ORIGINAL ARTICLE

Combination of Cytologic Findings and Circulating Tumor DNA From Cerebrospinal Fluid Revealed SCLC Transformation in Patients With Leptomeningeal Metastases of Lung Adenocarcinoma

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ABSTRACT

Introduction: Transformation to SCLC is a resistance mechanism to tyrosine kinase inhibitor in *EGFR*-mutated lung adenocarcinoma (LUAD). Nevertheless, the clinical and molecular features of SCLC transformation in LUAD with leptomeningeal metastases (LM) are scarce.

Methods: We retrospectively collected 237 patients with NSCLC who underwent lumbar puncture owing to suggestion of LM. All SCLC transformation in cerebrospinal fluid (CSF) was confirmed by two experienced pathologists using cytologic evaluation. CSF circulating tumor DNA (ctDNA) was tested by next-generation sequencing.

Results: Tumor cells in CSF samples were found in 111 patients (111 of 237, 46.8%), and eight cases (eight of 111, 7.2%) were identified as having SCLC cells in CSF. Seven patients carried the *EGFR* mutation, including four patients with *EGFR exon 19 deletion* and three patients with *EGFR exon 21 L858R* mutation. Another patient harbored *ERBB2* insertion. Seven of these patients were resistant to targeted therapy. CSF ctDNA analysis reported that *TP53* and *RB1* mutations were common. The median time from the diagnosis of advanced NSCLC to SCLC transformation found in CSF was 9.7 months (95% confidence interval [CI]: 4.0–17.5 mo). The median overall survival since the initial diagnosis of metastatic NSCLC was 15.3 months (95% CI: 1.2–29.4 mo). The median overall survival after SCLC transformation detected in CSF was 5.0 months (95% CI: 4.0–5.9 mo).

Conclusions: SCLC transformation may be revealed in CSF by both cytologic evaluation and ctDNA, not just in tissue that underwent rebiopsy. SCLC transformation of CSF is informative for resistance mechanism in patients with LUAD with LM on tyrosine kinase inhibitor progression, which was associated with poor survival.

Keywords: Leptomeningeal metastases; SCLC; Transformation; Cerebrospinal fluid; Lung adenocarcinoma

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Introduction

Leptomeningeal metastases (LM) with poor survival are still devastating complications of NSCLC.[1] Our previous study found that LM were more frequent in lung adenocarcinoma (LUAD) with *EGFR* mutation.[2] A series of EGFR-tyrosine kinase inhibitors (TKIs) have become the standard therapy for newly diagnosed *EGFR*-mutant lung cancer.[3–6] However, these patients are inevitably faced with the challenge of disease progression owing to drug resistance, including T790M-mediated resistance to first-generation TKIs, emerging *MET* dysregulation, and histologic transformations, etc.[7] SCLC transformation has been described in approximately 3% to 5% of patients with *EGFR* mutant who progressed on first- and second-generation EGFR TKIs,[8,9] and seems to increase to up to 15% in patients progressing on first-line osimertinib.[10] Whether SCLC transformation could present as the potential resistance mechanism in LM was not fully explored.

Patients with small cell transformation typically experience an aggressive clinical course, and their conditions worsen rapidly.[11] Hence, it is important to diagnose or predict the transformation. Nevertheless, the molecular pathogenesis of SCLC transformation is largely not known. Previous studies[12,13] have revealed that patients with *EGFR*-mutant NSCLC with baseline *RB1* and *TP53* alterations have high indication of SCLC transformation. Nevertheless, the mere detection of *RB1* and *TP53* mutations does not mean the occurrence of SCLC transformation, and small cell transformation does not necessarily need the inactivation of *RB1* or *TP53* mutation. To screen for transformation, it is critical to perform tissue biopsies. According to National Comprehensive Cancer Network guidelines, SCLC can be diagnosed on histologic samples through hematoxylin and eosin-stained sections or cytologic samples. Nevertheless, owing to the complexity of LM, the molecular basis of this fatal, increasingly prevalent condition and resistance to TKIs developing remained unclear, especially when LM tissue is very difficult to obtain, unlike extracranial tumors. In our previous study,[14] cerebrospinal fluid (CSF) circulating tumor DNA (ctDNA) could provide the unique genetic profiles of LM, which serve as a liquid biopsy medium for stratifying risk, predicting prognosis, and revealing resistance mechanisms for LM. Moreover, loss of heterozygosity (LOH) of *TP53* was detected in 73.1% of CSF ctDNA, and *RB1* mutation was captured in CSF of four patients, all of whom carried *TP53* LOH. Because *TP53* and *RB1* alterations universally occurred in classical SCLC and were the key to SCLC transformation, whether SCLC transformation could be one of the resistant mechanisms of LM has attracted our attention.

Given LM is traditionally diagnosed on the basis of cytologic finding of tumor cells in CSF, we used CSF in the present study to explore potential mechanisms of resistance to TKIs among patients with *EGFR*-mutated NSCLC who had primary suggestion of LM. Finally, to our knowledge, this study is the first to report that SCLC transformation was found in CSF from patients with *EGFR*-mutant LUAD and further reveals the clinical and molecular features of the transformation in patients with LM.

Materials and Methods

Patients and CSF Samples Collection

We performed a retrospective chart review in patients with *EGFR*-mutated NSCLC with confirmed LM on the basis of cytologic findings of CSF from June 2016 to May 2019 at the Guangdong Lung Cancer Institute. Cytologic slides of CSF were re-evaluated to confirm the pathologic classification of tumor cells. All patients provided signed informed consent, and this study was undertaken with the approval of the research ethics committee of Guangdong Provincial People's Hospital.

For patients with lung cancer suggestive of LM, lumbar puncture was performed, and CSF was collected. CSF samples were processed usually within 1 hour from collection in the pathology division of our hospital. CSF samples were centrifuged at 1000 rpm to spin the cells on microscopic slides. To prepare CSF ctDNA for next-generation sequencing (NGS) analysis, CSF was centrifuged, and the supernatant was preserved.

Cytologic Finding of CSF and Histologic Diagnosis of Primary Tumors

A Papanicolaou stain was used, and cell nuclei could seem darker than cytoplasms for further evaluating cytologic features. As an integral part of the diagnostic workup for LM, detailed morphologic evaluation of cellular composition in CSF to detect tumor cells was independently performed by at least two expert pathologists. Tumor cells of NSCLC typically seem as large cells with hyperchromatic nuclei with prominent nucleoli and dark, basophilic cytoplasm. Skewed nuclear-to-cytoplasmic ratio was observed. For adenocarcinoma cells, typical accumulation of mucin was presented in cytoplasmic vacuoles. SCLC was otherwise characterized by small, round, oval cells with scant cytoplasm, finely and evenly dispersed chromatin, absent or inconspicuous nucleoli, and high mitotic counts. Microscopic slides of primary tumor for histologic evaluations were compared at the same time.

Preparation of CSF ctDNA

An identical procedure was used for ctDNA extraction from CSF. The supernatant was centrifuged for 10 minutes at 16,000*g* at 4°C, then transferred to a new tube.

CSF ctDNA was extracted from the supernatant using the QIAamp Circulating Nucleic Acid kit (Qiagen, Valencia, CA). Quantification of ctDNA was performed using the Qubit 2.0 Fluorometer with the double-stranded DNA HS assay kit (Life Technologies, Carlsbad, CA). A minimum of 50 ng of cell-free DNA was required for NGS library construction.

NGS Library Preparation and Sequencing Data Analysis

DNA fragmentation was performed using the M220 Focused-ultrasonicator (Covaris, Woburn, MA), followed by end repair, phosphorylation, and adaptor ligation. Only tissue DNA was subject to fragmentation. Fragments of 200 to 400 base pairs in size were selected by AMPure beads (Agencourt AMPure XP kit; Beckman Coulter, Brea, CA), followed by hybridization with capture probe baits, hybrid selection with magnetic beads, and polymerase chain reaction amplification. Finally, a high-sensitivity DNA assay was performed to assess the quality and size of all fragments. Indexed samples were sequenced using the Nextseq500 sequencer (Illumina, Inc., Hayward, CA) with pair-end reads.

Sequencing data were mapped to the human genome (hg19) using Burrows-Wheeler aligner 0.7.10. Local alignment optimization, variant calling, and annotation were performed using the GATK 3.2 and MuTect (both from Broad Institute, Cambridge, MA) and VarScan (Genome Institute, Washington University, St. Louis, MO) software.

Data Collection and Analysis

Data were collected regarding demographic information, primary tumor histologic diagnosis, molecular pathologic characteristics, and clinical treatments and outcomes. Response and progression assessments were determined by the clinical physician's judgment and notes. Because the data of the patients included were retrospectively collected and analyzed, no defined or standard time interval for obtaining response assessment could be presented.

Time to SCLC transformation was defined as the time from diagnosis of metastatic NSCLC to the time of rebiopsy revealing SCLC phenotype in CSF. Overall survival (OS) was defined as the date of diagnosis of advanced disease to date of death or last follow-up of May 2023. We also calculated OS after transformation, which was the time elapsing from cytologic diagnosis of SCLC transformation to death or last follow-up.

Thus, descriptive statistics were developed. Survival curves and time-to-event end points were estimated using the Kaplan-Meier method. All statistical analysis and graph making were performed using SPSS version 22.0 (SPSS, Chicago, IL) and GraphPad Prism version 8 (GraphPad Software Inc., San Diego, CA).

Results

Patient Characteristics

We examined 237 patients with NSCLC, all of whom underwent lumbar puncture owing to suspicion of LM. NGS was performed simultaneously in CSF ctDNA, and some of the patients had dynamic CSF test. Among them, 111 patients had tumor cells found on microscopic slides of CSF. After detailed morphologic evaluation to discern pathologic diagnosis, eight cases were identified to have SCLC in CSF (eight of 111, 7.2%) with confirmed diagnosis of LUAD from primary tumor. This cohort included four women and four men, ranging in age from 27 to 64 years. Six patients were nonsmokers. As to primary tumor genotyping, *EGFR* mutations were detected in seven patients, including four with *EGFR exon 19 deletion* and three with *EGFR exon 21 L858R* mutation, and *ERBB2* insertion was captured in another patient. Seven patients were resistant to targeted therapy (Table 1).

Pretransformation and Posttransformation Course

Three of the eight patients (B196, B175, B176) with primary LUAD received at least two lines of EGFR TKIs before transforming to SCLC in CSF, including first-, second-, and third-generation TKIs. Four patients (B300, B224, B27, B22) received one line of EGFR TKIs including erlotinib, afatinib, and osimertinib, respectively, and were then diagnosed with SCLC transformation by CSF. One patient (B196) had tumor relapse with SCLC transformation in CSF after adjuvant EGFR TKI treatments. The remaining patient (B91) was diagnosed with LM with SCLC component in CSF at the initial diagnosis of advanced NSCLC. Thus, 87.5% of patients (7/8) had received an EGFR TKI at the time of transformation.

Given SCLC transformation was found retrospectively through re-evaluation of CSF cytologic findings, no patient in this small cohort received platinum-etoposide regimen as expected after transformation. In the subset of four patients, at least two lines of systemic therapies were administered. Four patients (B300, B27, B22, B176) received brain irradiation (Fig. 1).

Survival

The median time from the diagnosis of advanced NSCLC to SCLC transformation found in CSF was 9.7 months (95% confidence interval [CI]: 4.0–17.5 mo). The median OS since the initial diagnosis of metastatic NSCLC was 15.3 months (95% CI: 1.2–29.4 mo). The median OS

Table 1. Demographic Characteristics

PID	Age	Sex	Smoking Status	Gene Status	Initial Biopsy Histologic Diagnosis	Treatment Before SCLC Transformation	CSF Histologic Diagnosis	Treatment After SCLC Transformation	Survival
B91	60	F	Never	*EGFR L858R*	ADC	-	SCLC	Erlotinib Osimertinib	Death
B176	53	M	Ever	*EGFR 19DEL*	ADC	Gefitinib;pemetrexed combined;erlotinib/ combined; afatinib	SCLC	Afatinib combined: osimertinib	Death
B224	51	M	Never	*EGFR L858R*	ADC	Erlotinib Osimertinib	SCLC (dubious)	-	Death
B175	27	M	Never	*EGFR 19DEL*	ADC	Erlotinib/ combined; osimertinib PCB	SCLC	PCB	Death
B196	59	F	Never	*EGFR L858R*	ADC	Osimertinib	SCLC	PCB	Death
B300	64	F	Never	*HER2 20INS*	ADC	Afatinib	SCLC (dubious)	Pemetrexed; WBRT; pemetrexed+ anlotinib	Death
B22	46	M	Ever	*EGFR 19DEL*	ADC	Erlotinib	ADC + SCLC (dubious)	Erlotinib dose escalation; osimertinb combined	Death
B27	40	L	Never	*EGFR 19DEL*	ADC	Erlotinib PCB	NSCLC + SCLC	WBRT + bevacizumab	Death

19DEL, exon 19 deletion; 20INS, exon 20 insertion; ADC, adenocarcinoma; CSF, cerebrospinal fluid; F, female; M, male; PCB, paclitaxel in combination with carboplatin and bevacizumab; PID, patient identification; SCLC, small cell lung cancer; WBRT, whole brain radiation therapy.

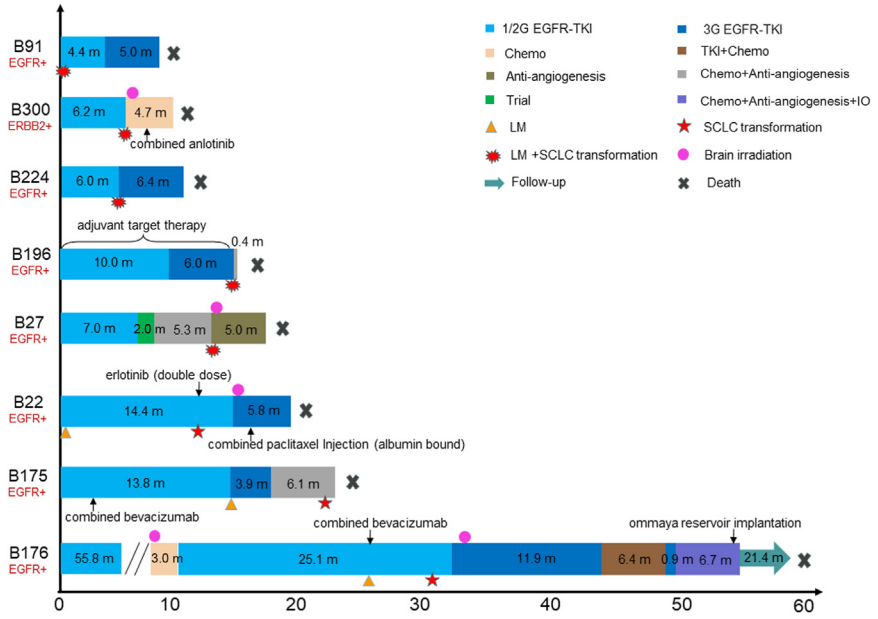

Figure 1. Treatment history on pre- and post-transformation detected by CSF in patients with leptomeningeal metastases. Treatment history of patients is described in timelines. Numbers indicate time (in months) from treatment to disease progression. Chemo, chemotherapy; G, generation; TKI, tyrosine kinase inhibitor; IO, immunotherapy; LM, leptomeningeal metastases.

after SCLC transformation detected in CSF was 5.0 months (95% CI: 4.0–5.9 mo) (Fig. 2).

Cytologic and Genetic Profiles of SCLC Transformation

At the time of transformation, CSF cytologic findings from these eight patients were reported as typical SCLC morphologic structure: clusters and sheets of densely packed, small, round or oval tumor cells with scarce cytoplasm, enlarged hyperchromatic nuclei, in obvious nucleoli, speckled chromatin, and focal nuclear molding. Matched primary tumor histopathologic characteristics were presented as typical adenocarcinoma (Fig. 3).

Limited tumor cells were found in CSF that were inadequate for immunocytochemistry (ICC) testing, but ctDNA from CSF was extracted and underwent NGS. The results confirmed driver mutations of *EGFR exon 19 del* and *L858R* and *ERBB2* insertion in all transformed cases. In patient B300, *ERBB2 p.V777delinsVGSP* and *TP53* mutation were detected in lung tissue at baseline and in CSF ctDNA after resistance to first-line afatinib. One transformed CSF sample harbored an *EGFR T790M* mutation, and another had *MET* copy number gain through ctDNA analysis. The frequency of *TP53* mutation and *RB1* inactivation was 62.5% (five of eight) and 50% (four of eight), respectively. Both altered genes were found in 37.5% of all cases (three of eight). However, it is not

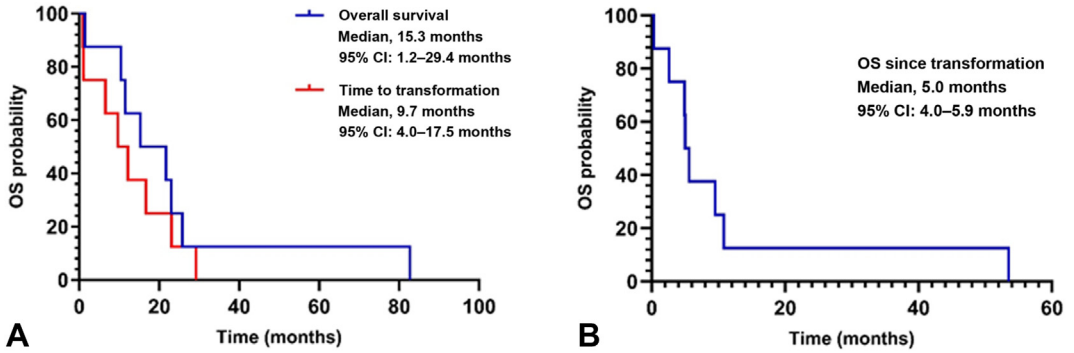

Figure 2. Kaplan-Meier curves of OS in patients with SCLC transformation in cerebrospinal fluid. (*A*) The median OS since the initial diagnosis of metastatic NSCLC and the median time from the diagnosis of advanced NSCLC to SCLC transformation found in cerebrospinal fluid. (*B*) The median OS after SCLC transformation. CI, confidence interval; OS, overall survival.

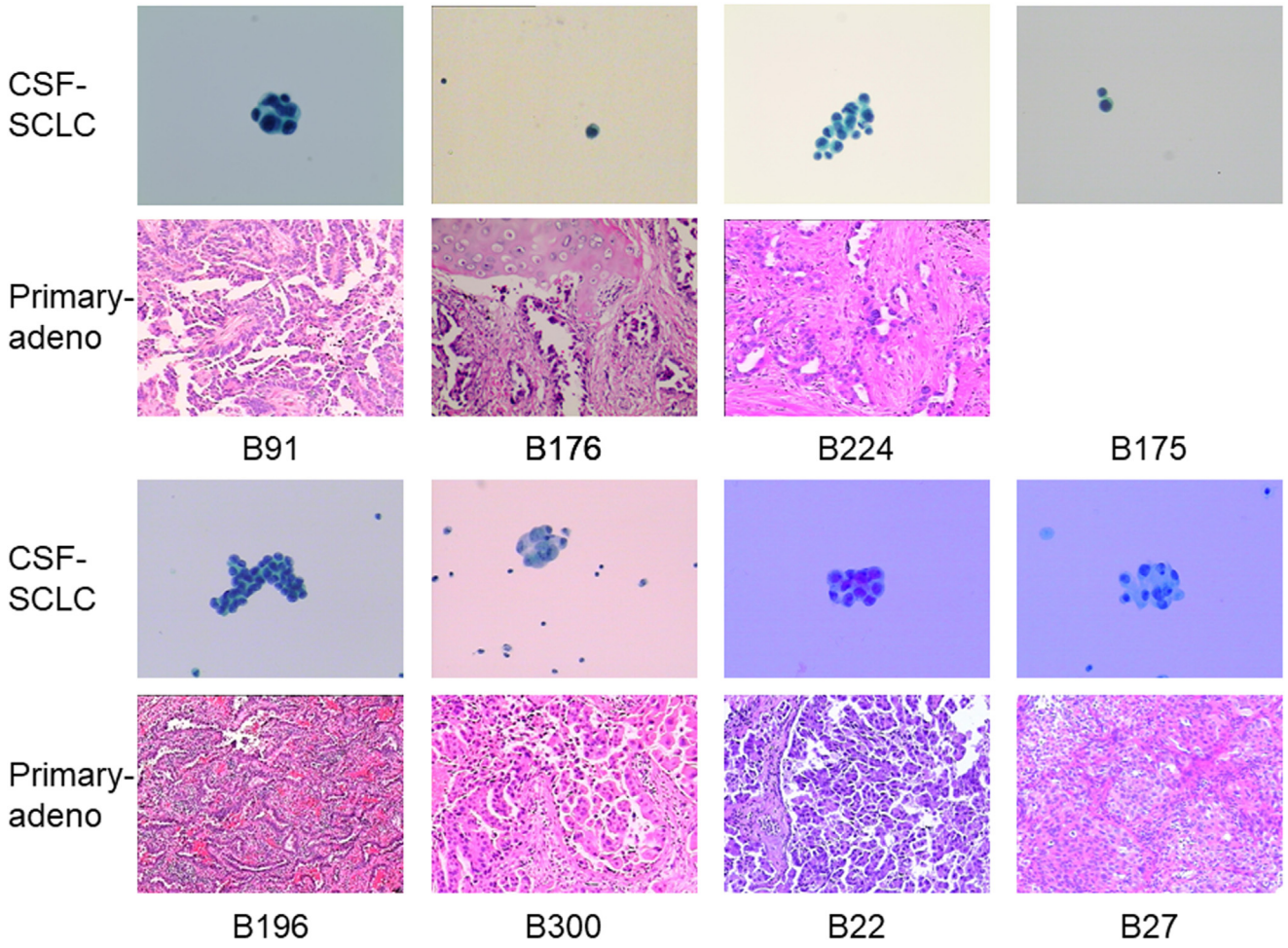

Figure 3. Cytologic evaluation (ThinPrep, Papanicolaou stain) of CSF and hematoxylin-eosin staining of matched primary tumors for eight patients with NSCLC with leptomeningeal metastases. adeno, adenocarcinoma; CSF, cerebrospinal fluid.

known whether *TP53* mutation and *RB1* inactivation coexisted with *EGFR* at baseline because seven of the eight patients did not receive NGS detection at baseline, except for patient B300. In addition, the copy number variants (CNVs) of *EGFR, CDKN2A, CDK4, FGF3*, and *FGF4* were relatively abundant in CSF ctDNA analysis (Fig. 4). Dynamic extraction for CSF was conducted in three cases (B22/B175/B176) with longer survival, when there was disease progression or symptom exacerbation. For all three patients, *EGFR 19del* with multiple CNVs was detected in CSF ctDNA throughout the disease course. Neoantigen burden was notably higher after TKI failure.

Discussion

SCLC transformation has been well described as one of the resistance mechanisms in *EGFR*-mutant NSCLC[8,9]; nevertheless, the presence of small cell transformation from LUAD has not been reported in CSF in those diagnosed with LM as potential intracranial resistance mechanisms to EGFR TKIs. In the present study, SCLC transformation was identified by cytologic evaluation and ctDNA of CSF for patients with LM in *EGFR*-mutated LUAD.

SCLC transformation is usually detected by hematoxylin and eosin staining and immunohistochemistry, using tissue testing or cytopathologic specimens such as cell blocks from malignant pleural effusion and pericardial effusion. Given a small number of tumor cells in CSF were insufficient for further ICC, it is challenging to diagnose SCLC transformation by CSF. Hence, we first re-evaluated the CSF cytologic finding of LM, which required pathologists rich in experience and at least two of these to verify SCLC in CSF. Furthermore, CSF ctDNA NGS was performed to testify the diagnosis. According to reports in the literature, CSF ctDNA could provide comprehensive genetic profiles of LM and has been widely used in clinical practice, especially those with only central nervous system progression and stable disease of extracranial lesions.[15–17] *TP53 LOH* and *RB1* mutation were detected far more frequently in CSF ctDNA than were those in plasma in our previous

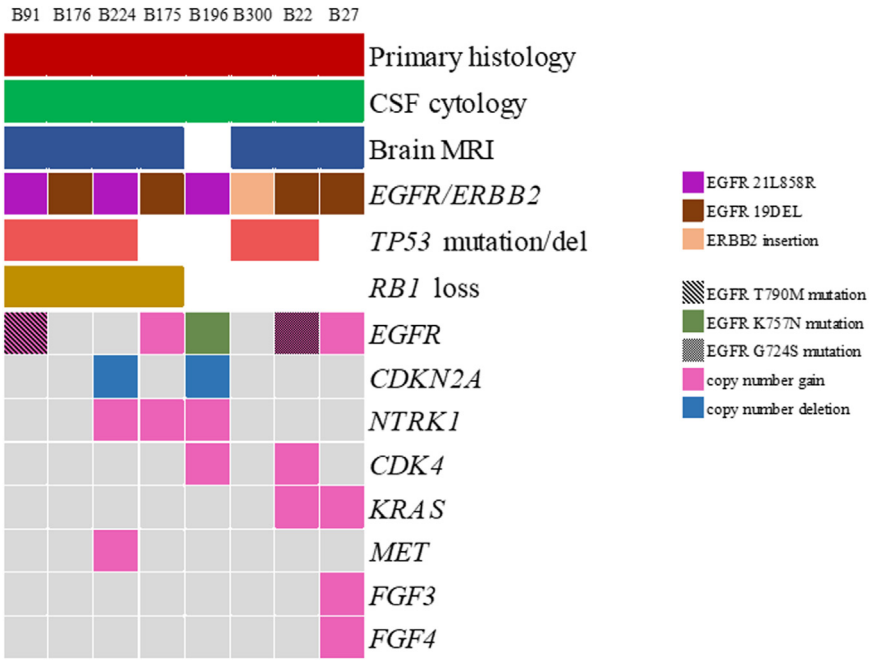

Figure 4. Genomic landscape of cerebrospinal fluid cell-free DNA in patients with leptomeningeal metastases. For each patient, the main clinical features and alterations in cancer genes are indicated with a color code. Clinical information is reported at the top of the panel. The type of genetic alteration (mutation, insertion, copy number gain, copy number deletion) is noted on the right. *19DEL, exon 19 deletion*; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging.

study,[14] indicating that CSF of LM may carry some of the molecular features of SCLC. In our study, *TP53* mutation and *RB1* inactivation were found in most of the cases, with the CNV of *EGFR, CDKN2A, CDK4, FGF3*, and *FGF4*. Of importance, given the increasing use of CSF ctDNA for patients with LM after acquired TKI resistance, our data emphasized the role of CSF for pathologic evaluation.

The histologic transformation from NSCLC to SCLC is one of the mechanisms of resistance to EGFR TKIs, the incidence of which was reported at 3% to 14%.[8–10] Concurrent *RB1* and *TP53* alterations were highly indicative of histologic transformation, with 25% presenting with de novo SCLC or eventual small cell transformation.[12] Moreover, central nervous system involvement was frequent after transformation, with a high rate of 64%,[18] suggesting constant vigilance was needed to identify SCLC transformation in those with LM. In this study, the founder *EGFR* mutations and *ERBB2* mutation were universally maintained; *TP53* alteration and *RB1* inactivation were 62.5% and 50%, respectively, and the triple-mutant population was 37.5% in CSF ctDNA of LM, further supporting the SCLC transformation in molecular profiling. In a previous study, combination of tissue and sequential ctDNA samples analyzed by NGS could find the presence of acquired gene alterations during tumor progression with SCLC transformation, suggesting the increasingly important role of liquid biopsy in this setting. In conclusion, the small cell transformation found in the CSF was credible, and patients with LM on EGFR TKI progression should undergo lumbar puncture to gain CSF for ctDNA NGS and cytologic evaluation in clinical practice.

Patients with NSCLC with small cell transformation experienced inferior clinical outcomes, and the median OS after SCLC transformation detected in CSF was 5.0 months (95% CI: 4.0–5.9 mo) in the present study, which was consistent with poor prognosis in transformed SCLC, but the poor prognosis may also be associated with LM. Nevertheless, there was an exception in patient B176. This patient had an initial diagnosis with stage IIIA adenocarcinoma with *EGFR 19del* and underwent radical resection and adjuvant chemotherapy. After more than 2 years, the patient relapsed owing to lung lesion enlargement and received pulmonary radiotherapy followed by gefitinib targeted therapy. Multiple brain metastases developed in the patient 5 years later, and he received cyber-knife radiosurgery of brain lesions and chemotherapy. During the next treatment with erlotinib, the patient reported headache and dizziness, and tumor cells were found in CSF. Erlotinib/afatinib plus bevacizumab were administered. After 25 months of progression-free survival, the patient was treated with osimertinib and whole-brain radiotherapy. Owing to worsening of LM symptoms, he was subsequently given osimertinib plus pemetrexed, aumolertinib, paclitaxel

combined with anlotinib, and atezolizumab, and underwent Ommaya reservoir implantation afterward. Notably, this patient had an uncommon course with a prolonged time of survival. The potential reasons may be related to cancer heterogeneity, which still needs further investigation.

Given small cell transformation was found through a retrospective re-evaluation of CSF cytologic findings, which was not reported in the initial diagnosis of LM, typical treatments for SCLC, such as platinum-etoposide regimen, were not administered as expected. Although the cohort of patients included in the study was from earlier years, most of these patients have received the third-generation EGFR TKI. Besides, patients with NSCLC with small cell transformation or LM have a poor prognosis. Hence, this population may have little benefit from improved treatment options after the development of small cell transformation.

The study had some limitations. Firstly, tumor cells in CSF were insufficient for ICC to further confirm SCLC transformation, and we failed to obtain leptomeningeal lesions to fully confirm SCLC transformation because they were invasive and difficult to access. Therefore, we combined the cell morphologic structure of CSF and ctDNA genetic profiles of CSF to support SCLC transformation. Secondly, the morphologic structure of CSF liquid biopsy was less typical than that of tissue biopsy, so the evidence for SCLC transformation in patients was limited, and it was greatly demanding of pathologists. Thirdly, this was a retrospective study mainly focused on patients with *EGFR*-mutated NSCLC with LM, and we did not collect the data of small cell transformation present in other metastases in all 237 patients. Hence, we were unable to compare prognostic differences among patients with small cell transformation at different sites. The cohort under study was limited in size and retrospective reassessment of CSF cytologic findings for evidence of SCLC transformation. Further validation through larger, prospective cohort studies is warranted.

In conclusion, a combination of CSF cytologic evaluation and ctDNA could reveal SCLC components in patients with LUAD with LM, identifying its suggested role as a resistance mechanism to SCLC transformation.

CRediT Authorship Contribution Statement

Jia-Xin Lin: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization.

Kai Yin: Investigation, Data curation, Visualization.

Li-Xu Yan: Investigation, Data curation, Visualization.

Mei-Mei Zheng: Resources, Data curation, Visualization.

Yang-Si Li: Resources, Data curation, Visualization.

Li-Xu Yan: Pathologic diagnosis, Visualization.

Qing-Ling Zhang: Pathologic diagnosis, Visualization.

Shi-Ling Zhang: Investigation, Data curation.

Kang-Hui Zeng: Investigation, Data curation.

Hong-Hong Yan: Formal analysis.

Zhi-Hong Chen: Resources, Supervision.

Xu-Chao Zhang: Resources, Supervision.

Hai-Yan Tu: Writing - review and editing.

Qing Zhou: Writing - review and editing.

Jin-Ji Yang: Writing - review and editing.

Ben-Yuan Jiang: Writing - review and editing.

Yi-Long Wu: Conceptualization, Methodology, Formal analysis, Resources, Writing - review and editing, Supervision, Funding acquisition.

Disclosure

Yi-Long Wu declares advisory services for AstraZeneca, Boehringer Ingelheim, Novartis, and Takeda; speaker fees from AstraZeneca, BeiGene, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, Merck Sharp & Dohme, Pfizer, Roche, and Sanofi; and grants from AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, Hengrui, and Roche outside of the submitted work. The remaining authors declare no conflict of interest.

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