Cell-Cycle and DNA-Damage Response Pathway Is Involved in Leptomeningeal Metastasis of Non-Small Cell Lung Cancer

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Abstract

Purpose: Leptomeningeal metastasis (LM) is a detrimental complication of non–small cell lung cancer (NSCLC) and associated with poor prognosis. However, the underlying mechanisms of the metastasis process are still poorly understood.

Experimental Design: We performed next-generation panel sequencing of primary tumor tissue, cerebrospinal fluid (CSF), and matched normal controls from epidermal growth factor receptor (EGFR) mutation-positive NSCLC patients with LM.

Results: The status of EGFR-activating mutations was highly concordant between primary tumor and CSF. PIK3CA aberrations were high in these patients, implicating an association with LM risk. Intriguingly, low overlapping of somatic protein-changing variants was observed between paired CSF and primary lesions, exhibiting tumor heterogeneity and genetic divergence. Moreover, genes with CSF-recurrent genomic alterations were predominantly involved in cell-cycle regulation and DNA-damage response (DDR), suggesting a role of the pathway in LM development.

Conclusions: Our study has shed light on the genomic variations of NSCLC-LM, demonstrated genetic heterogeneity and divergence, uncovered involvement of cell-cycle and DDR pathways, and paved the way for potential therapeutic approaches to this unmet medical need.

Introduction

Approximately 10% to 15% of patients with non–small cell lung cancer (NSCLC) in Western countries and 40% to 55% in Asia harbor epidermal growth factor receptor (EGFR)-activating mutations such as L858R and exon 19 deletion (19Del). EGFR tyrosine kinase inhibitors (TKIs) have opened up an era of target therapy in NSCLC and markedly extended progression-free survival (PFS) of patients with EGFR-activating mutations (1–6). However, there have been reports of cumulative incidence of central nervous system (CNS) metastases over time (7, 8). Diagnosis and treatment of leptomeningeal metastases (LM), a form of CNS metastases, remain particularly challenging.

LM is defined as the spread of malignant cells to the leptomeninges and throughout the subarachnoid space, and dissemination of tumors cells into the cerebrospinal fluid (CSF) compartment. About 9% to 10% of patients with NSCLC with EGFR-activating mutations will develop LM either at initial diagnosis or during TKI treatment (9, 10). LM is often associated with poor prognosis and represents a terminal event of NSCLC. The median overall survival (OS) after LM diagnosis is 3.1 to 11 months, with ~60% death due to LM or LM together with systemic lesions (9–13). So far, there is no effective treatment, though preliminary clinical activities of osimertinib and AZD3759 in patients with NSCLC with LM have been reported (14, 15), and other CNS-penetrant TKIs such as tesevatinib are being developed. Moreover, the mechanisms underlying the metastasis process are not elucidated yet. Further research is warranted to understand the genetic alternations driving the metastasis and to develop targeted strategies to improve the survival of patients with NSCLC-LM.

Plasma circulating tumor DNA (ctDNA) has been shown to effectively reflect tumor-specific genomic alterations and has been used to dynamically monitor tumor progression, response, and relapse (16). However, due to blood–brain barrier (BBB), patients with brain tumors do not present with or present with only low amounts of ctDNA in plasma (17). Recently, a few studies have demonstrated that tumor mutations are detectable in the CSF of patients with various primary and metastatic brain tumors, and ctDNA in CSF recapitulates the genomic landscape of brain tumors better than plasma (18–20). Therefore, CSF may serve as a potential biopsy to monitor brain tumor evolution. In this study, we performed next-generation panel sequencing of matched primary tumor tissues, CSF samples and normal controls from 11 patients with EGFR mutation-positive NSCLC to characterize genomic
aberrations during LM development and to understand signaling pathways implicated in this metastasis process.

**Materials and Methods**

**Patients and sample collection**

The 11 patients with NSCLC-LM were diagnosed at Zhejiang Cancer Hospital and Peking Union Medical College Hospital during 2009 to 2015. *EGFR* mutation status of their primary tumors was initially determined by amplification-refractory mutation system (ARMS) assay at the hospitals, as one of the inclusion criteria. The study was approved by the hospital research ethics committees, and all patients had signed the informed consent form. Formalin-fixed paraffin-embedded (FFPE) tumor and normal tissues and freshly frozen CSF and whole blood samples were collected for genomic profiling (Supplementary Table S1). All FFPE tissues were reviewed by histopathologic assessment. Tumor tissues contained ≥30% tumor percentage, and normal tissues had no tumor cell contamination under light microscopy.

**DNA extraction**

Genomic DNA from FFPE tumor and normal tissues was isolated using GeneRead DNA FFPE Kit (Qiagen) according to the manufacturer’s protocol. Total DNA from freshly frozen CSF was extracted by QIAamp Circulating Nucleic Acid Kit (Qiagen). Genomic DNA from whole blood was isolated using QIAamp DNA Mini kit (Qiagen). 2100 Bioanalyzer (Agilent) spectra and quantification of total CSF DNA were provided in Supplementary Fig. S1.

**Next-generation panel sequencing**

DNA samples were fragmented to approximately 200-bp peak size by sonication before library construction. The Illumina libraries were constructed with KAPA Hyper Prep Kit (Kapa Biosystems) according to the manufacturer’s protocol. For samples from patients P1 to 3, targeted panel sequencing was performed using SureSelect XT Target Enrichment System for Illumina Pair-end Multiplex Sequencing (Agilent), and captured by the LungPlasma panel (Burning Rock Biotech) covering 168 cancer-associated genes (Supplementary Table S2). Enriched libraries were sequenced on NextSeq 500 sequencing system (Illumina) with 150-bp pair-end reads. For specimens from patients P4-11, targeted panel sequencing was performed using SeqCap EZ System (Roche Nimblegen) and captured by the customized SeqCap EZ Choice Library (Roche Nimblegen) covering 403 cancer-associated genes (Supplementary Table S3). Enriched libraries were sequenced on HiSeq X10 sequencing system (Illumina) with 150-bp pair-end reads. On-target average sequence depth (before deduplication): FFPE tissues >500× except ~300× in P6T; blood >2,700×; CSF >4,000× except ~1,880× in PSC.

**Bioinformatics data analysis**

BWA (21) was used for mapping the pair-end reads to human reference genome hg19, samblaseter (22) for marking duplicate reads, and VarDict (23) for detection of single-nucleotide variants (SNV) and insertions and deletions (indels). Information on microsatellite instability (MSI) was extracted from the output of VarDict. Analysis of structural rearrangements was carried out using ArrayStudio (OmicSoft). SNVs and indels were called with the criteria of (i) variant allele frequency (VAF) ≥5% or ≥1% if associated with a COSMIC ID; and (ii) ≥3 supporting reads and ≥10 total reads after deduplication. Variant calls of hotspot mutations and structural rearrangements were manually inspected and rescued.

For tumor and CSF samples with patient-matched normal DNA, the somatic status was inferred by removing variants with VAF ≥10% in the normal control. For tumor and CSF samples without paired normal DNA, variants with VAF ≥10% in any of the normal samples from other patients were filtered.

Variants with ≥1% population prevalence in the Single Nucleotide Polymorphism Database (dbSNP; https://www.ncbi.nlm.nih.gov/snp/) were removed as common SNPs.

Protein-changing variants refer to SNVs and indels that fall into functional categories of frameshift, nonsense, start/stop lost, splice acceptor/donor, missense, and inframe insertion/deletion, with the first six types as likely deleterious.

**Results**

**Clinical characteristics of patients with NSCLC-LM**

The clinical characteristics of the 11 patients with lung adenocarcinoma with LM were detailed in Table 1. There were five males and six females, and the median age was 58 years old (range, 32–67). Their primary tumors carried either *EGFR* L858R or 19Del as determined by ARMS assay, and they received TKI treatment (gefitinib, erlotinib, or icotinib) 0.1 to 16.8 months (median, 1.8 months) after NSCLC diagnosis. One of the patients had LM at initial diagnosis, and 10 developed LM after 1 to 23.1 months (median, 7.3 months) of TKI treatment. Three patients had brain metastases (BMs) before they developed LM, and one was diagnosed with LM and BM simultaneously. The patients underwent systemic chemotherapy, intrathecal chemotherapy, or whole-brain radiotherapy (WBRT) during their disease courses.

**Profiling of primary tumor and LM-CSF by next-generation panel sequencing**

To understand the mechanisms underlying LM, we collected paired primary tumor and CSF samples from the 11 patients. The
<table>
<thead>
<tr>
<th>Patient</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
<th>P8</th>
<th>P9</th>
<th>P10</th>
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<tbody>
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<td>M</td>
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<td>M</td>
<td>M</td>
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<td>Age</td>
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<td>67</td>
<td>58</td>
<td>32</td>
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<tr>
<td>EGFR mutation type in primary tumor</td>
<td>19Del</td>
<td>L858R</td>
<td>L858R</td>
<td>L858R</td>
<td>L858R</td>
<td>19Del</td>
<td>L858R</td>
<td>L858R</td>
<td>L858R</td>
<td>L858R</td>
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<td>TNM staging at initial diagnosis</td>
<td>T2N2M1, IIIa</td>
<td>T1bN3M1, IV</td>
<td>T1bN3M1, IV</td>
<td>T4N2M1, IV</td>
<td>T4N2M1b, IV</td>
<td>T2N2M0, IIIa</td>
<td>T4N3M1b, IV</td>
<td>T2aN1M1a, IV</td>
<td>T4N3M1b, IV</td>
<td>T4N3M1a, IV</td>
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<td>Surgery</td>
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<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
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</tr>
<tr>
<td>BM diagnosis</td>
<td>0.2 mo</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
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</tr>
<tr>
<td>LM diagnosis</td>
<td>11.53 mo</td>
<td>8.6 mo</td>
<td>4.47 mo</td>
<td>14 mo</td>
<td>-0.03 mo</td>
<td>14.43 mo</td>
<td>-0.03 mo</td>
<td>5.07 mo</td>
<td>-0.03 mo</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>OS after NSCLC diagnosis</td>
<td>Alive (&gt;44.35 mo)</td>
<td>24.33 mo</td>
<td>5.63 mo</td>
<td>21.63 mo</td>
<td>11.77 mo</td>
<td>58.6 mo</td>
<td>13.97 mo</td>
<td>15.73 mo</td>
<td>23.7 mo</td>
<td>14.1 mo</td>
<td>47.8 mo</td>
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<td>OS after LM diagnosis</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
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<td>/</td>
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<td>EGFR-TKI</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>Icotinib</td>
<td>Gefitinib</td>
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<tr>
<td>Start</td>
<td>1.37 mo</td>
<td>187 mo</td>
<td>0.2 mo</td>
<td>0.1 mo</td>
<td>0.23 mo</td>
<td>1.77 mo</td>
<td>2.53 mo</td>
<td>15.03 mo</td>
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</tr>
<tr>
<td>End</td>
<td>Till now</td>
<td>Lost to follow-up</td>
<td>Until death</td>
<td>Until death</td>
<td>Until death</td>
<td>Until death</td>
<td>Until death</td>
<td>Until death</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PFS</td>
<td>10.17 mo</td>
<td>6.75 mo</td>
<td>4.27 mo</td>
<td>1 mo</td>
<td>9.4 mo</td>
<td>25.1 mo</td>
<td>7.9 mo</td>
<td>4.83 mo</td>
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<tr>
<td>Extracranial response</td>
<td>Bone metastasis, not evaluable</td>
<td>PR</td>
<td>PR</td>
<td>SD</td>
<td>31.5 mo</td>
<td>PR</td>
<td>PR</td>
<td>SD</td>
<td></td>
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<tr>
<td>Systemic chemo</td>
<td>Start</td>
<td>11.3 mo</td>
<td>0</td>
<td>0.07 mo</td>
<td>0.9 mo</td>
<td>/</td>
<td>42.17 mo</td>
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<td>/</td>
<td>1.17 mo</td>
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<tr>
<td>End</td>
<td>15.07 mo</td>
<td>17.4 mo</td>
<td>0.07 mo</td>
<td>17.33 mo</td>
<td>/</td>
<td>48.3 mo</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>11.17 mo</td>
<td></td>
</tr>
<tr>
<td>Intrathecal chemo</td>
<td>Start</td>
<td>11.53 mo</td>
<td>8.6 mo</td>
<td>/</td>
<td>0.13 mo</td>
<td>42.27 mo</td>
<td>8.03 mo</td>
<td>5.17 mo</td>
<td>21.47 mo</td>
<td>9.27 mo</td>
<td>35.53 mo</td>
</tr>
<tr>
<td>End</td>
<td>14.43 mo</td>
<td>12.33 mo</td>
<td>/</td>
<td>/</td>
<td>0.6 mo</td>
<td>43.4 mo</td>
<td>8.23 mo</td>
<td>5.4 mo</td>
<td>23.2 mo</td>
<td>9.75 mo</td>
<td>36.17 mo</td>
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<tr>
<td>WBRT</td>
<td>Start</td>
<td>11.7 mo</td>
<td>/</td>
<td>/</td>
<td>0.87 mo</td>
<td>14.87 mo</td>
<td>0.5 mo</td>
<td>/</td>
<td>21.93 mo</td>
<td>0.75 mo</td>
<td>/</td>
</tr>
<tr>
<td>End</td>
<td>12.17 mo</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>2.07 mo</td>
<td>16.72 mo</td>
<td>1.6 mo</td>
<td>/</td>
<td>22.07 mo</td>
<td>2.63 mo</td>
<td>/</td>
</tr>
<tr>
<td>Sample collection</td>
<td>Primary tumor</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
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</tr>
<tr>
<td>CSF</td>
<td>-0.2 mo</td>
<td>0</td>
<td>0.2 mo</td>
<td>-0.03 mo</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9.9 mo</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: Information on sex, age, EGFR mutation type in primary tumor (by ARMS assay), TNM staging at initial diagnosis, surgery, BM diagnosis (by MRI), LM diagnosis (by cytology), survival, EGFR-TKI treatment, systemic/intrathecal chemotherapy, WBRT, and collection of primary tumor/CSF specimens. The time points of surgery, BM, LM diagnosis, treatment start/end, and sample collection were calculated as months from NSCLC diagnosis (by pathology). Tumor responses were assessed according to Response Evaluation Criteria in Solid Tumors Version 1.1 (RECIST 1.1). Progression was defined as the development of a new lesion or relapse of any lesions during the treatment course. OS of patient P1 was calculated by data cutoff date (April 25, 2017). Abbreviations: Cmpd, compound; F, female; M, male; mo, months; OS, overall survival; PFS, progression-free survival; PR, partial response; SD, stable disease.
primary tumor tissues were obtained before EGFR-TKI therapy and CSF samples during TKI treatment (Table 1). Specimens were profiled by next-generation panel sequencing on cancer-associated genes using patient-matched whole blood or adjacent normal tissues as control if available. Somatic variants were called and annotated according to their functional impacts.

Concordance of EGFR-activating mutations in primary tumor and LM-CSF

We first investigated whether CSF carried the same EGFR mutation type(s) as the primary tumor. The status of EGFR-activating mutations was highly concordant between primary tumor and CSF pairs of all the patients, except in patient P10 whose primary tumor had undetectable EGFR mutation, while both L858R and 19Del were detected in CSF (Table 2). We reasoned that the negative EGFR testing results by panel sequencing in primary tumor of patient P10 were likely due to low amount of input and poor sequence coverage (only 20× and 29× effective depth around L858R and 19Del region, respectively). Indeed, 19Del was originally detected in her primary tumor by ARMS assay (Table 1). Taken together, there were eight patients with EGFR mutations present with NSCLC-LM cohort (five out of 11) is significantly higher than what was reported in general lung adenocarcinoma population—only five out of 371 and 44 out of 1552 patients with EGFR mutation-positive NSCLC in Western and Asian datasets, respectively, carried PIK3CA mutations (P < 0.001; Supplementary Table S5). These results implicated that aberrations in the PI3K pathway may be associated with NSCLC-LM risk.

Genetic divergence between primary tumors and LM

To better understand the underlying mechanisms of LM, we performed pair-wise comparison of somatic variants from primary tumors and CSF. Among the 11 patients, samples from patients P1 to 3 were profiled using a small gene panel with a ~260 kb coverage region (Supplementary Table S2), whereas specimens from patients P4 to 11 were sequenced with a large panel of 3.6 Mb region (Supplementary Table S3). Hence, to keep the consistency of data interpretation, we focused our analysis on the latter batch of results. Interestingly, low overlapping was observed in somatic protein-changing variants between matched primary tumor and CSF samples (Fig. 1A). Note that 3.2% to 25.7% (median, 5.8%) of variants from primary tumor were identified in paired CSF, and 5.4% to 32.4% (median, 17.9%) of variants in CSF overlapped with those in the primary site. Moreover, characterization of somatic SNVs within protein-coding regions revealed different mutational spectrum of matched primary tumor and CSF (Fig. 1B). There was a consistent trend toward elevated A>G and decreased C>T transitions in CSF samples comparing to paired primary tumors (P < 0.01). Even more intriguingly, there was a significant difference regarding size distribution of indels between primary tumor and CSF (Fig. 1C). CSF harbored more small indels (mostly 1 bp) either in overall or MSI regions. By contrast, tumor samples carried substantial numbers of large indels (>50 bp). These data suggested a high degree of tumor heterogeneity and genetic divergence during LM development.

Involvement of cell-cycle regulation and DNA-damage response (DDR) pathway in NSCLC-LM

To highlight genomic alternations implicated in the LM process, we combined the somatic protein-changing variants identified either from primary tumor or CSF samples of patients P4 to 11 and grouped them according to their occurrence. In total, there were 4246 unique variants, among which 129 in CSF and 47 in tumor showed ≥3 occurrence in each sample type, respectively (Supplementary Table S6). As the pair-wise comparison indicated that primary tumors and LM are highly divergent, we emphasized on the variants prevalent in CSF over primary tumors, or vice versa. Using the cutoff of n ≥3 in CSF and ≤1 in tumor, there were 55 CSF-recent variants mapping to 40 genes (Supplementary Tables S6 and S7). On the contrary, with the same criteria, we only had eight tumor-recurrent variants located to seven genes, among which four genes overlapped with those of CSF. When we classified these 43 genes based on existing biological pathway knowledge, 29 of them involved in cell cycle and DNA damage, chromatin remodeling and epigenetics, cytoskeleton remodeling, TGFβ, PI3K/AKT, Notch, Wnt or Hedgehog signaling, etc. (Supplementary Table S7). Noteworthy, cell-cycle regulation and DDR appeared to be the predominant pathway which harbored

Table 2. EGFR mutations and alterations of the PI3K pathway in patients with NSCLC-LM

<table>
<thead>
<tr>
<th>Patient</th>
<th>Primary tumor</th>
<th>LM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>EGFR 19Del (41.1%)</td>
<td>EGFR 19Del (18.2%)</td>
</tr>
<tr>
<td>P2</td>
<td>PIK3CA E545K (0.7%)</td>
<td>PIK3CA E545K (8.3%)</td>
</tr>
<tr>
<td>P3</td>
<td>EGFR L858R (54%)</td>
<td>EGFR L858R (37.3%)</td>
</tr>
<tr>
<td>P4</td>
<td>PIK3CA E545K (16.1%)</td>
<td>PIK3CA E545K (4.2%)</td>
</tr>
<tr>
<td>P5</td>
<td>EGFR L858R (63.4%)</td>
<td>EGFR L858R (17%)</td>
</tr>
<tr>
<td>P6</td>
<td>PIK3CA E545K (9.4%)</td>
<td>PIK3CA E545K (17.6%)</td>
</tr>
<tr>
<td>P7</td>
<td>PIK3CA E545K (17.6%)</td>
<td>PIK3CA E545K (17.6%)</td>
</tr>
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<td>P8</td>
<td>PIK3CA E545K (23.5%)</td>
<td>PIK3CA E545K (22.4%)</td>
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<td>P9</td>
<td>PIK3CA E545K (23.5%)</td>
<td>PIK3CA E545K (22.4%)</td>
</tr>
<tr>
<td>P10</td>
<td>EGFR L858R (28.8%)</td>
<td>EGFR L858R (63.6%)</td>
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<tr>
<td>P11</td>
<td>PIK3CA H1047L (20%)</td>
<td>PIK3CA H1047L (20%)</td>
</tr>
<tr>
<td>P12</td>
<td>PIK3CA E545K (9.4%)</td>
<td>PIK3CA E545K (9.4%)</td>
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</table>

NOTE: Mutation status of EGFR, PIK3CA, and PTEN in primary tumor and CSF samples from patients P1–11, as detected by panel sequencing. The VAF of each mutation was indicated in parentheses. Abbreviation: ND, mutation not detected.
CSF-recurrent alterations (Fig. 2 and Supplementary Table S7), implicating a role in LM development. Specifically, recurrent variants in DNA double-strand break (DSB) repair genes **BRCA2**, **RAD50**, and **RAD51AP2** and mismatch repair (MMR) genes **MLH3** and **MSH3** were enriched in CSF comparing to primary tumors (Fig. 2). Subsets of genes in chromatin remodeling such as **ARID1B** and **ATRX** also possessed variants more prevalently in CSF than primary tumor. There was no significant correlation between the presence of recurrent variants in cell-cycle and DDR pathway and systemic/intrathecal chemotherapy or WBRT (\( P > 0.49 \) by the two-sided Wilcoxon rank-sum test; Fig. 2).

**Discussion**

Our genomic analysis of paired primary tumors and CSF samples from patients with NSCLC-LM has revealed several aspects regarding the diagnosis and treatment of this devastating disease. First of all, the **EGFR** mutation type(s) in CSF were highly concordant with those in primary tumor (100% sensitivity and 90.9% concordance), suggesting that mutation testing in CSF is a potentially powerful molecular diagnostic approach for LM to guide precision medicine. There are two types of DNA sources in CSF—cell-free DNA (cfDNA) from supernatants and genomic DNA from cell pellets. It was reported that a higher fraction of cfDNA is tumor-derived than pellet DNA samples (20). However, in our experience, the **EGFR** mutation type(s) in CSF cfDNA and pellet DNA sometimes can be different and complement each other (Supplementary Table S8). Therefore, to gain a comprehensive view of the genomic landscape of LM, we used total CSF DNA for profiling in this study.

Among the 11 cases here, T790M was not detected in any of the CSF samples, even in patients who developed LM during EGFR-TKI treatment. Though we cannot fully exclude the possibility that concurrent systemic/intrathecal chemotherapy or WBRT may suppress acquiring of the T790M resistance mutation, our results are consistent with previous reports demonstrating that the emergence of T790M in CNS lesions was relatively rare comparing to extracranial sites (24–26). This can probably partially be explained by the low exposure of first-generation EGFR-TKIs in CSF due to poor BBB penetration property (26), so that intracranial tumor cells are shielded from the effects of compounds and thus will not readily acquire the secondary T790M gatekeeper mutation.

Patients with **EGFR** mutation-positive NSCLC develop CNS metastases more frequently than those with wild-type **EGFR** (9, 27). On the other hand, only about 10% of patients with **EGFR** mutation-positive NSCLC develop LM (9, 10), suggesting that there are other cofactors contributing to this metastasis process. We found a high prevalence of **PIK3CA** mutations in our patient cohort, comparing to the general **EGFR** mutation-positive lung adenocarcinoma population, implicating an association with LM risk. A similar finding was reported in squamous cell lung cancer (SqCLC) in which patients with PI3K-aberrant tumors had more aggressive disease marked by worse survival, higher...
metastatic burden, and greater incidence of BM (27% vs. 0% in others, \( P < 0.001 \); ref. 28). It remains to be seen clinically whether combination of PI3K inhibitors with EGFR-TKIs can treat or prevent CNS metastases.

Although LM and their lung primaries carried the same \( \text{EGFR} \) activating mutation(s), we observed a high degree of genetic divergence and clonal heterogeneity between the paired samples, as illustrated by the low proportion of shared variants, different mutational spectrum of SNVs and size distribution of indels. Studies of early-stage NSCLC reported a significant amount of mutational and copy-number heterogeneity across regions of primary lung tumors (29, 30). Greater heterogeneity was shown in the metastatic SqCLC, with as little as 15% of mutations shared between primary lung cancer and BM pairs (28). In our hand, 2.1% to 12.5% (median, 4.1%) of total variants overlapped between primary tumors and LM-CSF. The sources of genetic divergence can be multifold, for example, different originating leptomeningeal metastatic subclone from the predominant primary lung cancer clone, additional genomic alterations acquired during the metastasis process, and clonal evolution under tumor microenvironment and in response to various treatments.

In the light of the genetic heterogeneity observed in primary tumor-LM pairs, we asked whether CSF samples shared common genomic features across patients which differentiated them from tumor tissues. We observed more recurrence of variants in CSF than primary tumors, even though the latter carried a larger number of total variants. Moreover, when we cataloged genes with CSF-recurrent variants, the cell-cycle and DDR and chromatin remodeling showed predominance among all the mapped pathways, implicating that LM may harbor a greater degree of genome instability resulting from deficiency in DNA DSB repair, MMR, and chromosome maintenance. Although such genomic abnormalities might be induced by exposure of LM to various treatment regimens, it seemed that there was no significant association between the recurrence of variants in cell-cycle and DDR pathway and systemic/intrathecal chemotherapy or WBRT. Whether EGFR-TKI treatment played any role here requires further study. Nonetheless, even though these treatments may interfere with cell cycle and cause DNA damage in tumor cells, their immediate mutagenesis effects are likely random across the whole genome, rather than on particular sets of genes and coordinates. The latter event is more the product of selection and evolution, which requires relatively long time to develop. Therefore, the chance that enrichment of recurrent variants in DSB and MMR genes in CSF is induced directly by treatments per se is low, if any. Aberrations of \( \text{ATM}, \text{BRCA1} \) and \( \text{BRCA2} \) were observed at substantially higher frequencies in metastatic castration-resistant prostate cancer (mCRPC) compared with those in primary

![Figure 2.](image_url)

Involvement of cell-cycle regulation and the DDR pathway in LM development. Genes with variants recurrent in CSF (\( n \geq 3 \) in CSF and \( \leq 1 \) in primary tumor) or tumor (\( n \geq 3 \) in primary tumor and \( \leq 1 \) in CSF) were mapped to biological pathways. Cell-cycle and DNA damage presented as the most dominant pathway, followed by chromatin remodeling and epigenetics. The presence of recurrent variants in each gene/sample/patient was shown as light blue (variants of uncertain significance) or dark blue (likely deleterious) blocks, and potential exposure of specimens to each treatment regimen was indicated as black blocks. Exposure to treatment was defined as (i) lesion was diagnosed before or during the treatment and (ii) sample was collected during or after the treatment.
prostate cancers (31–33), suggesting that enrichment of alterations in a DDR pathway might be a shared feature of more advanced, metastatic cancer. It has been reported that mutational signature comprising of large numbers of small indels (mostly 1 bp) at nucleotide repeats, whose pattern is often termed MSI, is characteristic of cancers with defective DNA MMR (34). Consistently, we observed significantly more 1-bp size indels within MSI regions in CSF than primary tumors, supporting the fact that the genotype (e.g., aberrations in MMR genes) led to a phenotype (i.e., genomic instability).

Genomic instability, one of the underlying hallmarks of cancers (35), provides a platform for genomic alternations driving tumor formation but also represents an Achilles’ heel for the therapeutic opportunities of synthetic lethality. Recent advancements in PARP inhibitors have proven the clinical utility of DDR-based therapies in ovarian cancer, breast cancer, and mCRPC patients with deleterious BRCA and ATM mutations, or even beyond, DNA-repair defects in general (36–43). Deficiency of ARID1A, the paralog of ARID1B, has been shown to impair DNA-damage checkpoint and sensitize cancer cells to PARP inhibitors in vitro and in vivo (44). Trials in colorectal cancer have illustrated that MMR status predicted clinical benefit of immune checkpoint blockade (45), and PD-1 antibody has been approved for the treatment of unresectable or metastatic solid tumors having MSI-high or MMR defects. Thus, it will be of great interest to see besides BBB-penetrable EGFR-TKIs, whether NSCLC-LM exhibits therapeutic vulnerability to DDR inhibitors or immunotherapy.

One of the technical challenges in our study is the variant calling on FFPE tumor tissues. FFPE specimens tend to have more sequencing artifacts than freshly prepared samples due to fragmentation, cytosine deamination, and oxidative damage. In fact, the mutation load of primary tumors calculated from our panel sequencing is within the high range of what was reported for lung adenocarcinoma (34), implicating that some variants are probably FFPE artifacts. Taking that into consideration, the degree of heterogeneity we observed between primary tumor-LM pairs may be overestimated as the majority of artifact variants are random, and thus there will be low concordance across samples. So far, there is no optimal way to fully address this issue. However, we believe that our findings on cell-cycle and DDR pathway are not compromised by this imperfection. The particular conclusion came from recurrent variants (≥3 in one sample type), which were then mapped to genes and pathways. Given that artifacts are most likely random, the chance of the same artifact will show up in multiple samples is very low. Moreover, recurrent alternations of cell-cycle and DDR genes were more enriched in CSF, which were freshly frozen samples, further relieving the concern that they were illusions caused by FFPE artifacts.

In conclusion, we have demonstrated the value of CSF as liquid biopsy for monitoring LM evolution and directing precision medicine. While future study with larger sample size will be desired to cross-validate our findings, our study is the first exploration to show that cell-cycle and DDR pathway might play an important role in NSCLC-LM development and point to potential new therapeutic strategies targeting this unmet clinical need.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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