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Pleural effusion supernatant: a reliable resource for cell-free DNA in molecular testing of lung cancer

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Introduction DNA extracted from malignant pleural effusion (PE) sediments is the traditional source of tumor DNA for predictive biomarker molecular testing (MT). Few recent studies have proposed the utility of cell-free DNA (cfDNA) extracted from effusion cytology centrifuged supernatants (CCS) in MT. The aim of this study was to assess the feasibility and utility of molecular testing on cfDNA extracted from PE CCS in lung cancer patients.

Materials and methods The study was of prospective design. All PE CCS were collected and stored. Subsequently, in patients confirmed as primary lung adenocarcinoma (LUAD) and where patient matched effusion sediment/tissue biopsy/plasma was being tested for *EGFR* mutations, cfDNA extraction and *EGFR* MT by real-time polymerase chain reaction (qPCR) were performed. Custom panel targeted next-generation sequencing (NGS) (Ion Torrent; Thermo Fisher, Carlsbad, CA) was also performed wherever feasible.

Results Out of 299 PE CCS collected, 20 CCS samples were included in the study. Concordant *EGFR* mutations were detected in pleural effusion CCS of 10 of 11 (91%) *EGFR* mutant cases as per qPCR performed on the matched sediment DNA (n = 8), lung biopsy (n = 2), and plasma (n = 1) samples. In 1 positive sample, CCS detected additional *EGFR* T790M mutation. Among 10 CCS samples also tested by NGS, additional *EGFR* mutations missed by qPCR were picked up in 2 (2 of 10). Success of mutation detection in CCS cfDNA did not correlate with cfDNA quantity or tumor fraction in sediment.

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Conclusions cfDNA from effusion CCS is a reliable and independent source of tumor DNA highly amenable for MT and complement results from other tumor DNA sources for comprehensive mutation profiling in LUAD patients.

Introduction

Liquid biopsy refers to the sampling and analysis of body fluids for various tumor derived elements such as circulating cell-free DNA (cfDNA) and RNA, circulating tumor cells, and tumor-derived exosomes, microRNAs, proteins, and metabolites.[1] cfDNA are free circulating DNA fragments that vary in length from 50 to 450 base pairs, usually corresponding to the length of DNA wrapped around nucleosome along with linker (166 bp).[1] They are postulated to be derived from the degradation of DNA released from normal and cancer cells into blood and body fluids during cell death processes such as necrosis, apoptosis, or NETosis and may also be actively secreted as exosomes.[2,3] Despite the low concentrations of cfDNA in plasma and other body fluids such as urine,[4] saliva, cerebrospinal fluid, sputum, and respiratory secretions, it is possible to isolate cfDNA using highly sensitive kits and subject to molecular testing (MT).[5,6] The proportion of tumor-derived cfDNA as a fraction of the total cfDNA varies from patient to patient and we have a rapidly growing body of evidence showing that MT on cfDNA shows high concordance with results obtained on tumor tissue−derived DNA.[7]

Liquid biopsy offers many advantages over tissue biopsies for MT, most important among which is its potential for repeated sampling with minimal discomfort to patient allowing dynamic monitoring of the therapy efficacy, detection of resistance mutation emergence, and assessment of mutational heterogeneity, specifically in lung cancer, and with scope of usage in other malignancies as well.[6] Plasma represents the most commonly tested sample for such molecular analysis and mutation testing on plasma-derived cfDNA has been validated in multiple studies.[8] In lung cancer patients, plasma-derived cfDNA is currently approved for *EGFR* testing in 2 scenarios: lung adenocarcinoma (LUAD) patients with insufficient tumor tissue for baseline mutation testing and *EGFR*-mutant LUAD patients developing secondary resistance to tyrosine kinase inhibitors (TKIs).[9] The positive predictive value of plasma derived cfDNA testing is 100% and the negative predictive value ranges from 95.6% to 100% in various studies.[10] Positive detection rates are postulated to depend upon a number of factors including the sensitivity of the testing method and tumor-related variables such as size, degree of epithelial to mesenchymal transition, and clonal evolution.[11]

Malignant effusions are a common manifestation of advanced solid organ malignancies.[12] Particularly in lung cancer, a significant percentage of patients present with malignant pleural effusion (PE), and the effusion sediment that is processed into cell blocks or smears are the established sources of tumor-cell DNA that is used for predictive biomarker testing including high throughput molecular analysis,[13] while the effusion supernatant is usually discarded in most laboratories. Recently, studies have demonstrated that cytology centrifuged supernatants (CCS) from aspirates,[14] bronchial washings,[15] and effusions[7] are rich sources of tumor-derived DNA that can be effectively extracted using commercially available DNA[16] and cfDNA isolation kits.[17] Interestingly, the cfDNA yield from effusion CCS has been found to be comparable[18] or even superior to that of matched tumor tissue[5] and plasma samples[18-20] in the limited studies performed.

EGFR mutations are detected in nearly one-third of LUAD cases and are mandatory to be tested in all inoperable patients at baseline and at progression for treatment decisioning.[21] Any source of tumor DNA, including cfDNA, can be used for *EGFR* mutation testing by a suitable technique, most commonly real-time polymerase chain reaction (qPCR), with a sensitivity threshold of at least 5% mutant allele frequency. With the discovery of an increasing number of targetable driver alterations in *EGFR* wild-type lung cancer, the use of targeted next-generation sequencing (NGS) allows for their detection from a single test and NGS-based molecular analysis on cfDNA has been validated in a few studies.[20]

The present study was performed to analyze the accuracy and sensitivity of effusion CCS-derived cfDNA for *EGFR* mutation testing in comparison with results obtained with patient-matched tumor cell−derived DNA and plasma-derived cfDNA. In addition, the feasibility of performing NGS on PE CCS was also assessed in this study.

Materials and methods

The study was of prospective design spanning 9-months duration (January to September, 2022) and approved by the institute ethics committee (IECPG-740/23-12-2021). Informed consent was obtained.

Prospective sample collection

All PE samples received in the fresh state in the cytopathology laboratory are routinely processed within 4 hours of receipt by centrifugation at the rate of 1500 rpm for 5 minutes to obtain sediments that are then used in preparation of

smears and formalin-fixed paraffin embedded (FFPE) cell block (CB) for primary diagnosis and ancillary testing. Volume of effusion samples received for routine cytology ranges from 10 to 500 mL. Volume of CCS received for MT ranges from 5 to 15 mL. Five mL of the CCS was collected in all patients and was stored at −80°C in Falcon (Corning, Corning, NY) macro-centrifuge tubes until further use. No additional DNA precipitate, fixative, or media was added.

Case selection

Among the patients with stored CCS, only patients with (i) confirmed histo- or cytopathologic diagnosis of LUAD, and (ii) successfully subjected to *EGFR* mutation testing on tumor cell−derived DNA or plasma-derived cfDNA as part of routine predictive biomarker testing algorithms were selected for further analysis. The tumor cell−derived DNA may have been sourced either from the procedure-matched effusion sediment itself or from a non-procedure matched biopsy, provided the latter is sampled within a week of the thoracentesis.

Further analysis

The samples of the selected patients were thawed at 4°C and subjected to cfDNA extraction and downstream MT.

The effusion smears and cell block with immunohistochemical stained slides were reviewed for confirmation of diagnosis and assessment of tumor cell fraction. An average value of tumor fraction was calculated for each case based on assessment of cellularity on both smears and cell block sections. This was done independently by 3 pathologists (S.T., S.J., A.N.). In cases where there was greater than 5% variation in the assessment of tumor fraction among the pathologists, a joint consensus was reached after multiheader assessment.

DNA extraction and quantification (check for quality and quantity)

cfDNA extraction was performed using 5 mL of pleural effusions supernatant's using commercially available Maxwell RSC cfDNA plasma kit (Promega, Madison, Wis) according to manufacturer's instructions. Purification of extracted cfDNA was measured using Nanophotometer (Implen N60, Munich, Germany) and stored at −20°C until further use.

EGFR mutation analysis

qPCR was performed to detect clinically relevant *EGFR* hotspot mutations using the Therascreen *EGFR* RGQ PCR IVD kit (Qiagen, Hiden, Germany) on a Qiagen Rotor Gene 5plex HRM instrument. This is a ready-to-use kit enabling qualitative detection of 29 hotspot somatic mutations in exons 18, 19, 20, and 21 of the *EGFR* gene. The qPCR assay was performed with 10 ng/μL of tumor-cell DNA or 5 μL of cfDNA extracted from CCS/plasma (irrespective of DNA concentration), and the final analysis of samples *EGFR* mutation was assessed according to the manufacturers' instructions.

NGS

Targeted NGS with custom panel (Supplementary Table 1) was performed on the Ion Torrent platform (ThermoFisher, Carlsbad, CA) in all cases where *EGFR* mutation testing was negative on CCS by qPCR and in a subset of *EGFR* positive cases. Library preparation was done using Life Science Technology's Ion AmpliSeq custom target panel (ThermoFisher). This custom panel designed using Ion Ampliseq designer enables detection of 98 hotspots in 12 clinically relevant genes (*AKT1, BRAF, DDR2, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, PTEN, STK11)* in the context of non-small cell lung cancer. Sequencing reads obtained were mapped and aligned reads (BAM files) were further analyzed using IonReporter software with inbuilt Integrative Genomics Viewer. After passing the initial quality check, the variant calling file was additionally filtered by excluding intronic variants or synonymous variants. Followed by exclusion, each variant was assessed for phenotypic effect using prediction tools (SIFT, PolyPhen-2) to accurately detect somatic variants and the filtered variants were annotated according to the American College of Medical Genetics, Association for Molecular Pathology guidelines.

Statistical analysis

Statistical analysis was performed using the descriptive statistics methods in Microsoft Office 2021 (Redmond, WA). Concordance rates between CCS mutation results with patient matched FFPE/plasma results were calculated.

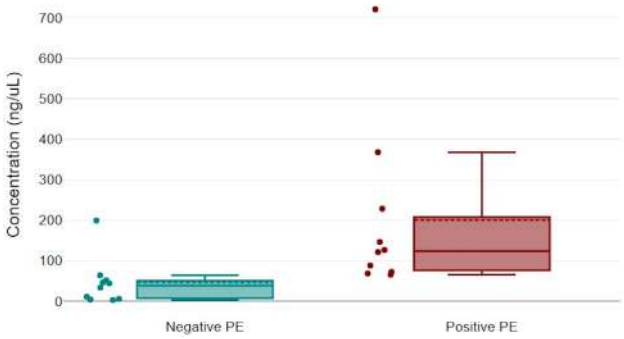

Figure 1 Significant difference in cfDNA quantification using Nanophotometer of supernatants from benign (mean 45.9 ng/μL; median 38.5 ng/μL) and malignant pleural effusions(mean 200.4 ng/μL; median 123.7 ng/μL); *P*-value 0.04 (Dotted line represents mean value, straight line represents median value).

Table 1 Summary of *EGFR* mutation testing results in the 20 patients included in the study.

Case no.	Age/Sex	TN/Post TKI	cfDNA quantity (ng/μL)	cfDNA quality ($A_{260/280}$)	Tumor fraction in effusion sediment (%)	*EGFR* mutation result on supernatant cfDNA	Matched tumor sample tested	DNA quantity (ng/μL)	DNA quality ($A_{260/280}$)	*EGFR* mutation result on CB/FFPE/Plasma
1	77/F	TN	68.12	1.7	5	Exon 19 del	CB	7.3	1.7	Exon 19 del
2	65/F	TN	11.5	1.5	5	Exon 21 L858R	CB	15	1.9	Exon 21 L858R
3	51/F	TN	228.45	1.9	5	Exon 20 ins	CB	5	2.9	Exon 20 ins
4	49/F	TN	33.15	1.6	15	Negative	CB	85.4	1.6	Negative
5	51/F	TN	14.5	1.4	40	Exon 19 del	CB	10	1.3	Exon 19 del
6	61/M	TN	146.1	1.8	10	Negative	CB	2.8	2.1	Exon 19 del
7	65/M	TN	60.5	1.8	70	Exon 20 ins	CB	189	2.0	Exon 20 ins
8	62/M	TN	29.7	1.7	20	Exon 19 del	CB	46.7	1.7	Exon 19 del
9	72/F	TN	65.3	1.7	40	Exon 19 del	Lung biopsy	24	1.8	Exon 19 del
10	49/M	TN	121.05	1.8	5	Exon 19 del	CB	7.4	1.4	Exon 19 del
11	69/M	TN	32.6	1.5	30	Negative	CB	44.8	1.6	Negative
12	61/F	Post TKI	10.53	1.0	30	Exon 19 del & T790M	Plasma	89.2	1.9	Exon 19 del & T790M
13	43/F	Post TKI	7.85	1.6	50	Exon 21 L858R & T790M	CB	52	1.8	Exon 21 L858R
14	35/M	TN	5	2.1	0	Exon 19 del	Lung biopsy	40.3	2.1	Exon 19 del
15	36/M	TN	41	1.9	30	Negative	CB	17.18	2.0	Negative
16	69/F	TN	19.25	1.8	40	Negative	CB	136	1.8	Negative
17	68/M	TN	20.1	1.7	40	Negative	CB	108	1.8	Negative
18	57/F	TN	34.5	1.8	20	Negative	CB	29.48	1.8	Negative
19	49/M	TN	28	1.8	15	Negative	CB	57	1.9	Negative
20	60/F	Post TKI	13.5	1.6	50	Negative	CB	15.15	1.6	Negative

Abbreviations: M, male; F, female; TN, treatment-naïve; CB, cell block; FFPE, formalin-fixed paraffin embedded; TKI, tyrosine kinase inhibitor; del, deletion; ins, insertion.

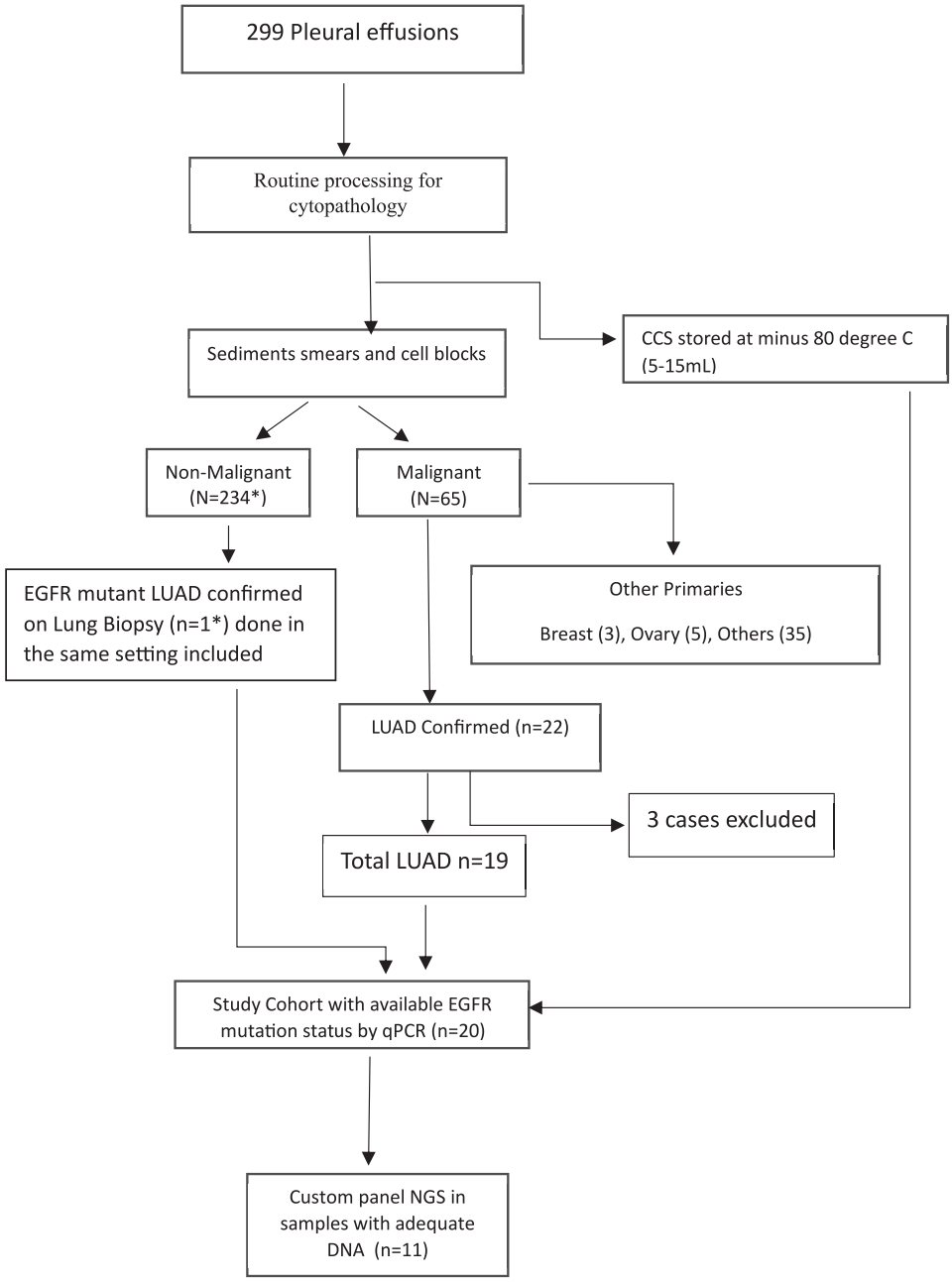

Figure 2 Flowchart showing sample selection and selection of study cohort for qPCR and NGS (*1 cytologically negative effusion with tissue diagnosis of *EGFR* mutant LUAD was included for comparison).

Results

During the study period, a total of 299 PE CCS were prospectively collected which included 65 malignant PE with confirmed primaries in lung (n = 22), breast (n = 3), ovary (n = 5), and others (n = 35).

Quantity and quality check of CCS cfDNA extraction

As a part of initial standardization of cfDNA extraction from effusion CCS, 10 cytologically malignant effusion samples and 10 cytologically benign effusion samples were randomly selected and compared for DNA quantity and quality. The quantification of isolated cfDNA from malignant effusions showed significantly higher yield (P value = 0.04) with a wide range of values ranging from 65.3 ng/μL to 720.75 ng/μL (median 123.68 ng/μL) in comparison with non-malignant effusions whose cfDNA yield ranged from 10.65 to 199 ng/μL (median 38.47 ng/μL) (shown in Fig. 1). The cfDNA quality was satisfactory with ratios of absorbance being ∼1.8 for $A_{260/280}$ and ∼2.0 for $A_{260/230}$ in all samples.

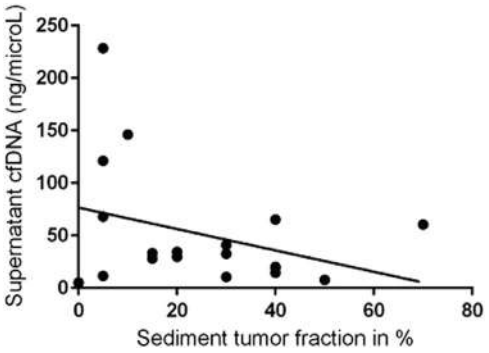

Figure 3 Linear regression analysis of sediment tumor fraction in correlation with supernatant cfDNA quantity in the 20 samples did not show any significant correlation ($R^2 = 0.1124$) with insignificant *P* value (0.1606).

Case selection

During the study period, 19 patients met inclusion criteria. A single cytologically negative sample (case 14) with matched biopsy-proven *EGFR* mutant LUAD diagnosis was also included (Table 1, Fig. 2).

The median age of the patients at diagnosis was 60.5 years with a female:male ratio of 1.2:1. Seventeen patients were treatment-naïve where the diagnosis of primary LUAD was confirmed by positive TTF-1 immunostaining on the effusion sediment smear/CB in all but 2 patients (cases 9 and 14), in which the diagnosis was confirmed on the synchronously obtained lung biopsy. Three patients were previously diagnosed *EGFR* mutant LUAD progressing on *EGFR* tyrosine kinase inhibitor therapy (cases 12, 13, and 20).

EGFR mutation testing was being performed as part of routine testing algorithm on tumor cell–derived DNA sourced from effusion sediment CBs (n = 17) or tissue biopsies (n = 2), while in 1 *EGFR* TKI progressed patient (case 12), only plasma was being subject to *EGFR* mutation testing as the effusion cell block was inadequate for MT. The median DNA quantity from these FFPE samples was 34.89 ng/μL (range: 2.8-189 ng/μL).

Effusion supernatant cfDNA yield and correlation with sediment tumor fraction

The median CCS cfDNA quantity was 31.15 ng/μL (range: 5-228.45 ng/μL).

On review of effusion sediment slides, tumor clusters were present on the smears and CB in 19 patients with tumor fraction varying from as low as 5% to maximum 70%. In case 14, no tumor cells were identified on light microscopy or on TTF-1 immunostaining of the effusion sediment preparations. There was no correlation between the CCS cfDNA yield with tumor fraction (*P* = 0.1606) (Fig. 3).

EGFR mutation results

EGFR mutations were detected in 11 CCS samples (Table 1). The mutations included exon 19 deletion (n = 6), exon 21 L858R (n = 1), and exon 20 insertions (n = 2) in 9 of 17 treatment-naïve patients and exon 20 T790M

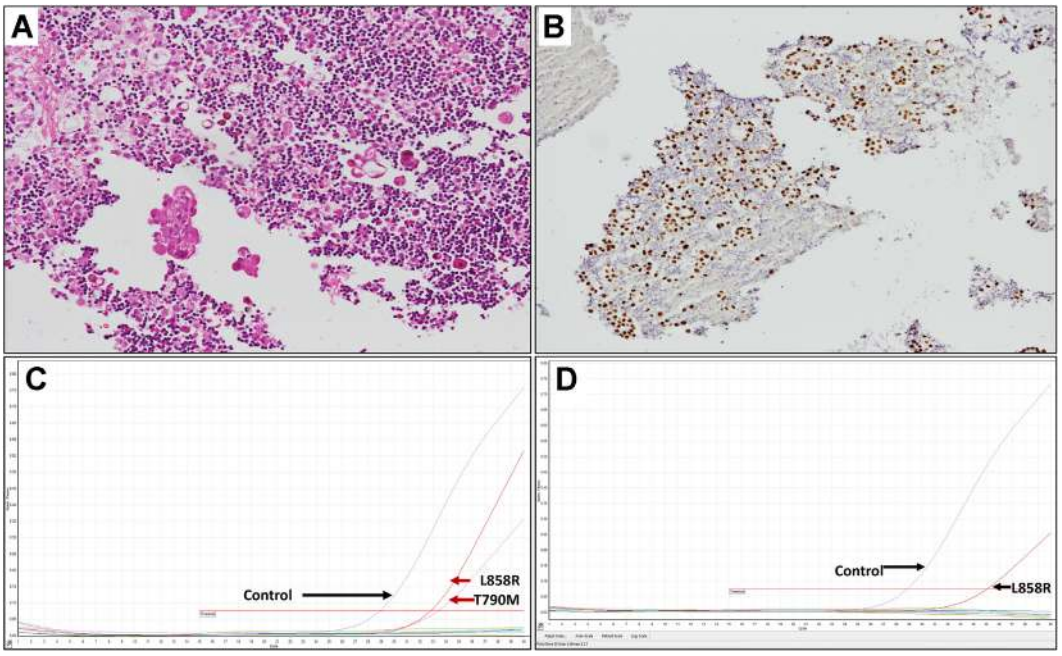

Figure 4 A, Microscopic view of cell block showing approximately 52% tumor fraction (hematoxylin and eosin stain, x100). B, TTF-1 positive tumor cells. C, qPCR curves showing exon 21 L858R as well as T790M mutation in supernatant cfDNA. D, qPCR curves showing exon 21 L858R mutation alone in tissue sample for case 13.

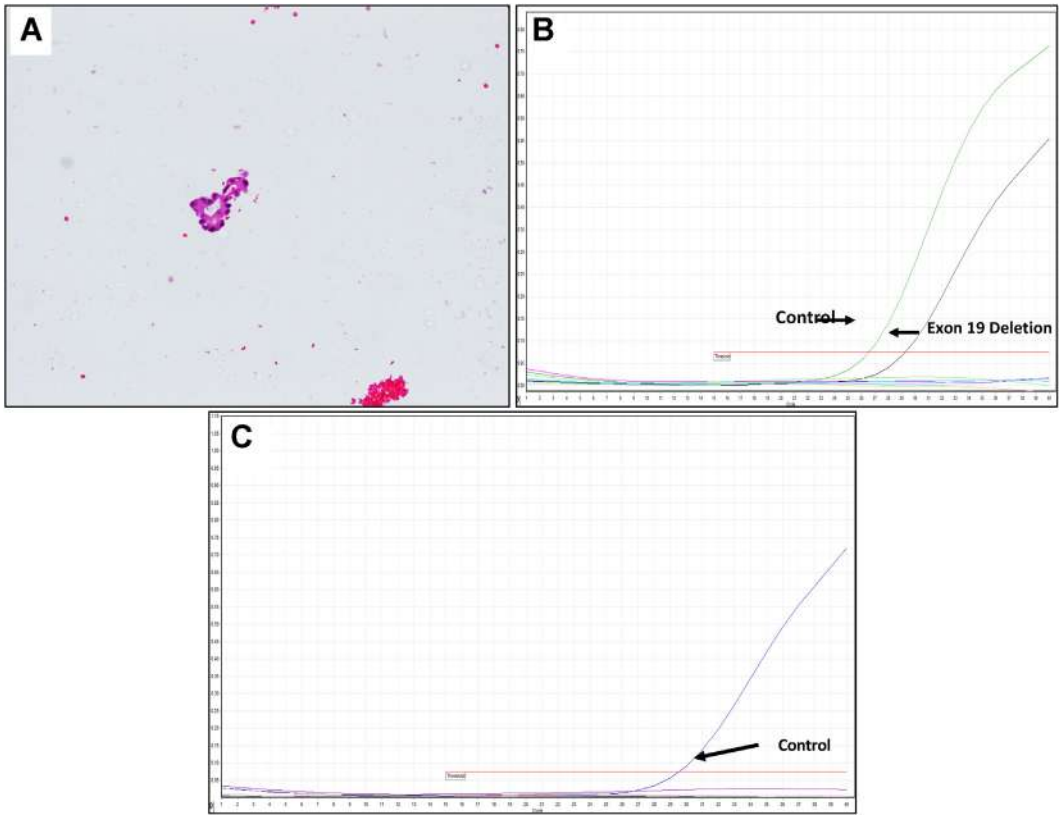

Figure 5 A, Microscopic view of cell block with low tumor fraction (approximately 10%) in case 6 (hematoxylin and eosin stain, x100). B, qPCR curve showing exon 19 deletion on cell block sediment DNA. C, qPCR curve of cfDNA obtained from supernatant pleural effusion that failed to detect the *EGFR* mutation.

mutations in addition to the founder mutation (L858R in 1, exon 19 deletion in 1) in 2 of 3 post-TKI progressed patients. There was no correlation between *EGFR* mutation results and cfDNA quantity ($P = 0.5488$) or tumor fraction ($P = 0.7172$) with *EGFR* mutation being detected in samples with cfDNA yield as low as 5 ng/μL (case 14).

EGFR mutation results on CCS were concordant with the results obtained on tumor tissue/plasma in 91% patients (10 of 11, 91% concordance). In 10 of 11 patients, the same hotspot mutations were detected in both CCS and tumor tissue/plasma. In case 13, additional T790M mutation was picked up on the CCS while the matched CB testing detected only the founder mutation despite a higher DNA quantity (52 ng/μL) and a high tumor fraction (52%) in the latter (Fig. 4).

One CCS (case 6) tested negative for *EGFR* mutation while the matched sediment showed presence of *EGFR* mutation (exon 19 deletion). This was despite a higher DNA quantity from the CCS (146.1 ng/μL) as compared to the matched CB tumor DNA quantity (2.8 ng/μL) (Fig. 5).

All other matched samples tested negative for *EGFR* mutations by qPCR on both tumor tissue and CCS.

The overall concordance of mutation results between CCS and tissue/plasma was 95% (19 of 20).

NGS results

Targeted NGS was performed in 11 effusion CCS (cases 6, 9, 11, 12, 13, 15-20). Of these, 1 CCS sample (case 12) with low DNA yield (1 ng/μL) failed quality control check. The reads generated in CCS samples, mapped to the GRCh38.p2 reference genome, exhibited a mapping efficiency exceeding 93.43%. Total generated reads were between 15,661,335 and 49,818,160 out of which mapped read of 14,632,580-47,294,208 were mapped. The mean depth of 1500X, with uniformity of 99.5% base coverage, was attained with a mean read length of 134bp. The results in comparison with qPCR results on paired tumor tissue/plasma and effusion CCS are tabulated as shown in Table 2. A comprehensive summary of MT results of all 20 cases is tabulated in Table 3.

EGFR mutation results were perfectly concordant between qPCR and NGS methods on CCS in 7 of 10 (70%) with the same *EGFR* mutations being detected in 1 (case 9) and the remaining 6 being negative (cases 6 and 16-20). In case 6, exon19del was detected on effusion sediment; both qPCR and NGS failed to detect the same on the CCS. *EGFR* exon 18 G719C mutation in case 11 (Fig. 6) and *EGFR* exon19del in case 15 were picked up exclusively by NGS

Figure 6 NGS reads of case 11 in which qPCR failed to detect *EGFR* mutation on tissue sample, whereas NGS on supernatant cfDNA detected *EGFR* mutation at exon 18 single nucleotide polymorphism G719C.

on the CCS, while qPCR on both the effusion CCS and effusion sediment in the same patient did not identify these alterations. On the other hand, in case 13, qPCR on CCS had identified an additional exon 20 T790M mutation, and the same was not identified by NGS (Table 3). Among the other gene alterations, single nucleotide polymorphisms in *BRAF* and *PIK3CA* genes were identified in three CCS by NGS (Table 2, Table 3).

Discussion

With increasing number of targetable alterations being reported in lung cancer, it is imperative that the same may be tested for in every patient presenting with advanced disease to ensure timely receipt of appropriate targeted therapy. qPCR for single gene mutation testing and targeted NGS for multiple gene testing are the molecular techniques that are usually applied on tumor DNA for this purpose. In recent years, plasma-derived cfDNA has been established as an alternate source of tumor DNA for such MT, particularly for *EGFR* mutation testing with excellent positive predictive values.[22] However, the utility and reliability of tumor cfDNA sourced from other body fluids, in particular, CCS of effusion samples, is less well established.[17] Effusion CCS−derived cfDNA differs from plasma-derived cfDNA as the former is more likely to contain a higher content of contaminating cellular DNA and extracellular vesicular DNA, which is not unexpected to be found in any cytology supernatant. Further, the efficacy rate for MT performed on cytology specimens also depends upon the additional factors such as method of acquisition, processing, preservation, and testing methods,[20] and may contribute to variable results across different laboratories. The present study, undertaken to analyze the feasibility and utility of effusion CCS for MT including NGS in lung cancer patients, demonstrates that

Table 2 Comparison of qPCR on CB/tissue and NGS of supernatant cfDNA.

Sample No.	Case No.	qPCR on tumor cell derived DNA or plasma cfDNA[a]	qPCR on supernatant cfDNA	NGS on supernatant cfDNA		Exon	Variant classification	VAF%	Functional change
				EGFR	Other				
1.	6	Exon 19 del	Negative	Negative	None	-	-	-	-
2.	9	Exon 19 del	Exon 19 del	Exon 19 del	None	EXON 19	Del	12.5	E746_A750del
3.	11	Negative	Negative	Exon 18 G719C	None	EXON 18	SNP	22.2	G719C
4.	12	Exon 19 del & T790M[a]	Exon 19 del & T790M	QC fail	-	-	-	-	-
5.	13	Exon 21 L858R	Exon 21 L858R & T790M	Exon 21 L858R	None	EXON 21	SNP	17.2	L858R
6.	15	Negative	Negative	Exon 19 del	None	EXON 19	In del	87.5	Leu747-thr751gelinsGln
7.	16	Negative	Negative	Negative	BRAF	EXON 15	SNP	18.1	V600E
8.	17	Negative	Negative	Negative	PIK3CA	-	VUS	-	-
9.	18	Negative	Negative	Negative	PIK3CA	EXON 21	SNP	4.7	p.H1047R
10.	19	Negative	Negative	Negative	-	-	-	-	-
11.	20	Negative	Negative	Negative	-	-	-	-	-

Abbreviations: qPCR, real time polymerase chain reaction; CB, cell block; cfDNA, cell-free DNA; NGS, next generation sequencing; VAF, variant allele frequency; QC, quality control; Del, Deletion; Indel, insertion with deletion; SNP, single nucleotide polymorphism; VUS, variant of uncertain significance; EGFR, epidermal growth factor receptor.
[a]In case 12 alone, plasma cfDNA was used as comparison.

Table 3 Summary of qPCR and NGS testing results in the 20 patients included in the study.

Case no.	Age/Sex	TN/Post TKI	Matched tumor sample tested	*EGFR* mutation status detected on CB/Lung biopsy/Plasma	*EGFR* mutation in CCS cfDNA by qPCR	*EGFR* mutation in CCS cfDNA by NGS	Variant detected in CCS cfDNA by NGS (Gene::%VAF)
1	77/F	TN	CB	Exon19 del	Exon19 del	[a]	[a]
2	65/F	TN	CB	L858R	L858R	[a]	[a]
3	51/F	TN	CB	Exon 20 INS	Exon 20 INS	[a]	[a]
4	49/F	TN	CB	Negative	Negative	[a]	[a]
5	51/F	TN	CB	Exon19 del	Exon19 del	[a]	[a]
6	61/M	TN	CB	Exon19 del	Negative	Negative	Negative
7	65/M	TN	CB	Exon 20 INS	Exon 20 INS	[a]	[a]
8	62/M	TN	CB	Exon19 del	Exon19 del	[a]	[a]
9	72/F	TN	Lung biopsy	Exon19 del	Exon19 del	Exon19 del	p.E746_A750del (*EGFR*::12.5%)
10	49/M	TN	CB	Exon19 del	Exon19 del	[a]	[a]
11	69/M	TN	CB	Negative	Negative	G719C	G719C (*EGFR*::22.5%)
12	61/F	Post TKI	Plasma	Exon19 del & T790M	Exon19 del & T790M	QC fail	[a]
13	43/F	Post TKI	CB	L858R	L858R & T790M	L858R	L858R (*EGFR*::17.2%)
14	35/M	TN	Lung biopsy	Exon19 del	Exon19 del	[a]	[a]
15	36/M	TN	CB	Negative	Negative	Exon19 del	p.L747_T751delinsGln (*EGFR*::87.5%)
16	69/F	TN	CB	Negative	Negative	Negative	V600E (BRAF::18.1%)
17	68/M	TN	CB	Negative	Negative	Negative	VUS (PIK3CA)
18	57/F	TN	CB	Negative	Negative	Negative	p.H1047R (PIK3CA::4.7%)
19	49/M	TN	CB	Negative	Negative	Negative	Negative
20	60/F	Post TKI	CB	Negative	Negative	Negative	Negative

Abbreviations: M, male; F, female; TN, treatment-naïve; CB, cell block; CCS, cytology centrifuged supernatants; QC, quality control check; TKI, tyrosine kinase inhibitor; exon19 del, exon 19 deletion; exon 20 INS, exon 20 insertion; VUS = variant of unknown significance; %VAF, percentage variant allele frequency.
[a]Not done.

CCS samples can be easily subjected to MT using both qPCR and NGS platforms at baseline and at tumor progression with high positive predictive values as compared to tumor DNA−based results.

EGFR mutation analysis by qPCR on effusion CCS showed excellent concordance (95%) with results obtained from patient matched tumor tissue testing, as in previous studies.[23,24] There were instances where certain mutations detected in tissue were not detected in CCS despite the presence of higher DNA quantity and use of the more sensitive NGS (exon19del in case 6). Such cases may be attributed to cfDNA contamination by non-tumor DNA shed from lymphocytes, histiocytes, and reactive mesothelial cells in effusions reducing both the fraction of tumor derived cfDNA and the resultant test sensitivity. On the contrary, certain mutations were detected only on the CCS but not in the tissue (T790M in case 13). This has been observed previously as well. Xiang et al compared molecular profiling by NGS between CCS and their matched cell pellets and identified T790M mutations exclusively in CCS in at least 3 samples.[23] Yang et al, using NGS, detected additional mutations in CCS samples of 3 of 11 patients who underwent paired CCS cfDNA and FFPE DNA testing.[25] Such events likely capture the spatial heterogeneity in tumor clonality.[24] The intact tumor cells being shed into the effusion that are more represented in the sediment may have a different clonal mutation profile as compared to tumor cells contributing to the cfDNA content in the effusion CCS.[26] Further supporting this hypothesis are our observations that the tumor fraction in the sediment/smears neither correlated with CCS cfDNA quantity nor with mutation detection rates with *EGFR* mutation being detected even in cytological negative effusions (exon 19 deletion in case 14). Thus, as highlighted previously,[21-24,26] CCS cfDNA appears to be an independent source of tumor DNA that can be used for MT even in patients with cytology-negative effusions. Cytologically negative effusion samples devoid of frank malignant cells obtained from patients with LUAD may contain tumor cfDNA, which can be tapped for MT.[27] MT on a larger cohort of lung cancer patients with cytologically negative effusions will throw light on the sensitivity and economic feasibility of such testing. With cfDNA monitoring being able to detect early recurrence in post surgically treated non-small cell lung cancer,[28,29] such studies are the need of the hour. In patients with malignant PE, testing both cell sediment and supernatant cfDNA increases the sensitivity of detecting activating *EGFR* mutations.[23] While this may not be practical or economically feasible in every case, CCS cfDNA testing should certainly be tested in patients with suboptimal tumor cellularity and/or tumor fractions in the sediment buttons.

Only one CCS sample (case 12) in the present study was directly compared with plasma cfDNA results and showed concordant results. Tong et al demonstrated increased mutation detection rate in PE supernatant compared to both PE sediment DNA as well as plasma cfDNA (98.4% in PE supernatant cfDNA versus 90.5% in PE sediment DNA versus 87% in plasma cfDNA) and attributed this increased sensitivity to the presence of higher mutant allele frequencies in effusion supernatants.[22] The close proximity to the lung tumor may possibly enhance the effective tumor DNA yield in effusions vis-à-vis plasma and this has been observed in other body fluids as well such as saliva for oral cancer[30] or urine for bladder cancer.[31] Further, previous studies have also highlighted that the cfDNA fragments from effusion samples tend to be longer (>300-500 bp) as compared to plasma (<200 bp),[32] likely contributing to better mutation detection rates. The longer-length cfDNA fragments are hypothesized to be derived from tumor cell necrosis with incomplete nucleic acid digestion.[32,33] The aforementioned points underscore the robustness of effusion supernatant cfDNA as a liquid biopsy sample and in a lung cancer patient undergoing thoracocentesis, MT on CCS cfDNA improves the sensitivity of driver mutation detection and complements the molecular information obtained from testing other tumor DNA sources such as tissue and/or plasma.

Use of various NGS platforms for cfDNA analysis performed in a few studies has demonstrated promising results.[19,24,34] Not only lung carcinomas but several other solid tumors can be analyzed using cfDNA derived from malignant effusions.[19] Yang et al demonstrated that good quality and quantity of cfDNA can be isolated from effusion supernatants with no differences in success rates of NGS between CCS cfDNA and FFPE DNA.[25] In the present study where cfDNA was subjected to targeted panel NGS using the same methods as for genomic DNA, all except 1 sample passed quality control with targetable molecular alterations identified on NGS in at least 7 of the 10 lung cancer patients. NGS showed superior sensitivity to qPCR with *EGFR* mutations being exclusively identified by NGS in 2 cases (cases 11 and 15). Surprisingly, in case 13, *EGFR* T790M mutation detected by qPCR on supernatant cfDNA was not detected on NGS of the same. This case was reviewed and was found to be unequivocally positive on qPCR and was considered a false-negative read with NGS. Similar events of false negative NGS reads have been observed in other studies as well.[35]

The routine incorporation of CCS cfDNA testing in MT protocols would eventually allow for reduction of turnaround time in cancer patients, allowing for early commencement of targeted therapy as it bypasses the steps of fixation and additional purification required for MT.[18] It can improve inadequacy rates for MT in cancer patients and reduce the need for repeat biopsies in patients with scant tissue on FFPE,[19] thus offsetting the disadvantage of higher costs associated with the use of separate kits for cfDNA isolation. Routine cold storage of effusion supernatants can be prospectively done in cytopathology laboratories with the only constraint being space available for sample storage. In the present study, supernatants were stored for up to a month at −80°C before cfDNA extraction, although longer

storage durations of up to 3 months are acceptable before any deterioration of cfDNA is expected to occur.[36] cfDNA once extracted can be stored compactly at −20°C and for much longer durations.[33,34] With a recent study demonstrating the superiority of extracellular vesicular DNA to cfDNA from effusion supernatants for *EGFR* mutation analysis,[25] it appears that the diagnostic value of supernatants is likely to increase exponentially in the coming years with the scope extending beyond lung cancer. Even though the concept of this study is not unique, this present study reinforces the utility of CCS in MT of LUAD. Through this study, it is evident that CCS can be regarded as a robust and reliable source of tumor DNA in LUAD obtained from pleural fluid. The feasibility of MT on CCS-derived cfDNA is also highlighted through this study.

The present study has certain limitations. In addition to small sample size, all samples could not be subjected to NGS due to financial constraints. The lack of testing on a resection sample that could have served as a "gold standard" contributed to difficulty in assessing discordant cases with regard to the different techniques (qPCR/NGS) used. Further, cfDNA fragment length assessment, which holds important preanalytical implications for appropriate selection of kits for sample purification and enrichment for NGS,[37] was not performed.

Conclusions

The present pilot study highlights that effusion supernatants are a rich source of tumor-derived cfDNA that can be easily exploited for mutational analysis in lung tumors. The results obtained complement the results from other sources of tumor DNA for a more comprehensive profiling of the molecular landscape of lung cancers, both at baseline and during treatment. The cfDNA obtained from effusion samples are of high quality and can be used for high throughput molecular analysis including NGS. Routine storage of effusion CCS and incorporation of CCS cfDNA into MT algorithms are recommended for any high volume cytopathology laboratory catering to cancer patients.

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Conflict of interest disclosures

None.

CRediT authorship contribution statement

Shilpi Thakur: Writing − review & editing, Writing − original draft, Data curation. **Amber Rathor:** Writing − review & editing, Writing − original draft, Data curation. **Surabhi Jain:** Writing − review & editing, Data curation. **Aruna Nambirajan:** Writing − review & editing, Visualization. **Sachin Khurana:** Writing − review & editing, Data curation. **Prabhat Singh Malik:** Writing − review & editing, Supervision, Project administration, Data curation. **Deepali Jain:** Writing − review & editing, Conceptualization.

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Data availability statement

Available to access from the corresponding author on request.

Supplementary data

Supplementary data accompanying this article can be found in the online version at https://doi.org/10.1016/j.jasc.2024.03.006.

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RESEARCH

'Plasma first' approach for detecting epidermal growth factor receptor mutation in advanced non-small cell lung carcinoma

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Abstract

Introduction The treatment approach for recently diagnosed advanced non-small cell lung cancer (NSCLC) with *EGFR* mutations primarily relies on confirming the tissue diagnosis as non-squamous NSCLC. This routine clinical practice of tissue diagnosis imposes several barriers and delays in turnaround time (TAT) for biomarker testing, significantly delaying the time to treatment. The objective of this study is to investigate the 'plasma first' approach for detection of *EGFR* mutation in advanced stage treatment naïve NSCLC patients.

Methods We prospectively collected blood samples of treatment naïve patients with clinical and radiological suspicion of advanced stage NSCLC prior to obtaining tissue biopsy. Plasma cfDNA was tested for *EGFR* mutation using two different methods. We compared the sensitivity and TAT of liquid biopsy with tissue biopsy.

Results In total, we analyzed plasma cell-free DNA (cfDNA) of 236 patients suspected of having advanced NSCLC for *EGFR* mutations. We observed a notably shorter turnaround time (TAT) of 3 days, which was significantly quicker compared to the 12-day TAT for tissue biopsy ($p < 0.05$). The ddPCR method had a sensitivity of 82.8%, which was higher than 66.34% sensitivity of ARMS-PCR. The current study also highlights that there is no significant difference in the clinical outcome of the patients whether treated based on liquid biopsy only or tissue biopsy (median progression-free survival of 11.56 vs. 11.9 months; $p = 0.94$).

Conclusions Utilizing a 'plasma first' strategy, given its shorter turnaround time, strong positive concordance and comparable outcomes to tissue biopsy, emerges as a highly specific and reliable method for detecting *EGFR* mutations in advanced-stage NSCLC.

Keywords NSCLC · EGFR · Liquid biopsy · Plasma first · Turnaround time

Introduction

Lung cancer is the primary cause of cancer deaths globally, often diagnosed at an advanced inoperable stage (Hung et al. 2019). Recent treatment advancements have shifted from the traditional chemotherapeutic approach to personalized targeted approaches (Chan and Hughes 2015; Jones and Baldwin 2018) based on identifying specific driver mutations like epidermal growth factor receptor (*EGFR*) and fusions in anaplastic lymphoma kinase (*ALK*) and ROS proto-oncogene 1 (*ROS1*) (Maemondo et al. 2010; Chuang and Neal 2015). The field of precision oncology revolves around the comprehensive molecular characterization of the most common adenocarcinoma subtype of non-small cell lung cancer (NSCLC). *EGFR* and its downstream signalling pathways have been the most extensively studied key player in the tumor development of NSCLC (Chan and Hughes 2015). *EGFR* mutations are more prevalent in Asia than other geographical regions and have been reported in up to 49.1% of Asian NSCLC patients with advanced stage (Benbrahim et al. 2018; Melosky et al. 2022). Studies have reported the occurrence of *EGFR* mutations in the Indian

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population ranging from 23 to 44% (Sahoo et al. 2011; Chougule et al. 2013; Singh et al. 2020). Tumor acquisition is vital and testing time for drivers is the current standard for the selection of treatment in *EGFR*-mutation positive advanced stage NSCLC (Lindeman et al. 2018; Panchard et al. 2018; Singh et al. 2022). However, difficulty in acquiring tumor tissue, delay in diagnosis, inadequate tumor tissue available for molecular testing and rapid deterioration of patient's general condition hinders timely molecular testing and early initiation of therapy (Pisapia et al. 2019).

Liquid biopsy provides a minimally invasive alternative for genotyping, overcoming limitations of conventional biopsies (Diaz and Bardelli 2014). Detecting targetable mutations from circulating tumor DNA (ctDNA), a component of circulating cell-free DNA (cfDNA) has opened up new possibilities in therapeutic decision making, offering the choice between the 'tissue first' versus 'plasma first' approach (Rolfo et al. 2021). In advanced NSCLC, ctDNA detection has been limited to patients who have either progressed on EGFR TKIs or have inadequate tumor tissue for molecular analysis (Canale et al. 2019). Liquid biopsy holds great potential for rapid diagnosis, prognosis and predicting treatment response (Kawahara et al. 2015). *EGFR* mutations are usually detected from tumor DNA in the form of formalin-fixed paraffin-embedded (FFPE) diagnostic blocks or ctDNA from plasma. The standard clinically applicable method of *EGFR* detection in FFPE is polymerase chain reaction (real-time PCR-based), while various methods have been developed for liquid biopsy samples. Next-generation sequencing based (NGS) approaches have significantly outperformed other methods with greater sensitivity but requires sophisticated computational methods and bioinformatic expertise (Lee et al. 2020). Real-time PCR-based and droplet digital PCR (ddPCR) based methods are the two most feasible clinically applicable methods that can be applied as a quick screening test for liquid biopsy samples for tumor genotyping. This strategy is more pragmatic in ethnic populations like Asians where the frequency of *EGFR* mutation is high. Hence, the aim of the present study is to evaluate the 'plasma first' approach using liquid biopsy in advanced stage treatment naïve NSCLC patients for early detection of *EGFR* mutation and compare it with 'tissue first' approach.

Subjects and methods

Sample collection and processing

This prospective study was approved by institute ethics committee (IECPG-740/23-12-2021) and all patients given written informed consent for blood collection. All patients enrolled ($n = 285$) in the study were presented to lung cancer clinic of the institute with clinical and radiological suspicion of lung cancer. 10mL of peripheral blood sample was collected aseptically in K2-EDTA vial prior to obtaining tissue biopsy.

Peripheral blood drawn was gently mixed by inverting the vial several times immediately and processed for plasma separation within 1 h of collection. To obtain plasma, the collected whole blood sample was centrifuged in an optimized two-step centrifugation process and stored at -80 °C until processed further for cfDNA extraction. 4mL of stored plasma was processed to isolate cfDNA using the Maxwell® RSC ccfDNA Plasma kit (Promega, USA) as per slight modifications in the manufacturer's instructions. DNA isolated was quantified using Nanophotometer (Implen N60, US) and Quantus Fluorometer (Promega, USA) using QuantiFluor® dsDNA kit.

Detection of EGFR mutations using ARMS-PCR

Real-time polymerase chain reaction (ARMS-PCR) was performed to detect clinically relevant *EGFR* hotspot mutations using EGFR RGQ PCR IVD kit (Qiagen, Manchester, UK). This is a ready to use kit which can qualitatively detect 29 clinically relevant hotspot mutations in the exon 18, 19, 20 and 21 of the *EGFR* gene. The qPCR assay was performed as per manufacturer's instructions and the final interpretation of data was done according to recommended kit guidelines.

Detection of EGFR hotspot mutations using droplet digital PCR (ddPCR)

All droplet digital PCR (ddPCR) consumables including droplet PCR supermix, droplet generation oil for probes, droplet generator cartridges and gaskets, droplet reader oil and ddPCR 96-well plates were procured from Bio-Rad Laboratories Inc. (Hercules, CA, USA). ddPCR was performed with three commercially available PrimePCR™ ddPCR™ Mutation Detection Assay Kit for E746_A750del, L858R and T790M (Bio-Rad; Hercules, CA). The PCR reaction was performed according to the manufacturers' instructions and the droplets were read by Bio-Rad QX200 ddPCR droplet reader system and finally analysed using Quantasoft version 1.7.

Statistical analysis

Data analysis was performed using Stata statistics version 14.2 (StataCorp LLC, USA). The Chi-Square test/Fisher Exact test was used to analyse the baseline categorical variables. Percentage of concordance between both techniques

was determined from matched *EGFR* positive patients only either by tissue or liquid biopsy. We estimated turnaround time (TAT) as the time defined between the registration of the samples (tissue or liquid biopsy) in the pathology or molecular biology laboratory and the *EGFR* molecular test performed. Progression free survival (PFS) was studied using Kaplan-Meier curves, defined as the period of time from the start of TKI therapy until disease progression or death of the patient from any reason.

Results

Patient selection

This was a prospective study with 285 newly diagnosed/suspected treatment naïve patients. Patients' peripheral blood was collected and plasma cfDNA isolated was quantified and checked for quality by using both spectrophotometer and fluorometer. All patients tested by liquid biopsy were diagnosed at an advanced metastatic stage of the disease with involvement of bone and brain (58.6%) as the most common metastatic sites followed by concurrent effusions and other sites such as liver, adrenal and pancreas.

Liquid biopsy for *EGFR* molecular testing using ARMS-PCR

All patients enrolled in the study were tested for *EGFR* mutation on liquid biopsy using ARMS-PCR prior to obtaining tissue biopsy (Fig. 1). Sixty-nine (24.2%) of total 285 treatment naïve patients with suspected NSCLC were found to be positive for *EGFR* mutation by liquid biopsy only using ARMS-PCR. In these 69 cases, 48 (69.5%) showed exon 19 deletions followed by 17 (24.6%) cases of exon 21 L858R mutation. Among the ten plasma only positive patients with exon 19 deletions, four samples had insufficient tissue for molecular analysis, and the tissue *EGFR* status was unknown for the remaining six cases. Similarly, for the cases where L858R mutation was detected in plasma, the tissue status was not known for two cases. There were a few uncommon mutations such as exon 20 insertions (2), exon

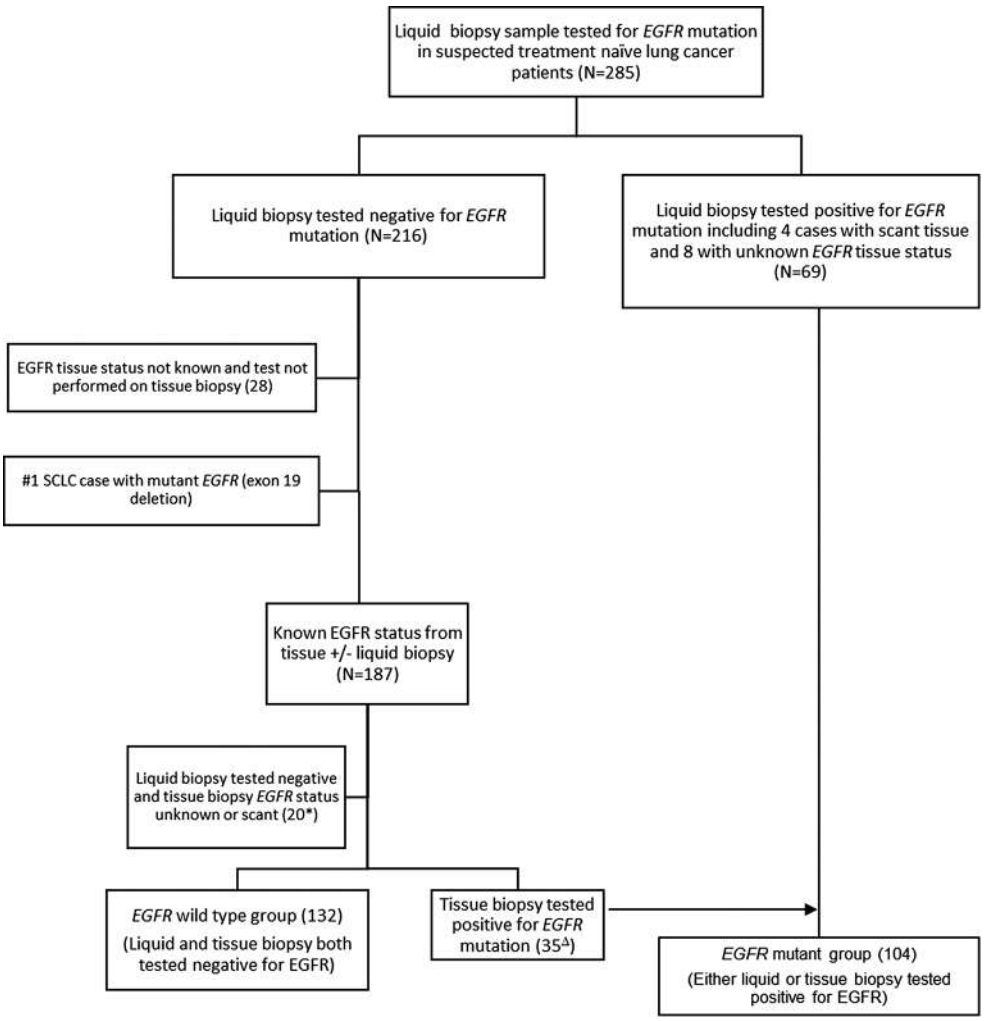

Fig. 1 Summary of samples collected for final analysis (*excluding cases in which tissue biopsy was scant for molecular testing; Δ patients tested negative on liquid biopsy by ARMS-PCR but tested positive on tissue biopsy; #excluded a case of small cell lung cancer tested positive for EGFR mutation by liquid biopsy)

21 L861Q (1) and 1 of compound mutation (exon 21 L858R and exon 20 T790M) (Fig. 2; Table 1). The median turnaround time (TAT) for detection of *EGFR* mutation using liquid biopsy was found to be 3 days (range 1 to 12 days).

Tissue sampling and *EGFR* molecular testing by ARMS-PCR

All 285 patients enrolled in the study were followed in the clinic for availability of matched tissue biopsy and confirmed *EGFR* mutation status. Twenty-eight (9.8%) cases were excluded because of non-availability of matched tissue biopsy, inconclusive biopsy, cancer of primary origin other than lung or patient lost to follow up after diagnosis without taking treatment. One case was additionally excluded diagnosed as small cell lung cancer on tissue biopsy which however detected to have *EGFR* mutation (exon 19 deletion) on liquid biopsy testing and then confirmed on tissue biopsy. Out of the remaining 256 patients, 187 (73%) tested negative for *EGFR* mutation using liquid biopsy. Among these, 132 (70.5%) cases were found to be *EGFR* wild type when tested using both tissue and liquid biopsy (Fig. 1). Twenty (10.6%) of 187 cases tested negative for *EGFR* mutation using liquid biopsy but their matched tissue biopsy *EGFR* status was scant for molecular testing. However, 35 (18.7%) of 187 were found to be positive for *EGFR* mutation on tissue biopsy using ARMS-PCR and plasma cfDNA of these cases was further tested for *EGFR* mutation using droplet digital PCR (ddPCR).

Overall, 236 treatment naïve patients tested for *EGFR* mutation and subdivided into two groups, 104 (44.06%) patients with *EGFR* mutations and 132 (55.94%) patients with wild-type *EGFR* (Fig. 1). Patients tested for *EGFR* mutation using tissue biopsy by ARMS-PCR showed exon 19 deletions in 59/104 (56.7%), exon 21 L858R in 25/104 (24%) and uncommon mutations in 5 (3 of exon 20 insertions, 1 of exon 18 G719x and 1 of exon 21 L861Q). Additionally, 3 cases showed compound mutation of which 2 had exon 21 L858R and exon 20 T790M while 1 had exon 21 L858R and exon 20 S768I. Of these 104 cases, tissue biopsy in 12 (11.5%) was either scant or not known for *EGFR* molecular testing but their matched liquid biopsy tested positive either using ARMS-PCR or ddPCR (Table 1). The median turnaround time (TAT) for detection of *EGFR* mutation using tissue biopsy was found to be 12 days (range 7 to 57 days).

Liquid biopsy for *EGFR* molecular testing using ddPCR

All 104 patients tested positive for *EGFR* mutations either using liquid or tissue biopsy had their plasma cfDNA tested for *EGFR* common mutations only using ddPCR except five cases with uncommon *EGFR* mutations. Using ddPCR,

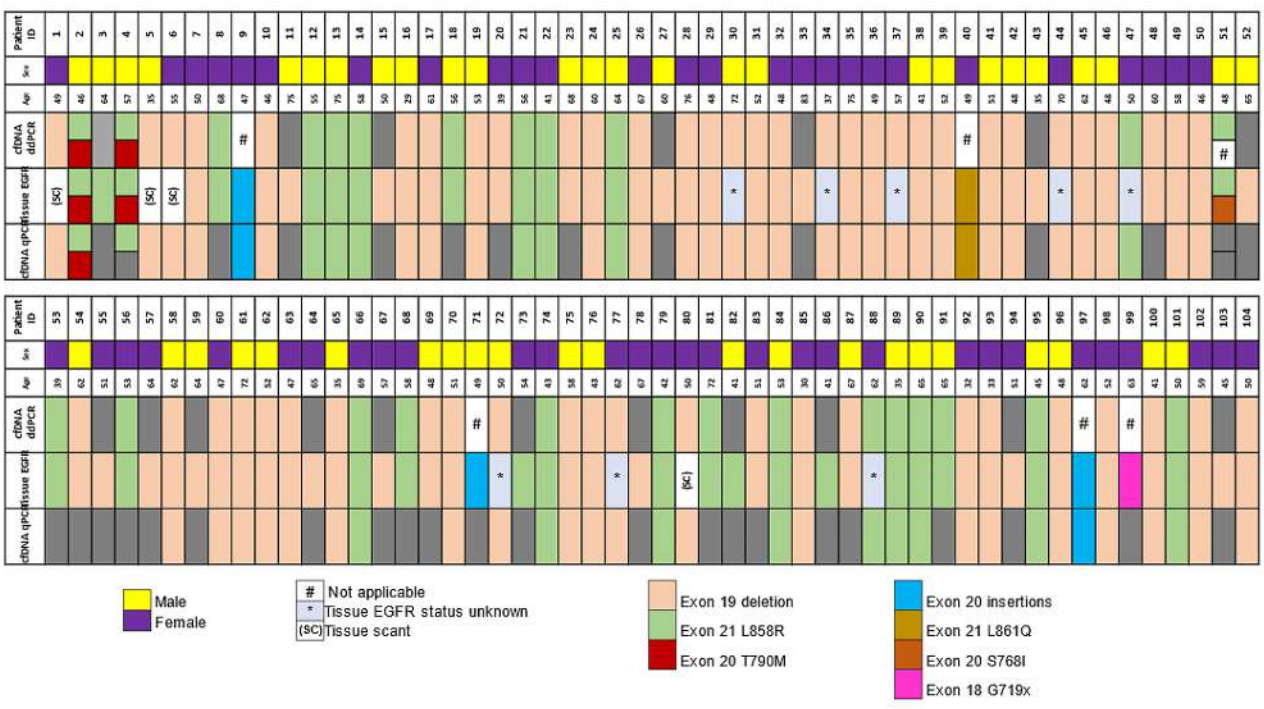

Fig. 2 *EGFR*-mutated patients' cohort and concordance of matched plasma cfDNA with tissue *EGFR* status as compared by ARMS-PCR and ddPCR

Table 1 Frequency distribution of *EGFR* mutation subtype as detected by tissue biopsy (ARMS-PCR only) or liquid biopsy (ARMS-PCR and ddPCR both)

Cases with EGFR mutation subtype	Tissue biopsy (ARMS-PCR)	Liquid biopsy (ARMS-PCR)	Liquid biopsy (ddPCR)
Common mutations			
Exon 19 deletions (69)	$59^{+4,\#6}$	48	55
Exon 21 L858R (27)	$25^{\#2}$	17	24
Uncommon mutations			
Exon 20 insertions (3)	3	2	**
Exon 18 G719×(1)	1	0	**
Exon 21 L861Q (1)	1	1	**
Compound Mutations			
Exon 21 L858R & Exon 21 T790M (2)	2	$2^{\$}$	2
Exon 21 L858R & Exon 20 S768I (1)	1	0	1^{Δ}

+ tissue scant for molecular analysis, # tissue EGFR status unknown, ** technically not applicable, $ L858R detected by both ARMS-PCR and ddPCR but T790M was detected by ddpcr only, $^{\Delta}$L858R was detected using ddPCR and exon 20 S768I was technically not applicable using ddPCR

55/99 (55.5%) cases showed exon 19 deletions followed by 24/99 (24.2%) of exon 21 L858R and 3 cases of compound mutation. Among the plasma negative cases, ddPCR identified 17 out of 33 false negative cases that were undetected by the less sensitive ARMS-PCR technique. However, two false negative cases, one with exon 20 insertions and one with exon 18 G719x could not be technically assessed using ddPCR. Detailed representation of matched tissue-plasma samples detected using ddPCR has been depicted in Fig. 2 and Supplementary Fig. 1.

Concordance and turnaround time (TAT) of tissue vs. plasma cfDNA *EGFR* mutation status

Matched plasma cfDNA from 104 *EGFR* mutant group when primarily tested using ARMS PCR, 69/104 (66.34%) cases positively correlated with tissue *EGFR* status. All 69 patients' plasma samples that tested positive for *EGFR* mutations with ARMS-PCR also showed positive results with ddPCR, except for mutations that were technically challenging to detect. We performed ddPCR further for common *EGFR* mutations and the assay sensitivity increased to 82.8% (82 out of 99 cases) when compared with ARMS-PCR (Fig. 2).

All patients tested for *EGFR* mutation using liquid biopsy had shorter TAT (the time when the patient is presented to the clinic till the revelation of *EGFR* mutation status to the treating clinician) with a median TAT of 3 days (range 1–12 days) in comparison to the tissue *EGFR* testing with a median TAT of 12 days (range 7–57 days) with a significant p-value of <0.05 (Fig. 3). There was no discordance in the target hotspot between matched plasma and tissue. The study showed that cfDNA testing for *EGFR* mutation detection using ddPCR had 82.8% sensitivity, 100% specificity, 100% positive predictive value (PPV) and 88.5% negative predictive value (NPV).

Clinical features of *EGFR* mutant and *EGFR* wild type patients

Demographic details of *EGFR* mutated and *EGFR* wild type patients (Table 2) showed overall male predominance (male to female ratio of 1.22:1) of treatment naïve patients tested for *EGFR* mutations. However, it was found that females with *EGFR* mutation were more prevalent than males ($p=0.01$). The median age of overall cohort was 55 years (range 28–84 years). Smoking history was available for 220/236 (93.2%) patients of which frequency of non-smoker was similar in both *EGFR* mutant and *EGFR* wild type group. However, the proportion of non-smokers in the *EGFR* mutant group was significantly higher than in the *EGFR* wild type group ($p=0.001$).

Treatment outcomes and survival of *EGFR* mutated patients

Out of 104 *EGFR* mutant patients, treatment details of 96 (92.3%) patients were available (Supplementary Table 1). Among these, 85/96 (88.5%) patients received EGFR TKIs of which 66 (77.6%), 10 (11.8%), 3 (3.5%) and 6 (7.1%) were treated with gefitinib, erlotinib, afatinib and osimertinib, respectively. In the remaining 96 cases, eight (8.34%) patients received combination of EGFR TKI (gefitinib) and chemotherapy while three (3.1%) received chemotherapy only. Kaplan-Meier survival analysis was performed based on *EGFR* status detected by liquid biopsy only and by tissue biopsy with or without liquid biopsy. After the median follow-up of 12.6 months, progression-free survival (PFS) of all patients undergoing EGFR TKI therapy was found to be 11.67 months (95% CI 9.34–16.24; Fig. 4A). Those patients treated only on the basis of liquid biopsy *EGFR* status had similar PFS (11.56 months 95% CI 5.26-NR) when compared with those where *EGFR* status was detected by tissue

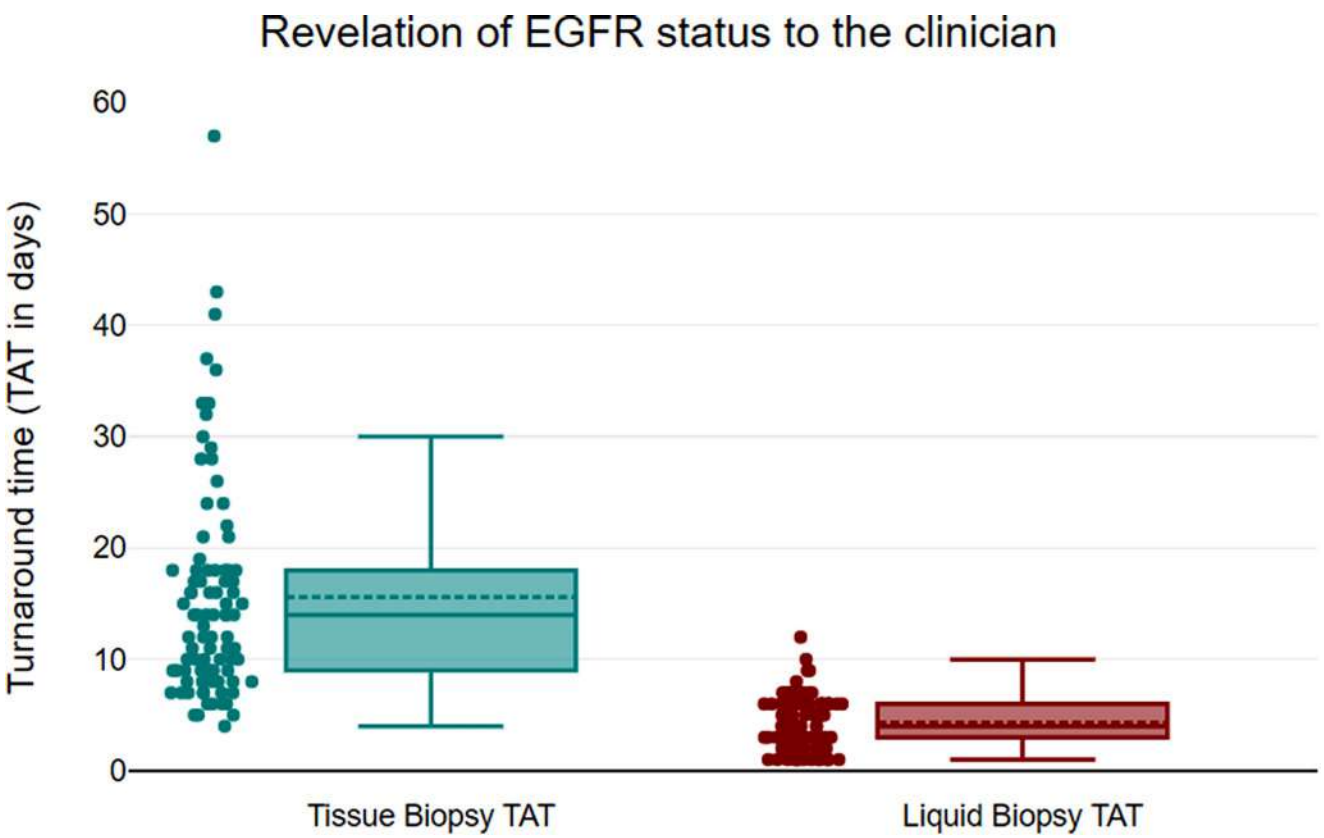

Fig. 3 Turnaround time (TAT in days) for detection of *EGFR* mutation from matched tissue and plasma primarily tested using ARMS-PCR

biopsy with or without liquid biopsy (median PFS of 11.9 months 95% CI 9.34–16.23) (log rank $p = 0.94$) (Fig. 4B).

At disease progression till the last date of follow-up, repeat liquid biopsy with or without tissue biopsy could be performed in 17 of 96 cases (17.7%). (Supplementary Table 1). Repeat tissue biopsy could be performed in 8/96 (8.3%) patients with paired liquid biopsy in six patients. Overall, 19 patients were available with repeat liquid or tissue biopsy and tested for presence of T790M resistance mutation or histological transformation. Among these, 4 patients showed presence of *EGFR* T790M resistance mutation by both, ARMS-PCR and ddPCR. Additionally, 2 patients showed small cell transformation (SCT) and 13 showed either only the founder mutation or ctDNA cleared for founder mutation.

Discussion

In recent years, treatment decisions of advanced stage unresectable NSCLC patients are mostly based on personalized medicine advancements. In advanced stage lung cancer, frontline liquid biopsy testing is recommended for *EGFR* mutation detection when tumor tissue is insufficient (Paweletz et al. 2016; Lindeman et al. 2018; Rolfo et al. 2021; Satapathy & Singh et al. 2021), while it was strongly recommended for detection in TKI resistance settings (Satapathy & Singh et al. 2021; Silveira et al. 2021; Filipits et al. 2023). The incidence of *EGFR* mutations in advanced stage NSCLC varies among different ethnicities, of which highest prevalence is observed among Asians (Benbrahim et al. 2018; Melosky et al. 2022; Hofman et al. 2023). Due to procedural and technical advantages of liquid biopsy, it has been widely accepted as an alternative to tumor tissue genotyping for detecting *EGFR* mutations in NSCLC (Paweletz et al. 2016; Leighl et al. 2019; Rolfo et al. 2021; Raez et al. 2023). Besides the limited availability of tissue biopsy for tumor genotyping, turnaround time is a critical factor for lung cancer patients with a heavy symptomatic disease burden. In such scenario, it is prudential to adopt testing strategies that help in quickly identifying patients eligible for targeted therapy by single-gene testing such as *EGFR* oncogenic driver mutations in comparison to the more comprehensive NGS based approaches. Furthermore, in regions of the world with high *EGFR* mutation rates, the initial molecular evaluation often involves limited PCR analysis for detecting *EGFR* mutations (Rolfo et al. 2021).

We performed single gene *EGFR* mutation testing by two methods, using real-time polymerase chain reaction based followed by ddPCR. Real-time PCR based methods have

Table 2 Patient demographic with clinicopathological details

Parameter	Patients with mutant EGFR	Patients with wild type EGFR	*p*-value
Median Age (range) in years	52 years (28–84 years)	57 years (28–82 years)	0.15
Gender	Total (*N* = 104)	Total (*N* = 132)	
Male	48 (46.2%)	82 (62.1%)	
Female	56 (53.8%)	50 (37.9%)	
Male: Female ratio	0.85:1	1.64:1	0.01
Smoking history			
Non-smoker	65	68	0.001
Smoker	18	51	
Tobacco	11	7	
Diagnosis			
NSCLC adenocarcinoma	94	116	-
NSCLC squamous cell carcinoma	-	2	
NSCLC-NOS	2	3	
NSCLC adenosquamous	-	6	
NSCLC (undifferentiated carcinoma)	4	-	
Poorly differentiated carcinoma	3	5	
Combined NSCLC and SCLC	1	-	

NSCLC = non-small cell lung carcinoma, SCLC = small cell lung carcinoma, NOS = not otherwise specified, EGFR = Epidermal growth factor receptor, N = numbers

been widely used due to their cost-effectiveness and reliable results (Hofman et al. 2023). In our study, *EGFR* mutation detection using ARMS-PCR method had a lesser sensitivity of 66.34% (69/104) as shown by other trials in comparison to ddPCR (Li et al. 2019; Satapathy & Singh et al. 2021; Douillard et al. 2014; Hsiue et al. 2016; Suryavanshi et al. 2018; Satapathy & Singh et al. 2021). Real-time PCR based assays have been widely used for detecting *EGFR* mutations in tumors and has shown limited clinical sensitivity when it comes to detecting *EGFR* mutations in liquid biopsy samples. We observed false-negative plasma results using ARMS-PCR in 35 cases with *EGFR* mutations. This further highlights the challenge of detecting *EGFR* mutations in liquid biopsy samples due to the low quantity of cfDNA. Liquid biopsy has proven to be an invaluable tool in identifying *EGFR* mutations in NSCLC patients. Through the utilization of highly sensitive ddPCR and NGS techniques, previous studies have demonstrated the remarkable sensitivity and specificity of this approach (Pawaletz et al. 2016; Wei et al. 2019; Soria-Comes et al. 2020; Satapathy & Singh et al. 2021).

However, various factors have been identified limiting the clinical sensitivity and false-negative results with plasma mutation analysis (Trigg et al. 2018; Markus et al. 2018; Aldae et al. 2020; Song et al. 2022). Plasma contains tumour-derived circulating tumor DNA (ctDNA), with the proportion of ctDNA in the bloodstream being influenced by the release from tumor cells undergoing apoptosis and necrosis. Aldae et al. have demonstrated a significantly lower shedding of ctDNA between NSCLC patients with central nervous system (CNS) metastases during disease progression and those without any CNS involvement. Various pre-analytical factors impact the quantity of cfDNA in the blood (Trigg et al. 2018; Markus et al. 2018). Additionally, patient related factors frequently contribute to the effectiveness of mutation detection, particularly in cases where there is a minimal presence of mutant DNA (Zhu et al. 2015).

The utilization of ddPCR assays to detect the low limit of detection enhances its suitability as a more sensitive

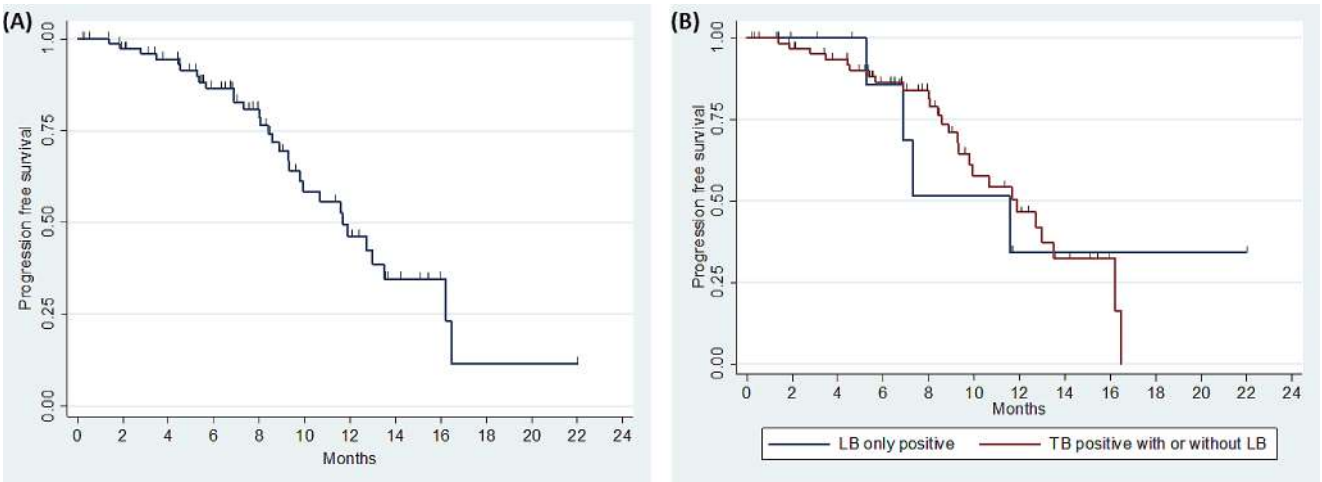

Fig. 4 Kaplan-Meier survival analysis showing, **A**) Progression free survival (PFS) in all patients treated with EGFR TKI; **B**) Progression free survival (PFS) comparison of patients with *EGFR* status detected from liquid biopsy only and tissue biopsy with or without liquid biopsy (LB = liquid biopsy, TB = tissue biopsy)

approach for identifying mutations in liquid biopsy samples. ddPCR assays have demonstrated a sensitivity in detecting *EGFR* mutation as low as 0.04%, with the detection limit depending on the sample DNA input and the ratio of mutant copies to wild-type DNA template (Zhu et al. 2015). We experienced failure in detecting one-third (35 out of 104 cases) of total *EGFR* positive cases using ARMS-PCR. Of these 35 cases, 2 cases were positive for exon 20 insertions and exon 18 G719x mutation which were not technically feasible using ddPCR. In the remaining 33 cases, ddPCR successfully detected mutations in approximately 51.5% (17/33) of the cases. Out of these, 12 cases were positive for exon 21 L858R mutation, and ddPCR was able to detect 9 out of these 12 cases. However, ddPCR identified only 8 out of 22 false- negative cases with exon 19 deletions.

We have observed cases that were initially false-negative but later tested positive in plasma using ddPCR, with mutant DNA fractions ranges from 0.1 to 0.9%. Such cases with very low tumor fraction may be attributed to cfDNA contamination by non-tumor DNA reducing the fraction of tumor derived DNA and thereby, false negative plasma results with less sensitive ARMS-PCR method. However, a significant number of false negative plasma samples (14 out of 22 cases) with exon 19 deletions went undetected using ddPCR. This could also be attributed to the utilization of the E746_A750del mutation assay in ddPCR, which is the common subtype for del 19, (Rossi et al. 2019; Zhao et al. 2020). It remains unclear whether other uncommon subtypes of exon 19 deletion mutation differ from the common one in terms of tumor DNA shedding. In a recently published study, we observed a case of exon 19 deletion with uncommon *EGFR* subtype (Leu747-thr751delinsGln) with a mutant fraction as high as 87.5%. Surprisingly, this uncommon *EGFR* mutation subtype went undetected by the less sensitive PCR-based assay but was successfully identified through NGS (Thakur & Rathor et al. 2024). Similarly, another study identified unusual L858R mutation identified using NGS in liquid-based cytology indicating that NGS based methods are superior than PCR-based methods in detecting more mutation sites within a target region (Wu et al. 2020). Furthermore, within a group of patients who tested negative on liquid biopsy results, we identified 2.8% (3/104) of cases with exon 20 insertions, with only one of these cases showing a negative result on liquid biopsy. EGFR Exon 20 insertions are heterogenous short in-frame insertions which are the third most frequent *EGFR* mutations in NSCLC (Burnett et al. 2021). While traditionally these mutations are associated with a poorer prognosis compared to classical *EGFR* mutations (Chouaid et al. 2021), the recent approval of selective inhibitors has sparked renewed interest in studying and targeting these specific mutations (Passaro et al. 2022). Detection of EGFR exon 20 insertions has been earlier limited to the use of multiplex-based PCR kits, which have shown significant false-negative results when compared to NGS (Shen et al. 2022; Rolfo et al. 2023).

The present study suggests that the plasma first approach can overcome a major implementation barrier for personalized medicine i.e. long waiting time of invasive tissue biomarker results (Aggarwal et al. 2019; Hofman et al. 2023). The estimated TAT in the clinical guidelines for *EGFR* testing using tissue biopsy is 7–10 days (Hofman et al. 2023) however significantly shorter TAT can be achieved using the 'plasma first' approach in comparison to tissue biopsy (median TAT of 3 vs. 12 days, respectively; p=<<0.05). We have shown how implementing 'plasma first' approach is linked to a significant improvement in the TAT as short as 1 day to reveal *EGFR* status to the clinician. Similar studies were performed using NGS based testing that demonstrated dispensability of liquid biopsy in determining front-line therapy decision with shorter TAT and greater test success rate in comparison to tissue biopsy (Aggarwal et al. 2019; Cui et al. 2022; Raez et al. 2023; García-Pardo et al. 2023; Russo et al. 2024). The challenge lies in obtaining matched tissue biopsy samples for patients with poor clinical conditions or when biopsies are not feasible. Consequently, this has led to a biased increase of 44.06% in the *EGFR* mutations.

Additionaly, we evaluated progression-free survival (PFS) of patients who received EGFR TKI therapy. The PFS did not significantly differ between patients treated based on liquid biopsy alone versus those treated based on tissue biopsy with or without liquid biopsy (median PFS of 11.56 vs. 11.9 months, respectively; $p = 0.94$). The observed PFS with EGFR TKIs was similar as reported in various studies and treatment decision based on liquid biopsy do not affect clinical outcomes (Huang et al. 2021; Lu et al. 2023). The 'plasma first' approach allowed clinician to treat patients with positive cfDNA results for *EGFR* single oncogene test. Although tumor tissue is the 'gold standard' for tumor genotyping, it still remains undergenotyped in many patients (Smolle et al. 2021). In the present study, tissue *EGFR* status was either unknown or not sufficient for *EGFR* molecular testing in twelve cases however, in these patients liquid biopsy was the only tool for predicting *EGFR* status. The study showcased the PFS of patients who underwent treatment solely based on their *EGFR* status, utilizing a single-gene testing approach. We also observed that nearly one-third of the *EGFR*-positive patients who received EGFR TKI therapy experienced a PFS duration of less than 5 months. Recent studies have shed light on the correlation between co-mutations and unfavorable outcomes, as well as the underlying mechanism that promotes resistance in *EGFR*-mutant lung adenocarcinoma (Vokes et al. 2022; Liu et al. 2022).

In addition, the NSCLC subtype is dynamically evolving with current recommendations suggesting testing with a multigene NGS based approach (Mosele et al. 2020; Ettinger et al. 2022). The primary limitation of the current study lies in exclusive testing of the *EGFR* gene through liquid biopsy, instead of conducting comprehensive multi-gene testing that includes comutations. Studies using NGS on liquid biopsy in metastatic advanced stage have shown the potential of using liquid biopsy as a standard of care to complement tissue genotyping (Paweletz et al. 2016; Aggarwal et al. 2019; Leighl et al. 2019). Some recent NGS based studies evaluated the potential of using plasma NGS approach in subjects with suspected lung cancer prior to obtaining tissue biopsy (Cui et al. 2022; Raez et al. 2023; García-Pardo et al. 2023; Russo et al. 2024). García-Pardo et al. and Cui et al. demonstrated similar median turnaround time (TAT) of approximately one week for plasma-based NGS compared to tissue diagnosis, which had a median TAT of around three weeks in advanced nonsquamous NSCLC. We are conducting an investigative study utilizing NGS based approach to evaluate the potential of using liquid biopsy in the management of treating patients with concomitant mutations. The current project served to evaluate the feasibility of integrating liquid biopsy into standard patient care for the most prevalent predictive biomarker, *EGFR*.

Conclusions

Liquid biopsy is non-invasive, offers high specificity and an efficiently quick testing method compared to tissue biopsy sampling, which may not always be feasible or sufficient for molecular testing. It can serve as an alternative for biomarker evaluation during initial diagnosis to detect *EGFR* mutations in advanced NSCLC. The turnaround time (TAT) for *EGFR* molecular analysis using liquid biopsy is significantly faster than tissue biopsy, thereby resulting in reduced delays in treatment. The present study indicates that survival outcomes are similar between liquid biopsy and tissue biopsy, suggesting that liquid biopsy is a promising modality for early detection of *EGFR* mutations in advanced NSCLC, especially in parts of globe where *EGFR* mutation rate is high.

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Author contributions A.R.: Methodology, Formal analysis, Investigation, Data curation, Project administration, Writing- Original draft. P.S.M.: Methodology, Formal analysis, Data curation, Resources, Project administration, Writing- Review & Editing. P.T.: Resources. S.K.: Resources, Funding acquisition. H.B.: Resources. D.P.: Resources. A.N.: Methodology, Formal analysis, Funding acquisition, Review & Editing. D.J.: Conceptualization, Methodology, Formal analysis, Data curation, Project administration, Writing- Review & Editing, Funding acquisition. All authors reviewed the manuscript.

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

Declarations

Ethical approval The study was ethically approved by the AIIMS Institute Ethics Committee (IEC) (Reference number IECPG-740/23-12-2021).

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

Consent to publish The authors confirm that informed consent was obtained from human research participants for publication. No patient identifying information is included in the article.

Competing interests The authors declare no competing interests.

Presentation at a meeting The work was presented as an e-poster at the World Conference on Lung Cancer (WCLC) 2022, organised by International Association for the Study of Lung Cancer (IASLC).

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