JCN Open Access ORIGINAL ARTICLE

pISSN 1738-6586 / eISSN 2005-5013 / J Clin Neurol 2020;16(4):581-585 / https://doi.org/10.3988/jcn.2020.16.4.581

High Cell-Free DNA Levels in Cerebrospinal Fluid Predict Leptomeningeal Seeding of Hematologic Malignancy

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Background and Purpose The main difficulty when diagnosing leptomeningeal metastases (LMSs) is the low sensitivity of cytology. Cancer cells release cell-free DNA (cfDNA) during proliferation and apoptosis, and so we analyzed the cfDNA level as a biomarker for LMSs in hematologic malignancy.

Methods This study prospectively enrolled 20 patients with hematologic malignancy who underwent cerebrospinal fluid (CSF) analysis. LMS was diagnosed based on both CSF cytology and clinical findings.

Results The CSF level of cfDNA was higher in patients with LMSs (108.17±84.84 ng/mL, mean±standard deviation) than in non-LMS patients (14.23±2.78 ng/mL). The sensitivity of cfDNA was higher than that of cytology (100% vs. 87%).

Conclusions The cfDNA level in the CSF can be used as a supplemental marker for diagnosing LMS in hematologic malignancy patients.

Key Words leptomeningeal metastases, hematologic malignancy, cell-free DNA.

INTRODUCTION

Leptomeningeal metastases (LMSs) have a highly variable clinical presentation, ranging from general symptoms such as headache to focal neurologic deficits, depending on the number, location, and size of lesions.[1] The prevalence of LMSs is reportedly 20−40% in leukemia, lymphoma, breast cancer, lung cancer, and melanoma patients, which is higher than that in patients with other types of cancer.[2,3] Hematologic malignancy is known to present as LMS without other systemic involvement, unlike solid tumors,[4] and its central nervous system (CNS) invasion is associated with a poor prognosis. However, LMS is often underdiagnosed, and also often shows a worse prognosis than other systemic malignancies, highlighting the need for faster and more-accurate diagnosis.

Diagnosing LMS has always been challenging due to the low sensitivity of testing modalities. Previous studies have attempted to develop biomarkers, but the current gold standard for diagnosing LMS is cerebrospinal fluid (CSF) cytology. Cytology analysis shows a diagnostic yield of 55% in a single test,[4] and an overall diagnostic yield of 71%. Moreover, the yield of the sample depends on the availability of an appropriate sample quantity (>10 mL) and its immediate processing.[1] Efforts to develop CSF biomarkers include chemical studies of tumor markers. In cases of negative cytology, CSF proteins and white blood cells (WBCs) in CSF can be the first clue for a diagnosis. Increased protein levels or WBC counts or decreased glucose levels in the CSF can be a clue for LMS. However, most LMS patients show CSF protein levels, WBC counts, and glucose levels that are within the normal ranges.[5]

Recent studies have progressed to looking for tumor markers in the CSF, such as vascu-

Received February 13, 2020
Revised May 15, 2020
Accepted May 15, 2020

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lar endothelial growth factor (VEGF) and stromal cell derived factor (SDF)-1.[6] These markers have shown diagnostic value in detecting LMS in breast cancer, lung cancer, and melanoma. One study analyzed carcinoembryonic antigen and cytokeratin-19 fragments (CYFRA21-1) in the CSF of LMS patients, and found that the level of CYFRA21-1 was higher in patients with unmatched pathology than in those with nonneurologic malignancy.[7,8] While these tumor markers in CSF have shown their potential as biomarkers for LMS, the investigations have been limited to a few specific tumors. Moreover, some tumor markers were increased in nontumorous conditions, with VEGF being increased in neurotuberculosis and moyamoya disease.[9,10]

The levels of circulating cell-free nucleic acids are often increased in blood. The cell-free DNA (cfDNA) level reflects the apoptotic and necrotic pathologic processes of malignant lesions. Recent studies of various malignancies have demonstrated the efficacy and efficiency of analyzing the cfDNA levels in various body fluids. cfDNA from the ascites and pleural effusions of metastatic cancer patients provide additional information that is not available from the plasma cfDNA level.[11] Urine cfDNA is a better diagnostic tool for cancer than urine cytology,[12] and so the possibility of using concentrated cfDNA from the CSF of patients with CNS malignancies is intriguing. Because the cfDNA level in the CSF has not been compared with cytology findings previously, we investigated the potential of using the level of cfDNA for diagnosing LMS in patients with hematologic malignancies and compared it with that of established diagnostic methods.

METHODS

Patient enrollment

CSF samples were prospectively collected from a single institution (Seoul National University Hospital) from June 2016 to May 2019. The patients had been previously diagnosed with hematologic malignancy and were hospitalized for evaluating the neurologic symptoms with a suspicion of LMS. CSF was collected during therapeutic or diagnostic procedures. The diagnosis of LMS was made based on both clinical evidence and cytology results as follows: 1) positive cytology findings at the initial or repeated lumbar puncture, with a clinical course of LMS, and 2) undetermined cytology (e.g., only atypical cells), but a further clinical course that was consistent with LMS, such as progressive focal neurologic dysfunction or abnormal neurologic examination without other possible cause.[13]

Demographic, conventional examination, and prognostic data were processed. All participating individuals provided written informed consents in accordance with the guidelines of the Institutional Review Board of the Seoul National University Hospital (1604-027-753), which approved the study.

Measurement of cfDNA

The levels of markers in CSF were measured using the MagMAX Cell-Free DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, 30 μL of 20% sodium dodecyl sulfate solution was poured into a nonstick RNase-free microcentrifuge tube (Thermo Fisher Scientific) with 600 μL of CSF and 12 μL of proteinase K solution, mixed well, and then incubated at 60°C for 20 minutes on a heat block. The tube was cooled on ice for 5 minutes and then mixed with 760 μL of MagMAX Cell-Free DNA Lysis/Binding Solution (Thermo Fisher Scientific) and MagMAX Cell-Free DNA Magnetic Beads (Thermo Fisher Scientific) at a 75:1 ratio. The solution mixture was gently mixed for 10 minutes using an Eppendorf Thermomixer (Eppendorf, Hamburg, Germany) at 2,000 rpm and 24°C. The centrifuged solution was placed on a DynaMag magnet (Thermo Fisher Scientific), and the supernatants were discarded. The processed solution was washed with MagMAX DNA Wash Solution (Thermo Fisher Scientific) and 500 μL of 80% ethanol. The Eppendorf Biospectrometer (Eppendorf) was used to quantify the cfDNA level in the final solution with the QuantiTPicoGreen dsDNA reagent (Thermo Fisher Scientific). The above-described test method was replicated using another 200-μL sample of CSF, with the results compared with those obtained using 600 μL of CSF to confirm the reliability of the measurement results.

Statistical analysis

Data were analyzed using SPSS software (version 25.0, IBM Corporation, Armonk, NY, USA) and were expressed as mean±standard-deviation values. The Mann-Whitney U test was used for statistical comparisons, with a p value of <0.05 considered to be significant.

RESULTS

Patient characteristics

This study enrolled 20 patients who were diagnosed with hematologic malignancy: 9 (45%) with acute lymphocytic leukemia, 2 (10%) with acute myeloid leukemia, 6 (30%) with diffuse large-B-cell lymphoma, 1 (5%) with Burkitt lymphoma, 1 (5%) with T-cell lymphoma, and 1 (5%) with mantle-cell lymphoma. The patients had mean and median ages of 47.15 and 51 years, respectively, 14 (70%) of them were male, and 8 (42%) had been diagnosed with LMS. Three patients showed atypical cytology: two patients were diagnosed with LMS due to a typical clinical course of LMS and deterioration, and the third was not diagnosed with LMS due to no clini-

Table 1. Demographic data, clinical information, and cfDNA levels of the LMS patients

Patient number	Age, years	Sex	Hematologic disease	Neurologic symptoms	Final diagnosis	Cytology	CSF WBC	Lymphocytes	Polymorphonuclear leukocytes	Others	CSF protein	cfDNA level (ng/dL)
1	53	M	ALL	Headache	LMS	LMS	26	18	0	8	97	291.74
2	33	M	ALL	Headache	Control	Control	0	0	0	0	80	13.88
3	54	M	AML	None	Control	Control	0	0	0	0	82	15.73
4	59	M	AML	None	Control	Control	1	1	0	0	60	16.19
5	65	F	ALL	Gait disturbance	LMS	LMS	1	0	0	1	60	21.98
6	38	M	DLBCL	Peripheral tingling sensation	Control	Atypical cells	4	1	1	3	49	15.73
7	26	M	ALL	None	Control	Control	0	0	0	0	52	16.19
8	29	F	ALL	None	Control	Control	0	0	0	0	34	13.42
9	63	F	ALL	None	Control	Control	2	0	0	2	31	15.96
10	49	F	DLBCL	None	Control	Control	0	0	0	0	34	14.34
11	64	M	MCL	None	LMS	LMS	1	1	0	0	49	123.77
12	19	M	DLBCL	Facial palsy	LMS	LMS	0	0	0	0	58	58.30
13	37	M	ALL	Headache	LMS	Atypical cells	2	2	0	0	51	56.25
14	70	M	TL	Weakness in both legs, LBP	LMS	LMS	312	14	4	82	115	161.49
15	58	M	DLBCL	None	Control	Control	Not tested	Not tested	Not tested	Not tested	46	5.55
16	31	M	BL	Headache	Control	Control	1	1	0	0	37	15.50
17	44	F	ALL	None	Control	Control	0	0	0	0	44	14.34
18	57	F	DLBCL	Binocular diplopia, right ptosis	LMS	LMS	0	0	0	0	75	131.18
19	19	M	ALL	None	Control	Control	1	1	0	0	65	13.89
20	75	M	DLBCL	Weakness in both legs	LMS	Atypical cells	10	6	0	4	124	20.63

ALL: acute lymphocytic leukemia, AML: acute myeloid leukemia, BL: Burkitt lymphoma, cfDNA: cell-free DNA, CSF: cerebrospinal fluid, DLBCL: diffuse large-B-cell lymphoma, F: female, LBP: lower back pain, LMS: leptomeningeal metastases, M: male, MCL: mantle-cell lymphoma, TL: T-cell lymphoma, WBC: white blood cell.

cal evidence or deterioration. The demographic data, clinical information, and cfDNA levels in all of the evaluated subjects are listed in Table 1.

cfDNA analysis

The level of cfDNA ranged from 5.55 ng/mL to 291.74 ng/mL. The CSF yield of cfDNA ranged from 20.63 ng/mL to 291.74 ng/mL (108.17±84.84 ng/mL) in LMS patients, and from 5.55 ng/mL to 16.19 ng/mL (14.23±2.78 ng/mL) in control patients (p<0.01) (Fig. 1). The diagnostic cutoff value for the cfDNA level was 18.41 ng/mL. Receiver operating characteristics curves were used to determine the optimal cutoff value of the cfDNA level. The sensitivity of the cfDNA analysis for the diagnosis of LMS was 100%, while that of cell cytology—which is the current gold-standard diagnosis method—was 87%. This difference in sensitivity between the cfDNA and cytology analyses was unable to show statistical significance due to the small number of patients.

Other CSF measures in LMSs

We also compared the diagnostic capabilities of other conventional factors, such as protein levels and WBC counts. The median and mean protein levels were 60.0 and 65.6 mg/dL, respectively, in the LMS group, and 49.0 and 56.7 mg/dL in the control group (p=0.047). The normal range of protein levels in CSF is 15–45 mg/dL, and seven control patients also showed protein levels that were higher than this normal range. The median and mean WBC counts were 1.00 and 10 cells, respectively, in the LMS group, and 15.73 and 42 cells in the control group (p=0.157).

Case presentation

Case 6

Patient 6 was a 38-year-old male diagnosed with diffuse large-B-cell lymphoma after radical orchiectomy of the right testis. He received chemotherapy including prophylactic intrathecal methotrexate, and had no neurologic symptoms or signs. A CSF study was performed at the same time as each intrathecal treatment, and the fourth and subsequent CSF cytology analyses showed atypical cells. The patient did not complain of any neurologic symptoms except for a peripheral tingling sensation and intracranial hypotension after the lumbar puncture. The headache was relieved after administering a blood patch. After six cycles of rituximab, cyclophosphamide, hydroxydaunorubicin, oncovin, predinosne regimen and intrathecal methotrexate, the patient achieved complete remission. The CSF cytology results continued to show atypical cells until intrathecal therapy was discontinued, but malignant cells were not found. This patient was diagnosed with non-LMS disease. The cfDNA level in this patient was 15.73 ng/mL, indicating the absence of LMS.

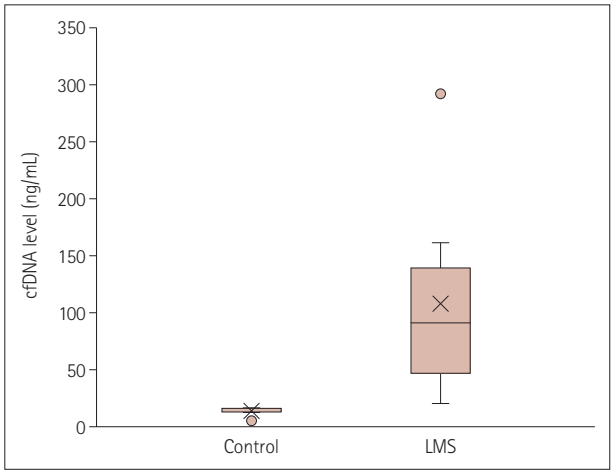

Fig. 1. Box plots of the cfDNA levels in control and LMS patients with hematologic malignancies. Each box plots show the median, first and third quartiles, and range. cfDNA: cell-free DNA, LMS: leptomeningeal metastases.

Case 13

Patient 13 was a 39-year-old male diagnosed with pre-B-cell acute lymphocytic leukemia, and he had a history of myalgia and night sweats. He was treated with chemotherapy and allogenic stem cell transplantation, and went through two remissions and three relapses. The patient complained of headache at the third relapse, and the CSF analysis showed atypical cells. The cfDNA level was 56.25 ng/mL, which exceeded our cutoff value for indicating LMS. The final clinical diagnosis was LMS, and the patient died 6 months later due to progression of the LMS.

DISCUSSION

Studies have shown that cfDNA can be present in many body fluids. The presence of cfDNA in urine and ascites has been suggested as a marker for tumor detection that does not require highly invasive tissue biopsies.[11,12] The use of CSF as a liquid sample for diagnosing the recurrence of brain tumors,[14] primary CNS tumors, and brain metastasis has been studied previously.[3] The present results suggest that the CSF cfDNA level is a simple, fast, inexpensive, sensitive, and efficient tool for detecting leptomeningeal seeding in hematologic malignancy patients, especially in comparison with the CSF cell cytology method that is the current gold standard for LMS diagnosis as well as other conventional methods such as CSF protein levels and pleocytosis.

Current National Comprehensive Cancer Network guidelines for diagnosing LMS are based on CSF cytology, but re-

liable sensitivity and specificity values are lacking for many of the current measures. For example, CSF proteins may be elevated in LMS patients, but this can also be the case in normal patients. While researchers attempted to validate CSF WBCs for diagnosing LMS, some studies of 1990s showed any numbers of lymphoblasts can be associated of CNS invasion regardless of increased CSF WBC counts in pediatric patients.[15] The WBC count in CSF is thus likely to be inappropriate for diagnoses. While repeated cytology analyses can increase the sensitivity, cfDNA is more sensitive than conventional measures.

Measuring cfDNA can be combined with measurements of other biomarkers for LMS. Biomarkers for LMS based on its primary source have emerged in recent years. Molecules such as CD19, ATIII, CD27, b2-microglobulin, interleukin (IL)-6, IL-10, serum chemokine ligand (CXCL)-13, neopterin, osteopontin, and microRNAs have shown efficacy and efficiency in diagnosing LMS in CNS lymphomas.[8] Evaluations of the levels of VEGF and SDF-1 showed that they were specific for LMS patients with melanoma, breast cancer, and lung cancer as the primary tumor.[6]

This study was subject to a few limitations. First, relatively few patients were enrolled, and so larger validation studies are needed. Second, the clinician was not blinded to the cfDNA level. Third, our microscopic diagnoses were made by the hospital's qualified pathologists, but the images were not validated. Although technology is being developed for increasing the accuracy of detecting markers in liquid biopsies, the normal range of cfDNA values also needs to be established. Further investigations of cfDNA in leptomeningeal disease are likely to broaden the spectrum of diseases in which cfDNA can be used as a biomarker.

Author Contributions

Conceptualization : all authors. Investigation : all authors. Writing—original draft : Eun Young Kim, Soon-Tae Lee. Writing—review & editing : all authors.

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

Acknowledgements

This study was supported by the SNUH Research Fund (0420180670).

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