

always feasible, and conventional cerebrospinal fluid (CSF) analyses via cytopathology or flow cytometry and diagnostic magnetic resonance imaging (MRI) have shown suboptimal sensitivity and discriminative capacity to enable the diagnosis of CNS involvement without tissue confirmation. The development of methods that overcome these limitations and allow reliable non-invasive identification of CNS involvement would be transformative for the clinical care of patients with suspected secondary CNS involvement by DLBCL.

Liquid biopsy, which involves analyzing circulating tumor DNA (ctDNA) in the blood or body fluid, is revolutionizing cancer diagnosis and surveillance. ctDNA originating from tumor tissue or lysed circulating tumor cells offers a noninvasive method for monitoring cancer progression and treatment responses [7]. Advances in next-generation sequencing (NGS) have enabled the analysis of variants in ctDNA, allowing tumor genotyping using blood samples [8]. High-throughput ctDNA sequencing can provide comprehensive genetic information regarding the tumor, serving as a surrogate for sequencing the entire tumor genome. This approach holds promise for the identification of therapeutic targets and early detection of relapse or residual disease. We have shown that ctDNA can be detected in plasma from patients with various subtypes of B-, T- or NK-cell lymphomas, correlating with tumor volume and patient outcomes [9-12]. However, only a minority of patients with primary CNS B-cell lymphomas have detectable ctDNA in their plasma, possibly because of the

blood-brain barrier [13]. Consequently, ctDNA from CSF has emerged as a promising biomarker for CNS involvement of B-cell lymphomas. In this study, we optimized a customized targeted sequencing approach to achieve ultrasensitive ctDNA profiling and investigated its potential for identifying CNS involvement without the need for biopsy.

We analyzed archived CSF samples collected between July 2020 and October 2022 from patients diagnosed as having large B-cell lymphoma who participated in a prospective cohort study (Samsung Lymphoma Cohort Study III, Institutional Review Board of Samsung Medical Center, File No. SMC 2017-12-068; ClinicalTrials.gov Identifier: NCT03117036). All patients were pathologically confirmed as having large B-cell lymphoma using immunohistochemistry [14]. As we monitored the patients enrolled, we identified those suspected of CNS involvement either based on clinical neurological symptoms and/or manifestations or radiologically by detecting parenchymal or leptomeningeal abnormalities in the brain or spine MRI scans. From these patients, we collected CSF samples in EDTA tubes via lumbar puncture or using an Ommaya reservoir (Integra LifeSciences, Inc., Princeton, NJ, USA). The samples were aliquoted into cryotubes and stored at -80°C until analysis. We selected a cohort of 17 patients with confirmed CNS involvement for a detailed study. CNS involvement was confirmed via brain biopsy in cases of parenchymal abnormalities and via cytological CSF examination for leptomeningeal involve-

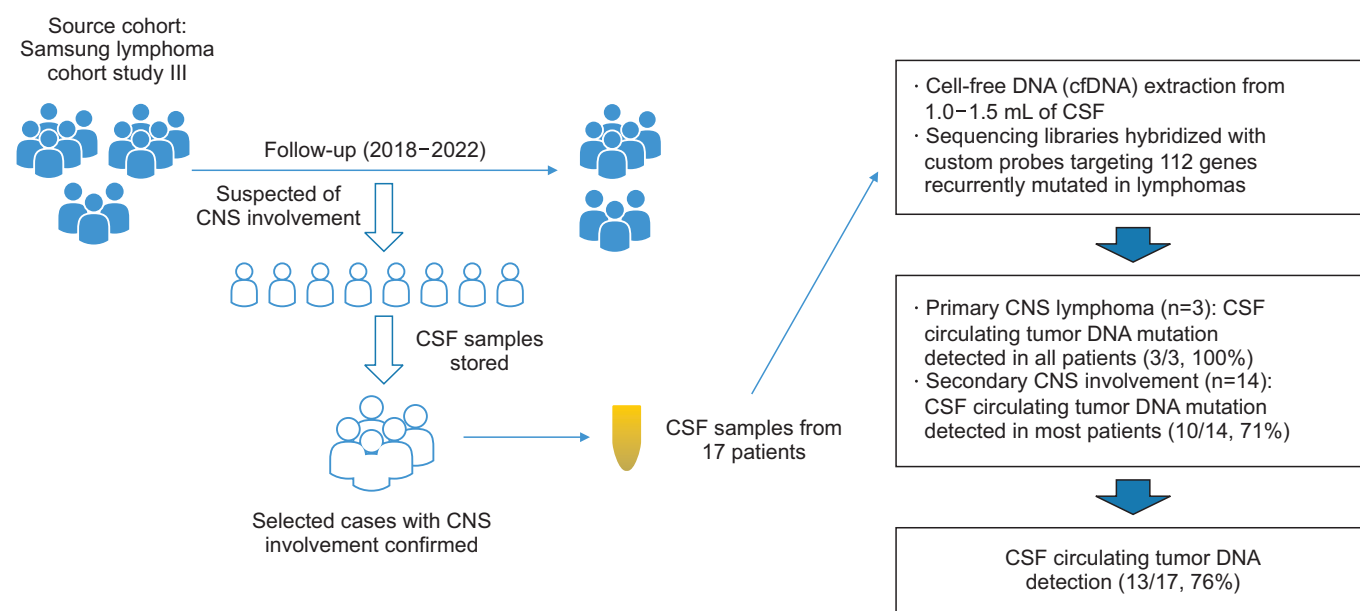


Fig. 1. Study flow.
Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid.

ment who were cytology-negative were identified based on their response to CNS-directed therapy and imaging findings. We monitored all patients' clinical outcomes, including survival status, via follow-up MRI.

We extracted cell-free DNA (cfDNA) from 1.0–1.5 mL of CSF using a MagMAX Cell-Free Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA yield and size distribution were assessed using a TapeStation 4150 instrument (Agilent Technologies, Santa Clara, CA, USA) and Qubit 4.0 fluorometer (Thermo Fisher Scientific). We utilized 0.04–40 ng of DNA for library construction. The DNA was ligated using a Twist MF Library prep Kit (Twist Bioscience, San Francisco, USA) with Illumina adapters and indexed with unique dual indices for duplex sequencing (Illumina, San Diego, CA, USA). The sequencing libraries were hybridized with custom probes targeting 112 genes known to be mutated in lymphomas (Supplemental Data Table S1). Pooled libraries were paired-end sequenced (2 × 150

bp) on the NovaSeq 6000 System (Illumina, San Diego, CA, USA). Single-nucleotide variants (SNVs) and insertions/deletions were called using PiSeq (Dxome, Sungham, Korea) to differentiate low-frequency variants from amplification artifacts and sequencing errors. The analytical sensitivity for SNVs was assumed to be 0.24% [15]. Copy number alterations were identified using ExomeDepth and a custom tool. Variants were visually confirmed using Integrative Genome Viewer (Broad Institute, Cambridge, MA, USA). The variant allele frequency (%) was calculated as the number of sequencing reads of a specific DNA variant divided by the overall coverage at that locus.

We analyzed 17 patients with CNS involvement who had CSF samples available for targeted sequencing (Fig. 1). Fourteen patients had systemic DLBCL with secondary CNS involvement, such as isolated CNS relapse or disease progression including the CNS, whereas three had primary CNS lymphoma (Table 1). However, their CSF cytology showed negative results although

Table 1. Patient clinical characteristics at CSF sampling time and CSF ctDNA detection

Case No.	Age (yrs)	Sex	Diagnosis	Clinical history of CNS involvement	Radiologic CNS involvement pattern	CSF cytology	CSF ctDNA	Survival
1	72	M	DLBCL, ABC type	RCHOP 6 cycles for stage I → Isolated CNS relapse	Leptomeninges	Negative	Not detected-	Dead
2	54	M	DLBCL, ABC type	RCHOP 6 cycles for stage 4 → Isolated CNS relapse	Parenchyma+Leptomeninges	Positive	Detected	Dead
3	41	M	DLBCL, ABC type	RCHOP 6 cycles for stage 1 → Isolated CNS relapse	Leptomeninges	Positive	Detected	Alive
4	34	M	Primary CNS DLBCL	Primary CNS involvement	Parenchyma	Negative	Detected	Alive
5	57	F	DLBCL, ABC type	RCHOP 6 cycles for stage 1 → Isolated CNS relapse	Parenchyma	Positive	Detected	Dead
6	39	F	DLBCL, GC type	RCHOP 6 cycles for stage 4 → Systemic relapse → ICED followed by ASCT → Systemic relapse with CNS involvement	Leptomeninges	Negative	Library construction failed	Dead
7	73	M	DLBCL, GC type	RCHOP 6 cycles for stage 3 → Isolated CNS relapse	Leptomeninges	Positive	Detected	Dead
8	61	M	DLBCL, ABC type	RCHOP 6 cycles for stage 2 → Isolated CNS relapse	Parenchyma	Positive	Not detected	Alive
9	40	M	DLBCL, GC type	RCHOP 3 cycles for stage 4 → Progression with CNS involvement	Leptomeninges	Positive	Detected	Dead
10	72	M	DLBCL, ABC type	RCHOP 6 cycles for stage 4 → Isolated CNS relapse	Leptomeninges	Negative	Detected	Dead
11	52	M	DLBCL, GC type	RCHOP 6 cycles for stage 4 → Systemic relapse with CNS involvement	Parenchyma+Leptomeninges	Negative	Not detected	Dead
12	72	M	Primary CNS DLBCL	Primary CNS involvement	Parenchyma	Negative	Detected	Alive
13	53	M	DLBCL, ABC type	RCHOP 2 cycles for stage 4 → Progression with CNS involvement	Parenchyma	Negative	Detected	Dead
14	73	F	DLBCL, GC type	RCHOP 3 cycles for stage 4 → Isolated CNS relapse	Parenchyma+Leptomeninges	Negative	Detected	Dead
15	71	M	Primary CNS DLBCL	Primary CNS involvement	Parenchyma+Leptomeninges	Negative	Detected	Dead
16	41	F	DLBCL, ABC type	RCHOP 6 cycles for stage 4 → Systemic relapse with CNS involvement	Parenchyma+Leptomeninges	Positive	Detected	Dead
17	68	F	DLBCL, ABC type	RCHOP 6 cycles for stage 4 → Isolated CNS relapse	Parenchyma	Negative	Detected	Dead

Abbreviations: CSF, cerebrospinal fluid; ctDNA; circulating tumor DNA; DLBCL, diffuse large B-cell lymphoma; ABC, activated B-cell like; GC, germinal center; CNS, central nervous system; RCHOP, rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone.

The feasibility of detecting ctDNA in the CSF of patients with large B-cell lymphoma involving the CNS is an area of active re-

A

Gene

2

3

4

5

7

9

10

12

13

14

15

16

17

ARID1A

ATM

B2M

BCL2

BTG1

CARD11

CCND3

CD58

CD70

CITA

CREBBP

DDX3X

DTX1

EBF1

ETV6

EZH2

FAS

FOXO1

GNA13

HIST1H1

ID3

IRF8

KDM6A

KLHL14

KLHL6

KMT2C

KMT2D

KRAS

MGA

MYC

MYD88

NF1

NOTCH1

NOTCH2

PIM1

PRDM1

PTEN

RHOA

SETD1B

SOC31

SPEN

TCF3

TET2

TP53

Amplification

Deletion

Multiple loci mutation

VAF <5%

VAF 5–50%

VAF >50%

B

Gene

No. 9

1st

2nd

3rd

No. 16

1st

2nd

BCL2

BTG1

CCND3

CREBBP

EZH2

GNA13

HIST1H1E

KMT2C

KMT2D

KRAS

MYC

NOTCH1

PTEN

RHOA

TCF3

TET2

C

DNA concentration (ng/ μ L)

Not detected

Detected

Library fail

ctDNA detection

Abbreviations: ctDNA, circulating tumor DNA; CSF, cerebrospinal fluid; CNS, central nervous system.

by deep sequencing (CAPP-Seq) for primary CNS lymphoma or secondary CNS involvement [19]. Despite its effectiveness, CAPP-Seq may not be universally available, making targeted sequencing of a large number of genes a more practical option in some settings. Our variant detection rate aligns with these findings, and our study provides a feasible approach for routine clinical application.

Despite its promising performance in variant detection, CSF ctDNA analysis still has some challenges. One challenge is the low concentration of ctDNA in CSF compared to that in peripheral blood, hampering detection [20]. Additionally, distinguishing tumor-derived DNA from background DNA released from normal cells in the CNS presents a challenge, especially considering the potential for contamination from blood or other sources during CSF collection. Despite these challenges, advancements in NGS techniques have improved the ctDNA detection sensitivity, enabling researchers to detect tumor-specific variants in CSF samples.

Our study demonstrated the feasibility of CSF ctDNA sequencing as a diagnostic tool for detecting CNS involvement in DLBCL despite most patients showing negative CSF cytological results. The development of early detection methods for identifying tumor cells in CSF may significantly impact treatment strategies for patients with systemic DLBCL. This would enable the initiation of targeted therapies for patients who are CSF cytology-negative yet suspected of CNS involvement, potentially altering their disease course. Advancements in CSF-based targeted sequencing may help in predicting the risk of CNS relapse or progression in patients with systemic DLBCL, facilitating the development of preemptive strategies for those at high risk. Given these potential benefits, there is a pressing need for further research to enhance the feasibility and accuracy of CSF targeted sequencing. This may ultimately lead to more precise and effective management of patients with DLBCL with or at risk of CNS involvement.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via <https://doi.org/10.3343/alm.2024.0257>

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AUTHOR CONTRIBUTIONS

Conception and design: Kim SJ; Resources: Yoon SE and Kim WS; Acquisition of data and experiments: Park MR, Park B, Ryu KJ, and Shin S; Analysis and interpretation of data: Kim SJ, Kim JJ, Shin S, and Lee ST; Drafting of manuscript: Kim SJ, Kim JJ, and Shin S. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

None declared.

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REFERENCES

1. Ghose A, Elias HK, Guha G, Yellu M, Kundu R, Latif T. Influence of rituximab on central nervous system relapse in diffuse large B-cell lymphoma and role of prophylaxis—a systematic review of prospective studies. *Clin Lymphoma Myeloma Leuk* 2015;15:451-7.
2. Jeong SY, Yoon SE, Cho D, Kang ES, Cho J, Kim WS, et al. Real-world experiences of CNS-directed chemotherapy followed by autologous stem cell transplantation for secondary CNS involvement in relapsed or refractory diffuse large B-cell lymphoma. *Front Oncol* 2022;12:1071281.
3. Thiel E, Korfel A, Martus P, Kanz L, Griesinger F, Rauch M, et al. High-dose methotrexate with or without whole brain radiotherapy for primary CNS lymphoma (G-PCNSL-SG-1): a phase 3, randomised, non-inferiority trial. *Lancet Oncol* 2010;11:1036-47.
4. Tomita N, Kodama F, Kanamori H, Motomura S, Ishigatsubo Y. Secondary central nervous system lymphoma. *Int J Hematol* 2006;84:128-35.
5. Kim SJ, Oh SY, Kim JS, Kim H, Lee GW, Won JH, et al. Secondary central nervous system (CNS) involvement in patients with diffuse large B-cell lymphoma: a therapeutic dilemma. *Ann Hematol* 2011;90:539-46.
6. Demirci U, Yüksel MK, Kırkızlar HO, Ateşoğlu EB, Mehtap Ö, Salim O, et al. A survey evaluating hematology physicians' perspectives on central nervous system prophylaxis. *Blood Res* 2023;58:99-104.
7. Jen J, Wu L, Sidransky D. An overview on the isolation and analysis of circulating tumor DNA in plasma and serum. *Ann N Y Acad Sci* 2000; 906:8-12.
8. Gerlinger M, Rowan AJ, Horswell S, Math M, Larkin J, Endesfelder D, et al. Intratumor heterogeneity and branched evolution revealed by multi-region sequencing. *N Engl J Med* 2012;366:883-92.
9. Shin SH, Kim YJ, Lee D, Cho D, Ko YH, Cho J, et al. Analysis of circulating tumor DNA by targeted ultra-deep sequencing across various non-Hodgkin lymphoma subtypes. *Leuk Lymphoma* 2019;60:2237-46.
10. Kim JJ, Kim HY, Choi Z, Hwang SY, Jeong H, Choi JR, et al. In-depth circulating tumor DNA sequencing for prognostication and monitoring in natural killer/T-cell lymphomas. *Front Oncol* 2023;13:1109715.
11. Kim SJ, Kim YJ, Yoon SE, Ryu KJ, Park B, Park D, et al. Circulating tumor DNA-based genotyping and monitoring for predicting disease relapses

- of patients with peripheral T-cell lymphomas. *Cancer Res Treat* 2023;55:291-303.
12. Yoon SE, Shin SH, Nam DK, Cho J, Kim WS, Kim SJ. Feasibility of circulating tumor DNA analysis in patients with follicular lymphoma. *Cancer Res Treat* 2024;56:920-35.
 13. Yoon SE, Kim YJ, Shim JH, Park D, Cho J, Ko YH, et al. Plasma circulating tumor DNA in patients with primary central nervous system lymphoma. *Cancer Res Treat* 2022;54:597-612.
 14. Cho J. Basic immunohistochemistry for lymphoma diagnosis. *Blood Res* 2022;57:55-61.
 15. Lee KS, Seo J, Lee CK, Shin S, Choi Z, Min S, et al. Analytical and clinical validation of cell-free circulating tumor DNA assay for the estimation of tumor mutational burden. *Clin Chem* 2022;68:1519-28.
 16. Rimelen V, Ahle G, Pencreach E, Zinniger N, Debliquis A, Zalmal L, et al. Tumor cell-free DNA detection in CSF for primary CNS lymphoma diagnosis. *Acta Neuropathol Commun* 2019;7:43.
 17. Watanabe J, Natsumeda M, Okada M, Kobayashi D, Kanemaru Y, Tsukamoto Y, et al. High detection rate of MYD88 mutations in cerebrospinal fluid from patients with CNS lymphomas. *JCO Precis Oncol* 2019;3:1-13.
 18. Bobillo S, Crespo M, Escudero L, Mayor R, Raheja P, Carpio C, et al. Cell free circulating tumor DNA in cerebrospinal fluid detects and monitors central nervous system involvement of B-cell lymphomas. *Haematologica* 2021;106:513-21.
 19. Mutter JA, Alig SK, Esfahani MS, Lauer EM, Mitschke J, Kurtz DM, et al. Circulating tumor DNA profiling for detection, risk stratification, and classification of brain lymphomas. *J Clin Oncol* 2023;41:1684-94.
 20. Song HH, Park H, Cho D, Bang HI, Oh HJ, Kim J. Optimization of a Protocol for Isolating Cell-free DNA From Cerebrospinal Fluid. *Ann Lab Med* 2024;44:294-8.