EGFR mutations and ROS1 and ALK rearrangements in a large series of non-small cell lung cancer in South India

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Abstract

Background: Driver mutations are seen in 80% of lung adenocarcinomas, and they influence prognosis and choice of therapy.

Aim: Aim of this study was to analyse the frequency of epidermal growth factor receptor (EGFR) mutations, ALK and ROS1 rearrangements and their association with age and gender in non-small cell lung cancer reported from a tertiary care center in South India.

Methods: Tumors from patients with non-small cell carcinoma of lung were evaluated for EGFR mutations, ALK and ROS1 rearrangements and their association with age and gender were studied.

Results: Two thirds of non-small cell carcinomas had driver mutations or rearrangements. EGFR mutation was common and seen in 34.1%, whereas ALK rearrangement was seen in 11.1% and ROS1 rearrangement in 2% patients. Among EGFR mutations, most common were Exon 19 deletion and L858R seen in 21.3% and 11% of patients, respectively. Adenocarcinoma was the histologic diagnosis in 81% to 85% of patients with exon 19 deletion and L858R mutation, respectively. EGFR mutation frequency in patients less than 36 years was 13.6%, whereas in older patients, it varied from 34% to 36%. Exon 19 deletion was seen in 29.8% females and 17.2% of males.

Conclusion: EGFR mutations are more common than ALK and ROS1 rearrangements. They are more common in females. Patients less than 36 years have reduced frequency of EGFR mutations. Exon 19 deletion and L858R are most common and are more prevalent in lung adenocarcinomas. Rare EGFR mutations are seen in patients aged more than 50 years.

KEYWORDS
ALK, EGFR, non-small cell lung carcinoma, ROS1
1 | INTRODUCTION

Epidermal growth factor receptor (EGFR) mutations have been reported in pulmonary adenocarcinomas and can be targeted by tyrosine kinase inhibitors. The most common EGFR mutations are exon 19 deletions and single-point substitution L858R in exon 21. These mutations are seen in 41% to 44% of non-small cell lung cancer. Less common mutations which are less responsive to tyrosine kinase inhibitors are G719X in exon 18, deletions in exon 20 (4% of cases) and L861Q in exon 21 (2% of cases). Acquired resistance to tyrosine kinase inhibitors is known to occur during progression of which the commonest reported is the T790M mutation. This mutation has been observed in 60% of patients.

There is variation in prevalence of EGFR mutations across countries and regions (20%-76% in Asia Pacific region, 6%-41% in Europe, 3%-42% in North America, 22%-27% in Indian subcontinent, and 9%-67% in South America). In Middle East and Africa, the reported prevalence is around 21.2%. In central Europe, the reported prevalence is 4.9%, and adenocarcinoma is the commonest histologic pattern. Worldwide prevalence of activating mutations as well as resistance mutations in EGFR is higher in Asia than other regions. In India, reported incidence of EGFR mutations is 31% to 51.8%. ALK rearrangements have been reported in nearly 3% to 8% with prevalence in younger patients. The reported prevalence in India is 2.7% to 3%. ROS1 rearrangements have been reported in 1% to 2% of non-small cell lung cancer. In India, the reported incidence of ROS1 rearrangement is around 2.8%

Currently, guidelines for non-small cell carcinoma recommend testing for EGFR, ROS1, ALK, BRAF, and PD-L1. However, new emerging biomarkers, namely, HER2, MET, RET, NTRK, and TMB, necessitate comprehensive molecular studies including next generation sequencing. Recent meta-analysis has investigated the role of other biomarkers such as Golgi phosphoprotein 3 and ERCC1 protein in non-small cell cancer of lung.

The aim of this study was to analyse the frequency of EGFR mutations, ALK and ROS1 rearrangements and their association with age and gender in non-small cell lung cancer.

2 | MATERIALS AND METHODS

Patients with histological diagnosis of non-small cell carcinoma lung were included in the study. Samples included biopsies from the primary site as well as metastatic sites.

Slides from paraffin-embedded tissue blocks were screened for presence of tumor. Once presence of tumor was confirmed, formalin-fixed paraffin-embedded (FFPE) sections were collected on glass slides. This was followed by proteinase K digestion. DNA was extracted from freshly cut FFPE tissue using QIAamp FFPE tissue DNA extraction kit (Qiagen) following the manufacturer’s instruction and eluted in ATE buffer. EGFR mutational analysis was performed using Therascreen EGFR RGQ PCR Kit (Qiagen). The Therascreen EGFR RGQ PCR kit allows the detection of 29 somatic mutations in the EGFR oncogene by combining Scorpions and Amplification Refractory Mutation System dual primer probes. Samples were processed according to the manufacturer’s protocol, using the Rotor-Gene Q real-time PCR cycler (Qiagen). The cycling times were 95°C for 15 minutes for 1 cycle, 95°C for 30 seconds and then 60°C for 40 cycles. The obtained data were analysed with the Rotor-Gene Q Series Software (Qiagen).

ALK gene rearrangement was detected by fluorescence in situ hybridisation (FISH) technique using Vysis ALK Break Apart FISH Probe Kit (CE-IVD marked, Abbot Molecular). Five micrometers thin paraffin-embedded tissue sections were mounted on positively charged slides, dehydrated in xylene and alcohol, hybridized with the probe and incubated overnight. They were then counterstained with DAPI (4,6 diamidino-2-phenylidole) and visualized under fluorescence (Cytovision system capture station software 7.4 v Leica fluorescent microscope) microscope. Signals from 50 cells were counted, and rearrangement was considered to be present if more than 25 cells recorded positive. If 5 to 25 cells were positive, then it was considered equivocal (Figure 3). ROS1 rearrangements were detected by FISH using 6q22 ROS1 Break Apart FISH Probe RUO Kit. Deparaffinized tumor sections were dehydrated and hybridized with the probe, counterstained and visualized under fluorescent microscope. Fifty cells were scored, and rearrangement was considered to be present if

### TABLE 1 Distribution of various EGFR mutations by gender in the study group

| EGFR mutation (n = 748) | Number (%) | Males n = 510 (%) | Females n = 238 (%) | Median age in years | P value |
|------------------------|------------|------------------|-------------------|--------------------|--------|
| No mutation            | 488 (65.5) | 359 (70.3%)      | 129 (54.2%)       | 62                 | <.001  |
| Exon 19 deletion       | 159 (21.3) | 88 (17.2%)       | 71 (29.8%)        | 60.5               | <.001  |
| L858R                  | 82 (11)    | 51 (10)          | 31 (13)           | 63                 | .2     |
| G719X                  | 12 (1.6)   | 6                | 6                 | 64                 | –      |
| Exon 20 mutation       | 3 (0.4)    | 2                | 1                 | 67                 | –      |
| L861Q                  | 3 (0.4)    | 3                | 0                 | 63                 | –      |
| S768I                  | 1 (0.1)    | 1                | 0                 | –                  | –      |

Note: Among 510 males, 151 (29.6%) had EGFR mutation of which commonest was exon 19 mutation seen in 17.2% males, followed by L858R mutation seen in 10.0%. The remaining four mutations (G719X, Exon 20 mutation, L861Q, and S768I) together were seen in 2.3% of males. Among 238 females, 109 (45.7%) had EGFR mutation of which commonest was exon 19 mutation seen in 29.8% females, followed by L858R mutation seen in 13.0%. The remaining four mutations (G719X, Exon 20 mutation, L861Q, and S768I) together were seen in 2.9% of females. Females significantly outnumber males in exon 19 deletion (P < .001).
25 or more cells showed positivity. If less than five cells showed positive pattern, then the tumor is negative for ROS1 rearrangement (Figure 4).

2.1 | Statistical analysis

The data analysis was generated using the Real Statistics Resource Pack software (Release 6.8). Copyright (2013-2020). Data were segregated into categorical and continuous variables. Categorical variables were expressed as percentages. Continuous variables were expressed as mean when normally distributed and as median when the distribution was not normal. Categorical variables were compared using Chi-square test, whereas continuous variables were compared using Kruskal-Wallis test (for data which were not normally distributed). NCSS2020 software was used for cluster analysis.

3 | RESULTS

There were 748 patients with histologically diagnosed lung cancer. 510 (68.1%) were males and 238 (31.8%), females. Age varied from 20 to 90 years with a median of 62 years. Primary tumor sites were tested in 684 (91.4%) and metastatic sites in 64 (8.5%) patients. Histologic diagnosis was adenocarcinoma in 602 (80.4%) patients, poorly differentiated/non-small cell carcinoma in 71 (9.4%), adenosquamous in 8 (1.0%) and squamous cell carcinoma in 3 patients (0.4%).

EGFR mutation (Table 1): 260 (34.1%) were positive for EGFR mutation. There were 151 (58.0%) males and 109 (41.9%) females with age varying from 29 to 90 years. 29.6% of males in the study (151/510) and 45.7% (109/238) females in the study had EGFR mutations. Among 260 patients, adenocarcinoma was the commonest histology in 213 patients (81.9%). Exon 19 deletion was detected in 159 patients (61.1% of patients with EGFR mutations), L858R in 82 (31.5% of EGFR mutations), G719X in 12 (4.6% of EGFR mutations), exon 20 insertion and L861Q in three patients each (1.1% of EGFR mutations) and S768I in one patient (0.3% of EGFR mutations) (Table 1). Among 159 patients with exon

| Mutation/deletion | Adenocarcinoma, n = 602 | Adenosquamous, n = 8 | Squamous, n = 3 | Metastases, n = 64 | Carcinoma/poorly differentiated/non-small cell, n = 71 |
|-------------------|--------------------------|-----------------|----------------|-----------------|---------------------------------------------|
| No mutation (n = 488) | 386 (79.0%) | 5 (1.02%) | 2 (0.40%) | 46 (9.4%) | 49 (10.0%) |
| Exon 19 (n = 159) | 134 (84.2%) | 1 (0.6%) | 0 | 7 (4.4%) | 17 (10.6%) |
| L858R (n = 82) | 70 (85.3%) | 1 (0.12%) | 0 | 8 (9.7%) | 3 (3.6%) |
| G719X (n = 12) | 8 (66.6%) | 1 (8.3%) | 0 | 2 (16.6%) | 1 (8.3%) |
| L861Q (n = 3) | 2 (66.6%) | | | | |
| S768I (n = 1) | 1 (100%) | | | | |
| Exon 20 mutation (n = 3) | 1 (33.3%) | | | | |

Note: 66.6% to 85.3% of patients with exon 19 deletion/L858R mutation/G719X mutation were diagnosed to have adenocarcinoma histologically. 9 to 22.5% of patients diagnosed with adenosquamous carcinoma/metastases/poorly differentiated carcinoma had either exon 19 deletion or L858R mutation or G719X mutation. Percentages in each cell have been calculated for the overall number of mutations in each row.

19 deletion, there were 88 males and 71 females with age varying from 29 to 90 years. In patients with exon 19 deletion, 84.2% were diagnosed to have adenocarcinomas, 10.6% were diagnosed as poorly differentiated carcinomas, 4.4% as metastases, and 0.6% as adenosquamous carcinoma (Table 2). Among patients with exon

| Mutation/EGFR/tumor type and demographic characteristics | ALK positive (number tested) | ROS1 positive (number tested) |
|----------------------------------------------------------|-----------------------------|-----------------------------|
| No mutation/deletion, n = 488 | 34 (198) | 4 (127) |
| Exon 19 deletion, n = 159 | 0 (71) | 0 (49) |
| L858R, n = 82 | 0 (26) | 0 (16) |
| G719X, n = 12 | 0 (5) | 0 (1) |
| L861Q, n = 3 | 0 (2) | 0 (2) |
| Exon 20 mutation, n = 3 | 0 (2) | — |
| Age range in years | 27-78 | 40-63 |
| Male: Female ratio | 1.8:1 | 4 (2%)* |
| Adenocarcinoma, n = 21 (%) | 19 (55.8%) | 2 (50%) |
| Metastases, n = 12 | 12 (35.2%) | 0 |
| Poorly differentiated carcinoma, n = 5 | 3 (8.8%) | 2 (50%) |

Note: ALK rearrangement by FISH was seen in 34/304 patients (11.1%). ROS1 rearrangement by FISH was seen in 4/195 patients (2.0%). Among 34 patients positive for ALK rearrangement, 55.8% were histologically diagnosed to have adenocarcinoma, whereas, 44.1% were diagnosed to have metastases or poorly differentiated carcinoma. In four patients with ROS1 rearrangement, two were diagnosed to have adenocarcinomas, and the remaining two were diagnosed to have poorly differentiated carcinoma. Among 64 patients with metastases, 30 (46.8%) had one of the driver mutations (28.1% with EGFR and 18.7% with ALK rearrangement). 38.0% of poorly differentiated carcinomas had one of the three driver mutations (30.9% with EGFR, 4.2% with ALK rearrangement and 2.8% with ROS1 rearrangement).

Abbreviation: FISH, fluorescence in situ hybridization.

*In this group, all four were males and no male female ratio could be calculated.
19 deletion, four (2.5%) patients had T790M mutation. Among the 82 patients with L858R mutation, there were 51 males and 31 females varying in age from 37 to 90 years. The histological diagnosis in these patients was adenocarcinoma in 85.3%, metastases in 9.7%, poorly differentiated carcinoma in 3.6% and adenosquamous carcinoma in 1.2%. Three patients (3.6%) had T790M mutation in addition to L858R mutation.

There were six males and six females with G719X mutation with age varying from 41 to 82 years. Among these 12 patients with G719X mutation, 66.6% had histologically diagnosed adenocarcinomas, 16.6% had metastases, 8.3% had poorly differentiated carcinoma, and 8.3% had adenosquamous carcinoma. L861Q mutation was seen in three male patients in the seventh decade. Among these patients, adenocarcinoma was the histological diagnosis in 66.6% and metastases in 33.3%. S768I mutation was seen in one male patient aged 63 years, and the histological diagnosis was adenocarcinoma. Exon 20 mutation was seen in three males in the seventh decade, and the histological diagnosis was adenocarcinoma in 33.3%, squamous cell carcinoma in 33.3% and poorly differentiated carcinoma in 33.3%.

EGFR mutation was detected in 260 (34.1%) patients. ALK was positive in 34 cases (11.1%), and ROS1 was positive in four cases (2.0%) (Table 3 and Figure 1). In 98 patients (76 male, 22 female with median age of 65 years), EGFR mutation and ROS1 and ALK rearrangement were absent. By hierarchical clustering, the study group formed nine clusters (Figure 2) with respect to tumor type (adenocarcinoma, poorly differentiated carcinoma, squamous cell carcinoma, and adenosquamous carcinoma). However, none of the clusters were specific for any mutation or rearrangement. Demographic characteristics, histological diagnosis and FISH images of cases with ALK and ROS1 positivity are summarized in Table 3 and Figures 3 and 4.
Among the different age groups, EGFR mutations were equally distributed among the various age group except in the 20- to 35-year age group (13.6%). Exon 19 deletion was most common in the 20-to 35-year age group (100%) which reduced with increasing age (Table 4 and Figure 2). L858R mutation was not seen in the 20-to 35-year age group and gradually increased with age. Other mutations G719X, L861Q, S768I, exon 20 insertion and T790M were not seen in the 20-to 35-year age group.

ALK and ROS1 rearrangements were most common in 36- to 50-year age group (23% and 7.4%, respectively). Exon 19 deletion frequency in males (17.2%) was less than females (29.8%), \( \chi^2 = 15.3, P < .001 \), whereas, absence of mutations in EGFR was significantly more common in males (70.3%) than females (54.2%), \( \chi^2 = 18.7, P < .001 \). L858R mutation was equally seen in males (10%) and females (13%), \( \chi^2 = 1.5, P = .2 \). Age distribution among the different groups (excluding exon 20 mutation, L861Q and S768I as

**FIGURE 3**  FISH analysis using ALK dual-color break apart FISH probes to detect ALK fusion as split orange and green signals. A, Sections were considered negative for rearrangement when orange and green signals appeared adjacent (indicated by arrow) to each other or yellow (fused) signals were seen. B, Rearrangement was considered to be present when the green and orange signals are two to three signals apart (indicated by arrows) or one orange signal without the corresponding green signal along with another fused signal is seen (magnification x630). FISH, fluorescence in situ hybridization

**FIGURE 4**  FISH analysis using ROS1 dual-color break apart FISH probes to detect ROS1 fusion. A, Negative patterns were identified as two fused signals per nuclei (marked by arrow). Positive patterns include one fused signal along with one separate orange and green signal (arrows mark the separated spectrum orange and spectrum green signal) or when one isolated green signal (indicated by arrow) and one fused signal is seen. B,C, Spectrum orange binds telomeric to ROS1 gene, and spectrum green binds centromeric to it (magnification x630). FISH, fluorescence in situ hybridization
the number of patients in these groups were very less) was not significantly different by Kruskal-Wallis test, $H = 4.48, P = .21$.

4 | DISCUSSION

As early as 2005, higher frequency of EGFR activating mutations had been reported in Asian females with no smoking history.\textsuperscript{29} In a large study including many Asian countries, EGFR mutation frequency was nearly 60%.\textsuperscript{30} Lower incidence of EGFR mutations were reported in non-Asians when compared to Asian patients (25% vs 39%).\textsuperscript{31} Exon 18 mutations of EGFR are seen more frequently in Asia. Exon 19 deletions and L858R mutations have been commonly reported in southern Asia, and L861Q mutations have been reported from northern Asia.\textsuperscript{12} In a European study on 552 patients, EGFR mutations were seen in 4.9%.\textsuperscript{11} In this study, exon 19 deletions were seen in 56%, exon 21 mutations in 30% and exon 18 mutations in 11%.\textsuperscript{11} Women had higher frequency of EGFR mutations than men (8.5% vs 2.8%). Among non-small cell lung cancers, the commonest histological pattern with EGFR mutations was adenocarcinoma (8.5%). A small proportion of squamous cell carcinomas (1.1%) showed EGFR mutations.\textsuperscript{11} No significant age difference was observed in patients with EGFR mutation (mean 70.3 years) and patients with wild-type EGFR (mean 66.7 years). In a large Spanish study on 2105 patients with non-small cell cancer, EGFR mutations were seen in 16.6%. EGFR mutations were more common in women (69.7%), and the predominant histological type was adenocarcinoma (80.9%). Among patients with mutations, 27.1% were less than 57 years, 30.1% were between 56.7 and 69.1 years and 42.8% were more than 69 years of age.\textsuperscript{32} In an Indian study by Sahoo and others, EGFR mutations were seen in 51.8% of non-small cell carcinoma. Commonest mutations were exon 19 deletion (52%) and L858R deletion (26%).\textsuperscript{14} In another Indian study, using immunohistochemistry, exon 19 and L858R mutations were seen in 26.6% patients.\textsuperscript{33} Among 907 Indian patients, EGFR mutations were seen in 23% with a female preponderance (29.8% vs 20.4% in males), and the predominant histological pattern was adenocarcinoma (25.9%).\textsuperscript{34} In the same study, EGFR mutations were seen in 3.8% squamous cell carcinoma. Data from Japan and East Asia indicate a prevalence of 27% to 30% EGFR mutation positivity in non-small cell lung cancers.\textsuperscript{35}

In our study, EGFR mutations were present in 34.1% which is nearly similar to the data from Japan and east Asia\textsuperscript{35} and Indian population.\textsuperscript{33} However, it is slightly higher than that reported in the study by Chougule A (23%) and is less than that reported by Sahoo et al.\textsuperscript{14} The prevalence of EGFR mutations is more than the Spanish population (16.6%) and European population (4.9%).\textsuperscript{11,32} However, this variation in prevalence and higher incidence in Asia have been previously reported in literature.\textsuperscript{9} In the present study, females had a higher prevalence of EGFR mutations (45.7%) compared to males (29.6%). This is similar to data from Europe, Spain and other Indian studies.\textsuperscript{11,32,33} Like other studies,\textsuperscript{11,32,34} adenocarcinoma was the commonest histological pattern in our study (81.9%). In the present study, the commonest mutations were exon 19 deletion and L858R mutation seen in 21.3% and 11% patients, respectively, which is similar to previous studies.\textsuperscript{11,14,33}

A few observers reported increasing incidence of EGFR mutations with age.\textsuperscript{36} Others reported a reduced frequency of EGFR mutations in patients less than equal to 50 years ($P = .04$) and a higher frequency of uncommon mutations ($P = .03$).\textsuperscript{37} However in our study, we found uncommon mutations of EGFR (G719X, L861Q, S768I, and exon 20 insertion) in patients aged above 50 years. This difference may be due to the fact that 83.5% of our patients were aged above 50 years.

Globally, ALK rearrangements have been reported in 2.7% to 8% of non-small cell lung cancers.\textsuperscript{15-19} In our study, we found ALK rearrangements in 11.1%. The slightly higher prevalence may be explained by the fact that we did not test the whole study group and we used FISH for detection of ALK rearrangements. ALK rearrangements have been seen more commonly in younger age group with median age of 51 years.\textsuperscript{17} Similarly, in our study, the median age group of patients was 48 years with a range from 27 to 78 years. In the study by Kwak et al, adenocarcinoma was the most prevalent histology in 96% of ALK positive lung cancers.\textsuperscript{17} In our study, adenocarcinomas were seen in 55.8% (Tables 2,3). We did not classify metastatic tumors histologically, which could be the reason for the lower incidence of adenocarcinoma as compared to previous study.\textsuperscript{17} Our prevalence of ROS1 rearrangements (2%) is similar to that reported elsewhere in literature.\textsuperscript{20,21}

CONFLICT OF INTEREST

The authors declare no conflicts of interest.
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ETHICS STATEMENT

Data were obtained from hospital information system and anonymized. Samples were collected after obtaining informed consent from patients. Approval for the study and publication was obtained retrospectively from the Institution Ethics Committee (approval number: AMH-C-S-013/07-20).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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