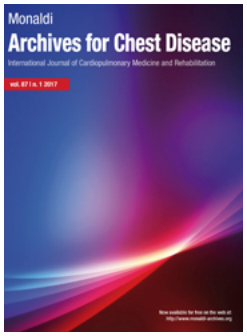

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Common epidermal growth factor receptor mutations in north Indian patients with non-small cell lung carcinoma: evidence from real-time polymerase chain reaction

Rakesh Behera,[1] Sheetal Arora,[1] Pranav Ish,[2] Geetika Khanna[1]

[1]Department of Pathology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi; [2]Department of Pulmonary Medicine, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi, India

Correspondence: Sheetal Arora, Department of Pathology, Vardhman Mahavir Medical College and Safdarjung Hospital, New Delhi - 110029, India.
Tel.: +91-0-9999049091. E-mail: sheetalaroragupta@gmail.com

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Abstract

Lung carcinoma was the ace cause of cancer deaths globally in 2022, with non-small cell lung carcinoma (NSCLC) accounting for 81% of the burden. Due to promising tyrosine kinase inhibitor (TKI) trials, NSCLC patients harboring epidermal growth factor receptor (*EGFR*) gene mutations are of interest. Our aim was to determine *EGFR* mutation prevalence in north India and its histologic and demographic correlations. We investigated the frequency of *EGFR* mutations in 40 patients with histologically confirmed NSCLC using real-time polymerase chain reaction. A 15% mutation frequency was observed in the study sample, involving 32 males and 8 females with a median age of 59 years. Squamous cell carcinoma (SCC) patients had only EXON20 (T790M, exon20 insertion) mutations, while adenocarcinoma patients had mutations in both EXON20 (T790M) and 21 (L858R) with mutation frequencies of 22% and 10%, respectively. 28% of the SCC patients were non-smokers, and 60% of these non-smokers had an *EGFR* mutation. South Indian and Asian studies have identified EXON19 (19-Del) and EXON21 (L858R) mutations as "common mutations" that account for nearly 80-90% of all mutations and respond well to TKIs. Interestingly, "common mutations" were found seldom in our study population, while the uncommon variants constitute 83% of all mutations, which we assume is due to diverse Indian genetics and ethnicity and co-existing signature mutations that involve the tyrosine kinase domain of EXON20. We suggest future genome-wide association studies to identify plausible genetic polymorphisms responsible for interethnic differences in *EGFR* mutation, which will contribute to better treatment and prevention of NSCLCs.

Key words: adenocarcinoma, squamous cell carcinoma, tyrosine kinase inhibitor, EXON20, T790M, ethnicity.

Introduction

GLOBOCAN 2022 reports lung carcinoma as the leading cause of cancer deaths worldwide, killing nearly 1.8 million patients and representing one in every ten newly diagnosed cancers amounting to nearly 2.5 million. This fatal cancer has an absolute burden of 81,748 in India for same year [1]. Lung carcinomas can be mainly categorised into two broad groups based on histology: small cell carcinoma and non-small-cell lung carcinoma (NSCLC). According to American society of clinical oncology, NSCLC is the commoner of these two, accounting for 81% of the incidence [2]. NSCLC is commonly diagnosed in its advanced stage of presentation and is not amenable to conventional curative treatments, resulting in a very poor prognosis [3,4].

In the modern era of precision oncology, EGFR gene mutation has become a focus of interest because of promising clinical trials involving tyrosine kinase inhibitors (TKIs) in NSCLC with EGFR mutations, which have led to significant improvements in lung cancer survival rates and quality of life. The mutation frequency of EGFR in NSCLC patients varies considerably from north to south in India and ranges between 20-39.6%. Noronha et al. reported 35% EGFR mutation frequency in NSCLC, and these mutations were seen frequently in adenocarcinoma histology, in females and non-smokers. Bhatt et al. found a higher mutation frequency of 39.6% in south Indian patients, with EXON19(76%) being most mutated and having a male predominance (64.3%). Tarigopula et al. studied 748 lung carcinoma patients from south India and reported mutation frequency of 36% in patients with adenocarcinoma histology with EXON19 deletion and L858R (EXON21) constituting 61% & 31%, respectively. Kasana et al. revealed a frequency of 35% in patients with lung adenocarcinoma from Kashmir, with EXON19 (55%) being most mutated, followed by EXON21 (30%) & EXON20 (15%). Bal et al. who tested EGFR mutations by RTPCR in adenocarcinoma lung patients from north India, found a lower mutation frequency of 15.4% with nearly equal distribution between males and females [5-9].

The EGFR gene spans over 192k base pairs, consists of 28 EXONs, and is situated in the short arm of chromosome 7. The gene has seven domains, including two large EGF-binding domains and another two cysteine-rich domains at the 5′ end. The transmembrane, tyrosine kinase, and regulatory domains are present in tandem after the cysteine-rich domain-2 towards the 3′ end. EXON 18-21, which are present in the tyrosine kinase domain of the EGFR gene, spans from codon 688 to 875 and has three special regions in them referred to as the P-loop, αC helix, and A-loop [10]. The EGF receptor protein consists of 1186 amino acids and includes an

extracellular ligand-binding region, a single hydrophobic transmembrane bridge linked to an intracellular juxta-membrane region, a tyrosine kinase domain, and an intracellular C-terminal tail containing numerous tyrosine residues. The extracellular amino-terminal end can be segmented into four domains, with domain III in charge of binding to ligands, hence activating the receptor for downstream signalling. The receptors, when bind to ligands induce their dimerization and subsequently cause autophosphorylation of tyrosine residues in the intracellular domain, which further activates the Ras/Raf/MAPK and PI3K/Akt/mTOR signalling pathways that regulate cellular functions like migration, proliferation, cell survival, etc [11,12]. EGFR can be aberrantly activated in several ways in NSCLC, including receptor overexpression, gene mutation, gene amplification, and polysomy, which are detected by various diagnostic methods like IHC, FISH, PCR, and NGS [13]. EGFR gene amplifications are predominantly linked to a solid histological pattern and more aggressive disease progression [14]. Detecting EGFR overexpression by immunohistochemistry is debated due to the complex scoring system and unavailability of novel antibodies that can detect all the EXON aberrations. FISH (Fluorescent in situ hybridisation) or CISH (Chromogenic in situ hybridization) are effective in detecting EGFR amplifications and polysomy, as well as identifying certain TKI-sensitive mutations, which may or may not include EXON 19 and 21 [13,15]. However, there is insufficient data to support the importance of distinguishing EGFR amplifications and polysomy and the inability to identify deletions and insertion mutations when there is an absence of gene amplifications by making FISH or CISH less helpful.

Direct sequencing of mutations and amplifications using PCR offers clear benefits due to faster detection period, and ability to detect specific mutations. However, a shortcoming of this technique is that it cannot detect certain sensitive, unknown mutations that respond well to TKIs [13]. NGS fills this gap by identifying somatic driver mutations, germline mutations, and measuring mutational burden. Even so, it can sequence circulating tumor DNA in liquid biopsy to screen and diagnose NSCLC early, eliminating the need for unpleasant and challenging lung biopsy procedures. A major challenge associated with NGS is the preparation of a gene library, which is tedious and extensive bioinformatics that requires experienced interpretation [16].

Almost 90% of EGFR mutations in USA are leucine-to-arginine substitution (L858R) in EXON21 or deletions in EXON19, which are generally referred to as "*common mutations*" [17]. A study conducted by Yoon et al. on the Korean population also found EXON19 (51%), and 21 (42%), to be most mutated and had better median overall survival than patients having EXON18 and 20 mutations among all the patients who responded to TKI therapy [18]. EXON19

Deletion results in a conformational shift in the EGFR helical axis, resulting in a narrowed ATP-binding cleft and increased ligand-dependent activation of the EGFR. The L858R (EXON21) mutations act by making the A-loop of the receptor more stable and enhancing the duration of ligand-dependent activation of EGFR. One of the resistant TKI mutations is the threonine-to-methionine mutation at codon790 (T790M) within EXON20, which severely affects the binding kinetics of the TKI to EGFR by enhancing the affinity of the receptor for ATP, resulting in a poor response to it [12]. Chiu et al. documented that, patients with uncommon mutations, including G719X (EXON18), S768I (EXON20), and L861Q (EXON21), benefited from TKI treatment, but with lower efficacy than those with common mutations in patients from Taiwan [19]. Apart from the above-mentioned, other uncommon mutations found in NSCLC are EXON18 (L692V, E709K, L718Q), EXON20 (exon20ins, C797S, L798I, G796D) and EXON21(L844V) [12,20].

In view of significant geographic variation of EGFR mutation worldwide, this study was aimed at determining the frequency and types of EGFR gene mutation by RT-PCR in primary treatment-naïve patients of NSCLC from north India. Besides, the correlation of EGFR mutations with socio-demographic parameters, smoking, histopathological subtypes, and stage of diagnosis was always a priority.

Materials and Methods

This study was conducted in a tertiary healthcare center in India, and biopsy samples were collected trans-bronchially via fibreoptic bronchoscopy from patients who were suspicious of lung cancer clinico-radiologically. After histopathological confirmation of NSCLC, only those patients were chosen for EGFR analysis who met our predefined inclusion criteria. We only included treatment-naïve primary NSCLCs who had at least 20% tumor tissue in their Haematoxylin & Eosin-stained tissue sections, and at the same time, extracted DNA must have ratio of absorbance at 260/280 nm in the range of 1.8to 2 as determined by a Nano-Drop spectrophotometer.

DNA was extracted from FFPE tissue sections using the TRUPCR® FFPE Tissue DNA Extraction kit, following the manufacturer's instructions. Extracted DNA samples were run using the TRUPCR EGFR kit on an ABI 7500 FAST Dx RTPCR instrument. The programme set up that the test samples underwent in the RTPCR instrument were one cycle at 94°C for 10 minutes, followed by second step of 10 cycles at 94°C and 68°C for 15 and 30 seconds, respectively. The final step was 40 cycles at 94°C for 15 seconds and 60°C for 60 seconds.

We analysed mutations in EXON18 (G719X), EXON19 (19-Del), EXON20 (ex20ins, T790M, S768I, C797S), and EXON21 (L858R, L861Q) of the EGFR gene. Cycle threshold (Ct) values for each test sample were calculated from the multicomponent plots, followed by ΔCt (Ct mutation - Ct reference). Positive for EGFR mutations were those samples having a ΔCt value less than or equal to the cutoff ΔCt value for that assay, and conversely, higher ΔCt values from the cutoff infer a negative mutation for that assay.

The presentation of the Categorical variables was done in the form of number and percentage (%). The association of the variables which were qualitative in nature were analyzed using Fisher's exact test. A p value < 0.05 was considered statistically significant. Data analysis was done using SPSS software, version 25.

Results

This cross-sectional study, involving 32 males (80%) and 8 females (20%), had a median age of 59 years. Besides socio-economic reasons, gender disparity in incidence (Male:Female-2.6:1, GLOBOCAN 2022) of lung cancers in India was a major reason for including 80% male patients in this study. All patients were grouped into four age groups: <=50, 51–60, 61–70, and >70 years. Overall mutation distribution revealed a 33.33%, 10%, 6.25%, and 20% mutation frequency across these age groups, respectively. Only 12.5% (1 patient) of all females and 15.6% (5 patients) of all included males had EGFR mutations.

EGFR mutation analysis by RTPCR demonstrated that 15.00% (6 cases) had an EGFR mutation, while 85.00% (34 cases) had no mutations or wild-type EGFR mutations. 10.00% (4 cases) exhibited *T790M* mutation, and 2.50% (1 case) had an EXON20 insertion mutation. Only 1 case (2.5%) had L858R (EXON21) mutation. All the test samples were negative for any mutations in EXON18 and 19 of the EGFR gene (Figure 1). Most patients (36) in this study presented at the advanced stage of lung cancer, i.e., stage IV, and mutations were found only in these patients. The rest of four patients were of stage III. Recent or past history of smoking was present in 29 patients, out of which three (10%) were positive for EGFR mutation involving EXON 20 & 21. Among the non-smokers three were positive for EGFR mutation involving only EXON 20, with a higher mutation rate of 27% (Figure 2).

This study involving eighteen patients with squamous cell carcinoma found EGFR mutation in four of them, with a mutation frequency of 22%, and involved EXON20 (T790M, exon20 insertion). Similarly, for adenocarcinoma histology, two were EGFR mutants out of twenty with a mutation frequency of 10% involving both EXON20(T790M) & 21(L858R). Two patients with

adeno-squamous carcinoma included were negative for EGFR mutation (Figure 3), (Table 1). Among the patients with squamous cell carcinoma, the majority were smokers, and five (28%) had no history of prior smoking. Out of these non-smokers three were EGFR mutants. Only six patients (30%) with the adenocarcinoma histology were non-smokers and all of them were EGFR non-mutants.

Discussion

Lung cancers have a very poor prognosis due to late diagnosis and metastasis, thus the requirement for personalized treatment strategies like targeted immunotherapy /chemotherapy which will help prevent this fatal epidemic. Hence, gathering population genetic information is very crucial. The present study observed that the EGFR mutation frequency (15%) in Indian patients with NSCLC was higher than that of patients from the USA, Italy, and African-American countries but comparatively lower than other Asian countries like China, Japan, Korea (Table 2). The mutation frequency among males was 15.6% and 12.5% in females, which is comparable and has a similar distribution pattern as reported by Bhatt & Bal et al. [6,9]. Mutations were seen to be prevalent in the age group of less than 50 years, which simply stresses the fact that mutations accelerate disease progression. Our study data matched global meta-analytic studies, which have previously reported that Asians have a higher mutation rate than Europeans (14%) [21,22]. However, being an Asian country, this study also reveals that the Indians have a lower EGFR mutation prevalence than the standard Asian average (38%). Studies done by Zhang et al. and Tanaka et al. reported that males have a lower EGFR mutation rate than female patients with NSCLC [21,23]. Since 80% of enrolments were males in this study cohort, this is one of the possible reasons for low mutation frequency in this study. But this also infers that the gender variation of EGFR mutations in North Indians is comparable to the global trend, even though there exist considerable genomic dissimilarities between countries and global regions.

Non-smokers have a higher mutation frequency of 27% compared to smokers (10%). 70% of patients with adenocarcinoma lung included had recent or past history of smoking. A possible reason behind this transition in the epidemiology of lung cancer from squamous histology to adenocarcinoma in India is attributed to change in smoking habits from “bidi” (a tobacco smoking product made in small-scale cottage industries) to cigarettes in the recent past, which have undergone changes in manufacturing processes to reduce nicotine content and include protective filters to minimize smoke exposure to the respiratory system. Secondly,

improvements in reporting by pathologists with the help of Immunohistochemistry helped in better cancer categorisation [24].

Herbst et al. concluded that environmental factors like smoking, air pollution, etc. are predominantly responsible for squamous cell carcinoma lung, and those who have never smoked usually have driver mutations in genes like EGFR, ALK, SOX2, etc. [25]. One-fourth of the squamous cell carcinoma patients included in this study were non-smokers, and 60% of these non-smokers have EGFR mutations, which reaffirms that driver mutations have a significant role behind carcinomas in non-smokers, and secondly, it infers that smoking is negatively associated with EGFR mutations ($p = 0.04$, Fischer's exact test). Overall EGFR mutation frequency observed in patients with squamous cell carcinoma was 22%, which was marginally higher than those of Joshi et al., Ling Ho et al., and Zhang et al., who have documented 5%, 9%, and 17% mutation frequency in squamous cell carcinoma lung, respectively [26-28]. Anusewicz et al. have reported accumulation of additional mutational burdens in patients with squamous cell carcinoma lung than those with adenocarcinoma, involving various cell cycle regulator genes and DNA repair genes, hence the existence of a significant difference in the downstream outcomes of the Notch, Wnt, Hedgehog, and ErbB signalling pathways between squamous and adenocarcinoma lung [29]. So, we are of the opinion that recent advances in molecular techniques, which are more sensitive and specific can easily detect many low allele frequency mutations which were previously reported rarely and hence an increased mutation frequency.

In their meta-analysis, Graham et al. identified EXON19 (19 Del) and EXON21 (L858R) mutations in NSCLC to be most common (80%–90%) globally as well as in South Asia and India [22]. Two Indian authors, Noronha et al. and Tarigopula et al. reported similar findings and found the '*common mutations*' more prevalent in patients with lung adenocarcinoma compared to other types of epithelial lung carcinomas in south Indians [5,7]. Surprisingly, most common mutation observed in our analysis was T790M (EXON20), accounting for 67% of all mutations, and an overall EGFR mutation frequency of 15%, which suggests the existence of interethnic variation in mutation frequency and types between north Indians and south. A very similar conclusion was drawn by Arrieta et al. who found that white Latin Americans had a 14% EGFR mutation frequency in NSCLC, but indigenous and mestizo populations had a 35% mutation rate, which they thought could be due to genetic polymorphisms present in these patients [30]. Although small sample size of the study group and infrequency of EXON20 in

other studies preclude us from asserting that this mutation is most common in the Indian population, the possibilities nevertheless exist.

The prevalence of pre-treatment T790M mutations in NSCLC has been variable and has a wide range of 0–38% depending upon sensitivity of the molecular method used [31,32]. According to Holt et al., roughly one-third of T790M mutations detected in real-world settings occur before EGFR TKI exposure and may be associated with germline inheritance. Ye et al. reported that a that a significant subset of patients with NSCLC and activating EGFR mutations also harbour low-level T790M (average 0.14% allele frequency) mutation in pre-treatment tumor samples. Pre-treatment T790M was detected in 35.9% of patients with activating EGFR mutations [33]. There is emerging evidence in support of the presence of low-level T790M mutations in pre-treatment tumors in patients with NSCLC with subsequent clonal expansion following exposure to first-generation EGFR TKIs [34,35]. Furthermore, diverse Indian genetics and the existence of molecular pathways & co-existing signature mutations that promote mutations in the tyrosine kinase domain of EXON20 in the north Indian population are other plausible causes.

The mutations found in patients in this study cohort are either primary or acquired resistance to TKIs such as gefitinib or erlotinib, making up 83% of all mutations. The rising popularity of TKI therapy due to improved patient survival outcomes makes the presence of resistant mutation patterns in Indians concerning. Although the sample size of our study is not large enough to draw any conclusions, especially in a country like India where the genetic makeup is vast and heterogeneous, the study results will aid in determining the necessity of implementing EGFR-TKIs therapy for NSCLC patients in India based on mutation and responsiveness. Apart from that, as we have already discussed, our study cohort interestingly showed some uncommon mutations, so follow-up with these patients could have given us some important data about the impact of these rarely studied mutations on overall survival as well as the effectiveness of different therapies on them, especially EGFR-TKIs. Lastly, due to the low occurrence of lung carcinomas in females, we could not include enough female patients, which could have provided more uniform results.

Conclusions

We observed that the EGFR mutation frequency in Indians, particularly from the north, is lower compared to other Asian countries and even many other Indian regions. Although the most common mutation observed in our study group was EXON20, considering its infrequency in

other studies and low sample size, we are not generalizing this to standard population till further studies prove same results. Nevertheless, recent advancements in molecular techniques and diverse genetics have always surprised with new mutation possibilities. Also, there has been emerging evidence in support of the presence of low-level T790M mutations in pre-treatment NSCLCs. Hence, reaching a common consensus across the diverse Indian population in the early stages of genomic studies is challenging and requires extensive genome analysis. North Indians have a high prevalence of EGFR mutations that are resistant to TKIs. The EGFR mutation is inversely correlated with smoking and the male gender. To identify plausible genetic polymorphism(s) responsible for interethnic differences in EGFR mutation, future genome-wide association studies are suggested that will contribute to better prevention and treatment of NSCLCs.

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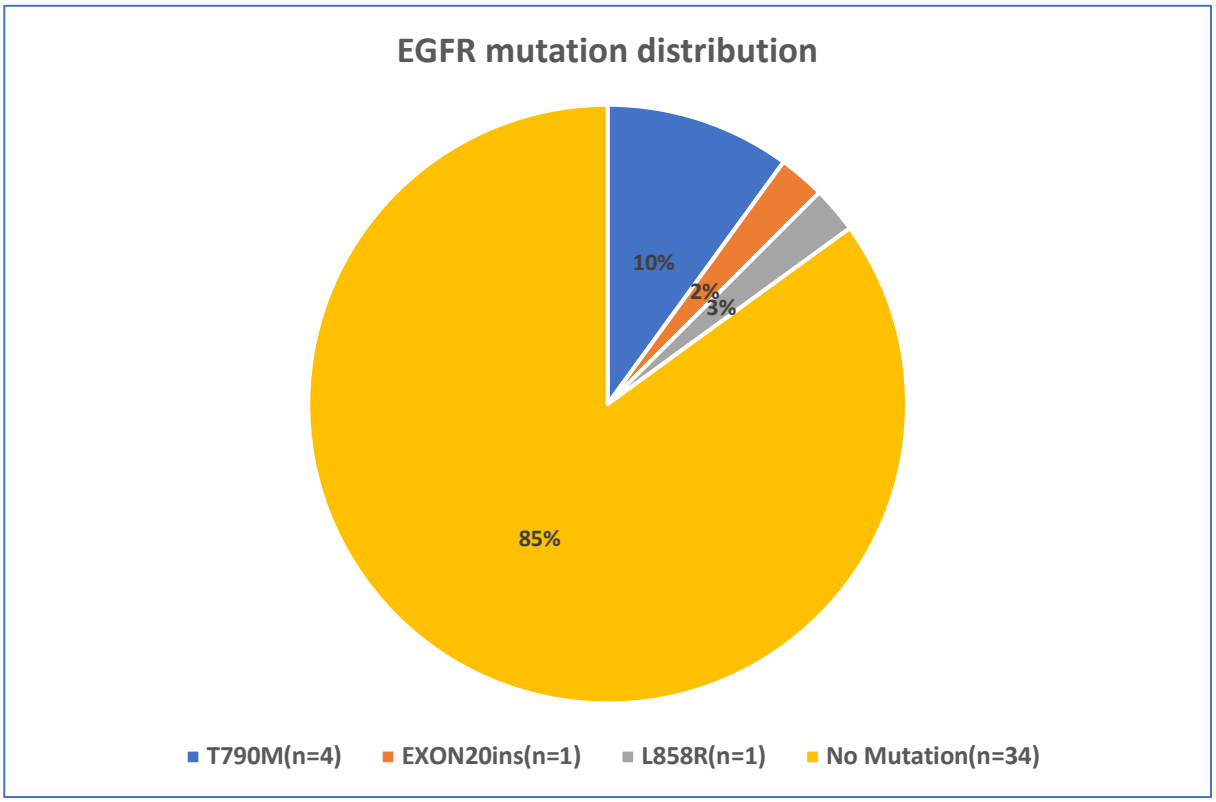

Figure 1. Distribution of epidermal growth factor receptor (EGFR) mutation across different EXONs individually in all patients.

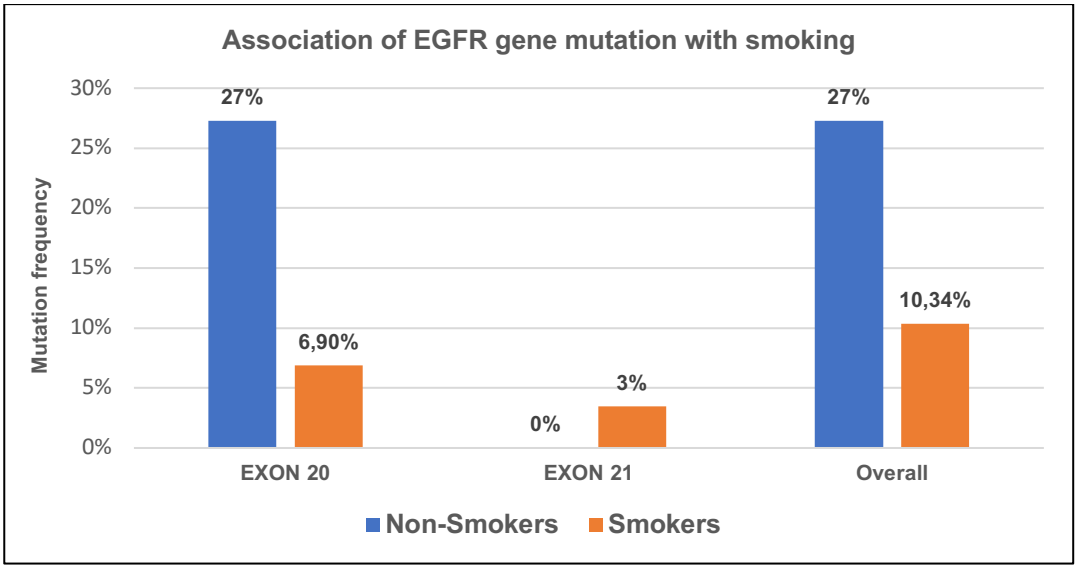

Figure 2. Association of epidermal growth factor receptor (EGFR) mutations with smoking. Patients with smoking history involved both EXON 20 and 21 with an overall frequency of 10.34%. Non-smokers showed a higher mutation frequency of 27%.

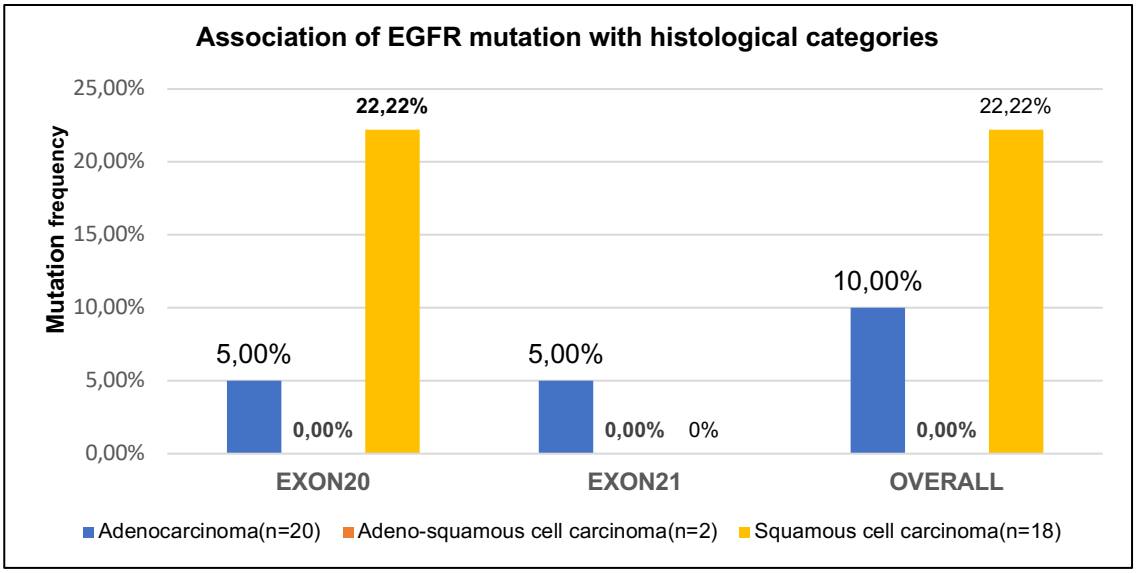

Figure 3. Association of epidermal growth factor receptor (EGFR) mutations with histological categories. Adenocarcinoma patients show 5% mutation frequency individually for EXON 20 and 21. Squamous cell carcinoma patients only involved EXON20 with frequency of 22.22%. EXON 18 and 19 mutation were not seen in any patient.

Table 1. Association of mutation frequency with relevant demographic and histological variables.

RELEVANT VARIABLES		MUTATION FREQUENCY (%)
GENDER	MALE(n=32)	16
	FEMALE(n=08)	13
SMOKING STATUS	PRSENT(n=29)	10
	ABSENT(n=11)	27
HISTOLOGY	Adenocarcinoma (n=20)	10
	Squamous cell Carcinoma (n=18)	22
	Adeno-squamous Carcinoma (n=02)	0

Table 2. Comparison of mutation frequency of our study with global studies.

Reference study	Patient group	Frequency			Detection method
		Overall (%)	ADC (%)	SQC (%)	
This study	Indian	15	10	22	RT-PCR
Tarigopula *et al.*	Indian	34	36	33	RT-PCR
Noronha *et al.*	Indian	35	35	25	RT-PCR
Leidner *et al.*	African-American	02	-	-	Direct sequencing
Yang *et al.*	USA	12	15	10	Direct sequencing
Zhou *et al.*	China	48	52	15	RT-PCR
Tanaka *et al.*	Japan	36	43	12	PNA-LNA PCR
Han *et al.*	Korea	19	21	05	Direct sequencing

RT-PCR, reverse transcription polymerase chain reaction; PNA-LNA-PCR, peptide nucleic acid-locked nucleic acid polymerase chain reaction.