

Research Article

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Diagnostic value of circulating tumor cells in cerebrospinal fluid

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Abstract: Objective: To assess circulating tumor cells in cerebrospinal fluid as a diagnostic approach to identify meningeal metastasis in patients with non-small cell lung cancer by using tumor marker immunostaining–fluorescence in situ hybridization (TM-iFISH). Methods: In 5 non-small cell lung cancer patients who were confirmed to have developed meningeal metastasis by cerebrospinal fluid cytology, 20 ml of cerebrospinal fluid was obtained through lumbar puncture, from which 7.5 ml was utilized for TM-iFISH to identify and quantitate circulating tumor cells, 10ml for cerebrospinal fluid cytology, and 2.5ml for detection of cerebrospinal fluid tumor markers. Results: TM-iFISH examination identified 18 to 1,823 circulating tumor cells per 7.5ml cerebrospinal fluid. In contrast, cytology assessment revealed tumor cells in only 2 cases. The expression levels of cerebrospinal fluid tumor markers were all increased in all 5 patients when compared with their respective serum levels. Contrast-enhanced MRI scans demonstrated presence of meningeal metastasis in all 5 cases. Conclusion: TM-iFISH may become a novel cerebrospinal fluid-based diagnostic strategy to identify circulating tumor cells and meningeal metastasis as compared to traditional diagnostic approaches, although its superior sensitivity and specificity needs to be confirmed through additional studies with a larger sample size.

Keywords: Leptomeningeal metastasis; non-small lung cancer; circulating tumor cells; CFS cytology

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1 Introduction

As a major type of central nervous system metastasis, leptomeningeal metastasis is defined as diffuse or focal infiltration of primary tumor cells into the meninges that bathe the brain and spinal subarachnoid, often occurring as a formidable complication for leukemia, lymphoma, lung cancer and breast cancer [1]. Patients with leptomeningeal metastasis have a median survival of only 4 to 6 weeks when untreated, which may be extended to 3 to 5 months upon combination therapy [2]. Unfortunately, diagnostic approaches allowing for early detection and evaluation of the disease remain far from effective. Currently, early diagnosis primarily depends upon cerebrospinal fluid cytology, symptomatic evaluation of the central nervous system and contrast-enhanced cranial MRI. In particular, cerebrospinal fluid examination has become the diagnostic gold standard; however, such strategy suffers from daunting pitfalls, such as poor sensitivity and inability to provide quantitative measures [3]. Therefore, it is greatly necessary to identify a more clinically efficacious strategy that enables sensitive detection of leptomeningeal metastasis [4-5].

Interestingly, multiple lines of recent studies have demonstrated that circulating tumor cells (CTCs), which have shed into the circulation from a primary solid tumor, are highly correlated with tumor metastasis, drug resistance, prognosis and recurrence. As non-hematopoietic epithelial cells, the majority of CTCs express epithelium-specific cytokeratin, accompanied by aberrant numbers of certain chromosomes (for example, chromosome 8 as haploid or polyploid). Clinical assessment of CTCs can be achieved by tumor marker immunostaining–fluorescence in situ hybridization (TM-iFISH), which effectively identifies and quantifies various non-hematopoietic epithelial cells through enrichment and analytic approaches, thus exhibiting great sensitivity and superior specificity. In the present study, to exploit new approaches to identify leptomeningeal metastasis, we interrogated the diagnostic values of CTCs through the TM-iFISH technique by studying 5 patients who were enrolled with confirmed

leptomeningeal metastasis in Tianjin Lake Hospital Cancer Intervention.

2 Methods and Materials

2.1 Inclusion Criteria

Enrolled patients were admitted for treating meningeal metastasis of non-small cell lung cancer from March to May, 2014, at Tianjin Lake Hospital. They met the following essential criteria: 1) non-small cell lung cancer patients as confirmed by histological or cytological diagnosis, 2) meningeal metastasis confirmed by cerebrospinal fluid cytology, 3) normal clotting time and platelet counts as confirmed by laboratory test, 4) controllable symptoms of intracranial hypertension after treatment with dehydration medications, 5) tolerance to lumbar puncture for cerebrospinal fluid collection, 6) confirmed exclusion of intracranial meningioma, ependymoma, meningioma and other brain lesions, and 7) signed informed consent.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

2.2 TM-iFISH

20 mL CSF was drawn from all patients by lumbar puncture, in which 7.5 mL was stored in the special tube of TM-iFISH detection at room temperature. TM-iFISH was used to detect CTCs within 3 days. Specific steps were as follows: (1) Cell enrichment (negative screening method of immunomagnetic heads): CSF was made into 100 μ L cell suspension after CD45 positive leukocytes were removed by immunomagnetic heads of envelope anti-CD45 antibodies; (2) Cell analysis (cell count and nucleic acid detection): 100 μ L cell suspension section was fixed first, and then centromeric probe 8 (CEP8) was adopted to detect the number of chromosome 8, anti-CK 18 (CK 18) antibody (manifesting that the captured cells derived from the epithelium) and CD45 antibody (showing that the captured cells were non-leukocytes) for immunofluorescence assay by FISH. Next, cell number was counted under an OLYMPUS-BX53 fluorescence microscope

(OLYMPUS Company, Japan) after staining (the captured cells were karyocytes) with 4-6-diamidino-2-phenylindole (DAPI). The count was repeated 5 times, and the mean was selected as the final value for each patient. The remaining 10 mL and 2.5 mL CSF were respectively used to conduct cytological and biochemical examinations after sample collection.

2.3 Identification and quantification of CTCs

CTCs did not express the surface markers of haematogenous cells, such as CD45, as they were non-haematogenous epithelial cells. Under fluorescence microscope, non-haematogenous naked nuclear cells (there was no red halo around cell nucleus) which did not express CD45 were examined under red channels. CEP8 FISH signal could be seen under orange channels as an orange light spot. The number of light spots was the number of chromosome 8, which in most of CTCs was polyploidy. Afterwards, CK18 expression in cells was observed under green channels, and DAPI staining under blue channels. Therefore, tumor cells originating from non-haematogenous epithelial cells were detected as DAPI⁺, CD45⁻, CK18⁺ or CK18⁻ and CEP8⁺. The chromosome could be haploid, diploid or polyploidy. However, circulating cells that were haematogenous leukocytes were identified as DAPI⁺, CD45⁺, CK18⁻ and CEP8⁺, in which the overwhelming majority of chromosomes were diploid.

3 Results

Among the 5 patients with confirmed meningeal metastasis enrolled in our study, there were 2 males and 3 females, with a median age of 52 years-old (ranging from 52 to 64 years-old). All patients developed lung cancer as the primary tumor, with 3 exhibiting parenchymal metastasis and all 5 meningeal metastasis. Clinical symptoms included persistent headache (5 cases), intracranial hypertension (3 cases), meningeal irritation (2 cases) and cauda equine syndrome (2 cases, Table 1). Contrast-enhanced MRI scans during hospital admission showed meningeal metastasis for all 5 patients. TM-iFISH-based examination demonstrated 18 to 1,823 tumor cells per 7.5ml (Figure 1). In contrast, cerebrospinal fluid cytology assessment only revealed 2 cases with tumor cells, which could not be quantified. The expression levels of cerebrospinal fluid tumor markers were all increased when compared with the reference range in the serum (Table 2).

Table 1: The clinical characteristics of 5 patients

No.	Gender	Age (Y)	Pathological types	Metastatic parts	Major clinical manifestations
1	Female	52	Adenocarcinoma	Meninx, brain	Headache, deaf
2	male	52	Adenocarcinoma	Meninx, brain	Headache, cauda equina syndrome
3	Female	52	Adenocarcinoma	brain	Headache, vomiting , dysopia
4	male	62	Adenocarcinoma	Meninx, brain	Headache
5	Female	64	Adenocarcinoma	Meninx	Headache, dysopia , cauda equina syndrome

Table 2: The results of the different testing methods for 5 patients

No	MRI enhancement scan	CSF TM-iFISH analysis	Cytological CSF analysis	CSF tumor marker analysis
1	Leptomeningeal metastasis	246/7.50ml	negative	CEA 13.16ng/ml NSE 10.21ug/l Cyfra21-1 2.65ug/l
2	Leptomeningeal metastasis	29/7.50ml	negative	CEA 0.20ng/ml NSE 18.81ug/l Cyfra21-1 5.31ug/l
3	Leptomeningeal metastasis	1823/7.50ml	positive	CEA 10.53ng/ml NSE 9.18ug/l Cyfra21-1 1.91ug/l
4	Leptomeningeal metastasis	18/7.50ml	negative	CEA 6.11ng/ml NSE 8.70ug/l Cyfra21-1 2.15ug/l
5	Leptomeningeal metastasis	720/7.50ml	positive	CEA 68.49ng/ml NSE 17.32ug/l Cyfra21-1 110.40ug/l

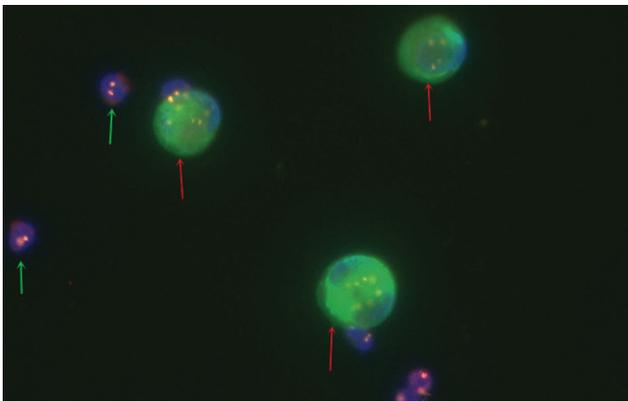


Figure 1: Circulating tumor cells (CTCs) (red arrow): positive DAPI staining (blue fluorescence), highly expressed PAN- CK (green fluorescence), CEP8 (polyploidy) , and CD45 (absent expression); white cells (green arrow): positive DAPI staining (blue fluorescence), PAN-CK (absent expression), CEP8 (diploid), and CD45 (red fluorescence). $\times 400$

4 Discussion

Leptomeningeal metastasis represents a challenging clinical complication that results from diffuse infiltration of primary tumor cells into the pia mater and subarachnoid and thereby causes severe damage to brain tissues, nerves and spinal cord [1]. Early diagnosis allows for timely intervention and treatment that can effectively delay disease progression and prevent neurological damage [2]. Currently available diagnostic approaches focus on the criteria below: 1) a clear history of cancer, 2) new

development of neurological symptoms, 3) typical MRI outcomes and 4) presence of tumor cells in cerebrospinal fluid. Patients who met the first two criteria and one of the last two are confirmed to have developed leptomeningeal metastasis. However, the negative rate for initial cerebrospinal fluid evaluation is up to 45%, and the sensitivity reaches up to 80% for the second inspection. Intriguingly, repeated inspections for 3 or more times are unable to help improve the positive rate [6]. Contrast-enhanced MRI scans offer nearly 100% specificity, which is accompanied by nodular meningeal thickening and linear, “tram-track” or diffuse meningeal enhancement. Sometimes prominent meningeal “tail sign” can be observed, together with decreasing brain parenchyma, cerebral and periventricular edema. However, contrast-enhanced MRI harbors a false-negative rate of 65% and a false-positive rate of 10% [7].

Tumor markers refer to biological factors secreted by cancer cells during development and proliferation of the host cells during reactive responses to tumorigenesis, which bear important diagnostic values for cancer patients. At present, well-established tumor markers for lung cancer include carcinoembryonic antigen (CEA), neuron specific enolase (NSE) and cytokeratin 19 fragment (Cyfra21-1). Wang et al investigated tumor markers in serum and cerebrospinal fluid from 35 patients that had developed meningeal metastasis from lung cancer, and identified that tumor markers were considerably effective for predicting meningeal metastasis, especially for those

refractory to cytology and MRI diagnosis [8]. However, important hurdles remain unsolved for cerebrospinal fluid tumor marker-based diagnosis, including poor specificity and high sensitivity, lack of standardized reference range, inability to determine the primary tumor and the damage to the blood-brain barrier resulting from cranial metastasis, brain injury and encephalitis. Therefore, we argue that cerebrospinal fluid-based cytology and contrast-enhanced MRI scans exhibit poor sensitivity for the purpose of early diagnosis of meningeal metastasis, whereas cerebrospinal fluid-based tumor markers have high sensitivity but low specificity. It is thus greatly important and helpful to identify novel diagnostic approaches with improved sensitivity.

In the current study, among the 5 cases that were previously confirmed with cerebrospinal fluid cytology as meningeal metastasis, only 2 showed the presence of tumor cells in the cerebrospinal fluid smears, suggesting the formidable inconsistency inherent to the approach of the cerebrospinal fluid evaluation. Intriguingly, contrast-enhanced MRI scans indicated meningeal metastasis for all 5 cases in the study, revealing superior diagnostic specificity. However, given its false-positive rate of 10%, contrast-enhanced MRI scans may not offer the most reliable diagnostic strategy. Notably, the expression levels of tumor markers were all elevated, albeit to varying extent, in all 5 enrolled patients, uncovering high diagnostic sensitivity. However, currently without a standardized reference range for tumor markers in the cerebrospinal fluid, our study instead focused on serum and identified 3 cases positive for meningeal metastasis, which may have occurred as a consequence of damage to the blood-brain barrier that adversely affects the diagnostic outcomes. This suggests that tumor marker-based diagnostic approaches from cerebrospinal fluids may be ineffective to accurately predict meningeal metastasis.

TM-iFISH serves as a well-established platform particularly designed to identify and quantify non-hematopoietic epithelial cells in biological fluid samples through enrichment and analytic techniques, thereby exhibiting high sensitivity and specificity for assessing CTCs. Previous studies have demonstrated that the superior diagnostic values of TM-iFISH in identifying CTCs for breast cancer and malignant melanoma [6,9]. In our study, all 5 cases were positive with CTCs as revealed by TM-iFISH, that is DAPI⁺CD45⁺CK18⁺ or CD18⁺CEP8⁺ at approximately 18 to 1,823 cells per 7.5ml, thus uncovering prominent sensitivity and specificity. Given its ability to quantify CTCs, TM-iFISH may harbor better diagnostic values than cerebrospinal fluid cytology, cerebrospinal fluid tumor markers and contrast-enhance MRI scan. In addition, our

results showed that the 2 patients who were diagnosed with cerebrospinal fluid cytology had higher numbers of CTCs than the other 3 patients; however, it remains to be fully determined whether there is a strong correlation between cerebrospinal fluid cytology and the number of CTCs in cerebrospinal fluid identified by TM-iFISH. The question may be answered by evidence from further studies with a larger sample size and a control group that do not develop meningeal metastasis.

In conclusion, TM-iFISH offers a potentially accurate and robust diagnostic approach to identify meningeal metastasis, although additional investigations are necessary to further characterize its diagnostic sensitivity and specificity.

Conflict of interests: The authors declare that they have no competing interests.

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