Comprehensive genomic analysis of predictive biomarkers for checkpoint inhibitor therapy from a limited amount of FFPE tissue and cfDNA

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Introduction

PD-L1 expression and MSI status are useful predictive biomarkers for checkpoint inhibitors (CIs) in some tumor types. However, studies have demonstrated that the identification of all potential patients who may benefit from CIs will require the analysis of both tumor cell and tumor microenvironment biomarkers. For example, recent data suggests that tumor mutation burden (TMB) and tumor mutation signatures as well as inflammatory signatures and T cell clonality in the tumor microenvironment may serve as independent predictive biomarkers of CI response. The need for evaluation of multiple biomarkers using IHC, PCR and/or NGS using limited amounts of tissue poses a challenge in the clinical setting. Here we conduct a study to evaluate whether a panel of tumor biomarkers can be accurately analyzed using low DNA input from FFPE specimens or using plasma-derived cfDNA. Multiple methods for assessing TMB and mutation signatures were compared. The impact of sample quality on biomarker analysis was also evaluated.

Materials and Methods

Samples and DNA/RNA extraction

A set of 11 CRC (Adenocarcinoma, Grade 2-4) and their matched plasma samples were used in this study. DNA and RNA were co-extracted from FFPE sections using RecoverAll™ kit. cfTNA was extracted from plasma using the MagMAX™ Kit.

DNA QC

KAPA hgDNA Quantification and QC Kit was used to evaluate FFPE DNA quality. A higher ratio of Q129 to Q41 (amplifiable molecules at sizes of 129bp and 41bp) indicates better sample quality. Q129 was chosen due to its closeness to OCAv3 amplicon size. An empirical metrics based on the ratio of Qubit to Nanodrop DNA concentration was also used for sample quality QC. Higher ratio indicates better sample quality as well.

Oncomine™ Tumor Mutation Load Assay (TML2.0)

The Thermo Fisher Oncomine™ Tumor Mutation Load Assay (TML) was used to assess the TMB level in the tumor samples selected (**Table1**). The TML assay evaluates TMB (mutations/Mb) by interrogating 409 cancer-related genes, spanning ~1.7 megabases of the genome. 20ng dsDNA, measured by Qubit, was used for library preparation as per the manufacturer's instruction. TMB analysis was performed using Oncomine Tumor Mutation Load - w2.0 – DNA workflow. The workflow reports the TMB result and Oncomine variants covered by the panel. For TMB, the workflow returns results for somatic SNVs based upon dbSNP, 5000Exomes, ExAC, and UCSC Common SNPs annotation source databases. The minor allele frequency range lies between 0.0 and 1.0E-6. It also filters out variants of homopolymer lengths greater than 7, coverage lower than 60, and allele frequency less than 5%. Besides the default mutant allele cutoff (AF=5%), a 10% AF cutoff was tested as well.

Whole Exome Sequencing (WES) for TMB

Whole exome sequencing was performed by Broad Institute on the same sample set. 200-250 ng (Qubit measured) DNA was used for library preparation for WES. Somatic variants (synonymous and non-synonymous of SNVs and indels within coding regions) were used to calculate TMB. Variant calling was based on mutant allele coverage without applying an allele frequency cutoff.

Oncomine™ Comprehensive Assay v3 (OCAv3)

The OCAv3 (ThermoFisher) is a targeted NGS assay that enables the detection of relevant SNVs, CNVs, gene fusions, and indels from 161 unique genes. The assay utilizes 20 ng DNA and 20 ng RNA input. Data analysis was performed using workflows i.e. Oncomine Comprehensive v3 - w3.1 - DNA and Fusions - Single Sample and Oncomine Comprehensive v3 - w3.2 - DNA - Single Sample with Mutation load (somatic SNV).

Oncomine™ Pan-Cancer Cell-Free Assay

The Oncomine Pan-Cancer Cell-Free Assay (ThermoFisher) was used to assess the cfTNA mutation and gene fusions found in 8 plasma sample (3 plasma samples were disqualified for analysis due to white blood cell lysis). The assay is designed to detect multiple targets (hotspot SNVs and short indels from 52 oncogenes and 4 tumor suppressor genes, gene fusion from 12 driver genes, MET ex14 skipping, CNV for 12 genes) in tumor-derived DNA and RNA isolated from the plasma fraction of whole blood. The assay employs a single pool of multiplex PCR primers for preparation of an amplicon library from cell-free total nucleic acid. Data analysis was performed using workflow-Oncomine TagSeq Pan-Cancer Liquid Biopsy w2.0 - Single Sample.

Results

Sample quality and sequencing metrics

Ratios of Qubit/NanoDrop and Q129/Q41 (**Table 1**) indicate good quality DNA was obtained from the 11 FFPE CRC samples. The DNA samples were further analyzed using three NGS assays for TMB and driver mutation detection, with OCAv3 and TML as targeted sequencing assays and WES as the reference result. The OCAv3 has coverage above 2000X, uniformity above 97% and MAPD at 0.2. 800X coverage and 97% uniformity were obtained from the TML assay. WES had at least 170X coverage and 80% bases above 50X coverage (**Table 1**). The high DNA sequencing metrics from the 3 NGS assays correlates with the sample quality prediction by Qubit/NanoDrop and Q129/Q41. In addition to the DNA component, OCAv3 has an RNA panel. Three out of the 11 RNA samples failed OCAv3 RNA sequencing QC due to the low expression of control genes (LRP1, TBP and MRPL13) in RNA pool 2.

Table 1. Tumor content, FFPE DNA QC and sequencing metrics

CRC tumor sample	Tumor content	FFPE DNA ratio (Qubit/Nano drop)	FFPE DNA ratio Kapa (Q129/Q41)	OCAv3 (DNA mean depth/DNA uniformity/ mapped DNA MAPD/ RNA fusion sample QC)	TML (DNA mean depth/DNA uniformity)	WES (Mean Target Coverage/Target Bases @ 50X%)
EXO-0000096	70%	30%	65%	2404/98.3%/0.19/ pass	1166/98.1%	186/87.7%
EXO-0000114	50%	25%	55%	2283/98.4%/0.2/ pass	1015/97.8%	244/90.3%
EXO-0000169	90%	38%	53%	2647/98.3%/0.2/ pass	926/97.7%	235/92.8%
EXO-0000172	80%	28%	51%	2583/98.2%/0.21/ fail[1]	844/97.7%	211/91.6%
EXO-0000183	50%	29%	53%	2673/98.2%/0.19 8/fail[1]	973/97.7%	173/87.7%
EXO-0000358	60%	27%	49%	3027/98.3%/0.20/ fail[1]	828/97.5%	204/91.5%
EXO-0001946	50%	26%	48%	2801/98.3%/0.19/ pass	1031/97.7%	149/81.7%
EXO-0002805	90%	36%	52%	2686/98.1%/0.20/ pass	1186/97.6%	165/82%
EXO-0003682	40%	32%	58%	3073/97.8%/0.20/ pass	1259/97.2%	178/90.4%
EXO-0004750	80%	28%	59%	3118/98.1%/0.19/ pass	1198/97.6%	254/93.2%
EXO-06336-K	70%	38%	58%	2983/97.8%/0.20/ pass	1179/97.5%	209/91%

NOTE: 1. OCAv3 pool2 failed. Total reads <100K.

TMB and driver mutation detection comparison

TMB Analysis

The TMB results generated from 3 different methods in this study are comparable with a range of 0-132 for OCAv3, 3-176.7 for TML (5% AF cutoff), 1.7-160.7 for TML 1(10% AF cutoff), 5.4-174.3 for WES in coding regions and 2.7-67.4 for WES in non-coding regions (**Table 2**). 10/11 samples have a TMB result below 20 and are considered as low mutation burden (**Fig. 1**,**ref. 1**). Sample EXO-06336-K has a significantly higher mutation burden than the rest of the samples. The correlation among the low mutation burden group is low, probably due to the variation of TMB detection and calculation in this group by different methods.

Two %AF cutoffs were tested in the TML workflow based on our previous observation that poor quality FFPE can artificially increase TMB in TML assay (**ref. 2**). We hypothesized 10% AF cutoff would reduce the false positive variant count. First, we tested these two cutoffs on this high quality sample set. As shown in **Fig. 1**, the AF cutoff has no difference on TMB result. We further tested the 10% cutoff on another FFPE sample set (n=22, variable quality: Qubit/Nano of 16 samples < 0.2 of and Q129/Q41 of 17 samples < 0.4), which had been analyzed using TML and MSI assay (**ref. 2**). **Fig. 2** showed 10% AF improves the accuracy of TMB calling by allowing a better separation of MSI high and MSS groups in contrast to the result from the 5% cutoff. The duplicate analysis workflow, an established workflow (**ref. 2)** for poor quality FFPE TMB call showed similar result to the 10% AF result. In summary, TML workflow with 10% AF cutoff allows for TMB detection from variable FFPE quality. Although OCAv3 can differentiate low and high TMB as shown in this study, the small size of the panel is expected to limit the detection of medium and high TMB levels.

Oncomine driver mutation detection by OCAv3 and TML

The mutation spectrum detected by OCAv3 showed KRAS and TP53 mutations are the most prevalent in this CRC sample set. 26 out of 27 SNV and indel mutations were confirmed with WES. One low frequency BRCA2 mutation could not be confirmed due to the low coverage of this region by WES. 4 CNV gains (2 in FGFR1, 1 in MYC and 1 in FLT3) reported by OCAv3 are confirmed by WES as well. Two fusions are to be confirmed. In addition to TMB, TML assay allows reporting of Oncomine variants. **Table 2** shows the Oncomine varaints reported by TML are concordant with OCAv3 and WES results, although TML lacks coverage on some genes.

Hypermutation sample

Both OCAv3 and TML assay identified a spectrum of mutations in DNA mismatch repair genes, DNA polymerase and other mutations associated with high mutation burden in the hypermutation sample EXO-06336-K.

Results continued

Table 2. Comparison of TMB results and mutation profiles from different assays

CRC tumor sample	TMB OCAv3	TMB TML2.0 -5% AF	TMB TML2.0 (10% AF)	TMB WES-coding regions	TMB WES-non-coding regions	OCAv3 variants	TML2.0 variants
EXO-0000096	4	6.66	6.66	8.13	5.48	KRAS G13D, TP53 P151fs, SMAD4 R361C, FGFR1 AMP. FNDC3B3-PIK3CA (189 reads)	KRAS G13D
EXO-0000114	0	5	4.17	11.14	6.29	KRAS G12D	KRAS G12D
EXO-0000169	12	5.01	3.34	7.26	4.71	MET E168D, KRAS G13D, TP53 R282W R196X, MYC AMP	MET E168D, KRAS G13D, TP53 R282W R196X
EXO-0000172	8	6.68	5.01	11.79	5.94	TP53 R280T	TP53 R280T
EXO-0000183	4	3.34	1.67	5.39	2.71	FGFR1 AMP	none detected
EXO-0000358	16	7.53	6.69	9.70	4.85	KRAS Q61H; CCND3 AMP	KRAS Q61H
EXO-0001946	12	6.68	6.68	6.59	3.07	RB1 E629fs, TP53 W91X	RB1 E629fs, TP53 W91X
EXO-0002805	4	10.85	8.34	7.79	3.84	KRAS G12V, SMAD4 R361H, GNAS R201H. PTPRK(1) - RSPO3(2) strong fusion.	KRAS G12V; GNAS R201H
EXO-0003682	0	6.68	5.84	8.30	3.73	KRAS G12V	KRAS G12V
EXO-0004750	4	4.17	4.17	6.94	3.88	KRAS G12V, FLT3 AMP	KRAS G12V
EXO-06336-K	132	176.59	160.71	174.28	67.40	MSH6 E1234X, ATR E1061X, PIK3CA M1043I, FBXW7 R479Q, PIK3R1 R386X, MET S1058P, PTEN E299X, KRAS A146T, POLE P286R, BRCA2 E881X (low freq at 3% for BRCA2, all others 15-20%)	MSH6 E1234X, ATR E1061X, PIK3CA M1043I, FBXW7 R479Q, PIK3R1 R386X, MET S1058P, PTEN E299X, KRAS A146T, RUNX1 R204Q, R166Q confirmed by Exome.

NOTE: Green colored mutations are confirmed by WES.

Figure 1. TMB measured by TML and WES (11 high quality FFPE CRC samples)

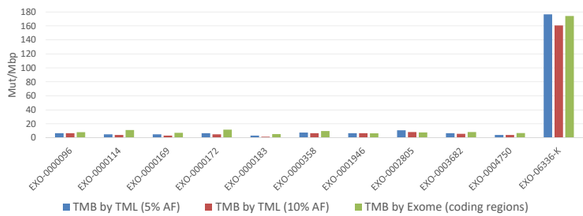
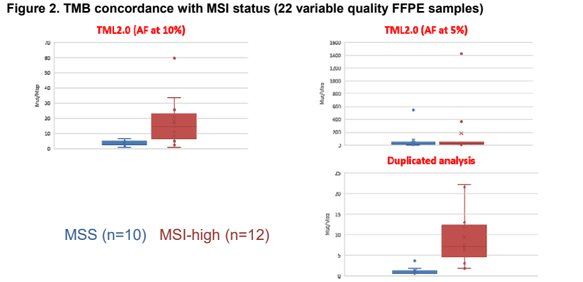


Figure 2. TMB concordance with MSI status (22 variable quality FFPE samples)



Analysis of matched plasma samples using the Oncomine Pan-Cancer Cell-Free Assay

Matched plasma samples were available for analysis for 8 cases. 19 variants (17 SNVs and indels and 2 CNV gain) are included in both the Pan-Cancer Cell-Free Panel and OCAv3. A comparison of the results from the matched plasma and FFPE tumor tissue samples is shown in **Table 3**. Nine SNVs and indels in KRAS, APC, TP53, SMAD4, FBXW7 and PIK3CA were detected in both plasma and tissue for 3 cases. An example of a TP53 R231Q mutation found in plasma and tissue is shown in **Fig. 3**. However, SNVs in KRAS, GNAS, TP53 and/or SMAD4 that were detected in 5 tissue samples were not detected in the matched plasma sample. Although the Pan-Cancer Cell-Free Assay is designed to detect gene amplification, CCND3 and FGFR1 amplification that was detected in 2 different tissue samples was not detected in the matched plasma sample. Interestingly, METex14 splice variants and skipping were detected at the DNA and RNA level, respectively, in 4 plasma samples but not the matched tissue sample. An example of a splice variant is shown in **Fig. 4**. These unexpected results are under further investigation. Fusions were detected in 2 tissue samples using OCAv3 but are not covered by the Pan-Cancer Cell-Free Assay.

Results continued

Table 3. Concordance of mutations in matched cfTNA and FFPE tumor tissue

CRC plasma sample (input)	Hotspots identified in matched tumor also covered by Pan-cancer cf Panel ROI and hotspot	Hotspot detected by Pan-cancer cf Panel (AF ≥ 0.5%)
EXO-0000096 (12.2 ng)	KRAS G13D; TP53 P151fs; SMAD4 R361C	KRAS G13D at 4%; TP53 P151fs at 3.5%; SMAD4 R361C at 1.2%; MET 2.5% splicing site mutation chr7:116412044 G>T (not in OCAv3 and exome) and RNA METex14 skipping at 538 Mol Cov. Mutant.
EXO-0000114 (20.0 ng)	KRAS G12D	MET 0.7% splicing site mutation chr7:116412044 G>T (not in OCAv3 and exome) and RNA METex14 skipping at 233 Mol Cov. Mutant.
EXO-0000169 (20.0 ng)	KRAS G13D, TP53 R282W, MYC AMP	None detected. (WBC contamination suspected due to high DNA yield).
EXO-0000183 (7.5 ng)	FGFR1 AMP. TP53 R231Q (non-hotspot)	TP53 R231Q at 1.76% in plasma are also in OCAv3 and exome. MET 11.4% splicing site mutation chr7:116412044 G>T (not in OCAv3 and exome) and RNA METex14 skipping at 1844 Mol Cov. Mutant.
EXO-0000358 (12.3 ng)	KRAS Q61H; CCND3 AMP	MET 11.4% splicing site mutation chr7:116412044 G>T (not in OCAv3 and exome) and RNA METex14 skipping at 5416 Mol Cov. Mutant.
EXO-0002805 (20.0 ng)	KRAS G12V, SMAD4 R361H, GNAS R201H	None detected
EXO-0003682 (20.0 ng)	KRAS G12V	CCND3 AMP (1.19)
EXO-06336-K (9.3 ng)	KRAS A146T, FBXW7 R479Q, PIK3CA M1043I, APC p.R1114* and p.R1450*	KRAS A146T at 2.1%, FBXW7 R479Q at 1.8%, PIK3CA M1043I at 1.2%, agreed with exome and OCAv3. APC p.R1114* (chr5:112174631 C>T) at 1.4% and p.R1450* (chr5:112175639 C>T) at 1.6% in plasma and also in exome, but not covered by OCAv3. TP53 p.E326* (chr17:7576870 C>A) at 1.5% in plasma, but not in exome and OCAv3.

Figure 3. Concordant TP53 R231Q mutation detection in plasma EXO-0000183

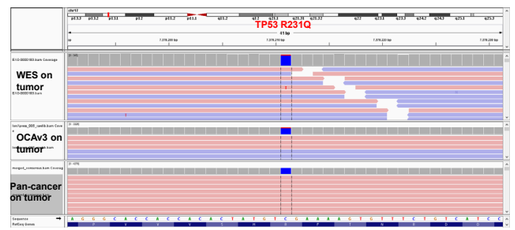
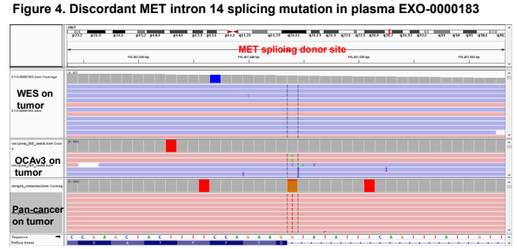


Figure 4. Discordant MET intron 14 splicing mutation in plasma EXO-0000183



Conclusions

- The Oncomine™ TML Assay generated TMB results that correlated with WES.
- There was a lower correlation between TMB results generated with OCAv3 Assay and WES.
- The Oncomine™ TML Assay can provide advantages over WES for TMB analysis such as lower FFPE DNA input requirements (20 ng vs. 250 ng) and faster TAT (5 days vs 30 days).
- The Oncomine™ TML Assay data analysis with a 10% AF cutoff can reduce the impact of poor quality FFPE DNA samples on TMB scores.
- Both the Oncomine™ TML Assay and OCAv3 Assay provide comprehensive mutation spectrum analysis using low FFPE nucleic acid input amounts.
- The Oncomine Pan-Cancer Cell-Free Assay successfully detected SNVs and indels with AF above 0.5% in some samples but the panel is not large enough to calculate TMB scores.

References

1. Chalmers ZR, et al., (2017) Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden. Genome Med., 9(1):34.
2. Peng F, et al., (2018) Evaluation of a commercial targeted NGS panel for tumor mutation burden assessment in FFPE tissue. AACR 2018 Abstract 3614.

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