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Original Article

Pathology Section

Detection of EGFR Mutations (Ex19Del, T790M, S768I, C797S, Ex20ins, G719X, L858R, L861Q) in Non-small Cell Lung Carcinoma and its Association with Various Clinicopathological Parameters: A Cross-sectional Study

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ABSTRACT

Introduction: Non-Small Cell Lung Cancers (NSCLC) is classified into squamous cell carcinoma and adenocarcinoma. Adenocarcinoma is commonly seen in non smoking females. Epidermal Growth Factor Receptor (EGFR) is a tyrosine kinase receptor which gets mutated in lung cancer. Tyrosine Kinase Inhibitors (TKI) like gefitinib are known to a have a good response in lung adenocarcinoma.

Aim: The aim of this study was to detect EGFR mutations in NSCLC and associate the mutation status with clinicopathological parameters.

Materials and Methods: The present cross-sectional study was conducted in the Department of Oncopathology and Transplant Immunology Molecular lab at Mahatma Gandhi Hospital, Jaipur, Rajasthan, India, from June 2023 to May 2024. All histopathological diagnosed cases of NSCLC were included in the study. Immunohistochemistry was further applied. EGFR mutation status (19Del, T790M, S768I, C797S, Ex20ins, G719X, L858R, L861Q) was checked using Real Time Polymerase Chain Reaction (PCR). An attempt was made to find out the association between the mutation status and various clinicopathological parameters. Statistical analysis was done using Statistical Package for Social Sciences (SPSS) statistics software windows version 22.0 released 2013.

Results: A total of 37 cases were included in the study of which 21 were males and 16 were females. There was near equal distribution when a comparison of smoking status was done. The most common clinical symptom was cough and the most common histological type was acinar adenocarcinoma. Thirteen out of 37 cases showed EGFR mutations. Exon 19 deletion was the most common mutation. EGFR mutation status showed statistical significance with non smokers and acinar adenocarcinoma.

Conclusion: Adenocarcinomas are more commonly seen in non smoking females. Exon 19 deletion is the most common type of EGFR mutation seen in lung adenocarcinomas. Mutations in EGFR are associated with good response to TKI.

Keywords: Adenocarcinoma, Epidermal growth factor receptor, Exon 19 deletion, Immunohistochemistry, RT-PCR

INTRODUCTION

According to Global Cancer Statistics 2022, Lung carcinomas are the most common cancer worldwide with the incidence of 12.4% and the most common cause for cancer related death with a mortality rate of 18.7% [1]. According to Global Cancer Observatory (GLOBOCAN) 2022, Lung carcinomas are the fourth most common cancer in India with an incidence of 5.8% and a mortality rate of 8.2%. Five year prevalence rate of lung cancers in India is 8.1 [2]. Lung cancers are broadly classified as NSCLC, Small cell lung cancer (SCLC) and Large cell Lung Cancers (LCLC). NSCLCs are further classified into adenocarcinoma and squamous cell carcinoma [3]. Tobacco smoking is widely recognised risk factor for lung cancer in squamous cell carcinoma and small cell lung carcinoma but smoke exposure seems to be less potent oncogenic factor for adenocarcinomas [4]. EGFR is a receptor tyrosine kinase present on the cell membrane. It has an extracellular ligand binding domain, a transmembrane domain, an intracellular tyrosine kinase domain and a regulatory region [5]. After binding to the ligands specific tyrosine residues of the intracellular domain, it becomes autophosphorylated and results in activation of downstream signalling cascade i.e., MAPK, PIK3-AKT, JAK-STAT pathway. This ultimately causes cell proliferation, cell differentiation, angiogenesis, metastasis and anti-apoptosis [6]. The TKI like erlotinib, gefitinib and afatinib have been targeted against EGFR and have shown dramatic response and progression free survival in lung adenocarcinoma patients [7]. Gefitinib is an orally active TKI which has been extensively used in multiple clinical trials and is an approved drug for treatment of advanced lung adenocarcinoma [8-10]. The use of TKI in the past decade in these patients and a response of the tumours to these molecules has built a new hope in making significant difference in survival of such patients [11,12]. The main objective of this study is to identify the EGFR mutation status in patients suffering from non-small cell lung carcinoma patients and to determine the mutation status with clinicopathological parameters i.e., age, gender, smoking status, clinical symptom, histological type, metastatic site and Immunohistochemistry (IHC) profile.

Detection of EGFR mutation status will help us to identify all those patients who can benefit from TKI therapy. Comparison of EGFR mutation status with various clinicopathological parameters will help us identify all those parameters which are routinely seen in EGFR mutated patients. This can particularly help us in resource restricted areas. Although, TKI therapy cannot be administered without knowing the EGFR mutation status.

MATERIALS AND METHODS

This study is a cross-sectional study conducted in the Department of Oncopathology and Transplant Immunology Molecular Lab at

Mahatma Gandhi Hospital, Jaipur, Rajasthan, India, from June 2023 to May 2024 (IEC No. MGMC&H/IEC/JPR/2024/3074).

Sample size calculation: The sample size was calculated using two formulas. First,

$n_0=Z^2\times P(1-P)/E^2$

$n_0=384$

Where, Z=Critical value of desired confidence level (95%, i.e., 1.96)

P=Maximum probability of variation (Which is maximum of 50%, i.e., 0.5)

E=Margin of error (5% or 0.05)

The second formula used was:

$n=n_0\times N/n_0+(N-1)$

n=36.61

Where, n=Sample size of known population

n_0=Proportion of unknown population

N=Known population size, i.e., 40.

Study Procedure

A total of 37 cases of histopathological diagnosed NSCLC were included in the study. The clinical data was retrieved from the requisition forms and the medical records. All those patients who received chemotherapy prior to tumour sampling were excluded from the study. Extra sections were taken on Poly-L-lysine coated slides to perform immunohistochemistry. Immunohistochemistry was performed using the Ventanna benchmark automated machine. TTF-1 and Napsin-A were the two primary antibodies used to confirm the diagnosis of adenocarcinoma. p40 and p63 were used to confirm the diagnosis of squamous cell carcinoma. More than 80% expression in the tumour cells was kept as the cut-off to confirm the diagnosis. Once the final diagnosis was made, extra 8-10 ribbon sections of 4-6 µm each were taken in microcentrifugation tubes and stored at 4°C. DNA was extracted from these sections using the TRUPCR DNA extraction kit. Once the DNA was extracted, the DNA content within the sample was quantified using the Invitrogen Qubit flourometer. Mastermix preparation was done using the substrate and different primers provided by TRUPCR EGFR kit and ultimately the DNA sample were added to this mastermix. Then this mix was transferred to the PCR wells and RT-PCR was performed. RT-PCR was performed to detect 8 mutations in the 4 exons of EGFR: Exon 18 (G719X), Exon 19 (Exon 19 deletion), Exon 20 (Exon 20 Insertion, T790M, S768I, C797S) and Exon 21 (L858R, L861Q). Once the RT-PCR run was completed, the cycle threshold value of the tests and reference curve were noted. The Δ Ct Value was calculated after subtracting the Ct values of the test from the reference. When the Δ Ct Value fell below the cut-off value, the sample was considered positive for that particular mutation.

STATISTICAL ANALYSIS

The data was entered in the excel sheet and then analysed through the SPSS statistics software windows version 22.0 released 2013. Armonk, NY: IBM Corp. Appropriate statistical tests like Pearson's Chi-square test were applied to establish the significant association between different variables.

RESULTS

As mentioned in [Table/Fig-1], out of the 37 cases included in the study, 21 were males and 16 were females. The age of the patients ranged from 32 to 86 years (Mean- 62.37 years). Among the 37 patients, there was an almost equal distribution among smokers and non smokers with a percentage of 43.2% (16 out of 37 cases) and 40.5% (15 out of 37 cases), respectively. Six out of 37 cases were ex-smokers (16.2%). The most common clinical symptom was cough followed by chest pain and dyspnoea. The most common histological type was acinar adenocarcinoma, 16 cases (43.2%) followed by solid adenocarcinoma (11 cases). IHC was further applied on all the cases. All the adenocarcinoma cases were either positive for TTF-1 or Napsin-A along with CK-7. A total of 30 out of 37 cases were positive for TTF-1 while 25 cases were positive for Napsin-A. Enteric adenocarcinoma was additionally positive for CK-20 and CDX-2. The IHC profile of mucinous adenocarcinoma was similar to the enteric type except that it was negative for TTF-1. Adenosquamous carcinoma was positive for p63 along with TTF-1. Squamous cell carcinomas were positive for p63 and negative for TTF-1 and Napsin-A.

Variables	Category	n	%
Age (years)	≤60	14	16.20%
	>60	23	83.70%
Gender	Male	21	56.80%
	Female	16	43.20%
Smoking status	Smoker	16	43.20%
	Ex-smoker	6	16.20%
	Non smoker	15	40.50%
Clinical symptoms*	Cough	17	45.90%
	Chest pain	15	40.50%
	Dyspnoea	12	32.40%
	Haemoptysis	12	32.40%
	Cachexia	2	5.40%
	Fracture	1	2.70%
Histological type	Lepidic adenocarcinoma	1	2.70%
	Acinar adenocarcinoma	16	43.20%
	Solid adenocarcinoma	11	29.70%
	Micropapillary adenocarcinoma	1	2.70%
	Enteric adenocarcinoma	1	2.70%
	Mucinous adenocarcinoma	1	2.70%
	Adenosquamous carcinoma	3	8.10%
	Squamous cell carcinoma	3	8.10%
Metastatic site	Pleura	7	18.90%
	Liver	2	5.40%
	Bone	1	2.70%
IHC profile**	TTF-1	30	81.10%
	Napsin-A	25	67.60%
	P63	6	16.20%
	CDX-2	2	5.40%
EGFR status	Exon 19 deletion	9	24.30%
	Exon 20 insertion	2	5.40%
	L858R	2	5.40%
	T790M	1	2.70%

[Table/Fig-1]: Frequency distribution of variables among study population.

From the 37 cases, 10 cases show metastasis, with pleura being the most common site (7 out of 10 cases) followed by two cases in the liver and one case to the right pelvic bone. IHC was again applied to confirm the diagnosis and all the cases showed positivity for either TTF-1 or Napsin-A.

The 37 cases were further processed for EGFR mutations of which 13 cases expressed positive mutations [Table/Fig-2]. The most common mutation was Exon 19 Deletions (9 of 13 cases) followed by two cases of Exon 20 insertion and L858R each, respectively [Table/Fig-3]. One of the 13 cases showed dual mutation which comprised of Exon 19 deletion and T790M.

When an association of positive EGFR mutations was made with various clinicopathological parameters, a statistical significance of <0.05 was found with smoking status and histological type [Table/Fig-2]. The statistical significance determines that EGFR mutations

Variables	Category	Mutation positive		Mutation negative		p-value
		n	%	n	%	
Age (years)	≤ 60 years	6	46.2%	8	33.3%	0.44
	> 60 years	7	53.8%	16	66.7%	
Gender	Males	5	38.50%	16	66.70%	0.1
	Females	8	61.50%	8	33.30%	
Smoking status*	Smoker	3	23.10%	13	54.20%	0.03*
	Ex-smoker	1	7.70%	5	20.80%	
	Non smoker	9	69.20%	6	25.00%	
Histological type*	Lepidic adenocarcinoma	0	0.00%	1	4.15%	0.04*
	Acinar adenocarcinoma	9	69.20%	7	29.20%	
	Solid adenocarcinoma	2	15.40%	9	37.50%	
	Micropapillary adenocarcinoma	1	7.70%	0	0.00%	
	Enteric adenocarcinoma	1	7.70%	0	0.00%	
	Mucinous adenocarcinoma	0	0.00%	1	4.15%	
	Adenosquamous carcinoma	0	0.00%	3	12.50%	
	Squamous cell carcinoma	0	0.00%	3	12.50%	
Metastatic site	Pleura	4	80.00%	3	60.00%	0.57
	Liver	1	20.00%	1	20.00%	
	Bone	0	00.00%	1	20.00%	

[Table/Fig-2]: Comparison of clinicopathological characteristics with EGFR mutation status using Chi-square test.

Variables	Category	Exon 19 Deletion		Exon 20 Insertion		Exon 19 Deletion and T790M		L858R		p-value
		n	%	n	%	n	%	n	%	
Age (years)	≤60 years	6	100%	0	0.00%	0	0.00%	0	0.00%	0.07
	>60 years	2	28.6%	2	28.6%	1	14.3%	2	28.6%	
Gender	Males	3	60.00%	2	40.00%	0	0.00%	0	0.00%	0.17
	Females	5	62.50%	0	0.00%	1	12.50%	2	25.00%	
Smoking status	Smoker	1	33.30%	2	66.70%	0	0.00%	0	0.00%	0.19
	Ex-smoker	1	100%	0	0.00%	0	0.00%	0	0.00%	
	Non smoker	6	66.70%	0	0.00%	1	11.10%	2	22.20%	
Histological type	Lepidic adenocarcinoma	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0.48
	Acinar adenocarcinoma	6	66.60%	1	11.10%	1	11.10%	1	11.10%	
	Solid adenocarcinoma	1	50.00%	1	50.00%	0	0.00%	0	0.00%	
	Micropapillary adenocarcinoma	1	100%	0	0.00%	0	0.00%	0	0.00%	
	Enteric adenocarcinoma	0	0.00%	0	0.00%	0	0.00%	1	100%	
	Mucinous adenocarcinoma	0	0.00%	0	0.00%	0	0.00%	0	0.00%	
	Adenosquamous carcinoma	0	0.00%	0	0.00%	0	0.00%	0	0.00%	
	Squamous cell carcinoma	0	0.00%	0	0.00%	0	0.00%	0	0.00%	
Metastatic site	Pleura	1	25.00%	1	25.00%	1	25.00%	1	25.00%	0.60
	Liver	1	100%	0	0.00%	0	0.00%	0	0.00%	
	Bone	0	0.00%	0	0.00%	0	0.00%	0	0.00%	

[Table/Fig-3]: Comparison of different clinicopathological parameters with types of EGFR mutations using Chi-square test.

were commonly seen in non smokers and all those who suffered from acinar adenocarcinoma of the lung. The same clinicopathological variables were further correlated with the type of EGFR mutation and no statistical significance was found [Table/Fig-3].

All the squamous cell carcinoma and adenosquamous carcinoma cases included in the study were identified in male smokers and no EGFR mutations were detected in these cases.

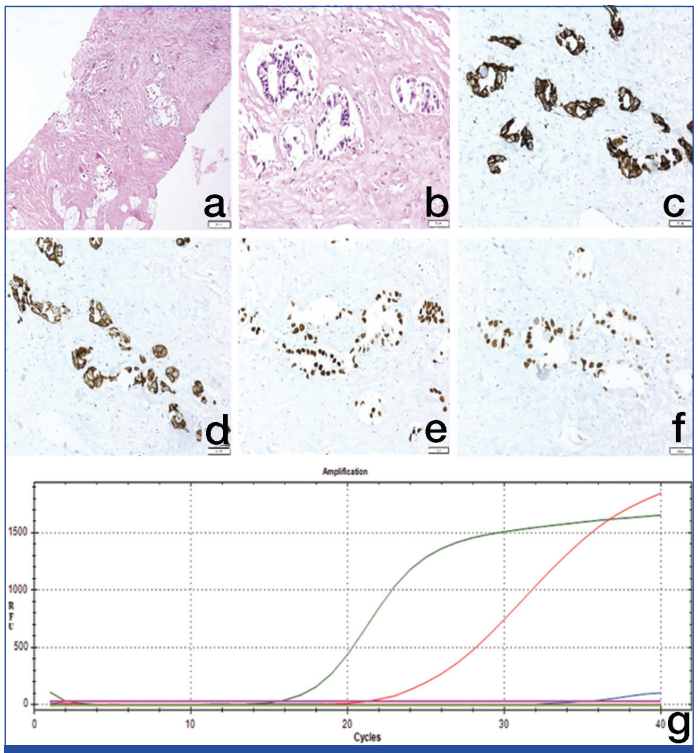
DISCUSSION

Lung carcinoma is the most common cancer and the most common cause of cancer related mortality worldwide [1]. In India, it is the fourth most common cancer and has a mortality rate of 8.2% [2]. Lung cancers are broadly classified as NSCLC, SCLC and LCLC. NSCLCs are further classified into adenocarcinoma and squamous cell carcinoma [3]. EGFR is a receptor tyrosine kinase present on the cell membrane [6]. Amongst all the lung cancer sub-types, EGFR is known to get mutated most commonly in adenocarcinomas. The four exons of EGFR involved in lung adenocarcinomas are Exon 18, 19, 20 and 21. The TKI like erlotinib, gefitinib and afatinib have been targeted against EGFR and have shown dramatic response and progression free survival in lung adenocarcinoma patients [7]. Gefitinib is an orally active TKI which has been extensively used in multiple clinical trials and is an approved drug for treatment of advanced lung adenocarcinoma [8-10].

EGFR mutation status is an important testing modality in NSCLC patients as there is availability of targeted therapy. According to the literature mentioned by the WHO, EGFR mutations are commonly seen in non smoker females [13].

In the present study, the patient who suffered from enteric adenocarcinoma was an 84-year-old non smoker female, who

presented with dyspnoea [Table/Fig-4]. Radiology revealed lesion in the left lung with pleural effusion. Examination of the pleural fluid showed metastatic carcinoma. Tru-cut biopsy was taken from the lung lesion and when IHC was performed over it, tumour cells showed positivity for TTF-1 and CDX-2. This case was one of the two cases with L858R mutations.



[Table/Fig-4]: (Case no. 26, Enteric adenocarcinoma): a) Presence of tumour cells in glands (Haematoxylin and Eosin, 40x); b) High power view of tumour cells in glands (Haematoxylin and Eosin, 400x); c) Tumour cells exhibiting positive cytoplasmic expression for Cytokeratin-7 (Immunohistochemistry, CK-7, 400x); d) Tumour cells exhibiting positive cytoplasmic expression for Cytokeratin-20 (Immunohistochemistry, CK-20, 400x); e) Tumour cells exhibiting positive nuclear expression for TTF-1 (Immunohistochemistry, TTF-1, 400x); f) Tumour cells exhibiting positive nuclear expression for CDX-2 (Immunohistochemistry, CDX-2, 400x); g) RT-PCR graph representing Ct value of the reference curve (Green dye curve) 16.06 and the Ct value of the Test curve (Red dye curve) 22.27 with a Δ Ct Value of 6.21 which falls within the cut off range of 11.5, hence positive for L858R mutation.

Another interesting case included in the study was of mucinous adenocarcinoma. The patient was a 59-year-old male smoker who presented with haemoptysis and cough. The lesion was found in the lower lobe of the right lung without any distant metastasis. The tumour cells were positive for CDX-2 and negative for TTF-1. No EGFR mutations were detected in this case.

In our study, majority of the EGFR mutations were seen in females when compared to males. Although due to equal distribution of EGFR mutation positive cases (8 out of 16 patients) and negative cases (8 out of 16 patients) in females, no statistical correlation was obtained.

Amongst the smoking status category, EGFR mutations were commonly found in non smokers. Nine out of thirteen EGFR positive cases were non smokers and a statistical correlation was found amongst them. The findings of our study are in concordance with the findings of the study previously conducted by Doval D et al., [14].

Our study also found that EGFR mutations were commonly seen in Adenocarcinoma. This finding was in concordance with the literature provided by WHO [13]. When we further classified the histological pattern of adenocarcinomas and correlated with the EGFR status, a statistical correlation was found between mutation positivity and Acinar adenocarcinoma.

Our study also found that the most common type of EGFR mutation was Exon 19 deletion. There were studies conducted previously by Sholl M et al., Mitsudomi T et al., and Yu L et al., who found that the most common type of EGFR mutations in lung adenocarcinoma was Exon 19 deletion [15-17]. The findings of our study were in concordance to the finding of their study.

Limitation(s)

This study does not include the survival data of those patients who received TKI therapy post detection of EGFR mutation status which is the foremost limitation of the study. Also, this study detected EGFR mutation status by PCR in an era of sequencing due to resource constraints, which is also a limitation. Sequencing has an edge over PCR because the same tissue can be used to detect multiple gene mutations.

CONCLUSION(S)

Lung carcinomas are more commonly seen in males when compared to females. Although, Adenocarcinomas are more commonly seen in non smoking females while squamous cell carcinomas are more prone in smokers irrespective of the gender. From this study, we also realised the importance of adequate tissue sampling for end to end reporting (morphology to molecular). With the support of immunohistochemistry, it was possible to classify majority of the unclassifiable NSCLC into Adenocarcinoma or squamous cell carcinoma. After RT-PCR examination, it was found that Exon 19 deletion was the most common type of EGFR mutation seen in lung adenocarcinomas. EGFR mutation status is the most valuable indicator for the screening of adenocarcinoma patients for TKI therapy. Mutations in EGFR are associated with good response of lung cancer patients to TKI. Although, there is a further requirement of molecular testing to improve the prognosis.

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