Screening of EGFR by IHC & FISH in Adenocarcinoma

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Abstract:

Background: Epidermal growth factor receptor (EGFR) is one of the targeted molecular markers in many cancers including lung malignancy. These mutations occur within EGFR exons 18–21, which encodes a portion of the EGFR kinase domain. EGFR mutations are usually heterozygous, with the mutant allele also showing gene amplification. Genetic modifications such as deletions, insertions and single nucleotide polymorphisms in the tyrosine kinase (TK) domain of EGFR is a common feature observed in most lung cancers. Immunohistochemistry (IHC) is a standard technique to detect the epidermal growth factor (EGFR) over expression in adenocarcinoma of lung.

Objective: The aim of this study is to look for over expression of epidermal growth factor receptor (EGFR) in adenocarcinoma of lung by immunohistochemistry technique and to detect EGFR positive mutations in adenocarcinomas lung by Fluorescent *in situ* hybridization (FISH).

Materials and Methods Retrospectively analysis of 58 cases of non-small cell lung cancer of the lung diagnosed on H &E sections at AP Government and General Chest Hospital, Hyderabad were taken for the study. Paraffin sections were immunostained with TTF-1, Napsin A and cytoketratin 5/6 to differentiate adenocarcinoma and squamous cell carcinoma among non-small cell lung cancer. EGFR expression was assessed in adenocarcinoma by immunohistochemistry. Fluorescent *in situ* hybridisation was performed on paraffin embedded block to look for positive EGFR mutations.

Results: 6o cases of lung cancer were analysed of which 58 were NSCLC and two were small cell lung carcinomas. Of the 58 cases of NSCLC, 23 cases (39.6%) were adenocarcinoma, 21 cases (35%) were squamous cell carcinoma and 14(23.3%) cases were poorly differentiated carcinoma. The peak incidence of NSCLC ranged between 50-70 years of age with a male predominance. All the 23 cases of adenocarcinoma diagnosed were submitted for TTF-1 and Napsin A staining by IHC. 16 adenocarcinoma cases were positive for both TTF-1 and Napsin A. 9 cases of poorly differentiated carcinomas also showed positivity for both (TTF-1 and Napsin A).These 25 cases of IHC proved adnocarciomas were submitted for EGFR over expression by IHC. 14(56.6%) EGFR IHC positive cases were then subjected to FISH, out of which, 5(35.7%)cases showed positive for EGFR mutations.

Conclusion: EGFR over expression is positive in adenocarcinoma by immunohistochemistry. Fluorescent in situ hybridization was performed on EGFR positive cases and EGFR mutations were confirmed.

Keywords: EGFR: Epidermal Growth Factor Receptor, NSCLC: Non small cell lung cancer, IHC: Immunohistochemistry, TTF-1: thyroid transcription factor-1, Napsin A.

Introduction: Lung cancer is the leading cause of cancer –related mortality worldwide. Approximately 85% of lung cancer cases are of the non-small cell lung cancer type (NSCLC) [(1)].It is estimated that about a million people die of cancer every year. Non-small cell lung carcinoma (NSCLC) accounts for 80-85% of all lung cancers [(2) (3)]. Thyroid Transcription Factor 1 (TTF-1) and Napsin A are both immunohistochemical stains that have been proven to stain a majority of lung ADCs .The prognosis of the patients remains poor, with an overall survival rate of less than 15% despite the advanced therapeutic options available. Recent studies suggest the existence of two distinct molecular pathways in the carcinogenesis of lung adenocarcinomas. K-ras oncogene is activated in association with the smoking and the EGFR oncogene is activated without the association of smoking [(3), (5)]. In targeted therapy, the epidermal growth factor receptor –tyrosine kinase inhibitors (EGFR-TKI) have revolutionized the treatment of adenocarcinoma lung [(4)]. The aim of the study is to screen EGFR by immunohistochemistry using biomarkers on adenocarcinoma lung. A very large numbers of studies on EGFR expression status in lung carcinomas are available in literature. But, studies on EGFR mutations, expressions are limited in Indian context [(5)].

Epidermal growth factor receptor (EGFR) belongs to a family of receptor tyrosine kinase (RTKs) that include EGFr/ERBB1, HER2/ERBB/NEU, HER3/ERBB3, and HER4/ERBB4.The binding of ligands, such as epidermal growth factor (EGF), includes a conformational change that facilities receptor homo or heterodimer formation, there by resulting in activation of EGFR tyrosine kinase activity[(24)]. Activated EGFR then phoshorylates its substrates, resulting in activation of multiplies downstream pathway within the cell, including the P13K-AKT-mTOR pathway, which is involved in cell survival, and the RAS-RAF-MEK-ERK pathway which is involved in cell proliferation [(24)].

FISH test can detect genetic abnormalities associated with cancer, it is also useful for diagnosing some types of unknown mutations. In some cases when the type of cancer has previously been diagnosed, a FISH test also can provide additional information to help predict a patient's outcome and whether he or she is likely to respond to chemotherapy drugs [(27)].

Materials and Methods: Paraffin blocks of all the cases NSCLC reported between 2012 and 2014 were retrieved from the archives of the Pathology Department of AP Chest Hospital and were included in the study. NSCLC were further classified into adenocarcinoma and squamous cell carcinoma based on morphology and immunohistochemistry (IHC). Five –micron-thick paraffin sections were cut and immunostained with TTF-1, Napsin A and Cytokeratin 5/6 biomarkers. Immunohistochemical staining for TTF-1 was performed using the biogenex monoclonal TTF-1 goat antibody against mouse antibody clone (BGX-397A) dilute in phosphate –buffer saline (PBS) [(7), (8), (9)].

All the adenocarcinoma proved by immunohistochemistry was immunostained with EGFR antibody and the results were analyzed. Immunohistochemistry staining for total EGFR protein was performed using the biogenex monocolonal EGFR goat antibody (primary antibody) against rabbit antibody; clone (Ep38Y) per-diluted in PBS on both control and test sections according to the manufacturer's instructions. Slides were scored on the cytoplasmic and /or membrane staining intensity as follows: a. No stain or faint staining intensity in <10% of tumor cells -0; b. faint staining in > 20% of tumor cells=2+, c. Moderate staining=3+, strong staining =5+ [(6)].

Fluorescence in situ hybridization (FISH):

Adenocarcinoma samples positive for EGFR by IHC and were subjected to EGFR FISH. Four µm- thick formalin-fixed, paraffin-embedded tissue were used for evaluation of EGFR genetic status. All cases had interphase FISH performed for EGFR rearrangement with the commercial EGFR dual color, break –apart rearrangement probe (SR Labs, Mumbai, India).Hybridization was carried out according to the protocol provided by the manufacturer.

Paraffin sections of 5µm were dewaxed and washed shortly with PBS.A pretretment the tissue sections were heated in citrate-buffer for 17 minutes and incubacted with Pronase E at 37°C for 3 minutes. Denaturation was performed by formamide 70% for 15 minutes at 75°C and afterwards stabilized by ethanol.The sextions were

then hybridise with Sigma EGFR/CEP7 Dual color probes for 20 hours at 37°C. After being washed, the air-dried slides were restained with 4', 6-diamindino-2-phenylindole before being analyzed. One hundred individual interphase nuclei per specimen were enumerated based on localization in the corresponding IHC sections samples were classified as positive for EGFR rearrangement when 15% or more of nuclei showed split signals (i.e., red and green signals were separated by ≥2 signal diameters) or single red signals (3′ EGFR) were observed. IHC and FISH slides for all cases were reviewed by two pathologists to confirm that scoring was carried out in the tumor cell population.

Results and Observation:

Sixty cases of carcinoma lung were diagnosed from 2012 to 2014 in AP Chest Hospital, Hyderabad. Of the 60 cases of lung cancer 37 (61.6%) were males and 25 (41.6%) were female with a male to female ratio of 1.4:1(Table: 1).Non-small cell carcinoma accounts to 96.6% (58 cases); small cell carcinoma (3.3%). Out of 58 cases, non small cell lung carcinoma 23 cases was (38.3%) adenocarcinoma (Fig-ia, ib) , 21 cases was (35%) squamous cell carcinoma, 14 poorly differentiated cases (Fig-iia, iib).The age incidence ranged from 50 to 70 years of age (Table-2, Graph-2,Chart-2).

TTF-1 and Napsin-A codes only for adenocarcinoma, cytokeratin 5/6 codes for squamous cell carcinoma (Table-3). Out of 23 cases of adenocarcinoma, 16 (69.5%) cases showed IHC positive for EGFR expression and in poorly differentiated carcinoma 9(64.2%) cases expressed EGFR expression (Table-4, Fig-iiia and iiib). Cytokeratin 5/6 was expressed in 7(33.3%) cases.

23 cases of adenocarcinoma were studies for TTF-I and Napsin A of which 16 (69.5%) cases were positive for both the markers and remaining 7 (30.4%) cases were negative for these markers.14 poorly differentiated cases, were subjected for IHC studies for TTF-1, Napsin A in which 9 (64.2%) cases were positive for TTF-1 and Napsin A. All the positive cases, which were positive for TTF-1 and Napsin-A was looked for EGFR over expression by IHC and was subjected to EGFR mutations.

EGFR over expressed by immunohistochemical studies were looked for EGFR mutations by Fluorescent in-situ hybridization. Dual-color break-apart fluorescent *in situ* hybridization was performed on paraffin-embedded tissue. FISH signals are seen as red (7p12 EGFR), green (5p15) (pericentromeric region of chromosome 60, and gold (8q24 CMYC).Out of 14 EGFR IHC positive only 5 cases (35.7%) showed EGFR mutation. These mutations were imparted color, for EGFR mutations (Table-5. Fig-iv a, iv b).

Table: 1

Gender distribution:

Gender	**No. of cases (60)**	**Percentage (%)**
Male	37	61.6%
Female	25	41.6%

Graph:1

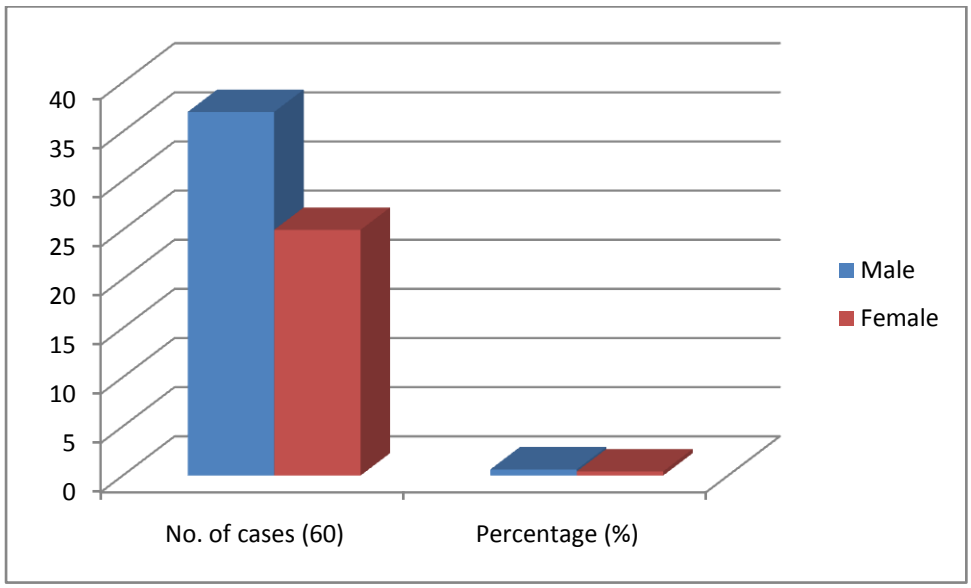


Table: 2

Different types of Lung carcinoma.

Tumor Types	**No. of cases (60)**	**Percentage (%)**
Adenocarcinoma (NSCLC)	23	38.3%
Squamous cell carcinoma (NSCLC)	21	35%
Poorly differentiated carcinoma	14	23.3%
Small Cell Carcinoma	2	3.3

Graph: 2

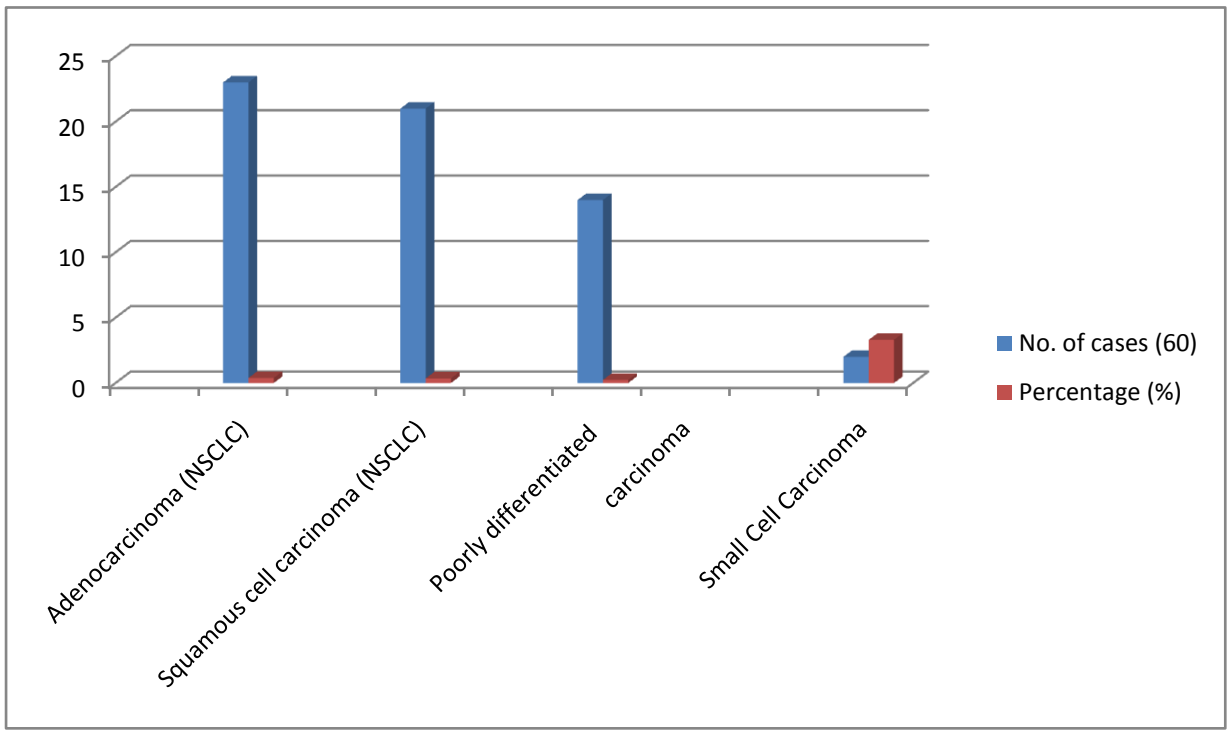


Chart:1

Prevalence of Lung carcinoma

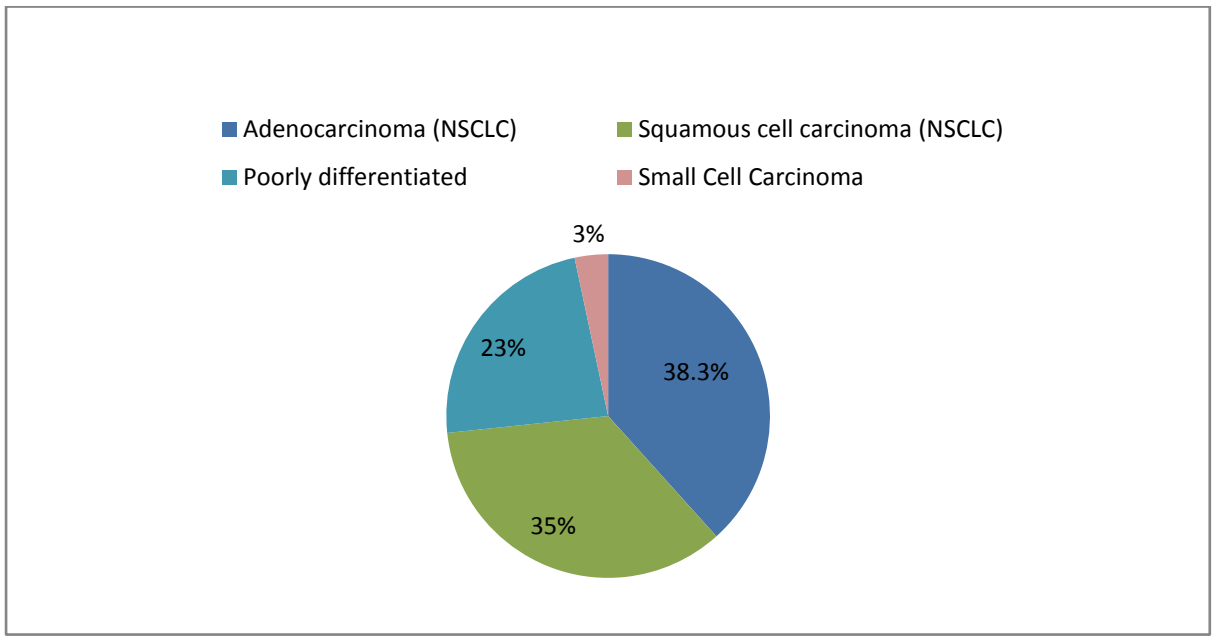


Table: 3

List of NSCLC and their immnunohistochemical expression:

NSCLC	**TTF-1**	**Napsin**	**Cytokeratin 5/6**
Adenocarcinoma	+	+	-
Squamous carcinoma	-	-	+
Poorly differentiated carcinoma	+	+	+
Adenosquamous	+	-	-

Table:4

List of IHC Positive Cases:

NSCLC	**Number of cases**	**TTF-1**	**Napsin**	**Cytokeratin 5/6**	**Percentage (%)**
Adenocarcinoma	23	12 (52.1%)	4 (17.3%)	-	69.5%
Squamous carcinoma	21	-	-	7	33.3 %
Poorly differentiated carcinoma	14	5 (35.7)	4 (28.5)	-	64.2%

Graph: 3

No. of Cases showing Immunohistochemical expression:

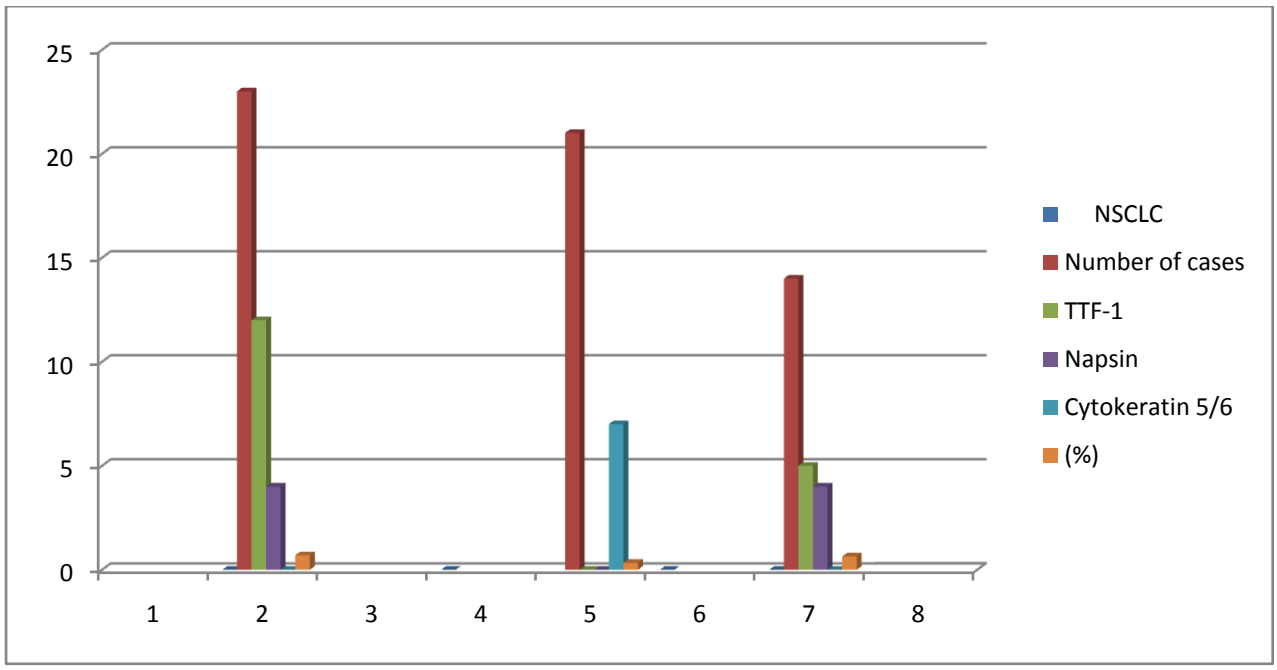


Table: 5

Fluorescent in-situ hybridization:

NSCLC	**No.of cases**	**EGFR IHC positive**	**EGFR mutations By FISH**	**Percentage (%) of FISH**
Adenocarcinoma	23	14 (60.8%)	5	35.7%

Chart: 2

EGFR expression and mutation (IHC & FISH)

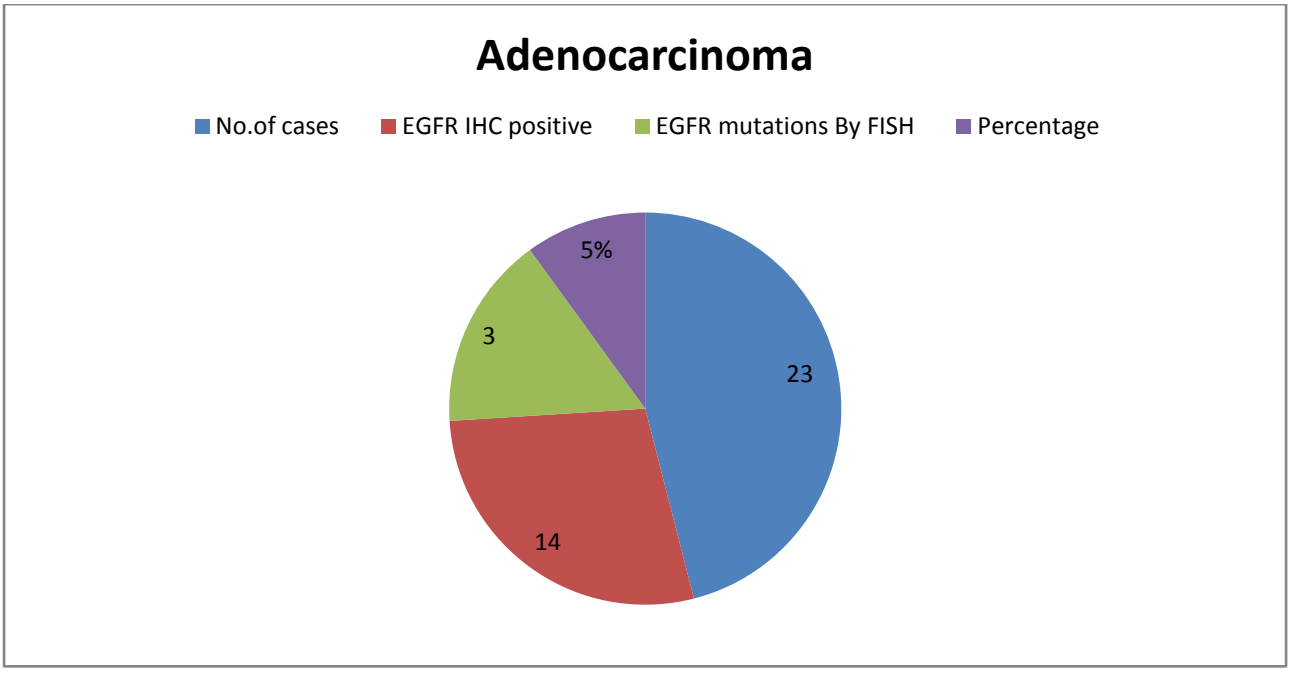


Fig: i a **Fig: i b**

Microphotograph of adenocarcinoma (thyroid transcription factor-1, ×100)

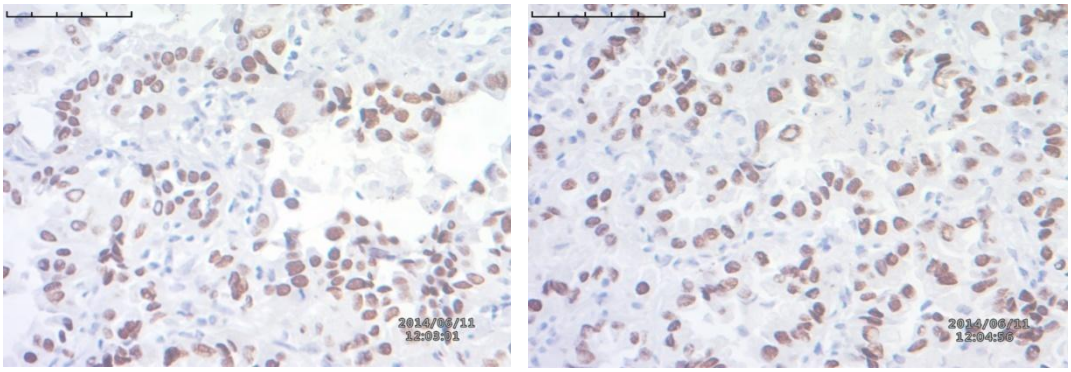


Fig : ii a **Fig: ii b**

Microphotograph of Poorly differentiated carcinoma (TTF-, Napsin A, ×100) (Moderate)

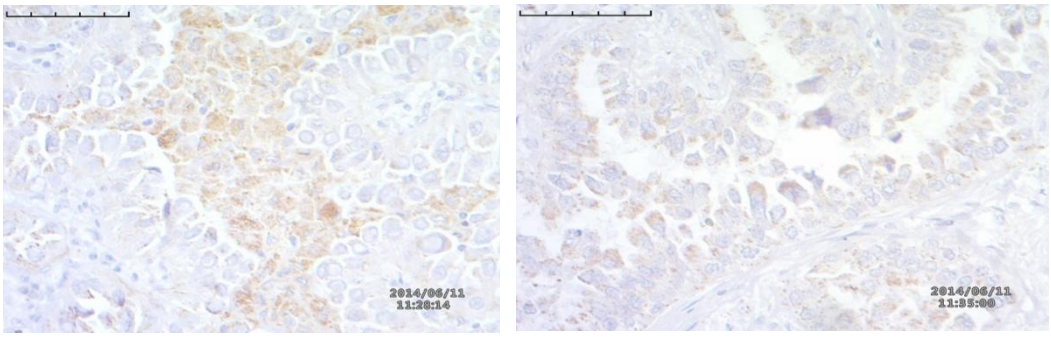
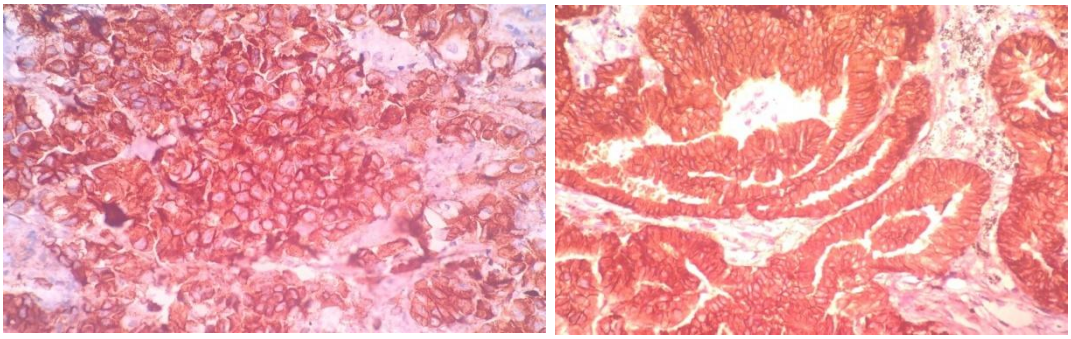


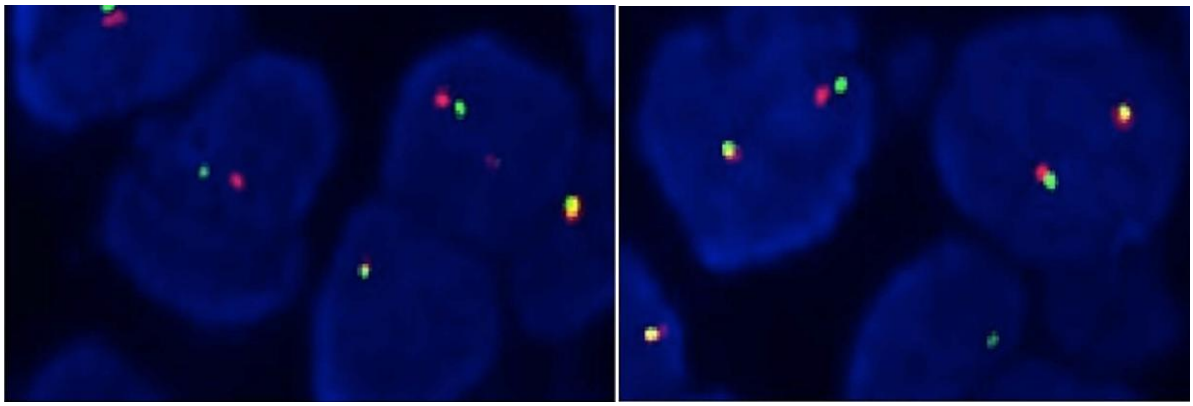
Fig: iii a **Fig: iii b**

Microphotograph of adenocarcinoma (Epidermal Growth factor receptor ×100)



Fluorescent in-situ hybridization:
Dual-color, break-apart fluorescent in situ hybridization was performed on paraffin-embedded tissue. FISH signals are seen as red (7p12 EGFR), green (5p15), aqua (pericentromeric region of chromosome 6), and gold (8q24 CMYC).

Fig: iv a **Fig: iv b**



Discussion: The incidence of lung carcinoma in India is rising day by day. Lung cancer is one of the leading causes of cancer –related deaths in India. NSCLC accounts for 80-85% of all lung carcinomas, and adenocarcinoma is the predominant histologic type with a male predominance [(10)]. In our studies 60 cases of lung carcinomas were diagnosed and were further classified as Non-small cell lung cancer (NSCLC) and Small cell lung carcinoma (SCLC) by routine H & E stain. NSCLC is further classified into Adenocarcinoma, Squamous cell carcinoma and poorly differentiated carcinoma based on H&E stain. In the diagnosed NSCLC, male cases 37 (61.6%) and female cases 25(41.6%) with the age difference of 50-70 years with the gender ratio (1.2:1). Justine A Barletta el at [(11)] conducted studies had 32 (36%) cases were male and 57 cases (64%) were female patients. The median age was 67 years (range 34-84 years). Peng Zhang et al [(12)] conducted studies among 324 diagnosed patients, out of which 222 cases were male and 102 cases were female patients with the gender ratio (2.2:1) and the median age was 61 years (range 30-84).

EGFR recently has attracted clinical attentation because of target therapies. Lynch et al [(13)] and Paez et al [(14)] reported that clinical responsiveness to gefitinib was associated with somatic mutations in the TK domain of the EGFR gene in NSCLC. Paez et al [(12)] found that the mutations were more frequent in adenocarcinoma, particularly in women of Asian origin. In our study we explore the EGFR protein expression assessed by IHC, EGFR mutations assessed by FISH, and their association to the prognosis. The majority of NSCLC tumors exhibit either intermediate (25%) or high (37%) levels of EGFR protein expression, and there was a significant correlation between EGFR protein expression and histologic sub-types. In our study showed that EGFR protein expression was more prominent in well-differentiated than in poorly differentiated histology. But, in poorly differentiated carcinomas most of the cases showed EGFR positive expression.

IHC is a well-established method routinely applied in lung cancer diagnosis in clinical laboratories. IHC also leads for the simultaneous analysis of expression level of other proteins and modifications. Immunohistochemical studies allows for the analysis of small tissue samples or cytological samples (body fluids, bronchial washing and fine needle aspirates samples) as well as circulating tumor cells [(13)].In our study two different markers TTF-1 and Napsin A are used for diagnosing adenocarcinoma lung by IHC.

TTF-1, a nuclear transcription factor that is expressed in the developing forebrain, thyroid epithelium, and fetal lung epithelial cells, is expressed in normal adult bronchiolar and alveolar epithelium as well as many pulmonary neoplasms, including most SCLCs and adenocarcinoma [(15, 16)].Suzuki et al [(17)] conducted studies, 84.4% of cases showed TTF-1 positivity of the lung adenocarcinoma specimens, its expression was noted in a large proportion. Justine A Barletta el at [(11)] demonstrated that TTF-1 expression was high in 48%, low in 24%

and absent in 28% of cases. In our study, 12 (52.1%) of adenocarcinoma and 5 (35.7%) of poorly differentiated carcinomas showed TTF-1 expression in NSCLC. TTF-1 staining in adenocarcinomas is generally strong and moderate in few cases and in poorly differentiated carcinomas shows moderate range of staining.

Napsin A is a newly discovered functional aspartic proteinase that is expressed in normal lung parenchyama in type II pneumocytes and is thought to be associated with primary lung adenocarcinoma. In 1998, Tatnell et al [(18)] reported the napsin A gene for the first time. Peng Zhang et al [(12)] studies demonstrated, napsin A expressed in 84.9% of primary adenocarcinoma with a granular cytoplasmic pattern. In our study only 8 (21.6%) cases expressed napsin A in adenocarcinoma and poorly differentiated carcinoma. When compared with the studies conducted by Tatnell et al, 84.9% of cases expressed positivity and in our study only 21.6% of cases expressed positivity for napsin A. Suzuki et al [(17)] showed that napsin A was expressed in almost all the tumor cells in most of the primary lung adenocarcinoma specimens (84.3%, 70/83).

In our study 58 cases of NSCLC, 25(67.5%) cases were positive for both TTF-1 and Napsin A by immunohistochemistry. The total of 25 (67.5%) cases which were positive for TTF-1 and Napsin A subjected to EGFR protein expression by immunohistochemistry. Reissmann et al [(19)] and Sanja Dacic et al [(20)] were able to detect EGFR protein expression in only 16% of the tumors analyzed. Pastorino et al detected 45% to 67% frequency of EGFR protein expression in NSCLC .In our study we are able to detect 14 (60.8%) cases of EGFR mutation in both adenocarcioma and poorly differentiated carcinomas (NSCLC).In our study, EGFR protein expression was determined on tumor tissue at the time of primary diagnosis. Sanja Dacic et al [(20)] investigated 28 cases of tumor samples, in which 24 (86%) cases showed an expression of EGFR on protein level. Zhiyong Lianh et al [(21)] studies showed 91/133 (68.4%) of lung adenocarcinoma were EGFR IHC-positive according to their interpreting criteria.

FISH has been established as the reference methods for assessment of gene amplifaction for many years.There are many ways to quantitate gene expression by FISH. EGFR gene amplification and gene mutations was seen only 10% in studies conducted by Hirsch et al [(22-24)] and Sanja Dacic et al [(20)]. Our study demonstrated that, out of 14 (60.8%) cases subjected for FISH only 5 (35.7%) cases showed positive for EGFR mutations. Zhiyong Lianh et al [(21)] studies found that 56/133 (42.1%) of lung adenocarcinoma showed EGFR FISH positive and EGFR FISH positivity was more frequent in late stages than in early stages of lung adenocarcinoma.

Conclusions:

The studies of lung cancer require accurate morphological differentiation between adenocarcinoma and squamous cell carcinoma. Adenocarcinoma is more common than squamous cell carcinoma in our study.TTF-1 and Napsin A was extremely useful in making a conclusive diagnosis, especially to look for EGFR protein expression by IHC. Immunohistochemical markers TTF-1, Napsin A are used to look for the desire EGFR protein expression in well and poor differentiated carcinomas. In our study 16 (69.5%) of adenocarcinomas showed positive for TTF-1 and Napsin A and 14cases of poorly differentiated carcinoma showed 9(64.2%) positive for both the markers. 14(23.3%) EGFR IHC positive cases were subjected to Fluorescent in situ hybridization (FISH) to look for EGFR mutations out which, 5 (35.7%) cases showed EGFR mutations by FISH.

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