Original Article

Circulating tumour DNA as a predictive biomarker for tumour response and prolonged clinical benefit with nivolumab in advanced non-small cell lung cancer

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ABSTRACT

Introduction: Immune checkpoint inhibitors (ICIs) have revolutionised the treatment of advanced non-small cell lung cancer (NSCLC). Quantitative monitoring of disease burden may prove predictive in these cases. We hypothesised that serial ctDNA molecular response assessment may be predictive of response to nivolumab. **Materials and Methods:** 44 NSCLC patients who received nivolumab were included. All the patients underwent response assessment by RECIST every four cycles. Peripheral blood samples were obtained at baseline and after four cycles of therapy, and the quantification of ctDNA was performed by qubit dsDNA HS assay. A variant allele fraction (VAF) of >0.3% was considered to be used for tracking. A cut-off change of 50% in VAF and ctDNA was considered for molecular response. Radiological evaluations were performed at baseline and every four cycles as per RECIST 1.1 criteria. **Results:** The median ctDNA concentration was higher in patients with time to progression (TTP) of <4 months ($P = 0.05$). Detectable alterations with >0.3% VAF were detected in 31 patients, and a molecular response of >50% was observed in patients with PR, stable disease (SD) and two patients with PD. The patients with <50%molecular response had a median PFS of 3.9 months vs. those with >50% had a median of 5.8 months ($P = 0.06$). A cut of 0.3 ng/microlitre baseline ctDNA concentration was predictive of PFS. **Conclusion:** This is an initial experience from India using liquid biopsy next-generation sequencing (NGS) for dynamic monitoring in immunotherapy-treated patients. A 50% cut-off for response as well as a baseline ctDNA 0f 0.3 ng/microlitre showed a trend for better PFS in these patients. This study highlights the need for serial monitoring. To our knowledge, this is among the first real-world Indian studies validating serial NGS-based ctDNA monitoring in immunotherapy-treated NSCLC. It demonstrates feasibility, predictive value, and real-world applicability in a lower-middle-income context.

KEY WORDS: Dynamic monitoring, liquid biopsy, nivolumab

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INTRODUCTION

Analogous to the targeted therapy paradigm, the development of immune checkpoint inhibitors (ICIs)

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has shown promise in the treatment of advanced non-small cell lung cancer (NSCLC), including in the first-line setting (Bronte G 2023).[1] However, despite the reported clinical benefit of these, acquired resistance eventually ensues, and no single predictive biomarker has been established which can predict responders versus non-responders.[2] This may be due to the dynamic nature of immune checkpoint interactions and the evolving tumour-immune microenvironment.[3] Currently, treatment monitoring relies on RECIST/iRECIST criteria, which may not capture early molecular changes predictive of response or resistance. These, however, are neither definitive nor sensitive. With the International Association for the Study of Lung Cancer (IASLC) statement recommendations advocating liquid biopsy in both diagnosis and monitoring of disease burden, appropriate selection of the patient in question as well as monitoring may be done using plasma-based ctDNA (circulating tumour deoxyribonucleic acid) levels in such patients.[4] Especially in cases of NSCLC, with the lung being an anatomically difficult site to access, repeated biopsies result in higher patient attrition as well as non-compliance.[5,6]

Recently, our group has reported the use of liquid biopsy for the detection of primary EGFR (epidermal growth factor receptor) mutation, using sensitive liquid biopsy techniques like amplification refractory melting system-polymerase chain reaction (ARMS-PCR) and droplet digital PCR.[7,8] However, next-generation sequencing-based detection of ctDNA levels and alterations with tracking of variants detected at baseline has also been demonstrated as a promising tool.[9] In a few real-world studies, next-generation sequencing (NGS)-based ctDNA monitoring has been reported both in the targeted therapy setting and the ICI setting, proving to be highly sensitive.[10] However, similar reports from this part of the world, especially the Indian subcontinent, are lacking, owing to socioeconomic restraints, lack of awareness in both patients and clinicians, as well as the nascence of the next-generation sequencing (NGS) technique in the country.[11]

Challenges with the implementation of liquid biopsy techniques include clarification of any potential predictive or prognostic associations with ICI therapy as well as validation in larger patient cohorts.[12] PDL1 (programmed death ligand 1) has been extensively evaluated in patients who receive immunotherapy with varying results.[13,14] No significant survival benefit was observed with tumours that did not express PDL1 in CheckMate.[15] Conversely, PDL1 expression was neither predictive of benefit nor prognostic in CheckMate 017 (NCT01642004). It was also seen that some patients with negative PDL1 expression responded to nivolumab.[16] As such, PDL1 seems to be an imperfect marker for predicting response, and the need of the hour is to identify better biomarkers.[17] Recently, Thompson *et al*. demonstrated the role of serial ctDNA as a biomarker of response and survival in NSCLC patients who were being administered pembrolizumab.[18]

This is a single-centre prospective study including patients with advanced NSCLC who have been treated with nivolumab.[19] We hypothesised that serial ctDNA molecular response assessment has the potential to be predictive of response to nivolumab.[18,20]

MATERIALS AND METHODS

Study design and patient accrual

This is a single-centre prospective observational study performed at Rajiv Gandhi Cancer Institute and Research Center, between 2020 and June 2023. Patients with histologically confirmed metastatic NSCLC scheduled to receive nivolumab (3 mg/kg) based therapy in any line were enrolled after informed consent. Patients with any other concurrent malignancy or presence of any actionable genomic alterations (*EGFR, ALK, ROS1, MET, NTRK,* and *BRAF* V600E) were excluded. PDL1 expression was assessed in the pretreatment specimens using the SP263 assay (VENTANA BenchMark system, Ventana Medical Systems, Inc., Tucson, AZ).

Response assessment

RECIST version 1.1 was employed for radiologic assessments. The efficacy of the drug was defined in terms of durable clinical benefit (DCB; complete response (CR), partial response (PR), and stable disease (SD) >6 months) or no durable benefit (NDB), progressive disease (PD) or SD <6 months).

Outcome definitions

The progression-free survival (PFS) was defined as the duration between the first nivolumab infusion and the date of first documented progression/death, whichever occurred earlier. The overall survival (OS) was defined as the duration between the date of diagnosis and the date of death/last follow-up.

Plasma collection and ctDNA extraction

Plasma was collected at the following timepoints:

- T0: Baseline (before first nivolumab dose)
- T1: After 4 weeks (prior to second cycle)
- T2: At first imaging response evaluation (after four cycles)
- T3+: Every six cycles or at radiological/clinical progression.

Blood was collected in either K2 EDTA tubes (BD Vacutainer® Blood Collection Tubes, Becton Dickinson, Franklin Lakes, USA) or Cell-Free DNA BCT® (Streck, La Vista, NE). The plasma fraction was separated from the blood cells by two consecutive rounds of centrifugation for 30 min at room temperature at 1600 × g. The collected plasma was then aliquoted and stored at − 80 °C until use. ctDNA was extracted from plasma volumes using a cell-free DNA extraction kit provided by Qiagen), according to the manufacturer's instructions. The ctDNA quantity was assessed with the dsDNA HS assay kit by the Qubit 2.0

Fluorometer (ThermoFisher Scientific). The final ctDNA concentration was assessed using real-time PCR.

NGS assay details

For all the samples, a targeted panel, Oncomine TagSeq Pan-Cancer Liquid Biopsy (Thermofisher Scientific, USA) comprising 52 genes and interrogating single-nucleotide variations (SNVs), indels, copy number alterations as well as gene rearrangements was used. NGS libraries were sequenced on an Ion S5™ instrument (Thermo Fisher Scientific) using semiconductor sequencing technology. Briefly, sequencing runs were planned on the Torrent Suite Software™ v5.8. Subsequently, the libraries were pooled and loaded on an Ion 540™ chip using the Ion Chef™ instrument (Thermo Fisher Scientific). The loaded chip was then sequenced using 500 flows. Raw data was processed automatically on the Torrent Server™ and aligned to the reference hg19 genome. Quality checks were performed manually for each sample based on the following metrics; number of reads per sample 15,000,000, on-target reads 90%, read uniformity 90%, median molecular coverage 5000× for a limit of detection of 0.3% variant allele frequency and a ctDNA input of 30-40 ng, median base coverage 30,000x.

NGS data analysis

Variant calling was performed on Ion Reporter™ Analysis Software v5.12 using the Oncomine™ TagSeq Pan-Cancer Liquid Biopsy w2.0 workflows. The analysis pipeline also includes signal processing, base calling, quality score assignment, adapter trimming, PCR duplicate removal, and control of mapping quality. Coverage metrics for each amplicon were obtained by running the Coverage Analysis Plugin software v5.10 (Thermo Fisher Scientific). Identified variants were considered if the variant had a molecular coverage of at least three, indicating that the variant was detected in three independent template molecules. Variants of clinical significance, potential clinical significance and uncertain significance were considered. Benign polymorphisms, potentially germline variants and clonal haematopoietic variants were filtered out. Finally, all candidate mutations were manually reviewed using the Integrative Genomics Viewer.

Mutation tracking and assessment of molecular response

To quantify ctDNA, the allelic fraction of mutant tumour-derived DNA was calculated with the help of NGS data. If more than one mutation is identified in a baseline sample, the mutation with the highest allelic fraction is used to track ctDNA levels over time. The assay has a sensitivity of 80% at a limit of detection (LOD) of 0.3% allelic frequencies (AF) and 99.9% at a LOD of 0.5% AF for single-nucleotide variants (SNV), insertions/deletions (INDEL). Specificity and accuracy are 99% for all variant classes and have a precision of 98% for SNV/INDEL and 99.9% for fusions. The molecular response was calculated using the SNVs, indels, and fusions, which had a minimum variant allele fraction (VAF) of 0.3%. The mean of all these in the baseline pretreatment sample was determined. This 0.3% criteria have been previously described by Raja *et al*., and was selected on the basis of 95-100% LOD.[21] The emergence of new variants or clonal evolution was not used in the assessment of molecular response, and only those present in the baseline sample were included for tracking and response assessments.

The molecular response is defined as the ratio of mean VAF on treatment to the mean VAF at baseline. The cut-off for responders has been taken as ≥49% and <49% for non-responders. This cut-off was deduced by receiver operator curve characteristics. Our cut-off is based on ROC characteristics, yielding a sensitivity of 92% and specificity of 90%. The 49% threshold was determined via ROC curve analysis, yielding a sensitivity of 92% and specificity of 90%. This aligns with prior studies, such as Zhang *et al*. (2021), who used similar VAF-based thresholds to dichotomise responders in serial ctDNA monitoring.[22,23]

Variants which may be a part of clonal hematopoiesis are well known when dealing with ctDNA. These variants, as reported earlier, are not affected by treatment, and their VAF largely remains unchanged during different therapeutic time points. Variants suggestive of clonal hematopoiesis (e.g. DNMT3A, TET2, and ASXL1) were filtered using known databases; however, paired WBC sequencing was not performed. Future studies should incorporate matched buffy coat analysis to delineate true tumour-derived ctDNA from CHIP variants.

Statistical analysis

Continuous variables were expressed as mean (SD) and categorical variables as count (%). Comparisons of baseline characteristics, ctDNA levels and molecular response were done using analysis of variance (ANOVA) for continuous variables and Chi-square or Fisher exact tests for categorical variables. Survival analyses on the basis of the median cut-off values are performed using the Kaplan–Meier method, providing median and *P* values. Comparisons were made using a log-rank test. PFS was defined as the time between the date of inclusion/date of starting nivolumab and the date of progression as determined by iRECIST/change of treatment due to any reason. OS for nivolumab was defined as the date of inclusion to date of last follow-up/death. Univariate and multivariate analyses were performed using logistic regression and Cox proportional hazard models. All statistical analyses were done using MedCalc QC (Ostend, Belgium) and conclusions made at 5% significance level. No formal correction for multiple testing was performed, given the exploratory nature of the study, but all *P* values are reported transparently.

RESULTS

Patient characteristics

A total of 44 patients were recruited who fulfilled the inclusion and exclusion criteria as per the study protocol.

The median age was 63 years, and there was a clear male preponderance. All were stage IV NSCLC after multiple lines of treatment and were treated with nivolumab. Adenocarcinoma and squamous histology were observed in 19 (%) patients each, and NOS in 6 (%) patients. The detailed demographic and clinical features are described in Table 1.

Molecular characteristics and ctDNA

Among the 44 patients, the median ctDNA at baseline was 0.63 ng/ul. This was higher than the median 0.23 ng/ul detected in 32/44 patients after four cycles of nivolumab ($P < 0.07$). The changes in ctDNA concentration of patients at baseline, after four cycles and the subsequent evaluation are depicted in Figure 1. The most frequent alteration detected was *TP53 (*36/44, 82%). The other alterations detected included *KRAS* (5/44, 11%), *PTEN* (3/44, 7%), *PIK3CA* (4/44, 9%), *BRAF* (1/44, 2.3%), *NRAS* (1/44, 2.3%), *EGFR* (exon 20 insertion in 1/44, 2.3%), and *RB1 (*2/44, 5%). The oncoplot depicting the distribution of variants detected is shown in Figure 2.

Table 1: Table depicting clinical and demographic features of the study cohort

Characteristic	*n* (Total)	%
Median age: 63 years (Range: 52-74)		
Gender		
Male	38	86%
Female	6	14%
Smoking status		
Ever	32	73%
Never	12	27%
ECOG PS		
0-2	35	80%
3-4	9	20%
Histology		
Adenocarcinoma	19	43%
Squamous	19	43%
NOS	6	14%
PDL1		
<1%	1	3%
1-49%	34	77%
≥50%	9	20%

ECOG PS: Eastern Cooperative Oncology Group Performance Status; NOS: Not otherwise specified; PDL1: Programmed death ligand 1

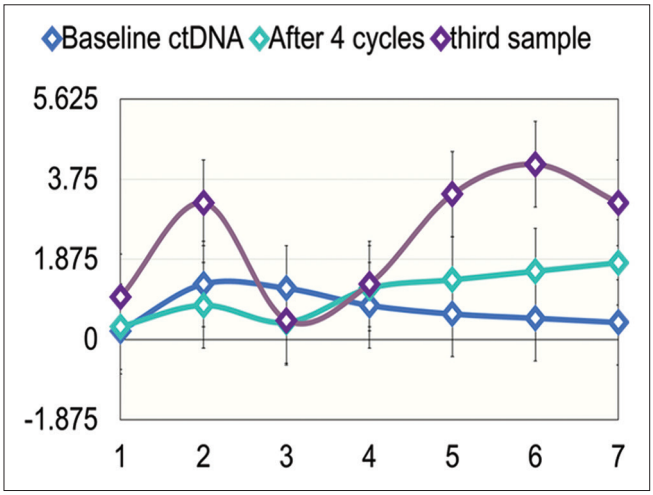

Figure 1: Graph depicting changes in ctDNA levels at different treatment timepoints in 7 representative patients from the study cohort. ctDNA: circulating tumour DNA

Survival parameters and molecular response

The median ctDNA concentration was higher in patients with PFS of <4 months than in those with PFS >4 months. ($P = 0.05$). Detectable alterations with >0.3% VAF were detected in 31 patients, and a molecular response of >49% was observed in patients with PR, SD and 2 patients with PD. The patients with <50%molecular response had a median PFS of 3.9 months vs. those with >50% response who had a median of 5.8 months ($P = 0.06$). Additional analysis revealed that a cut of 0.3 ng/microlitre baseline ctDNA concentration was predictive of PFS (>0.3 PFS of 4.3 months, vs. <0.3 PFS of 5.8 months). However, the ctDNA level at 4 weeks was also predictive of progression in those showing iUPD at first evaluation and PD subsequently. Waterfall depicting response in patients in relation to molecular response is illustrated in Figure 3.

The median overall survival (OS) in non-responders was 4 months (95%CI: 1.02-7.07) and was 13.3 months (95%CI: 9.2-24.3) in responders ($P < 0.004$). There were 23% long-term responders with median OS not reached at study endpoint (15 months). [Figure 4].

DISCUSSION

This is a single-centre experience of the utility of NGS-based liquid biopsy monitoring in patients of advanced NSCLC treated with nivolumab after initial therapy.[6] This study demonstrated that changes in the level of ctDNA and the mutation burden can be predictive of early progression, suggesting a potential for real-time monitoring of impending immunotherapy resistance.[22,23] Although a restricted sample size, the study was powered and has yielded demonstrable overall survival benefit, with

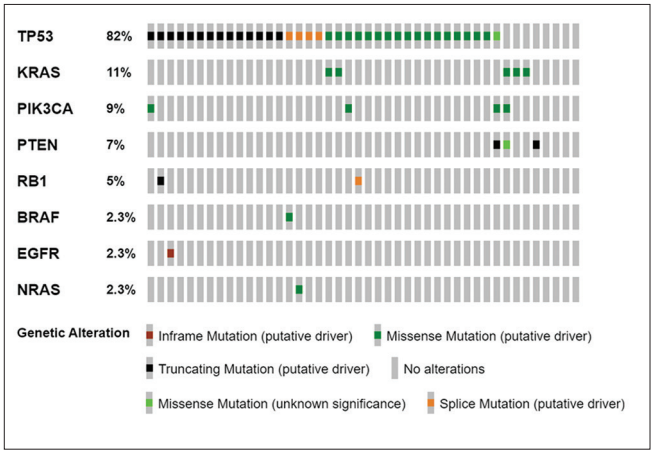

Figure 2: Oncoprint depicting the mutations detected in the study cohort at diagnosis

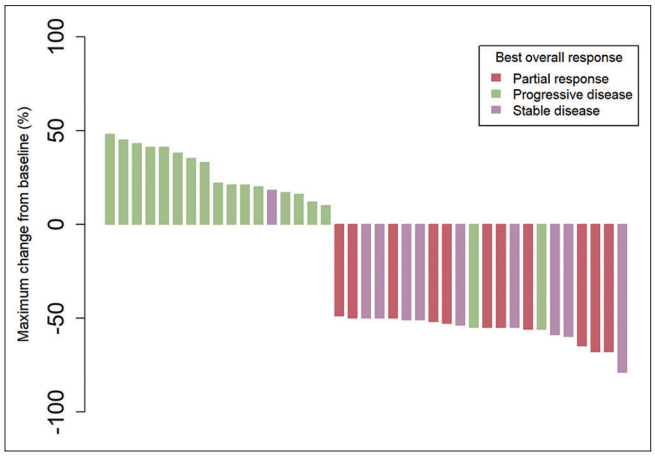

Figure 3: Waterfall plot depicting the response in the study cohort in relation to the maximum change in ctDNA from baseline

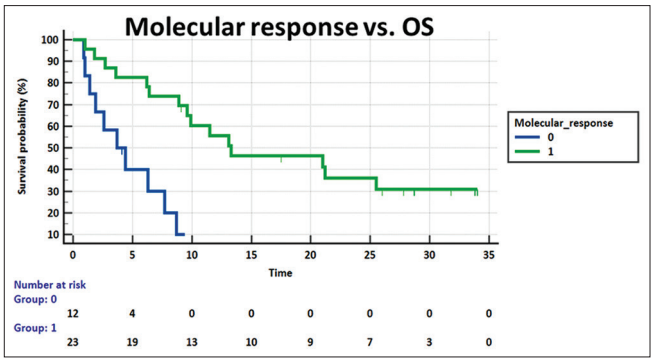

Figure 4: Kaplan Meier curve depicting the overall survival difference between those with ≥49% (green line coded 1) molecular response and those with <49% (blue line, coded 0) response. Median overall survival (OS) in non-responders was 4 months (95%CI: 1.02-7.07) and was 13.3 months (95%CI: 9.2-24.3) in responders ($P < 0.004$)

23% long-term responders in those showing a molecular response9.[24] The cut-offs determining the ctDNA and molecular response, although arbitrary were derived from ROC analysis.[25] However, Zhang *et al*. reported a cut-off of 50% based on weighted sum and residual mean VAF.[23] Routine ctDNA monitoring could enable early identification of non-responders to immunotherapy, potentially prompting timely escalation to alternative treatment lines before overt radiologic progression. This could be particularly impactful in resource-constrained settings where treatment cost-effectiveness is vital.

Passiglia *et al*. correlated changes in ctDNA concentration to NLR and disease burden; however, the same was not calculated in this study as NLR was not uniformly assessed in a single centre, leading to a cut-off bias based on reference ranges for each laboratory used.[26,27]

The median TTP in the study by Passiglia *et al*.[26] was 3.3 months at 6 weeks, corresponding to the PFS of 3.9 months in this study. In another study by Weber *et al*., patients with a ≥50% molecular ctDNA response had a similar PFS (median 10 months) and OS (median 18.4 months) like the non-shedders, whereas those with less than 50% response showed a median PFS of 2.0 months and an OS of 5.9 months.[22,27] This proportion is reflected in this study as well; however, numerical differences can be attributed to differences in sample size.

In the context of a resource-restrained society like India, where molecular diagnostics has recently witnessed an increased uptake, monitoring using NGS-based technology was elusive.[28] Nevertheless, ctDNA changes during treatment have a definite impact on the PFS and OS, and whether the analogy of BCR-ABL1 in CML can be applied for decision on therapy halt, is still to be validated in larger trials/studies, preceding clinical implementation.[29]

The limitations include the sample size and variable blood collection times, leading to missing data points. Additionally, the correction for clonal hematopoiesis was not definitive. Yet a major benefit in a setting like India is the fact that this is the largest cohort reported from the peninsula.[30] It is also a value-added tool in the armamentarium of the treating physician with a clear-cut clinical benefit. The inability to perform matched peripheral blood cell sequencing limited our capacity to fully exclude clonal hematopoiesis as a source of variant detection.

Our findings are consistent with Thompson *et al*. (2021),[18] who demonstrated ctDNA decline correlated with longer OS in NSCLC on pembrolizumab. Similarly, Weber *et al*. observed that >50% ctDNA decline was predictive of favourable outcomes.[27] Our study replicates this observation in an Indian real-world cohort treated with nivolumab, underscoring generalizability across populations and treatment lines.

Further work should focus on prospective multicenter validation in Indian and LMIC populations, integration of ctDNA with radiomics and tumour mutational burden data, and multi-omics approaches including T-cell receptor profiling and cytokine signatures to enhance predictive modelling.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Mansi Sharma, Shrinidhi Nathany, Rashi Sachdeva, Vanshika Batra, Priyanka Venkataraman, Abhinav Dewan, Sabeena Choudhary Anurag Mehta. The first draft of the manuscript was written by Shrinidhi Nathany and Ullas Batra and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of RGCIRC (RGCIRC/IRB/111/2020; 25/02/2020).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Conflicts of interest

There are no conflicts of interest.

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