Development of a Breast and Lung Cancer Research Panel To Target Therapeutically Relevant Copy Number and Gene Fusion Variants from Blood

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INTRODUCTION

With recent advances in next-generation sequencing (NGS) technologies, it is now possible to detect somatic mutations with allele frequencies in blood samples as low as 0.1% from circulating tumor DNA. A natural extension to this achievement is adding the ability to simultaneously detect copy number variants and gene fusions. A panel such as this addresses a full repertoire of variant classes found to be linked with certain tumors and would enable researchers additional to profile cancer samples more dynamically thus enriching current diagnostic tool sets. Here, we present progress on such an approach and apply current NGS technology to achieve our goals.

Results

Using control samples, we can reproducibly demonstrate detection of ERBB2 (HER2/neu) and FGFR1 gene amplifications with high statistical significance and as low as a 1.4 fold difference versus non-amplified loci in titration experiments. In addition, the FGFR1 gene amplification was detected in the context of a validated breast cancer somatic mutation panel in which no negative impact was exhibited and mutation detection specificity and sensitivity were both greater 90%. Lastly, we developed an additional panel to detect gene fusions relevant to lung cancer. Using the titration approach above, the EML4-ALK fusion variant was shown to have a limit of detection near 1% with no negative impact on detection sensitivity and sensitivity when combined with the validated lung cfDNA somatic mutation panel with a 0.1% limit of detection.

Conclusion

From the outcomes of these experiments, we have shown the ability to reproducibly and simultaneously detect copy number and gene fusion variants as well as somatic mutations at very low limits of detection in a cell free DNA background derived from blood samples.

MATERIALS AND METHODS

Lung panel (SNV + Fusions)

Plasma preparation:

Blood samples were collected into EDTA (BD) collection tubes following manufacturer's instructions. Plasma was obtained by centrifugation at 1600 x g for 10 min at 4˚C, followed by another spin at 6,000 x g for 30 min at 4˚C to remove any residual blood cells. For the fusion spike in control mix, (EML4-ALK, CCDC6-RET and SLC34A2-ROS1) nucleic acid was extracted from fusion positive cell lines and spiked into nucleic acid extracted from plasma from healthy donors at 1% by mass.

Nucleic Acid preparation:

Plasma: Nucleic acid was isolated from ~4 mL of plasma using a modified internal protocol with MagMAX™ Cell-Free DNA Isolation Kit.

Internal 0.1% SNV Control: AcroMetrix™ Oncology Hotspot Control in background GM24385 genomic DNA diluted to 0.1% allelic frequency. The control and background nucleic acid was fragmented to mimic the size of cfDNA.

In-house trifusion control mix: A control containing nucleic acid pool from fusion positive cell lines (EML4-ALK, CCDC6-RET and SLC34A2-ROS1) spiked into nucleic acid extracted from plasma from healthy donors at 1% by mass.

Breast panel (SNV + CNV)

Nucleic Acid preparation:

ERBB2 genomic DNA standards (SRM2373) component C (NIST) were used to evaluate ERBB2 CNV in our breast panel. DNA was fragmented to mimic cfDNA and calibrated with digital PCR for input amount . Wild type genomic DNA (NA24385,Coriell Biorepositories) was fragmented and used as background for titrations. ERBB2 Copy Number status of titrated DNA was confirmed with digital PCR. 6000 copies of these titrated DNA from each data point (CNV= 1.1~3.5) were used to generate libraries following current manufacture protocol. Sequence data were analyzed using an internal analysis pipeline. Plasma cfDNA from healthy blood donors (n=6) were also evaluated following same protocol and analysis.

To measure FGFR1 amplification, cell free media and cellular DNA extracts were prepared from the FGFR1 amplified cell line MDA-MB-134-VI (ATCC ® HTB-23™). cfDNA was extracted from cell free media using the MagMAX™ Cell-Free DNA Isolation Kit. Using internal cleanup procedures, any contaminant genomic DNA was removed from the cfDNA sample. Genomic DNA from cells was extracted using the PureLink® Genomic DNA kit.

All Panels

Library preparation:

Targeted libraries were prepared using the extended versions of already launched Oncomine™ Lung and Breast cfDNA assay and reagents containing additional Fusion and CNV designs.

Sequencing:

The Ion 520™ and Ion 530™ Kit-Chef were used for template preparation on the Ion Chef™, followed by sequencing on Ion S5™XL system using the Ion 530™ Chip.

Data analysis:

Data analysis was performed using the *variantCaller* plugin in Torrent Suite™ Software for SNV plus additional internally developed software tools for Fusion and CNV analysis.

RESULTS

Figure 1.Oncomine™ cfDNA Tagging Technology for rare mutation detection

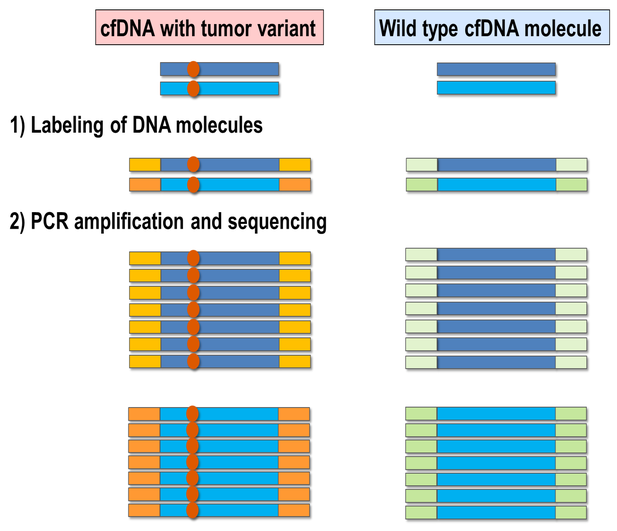

Fig 1. The Oncomine™ cfDNA Assay also applied to CNV and Fusion measurements for this study.

Table 1. Simultaneous Detection of known fusion and rare SNV targets

A.

	Library A1	Library A2
Target ID	0.1% SNV cntrl + 1% Trifusion Mix rep1	0.1% SNV cntrl + 1% Trifusion Mix rep2
SLC34A2-ROS1.S4R32.COSF1196	1	125
CCDC6-RET.C1R12.COSF1271	44	39
EML4-ALK.E6a/bA20	9	1
TBP.ProcessControl	17	22
HMBS.Process Control	28	19

B.

Gene ID	Variant Frequency	AA Change	Ref Allele	Variant allele
NRAS	0.09	p.Q61R	T	C
NRAS	0.09	p.T58T	T	C
ALK	0.08	p.R1275Q	C	T
ALK	0.18	p.F1174L	G	T
EGFR	0.27	p.V765M	G	A
EGFR	0.18	p.L792P	T	C
MET	0.08	p.Y1253D	T	G
KRAS	0.12	p.Q61H	T	G
KRAS	0.12	p.A59T	C	T
KRAS	0.09	p.G12D	C	T
KRAS	0.09	p.V8V	T	C
TP53	0.08	p.P278L	G	A
TP53	0.08	p.R273H	C	T
TP53	0.51	p.S241F	G	A
TP53	0.07	p.T125T	C	T
ERBB2	0.1	p.A775_G776insYVMA	-	ATACGTGATGGC

Table 1. Sequencing libraries prepared by combining the 0.1% SNV positive control + trifusion mix into a total nucleic acid background isolated from plasma from a normal donor. Fusion spike-in constitutes 1% of a 20 ng total input into library preparation. (A) Detection of ALK, RET and ROS1 fusion variants displayed by number of tagged molecules found using an in-house analysis pipeline. HMBS and TBP process control serve as quality control targets. (B) Expected SNVs at 0.1% detection levels found at acceptable levels. (FP=1, FN=1).

Figure 2.Detection of ERBB2 amplification at various spike in concentrations

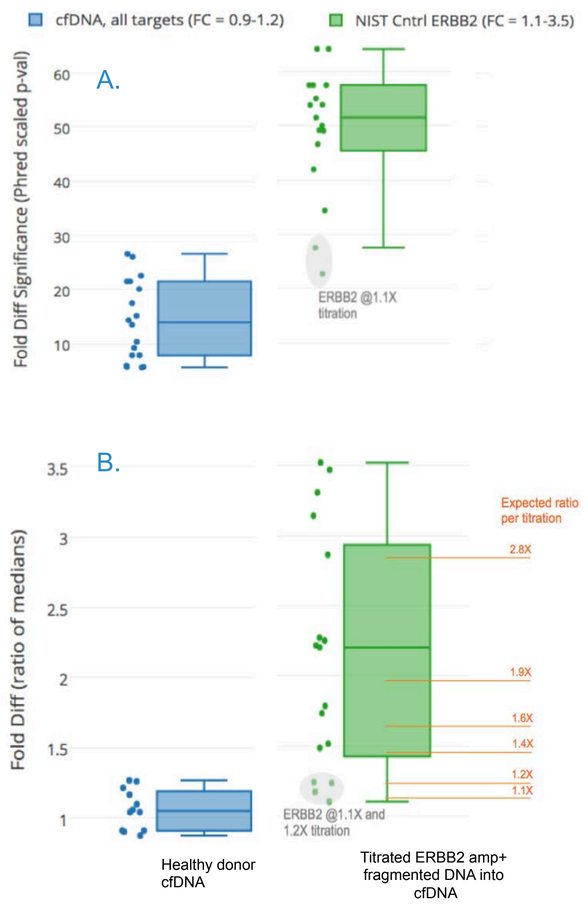

Fig 2. Fragmented NIST CNV control sample containing amplified ERBB2 amplified spiked into a cfDNA background (green boxes) at several titration levels compared to measurements using the same breast cancer panel with plasma derived cfDNA from healthy donors (blue boxes). A) Boxplot shows phred scaled p-values (-10*log10(p-val)) (Wilcox Rank Sum test) for significant fold difference of tagged molecule coverage between reference and ERBB2 amplicon sets in the panel. The same calculation was performed for the cfDNA sample. B) Fold difference of median tagged molecule coverage between reference versus ERBB2 amplicon sets. Observed fold differences closely match expected especially for the lowest titrations.

Figure 3. Tagged molecule coverage by amplicon variant class on breast cancer panel

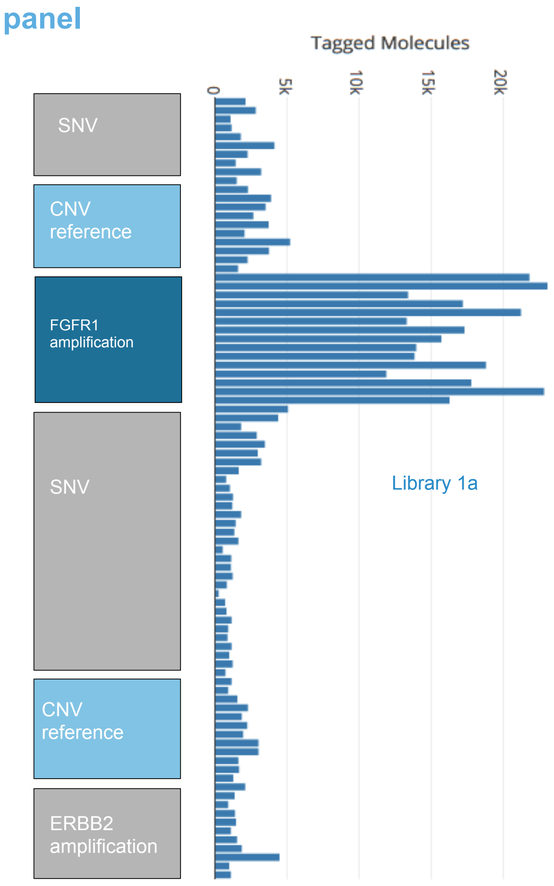

Fig 3. Tagged molecular coverage results from the breast cancer panel run on cfDNA derived from cell free media taken from the cultured HTB-23™ (FGFR1 amplification) cell line. From these results, we observe a 6-8 fold amplification of FGFR1 relative to reverence amplicon in the panel. Internal algorithms stabilize coverage variability across amplicons prior to calculating p-values and observed fold differences.

Table 2. Other known variants detected simultaneously with FGFR1 amplification

		Frequency (%)	
Paired Library Set	Variant	Cultured Cells gDNA	Conditioned Media cfDNA
Replicate 1a	KRAS p.G12R	0.43	1.16
Replicate 1b		0.59	0.87
Replicate 2a	TP53 p.E285K	0.87	0.8
Replicate 2b		1.11	6.68

Table 2. From cultured HTB-23™ cfDNA extracted from cell free media and genomic DNA were extracted from cells. SNVs known to be in this cell line were detected simultaneously with the FGFR1 amplification described in figure 3. Interestingly, in higher variant frequencies are observed in the cfDNA extraction from conditioned media versus genomic DNA extracted from the cells

CONCLUSIONS

- Oncomine™ cfDNA Assays provide an easy, quick, and reliable solution for research in detecting low frequency somatic variants (SNVs) in blood plasma and tissue
- A natural extension to the Oncomine™ cfDNA Assay is the addition of fusion and copy number variant classes to be detected in the same assays.
- Feasibility testing demonstrates simultaneous detection of at known fusion variants (ALK, RET and ROS1 driver genes) in lung cancer at a 1% titration level while maintaining high detection sensitivity for SNVs at minor allele frequencies close to 0.1% using contrived samples.
- In addition, we show that FGFR1 and ERBB2 copy number variants relevant in breast cancer and with known copy number amplification level, can be detected using spike in titrations of fragmented gDNA in cfDNA background as well as from cfDNA isolated from cell free media from cultured cancer cell lines. This extended panel also simultaneously detects known SNVs from these cell lines at low minor allele frequencies.

Please See Poster TS11 for more information about Oncomine™ cfDNA Assays