Original Article
The relationship between cerebrospinal fluid metastasis and gene mutations in non-small-cell lung cancer patients

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Abstract: Objective: The aim of this study was to use cerebrospinal fluid (CSF) cytology to give undiagnosed patients admitted to the hospital with severe neurological symptoms and without any anti-tumor treatment history a definitive diagnosis. Further, the aim was to explore the relationship between the frequency of gene mutations and mortality on the incidence of CSF metastasis in advanced non-small-cell lung cancer (NSCLC). Materials and methods: 30 patients diagnosed with NSCLC through CSF cytology were retrospectively analyzed. We analyzed 30 CSF metastasis patients and a control group of 20 advanced NSCLC patients without CSF metastasis. Results: 30 patients were diagnosed with CSF metastasis using CSF cytology and immunocytopathology. The frequencies of EGFR mutations and ALK fusion in the CSF metastasis group were higher than they were in the non-CSF metastasis group (80% and 50% respectively, $P<0.05$). The incidence of CSF metastasis with gene mutations was higher than it was with wild-type genes (70.6% and 37.5%, $P<0.05$), OR 4.0 (95% CI 1.14~13.99). The median survival time of the CSF metastasis group was 4.8 months (95% CI 4.2~5.3). However, the median survival time in the non-CSF metastasis group was 9.2 months (95% CI 3.3~15.1). The mortality of the CSF metastasis group (n=13, 43.3%) was significantly higher than it was in the non-CSF metastasis group (n=6, 30%). Conclusions: CSF metastasis in NSCLC patients has a higher frequency of gene mutations and mortality. EGFR mutation and ALK fusion patients have a higher incidence of CSF metastasis. EGFR mutations and ALK fusion may promote CSF metastasis and may be a predictor of prognosis in NSCLC patients.

Keywords: CSF metastasis, NSCLC, gene mutation, EGFR, ALK

Introduction

Malignant cells from malignant melanoma, lung, breast, and gastric cancer crossing the blood-brain barrier into CSF through hematogenous metastasis, endo- or perineural dissemination represents a poor prognosis. Nevertheless, in the era of targeted therapy, the median overall survival (OS) time of CSF metastasis is approximately less than 3 months [1]. Studies have found that leptomeningeal metastasis caused by NSCLC accounts for approximately 40-50% [2, 3].

Both tumor driver gene epidermal growth factor receptors (EGFR) and anaplastic lymphoma kinase (ALK) have been well studied and used for targeted therapy in tumor treatment. EGFR is a transmembrane tyrosine kinase receptor which can regulate DNA synthesis and cell proliferation by binding to the ligand for signal transduction. EGFR mutations can result in tyrosine kinase constitutive activation and then lead to tumorigenesis. EGFR tyrosine kinase inhibitors (EGFR-TKIs) such as gefitinib and erlotinib suppress tumor growth by binding to EGFR blocking signal transduction. And some clinical studies have showed that EGFR-TKIs improve the treatment response and progression free survival with light side effects in NSCLC compared to traditional chemotherapy [4-6]. In lung cancer, ALK rearrangement commonly translocated with the echinoderm microtubule-related protein 4 (EML4) gene is a subgroup of driver mutations which can benefit from an oral small-molecule inhibitor of ALK (crizotinib) treatment [7, 8].

Despite the improvements of targeted therapy like EGFR-TKIs in the treatment of NSCLC,

patients still have a high frequency of CSF metastasis. The relationship between EGFR mutation status and CSF metastasis is still controversial. Some studies found that CSF metastasis in NSCLC due to EGFR-TKIs prolongs survival [9-11]. However, some findings on the impact of EGFR mutations and the administration of EGFR-TKIs on the incidence of brain metastases are inconsistent [12-16]. But these relationships between the EGFR mutation status and CSF metastasis are based on EGFR-TKIs treatment. And there is a lack of data on the gene mutation status of CSF metastasis, especially on advanced NSCLC patients without any anti-tumor treatment before their diagnoses.

Previous studies on the relationship between EGFR mutation status, the brain, and leptomeningeal metastases of lung cancer were based on a series of treatments such as chemotherapy and radiation therapy [17-19]. But these studies ignored the function of the blood-cerebrospinal fluid barrier which can decrease the efficacy of lipid insoluble chemotherapy drugs [20, 21]. Under such conditions, lung cancer cells are prone to brain metastases [22, 23] and brain tissue becomes a plausible sanctuary for malignant metastasis [24].

In our study, we collected two groups of patients who did not undergo any anti-tumor treatments before their diagnoses to avoid any influential factors such as age, sex, pathology, anti-tumor treatment history, or blood-cerebrospinal fluid barrier function. Then we explored the frequency of gene mutations and mortality on the incidence of CSF metastasis in advanced NSCLC.

Materials and methods

Patient selection

A retrospective analysis was used to evaluate undiagnosed and untreated patients admitted to our hospital from August 1, 2017 to November 1, 2019 using CSF cytology. The study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University. The eligibility criteria were as follows: 1) patients admitted to the hospital with severe neurological symptoms; 2) patients with no history of pulmonary malignant tumors; 3) patients who had not undergone any prior anti-tumor treatments; 4) patients whose gene mutations were found using either next generation sequencing (NGS) or the amplification refractory mutation system (ARMS); 5) patients with confirmed NSCLC cells detected in their CSF using cytopathology and immunocytopathology; 6) the control patients had to have advanced NSCLC (stages III-IV at their initial diagnosis) and satisfy numbers 1-4 above but not number 5 above. Patients without gene mutation tests or with suspicious or atypical cells in their CSF were excluded.

50 patients who met the inclusion and exclusion criteria were enrolled in the study. All the patients read and signed the informed consent form to participate in this study. The age, sex, pathology, disease stage, EGFR and ALK mutation data were collected and recorded.

Lumbar puncture

In order to relieve the patients' neurological symptoms and get a definitive diagnosis, lumbar puncture was used to let out the CSF for the treatment and diagnosis. The CSF cells were centrifuged and analyzed using cytopathology.

Detection of the NSCLC cells in the CSF

The cells in the CSF were centrifuged on 3-4 slides for HE (hematein eosin) staining. If tumor cells were found, 2-3 slides were removed from the HE stained slides for immunocytochemical staining to determine the origins of the tumor cells. The immunocytochemical staining steps were as follows: 1) Remove the glass cover and degum using xylene until the gum was completely removed. Then dewater with gradient ethanol and fade the HE stains with 1% hydrochloric acid ethanol solution for 3 min. 2) Block and inactivate the endogenous peroxide enzymes using 0.3% H_2O_2 at 25°C for 15 min, then wash H_2O_2 and with distilled water for 3 min. 3) Repair the antigens with a citrate buffer at high pressure for 1.5 min, then let them cool naturally to room temperature and wash the slides three times with PBS (phosphate buffer saline) for 3 min. 4) Block the nonspecific antigens with normal sheep serum at 25°C for 10-15 min then remove the extra serum. 5) Add the specific antibodies like TTF1 (thyroid transcription factor 1) and NapsinA on the slides and incubate them overnight at 4°C. 6) Wash the extra antibodies three times with a PBS buffer for 3 min. Add biotin-labeled secondary antibody and incubate at 25°C for 15-20 min. 7) Add the DAB (diamino-

Table 1. Clinical characteristics of the 50 patients

	CSF metastases (N=30) n (%)	Non-CSF metastases (N=20) n (%)	P-value
Gender			0.341
Male	17 (56.7)	14 (70)	
Female	13 (43.3)	6 (30)	
Median age	56	59	0.881
Pathology			0.751
Adenocarcinoma	29 (96.67)	18 (90)	
Adenosquamous carcinoma	1 (3.33)	2 (10)	
Symptoms			
Cranial hypertension	0 (0)	1 (5)	0.837
Meningeal irritation	2 (6.67)	2 (10)	1
Dysfunction of extremities	4 (13.33)	2 (10)	1
Disorder of consciousness	4 (13.33)	1 (5)	0.63
Seizures	1 (3.33)	2 (10)	0.715
Headache	20 (66.67)	11 (55)	0.405
Nausea or Vomiting	11 (36.67)	9 (25)	0.556
vision disorder	4 (13.33)	2 (10)	1

benzidine) solution and observe the coloration under a microscope for about 3-5 min. Stop the reaction with running water. 8) Re-dye with hematoxylin for about 1-3 min then seal it with a neutral gum.

Gene mutation analysis

All 50 patients were tested to determine if they had any gene mutations, using samples of their CSF, blood, fresh cytology samples, and paraffin-embedded tissues before any treatment. Genomic DNA was extracted and purified using an AmoyDx® Tissue DNA Kit, FFPE DNA Kit, and a circulating DNA Kit (Spin Column, Amoy Diagnosis Co., Ltd, Xiamen, China) according to the kits' instructions. The gene mutation detection was performed using NGS (Amoy Diagnosis Co., Ltd, Xiamen, China) or ARMS (Amoy Diagnosis Co., Ltd, Xiamen, China).

Statistical analysis

The statistical analysis was performed using SPSS version 23.0. The frequencies and descriptive statistics of the patients' demographic and clinical variables were obtained. chi-square tests and Fisher's exact tests were used for the categorical variables. The overall survival was estimated using the Kaplan-Meier method and compared using log-rank tests. The Wald 95% confidence interval (95% CI) and the odds ratio (OR) were used as appropriate. And $P<0.05$ was considered statistically significant.

Results

Patient characteristics

The clinical characteristics of the 50 patients are listed in **Table 1**, of which 30 patients were diagnosed NSCLC through CSF cytopathology and immunocytopathology, as shown in **Figure 1**. Data from a total of 52 advanced NSCLC patients with non-CSF metastases were reviewed, of which 20 patients were selected according to the inclusion and exclusion criteria for the control experiment. They ranged in age from 40 to 74, and 62% of the patients were men. The most common symptoms and deficits that caused the patients to seek care at the hospital was headache in 31 patients (62%), nausea or vomiting in 20 patients (40%), then followed by vision disorders, dysfunction of the extremities, consciousness disorders, meningeal irritation, seizures, and cranial hypertension. And the clinical characteristics showed no significant differences between the CSF metastases group and the non-CSF metastases group ($P>0.05$).

Gene mutation analysis

All 50 patients underwent gene mutations, and their gene mutation statuses are shown in **Table 2**. 16 patients had wild-type genes, and 34 patients had gene mutations. The prevalent gene mutation was EGFR. 33 patients had
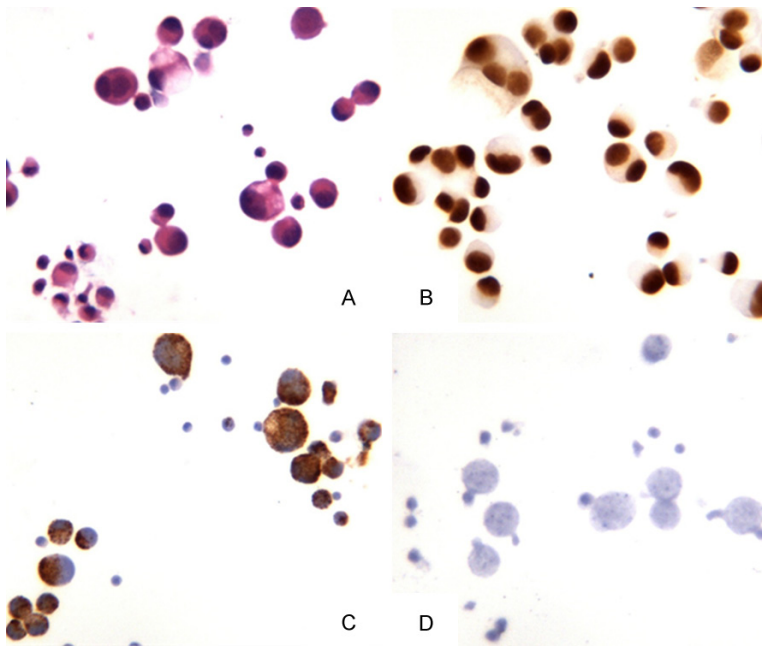
Figure 1. CFS cytological and immunocytochemical findings of patients with CFS metastasis. A. HE stain: large irregular shaped basophilic carcinoma cells with multiple nuclei and pointed cytoplasm borders. B. Immunocytochemical stain of TTF1: the nuclei were markedly brown-yellow. C. Immunocytochemical stain of NapsinA: the cytoplasms were markedly a coarse granular brown-yellow. D. Immunocytochemical stain of GFAP: there were no brown-yellow granules in the cytoplasms.

Table 2. Patient gene mutation statuses

gene mutation status	CSF metastases (N=30) n (%)	no CSF metastases (N=20) n (%)	P-value
Wild type	6	10	0.034
Gene mutations	24	10	
Exon 19	11	6	
Exon 21	11	3	
G719X	1	1	
ALK fusion	1	0	

In our study, we also analyzed the incidence of CSF metastases in the patients with gene mutations. 70.6% of the gene mutation patients had CSF metastases, and 37.5% of the wild-type gene patients had CSF metastases (P=0.034). The OR was 4.0, 95% CI was between 1.14 and 13.99.

Overall survival and mortality

Overall survival and mortality information was collected from the patients' medical records and the follow-up registry by telephone. OS was defined as the interval from the time of diagnosis to the time of death for any cause. The median OS was 4.8 months (95% CI, 4.2-5.3 months) in the CSF metastases group and 9.2 months (95% CI 3.3-15.1 months) in the non-CSF metastases group. And there was a statistically significant difference in the OS between the two groups (P<0.05; **Figure 2**).

The mortality of CSF metastasis group (n=13, 43.3%) was significantly higher than it was in the non-CSF metastasis group (n=6, 30%).

Discussion

EGFR mutations and 1 patient had ALK fusion. Among the 33 EGFR mutation patients, 17 (51.5%) had exon 19 deletions, 14 (42.4%) had an L858R of exon 21 mutation, and 2 (6%) had exon 18 alterations (G719X).

Among the 30 CSF metastasis patients, 6 had wild-type genes, 11 had exon 19 deletions, 11 had an L858R mutation, 1 had G719X, and 1 had ALK gene fusion. The frequency of gene mutations in the patients with CSF metastases was higher than it was in the patients without CSF metastases (80% and 50% respectively), which is statistically significant (P<0.05).

For undiagnosed patients without any previous anti-tumor treatment admitted to the hospital with severe neurological symptoms like cranial hypertension, delirium, and coma, lumbar puncture is not only a treatment that relieves the neurological symptoms but is also a method for getting a definitive diagnosis. The CSF cytology diagnosis is essential for diagnosing leptomeningeal metastasis, especially if the magnetic resonance imaging (MRI) is negative [25]. And some reports state that the CSF metastasis of lung adenocarcinoma for EGFR mutation detection is an alternative diagnostic tool to help guide the EGFR-TKIs treatment [22].
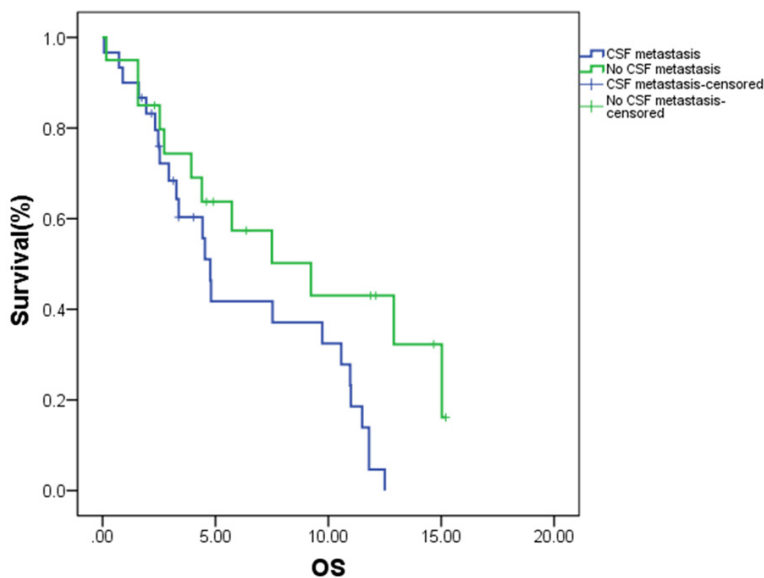
Figure 2. Overall survival times of the different groups. The median OS was 4.8 months (95% CI, 4.2~5.3 months) in the CSF metastases group and 9.2 months (95% CI 3.3-15.1 months) in the non-CSF metastases group.

tion patients had CSF metastases, and 37.5% of the wild-type gene patients had CSF metastases. The OR was 4.0, and the 95% CI was between 1.14 and 13.99. Perhaps our data volume was not big enough, so we excluded the effect of the tumor treatment on CSF metastasis. On the other hand, our results represent the original biological and genetic characteristics of the NSCLC cells in the CSF metastases.

Our results showed that the median OS in the CSF metastases group was significantly shorter than it was in the non-CSF metastases group. The median OS in the CSF metastases group was very close to

In our study we used the technique of fading HE staining for the immunocytochemical stains to determine the primary tumor of the cancer cells in the CSF. CSF cytology plays an important role in tumor diagnosis and treatment.

As we all know, the blood-cerebrospinal fluid barrier, which is a continuous, nonfenestrated endothelial microvasculature of the brain parenchyma, can keep malignant cells and chemotherapeutic drugs from infiltrating the brain. But this barrier can be disrupted by brain metastasis, leptomeningeal metastasis, chemotherapy, and radiotherapy. Consider this, we tried to limit the conditions of the advanced NSCLC patients who had not undergone any anti-tumor treatment until they were admitted to the hospital with severe neurological symptoms after a definite diagnosis in order to avoid the influential factors of their treatment histories and the blood-CSF barrier function. Furthermore, it makes it easier to observe the original biological and genetic characteristics of the NSCLC cells.

We analyzed the relationship between gene mutations and the incidence of CSF metastasis. In our research, 50 advanced NSCLC patients without any anti-tumor treatment before and their gene mutations were retrospectively analyzed. 70.6% of the gene muta-

the results of Emilie Le Rhun [1]. Thus, the early diagnosis of CSF metastasis is very important in improving the quality of life and prolonging the survival time using treatments with EGFR-TKIs, chemotherapy and radiotherapy. Considering the protection enabled by the blood-CSF barrier in anti-tumor treatment, some researchers studied the concentrations of different drugs. Britta Weber et al. reported that chemotherapy drugs hardly penetrate into the brain tissue or the CSF because of the blood-brain barrier which can decrease the efficacy of lipid insoluble chemotherapy drugs [20, 21]. However, the concentrations of chemotherapy drugs in normal brain tissue do not represent metastasis, for example cisplatin [26] and erlotinib [20]. Thus, gene mutation analyses like EGFR mutations and ALK fusion are essential for the NSCLC CSF metastasis population.

Our results revealed that the CSF metastasis of NSCLC patients has a higher frequency of gene mutations and mortality than those without CSF metastasis. In conclusion, EGFR mutations and ALK fusion may advance CSF metastasis and be a predictor of prognosis in NSCLC patients.

Disclosure of conflict of interest

None.

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