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ORIGINAL ARTICLE Lung Cancers

Predictive biomarkers in nonsmall cell carcinoma and their clinico-pathological association

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Abstract

Background: Lung cancer is the leading cause of cancer-related mortality worldwide. Genome-directed therapy is less toxic, prolongs survival and provides a better quality of life. Predictive biomarker testing, therefore, has become a standard of care in advanced lung cancers. The objective of this study was to relate clinical and pathological features, including response to targeted therapy (TT) and progression-free survival (PFS) with positive driver mutation. **Materials and Methods:** Archival data of nonsmall cell carcinoma patients with Stage IV disease were retrieved. Those who tested positive for one of the four biomarkers (epidermal growth factor receptor [EGFR], anaplastic lymphoma kinase [ALK], MET, and ROS) were included. Patient demographics and clinical features were reviewed. Tumor histomorphology was correlated with oncological drivers. Treatment response, PFS, and overall survival were studied in three subcohorts of patients who received computed tomography (CT), CT followed by TT and those who received TT in the first line. **Results:** A total of 900 patients underwent biomarker evaluation of which 288 tested positive. Frequency of the four biomarkers observed was 26.6% (229/860), 6.6% (51/775), 6.6% (5/75), and 5.1% (3/59) for EGFR, ALK, MET, and ROS-1, respectively. The median PFS for EGFR-mutated cohort was 12 months, whereas it was 21 months for ALK protein overexpressing cases. Patients treated with first-line tyrosine kinase inhibitors performed better compared to those who were switched from chemotherapy to TT or those who received chemotherapy alone ($P < 0.05$). **Conclusion:** Biomarker testing has improved patient outcome. Genome-directed therapy accords best PFS with an advantage of nearly 10 months over cytotoxic therapy.

Key words: Anaplastic lymphoma kinase 1, epidermal growth factor receptor, MET, nonsmall cell carcinoma, ROS

Introduction

Lung cancer is the global front-runner both in incidence and cancer-related deaths with a scorecard of 1.59 million deaths annually and 19.4% of the total cancer burden.[1] In India, lung cancer constitutes 6.9% of new cancer cases and 9.3% of all cancer related deaths in both sexes.[2] There is a rise in lung cancer incidence in developing countries like India and the overall 5 years survival of patients is still <15%.[3] Nonsmall cell cancer (NSCC) is the common subtype accounting for about 80% to 85% of all lung cancers with >70% being diagnosed with metastasis.[4] The 1st year survival rate for a patient with stage IV NSCC is approximately 50% which drops by half to 25% or even less by the end of 2nd year.[5] Numerous advances in recent years in terms of molecular diagnosis and genome-directed therapeutic interventions have resurrected hope of managing advanced lung cancer more effectively. Epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase 1 (ALK1), and ROS1 fusions are actionable lung cancer mutations targeted by US FDA approved tyrosine kinase inhibitors (TKI). MET amplification is also highly responsive to dual ALK and MET TKIs.

The present study has been conducted at a comprehensive cancer care center with aims of determining the incidence of sensitizing EGFR mutations, ALK1 and ROS1 rearrangement and MET amplification in advanced lung adenocarcinoma (ALADC). These driver alterations were correlated with clinical features, response to therapy and outcomes. The tumor histomorphology was also assessed in relation to specific mutation type.

Materials and Methods

The present study was carried out at a tertiary comprehensive cancer care center and archival data of NSCC patients diagnosed with Stage IV disease was retrieved. A total of 900 cases were analyzed from records of a 7-year period from January 2010 to December 2016. Patients were evaluated for mutational status which was correlated with demographic profile, histomorphological features, clinical features, and progression-free survival (PFS).

Sample collection

A total of 1769 mutational analysis/tests were carried out in 900 patients. This variation in the test counts is because in the initial years of biomarker testing was sequential and not simultaneous for all biomarkers. A total of 288 patients tested positive for one of the four driver mutations and were included in the study. These tests were performed either on core biopsy or wedge biopsy specimens. Review of histopathology slides was done, and histomorphology of NSCC was categorized as per the WHO 2015 classification into histological types and patterns (solid, glandular, papillary, lepidic, and micropapillary) by one of the authors (AM#) using H and E stained 4–5 μm sections. Limited immunohistochemistry (IHC) was performed using clone 8G7G3/1(Dako, Denmark) for thyroid transcription factor-1 and clone P40 (M) (Biocare, Netherland) for P40 immunostaining. Additional IHC was performed in 18 cases using clone MRQ-60 (Dako, Denmark) for Napsin A and D5/16 B4 (Dako, Denmark) for CK 5/6. All IHC were performed on Ventana Benchmark XT using ultraview labeling system. The patient population included in this study comprised cases diagnosed with NSCC-adenocarcinoma, NSCC favor adenocarcinoma and NSCC, not otherwise specified. In addition, five patients of squamous cell carcinoma also underwent testing for EGFR/ALK on account of young age, nonsmoking status and/or physician-specific request. Detailed

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treatment history and preceding data of treatment modality used in each case were curated from electronic medical records. In the survival analysis, only those patients for whom complete hospital visit records and follow-up data was available were studied to record PFS and overall survival (OS).

Mutational analysis for EGFR was performed using Qiagen EGFR therascreen RGQ polymerase chain reaction (PCR) KIT. Five sections of 4 µm each were collected in Eppendorf tube with manual macro-dissection to enrich tumor fraction wherever necessary. DNA was extracted using Qiagen DNeasy blood and tissue kit. The DNA was quality checked on the Qubit fluorometer. Multiplexed reverse transcription (RT) PCR was carried out on ROTORGENE thermal cycler in 8 tubes along with positive and no template control. Interpretation was done as per vendor's insert.

ALK1 protein was tested by IHC using anti-ALK (D5F3) Rabbit Monoclonal Primary Antibody with other proprietary component of the Ventana ALK assay on Ventana Benchmark XT Autostainer (using Ventana Optiview DAB and Amplification kit).

Fluorescence *in situ* hybridization (FISH) was performed on formalin-fixed paraffin-embedded lung tissue sections of 4–5 µM placed on positively charged slides. The specimens used for this study were hybridized using FISH assays with break apart probe set (ZytoLight SPEC ROS1 Dual Break Apart Probe ZytoVision GmbH, Germany), according to the manufacturer's instructions. FISH measurements were performed using fluorescent microscope Leica DM 6000 B (Leica, Japan) equipped with four filters (DAPI/Green/Orange/Aqua). The hybridized sections were examined under ×1000 for break apart signals. A distance of more than 2 signal diameter between red and green signals was considered positive. Lesser than 5 split signals were reported negative and >25 split signals were considered positive on count of 50 cells. In cases of 6–24 split signals, a second operator repeated the count. An average of ≥15% signals was considered positive. MET *in-situ* hybridization was done as per the manufacturer protocols (Zytolight directly labeled locus-specific identifier MET DNA probe; green and CEN-7 probe; orange). A centromeric 7 probe to MET signal ratio >5 was considered positive. Demographic, survival and other relevant clinical data were mined from electronic medical records.

Statistical analysis

Summary of all categorical variables is presented in frequency and percentages, whereas summary of continuous variable such as age was presented in mean ± standard deviation. Calculation of OS was in months and based on the date of start of the first-line systemic treatment for the metastatic disease until death from any cause. Patients were censored at their last follow-up visit if they were still alive or lost to follow-up. PFS was defined as months from the date of initiation of therapy to clinically determined disease progression or death from any cause.[6] Disease progression was observed by the date of radiographic imaging which demonstrated progression as noted by radiologist relying on response evaluation criteria in solid tumors 1.1. Patients were censored at the date of their last disease assessment if remained alive and progression free. Kaplan–Meier curves were used to estimate survival distribution for OS and PFS. Estimates of PFS and OS (median, 95% confidence interval [CI]) were reported using the Kaplan–Meier methods for censored data. Log-rank (Mentel-Cox) test was used to compare survival distributions for a different level of therapy. Reported *P* values are two-sided and no adjustments have been made for multiple comparisons. All analyses were performed using SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.).

Ethical clearance

The study does not carry any ethical implications, so institutional ethical committee waivered the study.

Results

Patient characteristics

Mean age of patients in the study was 58.0 ± 11.5 years (range: 24–94 years). EGFR sensitizing mutations, ALK1 protein overexpression, MET amplification, and ROS-1 rearrangement was observed in 26.6% (229/860), 6.6% (51/775), 6.6% (5/75), and 5.1% (3/59) patients. It is to be reiterated that variation in denominators of patient tested for all four mutations exists due to sequential testing as per clinician's orders. Of these, the survival data of EGFR mutated, and ALK overexpressing patients have been analyzed further using the Kaplan–Meier estimator.

Demography

The mutation-wise gender distribution of patients is shown in Figure 1. Of the 860 patients tested for EGFR, 534 were male and of these 133 (24.9%) tested positive whereas 326 were female and of these 96 (29.4%) were female. Table 1 demonstrates demographic and clinical characteristics of patients with EGFR mutation. EGFR mutations were observed in 26.7% (229/860) of the study participants. No statistically significant (*P* = 0.449) difference in the incidence of EGFR mutation was observed between genders (24.9% in males vs. 29.4% in females) in the study population. Smoking history was elicited in 24.8% of the EGFR-mutated cases and no significant difference was observed in mutational incidence between smokers and nonsmokers.

Patients had almost equal distribution in cohorts for age <40 years (29.9%) and 40–60 years (29.4%). About 30.4% of patients had NSCC involving the left lung and in 27.7% of the patients, right lung was involved. About 32.4% of the patients were not found to have any comorbidity. The most common EGFR activating mutation was deletion 19 (Del19) (54.3%) followed by L858R (27.2%). Distribution of patients with EGFR sensitizing mutations is shown in Figure 2. Out of the 20 dual mutations, most common observed was Del19 + T790M (40.0%; 8 patients) followed by Del 19 + L858R (20.0%; 4 patients). Other

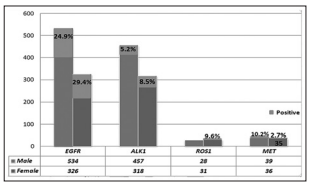

Figure 1: Gender-wise distribution of four types of mutations in nonsmall cell lung cancer

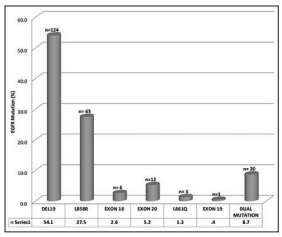

Figure 2: Distribution of epidermal growth factor receptor mutation in study subjects

were L858R + D761Y (5%; 1 patient), L858R + Exon 20 (5%; 1 patient), L858R + G719X (5%; 1 patient), L858R + T790M (10%; 2 patients), L861Q + G719X (5%; 1 patient), and G719X + Exon 20 (10%; 2 patients).

Table 1 also demonstrates the demographic and clinical characteristics of all patients with ALK1 protein positivity. Of the 51 patients who tested positive, 8.5% were women (P = 0.078). About 4.1% of the study participants were smokers (P = 0.0036). Young patients (20.3%) of age <40 years were the dominant population in ALK1 overexpressing cases. No comorbidities were found in 21.4% of patients.

ROS 1 was rearranged in 3/59 patients and all 3 of them were at least a decade younger than the mean age of presentation.

MET was amplified in 5/75 patients were amplified for C-MET oncogene, of which 4 were elderly smoker males of age >60 years.

Morphological distribution

Histomorphological pattern analysis in EGFR and ALK1 was done and revealed the primary pattern observed with EGFR sensitizing mutation was solid (47.6%; 109 patients) followed by acinar (34.5%; 79 patients), lepidic (9.2%; 21 patients) and papillary (5.2%; 12 patients) patterns. However, patients with ALK1 protein overexpression had predominant acinar configuration (66.7%; 34 patients) followed by solid growth pattern (29.4%; 15 patients), lepidic (2%; 1 patient), and papillary (2%; 1 patient).

Therapy administered

Type of therapy administered is summarized in Table 2. With regard to EGFR mutation, chemotherapy (Pemetrexed 500 mg/m^2 + Carboplatin AUC 5 IV) alone was exhibited to 49.8% whereas TKI (Erlotinib-150 mg PO once daily) was administered in the first line to 31.8%. Others received computed tomography followed by TKI (Switch-Platinum-based chemotherapy for four cycles followed by Erlotinib 150 mg PO once daily or earlier depending on the result of biomarker testing.).

Survival analysis

OS and PFS was calculated using Log-rank test among three types of therapies, i.e., TKI and switch [Table 3 and Figure 3]. OS and PFS were calculated only in patients where each visit was documented and confirmed at the time of data analysis. The median PFS and OS for EGFR cohort was 12 months (95% CI, 10.26–13.75 months) and 59 months (95% CI, 18.44–99.56 months), respectively. The median PFS for patients receiving TKI alone was 16 months while those who received upfront chemotherapy followed by TKI was 12 months, distantly followed by patients receiving chemotherapy alone, i.e., 6 months. In addition, median PFS for patients harboring Del 19 was 16 months (95% CI, 10.78–21.23 months) while that for patients with L858R mutation was 12 months (95% CI, 9.96–14.04 months).

However, median PFS and OS for ALK1 cohort were 21 months (95% CI, 13.55–28.55 months) and 30 months (95% CI, 23.16–33.56 months), respectively. Table 3 represents stratified survival analysis for three types of treatments received for EGFR and ALK1 Mutants. Statistically significant difference was observed in median PFS (P = 0.018). Median PFS was 6 months (1.68–10.32) for chemotherapy, 12 months (9.87–23.13) for Switch and 16 months (9.19–14.81) for TKI alone. Median PFS and OS for the stratified group were not calculated for ALK1 Mutants due to limited number of subjects.

Subgroup survival analysis for responders on EGFR-mutated patients shows median PFS 13 months (10.02–15.99)

Table 1: Demographic and clinical characteristics of all patients with positive EGFR mutation and ALK1 protein overexpression

Characteristic	Number of patients		
	Tested, *n*	EGFR positive, *n* (%)	ALK1 positive, *n* (%)
Total	860	229 (26.6%)	51 (6.6%)
Year treatment commenced			
2010	13	1 (7.69)	2 (1.6)
2011	80	22 (27.5)	-
2012	121	21 (17.4)	16 (12.3)
2013	100	30 (30.0)	13 (8.0)
2014	130	30 (23.1)	20 (7.9)
2015	163	51 (31.3)	
2016	253	74 (29.2)	
Age (years)			
<40	67	20 (29.9)	22 (7.3)
40-60	436	128 (29.4)	28 (5.7)
>60	357	81 (22.7)	1 (5.9)
Site*			
Left lung	303	92 (30.4)	22 (7.3)
Right lung	491	136 (27.7)	28 (5.7)
Bilateral	17	1 (5.9)	1 (5.9)
Smoking history*			
Present	326	81 (24.8)	13 (4.1)
Absent	434	148 (34.1)	38 (9.8)
Co-morbidity*			
Diabetes	65	14 (21.5)	4 (7.0)
Hypertension	97	32 (32.9)	5 (5.9)
Other	181	34 (18.9)	6 (3.6)
Nil	460	149 (32.4)	36 (21.4)

*Unknown figures are not included in the tables

Table 2: Treatment and response to therapy of nonsmall cell carcinoma patients of EGFR and ALK1 mutation

Treatment	EGFR, *n* (%)	ALK1, *n* (%)
Therapy		
Chemotherapy	114 (49.8)	8 (15.7)
TKI	42 (18.4)	17 (33.3)
Chemotherapy followed by TKI (switch)	73 (31.8)	24 (47.1)
Response		
Responders	90 (39.3)	18 (39.1)
Nonresponders	139 (60.7)	28 (60.9)

TKI=Tyrosine kinase inhibitors

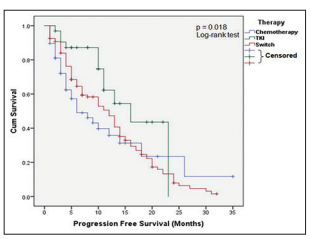

Figure 3: Kaplan–Meier curve comparing three types of therapy (epidermal growth factor receptor-mutated patients)

Table 3: Kaplan-Meier survival analysis of ALK overexpressing and EGFR-mutated patients

Time to an event	EGFR			ALK1	
	n	Median (months)	95% CI	Median (months)	95% CI
Progression free survival					
Overall	187	12	10.26-13.75	21	13.55-28.55
Chemotherapy	107	6	1.68-10.32	-	-
TKI	32	16	9.87-23.13	-	-
Chemotherapy with TKI	48	12	9.19-14.81	-	-
OS		59	18.44-99.56	30	23.16-33.56

OS=Overall survival, TKI=Tyrosine kinase inhibitors, CI=Confidence interval

for TKI alone, whereas median PFS was 12 months (8.21–15.79) and 10 months (3.60–16.40) for Switch and chemotherapy, respectively. Median PFS for nonresponders was 4 months (2.73–5.27) on chemotherapy and 10 months (6.30–13.70) on the switch while for TKI survival curve did not reach to the median and 1-year survival rate was 83.9%.

Discussion

Genome-directed therapy has become standard of care for stage IV ALADC. The mutational analysis, therefore, has gradually been adopted widely. The molecular testing for predictive biomarkers was performed in 900 cases over a period of 6 years. The EGFR positive rate was observed in 26.2% (860) of our population of NSCC patients. This rate of positivity is in consonance with that observed by Chougule *et al*.[7] (23%) and Kota *et al*.[8] (30.6%); two large center epidemiology studies published from India till date. While higher rates of EGFR sensitizing mutations have been observed in the far east and South-East Asia (36%–76%), the rates of EGFR positivity are intermediate in South Asian continent (22%–27%).[9] Our data show a twice higher (53.3% vs. 27.2%) rate of Del 19 mutations against L858R. While earlier studies have showed the only marginal difference in these frequencies, Del 19 has now been reported to have a much higher incidence as reported in several new studies (21%–49%)[10-12] including two from the Indian subcontinent.[13,14]

A combination of sensitizing and resistant mutations (dual mutation genotype) was observed in 8.7% (20 cases). Notable observation is the presence of T790M as one of the components in 10 cases (1.16%). This fact highlights that a subclone of T790M may exist from the very beginning of disease genesis and may not necessarily be a new mutation prompted by exposure to TKIs. A similar finding was seen in the study by Shi *et al*. who had 0.3% of patients harboring primary T790M mutation at the time of diagnosis.[11] In the present study, there was no gender bias among patients of lung cancer and mutation status.

Best survival was obtained in the group on the first-line TKI followed by those who received TKI after upfront chemotherapy (Switch) (P = 0.040). In general, patients who had TKI exposure had a better PFS than those who received chemotherapy alone (P = 0.027). These benefits obtained in PFS by use of TKIs is in line with results highlighted in the extensive meta-analytical review published by Greenhalgh *et al*.[15,16] The PFS of patients with Del 19 compared to L858R showed a significant survival benefit in the former (16 months vs. 12 months). Sutiman *et al*.[17] recently made a similar observation where exon 19 mutations showed the longest median PFS and significant survival benefit. In the present study, correlation of morphologic subtype with mutation pattern and survival advantage was insignificant reiterating little value of histology in the selection of patients for mutational analysis. In contrast to our result, Song *et al*.[18] observed that EGFR mutation frequency of micropapillary and lepidic predominant subtypes was more pronounced than that of other subtypes. Villa *et al*.[19] also found lepidic growth pattern to be the predominant pattern in their study population; however, EGFR mutants were distributed across all histologic subtypes. It was, therefore, agreed on, that histomorphology cannot be used to exclude patients from tyrosine kinase inhibitor therapy.

ALK-1 protein overexpression was noted in 6.58% (51/775) cases in this study. There was a significant bias toward the younger cohort with a mean age of 51.7 years. A similar statistically significant ALK-positive younger cohort was observed in a study published from across the globe.[20-22] This demographic profile resonates well with that observed by Noronha *et al*.[20] and Solomon *et al*.,[23] with the exception of slight female predominance and lower incidence of smokers with ALK-positive lung cancer observed in our study. A majority of patients in our study (47.1%, 24/51) received upfront chemotherapy followed by TKI. The overall median PFS in our study was 21 months which is much higher than that mentioned in another study from developing nations.[20] This could due to higher use of Cristine either upfront or as a part of switch therapy. In the present study, median PFS has yet not been obtained.

Our early experience with MET amplification and ROS1 gene rearrangement studies have showed positive response rate to crizotinib. More data are needed to ascertain the incidence of and responses to various forms of therapy.

Conclusion

Genome-directed therapy is the new standard of care. As observed in our study, approximately 40% of lung cancer patients can be benefited by testing for 4 genes. All patients of ALADC should be tested as no enrichment of patients is possible on the basis of histomorphology or clinical profile. Genome-directed therapy accords best PFS with an advantage of nearly 10 months over cytotoxic therapy (9.19–14.81; 95% CI) in EGFR-positive cases.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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