Detection of genes mutations in cerebrospinal fluid circulating tumor DNA from neoplastic meningitis patients using next generation sequencing

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Abstract

Background This study profiled the somatic gene mutations and the copy number variations (CNVs) in cerebrospinal fluid (CSF)-circulating tumor DNA (ctDNA) from patients with neoplastic meningitis (NM).

Methods A total of 62 CSF ctDNA samples were collected from 58 NM patients for the next generation sequencing. The data were blasted in GenBank and bioinformatically analyzed.

Results Cancer-associated gene mutations occurred in all 62 CSF ctDNA samples in TP53 (54/62; 87.10%), EGFR (44/62; 70.97%), PTEN (39/62; 62.90%), CDKN2A (32/62; 51.61%), APC (27/62: 43.55%), TET2 (27/62; 43.55%), GNAQ (18/62; 29.03%), NOTCH1 (17/62; 27.42%), VHL (17/62; 27.42%), FLT3 (16/62; 25.81%), PTCH1 (15/62; 24.19%), BRCA2 (13/62; 20.97%), KDR (10/62; 16.13%), KIT (9/62; 14.52%), MLH1 (9/62; 14.52%), ATM (8/62; 12.90%), CBL (8/62; 12.90%), and DNMT3A (7/62; 11.29%). The mutated genes enriched by the KEGG pathway analysis were the PI3K-Akt, which included the genes of TP53, EGFR, PTEN, KIT and KDR. After receiving intrathecal and systemic chemotherapy, the ERK1/2 signaling pathways of these CSF samples were activated. Furthermore, the CNVs of these genes were also identified in these 62 samples.

Conclusions This study identified gene mutations in all CSF ctDNA samples, indicating that such an approach could be useful as a second-line diagnostic strategy for NM patients. PI3K-Akt signaling may be the potential NM metastasis mechanism.

Background

Neoplastic meningitis (NM), also known as meningeal carcinomatosis, is one of the most significantly malignant subtypes of brain metastasis, and is associated with very poor survival of patients [1]. The primary cancers are mostly lung and breast cancers or brain tumors, such as medulloblastoma. In clinic, early NM detection and timely treatment could significantly impact the outcome of patients. However, the present diagnosis is primarily based on the clinical signs and symptoms plus cerebrospinal fluid (CSF) cytology and neuroimaging [2]. Furthermore, although the detection of tumor cells in the CSF is the key to make NM diagnosis, the CSF cytology may not be reliable due to insufficient sensitivity and specificity. Thus, the detection of cell-free circulating tumor DNA (ctDNA) could be another diagnostic strategy [3-5]. This can detect tumor-related genes and gene alterations in the plasma or CSF to monitor the tumor progression and/or treatment responses [3-5]. In brain tumor patients, the plasma ctDNA analysis has revealed either its absence, or extremely low levels [5, 6]. Fortunately, CSF ctDNA has been well demonstrated to be
present and even abundant in brain tumor patients [6, 7]. In order to better understand the molecular mechanisms underlying NM, the detection of mutated genes in the CSF could help medical oncologists identify the primary tumor and make effective treatment options for patients. Therefore, the present study aimed to investigate the gene mutations in the CSF ctDNA samples obtained from NM patients using the cutting-edge technique of next generation sequencing (NGS). This approach can help to characterize NM genetic profiles and perform NM molecular classifications, which can thereby be potentially applied for molecularly targeted therapy. Towards this end, a recent study [8] genetically profiled the CSF ctDNA obtained from NM patients with epidermal growth factor receptor (EGFR)-mutant non-small cell lung cancer (NSCLC). Upon completion of the present study, this is expected to pave a novel strategy for potential therapeutic approaches in the effective control of NM progression.

Methods

Study population and samples

In the present study, a total of 58 patients with NM, who received lumbar puncture for the CSF cytology examination between October 2014 and June 2018 in the Department of Neurology, The Second Hospital of Hebei Medical University (Hebei, China), were enrolled. The diagnosis of these 58 NM patients was entirely according to the clinical signs and symptoms, positive CSF cytology, and/or neuroimaging findings, such as contrast-enhanced brain magnetic resonance imaging (MRI) or computed tomography (CT), and the results from the CSF ctDNA. The NM clinical signs and symptoms included headache, nausea, vomiting, convulsion, lower back pain, cranial nerve paralysis, paresthesia, gait disturbances, vertigo and defects. The positive CSF cytology was defined by the distinctive pattern of the neoplastic cell morphology. That is, cells that had an irregular size and shape, and contained large and polymorphic nuclei with a lobulated state and malformed buds. The chromatin size increased with the basophilic coarse particles, and the mitotic activity was enhanced with aberrant mitosis. The nuclear membrane was usually thickened
with a saw-tooth-shaped and wear edge. In addition, the positive neuroimaging revealed the presence of leptomeningeal enhancement. The clinical data are presented in Table 1.

Table 1. Characteristics of 58 NM patients
| Clinical features                        | # of patients (%) |
|-----------------------------------------|-------------------|
| **Gender**                              |                   |
| Male                                    | 27 (46.6)         |
| Female                                  | 31 (53.4)         |
| **Age, year**                           |                   |
| Median (range)                          | 55 (23-77)        |
| **Primary cancer**                      |                   |
| lung cancer                             | 42 (72.4)         |
| gastric cancer                          | 4 (6.9)           |
| breast cancer                           | 3 (5.2)           |
| rectal cancer                           | 2 (3.4)           |
| prostatic cancer                        | 1 (1.7)           |
| parotid carcinoma                       | 1 (1.7)           |
| lymphoma                                | 1 (1.7)           |
| glioblastoma                            | 1 (1.7)           |
| unknown malignancy                      | 3 (5.2)           |
| **First symptom with NM**               | 10 (17.2)         |
| **Diagnostic basis**                    |                   |
| CSF cytology                            | 44 (75.9)         |
| neuroimaging findings                   | 8 (13.8)          |
| **CSF ctDNA**                           | 6 (3.6)           |
| **Treatment regimen (CSF samples)**     |                   |
| intrathecal chemotherapy and systematic therapy | 30 (48.4) |
| intrathecal chemotherapy                |                   |
| without systematic therapy              | 11 (17.7)         |
| systematic therapy without intrathecal chemotherapy | 12 (19.4) |
| no therapy                              | 9 (28.5)          |
| **Hydrocephalus**                       |                   |
| Yes                                     | 5 (8.6)           |
| No                                      | 53 (91.4)         |
The present study was approved by the Ethics Committee of the Second Hospital of Hebei Medical University (Hebei, China), and a written informed consent was obtained from each patient.

CSF samples and processing

The CSF samples were collected from each patient and placed into EDTA tubes, according to our hospital routine protocols. Then, these were centrifuged for five minutes at 1,000 g to pellet the cell contents, and stored at -20°C, while the supernatant was centrifuged at 10,000 g for an additional 30 minutes, according to a previous study [9], in order to collect the supernatant. Afterwards, the final supernatant was transferred into pre-labeled cryotubes and stored at -80°C. Next, the ctDNA was extracted from at least 5 mL of the CSF supernatant using a QIAamp Circulating Nucleic Acid kit (Qiagen, Valencia, CA, USA), according kit instructions, and the ctDNA was quantified using a Qubit2.1 Fluorometer and Qubit dsDNA HS Assay kit (Invitrogen, Carlsbad, CA, USA).

Preparation of the ctDNA library and the next generation DNA sequencing

The ctDNA samples were subjected to preparation of the Ion Proton library and DNA sequencing, according to the methodologies from previous studies [10-12]. Briefly, for each sample, an adapter-ligated library was generated using the Ion AmpliSeq Library Kit 2.0 (Invitrogen). That is, the pooled amplicons made from 10-20 ng of ctDNA samples were end-repaired and ligated to Ion Adapters X and P1, and purified using AMPure beads (Beckman Coulter, Indianapolis, IN, USA) to obtain the adapter-ligated products, followed by nick-translation and PCR-amplification for a total of five cycles. Then, the products were subjected to analysis using the Agilent 2100 Bioanalyzer and Agilent Bioanalyzer DNA High-Sensitivity LabChip (Agilent Technologies) to determine the concentration and size of the library, and sample emulsion PCR and emulsion breaking using the Ion OneTouch™ 2 system (Invitrogen) with the Ion PI Template OT2 200 Kit v3 (Invitrogen), according to manufacturer’s instructions. Afterwards, the Ion Sphere Particles (ISPs) were recovered,
and the template-positive ISPs were enriched with Streptavidin C1 beads (DynabeadsMyOne, Invitrogen) on the Ion One Touch ES (Enrichment System, Invitrogen) and quantified using the Qubit 2.0 Fluorometer (Invitrogen).

The ISP samples were sequenced using the Ion Proton System with Ion PI v2 Chips (Invitrogen) for 100 cycles, while the Ion PI Sequencing 200 Kit v3 (Invitrogen) was used for the sequencing reactions. Then, the SV-OCP143-ctDNA panel (San Valley Biotech Inc., Beijing, China) was used to detect the somatic mutations of 143 cancer-related genes. Since the CSF ctDNA samples contained short DNA fragments, the amplicons in the panel were specially designed for the efficient amplification of ctDNA, and the total read counts were more than 25 million to ensure an average base coverage depth over 10,000 folds. In addition, strict quality control criteria were used to ensure that the average uniformity of the base coverage is no less than 95.5% for the reliability of the DNA sequencing and mutation detection.

Processing and analysis of DNA sequencing data

The raw DNA sequencing data were processed and analyzed using the Ion Proton platform-specific pipeline (Torrent Suite v5.0) with a specific plug-in (Variant Caller v5.0), which included the readouts of the raw DNA sequences, the trimming of the adapter sequences, and the filtering and removal of poor signal sequences according to previous studies [10-12]. These three filtering steps were applied to eliminate the erroneous base calling, and the final variant calling was generated. That is, the first step evaluated the DNA sequences using the following criteria: the average total coverage depth is >10,000, each variant coverage is >10, the variant frequency for each sample is >0.1%, and the P-value is <0.01. The second step was to eliminate potential DNA strand-specific errors after visual examination of the gene mutations using the Integrative Genomics Viewer (IGV; http://www.broadinstitute.org/igv) or Samtools (http://samtools.sourceforge.net) software. For the third step, the total amplicon read counts from the Coverage Analysis Plugin were utilized to identify the copy number variants (CNVs). Then, the read counts per amplicon of each sample was normalized to the total number of reads for a given sample, and divided
by normalized counts from a composite normal male genomic DNA sample, which yielded a copy number ratio after correcting for GC content. The gene-level copy number was estimated through the determination of the coverage-weighted mean of the GC-corrected per-probe ratio, which was corrected with the expected error, according to the probe-to-probe variance [13]. Afterwards, genes with a copy number of <1 or >4 were regarded as loss or gain, respectively.

Functional annotation and pathway enrichment analysis

Next, the mutated genes were bioinformatically analyzed using the gene ontology (GO) terms [14] and the Kyoto Encyclopedia of Genes and Genomics (KEGG) pathway enrichment analysis with the Database for Annotation, Visualization, and Integrated Discovery (DAVID; v.6.8, https://david.ncifcrf.gov/tools.jsp). Then, the data were classified as the functional annotation and the KEGG pathway enrichment. A $P$-value of <0.05 was set to be statistically significant.

Statistical analysis

The statistical significance in gene mutation frequency between the two groups was analyzed using Fisher exact test with the SPSS 19.0 software (IBM, Armonk, NY, USA), and a two tailed $P$-value of <0.05 was considered statistically significant.

Results

Patient characteristics

The present study enrolled 58 NM patients, which included 27 male and 31 female patients, with median age of 55 years old (age range: 23-77 years old). Among these patients, 44 patients were diagnosed with NM based on the positive CSF cytology, eight patients were diagnosed with NM according to neuroimaging findings, and six patients were diagnosed with NM based on the presence of CSF ctDNA. The primary tumors were mostly lung cancer ($n=42$), followed by gastric cancer ($n=4$), breast cancer ($n=3$), rectal cancer ($n=2$),


prostatic cancer \((n=1)\), parotid carcinoma \((n=1)\), lymphoma \((n=1)\), glioblastoma \((n=1)\), and unknown primary malignancy \((n=3)\). For NM patients with lung cancer, the majority of these patients had lung adenocarcinoma \((27/42, 64.3\%)\), while of 55 patients had known primary malignancies and 10 patients \((18.2\%)\) had NM as the first clinical manifestation. These patients were followed up for two month or longer.

A total of 62 CSF samples were collected from these 58 NM patients, in which three CSF samples were collected from a single patient, while two CSF samples were collected from other two patients at distinct time points. Furthermore, among the 62 CSF samples, 30 CSF samples were collected from 28 patients who received intrathecal chemotherapy and systemic radiotherapy, chemotherapy, and/or molecule-targeted therapy, 11 CSF samples were obtained from 11 patients who received intrathecal chemotherapy, and 12 CSF samples were obtained from 12 patients who received systematic therapy. The remaining nine CSF samples were collected from nine patients who did not receive any anticancer therapy.

Cancer-associated gene mutations in the 62 CSF specimens, regardless of the origin of the primary cancer and the mutated genes functional enrichment analysis

The 62 CSF samples were all positive for ctDNA and mutations of cancer-associated genes. Specifically, 68 \((47.6\%)\) of the 143 cancer-associated genes analyzed in the present study had mutations in at least one NM CSF sample, and 62 \((100\%)\) of the NM CSF samples carried at least one mutated gene. The most commonly mutated gene was TP53 \((54/62, 87.10\%)\), followed by EGFR \((44/62, 70.97\%)\), PTEN \((39/62, 62.90\%)\), CDKN2A \((32/62, 51.61\%)\), APC \((27/62, 43.55\%)\), TET2 \((27/62, 43.55\%)\), GNAQ \((18/62, 29.03\%)\), NOTCH1 \((17/62, 27.42\%)\), VHL \((17/62, 27.42\%)\), FLT3 \((16/62, 25.81\%)\), PTH1 \((15/62, 24.19\%)\), BRCA2 \((13/62, 20.97\%)\), KDR \((10/62, 16.13\%)\), KIT \((9/62, 14.52\%)\), MLH1 \((9/62, 14.52\%)\), ATM \((8/62, 12.90\%)\), CBL \((8/62, 12.90\%)\), and DNMT3A \((7/62, 11.29\%)\). (Figure 1). Furthermore, these high frequency mutated genes were further bioinformatically analyzed, and it was found that the most enriched KEGG pathway was the PI3K-Akt signaling
pathway \((P = 7.0 \times 10^{-3})\), which included, but was not limited to, TP53, EGFR, PTEN, KIT and KDR (Figure 2).

Furthermore, the gene mutation frequency was divided into five groups: \(\geq 50\%\), 30\%-50\%, 10\%-30\%, 1\%-10\% and 0.2\%-1\%, respectively. Then, the gene mutation frequency was associated with the detectable tumor cells in the CSF samples, and it was found that when the mutation frequency was greater than 1\%, the tumor cells were extensively detected in the CSF, when compared to CSF samples without detectable tumor cells \((P<0.001\), Figure 3).

Copy number variations (CNVs)

Data on the CNVs in these CSF ctDNA samples were also obtained, and it was found that high CNVs occurred in 20 of 58 NM patients. Among these 20 patients, the primary tumors were 15 non-small cell lung cancers (13 tumors was lung adenocarcinoma, 86.7\%), four gastric cancers, and one each of breast cancer, parotid carcinoma, squamous cell carcinoma and unknown primary cancer. The deletion of the \textit{CDKN2A} copy number was the most frequent CNV that occurred in seven CSF ctDNA samples, and six of which were non-small cell lung cancers (6/20, 30\%). In the increase in \textit{CDK4} copy number that occurred in five lung adenocarcinomas, four of these exhibited an increase in \textit{MDM2} copy number. In addition, an increase in \textit{MDM2} copy number was also detected from another lung adenocarcinoma patient. Two CSF ctDNA samples had a gain of \textit{ERBB2 (HER2)} copy number from a parotid carcinoma patient, while an increase in \textit{CD44} copy number was identified in three patients, in which each patient has breast cancer, gastric cancer and unknown cancer, respectively. In addition, an increased \textit{EGFR} copy number occurred in three lung adenocarcinoma patients. Other CNVs of tumor-associated genes were detected in five patients (six positive CSF ctDNA samples) with decreased \textit{AR} copy numbers, five patients had decreased \textit{CD274} copy numbers, three patients each has a decreased \textit{PDCD1LG2} copy number, two patients each has an increase in \textit{FGFR2}, \textit{CCNE1}, or \textit{NKX2-1} copy numbers, respectively, and one patient had increased \textit{TIAF1}, \textit{GAS6}, or \textit{IL6} copy numbers, or reduced \textit{CSNK2A1}, \textit{JAK2}, \textit{MED12}, or \textit{SMAD4} copy numbers.
Association of gene mutations with intrathecal chemotherapy and systemic therapy

In the present study, 30 CSF samples were collected from patients who received intrathecal chemotherapy and systemic therapy, including radiotherapy, systemic chemotherapy and molecule-targeted therapy. Furthermore, 11 CSF samples were obtained from patients after intrathecal chemotherapy, 12 CSF samples were obtained from patients after systemic therapy, but without intrathecal chemotherapy, and the remaining nine CSF samples were collected from patients who did not receive any therapy. The data on these unique mutated genes were summarized for each group of patients, followed by a gene-annotation enrichment analysis. It was found that the ERK1/2 pathway was mostly enriched by the KEGG pathway analysis in patients who received both intrathecal chemotherapy and systemic therapy (Figure 4 and Table 2).

Table 2. Unique mutated genes in each treatment group
|                     | intrathecal chemotherapy and systematic therapy (30 samples) | intrathecal chemotherapy without systematic therapy (11 samples) | systematic therapy without intrathecal chemotherapy (12 samples) | Neither intrathecal chemotherapy nor systematic therapy (9 samples) |
|---------------------|-------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| ERBB3               | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| FGFR3               | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| FGFR4               | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| GAS6                | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| GNA11               | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| KNSTRN              | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| MED12               | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| MTR                 | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| MYCN                | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| NF1                 | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| TIAF1               | 5.26% WT                                                   | WT                                                            | WT                                                            | WT                                                            |
| AKT1                | 10.53% WT                                                  | WT                                                            | WT                                                            | WT                                                            |
| FBXW7               | 10.53% WT                                                  | WT                                                            | WT                                                            | WT                                                            |
| HRAS                | 10.53% WT                                                  | WT                                                            | WT                                                            | WT                                                            |
| ABL1                | 15.79% WT                                                  | WT                                                            | WT                                                            | WT                                                            |
| BAP1                | WT 12.50%                                                   | WT                                                            | WT                                                            | WT                                                            |
| MAPK1               | WT 12.50%                                                   | WT                                                            | WT                                                            | WT                                                            |
| SF3B1               | WT 12.50%                                                   | WT                                                            | WT                                                            | WT                                                            |
| PIK3R1              | WT 25.00%                                                   | WT                                                            | WT                                                            | WT                                                            |
| CSNK2A1             | WT 10.00%                                                   | WT                                                            | WT                                                            | WT                                                            |
| IL6                 | WT 10.00%                                                   | WT                                                            | WT                                                            | WT                                                            |
| MET                 | WT 10.00%                                                   | WT                                                            | WT                                                            | WT                                                            |
| SMAD4               | WT 10.00%                                                   | WT                                                            | WT                                                            | WT                                                            |
| APEXI               | WT 11.11%                                                   | WT                                                            | WT                                                            | 11.11%                                                        |
| BCL9                | WT 11.11%                                                   | WT                                                            | WT                                                            | 11.11%                                                        |

**Abbreviations:** WT, wide type.

The association of CSF ctDNA concentration with Karnofsky performance status (KPS) score, gene mutation and CSF tumor cells
Next, the CSF ctDNA concentration was associated with the KPS and gene mutations, and it was found that the CSF ctDNA concentration was not statistically associated with the KPS scores ($r = -0.038, P>0.05$; Figure 5) or the number of gene mutations ($r = -0.1950, P=0.1289$; Figure 5). However, it was found that the CSF ctDNA concentration was associated with tumor cells in the CSF, when compared to that without circulating tumor cells ($P=0.004; Figure 5$). Furthermore, the number of gene mutations was associated with the KPS score ($P<0.05$, Figure 5).

Cancer-associated gene mutations in the 45 CSF samples obtained from 42 NM patients with lung cancer

In the subgroup analysis, it was found that CSF ctDNA was detected in all 45 CSF samples obtained from 42 lung cancer patients with NM, and gene mutations were also detected in all patients. Specifically, **EGFR** mutations occurred in 39 of 45 patients (86.67%), followed by **TP53** (38/45, 84.44%), **PTEN** (27/45, 60.00%), **TET2** (18/45, 40.00%), **APC** (17/45, 37.78%), **CDKN2A** (14/45, 31.11%), **GNAQ** (14/45, 31.11%), and **NOTCH1** (11/45, 24.44%). A number of gene mutations previously reported with lung cancer were identified in CSF with NM, while EGFR, TP53, PTEN, TET2, APC, CDKN2A, GNAQ, NOTCH1, FLT3, VHL, BRCA2, PTCH1, CBL, MLH1, BRAF, NRAS, TSC2, CSF1R, KIT, MAP2K1, MSH2, TSC1, HRAS, IFITM1 and BCL9 mutations were statistically more common in the present cohort of NM, when compared to the lung cancer noted in the COSMIC database ([https://cancer.sanger.ac.uk](https://cancer.sanger.ac.uk)) (Table 3).

Enriched genes and gene pathways in NM patients with lung cancer

A total of 59 mutated genes were found in the 42 NM patients with lung cancer, and these were associated with various biological processes, which included the general signaling pathways underlying the progression of cancer ($P=5.21\times10^{-30}; q=5.05\times10^{-28}$), chronic myeloid leukemia ($P=7.01\times10^{-20}; q=3.40\times10^{-18}$), endometrial cancer ($P=3.82\times10^{-19}; q=1.24\times10^{-17}$), bladder cancer ($P=6.39\times10^{-19}; q=1.55\times10^{-17}$), melanoma ($P=1.78\times10^{-18}$);...
$q=3.44 \times 10^{-17}$), glioma ($P=1.62 \times 10^{-17}; q=2.61 \times 10^{-16}$), prostate cancer ($P=8.66 \times 10^{-17}; q=1.20 \times 10^{-15}$), and non-small cell lung cancer ($P=1.59 \times 10^{-15}; q=1.93 \times 10^{-14}$), while the related signaling pathways were the ErbB signaling ($P=4.92 \times 10^{-10}; q=3.67 \times 10^{-9}$), VEGF signaling ($P=6.00 \times 10^{-7}; q=3.06 \times 10^{-6}$), MAPK signaling ($P=1.33 \times 10^{-6}; q=5.88 \times 10^{-6}$), p53 signaling ($P=4.14 \times 10^{-6}; q=1.61 \times 10^{-5}$), and m-TOR signaling ($P=1.00 \times 10^{-5}; q=3.33 \times 10^{-5}$) pathways (Figure 6). Furthermore, among these 59 mutated genes, the KEGG pathway analysis revealed that EGFR, TP53, CDKN2A, CDK4, BRAF, NRAS, HRAS, JAK3, KRAS, MAP2K1, MAP2K2, PIK3CA and RB1 were strongly associated with non-small cell lung cancer.

The association of EGFR mutations between lung cancer tissues and NM CSF samples

Next, EGFR mutations were associated between lung cancer tissues and the NM CSF samples available in 10 patients (Table 4). Specifically, both samples were collected from N033, N063, N077, N1088, N156, N331 and N1286 during the TKI therapy, and N079, N090 and N355 at several months after the TKI. It was found that there were same EGFR active mutations between lung adenocarcinoma tissues and CSF of nine patients, except for N1088, in which the EGFR mutation was undetectable in the CSF sample.

Table 4. EGFR activating mutations in primary lung cancer and NM CSF samples

| CSF sample No. | Primary lung cancer | CSF       |
|----------------|---------------------|-----------|
| N033           | EGFR 19Del          | EGFR 19Del|
| N063           | EGFR L858R          | EGFR L858R, E709A, T790M |
| N077           | EGFR L858R          | EGFR L858R |
| N079           | EGFR L858R          | EGFR L858R |
| N090           | EGFR L858R          | EGFR L858R |
| N1088          | EGFR 19Del          | EGFR (-)  |
| N156           | EGFR 19Del          | EGFR 19Del, T790M |
| N331           | EGFR 19Del          | EGFR 19Del |
| N355           | EGFR L858R          | EGFR L858R |
| N1286          | EGFR L858R          | EGFR L858R |
A representative case

In the present cohort, there was a lung adenocarcinoma patient (#N156) who underwent surgical lung cancer resection, and tumor tissues had an $EGFR\, 19\text{Del}$ mutation detected by NGS. Thus, the patient orally received 125 mg of icotinib three times a day for six months and thereafter. However, the patient’s cancer spread into the leptomeninges, and the CSF sample revealed $EGFR\, 19\text{Del}$ and T790M mutations in the CSF ctDNA. Given such a situation, the patient was given 80 mg of AZD9291 once a day to replace the icotinib for 18 months, and the patient’s overall health condition improved. Furthermore, a complete response was confirmed by the contrast-enhanced brain MRI, CSF cytology, and undetectable $EGFR$ mutations in the CSF samples (Figure 7).

Discussion

In the present study, a total of 58 NM patients were enrolled for the detection of CSF ctDNA, gene mutations and copy number variations. The present data revealed that the primary tumors were lung cancer ($n=42$), gastric cancer ($n=4$), breast cancer ($n=3$), unknown primary malignancy ($n=3$), rectal cancer ($n=2$), and one each of prostatic cancer, parotid carcinoma, lymphoma and glioblastoma. The present cohort of patients had a rare primary tumor from the brain, and only one case of glioblastoma. Furthermore, the present cohort of NM patients revealed that the large majority of primary cancers ($27/42, 64.3\%$) was lung adenocarcinoma, and 10 patients ($18.2\%$) had NM as the first clinical manifestation, although seven of these 10 patients were clarified for their primary tumor. Afterwards, 62 CSF samples were acquired from 58 NM patients, and all samples contained detectable ctDNA, indicating that detection of CSF ctDNA is a sensitive biomarker for NM patients, since the ctDNA may not originate from benign tumors and non-neoplastic conditions, according to previous studies [6, 7]. Indeed, a previous study of ctDNA in 640 patients with different cancers [15] revealed that plasma ctDNA can be detected in at least 75% of patients vs. less than 50% patients with brain tumors, such as glioma, suggesting that CSF ctDNA can be an alternative source of samples for brain tumor
diagnosis, since the present data detected positive CSF ctDNA in all 62 CSF specimens. However, it may also be observed that all patients in the present study had NM, and tumor cells in NM can disseminate over the leptomeningeal surface, followed by neoplastic cell shedding into the CSF. Thus, it needs to be further determined whether the CSF could be used to detect early stage brain tumors. However, it is true that the CSF can be a best source to detect ctDNA in NM patients. Previous studies have also reported that all 26 patients [8] and three patients [6] had positive ctDNA in the CSF samples, and the present study further supports these previous studies. In addition, the present study further demonstrated that CSF ctDNA is a useful resource to analyze gene mutations, which can help medical oncologists identify primary tumors that can cause NM. It was found that the mutations of cancer-associated genes occurred in all 62 CSF ctDNA samples, with the highest frequency on TP53 (54/62, 87.10%), EGFR (44/62, 70.97%), PTEN (39/62, 62.90%), CDKN2A (32/62, 51.61%), APC (27/62, 43.55%), and TET2 (27/62, 43.55%). These mutated gene-related gene signaling was the PI3K-Akt and ERK1/2 signaling pathways. The ERK1/2 signaling pathway was significantly activated in NM patients who received intrathecal and systemic chemotherapy, indicating that intrathecal chemotherapy and systemic therapy might induce novel gene mutations in NM patients. The present study also identified the variation of gene copy numbers in these 62 samples. In conclusion, the data obtained from the present study demonstrates the following: (1) ctDNA is detectable in all CSF samples; (2) gene mutations are detectable in all CSF samples; (3) the gene copy number varies in all CSF samples; (4) the PI3K-Akt and ERK1/2 signaling pathways are the most altered signaling pathways for these mutated genes; (5) novel gene mutations are induced by intrathecal chemotherapy and systemic therapy in NM patients; (6) lung cancer (especially lung adenocarcinoma) is the major primary tumor in the present cohort of NM patients. Future studies would investigate the usefulness of the CSF and ctDNA for the early detection of brain tumors, and target these mutated genes for the therapy of NM patients or even patients with these primary tumors.

Indeed, the PI3K-Akt signaling pathway, including but is not limited to TP53, EGFR, PTEN, KIT and KDR, could be crucial or at least partially crucial in mediating primary cancer for meningeal metastasis. In particular, numerous isoforms and/or spliced variants
of PI3Ks participate in the regulation of various cell processes, such as cell cycle progression, cell polarization, migration, survival and metabolism, as well as tumor angiogenesis [16]. Furthermore, Akt is amenable to the vast majority of PI3K-mediated responses [17], and the alterations of Akt upstream regulators, elevated Akt expression, and/or Akt activation all result in the promotion of tumor metastasis [18]. For example, activated PI3K-Akt signaling could stimulate the translocation of α-actinin-4 from the nucleus to the cytoplasm and plasma membrane, which in turn induce changes in cell morphology and motility [19]. In human carcinogenesis, the PI3K-Akt signaling pathway inhibited the expression of tumor suppressor gene E-cadherin, which led to tumor cell epithelial mesenchymal transition and metastasis [20-22]. Previous studies have revealed that the PI3K-Akt signaling pathway plays a crucial role in the progression and metastasis of lung cancer [23], ovarian cancer [18], nasopharyngeal carcinoma [24], prostate cancer [25], colorectal cancer [26], and gastric cancer [27]. The present study further supports and confirms the important role of the PI3K-Akt signaling pathway in NM patients, which is novel, and to date, there has been no report in the literature. Hence, further studies are needed to verify the importance of this signaling pathway in NM. Furthermore, in the present study, ERK1/2 signaling was found to be enriched in NM patients after intrathecal chemotherapy and systematic therapy, indicating that the change in the ERK1/2 signaling could be associated with treatment resistance. Indeed, the inhibition of the ERK1/2 signaling pathway was caused by the Dioscorea bulbifera-induced apoptosis in human colorectal carcinoma cells [28]. Conversely, the GABAergic signaling facilitated breast cancer metastasis through promotion of the ERK1/2-dependent phosphorylation [29]. This speculation needs to be further studied to determine whether this is associated with treatment resistance to both intrathecal chemotherapy and systemic therapy.

In addition, the present study also identified the CNVs of different genes in the CSF ctDNA samples, and the most affected ones were CDKN2A, CDK4 and MDM2. As it is known, the deletion of tumor suppressor CDKN2A was associated with melanoma and pancreatic neuroendocrine tumors metastasis, and the reduced survival rate of patients [30, 31]. Indeed, during the DNA replication in cells, gene amplification could generally create a risk for gene overexpression, which could be involved in cancer initiation and
progression [32]. Furthermore, a previous study revealed that CDK4 and MDM2 mutations occurred in melanomas and liposarcoma, while ERBB2 mutations occurred in breast cancer, EGFR mutations occurred in astrocytoma, and MYCN mutations occurred in neuroblastoma [32]. Altered CD44 expression was associated with the aggressive clinicopathological characteristics of various human cancers [33]. In addition, the present study revealed the EGFR and TP53 mutations in the CSF samples of NM patients with lung cancer, in which the COSMIC database also confirmed that these were the most frequently mutated genes in the CSF of NM patients, when compared to primary lung cancer. Furthermore, a higher mutational rate was found from EGFR, TP53, PTEN, TET2, APC, CDKN2A, GNAQ, NOTCH1, FLT3, VHL, BRCA2, PTCH1, CBL, MLH1, BRAF, NRAS, TSC2, CSF1R, KIT, MAP2K1, MSH2, TSC1, HRAS, IFITM1 and BCL9 genes in NM with lung cancer, when compared to lung cancer patients without NM. A previous study revealed that EGFR mutations predisposed to the leptomeningeal metastases of EGFR-mutant non-small-cell lung cancer [34], and the present study further supports this notion. In spite of the relatively small sample size, the rate of the above mutated genes was much higher than what was reported in patients with lung adenocarcinoma from the COSMIC database, implicating that these gene mutations and the alteration of the signaling pathways are involved in and have became risk factors for NM.

After associating these EGFR activating mutations between primary lung cancer and NM, the present data shows that the EGFR activating mutations in the CSF samples were consistent with those of primary lung cancer. For example, the T790M in two CSF samples were sequentially collected during the TKI therapy. Furthermore, according to the gene set enrichment analysis, it was found that the ErbB, VEGF, MAPK, and m-TOR signaling pathways were significantly enriched in the 45 CSF samples obtained from NM patients with lung cancer, suggesting that the alterations of these signaling pathways might promote NM in lung cancer patients. In particular, the ErbB signaling pathway can regulate cell proliferation, migration, differentiation and apoptosis through the crosstalk with the PI3K-Akt, MAPK, or other signaling pathways [35].

Lastly, the representative patient in the present study provided a unique showcase. The genetic profiling of the CSF ctDNA clearly reflected the dynamic changes in these
identified driver genes and treatment responses. That is, this patient was detected to have the \textit{EGFR} 19Del mutation in the CSF sample, and thereby received icotinib. Thereafter, the CSF sample revealed the \textit{EGFR} 19Del and T790M mutations. Hence, the treatment was switched to AZD9291, which is a third generation EGFR TKI agent. A high response rate was exhibited by patients with tumors harboring the \textit{EGFR} T790M mutation, as well as a high capacity to penetrate into the CSF by crossing the blood-brain barrier \cite{36, 37}. The NGS allowed the physician to modify treatment option, in order to help the patient archive complete remission. As it is known, the \textit{EGFR} T790M mutation mediates the acquired resistance to EGFR TKI \cite{38, 39}, and a previous study reported that AZD9291 was designated as a powerful agent capable of overcoming the acquired \textit{EGFR} T790M resistance mutation \cite{40}.

However, the present study does have some limitations. For example, the cohort has a relatively small number of patients, and the different treatment options could not be separated to analyze the association of gene mutation, and the changes in gene copy number with treatment responses in these patients. The investigators were also unable to associate the gene mutations with the prognosis of these patients. Thus, future studies with a larger sample size from multiple institutions could help to solve these issues.

**Conclusions**

This study identified gene mutations in all CSF ctDNA samples, indicating that such an approach could be useful as a second-line diagnostic strategy for NM patients. PI3K-Akt signaling may be the potential NM metastasis mechanism.

**Abbreviations**

CNVs: copy number variations; CSF: cerebrospinal fluid; ctDNA: circulating tumor DNA; NM: neoplastic meningitis; EGFR: epidermal growth factor receptor; NSCLC: non-small cell lung cancer; MRI: magnetic resonance imaging; CT: computed tomography;

**Declarations**
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Availability of data and materials

The data that support the findings of this study are available from San Valley Biotechnology Incorporate but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request.

Conflict of interest statement

The authors declare that there is no conflict of interest in this work.

Authors’ contributions

Hui Bu and Chunyan Li conceived and designed the experiments. Yue Zhao prepared the manuscript. Junzhao Cui, Xin Chen, Xiaosu Guo, Weixin Han, Xueliang Wang and Yueli Zou performed the experiments. Junying He and Li Guo analyzed the data.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of the Second Hospital of Hebei Medical University (Hebei, China), and a written informed consent was obtained from each patient.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Table 3

Table 3 Gene mutations in CSF samples from NM patients with lung cancer compared with COSMIC database
| Gene   | NM (Our cohort) | Lung cancer (COSMIC database) | p-value |
|--------|-----------------|------------------------------|---------|
| ABL1   | 1/45 (2.22%)    | 64/5425 (1.18%)              | 0.417   |
| AKT1   | 1/45 (2.22%)    | 63/10898 (0.58%)             | 0.232   |
| APC    | 17/45 (37.78%)  | 200/5979 (3.35%)             | <0.001  |
| ATM    | 3/45 (6.67%)    | 288/5300 (5.43%)             | 0.974   |
| BAP1   | 1/45 (2.22%)    | 51/4574 (1.11%)              | 0.401   |
| BCL9   | 1/45 (2.22%)    | 0/2603 (0.00%)               | 0.017   |
| BRAF   | 4/45 (8.89%)    | 596/26989 (2.21%)            | 0.033   |
| BRCA1  | 2/45 (4.44%)    | 122/4786 (2.55%)             | 0.744   |
| BRCA2  | 7/45 (15.56%)   | 156/4753 (3.28%)             | <0.001  |
| CBL    | 5/45 (11.11%)   | 69/4871 (1.42%)              | 0.001   |
| CDH1   | 1/45 (2.22%)    | 50/5014 (1.00%)              | 0.367   |
| CDK4   | 1/45 (2.22%)    | 11/4572 (0.24%)              | 0.111   |
| CDKN2A | 14/45 (31.11%)  | 558/7621 (7.32%)             | <0.001  |
| CSF1R  | 3/45 (6.67%)    | 82/4775 (1.72%)              | 0.044   |
| CTNNB1 | 3/45 (6.67%)    | 157/6679 (2.35%)             | 0.161   |
| DDR2   | 2/45 (4.44%)    | 139/6918 (2.01%)             | 0.231   |
| DNMT3A | 2/45 (4.44%)    | 104/4644 (2.24%)             | 0.627   |
| EGFR   | 39/45 (86.67%)  | 26099/98618 (26.46%)         | <0.001  |
| ESR1   | 1/45 (2.22%)    | 64/4425 (1.45%)              | 0.484   |
| GNA11  | 1/45 (2.22%)    | 18/4780 (0.38%)              | 0.163   |
| GNAQ   | 14/45 (31.11%)  | 24/5036 (0.48%)              | <0.001  |
| HRAS   | 2/45 (4.44%)    | 33/7105 (0.46%)              | 0.020   |
| IFITM1 | 2/45 (4.44%)    | 3/2468 (0.12%)               | 0.003   |
| JAK2   | 1/45 (2.22%)    | 102/6912 (1.48%)             | 0.490   |
| JAK3   | 2/45 (4.44%)    | 88/5176 (1.7%)               | 0.181   |
| KDR    | 5/45 (11.11%)   | 218/5112 (4.26%)             | 0.060   |
| KIT    | 3/45 (6.67%)    | 120/6695 (1.79%)             | 0.048   |
| KRAS   | 2/45 (4.44%)    | 6809/42506 (16.02%)          | 0.034   |
| MAP2K1 | 3/45 (6.67%)    | 58/9328 (0.62%)              | 0.003   |
| MAP2K2 | 1/45 (2.22%)    | 23/4529 (0.51%)              | 0.212   |
| MLH1   | 5/45 (11.11%)   | 36/4969 (0.72%)              | <0.001  |
| MPL    | 2/45 (4.44%)    | 39/4946 (0.79%)              | 0.052   |
| MSH2   | 3/45 (6.67%)    | 54/4595 (1.18%)              | 0.017   |
| MYCN   | 1/45 (2.22%)    | 34/4544 (0.75%)              | 0.293   |
| NF1    | 1/45 (2.22%)    | 303/4814 (6.29%)             | 0.416   |
| NF2    | 2/45 (4.44%)    | 51/4865 (1.05%)              | 0.084   |
| NFE2L2 | 1/45 (2.22%)    | 212/5626 (3.77%)             | 0.881   |
| NOTCH1 | 11/45 (24.44%)  | 197/6456 (3.05%)             | <0.001  |
| NRAS   | 4/45 (8.89%)    | 128/16010 (0.8%)             | 0.001   |
| PIK3CA | 2/45 (4.44%)    | 719/16029 (4.49%)            | 1.000   |
| PTCH1  | 7/45 (15.56%)   | 86/5107 (1.68%)              | <0.001  |
| PTEN   | 27/45 (60.00%)  | 233/8576 (2.72%)             | <0.001  |
| PTPN11 | 2/45 (4.44%)    | 54/5910 (0.91%)              | 0.066   |
| RB1    | 2/45 (4.44%)    | 369/5651 (6.53%)             | 0.794   |
| RET    | 3/45 (6.67%)    | 142/5818 (2.44%)             | 0.181   |
| SMARCB1| 1/45 (2.22%)    | 31/4968 (0.62%)              | 0.251   |
| SMO    | 2/45 (4.44%)    | 72/5105 (1.41%)              | 0.136   |
| STK11  | 2/45 (4.44%)    | 594/8028 (7.4%)              | 0.646   |
| TET2   | 18/45 (40.00%)  | 90/4349 (2.07%)              | <0.001  |
| TP53   | 38/45 (84.44%)  | 4456/11831 (37.66%)          | <0.001  |
| TSC1   | 3/45 (6.67%)    | 70/4775 (1.47%)              | 0.030   |
|     |        |                  |       |       |
|-----|--------|------------------|-------|-------|
| TSC2 | 4/45  | (8.89%)          | 125/4800 | (2.6%) |
| VHL  | 9/45  | (20.00%)         | 23/5332 | (0.43%) |
| WT1  | 2/45  | (4.44%)          | 62/4634 | (1.34%) |

Figures
**Figure 1**

Profiling of gene mutations in the CSF samples obtained from NM patients, regardless of the primary cancer origin
Figure 2

The KEGG pathway analysis of mutated genes in the CSF obtained from NM patients regardless of the primary cancer origin.
**Figure 3**

The association of gene mutation frequency with detectable tumor cells in the CSF. MF, mutation frequency.
Figure 4

The KEGG pathway analysis of mutated genes in the CSF obtained from NM patients receiving both intrathecal chemotherapy and systemic therapy.
Figure 5 The association of CSF ctDNA concentration with the Karnofsky performance status (KPS) score, gene mutation and CSF tumor cells. (A) CSF ctDNA concentration vs. KPS. (B) CSF ctDNA concentration vs. the number of gene mutations. (C) The number of gene mutations and KPS scores. (D) CSF ctDNA concentration vs. the detectable circulating tumor cells in the CSF.
Figure 6

The data on the KEGG pathway analysis of mutated genes in the CSF obtained from NM patients with lung cancer
Figure 7

A representative case (N156). (A) The head contrast enhanced MRI. This shows the linear and strip abnormal enhancement of the cerebellar sulcus (red arrow) after the patient had a headache during the icotinib therapy for the primary tumor. (B) The May-Gruwald-Giemsa staining of the CSF sample. The data shows the tumor cells in the CSF (×1,000). (C) The brain MRI. This shows the dramatic improvement and complete response after received 80 mg of AZD9291 for 18 months, because the patient's CSF sample revealed EGFR 19Del and T790M mutations. (D) The May-Gruwald-Giemsa staining. The data shows fewer tumor cells (×1,000), when compared to that in B.