Mutation Profiling of cfTNA using Oncomine Pan-Cancer Cell-Free Assay

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Introduction

Liquid biopsy using cfDNA isolated from the plasma fraction of whole blood is less invasive for patients compared to tumor tissue biopsies. Targeted NGS analysis of cfDNA from cancer patients can be valuable in clinical trials for determining the presence of somatic mutations especially when a tumor tissue biopsy is not available. Cell-free RNA (cfRNA) analysis can also be used to detect the presence of gene fusions. The ability to analyze both cfRNA and cfDNA in a single assay provides an opportunity for comprehensive mutation profiling of cancer patients. The Oncomine Pan-Cancer Cell-Free Assay is designed to detect multiple targets in tumor-derived cfTNA (DNA and RNA) isolated from the plasma fraction of whole blood. The assay covers 52 genes, targeting hotspot single nucleotide variants (SNVs), short indels, gene fusions, copy number variations (CNVs), MET exon 14 skipping, and tumor suppressor genes (Table 1). Here we performed a proof of concept study to evaluate this TNA-based NGS assay using cfTNA controls and matched plasma and FFPE samples from late stage cancer patients.

Methods

cfTNA was isolated from the plasma using the MagMAX Cell-Free Total Nucleic Acid Isolation Kit. DNA and RNA from matched FFPE tumor tissue were isolated using the RecoverAll Total Nucleic Acid Isolation Kit. Control samples with 0.1% SNV/indel (positive control 1) and the CNV/Fusion/MET exon skipping (positive control 2) as well as the negative control were provided by the Thermo Fisher. The Structural Multiplex cfDNA Reference Standard and Multiplex I cfDNA Reference Standard Set were purchased from HorizonDx. 20 ng cfDNA or cfTNA was amplified using the Oncomine Pan-Cancer Cell-Free Assay following standard library construction to produce barcoded libraries. Four libraries were multiplexed for templating using standard protocols on the Ion S5 system with the Ion 540 chip. Sequencing data were analyzed with Torrent Suite software version 5.6 and Ion Reporter version 5.6 using Oncomine TagSeq Liquid Biopsy workflow. DNA and RNA from FFPE samples were analyzed using the Oncomine Comprehensive Assay v3 (OCAv3). Plasma and FFPE samples with discordant results between Pan-Cancer Cell-Free Assay and OCAv3 were further confirmed by the Oncomine Breast cfDNA Assay v2 and ddPCR assay by targeting PIK3CA and BRAF variants, respectively.

Results

Assay Performance and Limit of Detection

To validate the assay performance, we first analyzed 2 positive controls from Thermo Fisher, which include 87 SNVs and small indels in 25 genes at ~ 0.1% MAF (positive control 1), CNVs from 2 genes and fusions from 3 genes (positive control 2) (Table 1). The assay detected 76/87 (87%) SNVs from positive control 1. Two of the expected copy number gains were detected (MET and EGFR). All 3 expected gene fusions, i.e. EML4-ALK, SLC34A2-ROS1, CCDC6-RET as well as MET exon 14 skipping were also detected. No mutation was detected in the negative control.

We also challenged the assay using HorizonDx's Structural Multiplex cfDNA Reference Standard. The assay detected the expected copy number gain in MET and MYC as well as the SNVs in low GC and high GC regions. Interestingly, the assay also detected the expected fusion in SLC34A2-ROS1, CCDC6-RET from RNA portion of the assay although this standard is called cfDNA. This is likely caused by the inclusion of RNA in this commercial DNA standard (Table 2). In addition, 8/8 mutations in the HDx cfDNA reference standard were detected by the Oncomine Pan-Cancer Cell-Free Assay at 5%, 1% and 0.1%, except the 0.1% NRAS A59T (Table 3). In most cases, the limit of detection (LoD) was~0.1% MAF as expected (Figure 1) when 20 ng cfDNA was used.

Table 2. Variant Detection in the HorizonDx Structural Multiplex cfDNA Reference Standard

Variant type	Gene	Variant	Expected	MAF (SNV/Indel) Detected or CNV ratio (inferred copy number)	Mutant Molecular Coverage
SNV	EGFR	G719S	5.3%	5.72%	131
SNV	BRAF	V600E	18.2%	16.61%	202
SNV	PIK3CA	H1047R	16.7%	16.81%	419
SNV High GC	GNA11	Q209L	5.6%	5.70%	172
SNV High GC	AKT1	E17K	5.0%	4.77%	116
SNV low GC	PIK3CA	E545K	5.6%	5.39%	136
SNV low GC	KRAS	G13D	5.6%	5.36%	160
Long insertion	EGFR	V769_D770insASV	5.6%	4.70%	112
Long deletion	EGFR	E746_A750del	5.3%	6.01%	123
CNV	MET	AMP	4.5 copies	1.67 (3.34 copies)	NA
CNV	MYC	AMP	9.5 copies	3.19 (6.38 copies)	NA
Fusion	ROS1	SLC34A2(4)-ROS1(32)	5.6%	NA	36
Fusion	RET	CCDC6(1)-RET(12)	5.0%	NA	16

Table 3. SNV Limit of Detection based on HorizonDx's cfDNA Reference Standards

Gene	Variant	Wild Type		5%		1%		0.1%	
		Expected	Detected AF	Expected MAF	Detected MAF (Mutant Molecular Coverage)	Expected MAF	Detected MAF (Mutant Molecular Coverage)	Expected MAF	Detected MAF (Mutant Molecular Coverage)
EGFR	L858R	0.00%	ND	5.00%	3.33% (85)	1.00%	0.70% (15)	0.10%	0.29% (7)
EGFR	ΔE746-A750	0.00%	ND	5.00%	5.65% (143)	1.00%	1.43% (30)	0.10%	0.12% (3)
EGFR	T790M	0.00%	ND	5.00%	3.61% (127)	1.00%	0.87% (28)	0.10%	0.12% (4)
EGFR	V769-D770 insASV	0.00%	ND	5.00%	4.38% (138)	1.00%	0.57% (15)	0.10%	0.11% (3)
KRAS	G12D	0.00%	ND	6.30%	7.17% (202)	1.30%	0.91% (22)	0.13%	0.19% (5)
NRAS	Q61K	0.00%	ND	6.30%	6.97% (137)	1.30%	1.66% (29)	0.13%	ND
NRAS	A59T	0.00%	NC	6.30%	NC	1.30%	NC	0.13%	NC
PIK3CA	E545K	0.00%	ND	6.30%	6.94% (185)	1.30%	1.38% (31)	0.13%	0.09% (2)
ND, Not detected; NC, Not covered by the assay.									

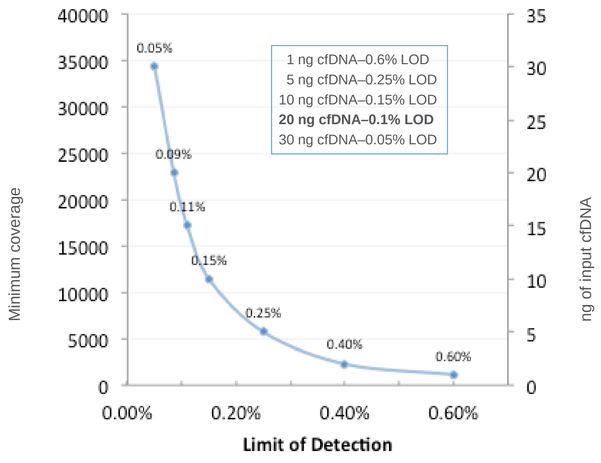

Figure 1. Expected Assay Limit of Detection (From Thermo Fisher)

Table 1. Oncomine Pan-Cancer Cell Free Assay
NOTE: Gene variants in bold are included in positive control 1 and 2

Hotspot												Tumor suppressor			CNV		Fusion	
Genes	Variants	Mut Freq % (mut mol count)	Genes	Variants	Mut Freq %(mut mol count)	Genes	Variants	Mut Freq %(mut mol count)	Genes	Variants	Mut Freq %(mut mol count)	Genes	Variants	Mut Freq %%(mut mol count)	Genes	Detection Copies	Genes	Detection (mut mol count)
AKT1	E17K	0.137 (6)	ESR1			**IDH2**	R172K	0.175 (11)		S566_E571delinsR	0.308 (11)		R805*	0.082	CCND1		**ALK**	ELM4(6)-ALK(20) (12)
ALK	F1174L	0.136 (8)	FGFR1				V530I	0.217 (8)	**PDGFRA**	N659K	0.065 (3)		R876*	0.154 (8)	CCND2		BRAF	
AR				N549K	0.119 (6)	**KIT**	K642E	0.212 (11)		D842V	0.087 (5)		S1234fs	0.130 (5)	CCND3		ERG	
ARAF				C382R	0.178 (8)		V654A	0.212 (11)		N848K	0.105 (6)	**APC**	Q1291*	0.113 (4)	CDK4		ETV1	
	V600E	0.206 (6)	**FGFR2**	Y375C	0.178 (8)		H697Y	0.162 (5)		P539R	0.206 (11)		Q1294*	0.113 (4)	CDK6		FGFR1	
	L597R	0.206 (6)		G305R	0.255 (10)		G12A	2.082	**PIK3CA**	E542K	0.206 (11)		E1353*	0.081 (3)	**EGFR**	3.1	FGFR2	
BRAF	D594G	0.206 (6)		S252W	0.230 (11)	**KRAS**	G12D	0.088 (5)		E545K	0.206 (11)		Q1378*	0.081 (3)	ERBB2		FGFR3	
	N581S	0.206 (6)		S249C	0.268 (11)		K117N	0.088 (5)		H1047R	0.107 (5)		Q1406*	0.100 (5)	FGFR1		**MET**	MET(13)-MET(15) (501)
	G464V	0.114 (6)		G370C	0.132 (5)		Q61H	0.165 (8)	RAF1				R505C	0.075	FGFR2		NTRK1	
CHEK2			**FGFR3**	G380R	0.132 (5)	MAP2K1				C618R	0.071 (4)	**FBXW7**	W526R	0.075 (4)	**FGFR3**	2.74	NTRK3	
	T41A	0.241 (11)		F384L	0.132 (5)	MAP2K2				C620R	0.071 (4)		R479Q	0.066 (4)	**MET**	2.5	**RET**	CCDC6(1)-RET(12) (35)
CTNNB1	S45F	0.241 (11)		A391E	0.132 (5)	**MET**	Y1253D	0.178 (7)	**RET**	E768D	0.150 (8)	PTEN			MYC		**ROS1**	SLC34A2(4)-ROS1(32) (343)
DDR2				G697C	0.273 (12)		M1268T	0.178 (7)		A883F	0.193 (7)	**TP53**	R248L	1.280				SLC34A2(4)-ROS1(34) (24)
	R108K	0.157 (8)	FGFR4			MTOR				M918T	0.366 (14)		P278L	0.067 (2)				
EGFR	A289V	0.151 (8)	**FLT3**	D835Y	0.217 (13)	**NRAS**	Q61R	0.255 (11)	ROS1				R273H	0.067 (2)				
	V292L	0.151 (8)	**GNA11**	Q209L	0.194 (12)		G12D	0.185 (10)	SF3B1				G245S	0.083 (3)				
	p.L755S	0.150 (7)	GNAQ			NTRK1			SMAD4				Y220C	0.112 (6)				
ERBB2	p.D769Y	0.150 (7)	**GNAS**	R201C	0.124 (7)	NTRK3				L412F	0.204 (11)		V216M	0.112 (6)				
	p.Glu770_Ala771insAYVM	0.129 (7)	**HRAS**	Q61R	0.085 (4)				**SMO**	W535L	0.113 (5)							
	p.V842I	0.201 (10)		G12V	0.142 (8)													
ERBB3			**IDH1**	R132H	0.230 (15)													

Assay Reproducibility

The HorizenDx 1% cfDNA Reference Standard was chosen to perform a repeat analysis to study the assay reproducibility (Table 4). The repeat analysis showed good assay reproducibility with similar MAF in each run.

Table 4. Assay Reproducibility

Gene	Variant	Expected	Detected MAF (Mutant Molecular Coverage)	
			A	B
EGFR	L858R	1.00%	0.7% (15)	0.2% (5)
EGFR	ΔE746 - A750	1.00%	1.4% (30)	0.8% (17)
EGFR	T790M	1.00%	0.9% (28)	0.5% (15)
EGFR	V769 - D770insASV	1.00%	0.6% (15)	0.6% (15)
KRAS	G12D	1.30%	0.9% (22)	1.4% (34)
NRAS	Q61K	1.30%	1.7% (29)	1.5% (25)
NRAS	A59T	1.30%	NC	NC
PIK3CA	E545K	1.30%	1.4% (31)	1.6% (34)
NC, Not covered.				

Profiling of Plasma Samples from Cancer Patients

cfTNA extracted from seven patient plasma samples was analyzed by the Oncomine Pan-Cancer Cell-Free Assay. The cfTNA input was based on yield and varied from 8-28ng. All variants detected at >0.1% were reported for the purposes of this pilot study. Plasma cfTNA results were compared to matched FFPE tissue results and/or to cfTNA results using the Oncomine Breast cfDNA Assay or ddPCR assay. No variants were detected in plasma cfTNA using either NGS assay for samples 5 and 6. Three variants that were detected in the plasma cfTNA at >1% MAF were also detected in FFPE tissue (**Bold**, BRAF V600K, NRAS Q61R, TP53 R280G). Three variants (*Italic*) that were detected in the plasma cfTNA at <0.5% MAF were not detected in the matched tissue sample. These variants were also not detected in the cfTNA sample using another NGS assay or ddPCR assay suggesting that they were reported as false positives. The cfTNA input was below 20ng for these samples, demonstrating that the LoD and cut-off for reporting positive mutation results may vary based on cfTNA input.

Table 5. Variants Detected in Plasma Samples from Cancer Patients

Sample	Cancer type	cfTNA input (ng)	Oncomine PanCancer Cell Free Assay		Second assay A, B, or C for plasma and/or FFPE
			Variant	MAF (mut mol count)	
1	Melanoma	8	**BRAF V600K**	11.0% (173)	Confirmed in FFPE by assay A.
			PIK3CA E542K	0.18% (4)	Not detected in FFPE by assay A and B. Not detected in plasma by assay B.
2	Melanoma	10	**NRAS Q61R**	1.5% (24)	Confirmed in FFPE by assay A.
3	Gastric	10	**TP53 R280G**	4.2% (48)	Confirmed in FFPE by assay A.
4	Breast	8	*BRAF V600E*	0.18% (3)	Not detected in FFPE by assay A and C. Not detected in plasma by assay C.
5	CRC	28	No variants detected		No variants detected in plasma by assay B.
6	Breast	27	No variants detected		No variants detected in plasma by assay B.
7	Ovarian	17	*PIK3CA E542K*	0.34% (12)	Not detected in FFPE by assay A and B. Not detected in plasma by assay B.
Assays: A, OCAv3; B, Oncomine Breast cfDNA; C, ddPCR					

Conclusions

- Oncomine Pan-Cancer Cell-Free Assay is able to detect multiple targets of interest in cfTNA including SNVs and indels, CNVs and fusions.
- SNVs and indels at 0.1% allele frequency can be successfully detected with 20 ng of input cfTNA.
- Additional validation is ongoing.

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For Further Information

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