Personalized Medicine and Imaging

Clinical Cancer Research

Hypermutated Circulating Tumor DNA: Correlation with Response to Checkpoint Inhibitor–Based Immunotherapy

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Abstract

Purpose: Tumor mutational burden detected by tissue next-generation sequencing (NGS) correlates with checkpoint inhibitor response. However, tissue biopsy may be costly and invasive. We sought to investigate the association between hypermutated blood-derived circulating tumor DNA (ctDNA) and checkpoint inhibitor response.

Experimental Design: We assessed 69 patients with diverse malignancies who received checkpoint inhibitor–based immunotherapy and blood-derived ctDNA NGS testing (54–70 genes). Rates of stable disease (SD) $\geq$6 months, partial and complete response (PR, CR), progression-free survival (PFS), and overall survival (OS) were assessed based on total and VUS alterations.

Results: Statistically significant improvement in PFS was associated with high versus low alteration number in variants of unknown significance (VUS, >3 alterations versus VUS $\leq$3 alterations), SD $\geq$6 months/PR/CR 45% versus 15%, respectively; $P = 0.014$. Similar results were seen with high versus low total alteration number (characterized plus VUS, $\geq$6 vs. <6). Statistically significant OS improvement was also associated with high VUS alteration status. Two-month landmark analysis showed that responders versus nonresponders with VUS >3 had a median PFS of 23 versus 2.3 months ($P = 0.0004$).

Conclusions: Given the association of alteration number on liquid biopsy and checkpoint inhibitor–based immunotherapy outcomes, further investigation of hypermutated ctDNA as a predictive biomarker is warranted. *Clin Cancer Res; 23(19); 5729–36.* ©2017 AACR.

Introduction

Checkpoint inhibitor–based immunotherapy has revolutionized treatment for malignancies across several histologies. For example, the PD-1 inhibitor pembrolizumab demonstrated a response rate of about 45% in a biomarker-selected population [defined as tumor immunohistochemical (IHC) PD-L1 staining >50%] in non–small cell lung cancer (NSCLC; ref. 1). Pembrolizumab is now approved in the first-line setting for biomarker-selected advanced NSCLC without sensitizing *EGFR* or *ALK* alterations. Other checkpoint inhibitors, such as the anti–CTLA-4 antibody ipilimumab and anti–PD-1 antibody nivolumab, as well as the anti–PD-L1 antibody atezolizumab, have demonstrated clinically significant efficacy for malignancies ranging from advanced melanoma to head and neck cancers, renal cell cancer, urothelial cancer, NSCLC, and colorectal cancer (2). Additionally, checkpoint blockade has demonstrated impressive response rates in hematologic malignancies, such as B-cell lymphomas (3).

Despite the undoubted efficacy of many of these immunotherapies, immune-related adverse effects are not negligible. For example, in a retrospective review of 14 phase I to III ipilimumab trials involving 1,500 patients, 64.2% of patients experienced some degree of toxicity, with 20% to 30% experiencing grade 3–4 immune-related side effects (including gastrointestinal, dermatologic, hepatic, endocrine, or pulmonary adverse events; ref. 4). Anti–PD-1 use has also been found to result in a rate of severe toxicity of approximately 6% (5). Furthermore, the majority of patients do not respond to therapy, and a subset of patients may experience hyperprogression (6, 7). For these reasons, it has become evident that predictive biomarkers of response are needed for these novel agents.

Recently, tumor mutational burden, as detected by tissue next-generation sequencing (NGS), has been shown to correlate with response to checkpoint inhibitors in several malignancies (8). This is not unexpected, because the immune system, once reactivated by checkpoint inhibitors, recognizes tumor cells because they present neoantigens derived from the mutanome. Presumably, the more neoantigens presented, the better the chances that the immune T-cell machinery will be triggered to eradicate the presenting cancer cell. Unfortunately, obtaining tissue biopsies in order to determine tumor mutational burden is at times difficult. We therefore investigated the utility of blood-derived circulating tumor DNA (ctDNA) in determining a hypermutated state and response to immunotherapy.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

This is the first analysis demonstrating a correlation between high alteration number detected in blood-derived circulating tumor DNA and favorable outcome, including overall response, progression-free survival, and overall survival with checkpoint inhibitor–based immunotherapy. These data provide the impetus to further investigate liquid biopsy as a viable, noninvasive, predictive biomarker for checkpoint inhibitor response across various histologies.

Materials and Methods

Patient data

Overall, 1,262 patients who had NGS (54 to 70 genes: Guardant Health) performed on cell-free, ctDNA derived from liquid (blood) biopsies were analyzed. Those who had solid malignancies and had received checkpoint inhibitor–based immunotherapy from December 2011 to December 2016 (N = 69 patients) were the subjects of this study. Of those 69 individuals, three were not evaluable for response (one whose baseline imaging was not traceable and two who were lost to follow-up before first restaging). The remaining 66 patients were evaluated for overall rate of stable disease (SD) ≥6 months, partial response (PR), or complete response (CR). All 69 patients were considered evaluable for progression-free survival (PFS) and overall survival (OS). In those situations where patients received multiple lines of immunotherapy, SD ≥6 months/PR/CR was assessed based on the first immunotherapy. Similarly, PFS and OS were assessed from cycle 1, day 1 of the first immunotherapy. In those situations where patients received multiple ctDNA analyses, data were used from the analysis closest to the date of initiation of first immunotherapy. Survival analyses were performed on variants of unknown significance (UVS) and total ctDNA alterations using cutoffs of 3 and 6 alterations (the mean values for numbers of these alterations across patients), respectively. All studies and analyses were performed in accordance with the ethical guidelines of the Declaration of Helsinki and the Belmont Report per a University of California San Diego, Internal Review Board-approved protocol (NCT02478931) and the investigational treatment protocols for which the patients gave written consent.

ctDNA NGS

Sequencing was performed by a Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP)-accredited clinical laboratory, Guardant Health, Inc. (http://www.guardanthealth.com). The Guardant360 (54 to 70 gene) test identifies characterized and VUS tumor-related genomic alterations within cancer-related genes. The data are analyzed from ctDNA extracted from plasma (two 10-mL blood tubes). This ctDNA assay has a sensitivity and specificity of 85%+ and >99.9999%, respectively, for detection of single-nucleotide variants in tumor tissue of advanced cancer patients (9).

Statistical analysis

For comparing rates of SD ≥6 months/PR/CR, the Fisher exact test was used to calculate P values with a 95% confidence interval (CI). For PFS and OS, Kaplan–Meier analysis was used with the log-rank (Mantel–Cox) test to generate P values. Hazard ratios and confidence intervals using log-rank analysis were also calculated. Those patients whose status was known and who had not progressed (for PFS) or died (for OS) at the time of last follow-up or the cutoff date for analysis (January 12, 2017) were censored at that date. Kaplan–Meier analyses for PFS and OS were also performed based on status at a 2-month landmark.

Results

Patient demographics

Overall, 66 patients were evaluable for SD ≥6 months/PR/CR; 69 patients were evaluable for PFS and OS (N = 18 tumor types; Table 1). Median patient age was 56 years (range, 22–85 years). Forty-three patients (62.3%) were men. The most common tumor types were melanoma, lung cancer, and head and neck cancer. The most common type of immunotherapy used was anti–PD-1 or PD-L1 monotherapy, which was administered to 54 patients (79.7%).

ctDNA findings

Overall, 63 patients (91%) had at least one ctDNA alteration. The median number of VUSs per patient was 2 (range, 0–20; mean = 3). The median number of characterized alterations per patient was 1 (range, 0–25; mean = 3). The median number of total alterations per patient (which include characterized and total VUSs) was 3 (range, 0–37; mean = 6).

Of 69 patients, 20 (29%) had >3 VUSs in their circulating-tumor DNA (ctDNA) versus 71% with ≤3 VUS ctDNA alterations. Twenty-three patients (33.3%) had ≥6 total ctDNA alterations (characterized alterations plus total VUS alterations) and 66.7% had <6 total ctDNA alterations.

Relationship of immunotherapy outcome and number of ctDNA alterations

Response (SD ≥6 months/PR/CR). Overall, 16 of 66 patients (24%) achieved SD ≥6 months/PR/CR. Rates of SD ≥6 months/PR/CR differed significantly in those with >3 VUS alterations versus those with ≤3 VUS alterations (45% vs. 15%, respectively; P = 0.014). Similarly, SD ≥6 months/PR/CR rates were 40.9% versus 15.9% (P = 0.025; ≥6 vs. <6 total ctDNA alterations, respectively; Table 1).

PFS

The median PFS for 69 patients in the study was 2.3 months (95% CI, 0.7–5.0 months; Table 1). The median PFS for patients with >3 versus <3 VUS was 3.84 versus 2.07 months (P = 0.019; HR, 0.52; 95% CI, 0.31–0.87; Fig. 1A). The median PFS for patients with ≥6 versus <6 total ctDNA alterations was also significantly different: 2.85 versus 2.19 months (P = 0.025; HR, 0.59; 95% CI, 0.35–0.99; Supplementary Fig. S1A).

Two-month landmark analysis for PFS

Those patients achieving ≥2 months PFS from start of immunotherapy were included in this analysis (N = 41). These patients were further subdivided into responders (those achieving CR or PR; N = 15) and nonresponders (SD, or progressive disease, PD; N = 26). A landmark comparing responders to nonresponders in the VUS >3 alterations group showed median PFS 23.2 months versus 2.3 months (P = 0.0004; HR, 0.15; 95% CI, 0.035–0.61;

Table 1. Demographics and baseline characteristics of patients who received ctDNA testing and checkpoint inhibitor–based therapy ($N = 69$ patients)

Variable	All patients *N* (%, if applies)	20 patients with VUS > 3 *N* (%, if applies)	49 patients with VUS ≤ 3 *N* (%, if applies)	*P*[c]
Age at diagnosis, years				
Median (range)	56.38 (21.89–85.32)	55.51 (33.63–75.05)	59.48 (21.89–85.33)	$P = 0.41$ (Mann–Whitney)
Mean ± SD	56.38 ± 14.15	54.21 ± 13.28	57.27 ± 14.25	
Gender, M (%) F (%)	43 (62.3%); 26 (37.7%)	8 (40%); 12 (60%)	18 (36.7%); 31 (63.3%)	
Diagnoses, *n* (%)				
Skin cancer	15 (21.7%)	6 (30%)	9 (18.4%)	$P = 0.34$
Melanoma	10 (14.5%)	3 (15%)	7 (14.3%)	$P = 1.0$
Squamous cell	3 (4.3%)	2 (10%)	1 (2%)	$P = 0.0073$
Basal cell	2 (2.9%)	1 (5%)	1 (2%)	$P = 0.71$
NSCLC	19 (27.5%)	1 (5%)	18 (36.7%)	
Head and neck cancer	9 (13.0%)	3 (15%)	6 (12.2%)	
Renal cell cancer	3 (4.3%)	0 (0%)	3 (6.1%)	
Gastrointestinal cancer	6 (8.7%)	2 (10%)	4 (8.2%)	
Colorectal	4 (5.8%)	2 (10%)	2 (4.1%)	
Appendix	1 (1.4%)	0 (0%)	1 (2%)	
Gastroesophageal	1 (1.4%)	0 (0%)	1 (2%)	
Bladder cancer (TCC)	2 (2.9%)	1 (5%)	1 (2%)	
Liver cancer (HCC)	3 (4.3%)	0 (0%)	3 (6.1%)	
Thyroid cancer	2 (2.9%)	1 (5%)	1 (2%)	
Breast cancer	3 (4.3%)	3 (15%)	0 (0%)	
Neuroendocrine cervical cancer	1 (1.4%)	1 (5%)	0 (0%)	
Unknown primary	3 (4.3%)	2 (10%)	1 (2%)	
Brain cancer (GBM)	1 (1.4%)	0 (0%)	1 (2%)	
Adrenal cancer	1 (1.4%)	0 (0%)	1 (2%)	
Uterine cancer	1 (1.4%)	0 (0%)	1 (2%)	
Type of immunotherapy[a], *n* (%)				
Anti-CTLA4 alone	3 (4.3%)	0 (0%)	3 (6.1%)	$P = 1.0$
Anti-CTLA4/anti–PD-1 combination	4 (5.7%)	2 (10%)	2 (4.1%)	
Anti–PD-1 or anti–PD-L1 alone	54 (79.7%)	16 (80%)	38 (77.6%)	
Other anti–PD-L1-containing combinations	8 (11.6%)	2 (10%)	6 (12.2%)	
Patients with ≥6 total (characterized + VUS) ctDNA alterations, *n* (%)	23 (33.3%)	19 (95%)	4 (8.2%)	$P = 0.0001$
SD ≥6 months/CR/PR, *n* (%)[b]	16/66 (24%)	9/20 (45%)	7/46 (15%)	$P = 0.014$
Median PFS, months[b]	2.30 (95% CI, 0.7–5.0)	3.84	2.07	$P = 0.019$ (HR 0.52; 95% CI, 0.31–0.87)
Median OS, months[b]	15.34 (95% CI, 6.80–15.68)	Not reached	10.72	$P = 0.042$ (HR 0.39; 95% CI, 0.18–0.83)
		23 patients with ≥6 total ctDNA alterations N (%)	**46 patients with <6 total ctDNA alterations N (%)**	
SD ≥6 months/CR/PR, N (%)[b]	16/66 (24%)	9/22 (40.9%)	7/44 (15.9%)	$P = 0.025$
Median PFS, months[b]	2.30 (95% CI, 0.7–5.0)	2.85	2.19	$P = 0.046$ (HR 0.59; 95% CI, 0.35–0.99)
Median OS, months[b]	15.34 (95% CI, 10.6–23.9)	Not reached	10.79	$P = 0.37$ (HR 0.69; 95% CI, 0.32–1.5)

Abbreviations: ctDNA, circulating tumor deoxyribonucleic acid; CI, confidence interval; GBM, glioblastoma multiforme; HCC, hepatocellular carcinoma; *P* = probability; SD, standard deviation; TCC, transitional cell carcinoma; VUS, variant of undetermined significance.

[a]Initial immunotherapy regimen received by the patient.

[b]$N = 66$ patients evaluable for SD ≥6 months/PR/CR; $N = 69$ patients evaluable for PFS and OS.

[c]*P* values calculated only when at least 10 patients were assessable in a category.

Fig. 2A). Similarly, 2-month landmark analysis (responders vs. nonresponders) in the ≥6 total ctDNA alterations group showed median PFS 23.2 versus 2.3 months ($P = 0.0006$; HR, 0.15; 95% CI, 0.038–0.63; Supplementary Fig. S2A).

Landmark analyses were also performed for responders versus nonresponders in the ≤3 VUS alteration group and <6 total alteration group. Median PFS was significantly improved for responders in both groups (Fig. 2B; Supplementary Fig. S2B).

Landmark analyses for responders in the >3 versus ≤3 VUS alteration groups showed a PFS of 23.2 versus 11.7 months, but this was not statistically significant. However, there were only 8 and 7 patients in each group, respectively (Fig. 2C). Analogous results were found for the ≥6 versus <6 total ctDNA alteration groups (Supplementary Fig. S2C).

Similar landmark analyses were done for nonresponders in the >3 VUS versus ≤3 VUS alterations groups ($N = 7$ vs. $N = 19$ patients, respectively), which showed a median PFS of 2.3 versus 3.6 months ($P = 0.82$; Fig. 2D); analogous results were found for the ≥6 versus <6 total ctDNA alterations groups (Supplementary Fig. S2D).

Overall survival. The median OS for 69 patients in the study was 15.3 months (95% CI, 10.6–23.9 months) from the start of immunotherapy (Table 1). The median OS for patients with >3 VUS alterations was not reached versus 10.72 months for patients with ≤3 VUS ($P = 0.042$; HR, 0.39; 95% CI, 0.18–0.83; Fig. 1B). The median OS for patients with ≥6 total ctDNA alterations was not reached versus 10.79 months for those with

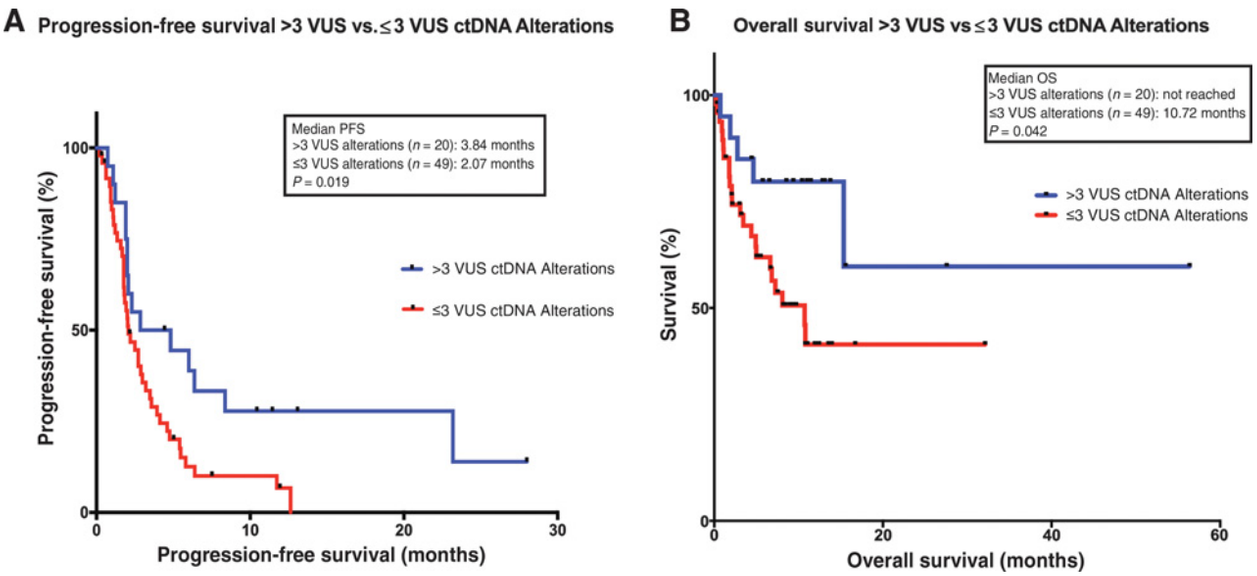

Figure 1.

PFS and OS for VUS >3 versus ≤3 groups. **A,** PFS is shown for 69 patients treated with checkpoint inhibitor–based immunotherapy. Comparison groups are those with >3 VUS ctDNA alterations (in blue) versus ≤3 VUS ctDNA alterations (in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients who are still progression-free at the designated time; they were censored at that point. **B,** OS is shown for 69 patients treated with checkpoint inhibitor–based immunotherapy. Comparison groups are those with >3 VUS ctDNA alterations (in blue) versus ≤3 VUS ctDNA alterations (in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients still alive at the designated time; they were censored at that point.

<6 total ctDNA ($P = 0.37$; HR, 0.69; 95% CI, 0.32–1.5; Supplementary Fig. S1B).

Two-month landmark analysis for OS. Those patients achieving ≥2 months OS from start of immunotherapy were included in this analysis ($N = 54$). These patients were also subdivided into responders (CR/PR, $N = 15$) and nonresponders (SD/PD, $N = 39$). The 2-month landmark comparing responders to nonresponders in the >3 VUS alterations group showed median OS was not reached for responders versus 15.34 months for nonresponders ($P = 0.11$; log-rank HR could not be calculated; Fig. 3A). Analysis at the 2-month landmark for responders versus nonresponders in the ≥6 total ctDNA alteration group showed median OS of not reached versus 15.34 months ($P = 0.02$; log-rank HR could not be calculated; Supplementary Fig. S3A).

Landmark analyses were also performed for responders versus nonresponders in the ≤3 VUS alteration group (not reached vs. 10.79 months; $P = 0.21$) and <6 total alteration group ($P =$ not significant; Fig. 3B; Supplementary Fig. S3B).

Two-month landmark analyses for responders in the >3 versus ≤3 VUS alteration groups ($N = 8$ vs. $N = 7$, respectively) and also ≥6 versus <6 total alteration groups ($N = 8$ vs. $N = 7$, respectively) were also performed. Median OS was not reached in any cohort and was not statistically different between cohorts (Fig. 3C; Supplementary Fig. S3C). Similar analyses were done for nonresponders in the >3 versus ≤3 VUS alteration groups ($N = 10$ vs. $N = 29$, respectively) and also ≥6 versus <6 total alteration groups ($N = 11$ vs. $N = 28$, respectively), without statistically significant differences (Fig. 3D; Supplementary Fig. S3D).

Discussion

To our knowledge, this is the first study to demonstrate that increased mutational burden, as reflected by the number of blood-derived ctDNA alterations, correlates with response to checkpoint inhibitor–based treatments. Previous studies have shown a correlation between tumor mutational burden, as measured in tissue, and response to immunotherapy in diseases such as lung cancer, melanoma, and urothelial cancer (8, 10–12). Because ctDNA can be obtained by a blood test without need for an invasive tissue biopsy, our current results may be clinically exploitable.

Biomarkers predicting response to immunotherapy have been investigated at the tissue DNA, RNA, and protein level. For example, at the DNA level, PCR detection of altered microsatellite foci in colorectal cancer has been predictive of response to anti–PD-1-directed therapy (13). Microsatellite instability is associated with defects in mismatch repair (MMR) genes that result in a hypermutated state, which presumably enhances the chance of response by increasing immunogenic neoantigen production by a hypermutated genome (8, 14–16). At the transcript level, mRNA sequences reflecting CD8-positive T cell and expanded immune signatures within the tumor microenvironment also predict response to checkpoint inhibitor therapy (17, 18). And, at the protein level, PD-L1 expression (IHC) in tumor and/or stromal cells is associated with higher response rates to checkpoint inhibitors, though IHC variability limits the precision of this assay (3, 19, 20). Specific mutational signatures, such as *kataegis*, a pattern of base mutations associated with APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide) family overexpression (correlating with viral presence) are also linked to PD-L1 overexpression (21).

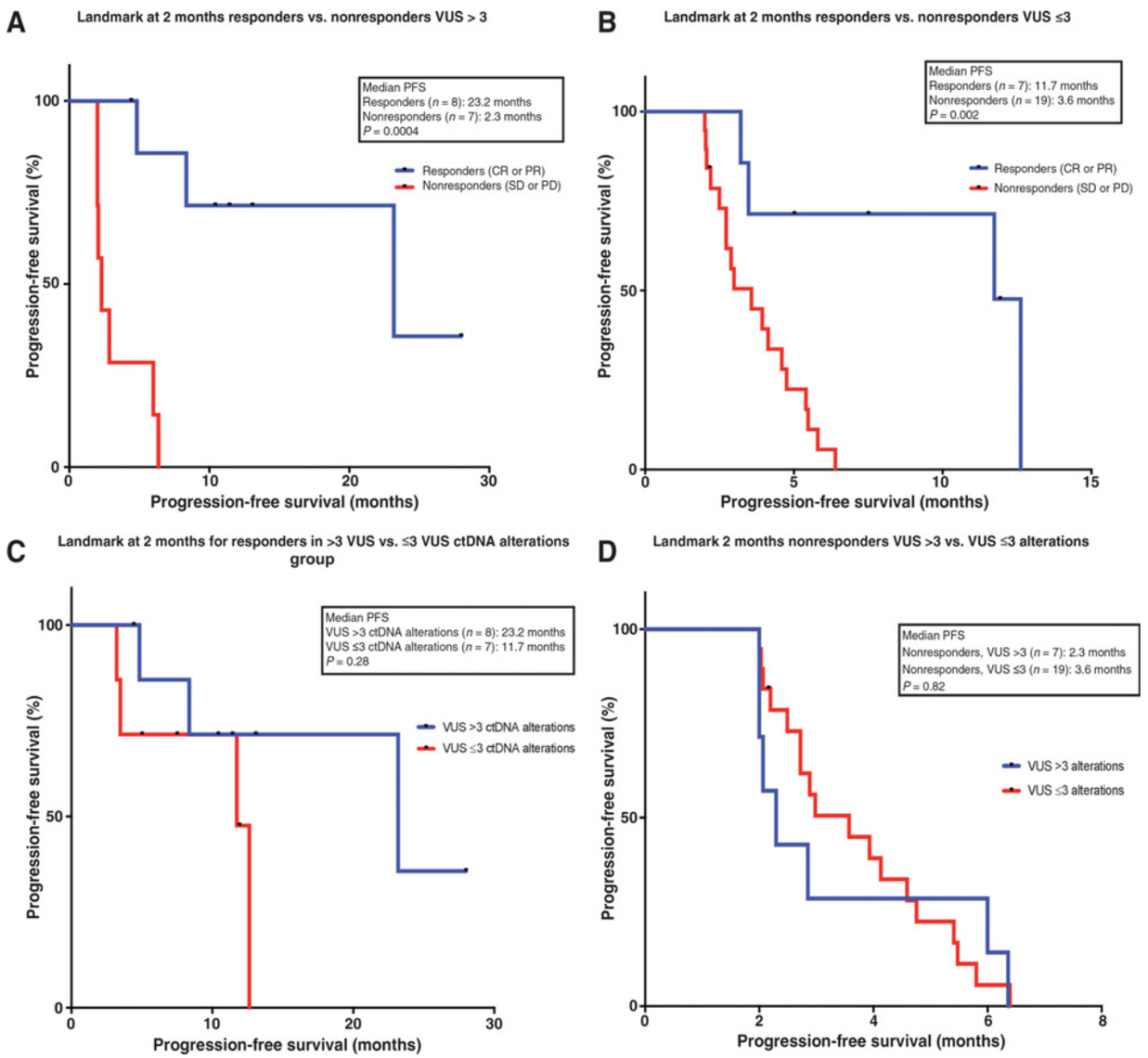

Figure 2.

Landmark analyses of PFS at 2 months for responders and nonresponders, VUS >3 versus ≤3 groups. **A,** A 2-month landmark study for PFS is shown for 15 patients treated with checkpoint inhibitor–based immunotherapy who had >3 VUS ctDNA alterations. Comparison groups are those who achieved response (CR or PR; in blue) versus those who did not achieve response (SD or PD; in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients who are still progression-free at the designated time; they were censored at that point. **B,** A 2-month landmark study for PFS is shown for 26 patients treated with checkpoint inhibitor–based immunotherapy who had ≤3 VUS ctDNA alterations. Comparison groups are those who achieved response (CR or PR; in blue) versus those who did not achieve response (SD or PD; in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients who are still progression-free at the designated time; they were censored at that point. **C,** A 2-month landmark study for PFS is shown for 15 patients treated with checkpoint inhibitor–based immunotherapy who had achieved response (CR or PR). Comparison groups are those with >3 VUS ctDNA alterations (in blue) versus ≤3 VUS ctDNA alterations (in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients who are still progression-free at the designated time; they were censored at that point. **D,** A 2-month landmark study for PFS is shown for 26 patients treated with checkpoint inhibitor–based immunotherapy who had not achieved response (SD or PD). Comparison groups are those with >3 VUS ctDNA alterations (in blue) versus ≤3 VUS ctDNA alterations (in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients who are still progression-free at the designated time; they were censored at that point.

As mentioned above, the role of hypermutated genomes in response to checkpoint inhibitors is illustrated by several studies. For instance, when comparing MMR-deficient versus MMR-proficient colorectal cancer, the overall response rate to pembrolizumab was about 60% versus 0% (22). Other factors contributing to hypermutation include dysfunction of DNA polymerase via germline mutations and molecular "smoking signatures" in NSCLC tumors that lead to differential mutational landscapes and

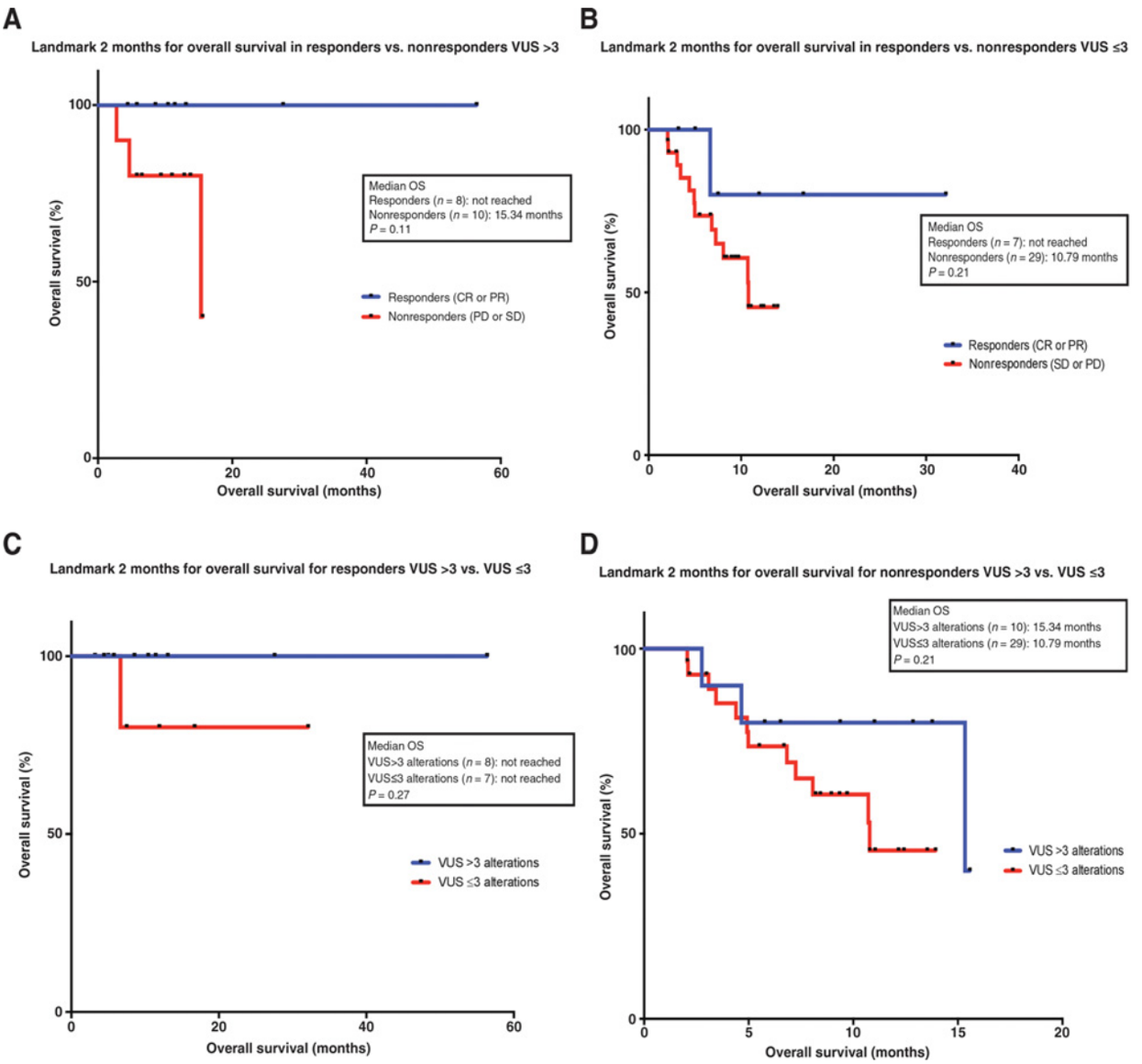

Figure 3.

Landmark analyses at 2 months for OS in responders and nonresponders, VUS >3 versus ≤3 groups. **A,** A 2-month landmark study for OS is shown for 18 patients treated with checkpoint inhibitor–based immunotherapy who had >3 VUS ctDNA alterations. Comparison groups are those who achieved response (CR or PR; in blue) versus those who did not achieve response (SD or PD; in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients still alive at the designated time; they were censored at that point. **B,** A 2-month landmark study for OS is shown for 36 patients treated with checkpoint inhibitor–based immunotherapy who had ≤3 VUS ctDNA alterations. Comparison groups are those who achieved response (CR or PR; in blue) versus those who did not achieve response (SD or PD; in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients still alive at the designated time; they were censored at that point. **C,** A 2-month landmark study for OS is shown for 15 patients treated with checkpoint inhibitor–based immunotherapy who had achieved response (CR or PR). Comparison groups are those with >3 VUS ctDNA alterations (in blue) versus ≤3 VUS ctDNA alterations (in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients still alive at the designated time; they were censored at that point. **D,** A 2-month landmark study for OS is shown for 39 patients treated with checkpoint inhibitor–based immunotherapy who had not achieved response (SD or PD). Comparison groups are those with >3 VUS ctDNA alterations (in blue) versus ≤3 VUS ctDNA alterations (in red). Data are calculated by the method of Kaplan and Meier, with log-rank *P* values. Course 1, day 1 of first immunotherapy represents starting point. Tick marks represent patients still alive at the designated time; they were censored at that point.

upregulation of PD-L1 expression (16, 20, 23, 24). As mentioned, other publications show a correlation between high tissue tumor mutational burden and checkpoint inhibitor response in several different tumor types (8, 10–12).

It therefore appears that tissue tumor mutational burden is a useful test for predicting response to immunotherapy. Still, presumably, mutational burden may change with time, and repeat biopsies may be difficult to obtain. Additionally,

obtaining tissue may be time consuming and invasive. Liquid biopsies that assess blood-derived ctDNA are noninvasive, easily acquired, and inexpensive. It is therefore of interest that, in our study, we demonstrate that overall response rates for patients in the high alteration groups (VUS>3 or total alterations $\geq$6) is significantly higher than in those in low alteration groups (VUS $\leq$3 or total alterations <6; 45% vs. 15%, respectively, for the VUS group, $P = 0.014$). Similarly, overall PFS and OS were significantly improved in patients with high VUS compared with low VUS alteration number. Analogous results for response rate and PFS were found when all alterations (not just VUS) were examined and dichotomized at $\geq$6 versus <6 alterations (Supplementary Fig. S1A and S1B). In the 2-month landmark analysis, PFS was best in those who had a response to checkpoint inhibitor–based therapy and also fell into the high alteration groups (PFS $\sim$23 months versus $\sim$2 months; responders vs. nonresponders; $P < 0.0006$; Fig. 2A; Supplementary Fig. S2A). In contrast, 2-month landmark PFS for responders versus nonresponders in the low alteration group (VUS <3) was 11.7 versus 3.6 months ($P = 0.002$; Fig. 2B). Similarly, 2-month landmark for OS showed longer survival in the responders versus nonresponders in the high alteration group (though not in the low alteration group). The small numbers of patients in each of the latter subgroups may have, however, precluded reaching statistical significance. Taken together, these data suggest that the hypermutated state, as assessed by ctDNA, correlates with better outcomes after checkpoint inhibitor–based immunotherapy, and that responders with hypermutated ctDNA had a median PFS that was close to two years.

There are several limitations to this study, including the fact that it is retrospective and the sample size is small. It will be important to perform larger prospective studies in the future. Furthermore, the number of genes assayed in our ctDNA analysis was only between 54 and 70. Unlike targeted NGS of tumor tissue, which often tests for hundreds of genes and allows a relatively accurate estimate of total mutational burden, targeted NGS of plasma cfDNA provides only a limited snapshot of the cancer genome. More extensive ctDNA gene panels merit investigation to determine if they increase the correlative value of our findings. Interestingly, Weiss et al. recently reported that chromosomal instability as reflected by copy number variation in cell-free DNA correlated with response to immunotherapy, consistent with our observations with number of alterations (25). Future studies should also compare cfDNA and tissue NGS mutational burden, preferably with biopsies and blood tests obtained on the same day. Yet another limitation is that, while the majority of cfDNA analysis was done prior to initiation on checkpoint inhibitor–based immunotherapy (56 of 66 evaluable patients), 10 patients began immunotherapy prior to cfDNA collection (median, 0.4 months). In the 56 patients who had cfDNA testing prior to or on the day of initiation of immunotherapy, the median time between cfDNA testing and start of immunotherapy was 1.6 months (range, 0–12 months). It is unknown at this time how treatment initiation may have influenced cfDNA results in these situations. Finally, we studied diverse tumor types and therefore the impact of hypermutated DNA in individual histologies was not assessable. Even so, the results may suggest generalizability across malignancies.

In summary, liquid biopsies provide several advantages in that they are easily obtained and less expensive than tissue biopsies. The ctDNA derived from blood may also represent shed DNA from multiple metastatic sites, whereas tissue genomics reflects only the piece of tissue removed. Our data suggest that ctDNA-determined hypermutated states predict improved response, PFS, and OS after checkpoint inhibitor therapy across histologies. Larger prospective studies are warranted to corroborate these findings.

Disclosure of Potential Conflicts of Interest

S.P. Patel reports receiving speakers bureau honoraria from Boehringer Ingelheim and Merck, and reports receiving commercial research support from Bristol-Myers Squibb, Eli Lilly, Genentech, GuardantHealth, Incyte, MedImmune/AstraZeneca, and Pfizer. A.G. Sacco is a consultant/advisory board member for Pfizer. R. Kurzrock is an employee of and has ownership interests (including patents) in CureMatch, Inc., is a consultant/advisory board member for Actuate Therapeutics and Xbiotech, and reports receiving commercial research grants from Foundation Medicine, Genentech, Guardant, Merck Serono, Pfizer, and Sequenom. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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