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Serial changes in liquid biopsy-derived variant allele frequency predict immune checkpoint inhibitor responsiveness in the pan-cancer setting

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ABSTRACT

Major immunotherapy challenges include a limited number of predictive biomarkers and the unusual imaging features post-therapy, such as pseudo-progression, which denote immune infiltrate-mediated tumor enlargement. Such phenomena confound clinical decision-making, since the cancer may eventually regress, and the patient should stay on treatment. We prospectively evaluated serial, blood-derived cell-free DNA (cfDNA) (baseline and 2–3 weeks post-immune checkpoint inhibitors [ICIs]) for variant allele frequency (VAF) and blood tumor mutation burden (bTMB) changes (next-generation sequencing) (N = 84 evaluable patients, diverse cancers). Low vs. high cfDNA-derived average adjusted ΔVAF (calculated by a machine-learning model) was an independent predictor of higher clinical benefit rate (stable disease ≥6 months/complete/partial response) (69.2% vs. 22.5%), and longer median progression-free (10.1 vs. 2.25 months) and overall survival (not reached vs. 6.1 months) (all $P < .001$, multivariate). bTMB changes did not correlate with outcomes. Therefore, early dynamic changes in cfDNA-derived VAF were a powerful predictor of pan-cancer immunotherapy outcomes.

BRIEF SUMMARY

Liquid biopsy to predict immunotherapy response.

Background

As a transformative cancer treatment, immunotherapies, especially checkpoint inhibitors, such as antibodies against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or the programmed death receptor 1 (PD-1) and its ligand (PD-L1), have achieved salutary anti-cancer effects among patients, even those suffering from advanced, metastatic disease.[1–5] However, across different solid tumors, the efficacy of checkpoint inhibitors is limited to a relatively small portion of patients. Recently, the Food and Drug Administration (FDA) authorized two tumor-agnostic approvals for the checkpoint inhibitor pembrolizumab,[6,7] based on deficient mismatch repair/microsatellite instability-high (dMMR/MSI-H) and tumor mutational burden (TMB) ≥10 mutations/Mb, respectively, because these two parameters predict better outcomes with immunotherapy.[6–12] Still, patients with MSI-H have a response rate of only about 40% across solid cancers with pembrolizumab.[7] The only other approved biomarker for predicting anti-PD-1/PD-L1 inhibitor response is PD-L1 expression by immunohistochemistry.[13,14]

Since most patients do not achieve durable benefit from the checkpoint inhibitors, even in the presence of approved response biomarkers, additional investigation is warranted. Liquid biopsy is a noninvasive method that captures circulating cell-free DNA (cfDNA) released into the bloodstream from tumor cells. With techniques such as next-generation sequencing (NGS), liquid biopsies can be exploited to detect somatic mutations in tumors. Mutation profile measured from cfDNA can help in selecting the right treatment for patients and assist in monitoring cancer progression and recurrence.[15–23]

To better predict immune response and resistance markers for anti-PD-1/PD-L1 inhibitors, especially in the early course of the treatment, we prospectively evaluated serial plasma samples for cfDNA in order to assess dynamic changes in variant allele frequency (VAF) and in blood TMB (bTMB), and the implication of these changes for checkpoint blockade responsiveness. Our results suggest that VAF changes in cfDNA provide an early forecast of outcomes after immunotherapy and therefore that implementation of early liquid biopsy may facilitate drug development in this field.

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Supplemental data for this article can be accessed on the publisher's website.

Methods

Study design and patient treatment

We prospectively enrolled 104 patients with diverse metastatic malignancies who received anti-PD-1/PD-L1-based therapies and obtained serial plasma to measure variants from tumor-derived cfDNA. Blood samples from timepoint A were obtained on the day of therapy, prior to the first treatment cycle. Subsequent blood samples prior to the second cycle of therapy (~3 weeks after the first treatment cycle) were collected as timepoint B samples. Blood samples were processed at Ambry Genetics (https://www.ambrygen.com/). The study was conducted under the UCSD IRB-approved PREDICT protocol (NCT02478931) and investigational therapies for which patients gave consent.

Next-generation sequencing

cfDNA was extracted from whole blood collected in 10 mL Streck tubes. Briefly, plasma was isolated from 10 to 20 mL of blood by centrifugation at 1,600 g for 10 minutes. cfDNA was extracted from plasma using QIAamp Circulating Nucleic Acid Kit (Qiagen) and quantified by Bioanalyzer (Agilent). Sequencing libraries were prepared using 10–30 ng of cfDNA with KAPA HyperPrep kits (Roche) according to manufacturer's instructions. Sequencing was conducted on the HiSeq2500 (Illumina Inc.) using 100 bp paired-end sequencing according to the manufacturer's workflow. Genomic alterations including single nuclear variations (SNVs), insertions and deletions from cfDNA samples were detected using a targeted NGS-based panel with 89 genes (Supplementary Table 1), including genes participating in antigen processing and presentation, immune response, and genes associated with cancer.[12] Germline variants are detected from whole blood samples using whole-exome sequencing as described previously.[24] Variants identified in cfDNA samples are used to calculate the changes in VAF and TMB.

Statistical analysis and outcome evaluation

A t-test was used for continuous data, and a logistic regression test was used for categorical data. OS was calculated from the start of treatment to last follow-up. PFS was calculated from the start of treatment until progression or death, whichever came first. Survival analyses were assessed by Kaplan–Meier analysis, and Cox's proportional hazard model was used to estimate hazard ratios (HRs) with 95% confidence intervals (CIs). Patients still alive (for OS) or still progression-free (for PFS) at last follow-up were censored at that point. Response was assessed by RECIST criteria per the physician. For multivariate analysis, variables with P values < .1 in univariate analysis were included in the multivariate regression model.

Average adjusted ΔVAF

With NGS, VAF is measured as the percentage of sequencing reads observed matching the variants divided by the overall coverage at that locus. Change in VAF (ΔVAF) after immunotherapy is calculated as $\Delta VAF = VAF_{timepointB} - VAF_{timepointA}$. To improve the prediction accuracy for patient response, we applied a machine learning algorithm to adjust ΔVAF calculation. We used an equation adjusted $\Delta VAF = VAF_{timepointB} - alpha*VAF_{timepointA}$ by adding an alpha value (weight) to $VAF_{timepointA}$. Average adjusted ΔVAF was defined as the average of each of the adjusted ΔVAFs for each alteration in any one patient (see Supplemental Methods for additional details).

ΔBlood TMB (ΔbTMB) calculation

TMB of blood (bTMB) was defined as the number of SNVs, insertions and deletions (indels) from coding regions per megabase of targeted regions. All base substitutions and indels in the coding region of targeted genes, including synonymous alterations, are counted. To calculate the TMB per megabase, the total number of mutations counted is divided by the size of the targeted regions (179 kb). ΔbTMB between timepoints A and B is calculated as $\Delta bTMB = bTMB_B - bTMB_A$.

Results

Patient characteristics

In this study, blood samples were collected from 104 patients to isolate cfDNA from plasma (Materials and Methods). DNA was isolated from 198 blood samples obtained from a total of 104 patients and sequenced with NGS. Twenty patients were excluded from the analysis: (i) 10 patients did not have blood samples from timepoint B and (ii) cfDNA samples from the other 10 patients failed NGS quality control. In total, a sample cohort of 168 distinct plasma samples obtained from 84 evaluable, immunotherapy-treated patients was included in this study (Figure 1 and Supplementary Table 2).

Patient characteristics of the treated patients are described in Table 1. The most common primary tumor sites were gastrointestinal (30.8%), genitourinary (16.3%) and gynecologic (13.5%), and head and neck (11.5%) cancers. All 104 patients were treated with immunotherapy, either as monotherapy, or in combination with a targeted agent, chemotherapy, or other immunotherapy. Overall, 102 patients received an anti-PD-1/PD-L1 agent in their regimen and two patients received anti-CTLA-4 monotherapy. Of the 84 evaluable patients, the best response from 79 patients was available at the time of data cutoff (Figure 1). Patients were considered as having clinical benefit if they showed stable disease (SD) for ≥6 months, partial response (PR), or complete response (CR). Patients with SD for <6 months or progressive disease were considered as non-responders (Table 1). Patients with ongoing SD for <6 months were considered too early to evaluate for response assessment. All 84 patients were evaluable for progression-free survival (PFS) and overall survival (OS).

Prediction of clinical benefit (SD ≥6 months/CR/PR) after immunotherapy with average adjusted ΔVAF in cfDNA

Cancer patients with higher tumor burden or more aggressive disease also have a higher concentration of cfDNA in their blood.[18–21] Drops in cfDNA level have also been shown to correlate with patient response to immune checkpoint inhibitors.[22,23,25] In cancer patients, cfDNA level can be
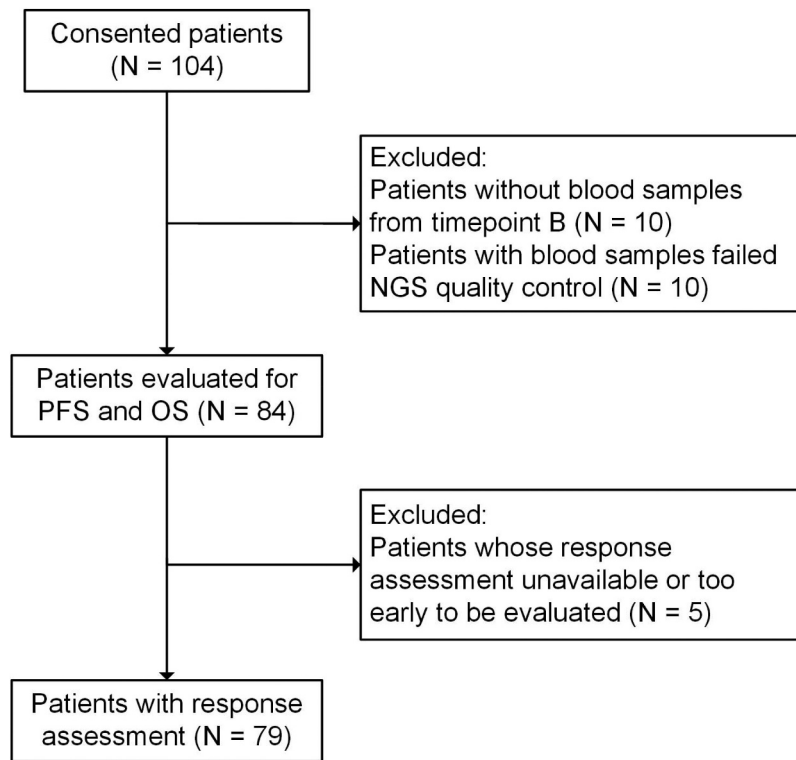
Figure 1. Consort diagram of cancer patients who were evaluated for progression-free survival (PFS) and overall survival (OS) (N = 84), and with response assessment (N = 79).
Abbreviations: OS, overall survival; PFS, progression-free survival.

measured with techniques such as NGS and droplet digital PCR. It is quantified by determining the allelic fraction of cfDNA fragments that contains cancer-associated variants.

In this study, we proposed that change in the VAF (which itself reflect the percent circulating tumor DNA) of the genomic profile of cfDNA from tumor is associated with patient response to immunotherapy. In order to measure the change, blood samples from timepoint A were obtained on the day of therapy, prior to the first treatment cycle. Subsequent blood samples prior to the second cycle of therapy (~3 weeks after the first treatment cycle) were collected as timepoint B samples. Variants with VAF $\geq 0.1\%$ including single nuclear variations (SNVs) and indels from cfDNA were detected using NGS (Methods). When comparing variants between timepoint A and timepoint B samples from each patient, we observed dynamic changes of VAF after the first treatment cycle, reflecting the change of both cfDNA level in blood and tumor burden in patients (Supplementary Tables 3–5).

Using timepoint A samples as baseline, we calculated VAF changes ($\Delta VAF = VAF_{timepointB} - VAF_{timepointA}$) between timepoint A and timepoint B for each variant. For each patient, an average of ΔVAF values from all variants (including somatic, synonymous, and variants of unknown significance) from the patient was calculated. Based on the data, patients with complete response are likely to have average $\Delta VAF < 0$, while patients with progressive disease are likely to have average $\Delta VAF > 0$. Variants from patient #67 and #81 were used as examples

for complete response and progressive disease, respectively (Supplementary Tables 3 and 4), with average ΔVAF of -0.32 and 3.35.

For patients who partially respond to immunotherapy, we began with the hypothesis that it is plausible that some tumor subclones respond to immunotherapy and reduce in size, while other subclones do not respond to treatment, so they do not shrink or keep growing. In this scenario, average ΔVAF may not be an optimal predictor for patient response, as ΔVAF for each variant may decrease or increase. Therefore, we developed a machine-learning model to adjust the calculation of average ΔVAF in order to improve its prediction accuracy for patient response. We applied maximum likelihood estimation (MLE) approach to find the best weighted difference to adjust ΔVAF calculation (Adj $\Delta VAF = VAF_{timepointB} - 0.37 \times VAF_{timepointA}$). For each patient, average adjusted ΔVAF was calculated by averaging Adj ΔVAF values from all variants from the patient. Further details of the average adjusted ΔVAF calculation are described in the Supplementary Methods. Average adjusted ΔVAF was examined per patient, and the median value was then calculated for all 84 patients (median = 0.11; $\leq$median [low] versus >median [high]). Patients with a low average adjusted ΔVAF (≤ 0.11) were predicted to have clinical benefit (SD ≥ 6 months/CR/PR) and patients with a high average adjusted ΔVAF (>0.11) were predicted to have no benefit from immunotherapy (SD <6 months/PD). As an example, there were a total 15 variants detected from patient #69 (Supplementary Table 5). Adj ΔVAF was calculated for each

Table 1. Patient characteristics (N = 104).*

Basic characteristics	N (%)
Age, median (range) (years)	61.5 (21.0–91.5)
Sex, N (%)	
Women	41 (39.4%)
Men	63 (60.6%)
Type of cancer, N (%)	
Gastrointestinal	32 (30.8%)
Genitourinary	17 (16.3%)
Gynecologic	14 (13.5%)
Head and neck	12 (11.5%)
Skin/melanoma	7 (6.7%)
Lung	6 (5.8%)
Breast	5 (4.8%)
Hematologic	4 (3.8%)
Central nervous system	3 (2.9%)
Others**	4 (3.8%)
Anti-PD-1/PD-L1-based therapy administered as, N (%)	
First line	36 (34.6%)
Second line	38 (36.5%)
Third line	13 (12.5%)
≥Fourth line	17 (16.3%)
Type of immunotherapy, N (%)	
Anti-PD-1/PD-L1 alone	51 (49.0%)
Anti-PD-1/PD-L1 with targeted agents	25 (24.0%)
Anti-PD-1/PD-L1 with chemotherapy	7 (6.7%)
Anti-PD-1/PD-L1 with anti-CTLA-4	15 (14.4%)
Anti-PD-1/PD-L1 with anti-CTLA-4 and targeted agents	2 (1.9%)
Anti-PD-1/PD-L1 with chemotherapy and targeted agents	2 (1.9%)
Anti-CTLA-4 alone	2 (1.9%)
Best response, N (%)	
Complete response	4 (3.8%)
Partial response	22 (21.2%)
Stable disease ≥6 months	12 (11.5%)
Stable disease <6 months	12 (11.5%)
Progressive disease	43 (41.3%)
Response assessment unavailable or too early to be evaluated***	11 (10.6%)

*The characteristics were curated on all 104 patients. N = 84 patients had treatment follow-up and passed cfDNA NGS quality control.
**Others: includes patients with sarcoma (N = 1), myxofibrosarcoma (N = 1), thymus squamous cell carcinoma (N = 1), and adrenal cortical carcinoma (N = 1).
***N = 8 had stable disease at the time of data cutoff; however, follow-up was less than 6 months and thus not included in the response analysis. N = 3 without adequate clinical information to assess the response.

of the 15 variants. Average adjusted ΔVAF was 2.25 from the 15 variants. The average adjusted ΔVAF of 2.25 is higher than the median value (0.11) from 84 patients, and patient #69 was predicted to be a non-responder of immunotherapy (and indeed the patient had progressive disease).

Patients with low cfDNA-derived average adjusted ΔVAF (≤0.11) have higher rate of clinical benefit (SD ≥6 months/CR/PR) from immunotherapy

We checked average adjusted ΔVAF distribution in 79 evaluable patients for response assessment. Average adjusted ΔVAF in patients with clinical benefit showed a range between –1.98 and 0.52, with a median value of 0.031; in contrast, average adjusted ΔVAF in patients without clinical benefit showed a range between −0.019 and 6.6, with a median value of 0.38 (P < .001) (Figure 2(a)). Checking patients with low average adjusted ΔVAF (≤0.11), there were 69.2% patients with clinical benefit (SD ≥6 months/CR/PR) as compared to 22.5% with clinical benefit (SD ≥6 months/ CR/PR) among patients with high average adjusted ΔVAF

(>0.11) (P value from logistic regression test <0.001) (Figure 2(b,c)). In multivariable Cox regression models, after adjusting for age, gender, cancer subtypes, treatment line, changes in blood TMB (ΔbTMB), tissue TMB, tissue MSI, and tissue PD-L1 status, low average adjusted ΔVAF remained as the most significant variable associated with patient clinical benefit (SD ≥6 months/CR/PR) (odds ratio [OR] for low versus high average adjusted ΔVAF, 9.65 [95% confidence intervals [CI]: 2.74–41.71, P value < .001]) (Table 2).

Patients with low versus high cfDNA-derived average adjusted ΔVAF have statistically better progression-free survival and overall survival after immunotherapy

Low average adjusted ΔVAF (≤0.11) was a predictor of longer PFS (Figure 3(a) and Table 3) and OS (Figure 3b and Table 3) according to the Kaplan–Meier analysis. All treated patients (N = 84) were included in the PFS and OS analyses of the low average adjusted ΔVAF (≤0.11) versus high average adjusted ΔVAF (>0.11) (median PFS, 10.1 versus 2.25 months, P value < .001; median OS, not reached after a median follow-up of 9.75 months versus 6.1 months, P value < .001) (Figure 3). In multivariable Cox regression models, including variables such as ΔbTMB, TMB from tissue, MSI and PD-L1 status, low average adjusted ΔVAF (≤0.11) remained as the most significant variable associated with a prolonged PFS (hazard ratio [HR] for low [≤0.11] versus high [>0.11], 0.35 [95% CI 0.19–0.64, P value < .001]) and with a prolonged OS (HR for low [≤0.11] versus high [>0.11], 0.35 [95% CI 0.19–0.63, P value < .001]) (Table 3).

Serial changes in blood TMB (ΔbTMB) were not associated with clinical outcome

Since tissue and blood-based TMB have been implicated as markers to predict response to checkpoint inhibitors,[10,26] we checked whether serial changes in blood TMB (ΔbTMB = bTMB$_{timepointB}$ − bTMB$_{timepointA}$) after treatment are associated with clinical outcomes. As shown in Supplementary Figure 1, there were no significant difference in the PFS and OS analyses between patients with decrease/no change (ΔbTMB ≤ 0) versus increase (ΔbTMB > 0) (N = 84) (median PFS, 6.7 versus 3.4 months, P value = .42; median OS, not reached after a median follow-up of 7 months versus 9.5 months, P value = .22). ΔbTMB was not used for multivariate analysis since P values from univariate analysis were not significant (Table 3). Similarly, baseline blood TMB (cutoff of 20 mutations/megabase) did not predict the outcome from immunotherapy.

Discussion

The introduction of immune checkpoint inhibition into the treatment arena for advanced cancers has provided significant clinical benefit and improved treatment outcomes for a subset of patients with cancer. Unfortunately, though, most patients do not respond to these agents. Various markers exist and continue to be developed in efforts to predict which tumors

Figure 2. Patients with low average adjusted ΔVAF (≤0.11) show higher rate of clinical benefit (SD ≥6 months/CR/PR) (N = 79). (a) Boxplot showing distribution of average adjusted ΔVAF from patients with SD ≥6 months/CR/PR (N = 36) and patients with SD <6 months or PD (N = 43). Line across the box = median, upper and lower edges of box = interquartile range of the average adjusted ΔVAF, upper and lower whisker = maximum and minimum average adjusted ΔVAF, dots = average adjusted ΔVAF from each patient. *P* values were computed using a t-test. (b) Bar graph showing the percentage of patients with SD ≥6 months/CR/PR versus SD <6 months/PD with average adjusted ΔVAF low (N = 39) versus average adjusted ΔVAF high (N = 40). *P* values were computed using logistic regression test. Patients (N = 79) with response assessment available and NGS data from timepoint A and B samples who passed sequencing quality control were included in the analysis. (c) Violin plot of average adjusted ΔVAF from patients with response assessment (N = 79). Patients with CR, PR, or SD ≥6 months are shown as green color. Patients with SD <6 months or PD are shown as red color. Patients are ordered by the average adjusted ΔVAF value (red lines) from low to high and dichotomized by the median (black dash line). Black dots represent adjusted ΔVAF.
Abbreviations: CR, complete response; NGS ,next-generation sequencing; PR, partial response; SD, stable disease; PD, progressive disease.

may be more susceptible to these drugs: PD-L1, TMB high, and MSI-high.[9,10,13] Despite these efforts, there are limited markers able to predict treatment responses early in the clinical time course. Striving to fill this unmet need, we investigated serial cfDNA samples among patients with diverse cancers treated with anti-PD-1/PD-L1 therapies.

Our patients who had serial cfDNA with lower average adjusted ΔVAF reflected the change in percent cfDNA in the blood post-treatment as compared to baseline and had significantly improved clinical outcomes (SD ≥6 months/PR/CR rates, PFS, and OS) (Tables 2, 3, Figures 2 and 3). These results are consistent with prior reports suggesting that serial cfDNA measurements can provide an early readout of outcome for

immunotherapy which could aid the radiographic assessment.[23,25,27] Radiographic evaluation of benefit from checkpoint inhibitors can occasionally be challenging, especially in the setting of pseudoprogression, since it is difficult to impossible to determine in such patients whether the tumor will eventually respond (and hence therapy should continue) or whether it is indeed growing (and therefore therapy should be stopped).[28] Our results suggest that the use of a second blood sample for cfDNA at weeks 2–3 after therapy can provide early assessment of the response. Therefore, serial cfDNA assessment may guide clinical decision that may allow patient to transition to the next line of therapy early on in case of progression.[23,27]

Table 2. Univariate and multivariate analyses for best response after immunotherapy (N = 79).

| | | | | Response (SD ≥6 m/CR/PR) | | | |
| | | | | Univariate | | Multivariate* | |
	Status	N	Response (SD ≥6 m/CR/PR)	OR (95% CI)	P value	OR (95% CI)	P value
ΔbTMB	≤0 mutation/Mb	56	29 (51.8%)	2.46 (0.90–7.25)	0.08	2.83 (0.73–12.08)	0.08
	>0 mutation/Mb	23	7 (30.4%)	-			
Average adjusted ΔVAF	Low (≤0.11)	39	27 (69.2%)	7.75 (2.93–22.24)	<0.001	9.65 (2.74–41.71)	<0.001
	High (>0.11)	40	9 (22.5%)	-			
Age (years)**	≤60.5	40	17 (42.5%)	1.29 (0.53–3.15)	0.58		
	>60.5	39	19 (48.7%)	-			
Gender	Female	32	14 (43.8%)	0.88 (0.35–2.18)	0.79		
	Male	47	22 (46.8%)	-			
GI cancers	YES	28	13 (46.4%)	1.06 (0.42–2.67)	0.91		
	NO	51	23 (45.1%)	-			
GU cancers	YES	12	2 (16.7%)	0.19 (0.03–0.81)	0.022	0.23 (0.02–1.39)	0.039
	NO	67	34 (50.7%)				
Treatment line	<2	24	13 (54.2%)	1.64 (0.63–4.38)	0.31		
	≥2	55	23 (41.8%)				
Tissue TMB high***	Yes (baseline)	9	6 (66.7%)	0.19 (0.03–0.85)	0.0042	0.27 (0.03–2.30)	0.025
	No	40	11 (27.5%)	0.86 (0.16–4.00)		0.86 (0.04–14.19)	
	Unknown	30	19 (63.3%)				
Tissue MSI high	Yes (baseline)	5	3 (60.0%)	0.30 (0.04–2.01)	0.02	2.84 (0.20–47.35)	0.58
	No	42	13 (31.0%)	1.11 (0.13–7.66)		2.39 (0.09–78.91)	
	Unknown	32	20 (62.5%)				
Tissue PD-L1 positive	Yes (baseline)	17	7 (41.2%)	0.68 (0.19–2.40)	0.099	1.28 (0.27–6.24)	0.28
	No	28	9 (32.1%)	2.04 (0.63–6.90)		3.83 (0.62–26.63)	
	Unknown	34	20 (58.8%)				

Patients (N = 79) with evaluable responses and NGS data from timepoint A and B samples who passed sequencing QC were included in the analysis.
All P values ≤0.05 are listed in bold.
*Variables with P value < 0.1 in the univariate analyses were entered into the multivariate analysis.
**Median age of the 79 patients is 60.5 years.
*** Tissue TMB high was defined by the laboratory; for instance, Foundation Medicine defined tissue TMB high as ≥20 mutations per megabase.
See Supplementary Table 6 for the vendor used to assess TMB, MSI, and PD-L1.
Abbreviations: ΔbTMB, serial changes in blood TMB; GI, gastrointestinal; GU, genitourinary; MSI, microsatellite instability; NGS, next-generation sequencing; QC, quality control; TMB, tumor mutational burden; VAF, variant allele frequency.
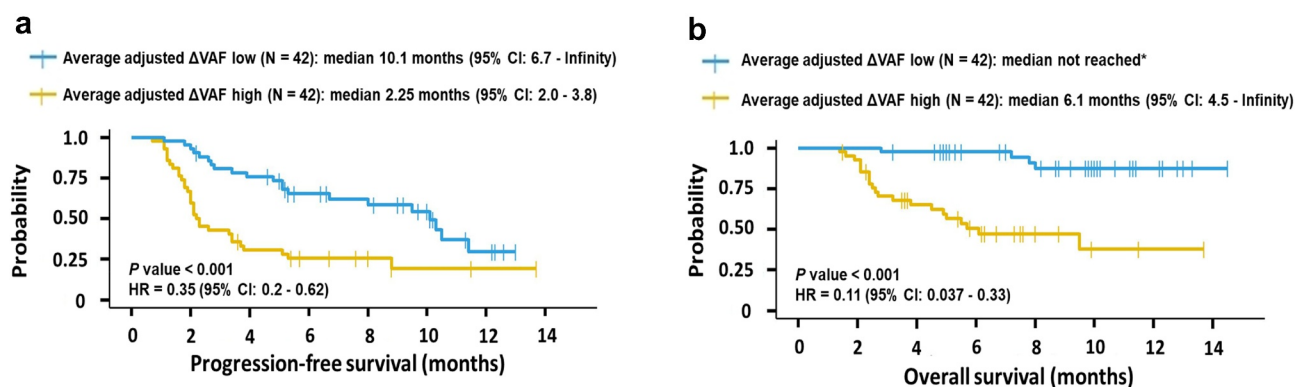
Figure 3. Patients with reduced average adjusted ΔVAF show better progression-free survival (PFS) and overall survival (OS) (N = 84). Kaplan–Meier curves displaying (a) PFS and (b) OS for patients with average adjusted ΔVAF low versus average adjusted ΔVAF high. Patients (N = 84) with timepoint A and B samples who passed NGS quality control criteria were used in the analysis. *P* values were calculated by the two-sided log-rank test. *Median overall survival not reached after a median follow-up of 9.75 months.

Table 3. Univariate and multivariate analyses for progression-free survival and overall survival after immunotherapy (N = 84).

			Progression-free survival				Overall survival			
			Univariate		Multivariate*		Univariate		Multivariate*	
	Status	N	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
ΔbTMB	≤0 mutation/Mb	59	0.79 (0.44–1.4)	0.42			0.61 (0.27–1.35)	0.23		
	>0 mutation/Mb	25								
Average adjusted ΔVAF	Low (≤0.11)	42	0.35 (0.2–0.62)	**<0.001**	0.35 (0.19–0.64)	**<0.001**	0.11 (0.04–0.33)	**<0.001**	0.35 (0.19–0.63)	**<0.001**
	High (>0.11)	42								
Age (years)**	≤60.5	43	1.24 (0.73–2.14)	0.43			1.01 (0.46–2.21)	0.98		
	>60.5	41								
Gender	Female	34	0.9 (0.52–1.56)	0.7			0.88 (0.39–1.95)	0.75		
	Male	50								
GI cancers	YES	29	0.95 (0.55–1.66)	0.87			1.04 (0.46–2.36)	0.92		
	NO	55								
GU cancers	YES	12	2.79 (1.39–5.62)	**0.0096**	2.47 (1.16–5.25)	**0.011**	2.06 (0.77–5.51)	0.18		
	NO	72								
Treatment line	<2	26	0.85 (0.47–1.55)	0.6			0.69 (0.27–1.72)	0.41		
	≥2	58								
Tissue TMB high***	Yes (baseline)	10		**<0.001**		**<0.001**		**0.0069**		**<0.001**
	No	41	5.30 (1.62–17.31)		4.36 (1.17–16.16)		6.75 (0.90–50.74)		4.96 (1.43–17.16)	
	Unknown	33	1.80 (0.52–6.24)		2.24 (0.48–10.47)		2.19 (0.26–18.23)		2.17 (0.48–9.85)	
Tissue MSI high	Yes (baseline)	5		**0.0081**		0.49		**0.032**		0.64
	No	44	2.24 (0.68–7.34)		0.71 (0.18–2.83)		3.29 (0.44–24.83)		0.73 (0.2–2.69)	
	Unknown	35	0.93 (0.27–3.19)		0.46 (0.09–2.47)		1.11 (0.13–9.22)		0.5 (0.11–2.36)	
Tissue PD-L1 positive	Yes (baseline)	17		**0.051**		0.97		0.19		
	No	28	1.23 (0.61–2.47)		0.97 (0.46–2.06)		2.47 (0.70–8.67)			
	Unknown	39	0.58 (0.28–1.22)		0.88 (0.32–2.40)		1.29 (0.35–4.76)			

Patients (N = 84) with timepoint A and B samples who passed NGS quality control criteria were used in the analysis.
All *P* values ≤ 0.05 are listed in bold.
*Variables with *P* value < 0.1 in the univariate analyses were entered into the multivariate analysis.
**Median age of the 84 patients is 60.5 years.
*** Tissue TMB high was defined by the laboratory; for instance, Foundation Medicine defined tissue TMB high as ≥20 mutations per megabase.
 See Supplementary Table 6 for the vendor used to assess TMB, MSI, and PD-L1.
Abbreviations: ΔbTMB, serial changes in blood TMB; GI, gastrointestinal; GU, genitourinary; MSI, microsatellite instability; TMB, tumor mutational burden; VAF, variant allele frequency.

Although higher tissue TMB can predict response to immune checkpoint inhibition as shown in the current report (Tables 2 and 3) as well as in previous literature,[10,29,30] and there is a pan-cancer FDA-approval for the anti-PD1 antibody pembrolizumab for patients with TMB ≥10 mutations/mb,[31] serial TMB dynamics in cfDNA in our study was not predictive of clinical outcome (Tables 2, 3 and Supplementary Figure 1). This might be due to the inherent mechanism of action of immune checkpoint inhibitors. These drugs are generally believed to cause tumor apoptosis via immune-mediated cell

death and caspase initiation, not via DNA alkylation, methylation, or other intranuclear/intracellular mechanisms that may lead to increases in TMB.[32]

There are several limitations to this study. First, the patients enrolled were heterogeneous (though this could also point to the generalizability of the results across cancers) and the sample size was moderately small. Thus, larger samples size will be required for further validation. Additionally, a variable treatment regimen with immunotherapies could be a confounder. Furthermore, the pattern of tumor DNA

shedding can vary among different histologies and requires further investigation. Lastly, ideal timing of serial cfDNA analysis is not established. Future evaluation with longitudinal study and different timepoints of blood sampling is required.

In conclusion, in a prospective study, we have shown that dynamic changes in VAF from cfDNA during the first 2 ~ 3 weeks of anti-PD-1/PD-L1 therapies were predictive of clinical outcomes. Therefore, these cfDNA changes may reflect a "molecular response"[23] that appears well before radiologic changes are measured. Implementation of early blood-derived liquid biopsy response markers from immunotherapy may facilitate drug development in this field.

Disclosure statement

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