



Evaluation of the OncoPrint Pan-Cancer Cell-Free Assay for Analyzing Circulating Tumor DNA in the Cerebrospinal Fluid in Patients with Central Nervous System Malignancies

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Available tools to evaluate patients with central nervous system (CNS) tumors such as magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) cytology, and brain biopsies, have significant limitations. MRI and CSF cytology have poor specificity and sensitivity, respectively, and brain biopsies are invasive. Circulating tumor DNA in CSF (CSF-ctDNA) could be used as a biomarker in patients with CNS tumors, but studies in this area are limited. We evaluated four CSF-ctDNA extraction methods and analyzed mutations in CSF-ctDNA with the OncoPrint Pan-Cancer cell-free assay. CSF-ctDNA was extracted from 38 patients with primary or metastatic CNS tumors and 10 patients without CNS malignancy. Commercial ctDNA controls were used for assay evaluation. CSF-ctDNA yields ranged from 3.65 to 3120 ng. Mutations were detected in 39.5% of samples. *TP53* was the most commonly mutated gene and copy number alterations were detected in *CCND1*, *MYC*, and *ERBB2/HER2*. Twenty-five percent of CSF-cytology—negative samples showed mutations in CSF-ctDNA. There was good concordance between mutations in CSF-ctDNA and matching tumors. The QIAamp Circulating Nucleic Acid Kit was the optimal method for extraction of CSF-ctDNA and the OncoPrint cell-free DNA assay is suitable for detection of mutations in CSF-ctDNA. Analysis of CSF-ctDNA is more sensitive than CSF-cytology and has the potential to improve the diagnosis and monitoring of patients with CNS tumors. (*J Mol Diagn* 2021, 23: 171–180; <https://doi.org/10.1016/j.jmoldx.2020.10.013>)

Currently used methodologies to evaluate patients with central nervous system (CNS) tumors include magnetic resonance imaging, detection of malignant cells in cerebrospinal fluid (CSF-cytology), and tissue biopsies/resections, which have several limitations. Magnetic resonance imaging and CSF cytology have poor specificity and sensitivity, respectively, and brain biopsies are invasive. Therefore, there is a critical need for better methodologies to diagnose and evaluate patients with CNS malignancies.

Liquid biopsies consist of analyzing circulating tumor cells or circulating nucleic acids in biofluids. In particular, several studies have addressed the utility of circulating tumor DNA (ctDNA) derived from plasma as a minimally invasive

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method of characterizing tumor mutations.^{1,2} However, studies have shown that plasma is suboptimal for the detection of ctDNA from CNS tumors.^{3,4} In contrast, CSF is a better source of cell-free DNA (cfDNA) to detect mutations derived from CNS tumors because of its proximity to the brain parenchyma.^{5–7} In addition, studies suggest that CSF ctDNA is more sensitive than CSF cytology for the evaluation of patients with CNS tumors.^{8,9} A recent study showed that CSF-ctDNA analysis can help clinical management by identifying actionable alterations and informing therapeutic decisions.¹⁰

Although the presence of ctDNA in CSF has been established previously, it is unclear which methodology is superior for optimal isolation of CSF-ctDNA.^{1,4,9,11,12} Similarly, it is unclear what the optimal sequencing assay is for identifying mutations in CSF-ctDNA. The OncoPrint cell-free assay (Thermo Fisher Scientific, Waltham, MA) is a next-generation sequencing (NGS) panel evaluating alterations in the hotspot region of 52 cancer-associated genes, as well as copy number alterations in 12 genes. The recommended cfDNA input amount for the OncoPrint assay is 20 ng. However, as low as 5 ng of cfDNA may be sufficient for evaluation of ctDNA with this assay (Thermo Fisher Scientific). Prior studies have performed successful NGS analysis of CSF-ctDNA starting with 0.75 to 7 mL of CSF to extract cfDNA with variable yields of approximately 1 to 100 ng.^{8,9,13,14} The objective of this study was to compare various methods for CSF-ctDNA isolation and to evaluate the utility of the OncoPrint Pan-Cancer Cell-Free Assay (OncoPrint) for the detection of CSF-ctDNA mutations.

CSF ctDNA from 38 patients with various types of primary and metastatic CNS tumors was also evaluated. CSF from patients with no history of CNS malignancy was used as control. Four different methods for CSF-ctDNA extraction were evaluated. Commercial cfDNA was used to evaluate the performance of the OncoPrint assay. The sensitivity of CSF-ctDNA analysis with the OncoPrint assay was compared with the results of CSF cytology. In addition, mutations detected in CSF-ctDNA were compared with those present in the corresponding tumor tissue.

Materials and Methods

CSF Samples

Thirty-eight CSF samples (Table 1) obtained from patients with a variety of primary or metastatic CNS tumors were selected for ctDNA analysis. The patient's diagnoses included the following: primary CNS tumors ($n = 10$), metastatic breast cancer ($n = 17$), lung cancer ($n = 8$), ovarian cancer ($n = 1$), ocular melanoma ($n = 1$), and uterine cancer ($n = 1$). CSF samples from patients without a history of CNS malignancy ($n = 10$) were used as controls (Table 1). Eight CSF samples were collected before treatment (Table 1) and the remaining 30 CSF samples were collected during treatment/follow-up evaluation. Patient

ages ranged from 35 to 73 years. There were 27 females and 11 males. Demographic information for the control samples was not available. The study was approved by the Committee for the Protection of Human Subjects from the University of Texas Health Science Center at Houston and MD Anderson Cancer Center (Institutional Review Board PA16-0766, HSC-MS-17-0407).

cfDNA Extraction from CSF

Ten additional CSF samples were selected for cell-free nucleic acid isolation using four different extraction kits [QIAamp Circulating Nucleic Acid (Qiagen, Hilden, Germany), Magmax Cell-free total Nucleic acid (Thermo Fisher Scientific), QIAasymphony DSP Circulating DNA (Qiagen), and Promega Maxwell RSC circulating cell-free DNA (Promega, Madison, WI)] with a starting volume of 1 mL for each kit. Extraction was performed according to the manufacturer's instructions. The final elution volume was 15 μ L. Isolated nucleic acids were evaluated using Qubit (Thermo Fisher Scientific) and Tape Station (Agilent Technologies, Santa Clara, CA) to select the optimal extraction kit. The optimal method (QIAamp Circulating Nucleic Acid; Qiagen) was used to extract cfDNA from all remaining study samples for ctDNA analysis with the OncoPrint assay (Figure 1).

DNA Extraction from Formalin-Fixed, Paraffin-Embedded Tissue and Comparison of Tissue and CSF Results

Corresponding tumor tissue blocks were available for 7 of the 38 patients (patients 13, 14, 17, 18, 22, 31, and 33) with CNS tumors included in the study. Ten 5- μ m slides were used to extract DNA. Hematoxylin and eosin slides were evaluated histologically by board-certified pathologists (L.Y.B. and H.C.), and the tumor-enriched region was marked for microdissection. AllPrep DNA/RNA formalin-fixed, paraffin-embedded (Qiagen) was used to isolate DNA for NGS according to the manufacturer's instructions. Deparaffination was performed using Hemo-De solution (Thermo Fisher Scientific) for 10 minutes, and air drying for 10 to 15 minutes before microdissection. The final elution was performed in 2 steps (25 μ L and 15 μ L). For the remaining 10 patients who were included in the comparison of tissue versus CSF ctDNA, the mutations were obtained from the patient's electronic medical record. Only genes that were examined in both tissue and CSF were included in the concordance analysis.

Targeted NGS Assay

NGS was performed using the OncoPrint Pan-cancer cell-free assay. At least 1.3 ng ctDNA was used for library preparation. Libraries were quantified via Tape station (Agilent), and templating was performed with the Ion 540

Kit-Chef (Thermo Fisher Scientific). Samples were sequenced at high depth on the Ion S5 XL sequencer and analyzed with Torrent Suite Software v.5.2 and Ion Reporter versions 5.6 and 5.10 (Thermo Fisher Scientific) using Human Genome Build 19 as the reference. The workflow for OncoPrint TagSeq Pan-Cancer Liquid Biopsy v2.1 was used with default parameters. OncoPrint variant annotator version 2.4 was used for variant annotation. The mean sequencing depth for all samples was 23,160x. Copy number variation (CNV) sensitivity was set to medium. Some CNVs were identified in control CSF samples without a history of cancer (range, 1.30- to 1.69-fold), these were considered nonspecific alterations. Therefore, a cut-off value of 1.7-fold or higher was set up to report a CNV alteration. Low allele frequency single-nucleotide variants (SNVs) were identified in the CSF from control patients (range, 0.07% to 0.20%). Therefore, a cut-off value of 0.3% was set to report a SNV. The recommended DNA input to achieve 0.1% SNV limit of detection is 20 ng, but not all CSF samples yielded 20 ng (Supplemental Figure S1). The range of cfDNA input for library preparation was 1.3 to 20 ng. The libraries were prepared and sequenced following the manufacturer's instructions.

Digital Droplet PCR

The digital droplet PCR *AKT1 p.E17K* assay (dHsaCP2000031) and *PIK3CA p.H1047R* assay (dHsaCP20000077) were used to confirm a subset of mutations identified with the OncoPrint Pan-cancer cell-free assay. Samples were run in duplicate with positive, negative, and no-template controls. Two microliters of the sample were used for setting up the digital droplet PCR reaction with 2 ng input. Droplets were generated using the Bio-Rad (Hercules, CA) automatic droplet generator, after which PCR amplification was performed according to the manufacturer's instructions. At least 10,000 droplets were required for droplet generation to be considered successful. Droplets were read with the QX200 droplet digital PCR system (Bio-Rad) and analyzed using QuantaSoft software version 1.7 (Bio-Rad).⁹

Results

Comparison of CSF-ctDNA Extraction Methods

To evaluate which commercially available cfDNA extraction kit provides the maximum yield and optimal sample quality, 10 CSF samples were used to extract cfDNA using an initial CSF volume of 1 mL for each kit. Two automated kits [QIASymphony DSP Circulating DNA (Qiagen) and cfDNA extraction Maxwell RSC system (Promega)], and two manual kits [QIAamp Circulating Nucleic Acid (QCNA; Qiagen), Magmax Cell-free Total Nucleic Acid (Thermo Fisher Scientific)] were tested using the same 10 CSF samples. On

average, the Magmax Cell-free Total Nucleic Acid kit gave the lowest yield while the QCNA kit gave the highest yield (Figure 1A). In addition, higher ctDNA concentrations were obtained with the QCNA (Supplemental Table S1). On average, the cfDNA yield from 1 mL CSF was 21.0 ng from the Magmax Cell-free Total Nucleic Acid kit, 47.4 ng from the QCNA kit, 32.0 ng from the cfDNA extraction Maxwell RSC system, and 21.2 ng from QIASymphony DSP Circulating DNA kit. Therefore, the QCNA kit was selected to extract patient samples because it produced a higher ctDNA yield. CSF ctDNA for the 38 study samples was extracted using the QCNA kit. The volume of CSF used for extraction ranged from 0.7 to 4.9 mL. The yield of cfDNA obtained ranged from 3.65 to 3120 ng. Although not significant, a trend was observed between larger CSF volumes and cfDNA yield (Table 1 and Figure 1B).

Performance Characteristics of the OncoPrint-cfDNA Assay

cfDNA Input for Library Preparation

The recommended amount of ctDNA input for the OncoPrint-cfDNA panel is 20 ng. A Seraseq ctDNA Complete Mutation Mix AF 0.1% (Seracare, Milford, MA) synthetic ctDNA control sample was used to determine if lower input amounts of cfDNA were sufficient to maintain a limit of detection of 0.1% for SNVs as the panel guidelines suggest. The expected mutant allele frequency (MAF) in the commercial control (provided by the manufacturer) was compared using 20 ng, 15 ng, 10 ng, and 5 ng of starting cfDNA. Dilutions were made up by adding nuclease-free water. As the cfDNA input decreased, the number of mutations identified also decreased. At 20 ng of input, 14 of 15 (93.3%) mutations were detected; while at 15 ng, 10 ng, and 5 ng, 8 of 15 (53.3%), 7 of 15 (46.7%), and 2 of 15 (13.3%) mutations were detected, respectively (Figure 2A).

DNA Library Input for NGS

The recommended ctDNA library input for the OncoPrint-cfDNA panel is 100 pmol. The optimal library input for maximum mutation detection was determined using the Seraseq ctDNA Complete Mutation Mix AF 0.1% synthetic ctDNA control. All of the libraries were made using the 20 ng ctDNA input. The expected MAF (provided by the manufacturer) was compared between the different DNA library input amounts (100 pmol, 50 pmol, and 30 pmol). It was observed that as the library input decreased, the number of mutations identified also decreased. At 100 pmol of input, 13 of 15 (86.7%) mutations were identified; while at 50 pmol and 30 pmol, 10 of 15 (66.7%) and 7 of 15 (46.7%) of the mutations were identified, respectively (Figure 2B). Because a reduction in mutation detection is seen at lower library inputs and a lower sample input, 100 pmol library input and 20 ng sample input were considered optimal for the analysis of CSF ctDNA.

Table 1 Characteristics of Patients and Samples

Sample no.	Age, years	Sex	Volume, mL	Total yield, ng	Primary site	CSF cytology	MRI	Parenchymal metastases	LMD	ctDNA genetic alterations (AF% or CNV ratio)
1 [†]	48	F	1.8	9.5	Brain [‡]	Negative	Positive	Yes	No	None detected
2	61	M	3	11.45	Brain [‡]	Negative	Positive	Yes	No	None detected
3 [†]	54	M	2.3	16.5	Brain [‡]	Negative	Positive	Yes	No	None detected
4 [†]	45	M	2.7	113.5	Brain [‡]	N/A	Positive	Yes	No	None detected
5 [†]	69	M	1.5	13.5	Brain [‡]	Negative	Positive	Yes	No	None detected
6	72	F	2.4	11.9	Brain [‡]	Negative	Negative	No	No	None detected
7	53	F	3	14.85	Brain [§]	Positive	Positive	Yes	Yes	None detected
8 [†]	44	M	1	11.25	Brain [¶]	Negative	Positive	Yes	No	None detected
9	24	M	1.3	9	Brain	Negative	Positive	Yes	No	None detected
10	19	M	2.5	110	Brain [¶]	Negative	Positive	Yes	No	None detected
11	73	F	2.5	12.3	Breast	Positive	Positive	No	Yes	<i>PIK3CA</i> p.H1047R (15.2%) <i>FGFR3</i> p.F384L (31.8%) <i>FGFR2</i> p.Y375C (6.8%)
12	71	F	4.9	8.45	Breast	Positive	Positive	No	Yes	<i>AKT1</i> p.E17K (31.8%)
13	52	F	N/A	3.63	Breast	Negative	Negative	No	No	None detected
14	54	F	3	4.35	Breast	Negative	Positive	Yes	Yes	<i>FBXW7</i> p.R505C (7.9%)
15	35	F	1.1	16.6	Breast	Positive	Positive	No	No	None detected
16	59	F	1	22.8	Breast	Negative	Positive	No	Yes	<i>ERBB2</i> ↑ (2.88)
17	43	F	N/A	38.45	Breast	Positive	Positive	No	Yes	<i>ESR1</i> p.D538G (78.5%) <i>MAP2K1</i> p.F129L (0.38%) <i>MYC</i> ↑ (3.9) <i>CCND1</i> ↑ (5.8)
18	54	F	0.7	33	Breast	Negative	Positive	Yes	Yes	None detected
19	71	F	2	4.4	Breast	Positive	Positive	No	Yes	<i>AKT1</i> p.E17K (15.4%)
20	71	F	3	8.4	Breast	Negative	Positive	Yes	Yes	None detected
21	59	F	3.6	42.25	Breast	Negative	Positive	No	Yes	None detected
22	44	F	4	24.6	Breast	Negative	Positive	N/A	N/A	<i>TP53</i> p.V157F (2.8%) <i>MYC</i> ↑ (1.8)
23	54	F	4	102	Breast	Negative	Positive	Yes	Yes	<i>TP53</i> p.H193R (75.7%) <i>ERBB2</i> ↑ (11.61)
24	47	F	3	19.9	Breast	Positive	Positive	Yes	Yes	<i>TP53</i> p.R196* (50.5%)
25	42	F	2.7	5.6	Breast	Atypical	Negative	No	Yes	None detected
26	71	F	4	48.25	Breast	Positive	Positive	No	Yes	<i>AKT1</i> p.E17K (27.7%) <i>CCND1</i> ↑ (1.96)
27	51	F	4	73.5	Breast	Positive	Positive	No	Yes	<i>PIK3CA</i> p.H1047R (24.0%)
28	54	F	2.8	7.25	Lung	Negative	Positive	Yes	Yes	<i>EGFR</i> p.S768I (14.5%) <i>EGFR</i> p.L858R (11.9%)
29	66	M	4	88	Lung	Negative	Negative	No	No	None detected
30	56	F	2	9.85	Lung	Negative	Positive	No	Yes	None detected
31 [†]	54	M	3	62.5	Lung	N/A	Positive	Yes	Yes	None detected
32 [†]	65	M	2.4	421.5	Lung	Negative	Positive	Yes	No	<i>TP53</i> p.G245C (21.6%)
33 [†]	58	F	2.1	3120	Lung	N/A	Positive	Yes	No	<i>TP53</i> p.C176R (73.1%)
34	65	M	3	36.4	Lung	Negative	Positive	Yes	No	None detected
35	53	F	3	10.95	Lung	Negative	Positive	Yes	No	None detected
36	47	F	3.1	104	Melanoma	Negative	Positive	No	No	None detected
37	64	F	3	4.95	Ovary	Negative	Positive	Yes	Yes	None detected
38	66	F	2.8	25	Uterine	Positive	Positive	Yes	Yes	<i>TP53</i> p.V272M (1.4%)
Sample no.			Volume, mL	Total yield, ng		Diagnosis		Parenchymal metastases	LMD	
C1			1.43	71.5		Hydrocephalus		No	No	
C2			1.55	77.5		Hydrocephalus		No	No	
C3			1.73	86.5		Hydrocephalus		No	No	
C4			0.516	25.8		Hydrocephalus		No	No	
C5			0.343	17.15		Hydrocephalus		No	No	

(table continues)

Table 1 (continued)

Sample no.	Volume, mL	Total yield, ng	Diagnosis	Parenchymal metastases	LMD
C6	0.736	36.8	Hydrocephalus	No	No
C7	1.19	59.5	Hydrocephalus	No	No
C8	5.3	265	Chiari malformation	No	No
C9	1.64	82	Chiari malformation	No	No
C10	3.16	158	Hydrocephalus	No	No

[†]Sample was collected before treatment.

[‡]Six glioblastomas.

[§]Two ependymomas/grade II.

[¶]One supratentorial primitive neuroectodermal tumor.

^{||}One subependymal giant cell astrocytoma/grade I.

↑, gene amplification; MAF%, Mutant Allele Frequency; CNV ratio, copy number ratio; CSF, cerebrospinal fluid; LMD, leptomeningeal disease; MRI, magnetic resonance imaging; N/A, cannot be evaluated.

cfDNA Dilution Study

The indicated limit of detection for MAF for the Oncomine-cfDNA panel is 0.1% with 20 ng input and 100 pMol library input. To test this we used the Seraseq ctDNA Reference Material v2 AF1% (Seracare) synthetic ctDNA control. Serial dilutions (1, 1:2, 1:4, and 1:8) were made using Seraseq ctDNA Reference Material. The dilutions were performed in duplicate and libraries were made and sequenced using the Oncomine-cfDNA panel with 20 ng sample input and 100 pMol library input. The expected allele frequency (provided by the manufacturer) was compared among the various dilutions: 1%, 0.5%, 0.25%, and 0.125%. It was observed that 23 of 24 (95.8%) mutations were detected in all of the diluted samples. All of the duplicates showed the same mutations with comparable MAF (Figure 2C).

Analysis of CSF cfDNA in Control Samples

NGS was performed using CSF-cfDNA obtained from patients with no history of CNS malignancy ($n = 10$). Low allele frequency SNVs (0.07% to 0.21%) and low-level amplifications (1.3- to 1.65-fold) were identified in 4 of the 10 control samples (Supplemental Table S2). Therefore, a cut-off value of 0.25% allele frequency for SNVs and 1.7-fold for CNVs were used as part of the criteria to report alterations in CSF ctDNA from patients with a history of CNS cancer.

Analysis of CSF ctDNA from Patients with CNS Malignancies

Circulating ctDNA from CSF samples was extracted using the QCNA extraction kit. Ten samples from patients without a history of cancer were used as controls; no mutations were detected in the CSF of control patients. The Oncomine-cfDNA panel was used to analyze CSF ctDNA and at least one genetic alteration was identified in 15 of 38 (39.5%) samples. Overall, 18 SNVs involving eight genes were identified. *TP53* was the most commonly mutated gene

observed in six patients, and six CSF samples showed the presence of copy number alterations in *CCND1*, *MYC*, and *ERBB2* (Table 1). Of the 17 CSF samples derived from patients with metastatic breast cancer, 11 samples showed at least one genetic alteration (11 of 17; 64.7%). Thirteen SNVs were found with common mutations in *TP53*, *AKT1*, and *PIK3CA*. Three of eight (37.5%) CSF samples derived from patients with metastatic lung cancer had at least one genetic mutation. Four SNVs were identified in *TP53* and *EGFR* (Table 1). Ten CSF samples derived from patients with primary CNS tumors were examined but no mutations were identified (0 of 10). A *TP53* mutation was identified in the patient with metastatic uterine carcinoma. No mutations were identified in 23 of 38 CSF samples (Table 1).

To confirm some of the results obtained with the Oncomine-cfDNA assay, five CSF samples, three with *AKT1 p.E17K* and two with *PIK3CA p.H1047R* mutations, also were analyzed using digital droplet PCR. All mutations (5 of 5) also were identified by digital droplet PCR, with comparable MAF (Figure 3).

Because the mean sequencing depth can influence the detection of variants with low allele frequency, the relationship between sequencing depth and mutation detection in the samples from patients with a magnetic resonance image positive for malignancy at the time of CSF collection was evaluated. A mutation was detected in CSF ctDNA in 26.3% of samples with a mean sequencing depth of $<10,000\times$. In contrast, mutations were detected in 52.6% of samples with a mean sequencing depth of $>10,000\times$. A relationship between mean sequencing depth and CSF volume was not observed. However, as expected, a correlation was observed between cfDNA input and mean sequencing depth, with an average depth of $38,321\times$ for samples with 20 ng cfDNA input and $18,132\times$ for samples with <20 ng cfDNA input.

Copy Number Variants

Six CNVs were identified in CSF-ctDNA from five samples with a ratio greater than 1.7-fold. The five samples were

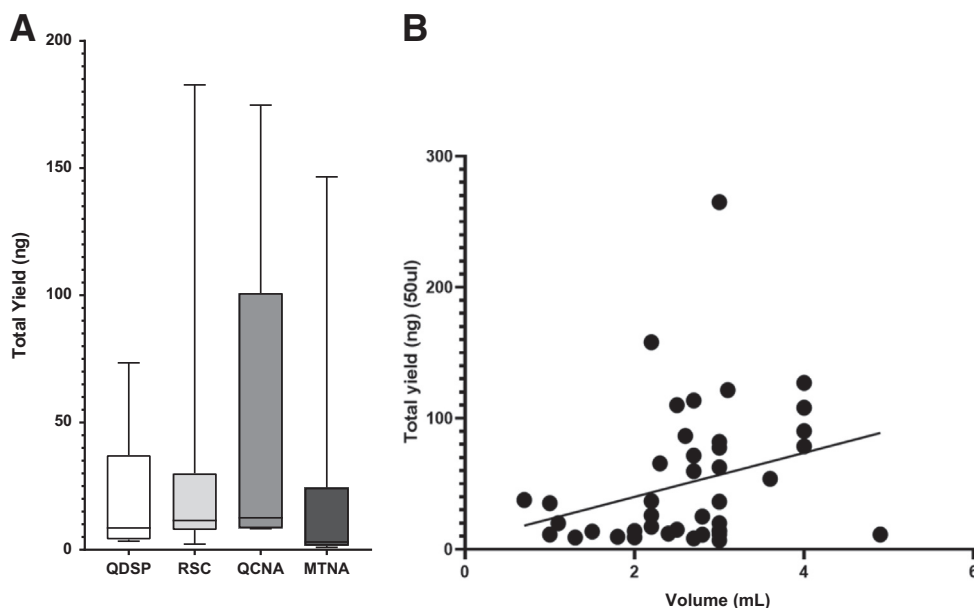


Figure 1 Comparison of DNA extraction kits. **A:** Bar graphs showing total yield (in nanograms) of cell-free DNA extracted from the same 10 samples using four different extraction kits. Yield (in nanograms) ranged from 0.95 to 182.70. The highest yield was obtained with the QIAmp Circulating Nucleic Acid kit (QCNA). **B:** A trend was observed when comparing the volume of CSF versus the total yield of circulating tumor DNA ($P = 0.06$; $r^2 = 0.08$). Samples 32 and 33 (outliers) were not considered for this analysis. MTNA, Magmax Cell-free Total Nucleic Acid; QDSP, QIASymphony DSP Circulating DNA; RSC, cell-free DNA extraction Maxwell RSC system.

from patients with metastatic breast cancer. The variants included two *MYC* amplifications, two *CCND1* amplifications, and two *ERBB2* amplifications (Table 1).

Concordance between Mutations in CSF ctDNA and Tumor Tissue

Of the 15 patients in whom mutations were detected in CSF-ctDNA, 8 patients also had the results of sequencing analysis from tumor tissue available in their electronic medical record and an Oncomine-cfDNA panel. A comparison of the mutations identified in tumor tissue with the mutations identified in CSF-ctDNA showed partial concordance (Table 2 and Supplemental Table S3). Complete concordance was seen in two patients, and six patients showed partial concordance between mutations identified in tumor tissue and CSF-ctDNA. Sixty-seven percent (10 of 15) of the mutations detected in tissue also were seen in CSF-ctDNA; although 33% (5 of 15) of the mutations detected in CSF-ctDNA were not detected in tumor tissue. Sample 28 had three *EGFR* mutations identified in tissue whereas two of three mutations also were identified in CSF-ctDNA analysis (Table 2). Copy number variants were detected in four samples, a total of five CNVs were identified with a ratio above the established cut-off value of 1.7-fold. Three CNVs were identified in three tissue samples, which also were detected in CSF. Two CNVs were detected in CSF ctDNA but not reported in the tumor tissue.

Comparison of CSF-ctDNA Analysis with CSF Cytology

To compare the sensitivity of the Oncomine-cfDNA assay with the results of CSF cytology two \times two contingency table analysis was used. Three of the 38 CSF samples had no CSF cytology results available and were excluded from the analysis (Table 1). Mutations in CSF-ctDNA were detected in all but two (samples 7 and 15) of the CSF samples with a positive CSF cytology result (8 of 10; 80%). In addition, mutations in CSF-ctDNA also were detected in 6 of 24 (25%) CSF samples with a negative CSF cytology result (Figure 4).

Discussion

Four different cfDNA extraction methods were compared using CSF samples (two automated and two manual methods). In our opinion, the QCNA extraction method provided the best overall performance in terms of yield and DNA integrity. One limitation of this extraction method was that it is a manual method with less throughput capacity than the automated methods. Although the Magmax Cell-free Total Nucleic Acid kit is recommended by the manufacturer for use with the Oncomine-cfDNA assay, we found the performance of this method for CSF-ctDNA extraction to be less optimal than the QCNA.

It is apparent from our results that the volume of the initial CSF plays an important role in the cfDNA yield. The CSF volume used in our study ranged from 0.7 to 4.9 mL

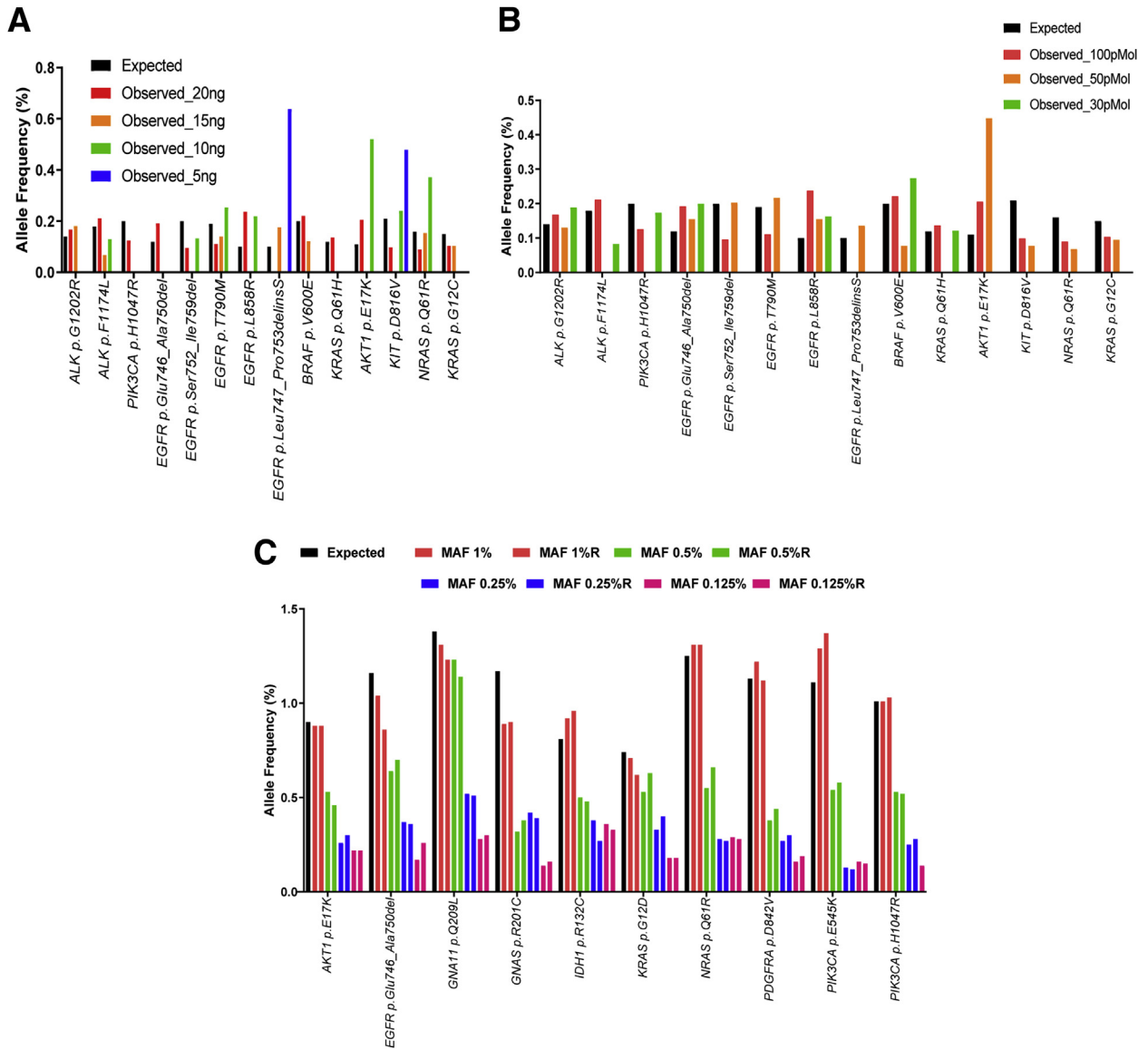


Figure 2 Performance of the Oncomine cell-free DNA assay. **A:** The commercially available Seraseq circulating tumor DNA (ctDNA) CompleteMutation Mix AF0.1% was used in varying amounts (20, 15, 10, and 5 ng) for library preparation. The mutant allele frequency (MAF) (%) for the various mutations detected is depicted in the y axis. At 20 ng of input, 14 of 15 (93.3%) mutations were detected; while at 15 ng, 10 ng, and 5 ng, 8 of 15 (53.3%), 7 of 15 (46.7%), and 2 of 15 (13.3%) mutations were detected, respectively. **B:** MAF (%) of variants detected in Seraseq ctDNA Complete Mutation Mix AF0.1% with varying library inputs of 100 pMol, 50 pMol, and 30 pMol. A starting amount of 20 ng was used for library preparation and various amounts of library input were used for sequencing. At 100 pmol of input, 13 of 15 (86.7%) mutations were identified; while at 50 pmol and 30 pmol, 10 of 15 (66.7%) and 7 of 15 (46.7%) of the mutations were identified, respectively. **C:** Seraseq ctDNA Reference Material v2 AF1% was serially diluted (1%, 0.5%, 0.25%, and 0.125%). The experiment was performed in duplicate. Twenty nanograms of ctDNA and 100 pmol of library were used for sequencing. The MAF of the variants detected is shown in the y axis. Ten mutations were detected for all dilutions. *APC p.1450**, *CTNNB1 p.T41A*, *EGFR p.T790M*, *EGFR p.L858R*, *ERBB2 p.Glu770_Ala771 ins AlaTyrValMet*, *FLT3 p.D835Y*, *GNAQ p.Q209P*, *KIT p.D816V*, *RET p.M918T*, *TP53 p.R248Q*, *TP53 p.R175H*, and *TP53 p.R273H* were missed at 0.125% dilution, and *BRAF p.V600E* was missed at 0.25% and 0.125% dilutions. This can be attributed to having an allele frequency lower than the limit of detection. *TP53 p.S90fs* was not detected because it is not covered by the panel.

and cfDNA yields ranged from 3.65 to 3120 ng. Seventy-six percent (76%) of samples yielded cfDNA amounts below the 20 ng recommended as input for the Oncomine-cfDNA assay (Supplemental Figure S1). Our experiments show that input amounts below 20 ng affect the ability of this assay to detect mutations in CSF-ctDNA and thus will affect assay sensitivity. It is possible that larger CSF volumes will

increase the ability of the assay to detect mutations in CSF-ctDNA.

The performance characteristics of the Oncomine pan-cancer cell-free assay for the detection of genomic alterations in ctDNA derived from the CSF of patients with CNS malignancies was analyzed. CSF-ctDNA from 38 patients with various types of primary CNS tumors, metastatic CNS

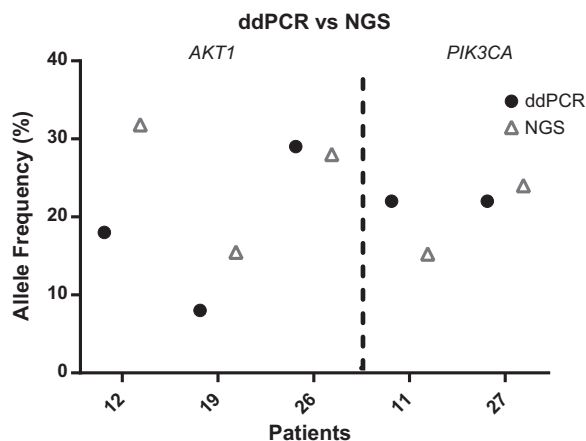


Figure 3 Orthogonal confirmation by digital droplet PCR (ddPCR). Five CSF samples with mutations in *PIK3CA* and *AKT1* detected by next-generation sequencing (NGS) were analyzed by ddPCR with similar results.

tumors, and controls (patients without a history of CNS malignancy) were evaluated. Overall, mutations in CSF-ctDNA were identified in 15 of 38 CSF samples from patients with CNS tumors, consistent with prior results showing that mutations in CSF-ctDNA can be identified in approximately 40% to 60% of samples.^{8,13} Variants were

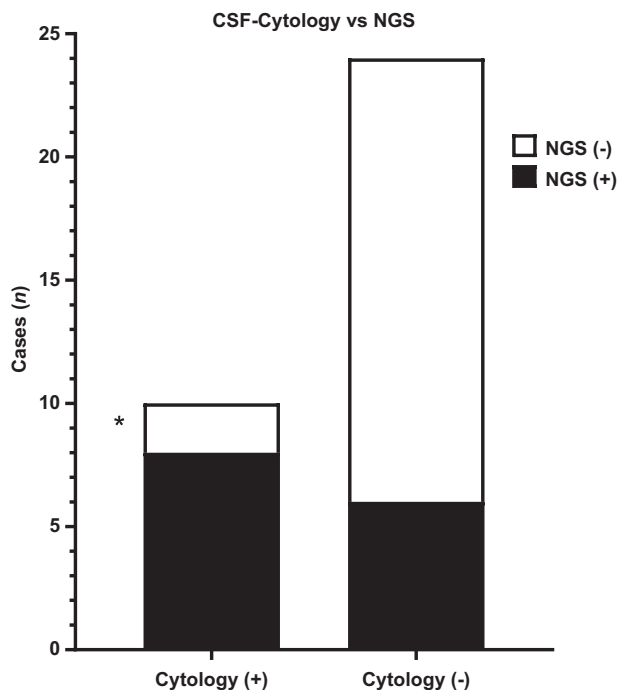


Figure 4 Comparison of cerebrospinal fluid (CSF) cytology and CSF circulating tumor DNA (ctDNA) analysis. Mutations were identified in 8 of 10 (80%) samples with a positive CSF cytology result. In addition, mutations in CSF-ctDNA were identified in 6 of 24 (25%) CSF samples with a negative CSF cytology result. Asterisk indicates low input.

Table 2 Comparison of Genomic Alterations Detected in Tissue and CSF ctDNA

Primary Tumor ID	Primary Tissue	Tissue analyzed	Tissue alterations	CSF ctDNA alterations
14*	Breast	Breast	<i>FBXW7</i> p.R505C <i>CTNNB1</i> p.S45F <i>HRAS</i> p. G12C <i>ERBB2</i> p.R896C	<i>FBXW7</i> p.R505C
16	Breast	Breast	<i>ERBB2</i> ↑	<i>ERBB2</i> ↑
17*	Breast	Pericardium	<i>ESR1</i> p.D538G	<i>ESR1</i> p.D538G <i>MAP2K1</i> p.F129L <i>MYC</i> ↑ <i>CCND1</i> ↑
22*	Lung	Brain	<i>TP53</i> p.V157F <i>MYC</i> ↑	<i>FBXW7</i> p.R505C <i>TP53</i> p.V157F <i>MYC</i> ↑
23	Breast	Breast	<i>ERBB2</i> ↑	<i>ERBB2</i> ↑
27	Breast	Bone marrow	<i>TP53</i> p.P48R	<i>PIK3CA</i> p.H1047R <i>TP53</i> p.P48R
28	Lung	Lung	<i>EGFR</i> p.S768I <i>EGFR</i> p.L858R <i>EGFR</i> p.T790M	<i>EGFR</i> p.S768I <i>EGFR</i> p.L858R
33*	Lung	Brain	<i>KIT</i> p.T500_S501insSA <i>TP53</i> p.C176R	<i>TP53</i> p.C176R

Bold entries in Table indicate genetic variations showing concordance between tissue and CSF ctDNA.

↑, gene amplification.

*Tissue sample sequenced with the OncoPrint PanCancer Assay. *ERBB2* amplification in the tissue was detected by fluorescence *in situ* hybridization.

CSF, cerebrospinal fluid; ctDNA, circulating tumor DNA.

identified in 4 of 10 CSF samples from controls. The majority of the variants in control samples were CNVs (ratio, ≤ 1.65) and mutations with a MAF of 0.21% or less (Supplemental Table S2). These variants could be easily identified and removed with the established cut-off values, therefore, the OncoPrint-cfDNA assay can achieve high specificity. Our results show that the OncoPrint-cfDNA panel can detect alterations successfully (SNV and CNV) in CSF-ctDNA with good specificity. However, the sensitivity of the assay is affected significantly by the low amounts of cfDNA obtained from many CSF samples.

The literature shows that mutations in CSF can be detected in approximately 50% of patients with a CNS tumor.^{13,15} Although there might be some contribution from the tumor's proximity to a CSF space, the reasons for this limitation in sensitivity in previous studies remain unclear. It is possible that assays with the ability to detect mutations with a low MAF could improve the sensitivity of CSF-ctDNA analysis in patients with CNS tumors. The OncoPrint-cfDNA assay detected 3 mutations with a MAF of $< 3\%$ in CSF-ctDNA [*TP53* p.V157F (2.8%), *TP53* p.V272M (1.4%), and *MAP2K1* p.F129L (0.38%)]. It is possible that these mutations would have been missed by less-sensitive NGS assays. Concordant CNVs in CSF and tissue were identified in 3 cases. Although this supports the idea that CNVs can be detected by analysis of CSF-ctDNA, the sample size was small and additional studies are required

to evaluate the utility of the CNV detection in the CSF of patients with primary or metastatic CNS malignancies.

It is apparent from our results that there are differences in the frequency of detecting mutations in CSF-ctDNA from patients with different tumor types. For example, no mutations were detected in CSF samples from patients with primary CNS tumors, whereas mutations were detected in 11 of 17 (64.7%) patients with metastatic breast cancer. This difference likely is multifactorial and is influenced by factors intrinsic to the biology of the primary or metastatic CNS tumors, and by factors intrinsic to the Oncomine-cfDNA assay. For example, one factor limiting the ability of the Oncomine-cfDNA assay to detect mutations in the CSF of patients with primary CNS tumors is that some of the genes frequently mutated in primary CNS tumors such as *TERT* promoter (*TERTp*), histone H3 (*H3F3A*), are not investigated by the assay. In addition to larger CSF volumes, using an NGS assay that investigates mutations in genes frequently altered in primary CNS tumors (such as *TERTp* and *H3F3A*)^{11,12,16} will increase the sensitivity of CSF-ctDNA analysis for patients with primary CNS malignancies. The Oncomine-cfDNA assay is better suited for the detection of genetic alterations in metastatic CNS tumors. This is evident by a detection rate of mutations in 64.7% and 37.5% of CSF samples from patients with metastatic breast cancer and lung cancer, respectively.

The mutations detected in CSF-ctDNA were compared with mutations present in matching tumor tissue from the same patients. Overall, there is relatively good concordance between mutations in CSF-ctDNA and tumor tissue, however, several discrepancies were identified. These discrepancies might reflect heterogeneity in the alterations present in primary lesions/systemic metastatic lesions versus CNS lesions because some of the tumor tissues used for comparison were outside the CNS.^{17,18} However, discrepancies were identified even when CNS tumor tissue was used for comparison, these cases might be owing to intratumor heterogeneity within the CNS tumor tissue or limited sensitivity of the NGS assay, which can be influenced by the amount of ctDNA.¹⁹ It also is possible that the low input of cfDNA used for some cases diminishes the sensitivity of the assay to detect all of the mutations present in CSF-ctDNA. Higher concordance between tissue and CSF-ctDNA is likely to be observed if larger cfDNA input is used for NGS analysis of CSF-ctDNA.

Previous studies have reported good concordance between mutations detected in CSF-ctDNA and tissue from primary or metastatic CNS tumors.^{3,13} In the present study mutations in CSF-ctDNA with a primary CNS tumor were not observed, therefore, only CSF tissue concordance could be examined in the setting of metastatic lesions. Our results show good CSF tissue concordance, which is superior to that reported in recent studies analyzing ctDNA in the plasma of patients with primary CNS tumors (ie, glioblastoma) in which none of the mutations in the tumor tissue were detected in plasma ctDNA.²⁰ These data and existing

literature support the idea that CSF is superior to plasma for the analysis of circulating tumor DNA in patients with CNS malignancies.^{3,13}

The Oncomine assay detected mutations in all patients with positive CSF-cytology with the exception of patient 7 (an ependymoma) and patient 15 (primary breast cancer metastasized to the brain). The lack of mutations detected in this sample could be attributed to a false-negative result of the NGS assay, owing to low cfDNA input amount (<5 ng) or to the absence of mutations in the investigated genes. Alternatively, the possibility of a false-positive CSF-cytology result also should be considered. The genes included in the Oncomine assay usually are not altered in ependymomas and a re-review of the CSF cytology confirmed the presence of tumor cells in this case. Therefore, this was interpreted as a false-negative result of the Oncomine assay, likely owing to inadequate coverage of genes altered in this particular tumor type.

In conclusion, our results show that the QCNA is an optimal method for the extraction of CSF-ctDNA and the Oncomine-cfDNA assay is suitable for the detection of mutations in CSF-ctDNA from patients with the most common metastatic CNS tumors (ie, breast and lung) including SNV and CNV such as *ERBB2* amplification. In addition, our results indicate that the analysis of CSF-ctDNA can be more sensitive than CSF cytology for the evaluation of patients with CNS malignancies and it could be a good complement in the evaluation of CSF from patients with suspected CNS disorders. Our results indicate that larger volumes of CSF to isolate enough amount of cfDNA (≥ 20 ng) are required for optimal assay sensitivity. Use of CSF-cfDNA for mutational analysis is informative and has the potential to improve the care of patients with primary or metastatic CNS tumors. This approach may allow for minimally invasive diagnosis and monitoring of patients by facilitating molecular characterization of tumors without the need for obtaining brain tumor tissue.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.jmoldx.2020.10.013>.

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