Development and validation of an accurate exome-scale cfDNA detection platform

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Introduction

Neoantigens are increasingly used as biomarkers for response to checkpoint blockade therapy and as therapeutic targets for neoantigen-based personalized cancer vaccines. Accurate identification of neoantigens requires comprehensive exome and transcriptome sequencing of both a tumor biopsy and a matched normal sample to enable identification of putative neoantigens, which occur across the genome. However, as tumor biopsy samples cannot always be obtained, and because tumor heterogeneity can result in an incomplete set of neoantigens from a single biopsy, we developed our ImmunoID NeXT cfDNA platform. Our aim was two-fold: (1) to identify neoantigens in cell free DNA (cfDNA) as a complement to tumor biopsy derived neoantigens and, (2) to track neoantigens in the cfDNA post immuno-therapy treatment. In contrast to most current tumor cfDNA tests, which are designed to assess a small number of variants or genes (often <100), our ACE ctDNA Exome platform covers ~ 20,000 genes at very high sequencing depth to accurately identify lower allele frequency variants, allowing for the detection of many putative neoantigens that could be missed when employing targeted approaches. In the present study, we assess the limit of detection (LOD) of our ImmunoID NeXT cfDNA platform assay for small nucleotide variants (SNVs) and demonstrate the utility of the assay for both monitoring and *de novo* identification of neoantigens directly from cfDNA.

Methods

Seracare ctDNA reference

We first wanted to estimate our assay's ability to detect clinically relevant SNVs. To accomplish this, we used Seracare ctDNA Standards that harbor 25 SNVs across 21 cancer relevant genes (Table 1) at allele frequencies (AFs) of 2%, 1% and 0.5%. Our assay and analyses do not include any unique molecular identifiers (UMIs) or duplex sequencing steps. We sequenced to an average depth of 1000X across the whole exome. We intersected identified somatic variants with 25 Seracare reference variants to obtain sensitivity at various AFs and input cfDNA amounts (Figure 1).

Allele frequency dilution using normal donor plasma

Seracare reference standards were not expressly designed to be used for specificity analysis, and are composed of a relatively small number of variants (25). To address these issues, we designed a study to generate a larger set of reference SNVs, and to assess specificity and sensitivity of our ImmunoID NeXT cfDNA platform. To do this, we used cfDNA from two healthy donors (D1 & D2) and identified a "gold set" of SNVs by performing somatic variant calling using D1 as tumor and D2 as matched normal. Gold set variants were defined as heterozygous (~90% of identified variants) private germline variants in D1. We then constructed two AF dilutions where varying amounts of cfDNA from D1 were spiked into the background cfDNA from D2 (Figure 2).

SNV detection assessment patient tumor samples

Finally, we obtained tumor (FFPE), matched normal (PBMC) and matched plasma from eight late stage (stage III/IV) colorectal cancer (CRC) cases, as well as a head and neck case with both pre- and post-treatment samples. Our first goal with these samples was to identify somatic variants that are shared between the tumor and cfDNA. Our second goal was to profile tumor evolution following checkpoint blockade therapy using paired tumor/plasma time point analysis.

Results

LOD assessment using ctDNA reference standards

Our analysis of somatic variants from Seracare standards revealed that the ImmunoID NeXT cfDNA platform successfully identified 24/25 (96%) gold set SNVs in 1% and 2% AF samples at 100ng, 50ng and 25ng starting DNA input amounts (Figure 1). At 0.5% AF we see a considerable loss in sensitivity for gold set variants using our somatic variant calling pipeline (Figure 1 green bars). By interrogating the pileups for the 0.5% AF samples and requiring >=3 reads of evidence supporting the gold set variants, we achieve 96% sensitivity (Figure 1 grey bars).

Gene ID	c.HGVS	p.HGVS	Gene ID	c.HGVS	p.HGVS
AKT1	c.49G>A	p.E17K	IDH1	c.394C>T	p.R132C
APC	c.4348C>T	p.R1450*	JAK2	c.1849G>T	p.V617F
BRAF	c.1799T>A	p.V600E	KIT	c.2447A>T	p.D816V
CTNNB1	c.121A>G	p.T41A	KRAS	c.35G>A	p.G12D
EGFR	c.2573T>G	p.L858R	MPL	c.1544G>T	p.W515L
EGFR	c.2369C>T	p.T790M	NRAS	c.182A>G	p.Q61R
FGFR3	c.746C>G	p.S249C	PDGFRA	c.2525A>T	p.D842V
FLT3	c.2503G>T	p.D835Y	PIK3CA	c.1633G>A	p.E545K
FOXL2	c.402C>G	p.C134W	PIK3CA	c.3140A>G	p.H1047R
GNA11	c.626A>T	p.Q209L	RET	c.2753T>C	p.M918T
GNAQ	c.626A>C	p.Q209P	TP53	c.524G>A	p.R175H
GNAS	c.601C>T	p.R201C	TP53	c.818G>A	p.R273H
TP53	c.743G>A	p.R248Q			

Table 1: Seracare ctDNA Standards, SNVs only.

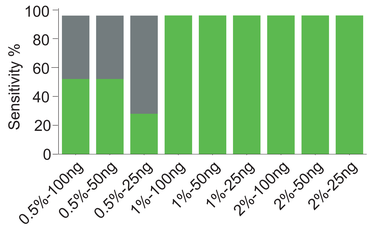

Figure 1: ACE ctDNA Exome Sensitivity with Seracare ctDNA Standards.

LOD assessment using normal donor plasma

In our healthy donor mixes, our ImmunoID NeXT cfDNA platform was able to detect *de novo* gold set SNVs with a sensitivity of >90% down to an allele frequency of 2%. For monitoring applications we were able to detect SNVs with a sensitivity of >92% down to an allele frequency of 0.5% (Table 2). When tested across a range of tumor types including melanoma, lung and colorectal, ImmunoID NeXT repeatedly detected highly concordant somatic events between tumor and cfDNA. In tumor samples allele frequencies ranged from 10% to 100%, and through cfDNA interrogation 60%-98% of these events were accurately detected in the plasma.

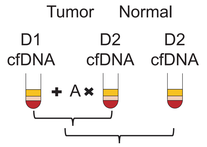

Figure 2: cfDNA dilution scheme for donors in LOD study. Somatic variant calling using D2 as matched normal; A = varying amounts of D2 cfDNA.

Allele Frequency	*De Novo* Detection		Monitoring
	TPR	FDR	TPR
3%	>92%	<3%	>99%
2%	>90%	<3%	>99%
1.5%	>80%	<5%	>98%
1%	>70%	<10%	>96%
0.5%	--	--	>92%

Table 2: Accuracy for *de novo* and monitoring applications across allele frequencies.

CRC tumor vs ctDNA SNV comparison

We analyzed eight late stage CRC tumor samples and identified between ~120-900 somatic variants with AFs >10%. We then queried the matched plasma from these same patients for the same variants. By requiring at least two polished reads supporting a variant in cfDNA, we observed between 43% and 90% of tumor variants in matched plasma (Figure 3, grey bars indicate variants unique to tumor). We also called somatic variants directly in plasma and set a cutoff of five polished reads of evidence. Even with this relatively stringent cutoff we observed variants that were unique to plasma (Figure 4, dashed circle). These observations show that a single tumor biopsy likely does not completely represent the mutational load of a cancer, and suggests a group of plasma derived variants (some of which may be neoantigens) could be missed.

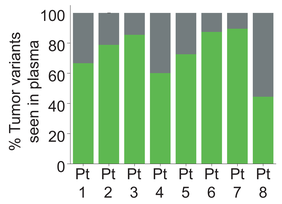

Figure 3: Variant concordance between tumor and plasma using a cutoff of two reads of evidence (monitoring).

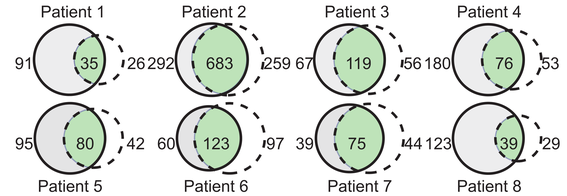

Figure 4: Variant concordance between tumor (solid) and plasma (dashed) using a cutoff of five reads of evidence (*de novo* plasma variant identification).

Tumor heterogeneity — head & neck cancer

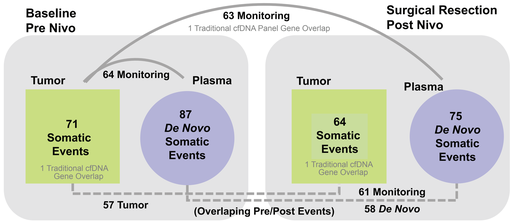

Figure 5: Variant concordance between tumor (solid) and plasma (dashed) using a cutoff of ten reads of evidence (*de novo* plasma variant identification). Patient information: cancer type: head and neck - oral cavity squamous cell; stage: T4aN3bM0; treatment: single dose Nivolumab prior to definitive resection; linicial outcome: early recurrence metastatic disease (deceased).

We are currently applying our cfDNA technology to profile pre- and post-treatment head and neck cancer samples from patients on checkpoint blockade therapy in collaboration with the Stanford School of Medicine (Figure 5). In this representative case, we observe strong concordance between pre- and post-treatment (~4 weeks post) in both the plasma and tumor, which could be indicative of a lack of response to therapy.

Conclusion

Our ImmunoID NeXT cfDNA platform has a sensitivity of > 95% to monitor and a PPV of >97% to detect *de novo* SNVs in plasma, down to an AF of 1.25%. The assay's high sensitivity is important in cases when monitoring of tumor variants in plasma is desired. The high PPV is useful when identifying variants derived solely from plasma without a tumor biopsy. We believe this assay can be used as a complement to the results of sequencing of tumor biopsy alone.

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