Brief Report

Brief Report: Methylation-Based ctDNA Serial Monitoring Correlates With Immunotherapy Response in NSCLC

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Abstract

This article will evaluate methylated circulating tumor DNA to monitor response to anti-PD1 based immunotherpy in non-small cell lung cancer.

Purpose: Circulating tumor DNA (ctDNA) can reflect the genetic and epigenetic composition of malignancies and can serve as a noninvasive biomarker for cancer diagnostics and monitoring. This study aimed to evaluate the utility of a methylation-based ctDNA assay as a predictive tool in non-small cell lung cancer (NSCLC) anti-PD1 based immunotherapy monitoring. **Methods:** We evaluated a cohort of 20 patients with NSCLC treated with anti-PD1 based immunotherapy that had both baseline and follow-up blood draws as well as outcome data available. Tumor Methylation Scores (TMS) were measured with an amplicon-based, multiplexed cfDNA assay that utilizes quantitative counting templates (QCTs) in conjunction with next-generation sequencing to count the number of methylated molecules at more than 500 genomic locations that are hypermethylated in cancer tissue. The association between TMS and real-world progression-free survival (rwPFS) on therapy was conducted using Cox proportional hazards model and plotted using the Kaplan-Meier method. **Results:** The change in TMS measured 4-10 weeks post-treatment initiation strongly correlated with immunotherapy response, as measured by rwPFS ($P < 0.0001$), compared to a weaker correlation of imaging RECIST v1.1 measurements with rwPFS ($P = 0.55$). Furthermore, TMS tracked with tumor burden on therapy in real-world cases. **Conclusions:** In this real-world dataset of NSCLC patients treated with anti-PD1 immunotherapy regimens, the TMS score measured within a 4-10 week window after treatment initiation can be predictive of response to therapy. Beyond this window, the TMS score can be associated with rwPFS and tumor dynamics. Early evidence suggests that changes in the methylation profile may be informative for monitoring occurrence of new somatic mutations. The cases presented demonstrate the application of using TMS for serial therapeutic response monitoring.

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Introduction

The use of circulating tumor DNA (ctDNA) from plasma has emerged as an important biomarker encompassing clinical applications which span screening, recurrence monitoring, and measuring responsiveness to therapies[1,2] However, tracking a limited number of somatic mutations may not accurately represent the tumor's composition, especially in late-stage cases where extensive evolution and clonal heterogeneity can be influenced by systemic therapies.[1,3,4] To address constraints, innovative ctDNA-based strategies for therapy response monitoring are being developed, including the quantification of methylated loci.[5–7] Enhancement in sensitivity can be achieved by interrogating more methylated ctDNA loci, reducing sample variability, limiting reliance on specific oncogenic variants, and enabling the detection of serial changes over time.[8,9] Employing ctDNA based strategies can refine upon a number of limitations in the clinic including variations in tumoral PDL1 levels, histology-based differences, and unique patterns of inflammation which may influence the expectations of response with anti-PD1 based antibodies.

Here, we utilized an approach leveraging a methylation assay tailored to track tumor-specific ctDNA signals. By targeting

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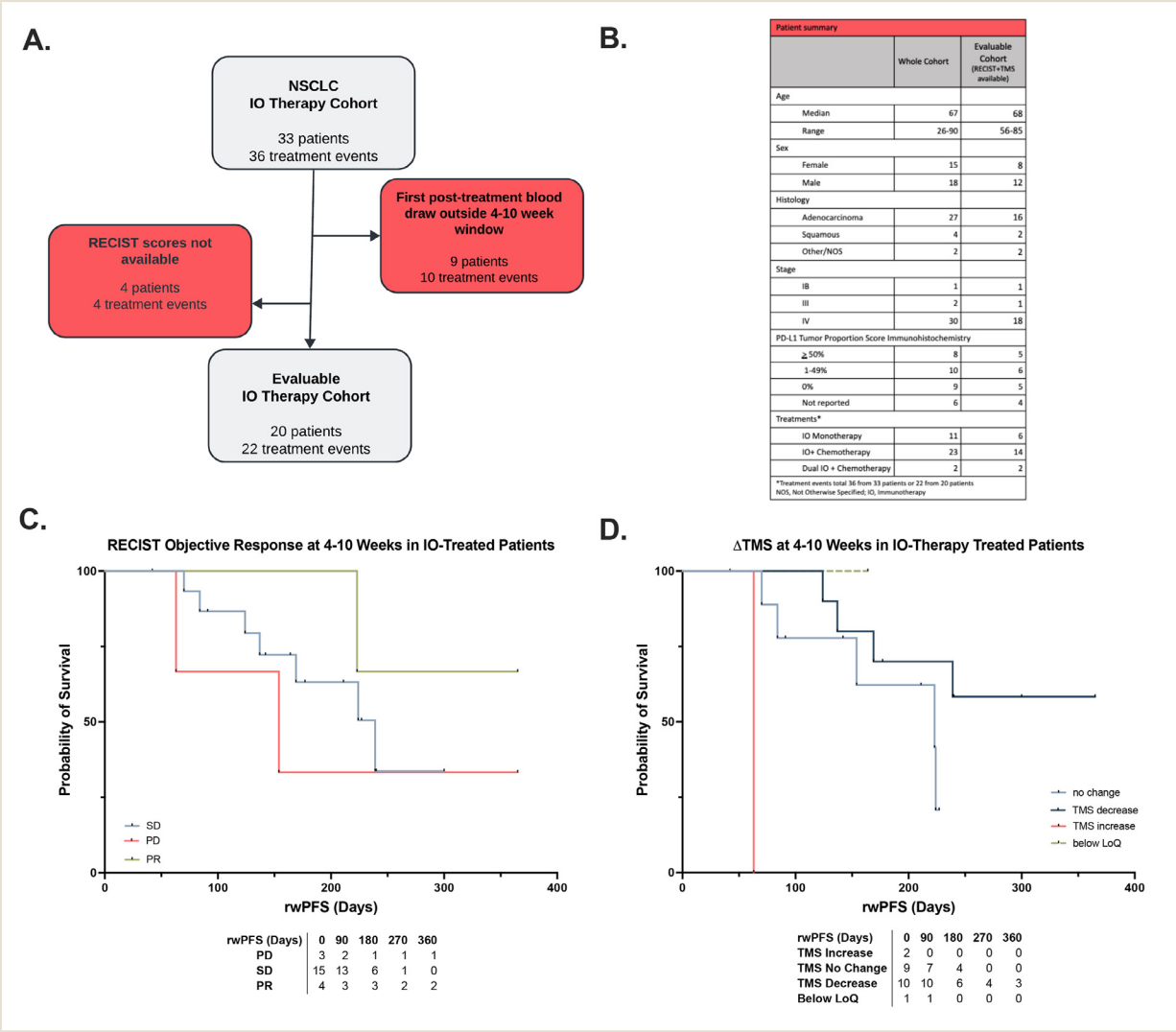
Figure 1 Tumor Methylation Score is predictive of treatment response. (A) CONSORT diagram of patients in the NSCLC cohort. (B) Table of patient demographics and clinical characteristics for the whole cohort ($n = 33$ patients, 36 events) and the evaluable cohort for whom both RECIST scores and TMS in 4-10 week window were available ($n = 20$ patients, 22 events). (C) Kaplan-Meier plot of the association between RECIST score and rwPFS for IO-treated patients ($n = 22$ events) $P = 0.55$. (D) Kaplan-Meier plot of the association between delta TMS and rwPFS for IO-treated patients in the 4-10 week window ($n = 22$ events), $P < 0.0001$.



Patient summary

	Whole Cohort	Evaluable Cohort (RECIST+TMS available)
Age		
Median	67	68
Range	26-90	56-85
Sex		
Female	15	8
Male	18	12
Histology		
Adenocarcinoma	27	16
Squamous	4	2
Other/NOS	2	2
Stage		
IB	1	1
III	2	1
IV	30	18
PD-L1 Tumor Proportion Score Immunohistochemistry		
≥50%	8	5
1-49%	10	6
0%	9	5
Not reported	6	4
Treatments*		
IO Monotherapy	11	6
IO+ Chemotherapy	23	14
Dual IO + Chemotherapy	2	2

*Treatment events total 36 from 33 patients or 22 from 20 patients
NOS, Not Otherwise Specified; IO, immunotherapy

genomic loci that are uniquely hypermethylated in cancer cells and by subtracting background methylation signal coming from the patient's buffy coat, we have demonstrated a predictive value of the change in Tumor Methylation Score (TMS) in association with real world progression free survival (rwPFS) for patients on immunotherapy regimens.

Materials and Methods

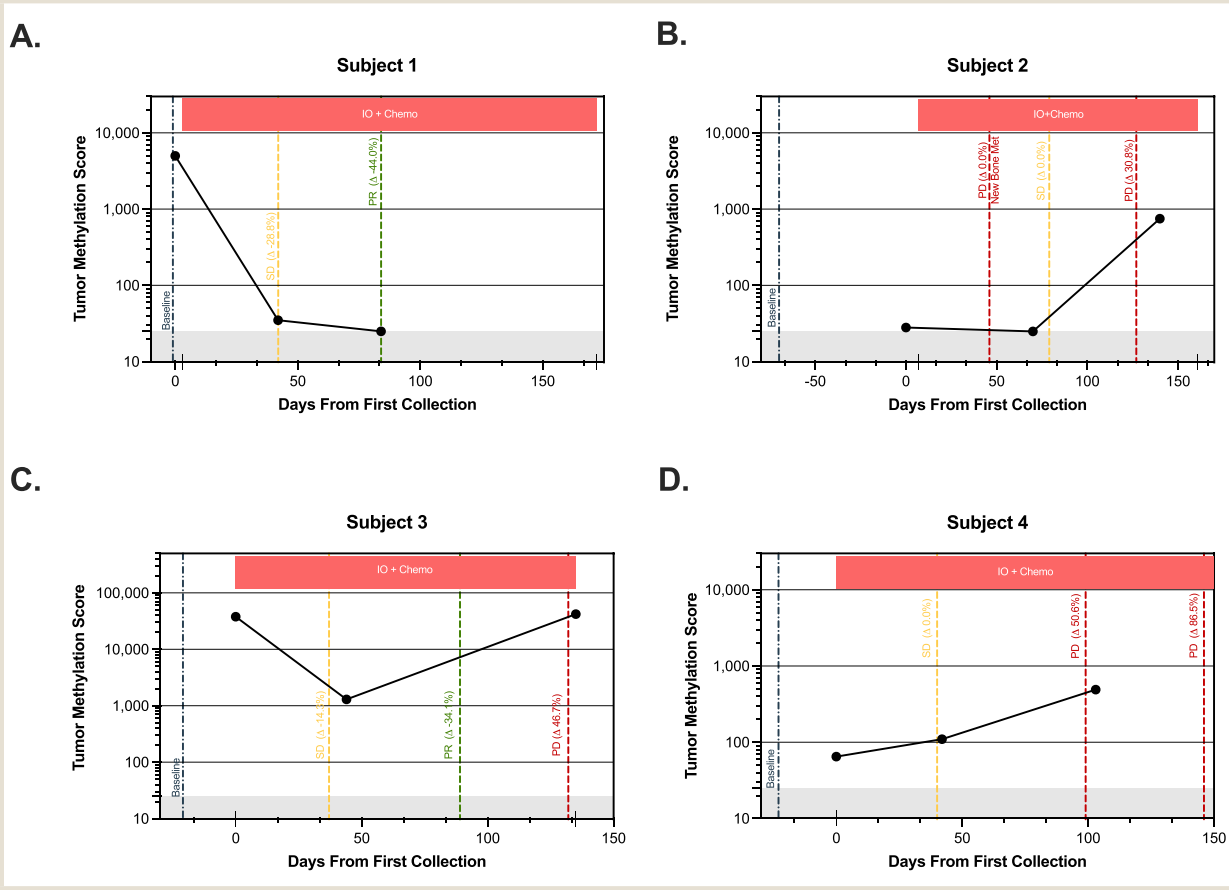
Cohort and Clinical Data Abstraction

This retrospective study was approved by the institutional review board of University of California San Diego. Thirty-three patients (36 treatment events across lines of therapy) were identified with advanced NSCLC treated with an anti-PD-1 immune check point inhibitor containing regimens who had longitudinally banked plasma samples, of which 20 patients (22 treatment events across therapy lines) fell within the analyzable 4-10 week window post-treatment initiation. Clinical data was extracted by retrospective chart review. Imaging data was retrospectively evaluated by RECIST v1.1 by a single investigator. Investigators were blinded to the clinical and imaging data until after TMS values were generated on all samples. Research was conducted in concordance with the Declaration of Helsinki.

Methylated ctDNA Monitoring

Patient plasma samples and buffy coat from baseline collections were analyzed using a validated next-generation sequencing-based

Figure 2 Clinical validation case studies. Tumor methylation scores correlate with disease outcomes across therapy types. (A-B) Clinical case studies of TMS corresponding with imaging assessments. (C-D) Representative clinical case studies in which the trend in TMS precedes the imaging outcome. Dashed lines represent RECIST evaluation objective responses.



assay that utilizes quantitative counting templates (QCTs).[10,11] Briefly, the assay quantifies the number of methylated molecules in both cfDNA and buffy coat at over 500 loci chosen to be hypermethylated in and specific to cancer, normalizes each measurement to 1000 molecular equivalents of universally methylated control loci, subtracts the buffy coat methylation from the cfDNA methylation, and sums across all 500 hypermethylated loci to generate the tumor methylation score (TMS) as a reflection of tumor burden. Compared to previous versions, the assay version employed here (v1.1) utilizes an updated bioinformatics pipeline that implements a weighted buffy coat background subtraction that corrects for survivorship/censoring bias of only observing non-negative values at each loci. This unbiased estimator reduces noise and allows for the limit of quantification to be set at TMS = 25. The change in TMS was calculated between the baseline draw and the first blood draw within the 4-10 week window after treatment. Changes in TMS were categorized as "increase," "decrease," or "no change" according to the following, pre-established, analytical and statistical specifications: an increase/decrease is called when (1) the percent change exceeds 15% and (2) the $\log_2$ likelihood of an increase/decrease compared to no change is more than 3. If the TMS at both timepoints was below the limit of quantification, the call was noted as below the quantifiable range.

Statistical Analysis

Real-World Progression Free Survival (rwPFS)

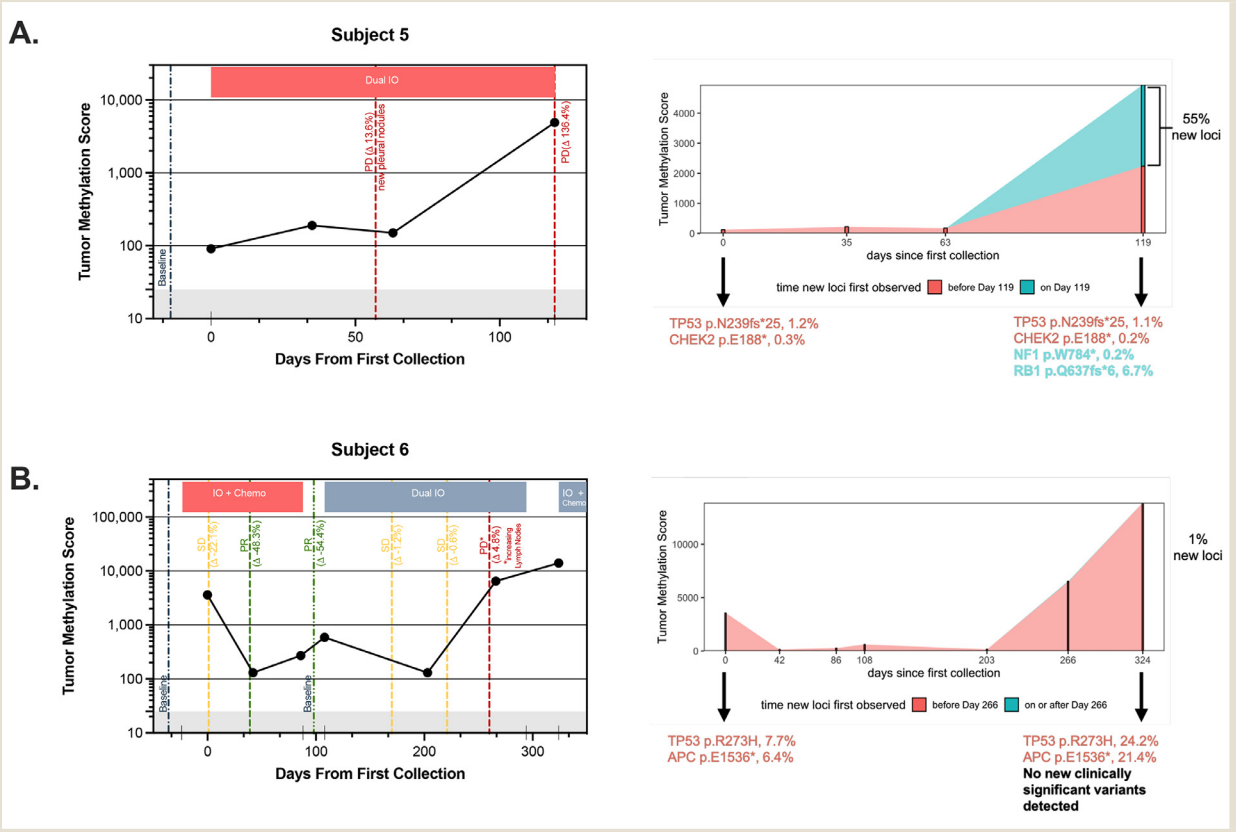
rwPFS was calculated as the difference, in days, between the start date and effective end date of the treatment line as previously described[12] All statistical analyses were conducted using R version 3.6.3 with packages survival (RRID: 3.2.13) and survminer (RRID: 0.4.9) To evaluate associations between variables and rwPFS, Cox proportional hazards were calculated with 95% confidence intervals. Statistical significance was assessed using the Wald test. Survival data was plotted using the Kaplan-Meier method. RECIST v1.1 was used to define objective response.

Results

Cohort

The cohort consisted of 33 patients (36 treatment events across lines of therapy) with NSCLC who had received immunotherapy (Figure 1A). For each treatment event, 3 longitudinal blood draws were collected representing a baseline (pre-treatment)

Figure 3 Changes in tumor methylation profile can correlate with appearance of new somatic mutations. (A) In Subject 5, TMS increased from 90 pretreatment to 4900 over 4 months (left). The overall TMS was divided by contributions from methylated loci that were detected before and after Day 119 (right). A total of 106 methylated loci were detected at any point before the last time point (Day 119). At the last timepoint, 113 newly methylated loci were detected contributing about 55% of total methylation, and indicated a significant change in tumor methylation profile. Somatic alterations were assessed with a comprehensive genomic profiling liquid biopsy assay using plasma collected at Day 0 and Day 119. We observed the appearance of new clinically meaningful alterations as noted in conjunction with the significant change in methylation profile. The VAFs of the alterations detected at baseline remained similar over time, concordant with the relatively unchanged amount of the baseline methylation profile (red). (B) A significant increase in TMS was also observed in Subject 6. However, very little change in tumor methylation profile was observed, with only 1% of methylation at Day 324 coming from loci that were never methylated before Day 266. Concurrently, there was no change in the diversity of somatic alterations measured at Day 0 and 324. The VAFs of the alterations detected at baseline increased, in concordance with the significant increase in the baseline methylation profile (red).



and 2 on-treatment samples. The majority of patients were advanced stage adenocarcinoma, and all patients received a regimen containing either immunotherapy or combination immunotherapy-chemotherapy (Figure 1B). At baseline, the percentage of samples with methylation signals above the assay's limit of quantification (LoQ) was 94.4% (34/36), with a median TMS value of 440.

On-Treatment Changes in Tumor Methylation Score is a Predictive Marker of Response to Immunotherapy

The current gold standard for assessing therapy response is evaluation of imaging results using RECIST v1.1 criteria. In this cohort of NSCLC patients treated with IO therapy, we examined the association between RECIST score and rwPFS in 20 patients for whom imaging data and TMS were available. There was an observed separation of curves when examining the association between objective response and rwPFS by imaging, however this trend did not reach significance ($P = 0.55$) (Figure 1C).

Previous studies have also shown that on-treatment changes in variant allele fractions (VAFs) are predictive of response in IO treated patients when using various thresholds, such as ctDNA clearance or 50% change within the first cycles of immunotherapy compared to baseline[13] As an additional biomarker of the tumor burden, we sought to similarly address whether the change in TMS from baseline was indicative of immunotherapy response. In the literature, iRECIST has documented cadence for imaging to be scheduled at 6-12 weeks for radiological assessment[14] In our study, we sought to understand whether a plasma collection at an earlier timepoint may provide additional response clues when scans were

completed. We measured the change in TMS obtained from patients who had available plasma samples at baseline and within 4-10 weeks post-treatment initiation. If patients had more than one blood draw in that window, the earlier time point was used. A total of 22 of the 36 treatment events had paired plasma samples collected within this predefined window with comparable RECIST scores. We first performed univariate analyses to determine which clinical factors in this cohort were associated with real world progression free survival (rwPFS). We found no significant associations between treatment type, histology, stage, or sex with rwPFS and were thus not included in further analyses. Given that this cohort includes both IO monotherapy and chemo-IO regimens, we examined the association between PD-L1 at ≥1% and rwPFS and, as expected, observed a significant association (HR 0.2, 95% CI, 0.05-0.92, $P < 0.05$)[15] When the treatment events were stratified by change in TMS (eg a statistically significant increase, decrease, or no change), there was a significant separation in rwPFS ($P < 0.001$) (Figure 1D). As such, compared to RECIST ($P = 0.55$), the increase or decrease as measured by TMS was a potentially more sensitive predictor of response to therapy in this cohort (Figure 1C).

Clinical Utility of Methylation-Based Monitoring

The assay correlated with tumor burden for many subjects through therapy, and representative realworld cases are demonstrated in Figure 2. In some instances, the assay can add additional evidence to support imaging evaluation, as exemplified by subjects 1 and 2 (Figure 2A and B). Blood draws were obtained near the time of scan and changes in TMS corroborated the imaging results. The TMS is also predictive ahead of scan, as evidenced in subjects 3 and 4. In subject 3, the TMS showed a rapid decline, suggesting response, while the scan nearest the draw remained stable. However, the subsequent scan later identified partial response. At the last blood draw in the series a TMS score increase corroborated the scan results at that time (Figure 2C). Similarly, subject 4 showed a rising TMS, yet the first scan reported stable disease, only for later scans to confirm progressive disease that was affirmed by an even higher TMS score (Figure 2D).

Discussion

Methylated circulating tumor DNA (ctDNA) was detectable in nearly all of the advanced NSCLC patients in our study. Through the quantitation of methylated loci, there is a large number of potential genomic regions that can be serially quantified in any given tumor, thus increasing the accuracy of the analysis for monitoring. We observed that variations in TMS during treatment, as assessed in the 4-10 week post-therapy initiation window, may provide additional clarity of early therapeutic response. The large panel of methylated loci in this assay do not directly quantify disease-associated somatic variants, and the quantitation of the unique methylation profile may allow for more broadly capturing tumor evolution that could otherwise be missed on a more limited panel. For example, in a case study of longitudinal response monitoring with paired comprehensive genomic profiling (CGP), an increased TMS resultant from an increase in the diversity of methylated loci showed previously undetected, clinically relevant alterations in a concurrent CGP assay (Figure 3A). Conversely, in another case where the increase in TMS was resultant from more abundance of the same previously detected loci, no new somatic alterations were detected by CGP assay (Figure 3B). Based on these preliminary observations, further studies can be conducted to understand how methylation-based dynamics could be linked to changes in tumor sub-clonal heterogeneity. While additional studies are needed to determine the most clinically useful implementation of this assay to clinical practice, noninvasive serial monitoring may provide insight into time to progression and changes in tumor heterogeneity. Limitations of the study include modest sample size and the real-world nature of this dataset (including sample collection timing and outcomes data availability). The selection of samples with imaging outcomes may bias the cohort in that rapid progressors were not captured and included in study. Cumulatively, these data may support the applicability of ctDNA TMS in therapeutic response monitoring and highlight the importance of serial assessment.

Conclusion

Clinical Practice Points

- Serial tumor methylation assessments may serve as a surveillance strategy to determine treatment response.
- Circulating tumor DNA dynamics as detected by methylation signatures can correlate with real world progression free survival.
- Tumor methylation dynamics may reflect heterogeneity and the development of somatic mutations.

Disclosure

Patrick Ye, Matthew G Varga, Naomi Searle, Robb Viens, Sydne Langpap, Zeqian Li, and Gary Palmer are employees of BillionToOne, Inc.

CRediT authorship contribution statement

Angela Hsiao: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Brian Woodward:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Patrick Ye:** Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Data curation, Conceptualization. **Matthew G. Varga:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ghaith Altaie:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Kevin Lu:** Writing – review & editing, Software, Data curation, Conceptualization. **Naomi Searle:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Robb Viens:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Sydne Langpap:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Zeqian Li:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Gary Palmer:** Writing – review & editing, Writing – original draft,

Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hatim Husain:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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