Original paper

Plasma biopsy by Tag-sequencing: an acceptable alternative to tumor tissue profiling in non-small-cell lung cancer

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Tag-sequencing is a modified next-generation sequencing (NGS) approach wherein targeted regions are tagged with unique molecular identifiers enabling error-free detection of rare genomic alterations. We aimed to perform this high-fidelity sequencing to identify actionable variants from the plasma of lung cancer patients.

Targeted sequencing was performed from plasma-derived cell-free nucleic acid in twenty-one advanced, treatment naïve, non-small-cell lung cancer (NSCLC) patients. Clinically significant genetic alterations were compared with matched tumor NGS profile for each patient (patient-level), and separately for each alteration (variant-level). Cross-platform validation was done for EGFR and KRAS mutations (real-time PCR) and ALK1 rearrangement (immunohistochemistry).

Forty-seven alterations (26 in plasma and 21 in tumor tissue) were detected in 19/21 tested cases. Overall-concordance between the two assays was 94.87% (κ of 0.71, 95% CI: 0.54-0.89). Patient-level and genic-concordance was 57.1% (12/21 cases) and 67.86%, respectively. Almost perfect agreement was reached for detecting actionable EGFR mutations and ALK1 rearrangement (κ of 0.89 and κ of 1, respectively), which was confirmed by single-gene testing.

Substantial agreement between the assays makes Tag-sequencing a viable option for identifying multibiomarkers from the plasma of advanced NSCLC patients in special circumstances where tissue has depleted/tumor is inaccessible/high risk of biopsy due to existing comorbidities.

Key words: driver mutations in lung cancer, free-circulating nucleic acids, Tag-sequencing, liquid biopsy.

Introduction

Lung cancer has been the leading killer across the globe [1]. Seeking driver mutation and inhibiting it with a corresponding inhibitor has improved survival in advanced non-small-cell lung cancer (NSCLC) [2, 3]. Predictive biomarker profiling of the core biopsy tissue is the current standard of care. However, inadequate material and potential hazards of a repeat biopsy have encouraged the use of liquid biopsy as a minimally invasive alternative. Tumor cell apoptosis and necrosis lead to extravasation of cell-free

nucleic acids (cfNAs) into peripheral blood and eventually into urine or cerebrospinal fluid. This cfNA being tumor-derived retains the molecular signatures of the cancer [4, 5]. However, the low abundance of tumor-derived nucleic acids requires high coverage which in turn can lead to errors of PCR duplicates combined with intrinsic errors of sequencing. Tag-sequencing is an error reduction technique that utilizes unique identifiers to remove errors facilitating error-free detection of low-abundant nucleic acid alterations [6, 7]. In this method, the target gene is first amplified and purified. The targeted amplicons are then amplified with barcode-adapted primers. About 10-15 base pair unique molecular identifier (UMID) sequences label the amplicons. The barcoded library is purified, size selected, quantified by qPCR, and sequenced. The sequencing reads sharing the same UMIDs are grouped together as one molecular family and read in consensus. A genuine mutation must be seen in the entire family. At least three molecular families should show the genetic alteration to be considered significant. This technique can considerably reduce the error rate of PCR and sequencing. Establishing the effectiveness of this strategy can make plasma biopsy an acceptable tool to detect molecular biomarkers in NSCLC in special circumstances where tissue has exhausted and fresh biopsy is impractical. Repeatability for mutation tracking over time and detecting variants derived through tumor heterogeneity shall further make cfNA testing an attractive proposition.

In a previous study, we validated tumor NGS testing in advanced/ metastatic NSCLC patients with respect to single-gene assay for detecting *EGFR* mutations and *ALK1* rearrangement [8]. Here, we have compared the ability of the novel Tag-sequencing NGS to identify actionable genomic alterations from the plasma sample of NSCLC patients and assessed the performance with tissue biopsy sequencing.

Material and methods

Research setting and patients

The study was initiated after obtaining ethical approval from the Institutional Review Board (RGCIRC/ IRB/ 168/ 2018, dated 1st June 2018) and was conducted in accordance with the Declaration of Helsinki. The study was conducted at a single center from 2018 until the end of 2019. Twenty-one advanced, treatment naïve, NSCLC patients, tested for predictive biomarkers by NGS on tumor tissue were simultaneously tested for biomarkers using plasma and were included in the study. There were nineteen cases of lung adenocarcinoma (LUAD) and two cases of squamous cell carcinoma (SCC) histology, the latter tested being young and non-smoker with a high probability of harboring an actionable mutation.

Tumor tissue sequencing

Tissue NGS was performed in the cohort a priori using Oncomine™ Solid Tumor DNA and Oncomine™ Solid Tumor Fusion Transcript kit on Ion-Torrent Sequencing platform. The procedure of isolation of nucleic acid from formalin-fixed and paraffin-embedded (FFPE) tumor tissue, library preparation, sequencing, and data analysis have been described earlier [8].

cfNA extraction and sequencing

To avoid temporal bias, the sampling of biopsy tissue and blood was performed simultaneously. Peripheral blood (~10 ml) was drawn in K^+EDTA tube and plasma was separated by two rounds of centrifugation (1200 g × 10 minutes followed by 16000 g × 10 minutes). Cell-free nucleic acid was extracted from 2-4 ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) as per the vendor's specification. The nucleic acid concentration was determined on Qubit® 3.0 Fluorometer (Invitrogen Life Technologies). The sequencing library was generated from 50 ng cfNA using Oncomine™ Lung Cell-Free Total Nucleic Acid Assay following the user guide (#A35864, MAN0017065). Tag sequencing barcode set (A31830) was employed for generating a barcoded library and ~50 pM was loaded on Ion 530™ Chip (8-plex pooled library). Sequencing was performed on the Ion-Torrent S5 Sequencing platform and the data was analysed in Torrent Suite™ Software 5.2 and Ion Reporter 5.10 using Lung Liquid Biopsy DNA and TagSeq Liquid Biopsy templates plugin. Sequenced reads were mapped to reference genome GRCh37/hg19 and the variants called on Ion reporter were reaffirmed on VarSome search engine [9].

The clinical significance of the alterations was investigated in the clinical decision support tool OncoKB [10].

Single-gene testing

Wherever tumor tissue was sufficient, testing for *EGFR* mutations and *ALK1* fusion rearrangement was performed by real-time PCR (using Qiagen EGFR Therascreen® RGQ PCR Kit) and immunohistochemistry (VENTANA ALK D5F3 CDx assay), respectively, as described previously [8]. *KRAS* mutation status (Codons 12/13) of the tumor tissue was confirmed by real-time PCR (using KRAS Mutation Analysis Kit – EntroGen) in selected cases; performed according to the manufacturer's recommendations.

Statistical analysis

Concordance was defined at both the patient-level (i.e. comparison of the set of all alterations seen in the individual patient) as well as at the variant-level (comparison of individual variants in the study cohort).

At the patient-level, finding an identical alteration in all the detected genes was called as concordant, while non-detection of the genomic alteration either in tissue or in plasma/ or a differing genomic alteration in the same gene was considered to be discordant.

At the variant-level, detecting identical or lack of an alteration (wild-type/wild-type) was considered to be concordant, while differing mutations observed in the same gene was considered to be partially concordant. Lack of an alteration by one of the two platforms in the same patient was considered to be a discordant result.

Descriptive statistics were used to summarize data. The agreement of the two techniques was measured by Cohen's κ statistics.

Results

The clinical characteristics of the patients have been presented in Table I. The list of genes and rearrangements interrogated by the respective panel has been shown in Supplementary Table I.

Table I. Characteristics of the study group (n = 21, lung cancer cases) [10]

	N	Percentage (%)
Age (years)		
Median (range)	54.00 (28-79)	
Mean ±SE	53.86 ±12.74	
Gender		
Male	14.00	66.67
Female	7.00	33.33
Smoking history		
Ever-smokers	10.00	47.62
Never-smokers	10.00	47.62
Unknown	1.00	4.76
Stage		
IIIb	1.00	4.76
IV	20.00	95.24
Tumor histology		
Adenocarcinoma (NOS)	19.00	90.48
Squamous cell carcinoma	2.00	9.52
Family history of cancer		
Yes	3.00	14.29
No	12.00	57.14
Unknown	6.00	28.57

Individual patient-level and variant-level concordance of the two NGS assays

Two LUAD patients had no pathogenic alteration detected by both platforms. In the remaining nineteen patients, deleterious variants were identified in seven genes and in one fusion transcript (Fig. 1). The assay results were in agreement for 12 (57.1%) cases. Five patients showed single gene discordance, three cases differed for two genes, and a single case for three genes (Fig. 1, Supplementary Table II).

A total of 47 pathogenic alterations were detected, including 21 in tumor tissue and 26 in plasma cfNA (Fig. 2A, Supplementary Table II). For the variants compared, 14/21 (66.7%) of tissue mutations were captured in plasma, and 14/26 (53.8%) of the cfDNA mutations were detected in the tissue (Fig. 2B, Supplementary Table II). The overall concordance between the two assays for the common thirteen genes/rearrangements interrogated by the panels was 94.87% (259/273). Tag-sequencing in the plasma sample identified seven deleterious/likely pathogenic variants [three in *KRAS* (two-p.Gly12Cys, p.Gly13Asp), single for *ERBB2* (p.Ala771_Tyr772insTyrValMetAla), *EGFR* (p.Leu858Arg), *MET* (p.Thr1010Ile) and *TP53* (p.Val157Phe)] that were not detected on tissue NGS. Whereas, two variants [*BRAF* (p.Leu597Gln) and *KRAS* (p.Gly12Asp)] were observed in the tumor but not in plasma (Fig. 2A and 2B). Notably, five partially concordant mutations, that showed genomic and functional concordance, but locational discordance was observed for the genes *KRAS* and *TP53* (Supplementary Table II). One non-targeted TRIM24-BRAF gene fusion was called for in the tissue sample of a single subject which we have excluded from the comparative analysis. At the genic-level, excluding 2 patients who had no driver mutations both on tissue and plasma biopsy, the estimates for concordance (both complete and partial) and discordance were 67.86% (19/28), and 32.14% (9/28), respectively (Supplementary Table II). A substantial agreement was attained between the two assays (κ = 0.71, 95% CI: 0.54-0.89). In particular, there was an appreciable agreement between tumor and plasma NGS results for detecting *EGFR* gene mutation in tumor tissue (95.24% concordance; κ = 0.89, 95% CI: 0.70-1.00).

Clinical implications of discordant gene alterations

Data mining for the therapeutic implication of the alterations was performed on Oncology Knowledge Base, OncoKB, which gives information based on the level-of-evidence to get benefit from a therapeutic agent. The filter was set for non-small-cell lung cancer (Supplementary Table II). Among the nine patients who showed discordant/partially concordant- solid vs. liquid biopsy

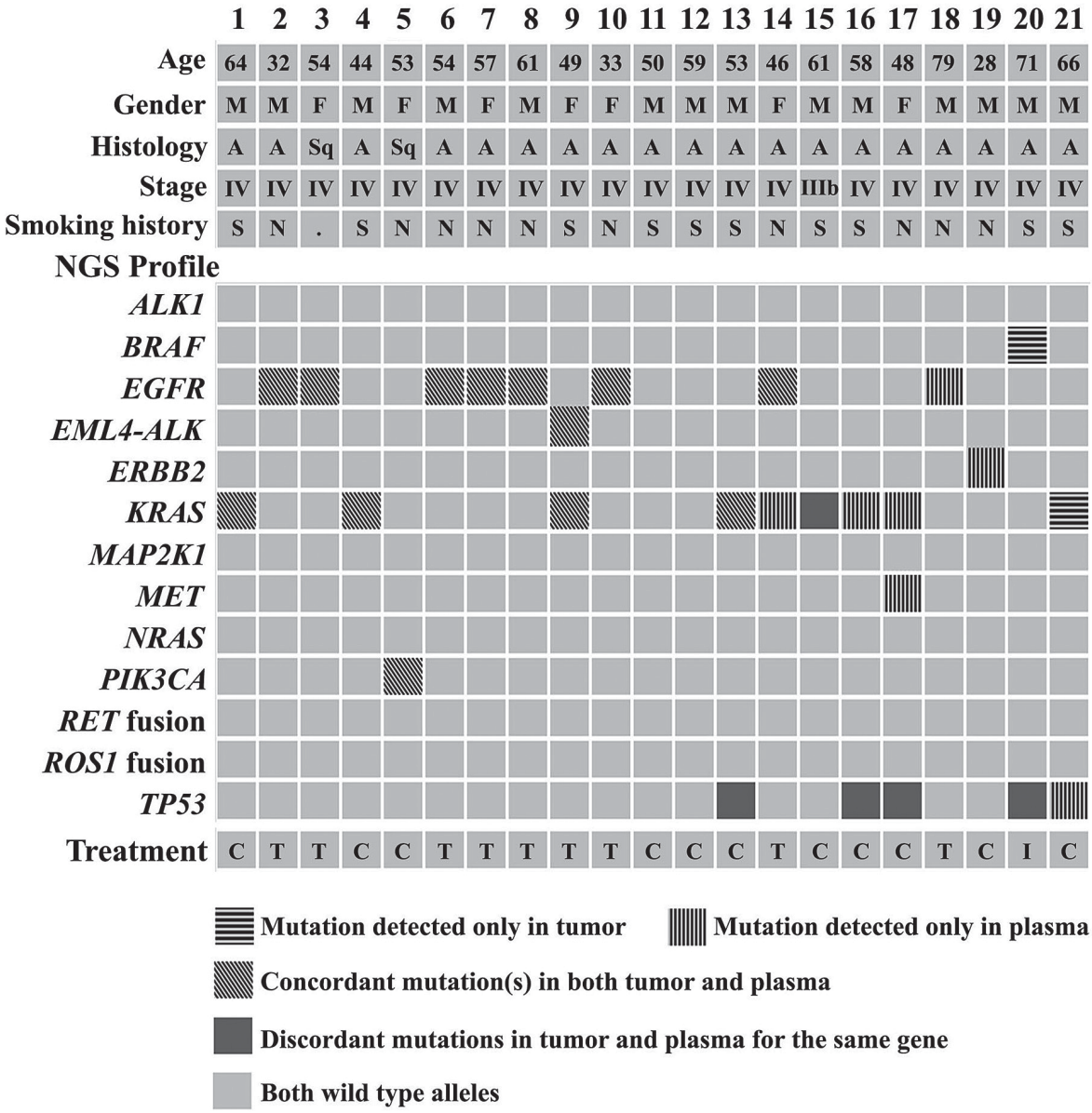

Fig. 1. Oncoprint chart depicting clinical features, alterations detected in tumor tissue and/or plasma, and treatment decision across 21 lung cancer subjects
M – male; F – female; A – adenocarcinoma, S – squamous cell carcinoma; S – smoker, N – non-smoker; . – unknown; C – chemotherapy; T – targeted therapy; I – immunotherapy

variants, the plasma biopsy could additionally pick actionable mutations in four patients. These are *EGFR* (p.Leu858Arg – level 1), *ERBB2* (p.Ala771_Tyr772insTyrValMetAla – level 2) in single patient each, and two cases with *KRAS* (p.Gly12Cys – level 3) mutations (Supplementary Table II, Fig. 2B). All other discordant oncogenic mutations in the genes *BRAF*, *KRAS, MET* and *TP53* that we observed were implicated as non-actionable in the OncoKB database.

Validation by single-gene assay

The results were validated by single-gene assay for detecting *EGFR* mutation in 19/21 cases (Table II, Supplementary Table III). The exon 18 *EGFR*delGlu709_Thr710insAsp variant observed in both tumor and plasma NGS was not tested by real-time PCR as the test kit lacks primers for the same. The single discordant (p.Leu858Arg) mutation that came positive in plasma NGS was confirmed by real-time PCR of tumor-derived DNA. Also, rearranged ALK1 was

Table II. Comparison of NGS and single-gene testing (SGT) results for detecting *EGFR* mutations, ALK1 rearrangement and *KRAS* mutations in the indicated number of cases

	NGS		SGT
	Tumor	Plasma	Tumor
EGFR		n = 19 (%)	
Mutated	6 (31.58)	7 (36.84)	6 (31.58)
Exon 18 (Indel)	1	1	_
Exon 19 deletion	3	3	3
Exon 19 deletion +p.T790M	1	1	1
Exon 21 (p.L858R)	1	2	2
Wild-type	13 (68.42)	12 (63.16)	13 (68.42)
ALK1		n = 18 (%)	
Rearranged	1 (5.56)	1 (5.56)	1 (5.56)
Wild-type	17 (94.44)	17 (94.44)	17 (94.44)
KRAS		n = 3 (%)	
Mutated	0 (0)	3 (100)	1 (33.33)
Wild-type	3 (100)	0 (0)	2 (66.67)

tested in 18/21 cases by immunohistochemistry, and showed perfect agreement with the NGS results in both tumor tissue and plasma cfNA (Table II, Supplementary Table III). In addition, we confirmed the three *KRAS* mutations called in plasma, but not in tissue NGS by real-time PCR of tumor DNA. Among them, one was positive for mutated *KRAS* whereas two were negative (Table II, Supplementary Table III). In this one case plasma result was true positive using single-gene assay as a reference while the tissue result was a false negative.

Discussion

This study explores the concordance between NGS multigene assay of plasma cfNA using molecular tagging approach and the routine tumor biopsy sequencing. Compared to a previous study that was conducted without the application of molecular barcodes [11], our study demonstrates considerable improvement in concordance. Ligating the region of interest with unique molecular identifiers significantly improved the performance and could pick mutations of low allele fraction in plasma with high accuracy. Substantial agreement between the two assays ($\kappa = 0.71$, 95% CI: 0.54-0.89) agrees with earlier studies by Kukita *et al*. and Tran *et al.* [6, 12]. However, these studies were undertaken in a lesser gene number as compared to the present study. In a recent study, Luca *et al*. demonstrated that sequencing based on molecular tagging technology achieves optimum performance for detecting driver gene mutations from the plasma of advanced NSCLC patients. But they mostly evaluated the performance metrics, and no comparison with mutations detected on tumor biopsy sample was undertaken [13]. In another study by Heeke *et al*. the impact of Tag-sequencing in liquid biopsy was evaluated by comparing the results with Foundation Liquid assay and tumor genotyping [14]. Our study has assessed the usefulness of Tag-sequencing in liquid biopsy in a treatment naïve setting for advanced NSCLC patients and a comparative analysis with tumor biopsy NGS has been made with respect to pathogenic variants as well as the individual patient.

Strong agreement was observed for detecting clinically actionable *EGFR* mutations and ALK1 fusion. For the detection of *EGFR* gene mutations, the plasma cfNA showed a 95.24% concordance with tumor NGS results. In addition, cross-platform evaluation by single-gene assay showed congruent results with plasma NGS. This observation agrees with the previous studies by Veldore *et al.* where plasma-derived cfNA was tested for *EGFR* mutations using deep sequencing in a large cohort of 163 patients and validated against allele-specific real-time PCR of tissue biopsy [15]. Supporting is the study by Steendam *et al*. that compares NGS and ddPCR for detecting primary activating *EGFR* mutations on cfNA, showing good agreement between the two methods [16]. Of note, in the present study, plasma NGS could also detect less common *EGFR* exon 18 deletion/insertion mutation. Among the *EGFR* mutated NSCLC cases, the *EGFR*delGlu709_Thr710insAsp variant shows less than 0.1% prevalence and is accepted as a tyrosine kinase inhibitor sensitive mutation [17]. Identifying this rare but potentially actionable mutation adds to the value of NGS-based testing as this

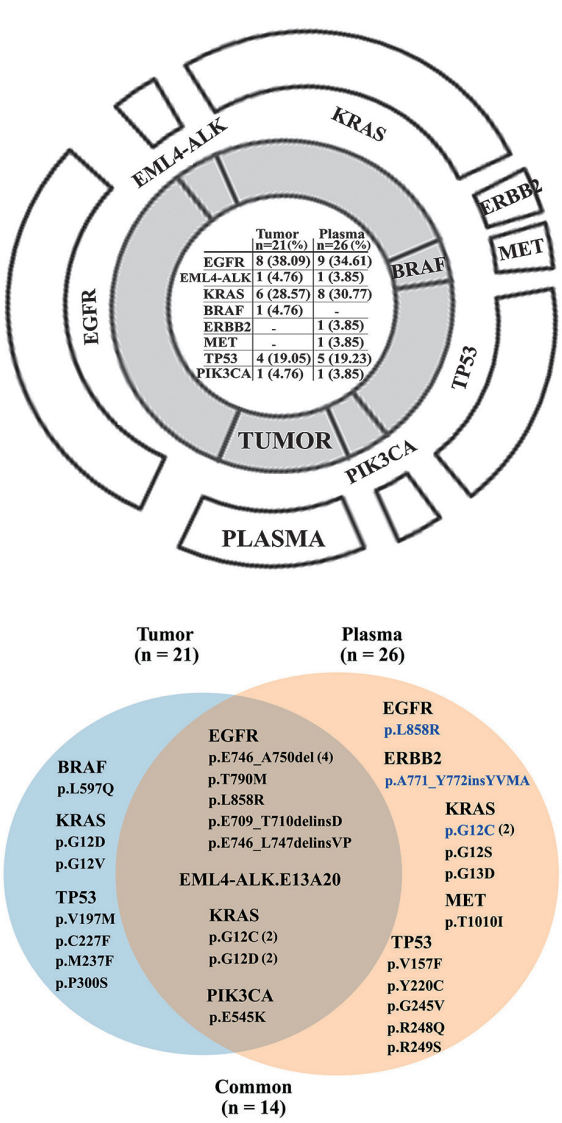

Fig. 2. Genomic alterations observed in tumor tissue and plasma. A) Doughnut-plot comparing frequency. B) Venn diagram depicting the overlapping variants. Tag-sequencing of plasma could detect additional four variants depicted in blue (n = 21, lung cancer patients)

mutation is not tested in the current FDA approved real-time PCR-based kit for *EGFR* sensitizing mutation detection (ie. Cobas® EGFR Mutation Test v2 and Therascreen® EGFR RGQ PCR Kit).

Additionally, as regards to *ALK1* fusion rearrangement and *PIK3CA*, absolute concordance observed, reinforces the suitability of cfNA based multigene assay as a valid testing methodology though the size of the cohort in the current study is small. Conventionally, *ALK1* testing is performed using fluorescence in situ hybridization (FISH) and/or immunohistochemistry on formalin-fixed, paraffin-embedded tissue [18, 19, 20]. The early NGS-based platform did not have the capability to identify fusion rearrangements. The contemporary assays, however, are capable of identifying fusions on NGS. The ability to identify fusions on cfNA can allow many more patients to obtain biomarker-based therapies as up to 33% to 53% of patients fail to have adequate tissue on core needle biopsies and fine needle aspirate, respectively [21].

The study revealed three additional *KRAS* mutations and single variants for *ERBB2*, *EGFR*, *MET* and *TP53* that was picked up on plasma Tag-sequencing NGS, but not by routine tissue NGS. One of the *KRAS* mutations was p.Gly12Cys, which is poised to become actionable [22, 23]. It has been observed that sometimes liquid biopsy shows additional mutations because of intratumoral heterogeneity that tissue biopsy may fail to detect. If the region outside the primary site harbors the mutant allele due to evolving branch mutations, it can be detected in plasma but not by tumor analysis [24]. Another plausible explanation is the 'Clonal Hematopoiesis of Indeterminate Potential' responsible for some of these calls [25]. The least plausible, yet possible cause could be second primary lung cancer that has a *KRAS* mutation but was not considered metastatic by clinical or radiological evaluation [26]. A similar explanation is likely for the *TP53* mutation identified only in cfNA. It is important to mention that Tag-sequencing of plasma has the potential to pick-up variants of low frequency due to high coverage, while in most tissue NGS the cut-off for filter is 3-5%. Therefore, plasma biopsy identifies additional mutations that are not detected on tissue biopsy sequencing, and so it is not appropriate to consider them as false positives.

At patient-level 9/21 (43%) cases showed discordance between plasma and tumor genotype profile, and the differences were mostly observed for missense variants in the *TP53* and/ or *KRAS* gene (in 7 cases). The *TP53* mutations are presently inconsequential for therapeutic decision making, however, they may have prognostic significance. We have not validated the discordant *TP53* mutations by single-gene testing as they are not relevant for therapy decisions. However, these may become useful over time for example the commutation of *KRAS* and *TP53* may provide the best results with checkpoint inhibitors [27, 28]. In the present study therapeutic decision was made according to tumor molecular profile. The sample size of our study is small and so we are not able to draw a conclusion regarding which assay results should be considered for selecting effective treatment.

Overall, the study shows that the Tag-sequencing of plasma cfNA achieves comparable performance to tumor NGS for detecting sensitizing *EGFR* mutations and *ALK1* translocation. Plasma biopsy was of value in identifying therapeutically actionable variants in four patients that tumor tissue sequencing failed to pick. Small sample size and lack of follow-up data is the limitation of the study. However, keeping in mind the substantial agreement of liquid biopsy with the tissue biopsy, its utility in tissue

deficient cases, in real-time monitoring of tumor status; Tag-sequencing of plasma could help in making treatment decisions, and understanding the mutational landscape in advanced NSCLC.

Conclusions

Using a small but focused gene panel to test cfNA that covers clinically relevant alterations, it is possible to offer biomarker-based therapies to the patients of advanced LUAD without available biopsy or cytology material. With improved outcomes from genome directed therapies, biomarker testing using cfNA in exceptional circumstances is a viable option with high predictive accuracy for biomarkers like *EGFR* gene mutations and *ALK1* rearrangement.

Supplementary Tables are available in online version of this article.

The authors declare no conflict of interest.

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