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Using cell free DNA (cfDNA) and RNA (cfRNA) in the diagnosis and monitoring of primary and metastatic CNS tumors

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ABSTRACT

Background: Cerebrospinal fluid (CSF) is a relatively accessible biospecimen, and liquid biopsy (LBx) testing using next-generation sequencing (NGS) provides actionable information on genomic abnormalities that can guide clinical decisions and therapeutic selection. To evaluate the potential clinical utility of CSF LBx, we analyzed real-world LB testing data from 1391 CSF samples.

Methods: CSF samples were submitted for clinical testing with various referring diagnoses, including primary brain tumors and metastatic tumors. NGS testing of cell-free DNA and RNA (cfDNA and cfRNA) as well as cellular RNA was performed using a targeted 302-gene DNA panel and a targeted RNA panel encompassing more than 1600 genes.

Results: Of all samples, 231 (16.6%) were completely negative for any abnormality, 69 (5.0%) showed findings consistent with clonal hematopoiesis of indeterminate potential (CHIP), 2 (0.1%) demonstrated B-cell clonality alone, and 14 (1.0%) showed chromosomal abnormalities without identified mutations. The remaining 1075 cases (78.4%) were positive for mutations with or without additional abnormalities, consistent with primary or metastatic malignancy. cfRNA levels were extremely low in the majority of cases, with expression levels near zero across a significant number of genes. Nevertheless, cellular and cfRNA were adequate for the detection of fusion genes in 68 cases (4.9%) and CAR-T constructs in 5 cases (0.4%) of lymphoid neoplasms previously treated with CAR-T therapy, including cases in which CAR-T cells were undetectable in peripheral blood.

Conclusions: These findings suggest that LBx using combined DNA and RNA NGS testing of CSF is a reliable approach that yields potentially practice-changing clinical information.

1. Introduction

Tumor DNA from primary or metastatic central nervous system (CNS) tumors often remain compartmentalized within the CNS because the blood–brain and blood–CSF barriers limiting the value of peripheral blood cell-free DNA (cfDNA) liquid biopsy (LBx) testing [1–7]. In contrast, cerebrospinal fluid (CSF) is in direct anatomic and physiologic contact with brain tissue, ventricular spaces, and leptomeningeal surfaces, enabling CSF cfDNA to more accurately reflect the genomic landscape of intracranial and leptomeningeal lesions [2–8]. Multiple studies have shown that CSF-based profiling can identify actionable alterations, capture spatial and temporal heterogeneity, and support longitudinal monitoring of CNS tumor burden and treatment response [9–11].

Furthermore, in certain circumstances obtaining CNS biopsy may be infeasible or high-risk and conventional CSF cytology has limited sensitivity and has high rate of failure or false negative [4,12]. In this setting, CSF cfDNA analysis has demonstrated diagnostic and clinical utility in both parenchymal brain metastases and leptomeningeal disease (LMD), including improved detection relative to cytology [4,12,13]. However, most of the published data is based on small number of cases for research purpose and there is very little data on real-world

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testing and screening in clinically challenging scenarios, especially when imaging is equivocal, cytology is negative, or biopsy is not readily pursued [6,7].

In our prior study of a limited cohort (n = 125) of patients tested because of suspected metastatic CNS involvement, CSF LBx confirmed metastatic disease in approximately 82% of cases, and tumor-associated findings were also detected in approximately 50% of cases with suspected primary CNS tumors [14]. Importantly, we observed chromosomal abnormalities (copy-number changes and/or large-scale genomic alterations) in approximately 54% of cases, supporting the inclusion of chromosomal aberration assessment as an essential component of CSF molecular analysis rather than restricting evaluation to single-nucleotide variants alone [14,15]. This concept aligns with broader liquid biopsy experience showing that cfDNA is particularly informative for chromosomal gains/losses and genome-wide copy--number changes, while complementary RNA-based approaches can enhance fusion detection [15].

In the current study, we expand upon these observations by reporting real-world results from a substantially larger series of consecutive CSF specimens submitted for liquid biopsy testing to **rule out or confirm** CNS neoplasm in patients with suspected primary or metastatic CNS disease. Using comprehensive profiling that integrates somatic mutations, gene fusions (when available), and chromosomal abnormalities, we aim to more precisely define the diagnostic yield of CSF LBx in the evaluation of suspected CNS malignancy and clarify how genomic findings contribute to clinical confirmation in routine practice.

2. Methods

2.1. Sample collection and study design

Cerebrospinal fluid (CSF) samples were submitted for clinical molecular testing between January 2024 and August 2025. Some of the samples were submitted with a confirmed CNS tumor, but majority were submitted for evaluation of suspected CNS neoplasm based on neurological symptoms, radiographic findings, or other clinical indications. Approximately 5-7 ml CSF fluid collected in clear tube without preservative were required for testing. Samples must be received in the laboratory and processed within 72 h of collection. Cells were isolated by centrifugation at 2000 × g for 10 min at 4°C. cfRNA, cfDNA and cellular RNA were extracted immediately.

Upon receipt, each CSF sample was centrifuged to separate the cellular fraction from the supernatant. The supernatant was used for extraction of cfDNA and cfRNA, while the cell pellet was used for extraction of cellular RNA.

cfDNA was analyzed by next-generation sequencing (NGS) using a targeted panel covering 302 cancer-related genes. Both cfRNA and cellular RNA were independently sequenced using a targeted RNA panel covering 1599 genes, 23 B-cell receptors gene families and 44 T-cell receptors gene families.

All test results were reported to the ordering clinicians and, when appropriate, to patients or their families as part of clinical testing. This retrospective analysis was conducted under an approved Institutional Review Board protocol (WCG IRB #1-1476184-1) and complied with the Declaration of Helsinki and its subsequent amendments.

2.2. cfDNA, cfRNA, and cellular RNA extraction

Cell-free nucleic acids were extracted from CSF supernatant using the Apostle MiniMax High-Efficiency Total Nucleic Acid Isolation Kit (Beckman Coulter, Brea, CA, USA) following the manufacturer's recommended protocol, as previously described [15].

Following extraction, the isolated cell-free total nucleic acid, half was treated with DNase to generate cfRNA and the other half was used for cfDNA analysis.

Cellular RNA (cRNA) was extracted from the cell pellet using the Agencourt FormaPure Total 96-Prep Kit (Beckman Coulter) on an automated KingFisher Flex system, according to the manufacturer's instructions.

2.3. Next-generation sequencing of cfDNA, cfRNA and cRNA

DNA libraries were prepared using the KAPA HyperCap workflow (Roche, Pleasanton, CA, USA). The targeted panel included 302 cancer-related genes relevant to hematologic malignancies and solid tumors. DNA quantification was performed using the Varioskan LUX microplate reader. Library preparation incorporated KAPA universal unique molecular identifier (UMI) adapters with KAPA Unique Dual-Indexed (UDI) Primer Mixes, enabling duplicate removal and sequencing error correction. Following library amplification and purification, target enrichment was performed using KAPA HyperChoice MAX custom probes together with the HyperCapture reagent kit. The final enriched libraries were quantified and sequenced on an Illumina NovaSeq 6000 system using 150 × 2 paired-end cycles. The average sequencing depth ranged between 25,000× and 30,000×.

Whenever possible, 50–100 ng of cfDNA was used for library preparation; however, lower input quantities were accepted in samples with limited cfDNA yield.

Sequencing data were processed using the DRAGEN v3.10.8 Somatic DNA-Seq pipeline with UMI analysis. Variant call format (VCF) files were subsequently annotated and analyzed through a rigorous review process that included manual inspection of BAM files for all reported variants.

Chromosomal copy-number alterations were evaluated using CNVkit, which estimates copy number changes by comparing binned read depths in both on-target and off-target regions against a pooled normal reference dataset.

Both cfRNA and cellular RNA (cRNA) libraries were prepared using a hybrid capture-based targeted RNA sequencing approach. The RNA panel included 1599 genes, 23 B-cell receptors gene families and 44 T-cell receptors gene families. Sequencing was performed on an Illumina NovaSeq 6000 platform. For quality control, RNA sequencing results were accepted only when ≥80 million sequencing reads were generated and the spliced reads were >20%.

3. Results

From January 2024 to August 2025, 1397 consecutive samples were analyzed, of which 6 (0.4%) yielded inadequate cfDNA or cfRNA for analysis and were excluded from further analysis. The remaining 1391 cases demonstrated adequate DNA and RNA results. However, the majority (approximately 90%) of cfRNA cases showed extremely low RNA levels for most sequenced genes, with values of zero. In contrast, cRNA was adequate in almost all analyzed cases.

Table 1
Testing results: Molecular finding and their prevalence in the tested samples.

	Number	Percentage
Total samples	1391	100.00%
SNV or CNV abnormality	1075	77.30%
CNV only	14	1.00%
B-cell clonality only	2	0.10%
CHIP only	69	5.00%
Negative for any abnormality	231	16.60%
Positive for CNV	535	38.50%
B-cell clonality	108	7.80%
Fusion genes	68	4.90%
CAR-T cells	5	0.40%
Cases with Germline mutations	445	32.00%
17p(TP53) deletion	167	12.00%
TP53 Mutation + Deletion	96	6.90%
EGFR Amplification	30	2.20%
ERBB2 amplification	31	2.20%

Of the 1391 cases, 16.6% were completely negative for any abnormality (Table 1). Five percent of cases showed low-level mutations in genes considered indicative of CHIP (clonal hematopoiesis of indeterminate potential), and 1% showed B-cell clonality without evidence of mutations or chromosomal abnormalities (Table 1). The submitted diagnosis types for cases positive for tumor are listed in Table 2. The most common specified diagnosis after brain tumor was breast cancer, followed by lung cancer and lymphoma. Among the negative cases, 35% were submitted with a diagnosis related to CNS pathology, including malignant neoplasm of unspecified brain or secondary neoplasm of the central nervous system. Fifteen percent of negative cases were submitted with a diagnosis of lung cancer, 6% with breast cancer, and 4% with lymphoma.

The detected abnormalities encompassed the full spectrum of molecularly specific findings, including single nucleotide variants (SNVs), copy number variations (CNVs), chromosomal translocations, and immunoglobulin clonality.

3.1. Disease specific mutation profiles

We used a DNA panel of 302 genes covering the majority of known cancer driver genes. In addition, mutations were evaluated across the 1600 genes tested by RNA sequencing. Not all mutations detected by DNA sequencing were detected by RNA sequencing and vice versa. When mutations lead to early termination codons, they were frequently absent from RNA sequencing results, likely due to nonsense-mediated mRNA decay (NMD). Conversely, when mutations were detected by RNA sequencing, they were frequently identified at a higher variant allele frequency (VAF) compared to DNA sequencing (Table 3), most likely reflecting preferential expression of the mutant allele due to silencing or imprinting of the wild-type allele.

Overall, 5029 mutations were detected across the analyzed samples. Table 4 shows the most frequently detected mutations in each disease category. As shown, *TP53* mutation is the most commonly detected abnormality across most of the listed neoplasms. Table 5 focuses on targetable mutations and their prevalence across various neoplasms. Germline abnormalities were determined based on VAF between 40% and 50%, especially when the co-detected mutations are significantly lower VAF and there is no evidence of deletion of the second allele by CNV analysis. Clonal hematopoiesis of indeterminate potential (CHIP) was determined based on the VAF and type of mutations (TET2, ASXL2, DNMT3A), clinical information and co-detected mutations. However, when relevant, peripheral blood testing was recommended and tested.

3.2. Chromosomal abnormalities and copy number variations

Chromosomal abnormalities were detected in 38% of all tested samples and in 50% of positive samples. Notably, 1% of all tested cases were positive for chromosomal abnormalities alone without mutations (Table 1). The majority of these cases were breast cancer or glioblastoma samples showing only *EGFR* amplification, likely due to extremely low tumor burden in the CSF sample. Beyond their diagnostic value, chromosomal abnormalities also provided clinically relevant information regarding the presence of monoallelic or biallelic alterations and gene amplification. Fig. 1 illustrates two representative examples: Panel A shows a breast cancer case with chromosomal abnormalities only, and Panel B shows a case with *EGFR* gene amplification and no detectable mutations or other abnormalities.

Table 2
Referring diagnosis and number of detected SNV. The number and percentage of the cancer diagnosis per referring physicians. The number of detected SNV per diagnosis is shown.

Clinical Diagnosis	Number of samples	Number of detected SNV
ALL	14 (1.3%)	14
AML	10 (0.9%)	22
Brain	325 (30.2%)	1885
Breast	138 (12.8%)	965
Carcinoma	62 (5.8%)	225
Colorectal	3 (0.3%)	13
Endometrial/Ovarian	9 (0.8%)	72
Lung	138 (12.8%)	715
Lymphoid	80 (7.4%)	447
Melanoma	6 (0.6%)	74
Neuroendocrine	7 (0.7%)	33
Sarcoma	5 (0.5%)	25
Other	278 (25.9%)	539
Total	1075	5029

Table 3
Example of VAF detected in DNA vs RNA: Significantly higher VAF detected in RNA.

Gene	HGVSc	HGVSp	DNA Alt Variant Freq	RNA Alt Variant Freq
BCL2	NM_000633.2: c.211C > T	NP_000624.2:p. Pro71Ser	20.29	100
BCL2	NM_000633.2: c.569A > T	NP_000624.2:p. Gln190Leu	44.93	100
SOCS1	NM_003745.1: c.192C > G	NP_003736.1:p. Tyr64Ter	82.27	100
SOCS1	NM_003745.1: c.239A > C	NP_003736.1:p. Tyr80Ser	34.39	100
SOCS1	NM_003745.1: c.416G > T	NP_003736.1:p. Gly139Val	51.96	100
SOCS1	NM_003745.1: c.447G > C	NP_003736.1:p. Glu149Asp	35.32	100
STAT3	NM_139276.2: c.1701T > A	NP_644805.1:p. Asn567Lys	32.71	75
HSP90AA1	NM_001017963.2: c.689G > A	NP_001017963.2: p.Gly230Asp	29.76	33.33

Table 4
Most common mutations detected in various neoplasms.

Neoplasm	Gene	Prevalence
ALL	CEBPA	14.3%
AML	NPM1	20.0%
Brain	TP53	16.9%
Breast	TP53	37.0%
Carcinoma	TP53	22.6%
CRC	APC	33.3%
Endommetrial/Ovarian	TP53	55.6%
Lung	TP53	29.7%
Lymphoid	KMT2C	18.8%
Melanoma	BRAF	50.0%
Neoroendocrine	KIT	28.6%
Sarcoma	CBL	40.0%
Other	TP53	22.7%

3.3. B-cell and T-cell clonality in CSF

The demonstration of B- or T-cell clonality in lymphoid neoplasms is critically important and can occasionally be more sensitive than mutation detection alone. Combining clonality assessment with mutation analysis significantly increases the diagnostic sensitivity for lymphoid neoplasms involving the CNS. None of the tested samples showed T-cell clonality, despite the presence of relatively high levels of T-cell markers across all tested samples. B-cell clonality was detected in 7.8% of tested samples (Table 2), and most of these clonal samples showed relatively low B-cell marker expression (CD19, CD20, and CD22 mRNA). In these cases, B-cell lymphoma or leukemia was confirmed only when supported by additional evidence, such as concurrent mutations consistent with a B-cell neoplasm. B-cell clonality as the sole abnormality was detected in 0.1% of cases (Table 1). In the absence of corroborating molecular evidence or prior documentation of the immunoglobulin gene family from

Table 5
Prevalence of targetable mutations detected in CSF samples in various neoplasms.

	PIK3CA	EGFR	BRAF	ESR1	IDH1	IDH2	ERBB2	BRCA1	BRCA2	AKT1
ALL	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
AML	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Brain	5.2%	6.8%	5.2%	3.4%	1.5%	1.2%	0.6%	2.2%	3.1%	1.2%
Breast	19.6%	2.9%	0.7%	11.6%	1.4%	0.7%	18.1%	4.3%	8.0%	4.3%
Carcinoma	4.8%	1.6%	0.0%	0.0%	0.0%	0.0%	9.7%	8.1%	1.6%	0.0%
CRC	0.0%	0.0%	33.3%	0.0%	0.0%	0.0%	0.0%	33.3%	0.0%	0.0%
Endommetrial/Ovarian	11.1%	11.1%	0.0%	11.1%	0.0%	0.0%	0.0%	33.3%	0.0%	0.0%
Lung	5.1%	27.5%	8.7%	0.0%	1.4%	0.7%	7.2%	2.2%	2.9%	0.0%
Lymphoid	1.3%	2.5%	2.5%	1.3%	1.3%	1.3%	0.0%	1.3%	2.5%	0.0%
Melanoma	0.0%	16.7%	50.0%	0.0%	0.0%	0.0%	0.0%	16.7%	0.0%	0.0%
Neoroendocrine	14.3%	0.0%	0.0%	0.0%	0.0%	0.0%	14.3%	0.0%	0.0%	0.0%
Sarcoma	20.0%	0.0%	20.0%	0.0%	0.0%	20.0%	0.0%	0.0%	0.0%	0.0%
Other	6.8%	0.0%	7.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

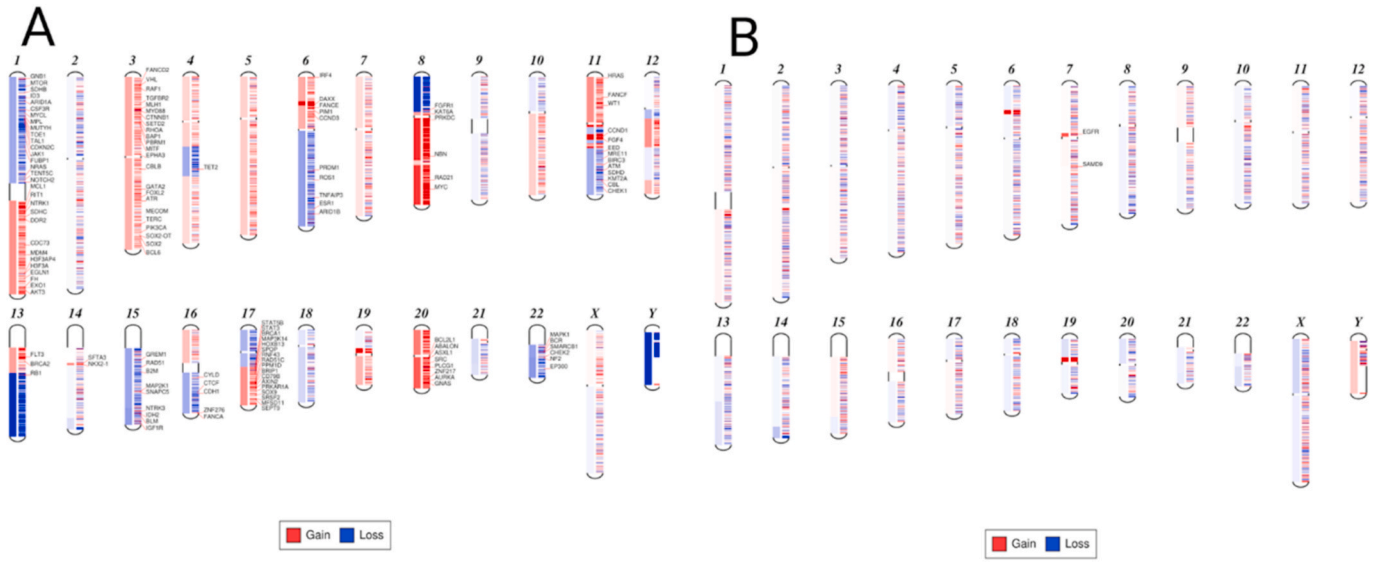

Fig. 1. Detecting chromosomal abnormalities using CSF liquid biopsy. Panel A illustrates the findings in a breast cancer sample with numerous gain, loss and gene amplification. Panel B shows an example of EGFR gene amplification in a patient with glioblastoma.

a diagnostic specimen (clonotype-informed), isolated B-cell clonality without accompanying mutations is considered insufficient to establish a conclusive diagnosis of lymphoma.

3.4. Detection of fusion genes and chromosomal translocations

RNA sequencing enabled the detection of fusion genes and chromosomal translocations. Some fusion genes were clinically relevant for treatment selection, such as *EML4::ALK* and *ESR1::RELA*, while others were diagnostically significant, such as *PAX3::FOXO1*. Similarly, in hematologic neoplasms, fusion gene detection was informative for both diagnosis and treatment decisions, including *BCR::ABL1*, *CBFB::MYH11*, *IGH::BCL2*, *RUNX1::RUNX1T1*, and others.

3.5. Quantitative measurement of CSF tumor load

Variant allele frequency (VAF) reflects the proportion of tumor-derived sequencing reads relative to total reads at a given locus but does not capture absolute tumor burden within the CSF compartment. To quantify the absolute number of mutant DNA molecules in CSF, tumor load was introduced as a metric expressed in molecules per milliliter (molecules/mL). Because a defined and consistent volume of CSF was used for DNA extraction and sequencing across all samples, tumor load was calculated using the following formula:

3.6. Tumor load = (alt read count/cfDNA loaded (ng)) × cfDNA concentration in the eluate (ng/mL)

Tumor load is a more representative and clinically interpretable measure than VAF for longitudinal monitoring of CNS disease burden, as it more accurately reflects both clonal and subclonal abnormalities (Fig. 2), is less susceptible to dilution by non-neoplastic background DNA, and more reliably captures disease trajectory over time (Fig. 3).

3.7. Detection of CAR-T cells

Chimeric antigen receptor T-cell (CAR-T) therapy is currently widely used for the treatment of multiple myeloma and lymphoid neoplasms. One of the major adverse effects of such therapy is immune effector cell-associated neurotoxicity syndrome (ICANS). The neurological symptoms of ICANS may overlap with those of CNS involvement by the underlying hematologic neoplasm, posing a significant diagnostic challenge. RNA sequencing analysis of CSF samples enables detection of the CAR-T construct, allowing distinction between ICANS and true CNS disease. Fig. 4 shows an example of an expressed CAR-T construct identified in a CSF sample, demonstrating *CD8A::CD247* and *CD8A::TNFRSF9* fusion transcripts.

Gene	VAF %	Chr
PIK3CA	59.26	3
CDKN2A	48.12	9
NF1	41.95	17
PAK3	32.02	X
GRIN2A	29.81	16
ESR1	24.41	6
ARID2	22.93	12
KMT2C	12.9	7
AXIN2	11.02	17
BCOR	4.84	X

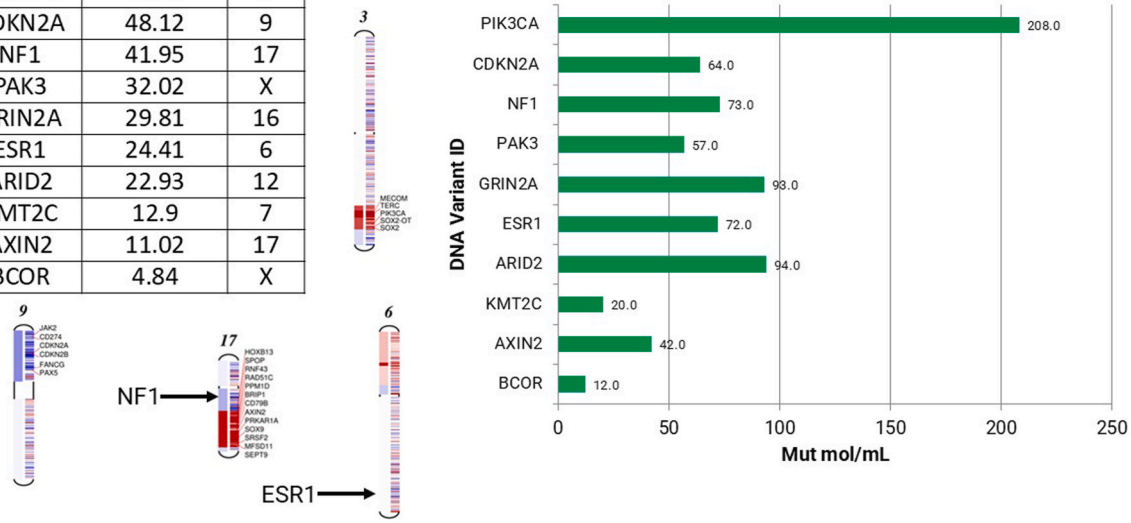

Fig. 2. Illustration of the difference between tumor load and VAF. The CSF sample shown is from a patient with breast cancer harboring the mutations listed in the table, along with their corresponding VAF values. The bars represent tumor load, expressed as the number of mutant molecules per milliliter of CSF. VAF values suggest that the *ESR1* mutation resides in a subclone relative to the clone harboring *PIK3CA*, *CDKN2A*, and *NF1* mutations, with the latter three exhibiting closely similar VAF levels. However, tumor load reveals that *PIK3CA* mutant molecules are present at more than threefold higher levels compared to the other variants, while *CDKN2A*, *NF1*, and *ESR1* mutant molecule counts are equivalent. The disproportionately elevated *PIK3CA* tumor load is attributable to gene amplification, as evidenced by copy number gain on the long arm of chromosome 3. Chromosomal analysis further demonstrates deletion of the second allele on the short arm of chromosome 9 (*CDKN2A*; 9p21.3) and the long arm of chromosome 17 (*NF1*; 17q11.2), with no copy number alteration detected on the long arm of chromosome 6 (*ESR1*; 6q25.1). Collectively, these findings indicate that tumor load more accurately reflects the underlying genomic complexity, and support the interpretation that *PIK3CA*, *CDKN2A*, *NF1*, and *ESR1* mutations co-occur within a single dominant clone — with *ESR1* additionally present or enriched in a subclone.

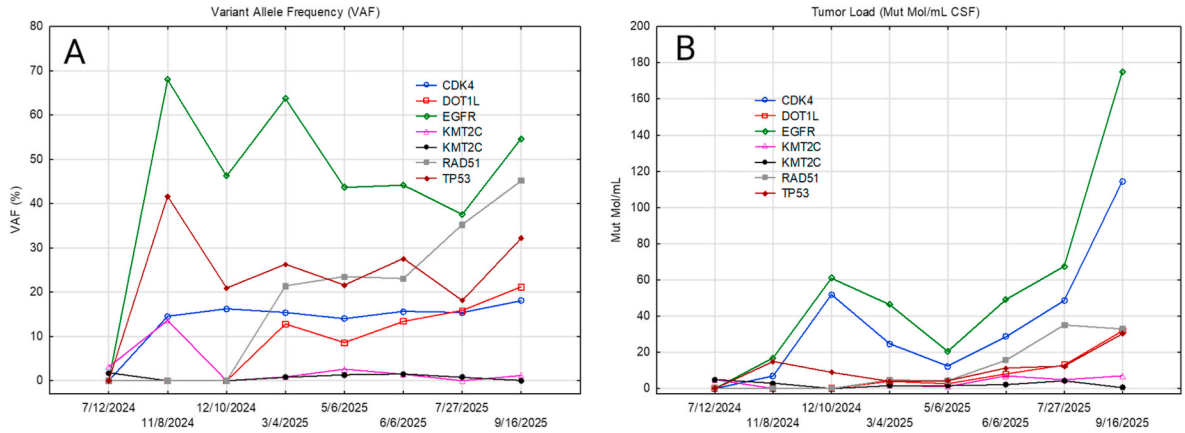

Fig. 3. Longitudinal monitoring of CNS disease burden using VAF versus tumor load in a patient with CNS metastatic lung cancer. Panel A depicts VAF trajectories for detected mutations across sequential CSF samples; Panel B depicts corresponding tumor load values. VAF-based monitoring fails to demonstrate a clear trend in disease burden over time, whereas tumor load plots reveal distinct and interpretable changes in mutant molecule levels, more reliably capturing disease trajectory across the monitoring interval.

4. Discussion

CSF-based liquid biopsy has emerged as one of the most sensitive and diagnostically informative tools for evaluating primary CNS tumors and leptomeningeal or metastatic involvement by systemic cancers. Unlike plasma, CSF directly bathes the leptomeninges and CNS parenchyma, providing a compartment-specific reservoir of tumor-derived DNA, RNA, and cells. CSF liquid biopsy is therefore considered highly reliable for both the diagnosis and longitudinal monitoring of CNS tumors, frequently outperforming conventional cytological examination and flow cytometry.

Here, real-world performance of comprehensive cfDNA and cfRNA evaluation by NGS is described across a large cohort of CSF samples, demonstrating that this approach provides clinically actionable information spanning diagnosis, therapeutic selection, and disease monitoring. CSF cfDNA and cfRNA profiling by NGS enables characterization of chromosomal structural alterations — including gene amplification and copy number loss — chromosomal translocations and fusion genes, expression of surface and cellular markers, somatic mutation profiles, and B- and T-cell receptor clonality.

Among 1391 samples tested, cancer-related markers were identified in 78.4% (77.30% SNV, 1% CNV, and 0.1% Clonality) of cases (Table 1). Fully negative results accounted for 16.6% of cases, while an additional 5.0% demonstrated low-level abnormalities interpreted as clonal hematopoiesis of indeterminate potential (CHIP). Distinguishing CHIP from disease-specific mutations can be challenging. Concurrent

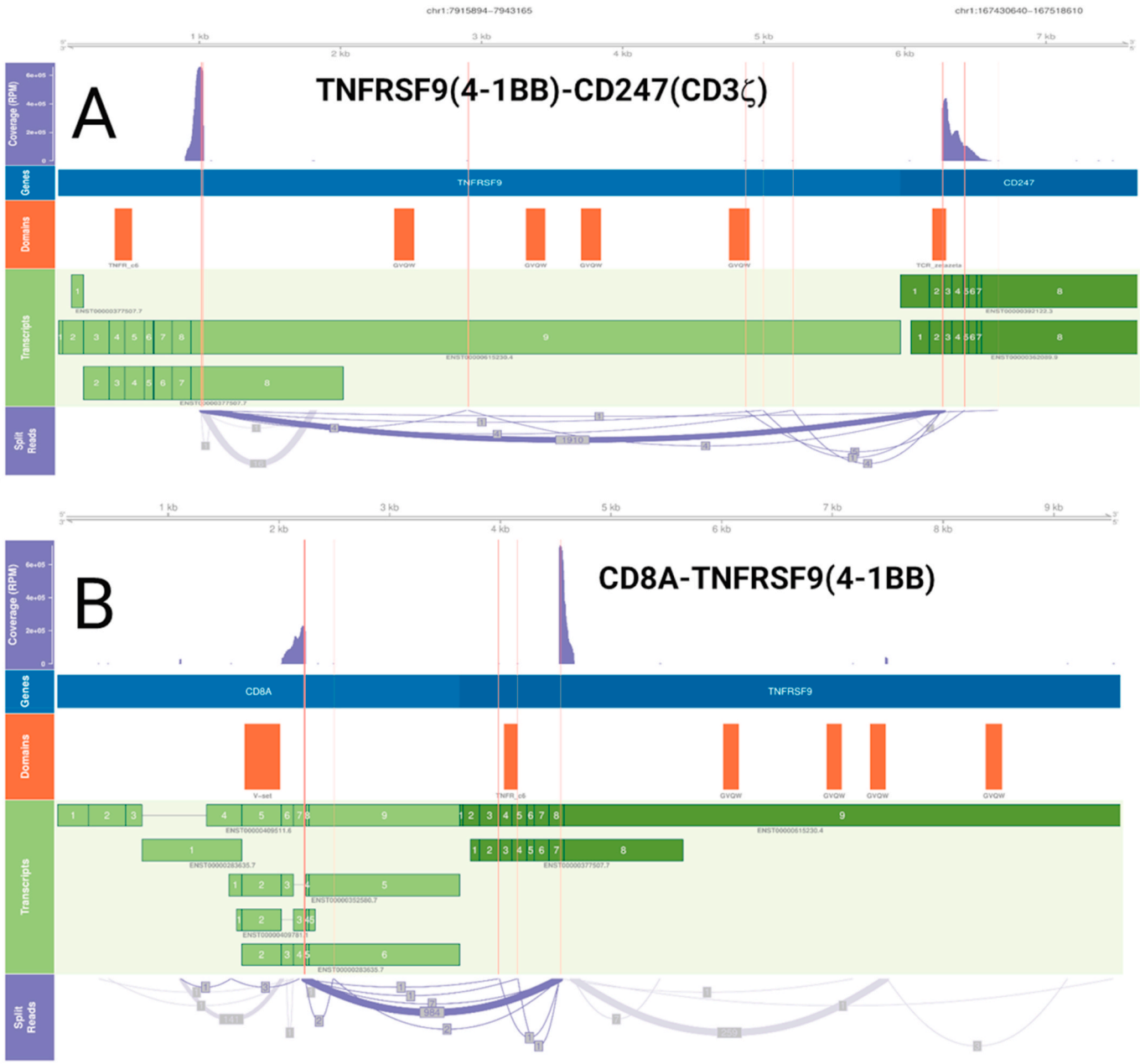

Fig. 4. Detection of expressed CAR-T RNA constructs. Panel A shows the expression of the fusion TNFRSF9(4-1BB)-CD247(CD3ζ) and panel B shows the expression of the fusion CD8A-TNFRSF9(4-1BB).

peripheral blood testing may be required for confirmation, and use of variant databases specific to both hematologic neoplasms and CHIP is essential for accurate interpretation. As with any liquid biopsy platform, negative results do not exclude CNS disease, whereas positive findings are highly informative. B-cell clonality was detected in 7.8% of cases (Table 2); among these, 7.7% harbored concurrent mutations or chromosomal abnormalities corroborating a lymphoma diagnosis. Clonality assessment was performed by RNA sequencing rather than DNA-based methods, on the basis that while each cell carries a single copy of the rearranged B-cell receptor locus, multiple RNA transcripts are generated per cell. Practically, this amplifies signal and enhances detection sensitivity and reliability.

One percent of cases demonstrated chromosomal abnormalities in the absence of detectable mutations, underscoring the importance of routine copy number analysis. Such analysis is critical not only for identifying therapeutically actionable amplifications or deletions, but also for detecting homologous recombination deficiency (HRD). Notably, gene amplification was detectable even in cases with extremely low tumor DNA input, as illustrated by *EGFR* amplification in glioblastoma specimens.

RNA-based sequencing enabled fusion gene detection across any gene partnering with any of the 1600 genes included in the targeted RNA panel. Fusion genes were identified in 4.9% of cases, several of which carry direct diagnostic and therapeutic relevance — including the *ESR1::RELA* fusion, which may arise in breast cancer following hormonal therapy. RNA sequencing further enabled identification of CAR-T construct sequences and the presence of CAR-T cells in CSF. The majority of CAR-T-positive cases demonstrated construct detection in CSF but not in peripheral blood, suggesting preferential homing to the CNS compartment and potentially prolonged CAR-T cell persistence within the CSF.

For longitudinal disease monitoring and assessment of therapeutic

response, quantification of tumor load rather than variant allele frequency (VAF) is essential. VAF reflects the proportion of mutant DNA within a sample and may remain stable or change disproportionately relative to actual tumor burden, as it is susceptible to multiple confounding variables independent of cancer load. These include fluctuations in non-neoplastic background DNA from hematopoietic and other normal cells, as well as copy number gains or losses — which occur frequently under therapeutic selective pressure — that alter the denominator of the VAF calculation. Tumor load is therefore reported as the absolute number of mutant molecules per milliliter of CSF, a metric that directly reflects disease level within the compartment and reliably captures therapeutic efficacy, disease progression, and the emergence of resistance.

5. Conclusions

This data demonstrates that although DNA-based profiling provides mutation and copy number information, RNA evaluation contributes a complementary and indispensable dimension of data critical for the diagnosis and management of primary and metastatic CNS malignancies. The findings presented here illustrate the value of a multi-analyte CSF liquid biopsy strategy integrating cfDNA, cfRNA, and cellular RNA analysis. Clonality assessment, chromosome-level genomic profiling, RNA-based fusion detection, and absolute tumor load quantification together transform CSF from a historically low-yield diagnostic specimen into a high-resolution molecular window into CNS disease biology. As the clinical landscape increasingly demands precision-guided therapeutic selection, monitoring of cellular immunotherapy, and characterization of complex CNS relapse patterns, this layered analytical approach is becoming very important part of CSF evaluation and diagnosis. While the CSF liquid biopsy as presented in this study shows that it can provide highly useful clinical information, we have not correlated with clinical outcome. More clinical studies are needed to decipher how these information can be used clinically and how it is improving patient care. The value of tumor load as a tool for evaluating measurable minimal disease (MRD) needs further clinical validation. Direct correlation of liquid biopsy with tissue biopsy is also needed and may provide information regarding inferiority or perhaps superiority of liquid biopsy.

Ethics declaration

The authors have NOT obtained informed consent from participants or their legal representatives. De-identified samples and data was used. Samples were obtained in the course of routine clinical care.

This study included organ or tissue donors. This study includes human biological material and consent was obtained by donors, or their next of kin or legal representatives, for use in this study and for publication of the article. The samples used in this research were not sourced from executed prisoners or prisoners of conscience.

The study protocol was approved by the Institutional Review Board (IRB) of the Western Copernicus Group (New England IRB, Aspire IRB, and Midlands IRB) (Number 1-1476184-1). The need for informed consent was waived due to the incidental collection and lack of risk. This study was conducted in accordance with the principles of the Declaration of Helsinki and its amendments.

Authorship

MA, AA, AC, and SA collected data, interpreted results and contributed to the writing of the paper. OB, NTG, AG and SK contributed to the concept, provided samples and reviewed paper.

Data availability

Data will be made available upon reasonable request to Malbitar@genomictestingcooperative.com.

Lay summary

Diagnosing brain and spinal cord cancers traditionally relies on examining spinal fluid under a microscope, which frequently misses cancer cells and cannot identifies genetic changes needed to guide treatment. We used a more powerful approach: analyzing tumor DNA and RNA shed into spinal fluid via a routine spinal tap. This "liquid biopsy" can detect cancer mutations, match patients to targeted therapies, and track treatment response. We are reporting the results of the largest real-world study of its kind with 1391 samples — demonstrating that this approach works broadly across patients and cancer types, and should be used a complementary testing to the old spinal fluid microscopy.

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Declaration of competing interest

MA, AC, SA, and AA are employed by a diagnostic company offers this type of testing. MA owns stocks in a diagnostic company offers this type of testing.

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