RESEARCH ARTICLE

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Non-Small Cell Lung Cancer: Targetable Variants in Concurrent Tissue and Liquid Biopsy Testing in a North Indian Cohort

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Abstract

Objectives: Testing for EGFR, ALK, ROS1 and MET alterations in paired tissue and plasma samples of treatment-naïve patients of NSCLC and correlating their status with overall survival. **Materials and methods:** One hundred treatment-naïve patients were recruited after obtaining informed consent. Ten ml of blood was collected within a period of two weeks from histological diagnosis, prior to the start of any treatment. DNA & RNA extraction was done from formalin-fixed paraffin embedded (FFPE) tissue and total cell-free nucleic acid extraction was done from plasma samples. EGFR mutation, ALK, ROS1 and MET rearrangements were tested by ARMS (Amplification Refractory Mutation System) PCR. All statistical analyses were conducted in R version 4.1.1. **Results:** A total of 61 cases showed molecular alterations in tissue samples which included EGFR mutations (47), ALK rearrangements (12), ROS1 fusion (2). MET alteration was not detected. Forty-three cases showed EGFR mutations in plasma, 26 of which were concurrently positive in tissue. Concordance observed was 62%. ALK-EML4 rearrangement, ROS1 fusion and MET were not detected in plasma samples. Sensitivity and specificity for detection of EGFR mutation in plasma were 55.3% and 67.9% respectively. Univariate Cox regression analysis showed a positive association between EGFR mutation in tissue and overall survival (HR = 0.4; 95% CI: 0.2-0.7; p = 0.003) and improved overall survival in those who received targeted therapy (HR = 0.29; 95% CI: 0.1–0.8; p = 0.02). **Conclusion:** Concurrent testing in tissue and liquid biopsy in NSCLC increased the detection of EGFR mutations (47% to 64%). This has substantial implications in deciding treatment and administration targeted therapy and the consequent overall survival.

Keywords: NSCLC-EGFR- ALK- targetable alterations- liquid biopsy-Concurrent testing

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Introduction

Lung cancer has been a significant cause for mortality all over the world including India (Sung et al., 2020). A significant challenge faced by clinicians is the limited treatment options available because the majority of cases are diagnosed at an advanced stage which makes it practically impossible to cover the wide landscape of therapies available. Hence, an effective and specific method of testing for therapeutically amenable molecular targets is the need of the hour to avail the benefits of targeted therapy especially in advanced stages of disease. Due to this fact, it has become almost indispensable to couple the histological diagnosis with molecular testing to identify therapeutically relevant biomarkers such as EGFR, ALK, ROS1, MET, BRAF etc. as this carries immense clinical and prognostic implications for the patient. Targeted therapies such as tyrosine kinase inhibitors show significant benefit in patients with genetic alterations in the form of better disease control and overall survival benefit. This favourable direction in treating lung cancer has prompted more precise and feasible testing strategies over the years (Brainard and Farver, 2019). However, although the testing process may be well-developed, a key issue is the procurement of adequate samples for testing (Zill et al., 2015). Conventionally, tissue samples (biopsy/FNA) were considered gold standard for molecular testing (Lindeman et al., 2018). But there are several challenges when it comes to performing a biopsy and obtaining an adequate sample for precise histological and molecular examination. These include the procedural complications in debilitated patients, inaccessible location or multiple sites of metastasis, poor repeatability for disease monitoring (Murtaza et al., 2013; Guibert et al., 2020). Moreover, a single-site biopsy offers no insight into intratumoral heterogeneity and fails to capture a complete snapshot of the molecular profile for the tumor (Gerlinger et al., 2012). Over the

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recent past, liquid biopsy has become an alternate option for testing when sufficient tissue material is unavailable. It is non-invasive, cost effective, easily repeatable and has less risk of procedural complications as compared to tissue biopsy (Rolfo et al., 2018; Guibert et al., 2020). Can both compensate for each other's lacunae so that the spectrum for detection of targetable alterations can be widened? This would prove as a tremendous leap towards providing the benefit of targeted therapies to those patients who may have been missed due to primary testing in either one of the samples. Here we explored this hypothesis and provide results that would lend strength to the proposition of concurrent molecular testing in tissue and cell-free samples.

Materials and Methods

Study design

One hundred treatment-naive patients of NSCLC were included in the study after obtaining written consent. Molecular analysis was done on paired tissue and plasma samples for EGFR, ALK, ROS1 and MET by real time PCR (Figure 1).

Mutation analysis in tissue samples

DNA and RNA extraction from formalin-fixed paraffin embedded (FFPE) tissue were done using QIAamp DNA FFPE tissue kit and RNeasy kit from Qiagen respectively. The nucleic acid samples were analysed for EGFR, ALK, ROS1 and MET. In one patient where a biopsy specimen was not available for molecular analysis, a cytology sample in the form of FNA was processed for molecular testing.

Mutation analysis in plasma samples

Ten ml of blood was collected in EDTA vials at the time of histological diagnosis or within two weeks from diagnosis prior to the start of any treatment following informed, written consent from patients. The blood was double centrifuged at 1,600g for ten minutes and supernatant plasma was carefully separated and stored at -80°C. Cell-free total nucleic acid extraction was done using MagMAX™ Cell-Free Total Nucleic Acid Isolation Kit from Thermo Fisher Scientific. Total nucleic acid from the plasma samples was tested for EGFR, ALK, ROS1 and MET.

Quantification of nucleic acids

The DNA, RNA and total nucleic acid were quantified using Nanodrop (Thermo Scientific) at 260/280nm wavelength and Qubit 3.0 spectrophotometer by fluorescent dyes (Invitrogen).

Real-time PCR

Reaction was performed on Quant Studio™ 6 Flex Real-Time PCR System. EGFR mutation was tested by using TRUPCR® EGFR kit from 3B BlackBio, Biotech India Limited, based on nested ARMS (Amplification Refractory Mutation System) where mutation specific reactions (exon 18, 19, 20 and 21) of the EGFR gene and a reference (wild type control in exon 2 without any known polymorphism/mutation) is amplified simultaneously. This kit is validated for formalin-fixed paraffin embedded (FFPE) and liquid biopsy (cell-free DNA). RNA rearrangements of ALK, ROS1 and MET were tested using the Lung cancer RNA panel from EntroGen, Inc. by real-time PCR with individual fluorescent probes.

Interpretation

EGFR mutation analysis was done based on the difference in cycle threshold (CT) of reference and mutation (CT mutation-CT reference= ΔCT). ALK/ROS1/MET alterations analysis was done based on detecting individual fluorescent probes and comparing cycle threshold (CT) of control and mutation.

Statistical analysis

All statistical analyses were conducted in R version 4.1.1.

Results

Targetable alterations

EGFR mutations: Among tissue samples, EGFR mutation was detected in 47 cases (47/100), whereas among cell-free samples it was detected in 43 cases (43/100). When concordance between the paired samples was compared, it was observed that in 26 cases (26/100), both tissue and liquid biopsy were positive for the mutation and in 36 cases (36/100), both were negative. Hence, overall concordance was 62%. Among the remaining cases, 17 cases (17/100) showed detectable EGFR mutation in the cell-free sample but not tissue, and vice versa in 21 cases (21/100) suggesting positive mutation status in tissue biopsy but not in liquid biopsy. (Figure 2 and Table 1, 2). Del 19 was the most common mutation detected in both tissue and plasma samples (53%, 60%) followed by L858R (32%, 21%). Other mutations included G719X, T790M, L861Q and Ex20 Ins. Complex mutations were detected in ten cases of tissue samples and six cases of plasma samples (Table 2).

ALK alteration

ALK-EML4 rearrangement was detected in 12 cases in tissue samples. However, none of the cell-free samples showed ALK rearrangement.

ROS1 fusion

ROS1 fusion was detected only in tissue samples of 2 cases while it was not found in any of the plasma samples.

MET alterations were not detected in any of the samples.

Table 1. Mutation Status and Concordance between Tissue and Plasma Sample

Plasma EGFR mutation status	Tissue EGFR positive	Tissue EGFR wild type	Total
Plasma EGFR positive	26	17	43
Plasma EGFR wild type	21	36	57
Total	47	53	100

Table 2. Spectrum of Molecular Alterations in Tissue and Plasma Samples

Tissue Mutations	Plasma Mutations										
	Del 19	Del 19, G719X, L858R	Del 19, T790M	L858R	G719X	T790M	L861Q	G719X, T790M	G719X, L861Q	Wild	Total
Del 19	6	1	1	-	2	-	-	-	1	8	19
L858R	-	-	-	5	1	1	-	-	-	4	11
G719X	-	-	-	1	-	-	-	1	-	3	5
T790M	-	-	-		-	-	-	-	-	1	1
Del19, L858R	1	-	-	-	-	-	-	-	-	-	1
Del 19, G719X	1	-	-	-	-	-	-	-	-	-	1
Del 19, T790M	1	-	-	-	-	-	-	-	-	2	3
Del 19, Ins 20	-	-	-	1	-	-	-	-	-	-	1
L858R, G719X	-	-	-	-	1	-	-	-	-	2	3
T790M, G719X	-	-	-	-	-	-	-	-	-	1	1
ALK, L861Q	-	-	-	-	-	-	1	-	-	-	1
ALK	2	-	-	-	-	-	-	-	-	9	11
ROS1	-	-	-	-	-	-	-	-	-	2	2
Wild	13	1	1	-	-	-	-	-	-	25	40
Total	24	2	2	7	4	1	1	1	1	57	100

Combinations of alterations

There was one case which harboured both EGFR mutation (L861Q) and ALK rearrangement in tissue biopsy whereas its plasma sample showed presence of the same subtype of EGFR mutation (L861Q). There were 3 cases where complex mutations were detected in cell-free samples but only a single mutation was present in the corresponding tissue sample. There were 10 cases where complex mutations were present in tissue, but their paired cell-free samples harboured only a single mutation. The sensitivity for detection of molecular alterations (EGFR, ALK, ROS1) in cell-free samples was 46.7%. For EGFR mutation alone, sensitivity was 55.3%.

Table 3. Cox Regression

Parameters	HR (95% CI for HR, L-U)	P value
Tumor Stage IV compared to stage III	7.8 (0.3-1.5)	0.47
EGFR mutant in Tissue	0.4 (0.2-0.7)	0.003
TKI	0.29 (0.1-0.8)	0.02

Therapy

Patients included in the study received either a single modality or in various sequential combinations of tyrosine-kinase inhibitors, chemotherapy and radiotherapy depending on the clinical indication, response, and economic constraints of the patient. The treatment was decided based on the mutation status in tissue. Among 47 EGFR positive patients, 31 cases received TKI based therapy. Majority were treated with geftinib, 3 cases received osimertinib as EGFR mutation T790M was detected in the primary site, afatinib was given in 1 case. Among 12 ALK positive patients, 3 patients received

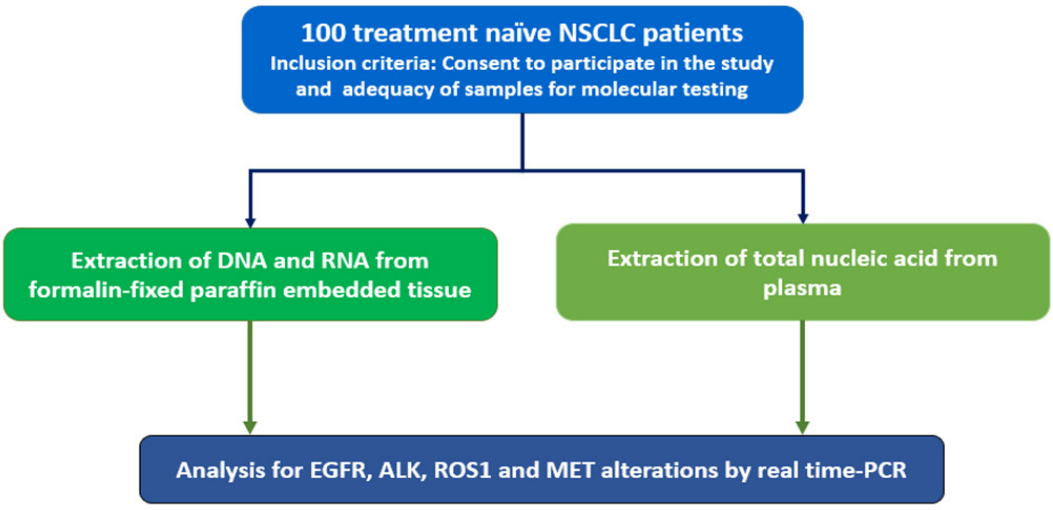
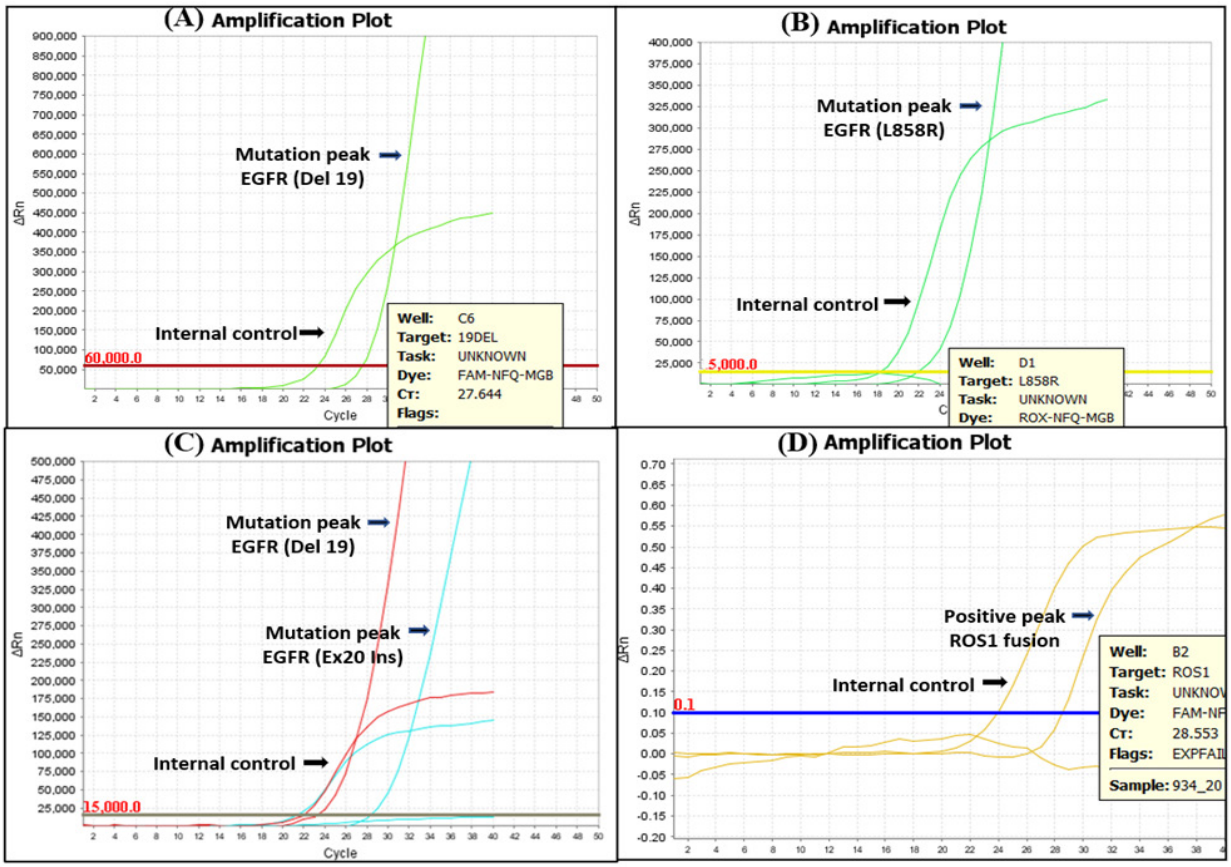


Figure 1. Study Design



(A) DEL 19 (B) L858R (C) Complex EGFR mutation, Del 19 and Ex20 Ins (D) ROS1 fusion.

Figure 2. The Horizontal Line Represents the Threshold. Curves crossing the threshold were considered and ΔCT, calculated by the difference in CT for internal control and sample, if less than the confirmed value for the specific mutation, was interpreted as positive for that mutation.

crizotinib therapy. Three of EGFR and ALK wild type cases received geftinib therapy. Fourteen EGFR positive cases as well as one ALK positive case received only TKI. Sequential chemoradiotherapy was given in addition to targeted therapy in 21 EGFR positive cases, two ALK positive cases and three wild type cases.

Survival outcome

The follow up period in this study of 100 patients varied between one and twenty-two months with 55% being dead at the end of the study. Overall survival (OS) was considered from diagnosis till the end of the study period. Progression free survival was not considered as progression could not be defined due to the lacunae in clinical and radiological information owing to the COVID-19 pandemic.

Survival based on mutation status

The Kaplan Meier survival analysis was done for 98 cases and the median overall survival was found to be 11 months (mean = 13.18 months) in NSCLC patients irrespective of mutation status. Patients with presence of EGFR mutation in tissue had a significantly higher median overall survival of 19 months followed by patients with ALK, ROS1 alterations with 11 months and 9 months for cases in which no mutations were detected (P-value = 0.006) (Table 3). One case each of poorly differentiated carcinoma and sarcomatoid carcinoma were excluded from the survival analysis as they skewed the results with less than one month of survival. However, when the plasma mutation status was considered, no significant difference in overall survival was observed between the EGFR positive cases and wild type cases with OS of 11 months and 10 months respectively and P value of 0.34 (Table 3, Figure 3). If the mutations found in plasma were considered as truly positive, the median overall survival dropped from 19 months (in the tissue EGFR mutant cases) to 12 months. No significance was seen in overall survival based on histological subtypes.

Survival based on therapy

Subgroup analysis with respect to different treatment regimens received by the patients was considered. Comparison between patients who received TKI based therapy versus other modalities (chemotherapy and/or radiotherapy) was done and the median survival was 19 months and 9 months respectively, which was significant P value = 0.02.

The Cox proportional hazard model

Cox regression analysis was applied to determine predictors of mortality. Hazard ratio and 95% confidence intervals of different variables were calculated. Univariate Cox regression analysis showed tissue mutation status,

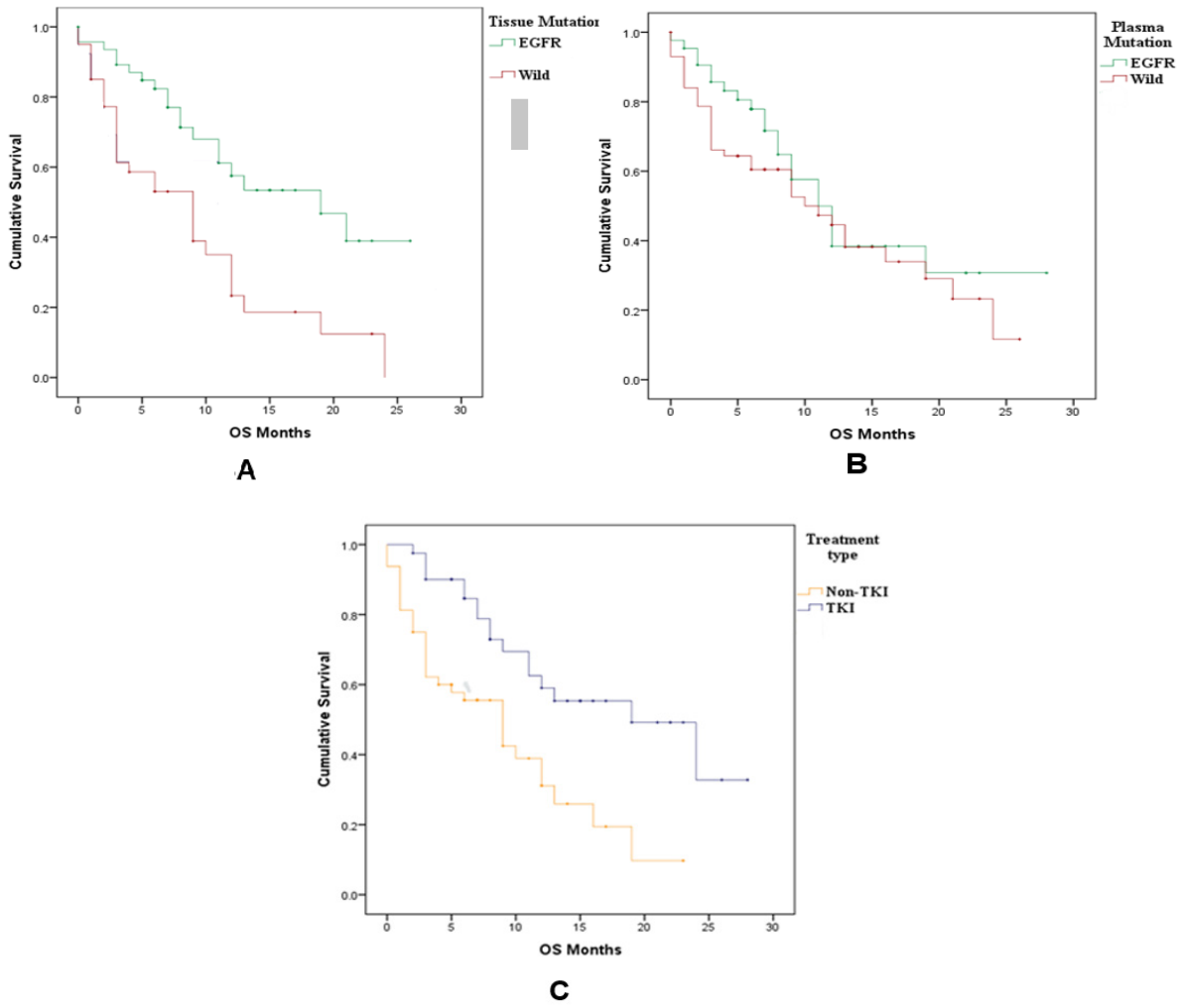


Figure 3. A, Mutated cases in tissue biopsy signifying better median OS (19 months) in EGFR mutated cases as compared to others (ALK/ROS1 and wild type, 11 months and 9 months respectively); B, EGFR mutated cases in plasma (11 months) as compared to wild type (10 months); C, In patients who received TKI (19 months) based therapy vs other modalities (9 months). TKI based therapy significantly increased the median overall survival

targeted therapy as the significant factors associated with overall survival (Table 3).

Discussion

Targetable molecular alterations such as EGFR, ALK, ROS1 etc., which are amenable to therapy have expanded the horizons of treatment in lung cancer with significantly improved survival and quality of life. This has become the basis for extensive search for newer alterations which could become potential targets for therapy (Kris et al., 2014). With this advancement, it also became imperative to explore methods that would detect these alterations with maximum sensitivity. This study explored the role of concurrent molecular testing in tissue as well as liquid biopsies and found that both are complementary to each other. The conventional molecular testing in lung cancer utilises tissue samples (trucut biopsy/FNA) for detection of amenable therapeutic targets but due to difficulty in procuring adequate sample in around 20-25% of patients due to inaccessible biopsy site, suboptimal condition of the patients, difficulty in serial sampling for monitoring therapeutic resistance etc, Liquid biopsy (plasma/serum) is recommended as an alternative when adequate samples are not available for testing or procurement of samples is difficult owing to suboptimal clinical conditions of the patient. It is non-invasive, cost effective and hence multiple samples can be procured for serial testing or monitoring response to therapy. Also, it may include a more comprehensive snapshot of the entire molecular profile of the disease including metastasis which makes it more practically applicable for therapeutic decisions

EGFR mutations occur at widely varying frequency across different geographic and ethnic regions. Midha et al (Midha et al., 2015) and others (Chang, 2007; Toh, 2010; Cooper et al,. 2013; Shi et al., 2014; Kim et al., 2015; Midha et al., 2015; yotsukura et al., 2017) have shown EGFR mutation frequency in tissue to be highest in Asia-Pacific region averaging 47% but with a very wide range of 11-76% in different parts of the region. European and Middle Eastern countries have shown a lower frequency comparatively (Colombino et al., 2019; Omar et al., 2022). The frequency of EGFR mutation in tissue in the Indian population was reported as 16 to 48% by various studies (Doval, 2013; Kasana et al., 2016; Maturu et al., 2016; Kumari et al., 2019; Kapoor

et al., 2021). A previous study from our centre reported a frequency of 31.6% in EGFR mutation in 250 cases of NSCLC in tissue biopsy with Del 19 and L858R being the most common mutations (Kumari et al., 2019). The same was observed in the present study with Del 19 (25 cases, 53%) and L858R (15 cases, 32%) accounting for 85% of all EGFR mutations in tissue biopsy (Chougule et al., 2013; Kate (2019). The uncommon mutations include G719X, which is the most frequent with a reported incidence of 2-4% (Beau-Faller et al., 2014), followed by Exon20 Ins (1-12%) (Burnett et al., 2021) and L861Q. The uncommon mutations detected in tissue were G719X (10 cases, 21%), Exon20 Ins (one case, 2%), L861Q (one case, 2%). Complex mutations constituted 10% of tissue EGFR mutations. Varying combinations of mutations such as Del 19+L858R (one case), Del 19+T790M (three cases), Del 19+G719X (one case), Del 19+Ex20 Ins (one case), L858R+G719X (three cases) and T790M+G719X (one case) were found. Complex EGFR mutations are reported in the range of 3-14% (Huang et al., 2004; Wu et al., 2011; Keam et al., 2014; Ramadhan, 2021). A previous study from our centre reported a frequency of 2% (Kumari et al., 2019). T790M was detected in 5 cases, four of which were in combination and one case showed isolated T790M. This mutation is known to be associated with resistance to tyrosine kinase inhibitors (Suda et al., 2009).

EGFR mutations were found in 43 of the 100 cell-free DNA samples. The maximum sensitivity achieved for detection of EGFR mutation in plasma by ARMS-scorpion PCR across different trials is 46% to 87% (Liu et al., 2013; Douillard et al., 2014; Duan et al., 2015; Karachaliou et al., 2015; Pasquale et al., 2015; Thress et al., 2015; Denis et al., 2019; Soria-Comes et al., 2019; Ulivi et al., 2021). Our study demonstrated a sensitivity of 55.3% which falls within the reported range. Further, it was observed that sensitivity for detection of molecular alterations in plasma increased with stage and metastatic nature of the disease. This observation is consistent with established literature that ctDNA shed into the circulation is proportional to the stage of the disease (Newman et al., 2014; Cohen et al., 2018). Among the plasma mutations, the most common was Del 19 (28 cases, 65%) followed by L858R (9 cases, 21%), G719X (8 cases, 19%), T790M (4 cases, 9%) and L861Q (2 cases, 5%). This trend is similar to the tissue mutations. Complex mutations were found in six cases of plasma (14%). Concordance between tissue and plasma samples was 62% (26 cases), where both had the same mutation status. Overall concordance in other studies was reported in the range of 60 to 94% (Liu et al., 2013; Douillard, 2014; Duan et al., 2015; Karachaliou et al., 2015; Pasquale et al., 2015; Thress et al., 2015; Ulivi et al., 2021; Denis et al., 2019; Soria-Comes et al., 2020). EGFR mutation was present in the plasma but absent in the corresponding tissue samples in 17 cases. These are the "false positives" in this study. This can be attributed to the molecular heterogeneity of the tumour at different sites (Gerlinger et al., 2012) which may not have been represented in a single site tissue biopsy. However, when a negative result is observed in a cell-free sample, it must be confirmed in the tissue sample if the latter is available (Oxnard et al, 2014). Oxnard et al., (2014) showed that 31% of cases who tested negative for T790M mutation in tissue had a positive result for this mutation in plasma, similar to the results in our study.

ALK frequency is reported between 1 to 10% around the world (Fernandez-Bussy et al., 2016; Lee et al., 2018; Maturu et al., 2016; Martin et al., 2016; Tian, 2017; Ramadhan, 2021). Indian data reported a frequency in the range of 4 to 11.5% (Bal et al., 2016; Rana et al, 2018; Mohan et al, 2020; Kishore RR and Pan V, 2023). The method used for ALK detection in these studies was immunohistochemistry or FISH or both. We detected the same by real-time PCR using an RNA panel kit. In the present study, the frequency of ALK-EML4 rearrangement was 12% in tissue biopsies. One case showed concurrent EGFR (L861Q) mutation and ALK rearrangement in tissue biopsy and 2 cases showed ALK rearrangement in tissue biopsy and EGFR (Del 19) mutation in the corresponding plasma sample. ALK rearrangements were not found in cell-free nucleic acid samples. Detection of ALK rearrangements in cell-free nucleic acid samples needs to be validated and more prospective studies are needed to mark the reliability of real time PCR as a platform for it (Chu et al., 2020; Nilsson et al., 2016; Dagogo-Jack, 2018).

Frequency of ROS1 is reported as 1-2% in world data (Clave et al., 2016; Scheffler, 2015). However, data from the Indian population is scant. Mehta et al., (2020) reported a frequency of 2.8% in the North Indian population. Another recent study reported 3.85% ROS1 positivity on immunohistochemistry (Kishore and Pan, 2023). The current study reports an incidence of ROS1 fusions in 2% cases tested in tissue biopsy. ROS1 fusion was not found in corresponding cell-free nucleic acid samples.

MET is one of the least common alterations found with a reported prevalence of 1-10% (Drilon et al., 2017; Song, 2019). However, in our study, we did not find any case with MET alterations. This is probably due to the test being performed on a real time PCR based platform which is less sensitive and specific for the detection of MET alterations (mutations and amplifications) as these are better detected by the NGS platform. Studies have shown that frequency of MET alterations are higher if hybrid capture based sequencing is used over amplicon-based sequencing platforms (Subramanian and Tawfik, 2021).

The results in the plasma samples indicate an increased prevalence of EGFR mutation at baseline from 47% to 64%. Also, different mutations (one or more) may be detected in tissue and plasma samples. These aspects highlight the relevance of plasma mutation status. It is likely that different molecular clones may be present in the primary tumour which may escape detection in first testing. A different clone may be present in the metastatic sites which may be inaccessible for biopsy. Such cases are likely to prove as a therapeutic challenge because minimal or negligible clinical response may be observed when treated according to the molecular profile of the primary single-site biopsy. However, if the liquid biopsy results are considered, there could be a dramatic improvement in treatment and control of the disease. In this study, 47 cases received targeted therapy based on the primary

tissue biopsy results (EGFR positive). The rest of the cases (53/100) were assumed to be wild type and hence did not receive any targeted therapy. Seventeen of these 53 cases showed EGFR mutation in the cell-free samples. As the therapy was initiated based on the tissue results, these cases missed the benefit of targeted therapy. It is likely that some of them may have an improved quality of life and better overall survival, had they been treated with anti-EGFR therapy.

When the overall survival in plasma EGFR positive cases was compared with plasma wild type cases, there was no significant difference. This contrasts with considerably improved survival in tissue EGFR positive cases when they were compared against tissue wild type cases (19 months vs 9 months). Thus, a positive mutation status may favourably influence survival based on the fact that these patients are likely to receive targeted therapy and hence have an improved disease outcome (Sequist, 2013; Yang et al., 2015). When the overall survival was compared between subgroups based on mutation type (EGFR vs ALK, ROS1 vs wild type), it was found that those with EGFR mutations had better overall survival as compared to ALK, ROS1 alterations and wild type cases (19 months vs 11 months vs 9 months). The reason for this significant difference in survival can be attributed to two facts. Firstly, the majority of ALK, ROS1 patients (79%) did not receive targeted therapy due to financial constraints or other reasons. In comparison, 70% of EGFR tissue mutated cases received targeted therapy. Secondly, ALK rearrangement is associated with advanced disease and metastasis, both poor predictors of survival (Shaw, 2009; Shaw and Engelman, 2013). It was seen that patients who received targeted therapy had a significantly higher median of 19 months as compared to those who received other modalities of treatment which was 9 months ($P = 0.002$). This highlights the definite benefit of targeted therapy for improved disease outcome in patients, especially in those harbouring targetable mutations. Aggarwal et al., (2019) demonstrated detection of therapeutically relevant mutations in plasma who had successful disease control when treated based on the plasma mutation result. Their study further elucidated that concurrent testing in tissue and plasma increased the frequency of detecting targetable mutations as compared to testing in tissue only.

In conclusion, the observations in this study highlight the significance of concurrent testing in tissue and liquid biopsy samples, as several cases of EGFR mutations were missed in tissue but picked up in liquid biopsy at baseline testing. A fair hypothesis can be made that these patients would benefit from targeted therapy. Therapeutic decisions based on the results of concurrent testing will broaden the horizon and include more cases that can benefit from targeted therapy. Thus, although liquid biopsy is not a replacement for tissue, it has a complementary role in the detection of targetable alterations at baseline when tested concurrently.

Author Contribution Statement

This study was designed and supervised by Dr. Niraj Kumari, Dr. Narendra Krishnani and Dr. Raghavendra Lingaiah. Sample collection and experimental activities were performed by Dr. Paturu Radha and Shreya Srivastava. Dr. Shalini Singh and Dr. Alok Nath contributed to data curation and clinical correlation. Result analysis and statistics was performed by Dr. Paturu Radha, Dr. Raghavendra Lingaiah and Dr. Saima Haleem Siddiqui. The preliminary manuscript was written by Dr. Paturu Radha with the aids of Dr. Raghavendra Lingaiah and Dr. Niraj Kumari, revised by Dr. Narendra Krishnani and finalised by Dr. Niraj Kumari.

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Ethical statement

The study objectives were explained to subjects before they signed an informed written consent. The study protocol and procedures were ethically approved by the Institutional Ethics Committee (vide IEC code No.: 2020-1-MD-EXP-14).

Limitations of the study

In this study, paired samples of tissue and plasma were tested for molecular alterations by real-time PCR. However, more sensitive techniques such as digital PCR and NGS platforms are recommended for detection. Confirmatory testing for ALK rearrangements was not performed by FISH and the kit used for ALK and ROS1 alterations has not been validated on cell-free/plasma samples. Hence negative results in plasma may need to be confirmed by other methods.

Conflict of interest

The authors declare no conflict of interest.

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