Detection of Driver and Resistance Mutations in Leptomeningeal Metastases of NSCLC by Next-Generation Sequencing of Cerebrospinal Fluid Circulating Tumor Cells

Ben-Yuan Jiang, Yang-Si Li, Wei-Bang Guo, Xu-Chao Zhang, Zhi-Hong Chen, Jian Su, Wen-Zhao Zhong, Xue-Ning Yang, Jin-Ji Yang, Yang Shao, Biao Huang, Yan-Hui Liu, Qing Zhou, Hai-Yan Tu, Hua-Jun Chen, Zhen Wang, Chong-Rui Xu, Bin-Chao Wang, Shu-Yu Wu, Cun-Yi Gao, Xian Zhang, and Yi-Long Wu

Abstract

Purpose: Leptomeningeal metastases are more common in non–small cell lung cancer (NSCLC) with EGFR mutations. The diagnosis is difficult by traditional imaging only, and leads to poor understanding of resistance mechanisms of leptomeningeal metastases.

Experimental Design: We compared the CellSearch Assay, the Thinprep cytologic test (TCT), and brain magnetic resonance imaging (MRI) in 21 NSCLC patients with suspected leptomeningeal metastases. Next-generation sequencing that included 416 cancer-associated genes was also performed on cerebrospinal fluid circulating tumor cells (CSFCTCs) of 19 patients.

Results: Twenty-one patients were diagnosed with leptomeningeal metastases, and CSFCTCs were captured by CellSearch in 20 patients (median, 969 CSFCTCs/7.5 mL; range, 27–14,888). CellSearch had a sensitivity of 95.2% for leptomeningeal metastases diagnosis, which was higher than that of TCT (12/21, 57.1%), MRI (10/21, 47.6%), and MRI plus TCT (19/21, 90.5%), respectively. CTCs were found only in 5 of 14 patients (median, 2 CTCs/7.5 mL; range, 2–4), which was a much lower ratio than CSFCTCs. Genetic profiles of CSFCTCs were highly concordant with molecular mutations identified in the primary tumor (17/19, 89.5%). The resistance gene EGFR T790M was detected in 7 of 9 patients with extracranial lesions, but was detected in only 1 of 14 CSFCTC samples. Other potential resistant mutations, such as MET amplification and ERBB2 mutation, were also identified in CSFCTCs.

Conclusions: CSFCTCs captured by CellSearch may be a more sensitive and effective way to diagnose leptomeningeal metastases, and may serve as a liquid biopsy medium for gene profiles in NSCLC patients with leptomeningeal metastases.

Introduction

Leptomeningeal metastases remain devastating complications and they occur in 1–9.1% of patients with solid tumors. The incidence of leptomeningeal metastases in non–small cell lung cancer (NSCLC) patients has increased in the last 10 years (1); the current estimated incidence is 3.4%–3.8% (2, 3), and much more common in those with EGFR mutations with an incidence of 9.4% (2). Leptomeningeal metastases can be diagnosed by identifying tumor cells in the cerebrospinal fluid (CSF) or through magnetic resonance imaging (MRI) according to the National Comprehensive Cancer Network guidelines. However, the sensitive and specific diagnosis of leptomeningeal metastases is still challenging (4–6). It is sometimes difficult to identify leptomeningeal metastases as neurologic symptoms can be complicated and atypical; and the sensitivity of brain MRI was only 53% in a previous large study (7). CSF cytology can confirm leptomeningeal metastases diagnosis, and has high specificity (about 95%) but low sensitivity (about 50%) at the first lumbar puncture, leading to the deferred diagnosis of leptomeningeal metastases (8–10).

The CellSearch Assay, which is based on the assessment of nuclear and cell surface markers, has been validated to detect and isolate CTCs for follow-up analysis and diagnosis of lung, breast, prostate, and colorectal cancers that strongly express epithelial cell adhesion molecule (EpCAM; refs. 11–14). This technology has also been applied to diagnose leptomeningeal metastases by capturing malignant cells in the CSF of patients with solid tumors (15–18). However, the potential role of
CSFCTCs in Leptomeningeal Metastases of NSCLC

Translational Relevance

This study demonstrates the feasibility of cerebrospinal fluid circulating tumor cells (CSFCTCs) for sensitive diagnosis and "liquid biopsy" of leptomeningeal metastases in advanced non–small cell lung cancer (NSCLC). Leptomeningeal metastases are more common in patients with EGFR mutations, but tissue samples are very difficult to obtain, which leads to poor understanding of resistant mechanisms of leptomeningeal metastases. Here we perform next-generation sequencing including 416 cancer-associated genes on CSFCTCs in leptomeningeal metastases patients. Results showed that CSFCTCs captured by CellSearch may be a more sensitive and effective way to diagnose leptomeningeal metastases, and more importantly, genetic profiles of CSFCTCs were highly concordant with molecular mutations identified in the primary tumor (89.5%), and resistant genes could also be identified in CSFCTCs. These data demonstrate the feasibility and clinical utility of CSFCTCs as a liquid biopsy method for the detection of driver genes and resistance mutations of leptomeningeal metastases in patients with NSCLC.

Identification of CSFCTCs and CTCs

The CellSearch CTC kit (Veridex LLC) was used to count the number of tumor cells of epithelial origin using immunomagnetic enrichment of cells expressing epithelial cell adhesion molecule (EpCAM) followed by nuclear staining with DAPI (4,6-diamidino-2-phenylindole) and immunofluorescence detection of cytoplasmic cytokeratins (CK) 8, 18, and/or 19. Membrane CD45 staining was also performed to detect contaminating leukocytes. Samples collected in Cellsave preservative-containing tubes were processed within 96 hours. In total, 7.5 mL of peripheral blood and 5–7.5 mL of CSF were collected for the tests. When the CSF was less than 7.5 mL, PBS was mixed with CSF to a volume of 7.5 mL. The samples were tested by CellSearch standard procedures, and CSFCTCs were captured in the CellSearch cartridge, then transferred to the sterilized centrifuge tubes with pipette and preserved in −80°C refrigerator for further explorations.

Results are reported as the number of CSFCTCs/7.5 mL of CSF and CTCs/7.5 mL. Figure 1 shows representative peripheral epithelial tumor cells captured by CellSearch in peripheral blood and CSF of a patient. CTCs (Fig. 1A) and CSFCTCs (Fig. 1B) were identified using the following criteria: the cell was positive for the epithelial cell marker (CK-PE) and the nuclear dye (DAPI), negative for the leukocyte marker (CD45/APC), and negative in the blank channel.

Next-generation sequencing of CSFCTCs

We performed next-generation sequencing (NGS) using a capture-based targeted sequencing panel on the CSFCTCs of 19 patients. Human genomic regions totaling 1.4 megabases in size, including selected exons and introns of 416 genes, were captured using 120-base pair (bp) probes. DNA was fragmented into 200–250-bp segments, captured by the 120-bp probes, and sequenced by obtaining paired 2 × 150-bp reads.

To obtain DNA from CSFCTCs, cells were digested in 100-µL proteinase K for 10 minutes at 37°C, followed by extraction with QIAamp DNA FFPE Tissue Kit (Qiagen) with modified protocols. After DNA extraction from CSFCTCs, DNA concentrations were measured using the Qubit dsDNA assay (Invitrogen). DNA quality was confirmed by checking that the A260/A280 ratio was 1.8–2.0. DNA amounts varied after extraction from CSFCTCs. Total DNA was subjected to whole genome amplification (WGA) followed by library construction. Libraries were quantified using the Qubit dsDNA assay (Invitrogen), and same amount of amplification products were used for capture-based targeted sequencing. DNA was hybridized with the capture probes (the baits), selected using magnetic beads, and amplified by PCR. A bioanalyzer [Qubit and Agilent 2100 (Agilent Technologies)] was then used to perform high-sensitivity assays assessing DNA quality and size range. All samples were sequenced on a HiSeq 4000 platform (Illumina, Inc.), and paired-end reads were obtained. We aimed to achieve an average sequencing depth of 2000× for all targeted regions.

Mutant allele frequency (MAF) analysis was performed using Genome Analysis Toolkit and VarScan 2.3.9. Notably, single-nucleotide polymorphism (SNP) analysis was also performed with the above two software in this work, and the MAFs were around 50% or 100%, suggesting that the bioinformatics pipeline was performing as we had expected.
Characteristics of patients with leptomeningeal metastases

All of the patients were Chinese and had been diagnosed with lung adenocarcinoma. The median age was 53 years old (range, 32–76 years) and 10 of the 21 patients were male. Most patients (20/21) were diagnosed with stage IV disease, and the majority (16/21) classified as poor Eastern Cooperative Oncology Group performance status (ECOG PS > 1). Dizziness and headache were the most common neurologic symptoms, and 14 patients had concurrent brain metastases (Table 1).

Sensitivity and specificity of three methods during the initial diagnosis of leptomeningeal metastases

Leptomeningeal metastases was diagnosed by typical brain MRI or tumor cells found in the CSF (TCT or CellSearch) with or without neurologic symptoms, and all 21 patients were

Results

Characteristics of patients with leptomeningeal metastases

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Sensitivity and specificity of three methods during the initial diagnosis of leptomeningeal metastases

Leptomeningeal metastases was diagnosed by typical brain MRI or tumor cells found in the CSF (TCT or CellSearch) with or without neurologic symptoms, and all 21 patients were
identified as having leptomeningeal metastases (Fig. 2). The sensitivity of CellSearch was 95.2%, while the sensitivity of TCT from the same CSF puncture, MRI, and combined MRI plus TCT were 57.1%, 47.6%, and 90.5%, respectively. The specificity of CellSearch was 100% (Table 2).

The relationship between CSFCTCs and CTCs, MRI, and TCT CSFCTCs were found in 20 of 21 patients (median, 969 CSFCTCs/7.5 mL; range, 27–14,888), while CTCs were captured in only 5 out of 14 patients (median, 2 CTCs/7.5 mL; range, 2–4), a much lower ratio than the CSFCTCs. In addition, 12 patients had positive conventional cytology (TCT) CSF results, and MRI revealed typical leptomeningeal metastases in 10 patients (Fig. 2). Cytology-positive cases (median, 1146 CSFCTCs/7.5 mL) were prone to have a greater number of CSFCTCs than cytology-negative cases (median, 156 CSFCTCs/7.5 mL), but there was no significant difference between the two arms ($Z = -1.492; P = 0.136$).

Interestingly, 11 patients that experienced neurologic symptoms presented with no leptomeningeal metastases by MRI, but had carcinoma cells in their CSF samples. Two of these patients (patient 6 and patient 15) were negative in both traditional brain MRI and TCT, but metastatic CSFCTCs were captured by CellSearch (Fig. 2). Moreover, EGFR L858R in patient 6 and EGFR del 19 in patient 15 were identified in the CSFCTCs (Table 3).

Of the 10 patients with typical MRI images, 7 patients had no tumor cells in their CSF samples by TCT method, while 6 of them were found positive by CellSearch (Fig. 2) and driver genes were identified in their CSFCTCs (Table 3). Clinically significant gene profiles of CSFCTCs To interpret the genetic alterations in CSFCTCs with regard to disease development, the gene profiles of primary and re-biopsy specimens taken from the same patient were compared.

Table 1. Characteristics of 21 patients with leptomeningeal metastases

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, y</th>
<th>Gender</th>
<th>Histology</th>
<th>Disease stage before LM</th>
<th>ECOG PS at LM</th>
<th>Neurologic symptoms</th>
<th>Concurrent brain metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>53</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>3</td>
<td>Dizziness</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>3</td>
<td>Dizziness, headache, and numbness</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>1</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>Female</td>
<td>AC</td>
<td>IIA</td>
<td>3</td>
<td>Dizziness and hearing loss</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>46</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>1</td>
<td>Vomiting</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>3</td>
<td>Dizziness</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>59</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>4</td>
<td>Facial paralysis and hearing loss</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>51</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>1</td>
<td>None</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Dizziness</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>54</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Headache and visual loss</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>35</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Headache</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>53</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Headache</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>55</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Dizziness</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>54</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Anomia and dyslexia</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>32</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Blindness and anosmia</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>67</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>1</td>
<td>Dizziness</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>65</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Dizziness and vomiting</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>69</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Dizziness</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>76</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>1</td>
<td>Dizziness</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>46</td>
<td>Male</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Dizziness</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>46</td>
<td>Female</td>
<td>AC</td>
<td>IV</td>
<td>2</td>
<td>Headache</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviations: AC, adenocarcinoma; ECOG PS, Eastern Cooperative Oncology Group performance status score; LM, leptomeningeal metastases; +, positive; −, negative.

Figure 2. Relationship between CSFCTCs and CTCs, MRI. CSFCTCs, cerebrospinal fluid–circulating tumor cells; CTCs, circulating tumor cells; TCT, Thinprep cytologic test; MRI, brain magnetic resonance imaging; LM, leptomeningeal metastases; UA, unavailable.

No. Definitive LM CSFCTCs (cells/7.5 mL) TCT MRI Combined MRI and TCT CTCs (cells/7.5 mL) UA UA UA UA UA UA UA

1 1 1 1 1 1 1 1
2 1 1 1 1 1 1 1
3 1 1 1 1 1 1 1
4 1 1 1 1 1 1 1
5 1 1 1 1 1 1 1
6 1 1 1 1 1 1 1
7 1 1 1 1 1 1 1
8 1 1 1 1 1 1 1
9 1 1 1 1 1 1 1
10 1 1 1 1 1 1 1
11 1 1 1 1 1 1 1
12 1 1 1 1 1 1 1
13 1 1 1 1 1 1 1
14 1 1 1 1 1 1 1
15 1 1 1 1 1 1 1
16 1 1 1 1 1 1 1
17 1 1 1 1 1 1 1
18 1 1 1 1 1 1 1
19 1 1 1 1 1 1 1
20 1 1 1 1 1 1 1
21 1 1 1 1 1 1 1

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Table 2. Sensitivity and specificity of three methods for the initial diagnosis of leptomeningeal metastases

<table>
<thead>
<tr>
<th>Diagnostic method</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCT</td>
<td>57.1%</td>
<td>—</td>
</tr>
<tr>
<td>MRI</td>
<td>47.6%</td>
<td>—</td>
</tr>
<tr>
<td>Combined MRI and TCT</td>
<td>90.5%</td>
<td>—</td>
</tr>
<tr>
<td>CSFCTCs</td>
<td>95.2%</td>
<td>100%</td>
</tr>
</tbody>
</table>

(Table 3). The primary and rebiopsy tumor samples were examined using a range of clinically approved laboratory tests. In the 19 patients whose CSFCTCs DNA was detectable by NGS, gene profiles were highly concordant with the primary identified molecular mutations (17/19, 89.3%; Table 3). There was no direct evidence that the number of CSFCTCs would affect the identification of tumor-associated genetic alterations in their NGS assay, for the two patients (patient 7 and patient 12) whose CSFCTCs were negative in NGS assay have a bit higher number of CSFCTCs than median.

An important potential application of CSFCTCs is as a "liquid biopsy" to identify driver genes and drug-resistant mutations in patients with leptomeningeal metastases (Table 3). Of the 16 patients who experienced leptomeningeal metastases progression during treatment with EGFR/ALK inhibitors, the common resistance mutation EGFR T790M was detected in 7 of 9 patients who underwent extracranial lesion rebiopsy. However in CSFCTCs, 14 patients were found to have EGFR common mutations, while only one was diagnosed with EGFR T790M (patient 2, Table 3; Fig. 3A). CSF collection was performed at the same time as rebiopsy, so no additional treatment between rebiopsy and CSF sampling was given in most of the cases; those whose CSF collection was after rebiopsy, their treatments are shown in Table 3. Other potential resistance mutations were also seen in CSFCTCs. MET amplification was detected in patient 3 with EML4-ALK (44%) and patient 14 with EGFR del19 (35%). PIK3CA exon2 N107S and MET exon11 F839L were identified in patient 18 with EGFR L858R (70%; Fig. 3B). ERBB2 exon6 T328fs and ROS1 exon7 W215X were detected in patient 9 with EGFR del19 (18%). However, the relationship between these mutations and clinical response to treatment remains unknown due to the small number of patients in this study.

Discussion

The diagnosis of leptomeningeal metastases is currently dependent on the identification of tumor cells in the CSF

Table 3. Gene profiles in primary tissue and CSFCTCs in 19 patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Primary gene profile</th>
<th>Targeted therapy before LM</th>
<th>Rebiopsy gene profile</th>
<th>Treatment between rebiopsy and LM</th>
<th>CSFCTCs gene profile (NGS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Erlotinib</td>
<td>EGFR L858R (lung, ARMS); MET (IHC); 80% (3+/3+) 20% (2+)</td>
<td>Gefitinib+INC280, chemotherapy</td>
<td>EGFR L858R (26%)</td>
</tr>
<tr>
<td>2</td>
<td>EGFR del19 (lung node, ARMS)</td>
<td>Icotinib</td>
<td>UA</td>
<td>UA</td>
<td>EML4-ALK (6%); EGFR T790M (2%)</td>
</tr>
<tr>
<td>3</td>
<td>EML4-ALK (lung, FISH, 48%)</td>
<td>Crizotinib</td>
<td>EML4-ALK (pleural effusion, FISH, 64%)</td>
<td>UA</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>EGFR del19 (pleura, snapshot)</td>
<td>Gefitinib</td>
<td>EGFR del19 and T790M (lung, ARMS)</td>
<td>UA</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Erlotinib</td>
<td>EGFR L858R and T790M (lung, snapshot)</td>
<td>Avitinib</td>
<td>EML4-ALK (3%)</td>
</tr>
<tr>
<td>6</td>
<td>EGFR, ALK (WT)</td>
<td>None</td>
<td>Snapshot, MET, KIT (WT)</td>
<td>None</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>7</td>
<td>EGFR, ALK (WT)</td>
<td>Gefitinib</td>
<td>EGFR del19 and T790M (lung, ARMS); MET:100% (3+/3+) (IHC)</td>
<td>None</td>
<td>Common driver gene (WT)</td>
</tr>
<tr>
<td>8</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Gefitinib, erlotinib</td>
<td>EGFR del19 and T790M (lung, ARMS)</td>
<td>Erlotinib</td>
<td>EGFR del19 (56%)</td>
</tr>
<tr>
<td>9</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>UA</td>
<td>EGFR L858R (1%)</td>
</tr>
<tr>
<td>10</td>
<td>EML4-ALK (lung, ARMS)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>EML4-ALK and T790M (plasma, ARMS)</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>EGFR, ALK (WT)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>UA</td>
<td>EGFR L858R (11%)</td>
</tr>
<tr>
<td>12</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>UA</td>
<td>EML4-ALK (1 reads)</td>
</tr>
<tr>
<td>13</td>
<td>EML4-ALK (lung, FISH)</td>
<td>Erlotinib, afatinib</td>
<td>CRizotinib</td>
<td>UA</td>
<td>EML4-ALK (15%); MET amplification (4.4 times)</td>
</tr>
<tr>
<td>14</td>
<td>EGFR del19 (lung, ARMS)</td>
<td>Erlotinib</td>
<td>Erlotinib</td>
<td>UA</td>
<td>EGFR del19 (35%); MET amplification (4.4 times)</td>
</tr>
<tr>
<td>15</td>
<td>EGFR del19 (lung, ARMS)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>UA</td>
<td>EML4-ALK (15%); MET amplification (4.4 times)</td>
</tr>
<tr>
<td>16</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>EML4-ALK (35%); MET amplification (4.4 times)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>EGFR L861Q (lymph node, ARMS)</td>
<td>Erlotinib</td>
<td>Erlotinib</td>
<td>UA</td>
<td>EML4-ALK (15%); MET amplification (4.4 times)</td>
</tr>
<tr>
<td>18</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>EML4-ALK (15%); MET amplification (4.4 times)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>EML4-ALK (15%); MET amplification (4.4 times)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>EGFR L858R (lung, ARMS)</td>
<td>Gefitinib</td>
<td>Gefitinib</td>
<td>EML4-ALK (15%); MET amplification (4.4 times)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: 20 INS, exon 20 insertion; Del 19, exon 19 deletion; UA, unavailable; WT, wild type.

*Sequencing on CSFCTCs was not performed on two patients, including patient 8, for whom no CSFCTCs were identified by CellSearch, and patient 20, whose cells were not preserved after CellSearch analysis.

*More details of Patient 2 were shown in Fig. 3A.

*More details of Patient 18 were shown in Fig. 3B.
and/or typical brain MRI findings, but both methods have disadvantages. In this study, we adapted the CellSearch Assay to capture CSFCTCs in 21 NSCLC patients to diagnose leptomeningeal metastases. The results showed significantly higher sensitivity and specificity of CSFCTCs as compared with traditional methods. NGS was further performed on CSFCTCs, which revealed driver genes that highly correlated with that in primary tumor and potential resistance mutations. This suggests that CSFCTCs provide the potential means for liquid biopsy in leptomeningeal metastases of NSCLC.

Identification of CTCs by CellSearch is popular for follow-up studies and determination of prognosis in cancer patients (11–14), and its application to detect CSFCTCs had also been examined with increasing interest (15–18). In this study, we confirmed that CellSearch had a specificity of 100% and a sensitivity of 95.2%, which is significantly better than traditional diagnostic methods including TCT and MRI. The following characteristics of CellSearch could explain the superior sensitivity and reliability in identifying CSFCTCs (15). CellSearch with anti-EpCAM coated nanoparticles could enrich malignant cells with EpCAM expression, which is also found
on tumor cells in CSF, thus greatly improving sensitivity. In addition, the CellSearch assay's semiautomated procedure and precise criteria (CK+/CD45∓/DAPI+) confer additional benefits over TCT, which mainly depends on the opinions of experienced pathologists. Overall survival of advanced NSCLC patients has improved with the development of new agents, but the prognosis of patients with leptomeningeal metastases remains poor (2, 3). Early identification of leptomeningeal metastases results in more treatment options, and CellSearch has proven to be more sensitive at diagnosing leptomeningeal metastases than traditional methods.

To the best of our knowledge, this is the first study to demonstrate that CTCs in peripheral blood were found at much lower concentrations than CSFCTCs in CSF detected by CellSearch in NSCLC, suggesting that CSFCTCs may be a better way to represent leptomeningeal metastases than CTCs. It is quite clear that CTCs could serve as valuable biomarkers in NSCLC (21–24); however, it is unclear whether these actual CTCs are indeed the source of metastases, and it could be logical to assume that there are heterogeneity and variations in the temporal and spatial distributions of CTCs within both peripheral blood and CSF circulation (21). Malignant cells progressed and proliferated in CSF after leptomeningeal metastases, whereas these cells were less likely to circulate to peripheral blood due to the blood–brain barrier. Therefore, CTCs may not accurately reflect the "real world" of tumor burden in intracranial conditions of NSCLC as current data suggested. Ahn and colleagues found that EpCAM overexpression was detected in 120 of 234 (51.3%) surgically resected lung adenocarcinoma tissues (25), and EpCAM-positive CTCs were less commonly identified in NSCLC patients as compared with other epithelial tumors, with a highly negative rate (21%) of EpCAM expression in both primary and metastatic tissues (26). Moreover, CTCs in NSCLC showed high heterogeneity with different epithelial features, and they could acquire mesenchymal molecular features during tumor development, and those with epithelial-to-mesenchymal transition (EMT), a widely reported prerequisite for metastases, would result in a more mesenchymal phenotype that might lose the expression of EpCAM, and EMT was not occurred homogeneously in CTCs within the circulation of NSCLC (21, 27–31). As the CellSearch assay only played a limited role in capturing EpCAM-negative CTCs of NSCLC, it would result in some CTCs remaining undetected by the technique (26, 30, 32). CSFCTCs were much higher than CTCs in peripheral blood in current study, which may also indicate that EpCAM expression of tumor cells in CSF might be much higher than that in extracranial lesions, and more explorations are needed to evaluate its role of CSFCTCs in leptomeningeal metastases. CTCs were identified as cells with CK-PE and DAPI positivity in this study, and there was a possibility to estimate the total cells that have been coenriched with the CellSearch system to compare the difference between CSFCTCs and CTCs in future researches.

Repeated biopsy of the tumor is essential to tailor treatment of NSCLC, especially in patients with stable primary lesions but CNS progression. In these cases, CSF is the best choice for biopsy due to its easy access, and it plays an important role in the investigation of resistance mechanisms and determination of optimal treatment (20, 33, 34). Leptomeningeal metastases were much more common in those with EGFRT790M mutations in our previous study (2). In this study, EGFRT790M, the most common resistance mutation in NSCLC (35), was identified in 7 of 9 patients who underwent extracranial lesion rebiopsy. In contrast, this mutation was only found in the CSFCTCs from 1 of 14 patients. These findings are consistent with previous small sample studies in which EGFRT790M mutation was less common in CNS lesions or CSF cfDNA in patients with CNS metastases compared with extracranial recurrent lesions (36–40). This may be related to the incomplete penetration of the TKIs into the CSF and the heterogeneity of T790M status both spatially and temporally (40–43). T790M-negative tumor cells consistently have increased growth potential and are commonly found in extrathoracic sites, including in the CNS (39), suggesting that additional mechanisms may contribute to leptomeningeal metastases resistance to TKIs. What’s more, CellSearch can only detect EpCAM/keratin-positive CSFCTCs, which might underestimate those EpCAM-negative tumor cells in CSF that might carry EGFRT790M mutations, and further explorations are needed. NSCLC patients with EGFRT790M mutations were more frequently CTC-positive compared with those with EGFWR wild-type tumors in previous study (26), and EGFRT790M mutations could also be detected in CTCs in peripheral blood in previous studies (44, 45); however, only a small handful of patients with leptomeningeal metastases were able to be identified with positive CTCs in this study, suggesting that CSFCTCs could be more representative than CTCs in NSCLC patients with leptomeningeal metastases. Other potential resistance mutations were also seen in CSFCTCs in this study. MET amplification was detected in patient 14 with EGFRT790M. PIK3CA exon2 N107S and MET exon11 F839L were identified in patient 18 with EGFRI858R. ERBB2 exon8 T328fs and ROS1 exon7 W215X were detected in patient 9 with EGFRI858R. However, the relationship between these mutations and clinical drug resistance were not elucidated in this study due to the small sample size, thus, additional studies are needed to determine the importance of these mutations. Together, these results suggest that CSFCTCs could serve as a source of "liquid biopsy" to determine the genetic profile and diagnosis of leptomeningeal metastases in patients with NSCLC.

There were some limitations to this study. This was a retrospective study with a small number of patients. Most patients refused or did not present for repeated lumbar puncture; thus, we were unable to determine what, if any, changes were induced in CSFCTCs by therapy. There are also some disadvantages to CellSearch. Currently, this method is too expensive to use in daily clinical practice, and large amounts of CSF (about 5 to 7.5 mL) are needed to run the test. Additional development is needed to increase the application of this method to capture CSFCTCs.

In conclusion, CellSearch could be a better sensitve method for detecting tumor cells in CSF, and potentially provides earlier diagnosis of leptomeningeal metastases. More importantly, CSFCTCs could be an important and new way of "liquid biopsy" for genetic profiles of metastatic tumor cells in leptomeningeal metastases patients of NSCLC.

Disclosure of Potential Conflicts of Interest
C.-R. Xu has ownership interests (including patents) in Illumina, and reports receiving speakers bureau honoraria from AstraZeneca, Eli Lilly, Pfizer and Roche. No potential conflicts of interest were disclosed by the other authors.
Authors’ Contributions
Writing, review, and/or revision of the manuscript: B.-Y. Jiang, Y.-S. Li, Z.-H. Chen, W.-Z. Zhong, J.-J. Yang, B. Huang, Y.-H. Liu, Q. Zhou, C.-R. Xu, C.-Y. Gao, Y.-L. Wu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.-S. Li, W.-B. Guo, X.-C. Zhang, Z.-H. Chen, W.-Z. Zhong, J.-J. Yang, B. Huang, Q. Zhou, C.-R. Xu, C.-Y. Gao, Y.-L. Wu
Study supervision: Y.-S. Li, Z.-H. Chen, J.-J. Yang, B. Huang, C.-Y. Gao, Y.-L. Wu

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References


Detection of Driver and Resistance Mutations in Leptomeningeal Metastases of NSCLC by Next-Generation Sequencing of Cerebrospinal Fluid Circulating Tumor Cells

Ben-Yuan Jiang, Yang-Si Li, Wei-Bang Guo, et al.


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