, MET*, and *BRAF*) are located on chromosome 7q. The presence of *EGFR* T790M within the CSF was linked to LM patients with a survival time of <6 months ($p = 0.163$) (Fig. 2B). However, while *CDK6* CNG and higher TMB were correlated with LM patients who received ITP with a survival time > 6 months, these associations did not reach statistical significance ($p = 0.094$) (Fig. 2C) or ($p = 0.067$) (Supplementary Fig. S4B), respectively.

Therefore, we conducted an additional analysis, delving into the Kaplan-Meier curve for these specific genomic alterations. Patients with *EGFR* T790M in CSF cfDNA had significantly shorter survival times than those without *EGFR* T790M, with a median survival time of 1.9 versus 9.9 months following ITP ($p = 0.010$) (Fig. 2E). However, the presence of *MET, CDK6, MYC* CNG, and TMB above or below the median value of 9.4 did not yield significant differences in survival time (Fig. 2D&F and Supplementary Fig. S4C& D).

Among the four patients with *EGFR* T790M in CSF cfDNA, two patients developed LM while undergoing osimertinib treatment. The remaining two patients encountered LM progression while receiving osimertinib treatment. Genomic profiling of CSF cfDNA from the four patients revealed the coexistence of *EGFR* T790M and EGFR-independent resistance mechanisms (Table 3).

Regarding whether NGS results guided clinicians' treatment strategies, 18 (72.0 %) of the 25 enrolled patients underwent changes in their systemic treatment approach following the findings of CSF cfDNA NGS study (Table 3). Of the 18 patients who underwent changes in their systemic treatment approach based on the CSF cfDNA NGS results, seven did not receive ITP. Among these, three patients experienced improvements in neurological symptoms (one also underwent ventriculoperitoneal shunting simultaneously), while the remaining four patients had progressive symptoms.

Survival outcomes of NSCLC patients with LM

Among the 25 enrolled patients, the median time interval between LM diagnosis and death (LM-death) was found to be 10.4 months (95.0 % CI, 5.5–15.3) (Supplementary Fig. S5A). Among the 18 patients who received ITP, there was a slightly longer time interval of LM-death compared to those patients without ITP, with a median interval of 10.4 versus 7.0 months ($p = 0.409$), respectively (Supplementary Fig. S5B). Furthermore, among patients with brain metastasis when LM diagnosis was detected from CSF cfDNA, there was a trend of having a shorter time interval of LM-death compared to those without brain metastasis, with a median interval of 7.0 vs. 10.9 months ($p = 0.105$), respectively (Supplementary Fig. S5C).

Further, we investigated the clinical outcomes of 17 patients who received osimertinib as systemic treatment after being diagnosed with LM. Fourteen of 17 opted for ITP for LM management. The median LM-death interval for the 14 patients with ITP was longer than for the remaining three patients who did not undergo ITP, with median intervals of 10.4 vs. 4.8 months ($p = 0.184$), respectively.

The median OS of total 25 enrolled patients was 45.2 months (95.0 % CI, 26.2–64.2) (Supplementary Fig. S6A). The median OS among patients with or without ITP was 49.1 vs. 45.2 months ($p = 0.345$), respectively (Supplementary Fig. S6B).

Serial monitoring of CSF cfDNA

Case CSF-03, a 41-year-old man with recurrent *EGFR*-mutant NSCLC, initially responded well to first-line afatinib and bevacizumab therapy, lasting a 28-month period of clinical improvement (Fig. 3). The development of new brain metastases and LM through follow-up brain MRI during this combination therapy led to discontinuation. CSF cytology showed no evidence of malignancy. After switching to osimertinib and whole-brain radiotherapy, he remained stable for seven months but then developed progressive symptoms including headaches, vertigo, and nausea. Follow-up brain MRI revealed abnormal leptomeningeal signal intensity in the bilateral cerebral and cerebellar hemispheres. CSF cytology revealed an adenocarcinoma. Restaging CT demonstrated progressive disease with enlargement of the mediastinal lymph node and new metastatic bone lesions. The patient received a combination of intravenous chemotherapy (cisplatin–pemetrexed), ITP, and osimertinib, yielding a five-month response.

Before starting ITP, CSF cfDNA analysis revealed *EGFR* T751_I759delinsD, *SMAD4* D537Kfs*14, and *EGFR* CNG. Five months after ITP, the patient experienced progressive cognitive impairment, recent memory loss, and general weakness. Follow-up brain MRI demonstrated progressive leptomeningeal enhancement in both the supratentorial and infratentorial brain regions. Intravenous cetuximab was then incorporated into the treatment regimen, along with osimertinib, intravenous pemetrexed, and ITP. After three months of this combination therapy, CSF cfDNA analysis indicated a reduction in CSF cfDNA concentration (from 12.4 to 6.7pg/ul), *EGFR* and *SMAD4* mutant allele frequency, as well as *EGFR* copy number. The patient's neurological status remained stable for eight months after the commencement of combination therapy. Unfortunately, he died of pneumonia with an OS of 20 months from the LM diagnosis.

Discussion

Our study aimed to investigate the clinical utility of CSF cfDNA in NSCLC patients with LM and explore the efficacy of ITP as a treatment strategy. Our findings revealed the potential benefits of ITP, with a median survival time of 7.4 months after ITP initiation. The coexistence of *EGFR* T790M and EGFR-independent resistance alterations in CSF cfDNA was associated with shorter survival times after ITP, whereas *MET* CNG in CSF cfDNA was associated with survival times exceeding 6 months following ITP. Furthermore, serial monitoring of CSF cfDNA revealed changes in genomic alterations over time in response to treatment.

It is frequently observed that even with significant LM, tumor cells can still be scant or absent in the CSF or difficult to diagnose due to abnormal morphology. Conversely, the CSF fluid supernatant has been recognized as a valuable source of tumor-derived DNA in previous studies [10,23–25]. In our study, all original driver mutations, including *EML4-ALK* and *KIF5B-RET* fusions, were detected in 27 CSF cfDNA (including six with negative cytology), confirming the molecular diagnosis of leptomeningeal disease. Our results were similar to previous findings that genotyping CSF supernatant is a more sensitive method

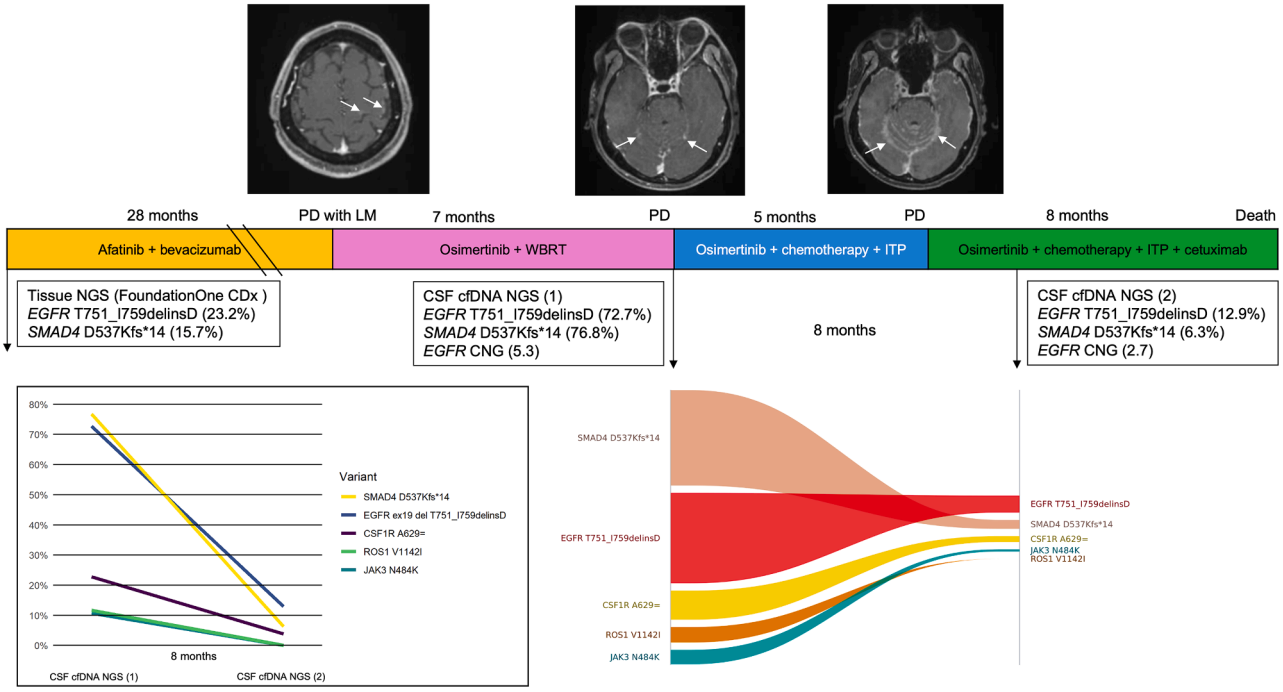

Fig. 3. A case's treatment trajectory with two instances of CSF cfDNA results, showcasing genomic alterations and their variations, along with corresponding treatment interventions. CNG, copy number gain; PD, progressive disease; LM, leptomeningeal metastasis; ITP, intrathecal pemetrexed; WBRT, whole brain radiotherapy.

(100 % sensitivity for six cytology-negative samples) for detecting mutations and confirming leptomeningeal disease involvement, disregarding the cytological results, even at low cfDNA concentrations [11,26]. Among the seven patients who did not receive ITP and adjusted their systemic treatment based on CSF cfDNA NGS results, three (42.9 %) achieved a state of neurological response. While NGS technology is commonly recommended for guiding precision treatment in advanced non-squamous NSCLC patients at diagnosis by utilizing tumor or plasma samples [27,28]. LM exhibits unique growth patterns and molecular distinctions compared to brain parenchymal metastasis [10,29], and can yield different genomic profiling results from primary lung tumors [10,30]. Therefore, the therapeutic implications arising from NGS findings via rebiopsy of tissues sample or plasma cfDNA for LM treatment may not be fully effective [31,32].

In our study, 18 patients received ITP, and all of them had experienced symptomatic LM progression on targeted therapy before undergoing ITP. Among the 18 patients, 14 (77.8 %) achieved effective control of neurological symptoms with ITP. Only four (22.2 %) patients experienced grade 2 or higher neutropenia as an adverse event. The median survival time after ITP was 7.4 months, which is consistent with the findings of previous reports [17,33]. Therefore, our results support the clinical benefit and safety of ITP in extensively treated NSCLC patients who experience progressive LM after undergoing targeted therapy [17,34].

In our exploratory analysis, *MET* CNG served as a predictor of survival times exceeding 6 months after ITP. Focal CNG in the *MET*, which results in oncogene addiction, is an actionable driver alteration in NSCLC. This alteration can occur either as a primary driver or as a mechanism of acquired resistance following treatment with tyrosine kinase inhibitors (TKIs) [35]. Cohort B of the phase II VISION trial, which investigated the efficacy of tepotinib in patients with NSCLC and *MET* amplification, analyzed plasma cfDNA biomarkers at baseline [36]. These analyses indicate that focal *MET* amplification is associated with more favorable outcomes than non-focal MET amplification. However, in the phase II INSIGHT 2 study, which assessed the combination of tepotinib and osimertinib in *EGFR*-mutant NSCLC patients with *MET* amplification who had been previously treated with osimertinib, the efficacy of the combination therapy was not analyzed based on the focality of *MET* amplification [37].

Moreover, no clinical studies have explored the efficacy of combining EGFR and MET inhibitors in patients with LM who have acquired *MET* amplifications. In our study, all six patients with *MET* CNG who received ITP exhibited a survival time exceeding 6 months. Among these *MET* CNG cases, five were identified as non-focal based on the concurrent presence of *CDK6/BRAF* CNG on chromosome 7q. Based on the findings from Cohort B of the phase II VISION trial, the addition of a MET inhibitor may not confer clinical benefits to patients with non-focal *MET* CNG. In accordance with our findings, ITP could be considered a potential treatment option for NSCLC patients who have LM and acquired *MET* CNG, regardless of the focal nature of *MET* CNG.

In our analysis, four patients who had *EGFR* T790M detected in their CSF cfDNA had significantly shorter survival times than those who did not exhibit *EGFR* T790M. Among the four patients, one individual with *EGFR* G719A mutation and de novo T790M exhibited concurrent *EGFR, MET, MYC, CDK4, CDK6, CCNE1*, and *BRAF* CNG, in addition to the detection of subclonal *BRAF* V600E within the CSF cfDNA. The remaining three patients with *EGFR* exon 19 deletion and acquired T790M mutations exhibited co-occurring alterations in CSF cfDNA, including *NF2* Y144*, *PTEN* T319*, and *PIK3CA* H1047R, respectively. The coexistence of *EGFR* T790M and EGFR-independent resistance alterations emerges because of tumor heterogeneity following the administration of EGFR-TKIs [38]. Patients with multiple pre-existing resistance mechanisms, such as T790M and *MET* alterations, tended to exhibit less favorable responses when treated with a third-generation *EGFR* TKI, rociletinib [39]. Similarly, both initial and subsequent ctDNA profiling revealed the enrichment of *PIK3CA* alterations in subclonal tumors with *EGFR* T790M mutations, which were demonstrated to contribute to osimertinib resistance [40]. Our findings emphasize the significance of tumor heterogeneity in *EGFR*-mutant NSCLC with LM

This study had some limitations. Although a small sample size was sufficient for a preliminary investigation, larger cohorts could provide more robust insights. Furthermore, the diversity of mutations in our

cohort could lead to confounding factors influencing survival outcomes. This retrospective study has inherent limitations in data collection and potential selection bias. However, the strength of our study is that broader patient populations and treatment settings were included from five hospitals in Taiwan and truly reflected real-world practice, including comprehensive analyses of ITP treatment in NSCLC patients with LM and the variability of cytology results from CSF samples leading to successful NGS analyses.

Conclusions

Our study focused on recurrent or advanced NSCLC patients with LM and highlighted the efficacy of ITP and the significance of specific genomic alterations in predicting survival outcomes. The results revealed important insights into the management of LM in NSCLC patients, providing personalized treatment approaches, and the potential of CSF cfDNA analysis for monitoring disease progression and treatment response.

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Data availability

This article and the supplementary materials contain all the data presented in this study.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 3.5 in order to enhance English writing and editing. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Sheng-Kai Liang: Data curation, Formal analysis, Resources, Visualization, Writing – original draft. **Wei-Yu Liao:** Conceptualization, Data curation, Resources, Visualization, Writing – original draft, Writing – review & editing. **Jin-Yuan Shih:** Data curation, Resources, Writing – review & editing. **Chia-Lin Hsu:** Data curation, Resources. **Ching-Yao Yang:** Data curation, Resources. **Shang-Gin Wu:** Data curation, Resources. **Yen-Ting Lin:** Data curation, Resources. **Yueh-Feng Wen:** Data curation, Resources. **Lun-Che Chen:** Data curation, Resources. **Yen-Fu Chen:** Data curation, Resources. **Ya-Fang Chen:** Data curation, Resources. **Yen-Heng Lin:** Validation. **Chong-Jen Yu:** Data curation, Resources, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

SKL received speaking honoraria from ACTgenomics, Boehringer Ingelheim, AstraZeneca, Pfizer, Novartis, Bristol-Myers Squibb, and Chugai Pharma Taiwan. WYL reported receiving speaking honorariums from AstraZeneca, Roche, Boehringer Ingelheim, Eli Lilly, Pfizer, MSD Oncology, Novartis, TSH Biopharm, Bristol-Myers Squibb, Johnson & Johnson, Bayer, and Chugai Pharma Taiwan outside of the submitted work. JYS received speaking honoraria from ACTgenomics, Amgen, AstraZeneca, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, Chugai Pharma, CStone Pharmaceuticals, Daiichi Sankyo, Eli Lilly, Genconn Biotech, GSK, Janssen, Lotus Pharmaceutical Co., Merck Sharp & Dohme, MundiPharma, Novartis, Ono Pharmaceutical, Orient EuroPharma, Pfizer, Roche, Takeda, TSH Biopharm, TTY Biopharm; received support for attending meetings from AstraZeneca, Roche, and Chugai Pharma; as well as grant from Roche. The other authors have no relevant conflicts of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2024.101113.

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