HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS OF NON SMALL CELL LUNG CARCINOMA WITH SPECIAL EMPHASIS ON EGFR EXPRESSION

By

Dr. R. SHWETHA RAJ

A thesis submitted to

THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY, CHENNAI

In partial fulfillment of the requirements forthe award of the degree of

M.D in PATHOLOGY

DEPARTMENT OF PATHOLOGY

PSG INSTITUTE OF MEDICAL SCIENCE & RESEARCH

PEELAMEDU, COIMBATORE- 641 004

TAMILNADU, INDIA

CERTIFICATE

This is to certify that the dissertation work entitled, **"HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS OF NON SMALL CELL LUNG CARCINOMA WITH SPECIAL EMPHASIS ON EGFR EXPRESSION"** Submitted by **Dr. R. Shwetha Raj,** is a work done by her during the period of study in this department from 30/06/2015 to30/06/2017. This work was done under the guidance of **Dr. G Umamaheswari** Associate Professor, Department of Pathology, PSG IMS&R.

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CERTIFICATE

This is to certify that the thesis entitled, **"HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS OF NON SMALL CELL LUNG CARCINOMA WITH SPECIAL EMPHASIS ON EGFR EXPRESSION"** submitted by **Dr. R. Shwetha Raj** to The Tamilnadu Dr MGR Medical University, Chennai, for the award of the degree of **Doctor of Medicine in Pathology,**is a bonafide record of research work carried out by her under my guidance. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

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DECLARATION

I **Dr. R. Shwetha Raj**, do hereby declare that the thesis entitled, **"HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS OF NON SMALL CELL LUNG CARCINOMA WITH SPECIAL EMPHASIS ON EGFR EXPRESSION"** is a bonafide work done by me under the guidance of **Dr. G Umamaheswari**, Associate Professor Department of Pathology, PSG Institute of Medical Sciences & Research.This study was performed at the PSG Institute of Medical Sciences & Research, Coimbatore, under the aegis of the The Tamilnadu Dr MGR Medical University, Chennai, as part of the requirement for the award of the MD degree in Pathology.

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November 25, 2016

To
Dr R Shwetha Raj
Postgraduate
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Guide: Dr G Umamaheswari
PSG IMS & R
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore - 4, has reviewed your proposal on 25th November 2016 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your request to renew the approval for the study entitled:

"Histopathological & immunohistochemical analysis of non small cell lung carcinoma with emphasis on EGFR expression"

The following documents were received for review:

1. Request for renewal dated 23.11.2016

After due consideration, the Committee has decided to renew the approval for the above study.

The members who attended the meeting held on at which your proposal was discussed, are listed below:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr R Nandakumar (Chairperson, IHEC)	BA., BL	Legal Expert	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr S Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr D Vijaya	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The approval is valid for one year (29.12.2016 to 28.12.2017).

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Yours truly,

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr S Bhuvaneshwari
Member – Secretary
Institutional Human Ethics Committee

Proposal No. 15/400

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To
Dr Shwetha Raj R
Postgraduate
Department of Pathology
Guide: Dr G Uma Maheswari
PSG IMS & R
Coimbatore

Ref: Project No.15/400

Date: December 29, 2015

Dear Dr Shwetha Raj,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 18.12.2015 to conduct the research study entitled *"Histopathological & immunohistochemical analysis of non small cell lung carcinoma with emphasis on EGFR expression"* during the IHEC meeting held on 24.12.2015.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol (Version 1 dated 19.12.2015)
3. Confidentiality statement
4. Application for waiver of consent
5. Data collection tool (Version 1 dated 19.12.2015)
6. Current CVs of Principal investigator, Co-investigators
7. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 24.12.2015 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr. R. Nandakumar	BA., BL	Legal Expert, Chairperson	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr. S. Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr D Vijaya	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee
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Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr Sudha Ramalingam
Alternate Member - Secretary
Institutional Human Ethics Committee

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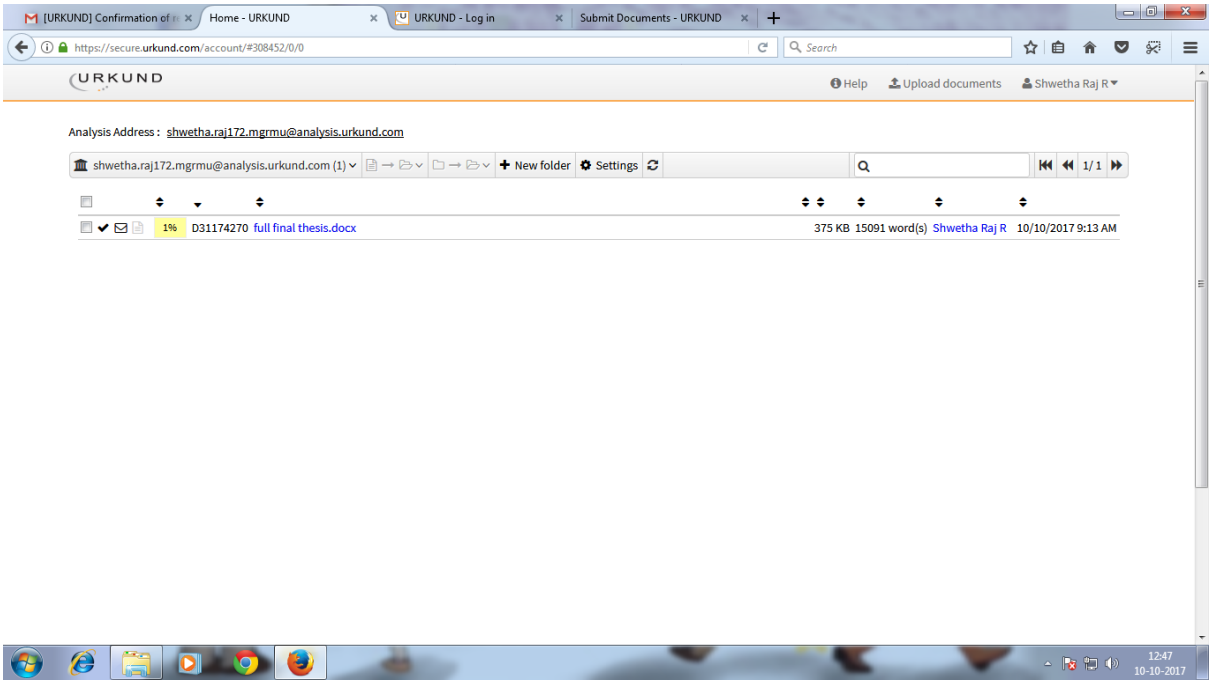

ACKNOLWEDGEMENT

First and foremost, I would like to render my heartfelt thanks and prayers to the Almighty.

I owe a debt of gratitude to my thesis guide, advisor and mentor ***DR UMAMAHESWARI*** for her invaluable insight, meticulous guidance and constant encouragement without which this dissertation would have not come to friution. I choose this moment to acknowledge her immense contribution.

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TABLE OF CONTENTS

INTRODUCTION

Lung malignancies are the commonly diagnosed cancers since 1985 and is the leading cause of cancer related mortality in the industrialized countries. An estimated 1.61million new cases of lung malignancies are diagnosed worldwide , of which 1.38 million deaths occur per year, thus surpassing the other common causes of cancer deaths.(1)

In India approximately 63,000 new lung cancer cases are being reported each year, with a five-year survival rate of (17.7 percent). The five-year survival rate for lung malignancies is 55 percent for cases detected when the disease is still localized. Only 16 percent of cases are diagnosed at an early stage. More than half of people with lung malignancies die within one year of diagnosis.(2)

The etiology of lung cancer is multifactorial. Tobacco smoking is considered the leading cause for lung malignancies but the proportion of lung cancer in never smokers is also increasing and shows a gender bias globally, occurring more commonly in women thus confirming the role of both genetic and environmental factors in the causation of lung cancer. In specific, high proportion of Asian women are being diagnosed of lung cancer recently. (3)

Traditionally, lung malignancies are classified as Non Small cell Lung Carcinoma and Small Cell Lung Carcinoma. Recently, 2015 onwards,

the sub typing of lung cancers has been revised with the need for specific sub typing of NSCLC. New insights regarding molecular targeted therapy in particular, has necessitated the revision to the traditional classification. Differentiating between squamous cell carcinoma and adenocarcinoma is now recommended, because of the availability of targeted therapy (tyrosine kinase inhibitors) in cases of adenocarcinoma. Most of the well to moderately differentiated NSCLC can be easily sub typed by using H and E alone, but the difficulty arises in small biopsies with poorly differentiated morphology. Various IHC markers play a significant role in the sub typing of NSCLC.

Molecular techniques in the identification of the mutational status in the NSCLC has significant implication in the treatment of patients with adenocarcinoma in which various mutations such as EGFR,ALK, KRAS are identified.

In contrast to resection specimens where we can differentiate the morphology precisely, in small biopsy specimens sub typing becomes challenging. With the advent of new imaging guided biopsy techniques and the unresectable stage at presentation in most of the lung cancer cases, the small biopsies become the sole available tissue for the diagnosis. So it seemed necessary to assess IHC markers in addition to H and E sections for small lung biopsy for the sub typing of poorly differentiated NSCLC.

Since identification of mutation by using IHC has recently developed, the detection of EGFR mutation provides scope for targeted therapy and has become mandatory to assess the mutational status in lung adenocarcinomas.

Our study, deals with the sub typing of NSCLC into adenocarcinoma and squamous cell carcinoma with the help of a panel of immunohistochemical markers. And identification of the mutational status (EGFR) in all IHC proven adenocarcinomas. The identification of the EGFR mutation is done immunohistochemically. We have also studied and correlated clinical characteristics of the patient (Age, Sex, radiological location of tumor and smoking status) with our immunohistochemical / histopathological diagnosis.

We hope, that the results of our study will increase the understanding of this entity lung cancer in particular NSCLC and further efforts in developing successful strategies for presentation, early diagnosis and targeted therapy.

AIM AND OBJECTIVES

- To immunohistochemically subtype Non small cell lung carcinoma.
- To assess the histological features in lung adenocarcinomas, squamous cell carcinoma and Non small cell lung carcinoma.
- To assess the expression of TTF-1, NAPSIN-A,p63 and CK5/6 in lung carcinoma diagnosed as adenocarcinomas, squamous cell carcinoma and non small cell lung carcinoma.
- To evaluate the EGFR mutational status in all IHC proven adenocarcinomas with the use of immunohistochemistry.
- To correlate and evaluate the clinical features with the morphology and immunohistochemistry of NSCLC.

REVIEW OF LITERATURE

Lung cancer has remained to be the leading cause for cancer incidence and mortality worldwide.

Despite the arrival of new treatment modalities the five year survival rate for lung cancer remains relatively poor.By the time the diagnosis is made lung cancer is well advanced and the treatment options becomes limited. A few co-existent non neoplastic lung diseases such as COPD also make it difficult.

It is noted that lung malignancies have a higher incidence among males worldwide than any other type of cancer, followed by prostatic cancer and gastric cancer. And among females it was the fourth most diagnosed cancer after breast cancer and colorectal cancer. But off late the incidence in females also show a rising trend (more off in developed countries) which may be attributed to the life style changes such as smoking.[5,6,7]

Lung malignancies have recently gained more importance due to the need for its subtyping, identification of the mutation status and the newly developed treatment modalities. The evaluation of lung cancer including the diagnosis, subtyping and the therapeutic options is a challenge to both the pathologist and the clinician.

EMBRYOLOGY

The respiratory system arises or develops from a" median diverticulum of the foregut."

It is said that the lining epithelium is of "endodermal origin" whereas the connective tissue i.e the cartilage and muscle are all derived from "splanchonopleuric mesoderm". The diverticulum forms the respiratory system which begins as a midline groove and is called the tracheobronchial groove. The separated portion of the groove or the free caudal end of the groove/diverticulum in said to form the "lung bud". The portion of the diverticulum cranial to the bifurcation forms the 'trachea & larynx '.The bifurcated lung bud undergoes primary division to form the right and left primary or principal bronchi further subdivisions give rise to two lobar bronchi of left lung & three lobar bronchi of right lung. The parenchyma of the lung is said to be formed by repetitive, continuous branching of the bronchial tree which ultimately forms the terminal bronchial tree. Later the alveoli is formed by the expansion of the terminal parts of the tree.

During fetal life, the entire subdivision of bronchial tree is lined by cuboidal type of epithelium which postnatally on respiration becomes thinned out.

The lung parenchyma, the developing lobar bronchi are all separated from one other by layers of mesoderm, which ultimately forms the

connective tissue basis of lung and the pleura. The pleura lines the surfaces of each lobe separating one another by forming fissures.

The embryological development of respiratory system is divided into stages of development i.e embryonic, pseudoglandular, canalicular, terminal sac phase and alveolar phase.[8]

ANATOMY

Lungs are a pair of organs situated in the thoracic cavity of as either sides of the mediastinum. Which are essential organs of respirations. Each lung innervates the respective pleural cavity. The adult right lung weighs 625gms and left lung weighs 50-100gms lesser or approximately 525gm.

Right lung has 3 lobes – Superior lobe, Middle lobe, Inferior lobe, 2 fissures -oblique (divides the superior and inferior lobe), horizontal (divides the superior and middle lobe).

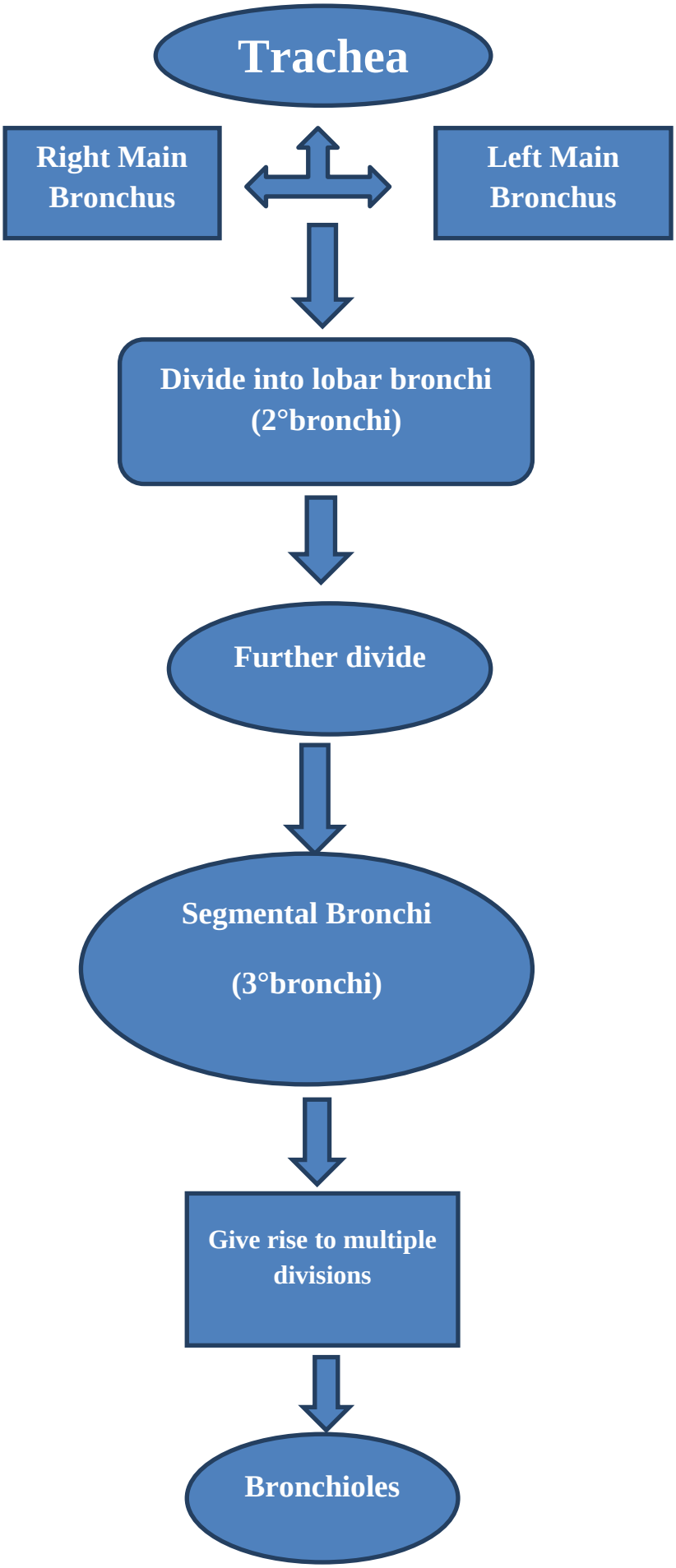
Left lung has 2 lobes-Superior lobe, Inferior lobe, 1 fissures-oblique (dividing the two lobes).

To mention in specific, in the left lobe, a tongue shaped projections is seen below the cardiac notch and is called LINGULA. It corresponds to the middle lobe of right lung.

Root and hilum-The root of the lung is a short pedicle or tube like structure which connects the medial surface of the lung to the mediastinum.

It is covered by a patch of mediastinal pleura which reflects onto the surface of the lung as visceral pleura. The region outlined by this pleural reflection on the medial surface of lung is called the hilum.

The contents of root / hilum are:- One Pulmonary artery, two Pulmonary vein, bronchial artery(one on the right and two on the left),bronchial vein,the main / principal bronchus , nerves (pulmonary plexuses), lymphatic's, bronchopulmonary lymph nodes and the areolar tissue.


Trachea
Right Main Bronchus
Left Main Bronchus
Divide into lobar bronchi (2°bronchi)
Further divide
Segmental Bronchi
(3°bronchi)
Give rise to multiple divisions
Bronchioles

BRONCHOPULMONARY SEGMENT

Each bronchopulmonary segment is an irregular cone with its apex at the origin of segmental bronchus and base onto the surface of the lung.

This is an area of lung supplied by segmental bronchus and its accompanying pulmonary artery branch. Branches of pulmonary vein pass between and around the segments.

There are about 10 bronchopulmonary segments in each lung.

BLOOD SUPPLY

Arterial supply- Bronchial artery and vein are the nutritive supply or source to the pulmonary tissue.

BRONCHIAL ARTERY

Right lung - bronchial artery which arises from either 3rd posterior intercostal artery or from upper left bronchial artery.

Left lung –two bronchial arteries which arise directly from thoracic aorta.

BRONCHIAL VEINS

Drain into either:

1. Pulmonary vein or into left atrium.

2. Azygos vein or right side superior or intercostal vein or hemiazygos on left side.

PULMONARY ARTERY

The pulmonary artery originates from pulmonary trunk and they carry deoxygenated blood from the right ventricles of the heart.

PULMONARY VEIN

On each side through the superior pulmonary vein and inferior pulmonary vein. They carry Oxygenated blood to the heart (left atrium) from the lungs.

LYMPHATIC DRAINAGE

Superficial, sub pleural and deep lymphatic's of lung drain into lymph nodes called 'tracheobronchial nodes'.[9,10]

HISTOLOGY

Trachea- Lined by ciliated columnar admixed with numerous mucus secreting goblet cells.

Bronchi-As the bronchi divides as right and left from the trachea and under goes consecutive divisions, its diameters also decrease with it. The bronchus is lined by respiratory epithelium which is cusped by a collar of

hyaline cartilage. The cartilage part also diminishes as the bronchi divides to form bronchioles.

Bronchioles- Bronchioles lack cartilage and submucosal glands. Rather the lining is (mucosa) is supported by elastic fibers and submucosa has incomplete bundles of smooth muscles which encircle the mucosa.

In specific, the type of epithelium in the bronchioles is a little different, rather shows some transitions. The lining is simple columnar, gradually becoming cuboidal as the diameter decreases. Ciliated cells persist but goblet cells disappear at the level of terminal bronchioles. In addition at the terminal bronchioles “Clara Cells” are noted at the level of terminal bronchioles. They are non ciliated cells with domed apical surface. It is said that the Clara Cells function is to secrete surfactant proteins. It is also said that their secretions may be involved in xenobiotic metabolism of carcinogens and lung toxicants.

Alveoli-The alveoli, alveolar ducts, respiratory bronchioles and blood vessels form the chief unit of lung function, also called the acini.

On paraffin sections, the alveoli resemble honeycomb structure and appear as empty spaces bordered by thin walls which form open sacs or closed polygons. The alveoli resemble honey comb structure. They are lined by squamous epithelium (type 1 alveolar cells) whose function is to allow gas exchange with capillaries of alveolar wall. Cuboidal cells (type 2

alveolar cells) whose main function is in production and clearance of surfactant. Also seen are many macrophages lining the alveolar surface. The alveolar interstitium contains many capillaries, fibroblasts, collagen and elastic fibers.[11

LUNG MALIGNANCY

ETIOLOGY

1. SMOKING

Smoking is the single and most major cause of lung cancers all over the world.

The association between smoking and development of lung cancer has been established from both epidemiological studies as well as from experimental results.

Tobacco association with cancer has been noted for at least 200 years now. Tobacco smoking has been clearly defined the most important cause for the development of lung cancer. Various studies since ages have also proved the same. Apart from cigarettes or tobaccos, bidi and hookah smoking also increases the risk of developing lung cancer.

One of the oldest and earliest studies on the relationship between smoking and lung cancer is by “Muller in 1940”.According to the study Muller classified smokers as:

Moderate smokers – (1-3 cigars / 1-15 cigarettes, 1-20gm tobacco per day)

Heavy smokers – (4-6 cigars/16-25 cigarettes, 21-35gm tobacco per day)

Very heavy smokers – (7-9 cigars/26-35 cigarettes, 26-50gm tobacco per day)

Excessive smokers - (10-15 cigars/ over 35 cigarettes, over 50gm tobacco per day)[19]

Another study by "DOLL & MILL" highlighted the importance of the duration of smoking and development of lung cancer.

Based on this, to establish the relationship, an index has been developed called the "smoking index".

Smoking index = Number of bidis/ cigarettes smoked / day x Number of years smoked.

This is graded as mild when it is below 100, moderate when it is 100-299 and severe when it is 300 or more. As the smoking index increases the risk of developing lung cancer also increases.[20]

The most important content in the cigarettes which is carcinogenic is "TAR" apart from various other chemicals (about 108 chemicals) such as polynuclear aromatic hydrocarbons, 2- naphthylamine and 4 alphaminobiphenyl are noted.

The amount of tar is proportional to the risk level of developing lung cancer. It is said that less than 17.6 mg tar is associated with lower risk of lung cancer whereas 25.7mg tar is associated with higher risk of developing cancer. [21]

Passive smoking also plays a significant role is the development of lung cancer. Non-smoking women whose husband's smoke or vice-versa tends to have a risk of 1.4 to 1.9 times than that of non-smokers.[22-34]

2. OCCUPATIONAL RISKS.

Occupational hazards / risks have always been associated with many respiratory diseases. There are major forms of carcinogens which significantly plays its role in the development of lung cancers. The major occupational carcinogens are: Asbestos, Arsenic, Nickel refinery workers , Radiation, Chromium , Mustard gas , Ether, tars etc.

3. DIETARY FACTORS:

It is said that dietary factors have both protective and increased risk for developing lung cancer.

Many studies have shown that β-carotene has a protective effect which is found in vegetables. In contrast is it said that cholesterol and animal fat increases the risk of lung cancer.[35]

4. AIR POLLUTION:

The difference in the incidence and mortality among the urban-rural areas prove the role of air pollution in the development of lung cancer. Urban air contains several carcinogens including asbestos, arsenic and polycyclic aromatic hydrocarbons from incomplete combustion of fuel.

Some forms of indoor air pollutions also add to the cancer burden. Few examples are burning of coal at home causes increase in levels of sulphur dioxide, carbon monoxide, total suspended particles etc. Cooking fumes, burning of incense sticks at home all add up to the causes for the development of lung cancer.

5. CHRONIC LUNG DISEASES AND LUNG CANCER

People with chronic non-neoplastic lung diseases such as tuberculosis, pneumonia, emphysema, tend to have increased risk of lung cancer.

It is said that an individual with chronic lung disease, due to the impaired lung ventilation there is increased pulmonary carbon dioxide, which in turn causes hyperplasia of cells (pulmonary neuroendocrine cells). All these are receptor mediated activity. These cells produce a lot of growth factors which contributes to the increased load of lung cancer in individuals with chronic non-neoplastic lung disease.

6. HOST FACTORS AND LUNG CANCER

It is observed that the risk of lung cancer in specific adenocarcinoma is associated with (i). Short menstrual cycles in women [36] (ii). Age of menopause [37]. Basically, the estrogen and progesterone receptors also play a role in controlling the growth of lung cells.

7. ONCOGENES :

Recently it has been identified that 'genes' play an important role in the process of malignant transformation.

In lung cancer, many recent cytogenetic studies have revealed numerical abnormalities and few structural alterations which include deletions and transformations. Over all action of these mutations are activation of " proto-oncogenes" promote oncogenesis, such as "ras and myc family" and inactivation of recessive tumor suppressor genes (suppress tumor development)[38,39,40,41]

Frequently involved proto with oncogenes are:

C- myc, N- myc,. L- myc – (deregulated expression)

K-ras, H- ras, N- ras – (activating mutation)

Her-2/ neu (deregulated expression)

Tumor suppressor genes are3p14,3p21,3,3p24-25,5q,9p,11p15.13q14 and 17p13 (p53 gene)[42,43,44] ras gene, point mutation are noted in NSCLC, predominantly in adenocarcinoma whereas bcl-1 locus is affected and plays its role in development of squamous cell carcinoma.

PATHOGENESIS OF LUNG CARCINOMA

Lung cancers are broadly divided into "non small cell lung carcinoma" and "small cell lung carcinoma". All types of lung cancer tend to arise from a common stem cell and this "common stem cell theory for histogenesis of lung cancer is now widely accepted".

In relation to lung, there are two different stem cell populations which are involved in renewal of epithelial cells. i.e.

a) The basal cell in trachea and bronchi b) Clara cells of bronchioles.

The carcinogens which initiate the malignancy could be in the form of smoke or occupational exposure. Ultimately targets the DNA. But for which these carcinogens have to undergo some amount of modification, transform into chemically active forms of carcinogens and then bind to macromolecules (could be DNA).

For eg: N- nitroso compounds which are a part of few carcinogenic substances, it requires metabolism to form a chemically active / reactive intermediate then these active forms transfer a alkyl group to DNA and

shows affinity for respiratory tissues. After which there is initiation of carcinogenesis affecting the stem cells and finally malignancy.[45]

CLASSIFICATION:

The diagnosis and treatment of lung cancer solely depend on correct histologic classification and staging of the tumor. Earlier i.e. before the 2004 WHO classification there was no therapeutic implication involved in the histological subtyping of lung malignancies such as squamous cell carcinoma or adenocarcinoma. They were all rather grouped as non small cell lung cancer. However due to the recent therapeutic advances(targeted therapy) histopathological subtyping both morphologically and immunohistochemically and mutation identity has gained profound importance.

The recent WHO (2015) classification has brought in new entities, deleted few and have also made changes in reporting biopsies and resection specimens. [4,5]

TABLE 1. 2015 WHO Classification of Lung Tumors[a,b,c]

Histologic Type and Subtypes	ICDO Code
Epithelial tumors	
Adenocarcinoma	8140/3
Lepidic adenocarcinoma[e]	8250/3[d]
Acinar adenocarcinoma	8551/3[d]
Papillary adenocarcinoma	8260/3
Micropapillary adenocarcinoma[e]	8265/3
Solid adenocarcinoma	8230/3
Invasive mucinous adenocarcinoma[e]	8253/3[d]
Mixed invasive mucinous and nonmucinous adenocarcinoma	8254/3[d]
Colloid adenocarcinoma	8480/3
Fetal adenocarcinoma	8333/3
Enteric adenocarcinoma[e]	8144/3
Minimally invasive adenocarcinoma[e]	
Nonmucinous	8256/3[d]
Mucinous	8257/3[d]
Preinvasive lesions	
Atypical adenomatous hyperplasia	8250/0[d]
Adenocarcinoma in situ[e]	
Nonmucinous	8250/2[d]
Mucinous	8253/2[d]
Squamous cell carcinoma	8070/3
Keratinizing squamous cell carcinoma[e]	8071/3
Nonkeratinizing squamous cell carcinoma[e]	8072/3
Basaloid squamous cell carcinoma[e]	8083/3
Preinvasive lesion	
Squamous cell carcinoma in situ	8070/2
Neuroendocrine tumors	
Small cell carcinoma	8041/3
Combined small cell carcinoma	8045/3
Large cell neuroendocrine carcinoma	8013/3

TABLE 1. (*Continued*)

Histologic Type and Subtypes	ICDO Code
Papillomas	
Squamous cell papilloma	8052/0
Exophytic	8052/0
Inverted	8053/0
Glandular papilloma	8260/0
Mixed squamous and glandular papilloma	8560/0
Adenomas	
Sclerosing pneumocytoma[e]	8832/0
Alveolar adenoma	8251/0
Papillary adenoma	8260/0
Mucinous cystadenoma	8470/0
Mucous gland adenoma	8480/0
Mesenchymal tumors	
Pulmonary hamartoma	8992/0[d]
Chondroma	9220/0
PEComatous tumors[e]	
Lymphangioleiomyomatosis	9174/1
PEComa, benign[e]	8714/0
Clear cell tumor	8005/0
PEComa, malignant[e]	8714/3
Congenital peribronchial myofibroblastic tumor	8827/1
Diffuse pulmonary lymphangiomatosis	
Inflammatory myofibroblastic tumor	8825/1
Epithelioid hemangioendothelioma	9133/3
Pleuropulmonary blastoma	8973/3
Synovial sarcoma	9040/3
Pulmonary artery intimal sarcoma	9137/3
Pulmonary myxoid sarcoma with *EWSR1–CREB1* translocation[e]	8842/3[d]
Myoepithelial tumors[e]	
Myoepithelioma	8982/0
Myoepithelial carcinoma	8982/3

The overall relevant changes are noted below:

- Use of immunohistochemical markers for classification/subtyping of lung malignancies including biopsy and resected specimens.
- It has highlighted the importance of genetic workup, in particular the molecular studies to help in the targeted therapy for advanced cases of lung malignancies.
- 2011 IASLC/ATS/ERS have put a new way of classifying biopsy and cytology specimens and a different approach to classify resected specimens.
- The new classification has completely brought in a new approach to lung adenocarcinoma classification, according to the 2011IASLC/ATS/ERS.
- The classification of squamous cell carcinoma has changed to keratinizing, non keratinizing and basiloid squamous cell carcinoma, with non keratinizing requiring immunohistochemical panel.[5,13,14.15]

ADENOCARCINOMA

This is an epithelial tumor of the lung exhibiting glandular differentiation. Initially in the 1950's it was noted that squamous cell carcinoma is the predominant subtype among the lung malignancies. But, later in 1960's there was near shift and adenocarcinoma showed a rising trend in its predominance. It is said that adenocarcinoma occur in non-

smokers. Other risk factors like occupational hazards, air pollution etc. also contributes in the development of adenocarcinoma. It usually manifests as a nodule (<3cm in size). They can occur anywhere in the lung, but is commonly seen in the periphery of the lung, and affects the upper lobe more than any other lobes.

2004 adenocarcinoma classification:

- Adenocarcinoma mixed subtype
- Acinar adenocarcinoma
- Papillary adenocarcinoma
- Bronchoalveolar carcinoma
  - Nonmucinous
  - Mucinous
  - Mixed/ intermediate

  } Removed in 2015 classification
- Solid adenocarcinoma with mucin production
- Fetal adenocarcinoma
- Colloid adenocarcinoma
- Mucinous cyst adenocarcinoma
  Signet ring adenocarcinoma
  Clear Cell Adenocarcinoma

  } Removed in 2015 classification

2015 additions

- Adenocarcinoma in situ (as a pre invasive lesion)
- Minimally invasive adenocarcinoma
- Classifying invasive adenocarcinoma based on subtype
- Using the term lepidic for the non-invasive component present as a part of invasive adenocarcinoma
- Mucinous bronchoalveolar carcinoma is renamed as invasive mucinous adenocarcinoma
- Undifferentiated carcinoma – can either be diagnosed as large cell carcinoma or if IHC (+), mucin (+) put into solid variant of adenocarcinoma[5,46]

THE VARIANTS OF ADEOCARCINOMA:

LEPIDIC ADENOCARCINOMA:

The tumor cells are bland pneumocytes, growing along the alveolar wall.

This lepidic pattern of adenocarcinoma can also show invasive foci (>5mm in greatest dimension) and termed as “lepidic predominant adenocarcinoma”. Non mucinous adenocarcinoma with lepidic growth pattern as the major component should be distinguished from the (I) invasive mucinous adenocarcinoma with lepidic growth pattern.(II) minimally invasive adenocarcinoma. The features differentiating these are (a) Invasion of lymphatic’s and blood vessels (b)Tumor necrosis (c)Invasive component >5mm (d) should show spread through alveolar wall.[5]

ACINAR ADENOCARCINOMA:

Neoplastic cells arranged in glandular / acinar pattern. They are round to oval with a central lumen which may contain mucin.

The cells lining the glands are columnar, may show peripheral nuclear polarization with central cytoplasm. Cribriform pattern can also be included and they tend to have poor prognosis .To define invasion, the presence of myofibroblastic stroma is required.[5]

PAPILLARY ADENOCARCINOMA:

The neoplastic cells are arranged in a papillary pattern with a prominent presence of fibro vascular core. Myofibroblastic stroma is not required to detect invasion.[5]

MICROPAPILLARY ADENOCARCINOMA:

Micro papillary variant shows tumor cells arranged in small papillary tufts without a fibrovascular core. They rather show focal florets and appear detached from or appear connected to the alveolar wall. Vascular and stromal invasion are commonly noted.[5]

SOLID ADENOCARCINOMA:

This variant shows polygonal cells arranged in sheets with an absence of identifiable pattern of adenocarcinoma.

The diagnostic criteria for solid adenocarcinoma is to identify mucin between $\geq$ 5 tumor cells in at least 2 hpf and should be confirmed with mucin stain (ABPAS).This variant should be distinguished from large cell carcinoma and squamous cell carcinoma both of which may rarely show intracellular mucin.[5]

OTHER VARIANT OF ADENOCARCINOMA

INVASIVE MUCINOUS ADENOCARCINOMA:

This variant is the newer version of the formerly classified mucinous bronchoalveolar carcinoma (WHO 2004)

These tumors have a tendency to be multicentric and bilateral.

Histologically these tumor cells show a goblet / columnar cell morphology with abundant intra cellular mucin and basally located small nuclei. Nuclear atypia is inconspicuous .Surrounding alveolar spaces may also be filled with mucin. They may have any pattern such as lepidic, papillary, micro papillary, glandular except the solid pattern.

In resection specimens, if the tumor may also show a non mucinous component (adenocarcinoma in situ or a minimally invasive adenocarcinoma) in such scenario, if that (non mucinous) component is >10% it should be classified as mixed invasive mucinous and non mucinous adenocarcinoma.

Immunohistochemistry differs, these tumors are CK7 positive and are negative for TTF1 and Napsin.[5]

COLLOID ADENOCARCINOMA:

Colloid adenocarcinoma is variant with abundant mucin pools replacing the air spaces. Here, the mucin pools distend the alveolar spaces and destroy their alveolar wall and appear as an invasive growth pattern into the alveolar spaces. The tumor cells are columnar with goblet like appearance; they grow in a lepidic pattern. They can also be glandular and are seen floating in the mucin pools.

This variant is weakly positive for TTF 1, Napsin and rather express MUC 2, CK20 and should be differentiated from invasive mucinous adenocarcinoma[(5)]

FOETAL ADENOCARCINOMA:

This variant of adenocarcinoma resembles the fetal lung. There are two grades, the low grade and the high grade. In the low grade fetal adenocarcinoma, there is low nuclear atypia, morule formation and the neoplastic glands are surrounded by loose fibromyxoid stroma whereas the high grade shows prominent nuclear atypia with absence of morule formation.[(5)]

ENTERIC ADENOCARCINOMA:

This variant is so named because of its resemblance to colorectal carcinomas. Histology wise, they have an acinar or cribriform pattern or

sometimes papillotubular structures. The neoplastic cells are tall columnar with eosinophilic cytoplasm, vesicular nuclei. They tend to have a brush border. Central geographic or dotted necrosis and scarring can also be noted.[5]

MINIMALLY INVASIVE ADENOCARCINOMA:

This is a new entity in the 2015 classification

The diagnostic criteria include: should be a small solitary adenocarcinomatous tumor (≤ 3cm) with predominance of lepidic pattern, invasion should be ≤ 0.5cm is greatest dimension and should include all patterns of adenocarcinoma (acinar , papillary, micropapillary, fetal, colloid etc) should also infiltrate the myofibroblastic stroma.

Exclusion are :If tumor invades lymphatic's, blood vessels air spaces or pleura or if it shows any tumor necrosis or spread through air spaces or if the cell are usually non mucinous (type II pneumocytes) or could rarely be mucinous (tall columnar cells)[5]

PREINVASIVE LESIONS

This is again has a new entity in the 2015 classification which includes

- Atypical adenocarcinomatous hyperplasia
- Adenocarcinoma in situ (the new entity)

ATYPICAL ADENOCARCINOMATOUS HYPERPLASIA:

These are small localized lesion (≤ 0.5cm) seen arising from the centriacinar region near the respiratory bronchioles. They have mild to moderate atypical type II pneumocytes and are cuboidal to dome shaped with clear to foamy cytoplasm. Intranuclear eosinophilic inclusions may also be present. Double nuclei may be seen with occasional mitosis.

ADENOCARCINOMA IN SITU:

Small localized lesions (≤ 3cms), with the growth restricted to cells along the preexisting alveolar structures (pure lepidic pattern). They lack stromal, pleural or vascular invasion.Invasive pattern such as acinar, papillary, solid or micropapillary are absent.

These adenocarcinoma in situ are mostly non-mucinous, rarely mucinous cases can also occur.

SQUAMOUS CELL CARCINOMA

2004	2015
Squamous cell carcinoma	Squamous cell carcinoma
- Clear cell variant	- Keratinizing squamous cell carcinoma
- Papillary variant	- Non-keratinizing squamous cell carcinoma
- Small variant	- Basiloid squamous cell carcinoma
	- Pre invasive lesion squamous cell carcinoma

Squamous cell carcinoma, is a malignant epithelial tumor which exhibits keratinization and /or intercellular bridges or if they are morphologically undifferentiated but express squamous cell markers (IHC). This epithelial neoplasm is strongly associated with smoking than adenocarcinoma. It is dependent on the amount , duration, starting age and the tar level of cigarettes smoked. Other occupational agents are also associated with the development of squamous cell carcinoma of which arsenic exposure is most important.[5,46]

The clinical features of keratinizing and non-keratinizing squamous cell carcinoma are similar to other NSCLC except that they are more aggressive tumors (superficial spreading) squamous cell carcinomas arise in a main or lobar bronchus and are predominantly centrally located tumors (2/3rdof the cases).

Histopathologically, keratinizing squamous cell carcinomas are identified by the presence of keratinization, keratin pearls and intercellular bridges. These features are better noted in well differentiated tumor than the poorly differentiated ones.

In case of non-keratinizing squamous cell carcinoma immunohistochemistry is mandatory to differentiate it from large cell carcinoma. The immunohistochemical markers used are P40, p63, CK5 or CK5/6.

Architectural pattern described earlier in the 2004 classification like the papillary, small cell, clear cell are no longer used. The only pattern retained is the “Basiloid squamous cell carcinoma.

BASALOID SQUAMOUS CELL CARCINOMA:

These are poorly differentiated epithelial tumors composed of proliferating small cells arranged in lobular pattern and shows peripheral palisading. They lack the typical squamoid morphology but exhibit the immunohistochemical markers for squamoid differentiation. If the keratinizing and non keratinizing components are present, then >50% of the tumor should exhibit the basiloid features to call it asbasiloid squamous cell carcinoma. These tumors were initially considered as large cell carcinoma’s but later in 1999 and 2004 classification they were recognized as special entity.

This variant has similar clinical features, gross appearance as that of the other squamous cell carcinomas. Histopathologically, they are arranged in anastomosing trabecular pattern with multiple layers and peripheral palisading. The tumor cells are small, cuboidal or fusiform, monomorphic with hyperchromatic to finely granular chromatin and absent or focal nucleoli. They tend to have scant cytoplasm and have high mitotic rate. Comedo necrosis is also present.

These tumor's express the same panel for immunohistochemical markers as that of any other squamous cell carcinoma (positive for P40, P63 or CK5 and Negative for TTF1). They should be differentiated from large cell carcinoma, small cell carcinoma, adenoid cystic and NUT carcinoma.

PRE-INVASIVE LESION:

The new entity in 2015 classification is "squamous cell carcinoma in situ". This entity is a part of the sequence in the development of squamous cell carcinoma. It starts with squamous dysplasia --- Squamous cell carcinoma in situ --- Squamous cell carcinoma.

Dysplasia can occur in a single location or can also be multifocal. It basically involves the tracheobronchial tree. Generally the patients with dysplasia are asymptomatic and occur in patients with heavy tobacco exposure (>30 pack years of cigarette smoking) and in patients with obstructive airway disease. The preinvasive lesions are seen more commonly in men than women. The diagnostic techniques used to identify the preinvasive lesions include white light microscopy, auto fluorescence bronchoscopy, narrow band imaging and optical coherence tomography. Histopathologically, as a response to the irritants the bronchial epithelium may show squamous metaplasia or basal cell hyperplasia with loss of the normal cilia or goblet cells. The dysplasia is graded (mild, moderate, severe) and the other preinvasive lesion, carcinoma in situ is differentiated based on

cell size, nuclear features, cell orientation, maturation and epithelial thickness.

ADENOSQUAMOUS CARCINOMA:

This tumor shows both components, the adenomatoid and the squamoid, with each component constituting at least 10% of the tumor. For an accurate and definitive diagnosis resected specimen are required.

Histopathologically, it is important to have10% of each component to make the diagnosis. If there is a minor component of any other subtype even that should be made note of (i.e<10%), because the mixed histology reflects the genetic status of either component regardless of the proportions.

The two main components may be separate or merged. These tumors tend to behave more aggressively. It is said to have a poorer prognosis in comparison to other NSCLC.[5]

DIAGNOSIS OF LUNG CARCINOMA

Diagnosis lung cancer is a variable process. The tests and the order of tests depends on various factors such as history, presenting complaints and physical examinations finding's. For a few, diagnosis is straightforward. For other's the process is quite complex due to the lack of symptoms or due to late presentation. Like any other disease the diagnosis starts with medical history, physical examination imaging and then the laboratory testing.

MEDICAL HISTORY:

Medical history plays a significant role in the diagnosis. It may also help in having a provisional diagnosis. Things to be included in the history part are: (i). Personal history, which includes smoking or also about passive smoking (ii). Occupation history, any exposure to any sort of carcinogens (iii).Most importantly family history of lung carcinoma or any other epithelial cell cancers. (iv). The symptom's should also be asked in elaborate.[47]

PHYSICAL EXAMINATION:

This is also a crucial step in the diagnostic process. Some of the findings are would find in a patient with lung malignancy are: fever, abnormal breath sounds, swollen lymph nodes, swelling of hands, feet, face or ankles, enlargement of liver, kidney, bony tenderness etc.[47]

IMAGING STUDIES:

In the recent few decades, with the introduction of new technologies such as like CT, MRI, PET and more recently PET-CT etc. have helped in various medical fields, imaging plays a significant role in diagnosis, staging and follow up of malignancy.

Lung malignancies are presumed to be difficult to detect at early stages of the disease due to which this malignancy is considered to have a

high death rate. Because lung tumors are encased within the rib cage, early diagnosis by physical examination is not possible. Chest radiographs are simple and ideal for demonstrating pulmonary abnormalities.

Uses of these imaging techniques are: -Helps in early detection, for targeted sampling, it detects the size etc., which helps in staging and helps in treatment (radiation dose)

Methods used for diagnosis of lung malignancies:

- Chest radiography - For large nodules but is unable to differential benign from malignancy.
- CT – For small nodules, for margins (i.e. speculated margins not pathognomic for a malignant nodule),for internal characteristics of tumor (i.e. detects infiltration of tumor into adjacent lung parenchyma or localized lymphatic spread)
- MRI – MRI in considered superior to CT. But this method is exploited once cancer is diagnosed, because MRI helps in evaluation of soft tissue involvement, chest wall involvement or nerve involvement. However, it does not serve effectively in early identification of lung cancers.
- Pet emissions tomography - This method helps in detecting nodules which are indeterminate on CT. PET with F-fluorodeoxy glucose

assesses use of glucose by different body structures based on the uptake of F-FDG by metabolically active tissue. Eg:- NSLLC have high metabolic rate, so these tend to take up F- FDG more intensely and appear hot on PET imaging.

Radiographic examination gives clue to the histopathological diagnoses of lung cancer. It is important to know the relationship between the radiological location and histological subtypes, as subtyping has apparently gained importance due to the new modality of treatment which has been introduced.

Years ago, a study was done in which radiographs were examined based as location, number, distribution and other characteristics of the lesions which include cavitation, calcification etc. In which they came up with a conclusion that majority of patients have their right lung involved, especially their upper lobe being affected and more of the anterior segment. In the same study it is said that squamous cell carcinoma diagnosed histopathologically present commonly as central tumors which is similar to the mayo clinic study[7]. And that of adenocarcinoma is seen as a peripheral mass which is in concordance with various studies in the west[7,13, 14]

With all this were gain an information regarding the importance of radiology, in specific the radiologic location, pattern and its correlation with the histopathological diagnosis.[48,49]

CYTOLOGY:

SPUTUM ANALYSIS:

The historical, sputum cytology also has its own minor role in the diagnosis of lung malignancy. Spontaneous sputum production itself indicates a pulmonary disease. And particularly, smokers (with or without bronchogenic carcinoma) readily cough out sputum. However, patients must be well instructed as to how they have to collect the sputum. The collected sputum should be of deep cough, if they are unable to get that sputum, they can inhale some nebulizing agents or steam or some chemicals such as propylene glycol.

To detect malignancy, the optimum numbers of specimens should be of minimum three and maximum five. Generally the location, size of tumor also plays its role in the sputum cytology positivity. It is said that central lesions (squamous cell carcinoma, small cell carcinoma) can be identified on sputum cytology tumor adenocarcinoma which are peripheral coin lesions. Similarly larger lesions can be identified than small ones. Overall, sputum cytology is good for a basic typing of tumors and can be used as a screening test. The techniques involved in sputum cytology are: (i) pick and smear (ii) Saccomanno technique (iii) liquid based cytology.[50,51]

BRONCHIAL BRUSHINGS / WASHING:

Bronchial cytology is usually recommended for endobronchial and centrally located lesions, but sometimes it may also help in the diagnosis of peripheral lung lesions.

Bronchial cytology is considered satisfactory if evidence of any pathogenic process is noted such as malignant cells, any infectious agents etc. Other than this to call it satisfactory, it should contain plenty of respiratory epithelial cells. If the smears are obscured by many squamous cell, blood, inflammatory cells or saprophytes it's considered unsatisfactory for evaluation.[50,51]

Bronchial washings is preferable in lesions which are beyond the reach of the bronchoscope, they help in the diagnosis of peripheral lesions.

BRONCHOALVEOLAR LAVAGE (BAL):

Earlier round the 1920's BAL was used to treat alveolar proteinosis, phosgene gas poisoning and asthma. Recently it is used for the diagnosis of infection's, inflammation and malignancies etc. In comparison to other bronchial cytology (brushings and washings) BAL help us to visualize and sample peripheral lung which is beyond the reach of the bronchoscope.

BAL is said to be satisfactory if it is rich in macrophages and poor in ciliated respiratory epithelial cells or squamous cells or any other debris.

BAL has a high diagnostic yield for malignant cells. It is less dependent on the tumor location, tumor size than bronchial brushings or washings.[50,51]

FNACBIOPSY:

Fine needle aspiration biopsy is a useful tool in the diagnosis of lung malignancies. Transthoracic FNA biopsy is considered the best – for diagnosing small peripheral lesions. Contraindications to the transthoracic FNA biopsy are uncontrollable cough, unconscious patient, bleeding abnormalities, poor lung function etc. Post the procedure it is said that the patient should be observed for few hours and in this technique bilateral biopsies are not recommended. Pneumothorax is one of the most dreaded complications associated with this procedure. The other may include hemoptysis, air embolism and sepsis. But the diagnostic benefits outweigh the risk factors and complications. FNA biopsy helps in differentiating small cell lung carcinoma from nonsmall cell lung carcinoma in about 95% of the cases. FNA biopsy can be done guided with ultrasound, including endoscopic ultrasound (EUS) or endobronchial ultrasound EBUS. Other methods which can be employed are CT guided, PET guided, fluroscopy etc. To evaluate the adequacy, it is better to have an onsite cytologist or a technician. In comparison with exfoliate respiratory cytology (sputum, washing or brushings) FNA biopsy (transthoraic) is said to be more sensitive and helps in diagnosing more of the peripheral lesions. [50,51]

HISTOPATHOLOGY:

Lung biopsies are considered the most important diagnostic tool for the definitive diagnosis of lung malignancies and other pulmonary disorders.

There are three methods used for the evaluations of lung tissue:

- Endoscopic biopsies.(by using fibroptic or rigid bronchoscope)
- Transthoracic (percutaneous) core needle biopsy.
- Video-assisted thoracoscopic surgery (VATS) lung biopsy.

- Indications:
  - To evaluate the lung lesions noted on radiological examination
  - To evaluate the patency of the airway and also to assess any unexplained hemoptysis and cough.
  - For staging lung cancer pre operatively and to evaluate the response to therapy[52]

ROLE OF IMMUNOHISTOCHEMISTRY IS THE DIAGNOSIS OF LUNG MALIGNANCIES:

Lung biopsies can be classified as benign or malignant on H&E examination. Further subtyping of lung malignancy requires the use of immunohistochemistry. The main idea of performing immunohistochemistry is to identify the cell constituents (ANTIGENS) and further recognise and

classify the specific cells within a cell population whose morphology is heterogeneous or homogenous. Recently, 2015 WHO classification has thrown great importance on the subtyping of malignancies due to the new therapeutic approaches (targeted therapy) which has bloomed up. In the present classification, the reporting criteria has also been mentioned in which immunohistochemistry plays a significant role. In any tumor in which morphology is doubtful a panel of immunohistochemical markers should be runned.

Overall, immunohistochemistry has three significant roles (i).Diagnosis (ii). Prognosis and (iii).Identifying the predictive markers for lung cancer therapy.

To emphasis on the differentiating panel for NSCLC.

- In adenocarcinoma the markers are:[5]
    - TTF-1 (75% of adenocarcinoma)
    - Napsin
    - CK7
    - CK20 (positivity in invasive mucinous adenocarcinoma)
    - Other markers are CDX2,MUC2 etc.
    -

- In squamous cell carcinoma the markers are:[5]

  - Non keratinizing squamous cell carcinoma are positive for p40, p63, CK5, CK5/6.

  - In keratinizing squamous cell carcinoma TTF should be negative for its diagnosis.

  - In basiloid squamous cell carcinoma, other cytokeratin's like CK1, CK5, CK10 and CK14 should be positive.

- Adenosquamous carcinomas[5]

  - Here, the respective adenocarcinoma markers (TTF1) and squamous cell carcinoma markers (p40) should be expressed to call it Adenosquamous carcinoma.

  - The TTF 1 positive cells should not take up the squamous markers and vice versa.

PNEUMOCYTE MARKERS:

THYROID TRANSCRIPTION FACTOR 1 (TTF1)

It is a 35-kDa homeodomain containing DNA binding protein of NKx-2 gene family. This marker is expressed in the thyroid and lungs. As it is noted in the early stages of human development it plays an important role in the cell differentiation and morphogenesis of both thyroid and lung.

As the expression of TTF1 happens early during lung differentiation. The nuclear expression of TTF-1 is helpful in the diagnosis and management of lung neoplasms.

TTF 1 is used for the subtyping of lung malignancies. It is said that 62.5% to 90% of adenocarcinoma's express TTF-1 and they are found at the very low levels in squamous cell carcinomas.

TTF1 are also expressed by normal pneumocytes. Therefore a clear differentiation should be made between the normal pneumocytes and malignant cells. [53-,61]

NAPSIN[62,63]

Napsin is an aspartic proteinase, it is expressed as a cytoplasmic marker is lung parenchyma. It is homologous with the polypeptide $TA0_2$ and is involved in maturation of the biologically active surfactant protein B. It also consists of a 38-RDA protein, a protein which is expressed in type II pneumocytes, alveolar macrophages, renal tubules and ducts of the pancreas.

Napsin was used to differentiate primary lung carcinomas from metastatic. Napsin shows a cytoplasmic granular positivity/ staining. Few studies suggested that Napsin is a more sensitive marker than TTF in differentiating lung primary from other metastatic adenocarcinomas and to differentiate it from squamous cell carcinoma.

P63[66,67]

It is a very specific and sensitive antibody for myoepithelial cells and has nuclear affinity especially for the basal layer of stratified squamous epithelium. As the cells gets differentiated its reaction / positivity begins to diminish. Its expression can also be noted in basal cells of respiratory epithelium, germinal center cells, basal cells of prostate, placental cytotrophoblasts, myoepithelial cells of breast.

Squamous cell carcinoma's of lung, cervix, head and neck and basal cell carcinoma of skin shows strong nuclear positivity for P63.

CK5/6[64,65]

CK 5/6 are intermediate sized keratins. They are mainly expressed in keratinizing (epidermis) and non-keratinizing (mucosa) squamous epithelium. They can also be seen in the basal myoepithelial cell layers of the prostate, salivary glands and breast.

This marker is expressed in benign and malignant tumors of epidermal squamous mucosal and myoepithelial origin.

They usually show diffuse cytoplasmic staining with perinuclear enhancement. They are a part of the panel used to subtype the NSCLS (ie). Adenocarcinoma and squamous cell carcinoma. These are diffusely and strongly expressed (cytoplasmic staining) in squamous cell carcinoma.

ROLE OF MOLECULAR TESTING IN LUNG CANCER

Generally, tumor cells contain many genetic abnormalities the most important ones are abnormalities of driver genes and are required for the tumor cell survival. These driver mutations play is significant role in tumor genesis, thereby analyzing these mutations help in understanding the complex molecular pathogenesis of lung cancer.[5]

In lung cancer the known driver mutations are EGFR, KRAS and ALK. These are most specifically seen in lung adenocarcinomas. EGFR & ALK mutations are usually noted in never smokers, and are seen as a peripheral lung lesion. Whereas KRAS mutation is noted in smokers with lesions in the hilar region. This relationship is attributed to the "anatomical compartment model", as already discussed in the pathogenesis earlier. In this concept it is said that "basal cell are the putative stem cells of the bronchus for central airway compartment and type II pneumocytes are putative stem cells for the terminal respiratory unit of the peripheral compartments" .[5]

EGFR

EGFR is a member of tyrosine kinase receptors, has a significant role in the regulation of cell proliferation, survival and differentiation. EGFR are over expressed in a number of solid tumors such as lung, breast, prostate, colon, bladder and few ovarian carcinomas. In NSCLC, EGFR is over expressed due to either amplification or mutation.

Since there has been much development in the therapy modalities (targeted therapy). It has become important to identify the EGFR status and is included as a protocol in the diagnosis of lung cancer (mainly adenocarcinoma). New drugs such as Cetuximab, Erbitux, Gefitinib and Ercotinibetc have been discovered which helps n the targeted therapy against lung adenocarcinoma[68,69]

The driver mutations which stimulate EGFR, activates intracellular signaling and influences the proliferation, mobilization, angiogenesis and other mechanisms of the cell affected.

In NSCLC either there is overexpression of the receptors for EGFR ligand or the mutations have targeted the intracellular domain with tyrosine kinase activity, (i.e. between exon 18-21) and this influences the prognosis, survival and response to chemotherapy.

RECEPTOR

This receptor belongs to the ErbB family which also includes other receptors such as HER2/neu, HER3, HER4 etc.

These are basically transmembrane glycoprotein which is encoded by a gene located on chromosome7. It has three parts, the extracellular domain, a hydrophobic transmembrane domain and an intracellular domain which is associated with intrinsic tyrosine kinase activity.

These receptors are basically inactive monomers, when the ligand (epidermal growth factors, EGF) attaches itself to the extracellular domain of the receptors,there is receptor dimerization. Dimerization can be homodimerization, when two similar receptors dimerize (ErbB1-ErbB1) or could be hetrodemerization, ie two different receptors(ErbB1-ErbB3). Overall, once the receptor are dimerized (activated) further it leads to auto phosphorylation which then activates the tyrosine kinase (i.e. The intracellular domain) and further as a cascade of biochemical processes goes on which ultimately leads to activities like proliferation, differentiations, apoptosis and cell migration.[69]

REGULATIONS OF ACTIVITY OF TYROSINE KINASE

The tyrosine kinase activity depends on the conformational state of the domain. Either active or inactive the ability of the kinase to transfer a phosphate group from adenosine triphosphate to a peptide substrate is what helps in regulating the signaling pathway.[69]

INTRACELLULAR SIGNALING PATHWAY

After activation of tyrosine kinase post conformational change and transfer of phosphate group to the peptide substrate. There are four intracellular pathways involved in the activation of EGFR.[69]

The pathways involved are:

1. Ras/mitogen activated protein kinase(MAPK)

2. Phosphatidylinositol 3 kinase (PI3K)

3. Phospholipase C gamma(PLCgamma)

4. STAT- signal transducer and activator of transcription.

Once Ras is activated EGFR , leads onto a series of events which ultimately causes nuclear transcription of genes and this results in proliferation, differentiation, migration, angiogenesis and anti apoptosis, and finally causes genesis of carcinoma.[69]

ASSESSMENT OF EGFR MUTATION STATUS IN LUNG ADENOCARCINOMA

There is an immense need for subtyping of NSCLC by using immunohistochemistry. New protocol of identifying the EGFR mutational status in the diagnosis of lung carcinoma as it helps in the treatment methodologies (targeted therapy). It is said that in about 20% of the lung adenocarcinoma patients, somatic mutation with in the tyrosine kinase domain are found and are the most useful predictors of response to the use of EGFR tyrosine kinase inhibitors such as Gefitinib or Erlotinib. It is also noted that patients on ERFR TKI survive longer than the ones on cytotoxic chemotherapy.

There is usually an inframe deletion in exon19 (E746_A750)and the exon 21 (L858R) substitution (point mutation) which are the most common EGFR mutations identified . They represent about 90% of all mutations. Analysis of these mutations is done by many methods which helps in the further management of the patient (targeted therapy). Generally a common mutation analysis is done .The methods employed can be molecular (ie) by direct DNA sequencing (low sensitivity) or by real time PCR, using specific probes or amplified refractory mutation system technology (ARM). Other easier techniques is the use of immunohistochemistry, EGFR mutant specific antibodies are used to identify the mutation.[70]

These methods have their even advantages and disadvantages .In case of direct sequencing of PCR amplified genome, it can detect all mutation in the region analyzed, but it has limited sensitivity if the tumor cells are of smaller fraction in the specimen obtained. Whereas in (ARMS) amplification refractory mutations system is more sensitive, but detects only few mutations, only few per reaction. And direct sequencing of DNA is more expensive and needs well equipped laboratories, reagents and technicians.

The immunohistochemical method (i.e.) with the use of specific rabbit monoclonal antibodies against the two most common EGFR mutations seemed to be more specific but lacked the sensitivity due to

inconsistency related to the methodology and interpretation. And this method is said to be cost effective too.

Generally these should be proper communication, collaboration and co-ordination between the departments involved in the management of lung cancer. This process involves clinicians, radiologists, pathologists and molecular biologists. The decision to test for EGFR mutation should be that of the treating physician at the time of diagnosis. And for the testing procedure sufficient biopsy material should be obtained and should be submitted for histopathological analysis and for molecular analysis. All patients with NSCLC should be tested, but it's a must for those histopathologically diagnosed as adenocarcinoma and/or for patients with negative history for smoking to be tested for EGFR as the frequency of mutation are high in such patients.[70]

CHARACTERISTICS OF EGFR MUTATION IN LUNG ADENOCARCINOMA.

EGFR mutation is seen in the kinase domain of the receptor tyrosine kinase, which leads to constitutive activation for downstream signaling The two most common mutations are: Inframe deletions in exon 19 seen in 90%of the cases and point mutation in exon 21 (L858R).

EGFR mutation are highly specific for lung adenocarcinoma, frequently detected in cases with lepidic and papillary growth and is

associated with TTF1 positivity ,seen in nonsmokers and in males predominantly.

This mutation in lung adenocarcinoma shows ethnic differences with a prevalence in Caucasians (10-15%) and in Asians(30-40%).EGFR mutation acts as a prognostic factor as well as a predictive factor, i.e. the patient's response to tyrosine kinase inhibitors.[5]

NOTE ON THE EGFR MUTATION STATUS IN SQUAMOUS CELL CARCINOMA

It is also important to note that EGFR mutation can also be present squamous cell carcinoma's of lung. Few studies have thrown light onto the incidence and clinical significance of EGFR mutations in squamous cell carcinoma of lung. The results of their study showed that EGFR mutation in patients with squamous cell carcinoma had better outcome once detected and on TKI therapy, but they have also said that the response is anytime inferior to the outcomes seen in EGFR positive adenocarcinoma treated with TKI.

To mention, in one another study 639 patients with squamous cell carcinoma were tested for EGFR mutation, out of which 29 were positive (4.5%) for the mutation. The authors concluded that, many of these patients respond to TKI but is comparison to adenocarcinoma their response is somewhat lower.

In lung squamous cell carcinoma, the role of EGFR testing becomes controversial. However, EGFR mutations are rarely found in squamous cell carcinoma and more importantly it is not a predictive marker for the use of TKI as in adenocarcinoma.

Therefore, EGFR mutation testing is mandatory for lung adenocarcinoma and not in lung squamous cell carcinoma.[71]

MATERIALS AND METHODS

Lung biopsies which were received in the histopathology department from the year 2013 to 2015 were retrieved from the archives of department of pathology, PSGIMSR.

Post the institutional ethical clearance cases reported as adenocarcinoma, squamous cell carcinoma and NSCLC were obtained.

Samples for the study were selected according to the following criteria: All H & E diagnosed adenocarcinomas , squamous cell carcinomas and NSCLC. The total number of cases finalized were 50 (which was our sample size) and obtained from the departmental archives both retrospectively and prospectively from the year 2013 (June) to 2015(June) .

Simultaneously, patient's clinical data (i.e. age, sex, smoking status, clinical presentation and radiological presentation) and other relevant data were also collected and recorded.

Exclusion criteria : Other biopsies which were reported as tuberculosis, bronchiectasis, pneumonia ,few other non specific condition, small cell carcinoma, neuroendocrine carcinoma and biopsies with scattered atypical cells or inadequate material for diagnosis or any other metastatic malignancies ,cases with previous history of chemotherapy or radiotherapy were all excluded from the study.

All the H&E slides were reviewed again and a tentative morphological diagnosis was made and recorded.

Fifty tissue blocks were cut into 4mm thick sections, these sections were taken on a poly-l-lysine coated slides for further immunohistochemistry staining.

IMMUNOHISTOCHEMISTRY: [72]

STEP 1: Post H&E examination for adequacy and morphology, the cases were finalized. Total of 50 cases were selected. Five sections were cut at approximately 4 micron thickness, were taken onto a poly-L-lysine coated slides. These slides are incubated at 37^{o}c for one day and further incubated at 58^{o} c for over night Once the sections are ready the antibodies can be used.

The antibodies used were:

Antibody	Source	Clonality	Clone	Species	Dilution	Pretreatment
TTF-I	Pathinsitu	Monoclonal	EP229	Rabbit	Pre-diluted	None (Normal IHC procedure)
Napsin	Pathinsitu	Monoclonal	EP229	Rabbit	Pre-diluted	None
P63	Pathinsitu	Monoclonal	4A4	Mouse	Pre-diluted	(Normal IHC procedure)
CK 5/6	Dako	Monoclonal	D5/16 B4		Pre-diluted	None (Normal IHC procedure)

STEP 2:

Deparaffinization - Xylene 1 – 15min, Xylene 2 - 15min

STEP 3:

Dexylenisation - Absolute alcohol 1 – 1min, Absolute alcohol 2 – 1 min

STEP 4:

Dealcoholisation - 90% Alcohol – 1min, 70% Alcohol – 1min

STEP 5:

Rehydration - Tap water - 10min

STEP 6:

Rinsing - Distilled water – 5 min

STEP 7:

Antigen retrieval (heat induced) – pressure in EDTA buffer (pH-9)-10min

Leave the pressure cooker in the sink with running tap water – 20min (to let it cool at room temperature.

STEP 8:

Rising - Distilled water – 5min

STEP 9:

Washing - Trisodium (TBS), Phosphate buffer -5 min x 2times (pH – 7.6)

STEP 10:

Blocking of endogenous peroxidase enzyme – peroxidase block – 10-15min

STEP 11:

Washing – TBS buffer -5min x 3 times

STEP 12:

To block nonspecific reaction with other tissue antigen – power block – 15min

STEP 13:

Antigen-antibody reaction - drain and cover the sections with markers – 1hr

Primary antibody - covered primary antibody TTF-I, Napsin, P63, CK 5/6 onto the respective sections.

STEP 14:

Washing – TBS buffer (To wash unfound antibodies) – 5 min x 3times

STEP 15:

Super enhancer – to enhance the reaction - 3omin

STEP 16:

Washing - TBS buffer (To wash the unbound antibodies)- 5 min x 3times

STEP 17:

Super sensitive ply-HRP - done to label the enzyme and to elongate the chain - 30min

STEP 18:

Washing – TBS buffer (to wash the unfound antibodies) – 5min x 3 times

STEP 19:

Chromogen (color development with working color development solution – to give color to the antigen – 5-8min

STEP 20:

Washing (i) TBS buffers (to wash) -5 min x 3 times

Washing (ii) Tap water - 5min

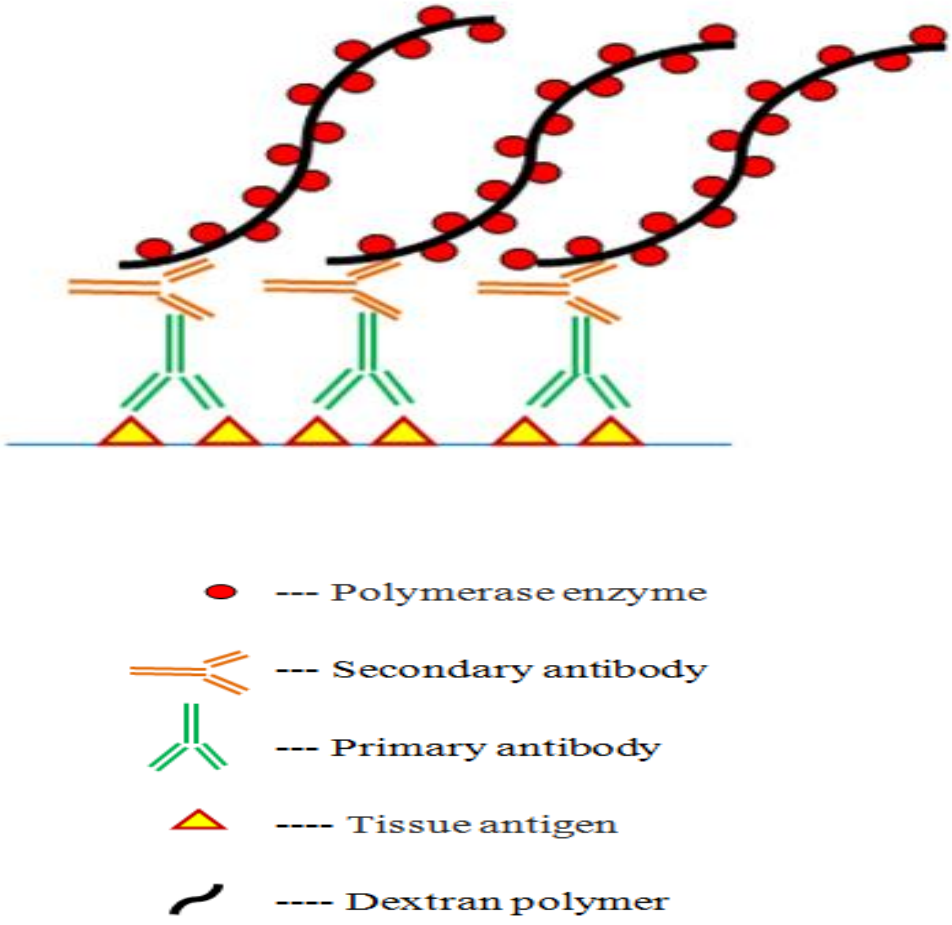
STEP 21:

Counter stain Hematoxylin (Haris Hematoxylin) - 1min

STEP 22:

Washing - tap water (to remove the excess stain) - 5 min

Once all steps are done, the slides should be air dried, followed by xylene (cleaning) and mount with DPX.



RESULTS:

- Tumor cells take up brown color
- There could be nuclear and cytoplasmic expression.
- TTF1 and P63 show nuclear expression
- CK 5/6 and Napsin show cytoplasmic expression.

Once the slides were ready, they were reviewed in the following methods.

- They (TTF-I, Napsin, CK5/6, P63) were all viewed under "NIKON Eclipse E-200" microscope.

IMMUNOHISTOCHEMISTRY SCORING:[74,75]

Intensity score		**Proportion score**	
Score		**Score**	
0	None	0	None
1+	Weak presence of tumor cells	1	1% to 10%
2+	Moderate presence of tumor cells	2	11% to 30%
3+	Strong presence of tumor cells	3	31% to 50%
		4	51% to 70%
		5	>70%

- Final tabulation was prepared along with all the findings.
- The representative photographs were taken in the LEICA microscope with the use of LEICA application suite.

One the H&E diagnosis of NSCLC were subtype with the help of IHC markers (TTFI, Napsin, CK5/6, P63) into adenocarcinoma and squamous cells carcinoma, the next step of analysis of EGFR mutation status was taken up. All adenocarcinoma cases were selected and EGFR was runned 21 adenocarcinoma cases of the 28 cases.*(Out of 28 adenocarcinoma cases only 21 blocks were available. So cases which had no block for sections were excluded.)*

The same steps were followed (step -1 to 22) as those of the other few markers. The primary antibody used here was EGFR.

Antibody	**Source**	**Clonality**	**Clone**	**Species**	**Dilution**	**Pretreatment**
EGFR	Pathinsitu	Monoclonal	EP229	Rabbit	Pre-diluted	None (Normal IHC procedure)

RESULTS:

Note: The antibody EGFR, help in analysis of mutation in common, it could be either the exon19 mutation (E746-A750del) or the exon21 mutations (L858 R point mutation). It does not specifically identify any of the above

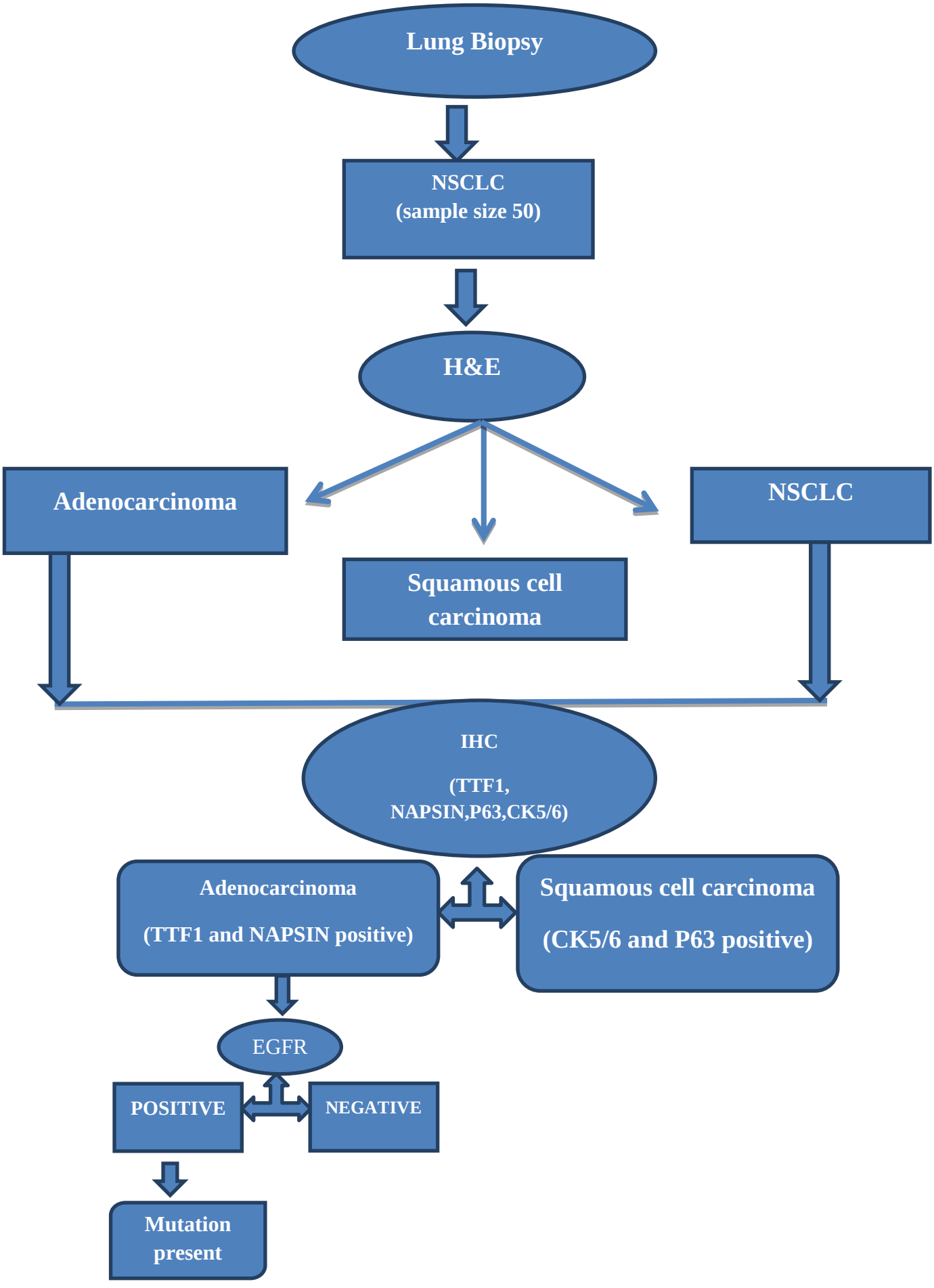
mentioned mutation individually (for such individual identification if required molecular techniques can be adapted)

The slide were scanned (in a NIKON eclipse E200 microscope) and scored based on cytoplasmic or nuclear staining.[81]

0	-	No staining or faint staining intensity > 10% of tumor cells
1+	-	faint staining > 10% of tumor cells.
2+	-	Moderate staining
3+	-	Strong staining

A case was considered positive if at least 1 core showed 2+ or 3+ staining or if 2 cores showed 1+ staining. If all the cores showed 1+ or if the 3 cores showed 1+ it was considered negative.

Once the slides was evaluated and scored, considered positive or negative, they were tabulated.


Lung Biopsy
NSCLC
(sample size 50)
H&E
Adenocarcinoma
Squamous cell carcinoma
NSCLC
IHC
(TTF1, NAPSIN,P63,CK5/6)
Adenocarcinoma
(TTF1 and NAPSIN positive)
Squamous cell carcinoma
(CK5/6 and P63 positive)
EGFR
POSITIVE
NEGATIVE
Mutation present

RESULTS & OBSERVATIONS

HISTOPATHOLOGICAL DIAGNOSIS (H&E):

Fifty blocks of NSCLC diagnosed cases were taken from the archives of the department of pathology. The exclusion being cases diagnosed as small cell lung carcinoma/neuroendocrine carcinoma/large cell carcinoma & others.

The first sections from these blocks were stained with H&E and a diagnosis was made based on the morphology. These cases were earlier confirmed by two pathologists. Their diagnoses were re-confirmed and the H&E slides were reviewed once again after the commencement of the study.

Based the H&E slides a diagnosis was made. Majority of the cases were classified into adenocarcinoma and squamous cell carcinoma excepting a few cases which lacked any convincing morphology to subtype it on H&E, such cases were labeled as NSCLC.

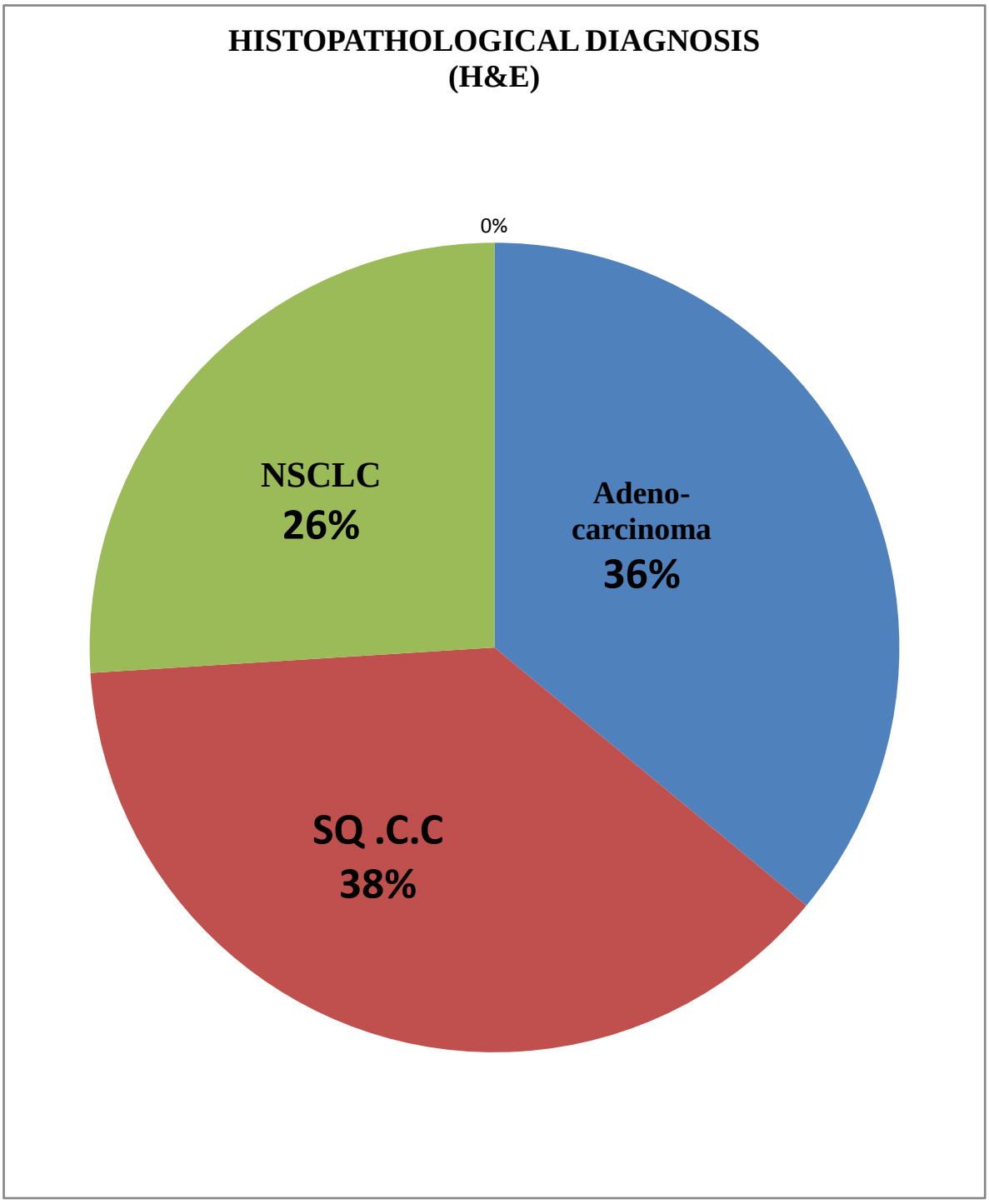


FIGURE 1-DEPICTS THE NUMBER OF CASES DERIVED AFTER MORPHOLOGICAL DIAGNOSIS (H & E)

ADENOCARCINOMA CASES : (N =18)

There were 18 number of cases diagnosed as adenocarcinoma on H& E(morphology based) which also correlated with the diagnosis made by the other two pathologists at the time of reporting. There were 10 males and 8 females between the ages of 40years and 80 years. The clinical features consisted mainly of cough the other symptoms were dyspnea, weight loss, loss of appetite etc. Most of the cases were clinically diagnosed as? Malignancy but in some cases the diagnosis of? COPD/emphysema had been offered. The CT scan findings included generally showed lesions in the lung, majorities were peripheral lesions and others were central (hilar, perihilar). Few cases had no CT findings.

Microscopically, almost all the cases were moderately differentiated with the tumor showing gland formation.*(fig 2-shows the H & E section of a typical glandular pattern of adenocarcinoma)* Among which a few cases showed intracytoplasmic mucin (fig 4- shows the mucinous variant of adenocarcinoma). Few had infiltrating lymphocyte, some showed necrosis and few had just a few scattered atypical cells which were considered inadequate.

SQUAMOUS CELL CARCINOMA: (N=19)

There were 19 number of cases diagnosed as squamous cell carcinoma on H&E(morphology based) which also correlated with the diagnosis made by the other two pathologists at the time of reporting. There

were 18 males and 1 female between the ages of 40 years and 80 years. The clinical features were similar to that of adenocarcinoma and consisted of cough, dyspnea etc. The CT scan findings included mass/lesion in the lung(few were peripheral and few were central).Microscopically, the cases were well to moderately differentiated with about 50 to 60% of the cases showing keratinization or intercellular bridges or keratin pearls etc.*(fig 18- illustrates the H & E section of SQ.C.C with individual cell keratinization and few other features of SQ.C .C)*

CASES DIAGNOSED AS NSCLC (N=13)

Cases which were difficult to be classified as either adenocarcinoma or squamous cell carcinoma were put under this category NSCLC. Overall about 13 such cases were identified of which 11 cases were males and 2 were females and were seen between the age group of 40 to 80 years. Microscopically, there was no evidence of gland formation, mucin, keratinization or intercellular bridges etc. There were just malignant cell which could not be subtyped on H&E.*(fig 4- depicts the morphologically unclassifiable NSCLC).*

In this study, we have retrospectively studied 4 most commonly used IHC markers, including TTF-1,NAPSIN,CK5/6,P63 in the subclassication of NSCLC (n-13) cases and reconfirmed the H & E diagnosis for the earlier diagnosed adenocarcinomas(n-18) and squamous cell carcinomas (n-19) in small biopsy samples.

TABLE 1- ILLUSTRATES THE CORRELATION BETWEEN MORPHOLOGICAL AND IMMUNOHISTOCHEMICAL DIAGNOSIS:

	HP DIAGNOSIS	**IHC DIAGNOSIS**	**% OF CORRELATION**
ADENOCARCINOMA	**18**	**18**	**100%** (ALL CASES MATCHED)
SQUAMOUS CELL CARCINOMA	**19**	**18**	**94%** (ONE CASE DID NOT MATCH,TURNED OUT TO BE ADENOCARCINOMA)
NSCLC	**13**	**ADENO: 9 SQ.C.C: 4**	**-**

Morphologically (H & E) diagnosed adenocarcinoma's show 100% correlation with the respective panel of markers (IHC), whereas squamous cell carcinoma's show 94% correlation. Of the unclassified NSCLC's we derived 9 adenocarcinomas and 4 squamous cell carcinoma's cases post IHC.

TABLE 2 – IMMUNOHISTOCHEMICAL SUBTYPING OF MORPHOLOGICALLY DIAGNOSED NSCLC(n-13)

ADENOCARCINOMA ***(n:9)***	**NUMBER OF CASES**	**PERCENTAGE**
TTF-1 AND NAPSIN POSITIVE	**5**	**60%**
ONLY TTF-1 POSITIVE	**2**	**20%**
ONLY NAPSIN POSITIVE	**1**	**10%**
TTF-1 AND MILD P63 POSITIVE	**1**	**10%**
SQUAMOUS CELL CARCINOMA ***(n:4)***		
CK5/6 AND P63	**ALL 4 cases were CK5/6 AND P63 POSITIVE**	**100%**

Of the 13 morphologically diagnosed NSCLC'S we obtained 9 adenocarcinomas which showed 60% TTF-1 and NAPSIN positivity, 20% of only TTF-1 positivity,10% of only NAPSIN and another 10% of strong TTF-1 with weak P63 positivity. Similarly for squamous cell carcinoma's all 4 cases were CK5/6 and P63 positive.

TABLE 3- SHOWS THE SCORING PATTERN IN IHC DERIVED ADEOCARCINOMAS

N=28		INTENSITY SCORE				PROPORTION SCORE					
	NUMBER OF CASES	0 (NONE)	1 (WEAK)	2 (MODERATE)	3 (STRONG)	0 (0%)	1 (1-10%)	2 (11-30%)	3 (31-50%)	4 (51-70%)	5 (>70%)
TTF-1	24 (86%)	3 (11%)	0	5 (18%)	20 (71%)	3 (11%)	0	0	5 (18%)	5 (18%)	15 (54%)
Napsin	25 (89%)	3 (11%)	1 (4%)	2 (7%)	22 (79%)	3 (11%)	0	2 (7%)	2 (7%)	5 (18%)	16 (57%)
Ck5/6	0	0	0	0	0	0	0	0	0	0	0
P63	3 (11%)	0	1 (4%)	2 (7%)	0	0	3 (11%)	0	0	0	0

- In the diagnosed adenocarcinoma cases(n-28),71% of the cases showed positivity for both TTF-1 and NAPSIN 11% showed only TTF-1 positivity, 11% showed only Napsin positivity and 7% had weak P63 positivity along with the dual marker.*(fig 23-25 denotes the focal p63 positivity in adenocarcinomas).*

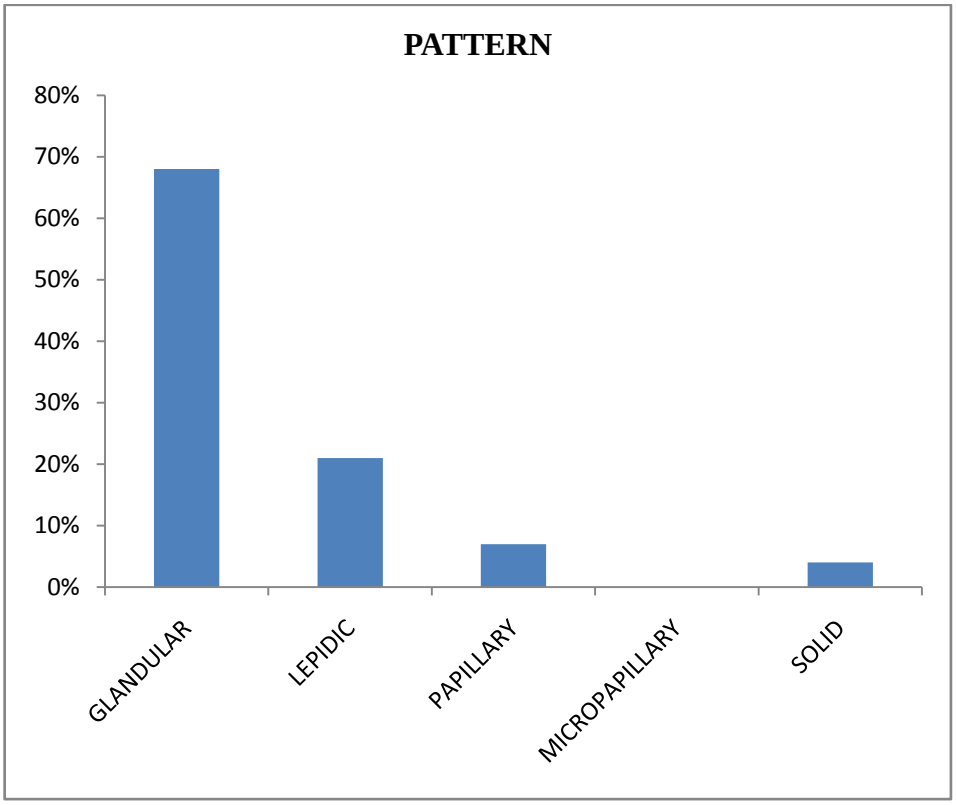
- Majority of the TTF-1 positive cases(71%) showed an intensity score of 3+(strong) and 54% of the cases (fig 5 to 8 shows the intensity score for TTF-1 & NAPSIN)showed a proportion score of 5(>70% of tumor cells had taken up the stain) similarly NAPSIN also showed strong 3+ positivity in 79% of the cases and a proportion score of 5 in 57% of the cases.

TABLE 4 – CLINICAL AND IMAGING CHARACTERISTICS IN IHC PROVEN ADENOCARCINOMA'S

Adenoca	**AGE (years)**					**SEX**		**SMOKING STATUS**		**RADIOLOGICAL LOCATION**					
N=28										**SIDE**			**SITE**		
	30-40	**41-50**	**51-60**	**61-70**	**> 70**	**M**	**F**	**Smoker**	**Non smoker**	**Right**	**Left**	**Not known**	**Peripheral**	**Central**	**Not known**
Number of cases	2	6	8	8	4	18	10	11	17	18	4	6	15	8	5
Percentage (%)	7	21	29	29	14	64	36	39	61	64	14	21	54	2	18

In this study, adenocarcinomas were noted in elderly age groups (51-70years) of age. Cases showed a male preponderance and were noted to be more in non-smokers. On imaging studies the tumor affected the right lung more and the lesions were located peripherally.

FIGURE 2 – HISTOPATHOLOGICAL PATTERN ANALYSIS IN IHC PROVEN ADENOCARCINOMA'S (n-28)



The predominant pattern noted in the study was glandular about 70% of the cases showed this pattern. The other patterns seen were lepidic (21%),papillary(7%) and (4%) of solid pattern.*(fig 1-4 shows the various patterns noted in the adenocarcinoma cases of our study)*

TABLE 5- SHOWS THE SCORING PATTERN IN IHC DERIVED SQUAMOUS CELL CARCINOMAS

N=22		INTENSITY SCORE				PROPORTION SCORE					
	NUMBER OF CASES	0 (NONE)	1 (WEAK)	2 (MODERATE)	3 (STRONG)	0 (0%)	1 (1-10%)	2 (11-30%)	3 (31-50%)	4 (51-70%)	5 (>70%)
Ck5/6	21 (95%)	2 (9%)	1 (5%)	0	19 (86%)	2 (9%)	4 (18%)	0	0	2 (9%)	14 (64%)
P63	22 (100%)	0	0	5 (23%)	17 (77%)	0	1 (5%)	0	1 (5%)	4 (18%)	16 (73%)
TTF-1	1 (5%)	0	0	1 (5%)	0	0	1 (5%)	0	0	0	0
Napsin	0	0	0	0	0	0	0	0	0	0	0

- In the diagnosed squamous cell carcinoma cases(n-22),90% of the cases showed positivity for both CK5/6 andP63%.5% showed only p63 positivity, 5% showed weak TTF-1 positivity with the other two squamous cell markers.
- Majority of the CK 5/6 positive cases (86%) showed an intensity score of 3+(strong) *(fig 9 to 12 shows the intensity score for P63& CK5/6)*and 64% of the cases proportion score of 5(>70% of tumor cells had taken up the stain) similarly P63 also showed strong 3+ positivity in 77% of the cases and a proportion score of 5 in 73% of the cases.

TABLE 6 – CLINICAL AND IMAGING CHARACTERISTICS IN IHC PROVEN ADENOCARCINOMA'S

Sq c c	AGE (years)					SEX		SMOKING STATUS		RADIOLOGICAL LOCATION					
N=22										SIDE			SITE		
	30-40	41-50	51-60	61-70	>70	M	F	Smoker	Non smoker	Right	Left	Not known	Peripheral	Central	Not known
Number of cases	0	3	5	9	5	20	2	17	5	12	5	5	11	7	4
Percentage (%)	0	14	23	41	23	91	9	77	23	55	23	23	39	32	14

In this study, squamous cell carcinomas were noted in elderly age groups (51-70years) of age. Cases showed a male preponderance and were noted to be more in smokers. On imaging studies the tumor affected the right lung more and they were seen as both peripheral and central lesions.

EGFR

With the advent of new therapy modalities(targeted therapy), identification of the mutational status has become significant. In this study our interest lies in the identification of EGFR mutational status in the already diagnosed adenocarcinoma patients. Though various methods are available the method used here is “immunohistochemistry”.

Post immunohistochemistry, 28 cases adenocarcinoma cases were identified. Out of these cases only 21 blocks were available.

TABLE 7 – CASES SELECTED FOR EGFR MUTATIONAL STUDY

TOTAL NUMBER OF (IHC) ADENOCARCINOMAS	**28**
BLOCKS AVAILABLE	**21**
NUMBER OF BLOCKS NOT AVAILABLE	**7**

TABLE 8 – DEPICTS THE EGFR STAINING PATTERN

SCORE	NUMBER OF CASES	PERCENTAGE
0	1	5%
1+	3	14%
2+	3	14%
3+	14	67%

NOTE: 2+ AND 3+ IS CONSIDERED POSITIVE FOR EGFR MUTATION.

INTERPRETATION: 17 EGFR POSITIVE CASES.

81 % OF THE ADENOCARCINOMA

POSITIVE CASES SHOWS EGFR MUTATION.

TABLE 9 – ILLUSTRATES THE BASELINE CLINICAL CHARACTERISTICS OF PATIENTS WITH "EGFR MUTATION"

BASELINE PATIENTS CHARACTERICTICS	NUMBER OF CASES	PERCENTAGE
SEX		
Males	9	53%
Females	8	47%
AGE(years)		
40-50	3	18%
51-60	6	35%
61-70	6	35%
>70	2	12%
SMOKING STATUS		
Smokers	7	41%
Non smokers	10	59%
RADIOLOGICAL LOCATION		
Peripheral	12	70%
Central	3	18%
Not known	2	12%
PATTERN		
Glandular	12	70%
Lepidic	2	12%
Papillary	2	12%
Micropapillary	0	0
Solid	1	6%

EGFR mutation positivity is seen predominantly in males(53%),it is seen within an age group of 51 years to 70 years. Nonsmokers seem to harbor the mutation. The predominant pattern observed in the EGFR mutated adenocarcinomas is the glandular pattern.

DISCUSSION

Lung cancers are usually and broadly divided to non small cell lung carcinoma and small cell carcinoma. In the present 2015 WHO classification they have mentioned the subtyping of NSCLC to be very essential, due to the new treatment modalities which have come up off late. Similarly the importance of the use of IHC in the subtyping of NSCLC has also been highlighted. Reporting of small biopsy specimens, EGFR and other mutations have also been given importance. The identification of the mutation within any NSCLC has more or less become mandatory as this helps in the therapy of lung carcinoma patients.[5]

Usually most non small cell lung carcinoma's can be subtyped as adenocarcinoma and squamous cell carcinoma on biopsies without the use of any additional special stain or immunohistochemistry. Moreover, the 2011 WHO classification is based only as the H&E morphology. Difficulty in subtyping usually never arises in biopsies as far as if it is inadequate due to poor sampling or due to the presence of only small amounts of tumor or in any poorly differentiated tumor. Many studies have examined resection specimen's, tissue micro arrays or a mixture of specimen types, but we focused on biopsy specimen's as most lung cancers are unresectable at diagnosis, and the only tissue available for diagnosis is through biopsy for a large percentage of cases.

In this study, we have identified 50 NSCLC majority on small biopsies (TBLB),endoscopy assisted biopsy(EBLB) and few CT guided biopsy. These cases were subtyped both morphologically (i.e. H&E) and immunohistochemically.

Apart from the subtyping with the help IHC markers (TTF-I, Napsin, CK5/6, P63),this study also includes the identification of the mutation status of EGFR in all IHC proven adenocarcinoma's with the help of monoclonal antibodies (IHC). Similarly, the patient's baseline characteristics of EGFR positive adenocarcinomas were compared, correlated and studied.

Of the 50 cases, 18 cases (36%) were adenocarcinomas,19 cases(38%) were squamous cell carcinomas these cases were readily diagnosed only on H&E (morphology based). But in 13 cases(26%) the sub classification of NSCLC by morphology alone was difficult. Due to its poor differentiation, scanty malignant cells and inadequate sampling. These cases (13 cases) were broadly classified as NSCLC on H&E.

Well to moderately differentiated adenocarcinoma's were identified based on its pattern i.e. either glandular or lepidic or solid or papillary etc. The cytology predominantly showed cells with moderate amount of cytoplasm with round to oval vesicular to hyperchromatic nuclei and conspicuous nucleoli. Few cases evidently showed mucin production, because of which in this study we did not perform mucin stain. [73]

Similarly, Well to moderately differentiated squamous cell carcinoma were identified based their keratinization, pattern of arrangement mostly in sheets and nests. The cells are generally polygonal with abundant eosinophilic cytoplasm, vesicular nuclei and prominent nucleoli. Keratinization is noted in the form of individual cell keratinization, keratin pearls and intercellular bridges. [73]

To subtype the H&E diagnosed NSCLC (13 cases 26%) and to further reconfirm H&E diagnosed adenocarcinoma's(18cases,36%) and squamous cell carcinoma's (19cases, 38%) a panel of IHC markers were used.

The panel composed of four markers, two to diagnosis adenocarcinoma (TTF-I & Napsin) and two to diagnose squamous cell carcinoma (CK5/6 & P63).

CORRELATION BETWEEN MORPHOLOGICAL DIAGNOSIS AND IMMUNOHISTOCHEMICAL DIAGNOSIS:

After applying the panel of four markers to all 50 cases, i.e. TTF-I, Napsin, CK5/6 and P63, the initial 18 cases of H&E diagnosed adenocarcinomas still remained adenocarcinoma with TTF-I and Napsin positivity. This showed 100% correlation. Similarly, the initial 19 cases which was diagnosed as squamous cell carcinoma on H&E, showed 18 cases as squamous cell carcinoma whereas one turned out to be adenocarcinoma. The 18cases which correlated with H&E diagnosis showed CK5/6 & P63

positivity. For squamous cell carcinoma the percentage of correlation between H&E (morphology) and IHC was 94 %.*(Table 1)*

In our study, the 13 cases of unclassifiable NSCLC on H&E were subtyped into 9 adenocarcinomas and 4 squamous cell carcinomas with the help of immunohistochemistry.

So finally, we derived 28 IHC proved adenocarcinomas and 22 squamous cell carcinomas.

ADENOCARCINOMA:(n:28)

CLINICAL FEATURES:

Adenocarcinoma was the commonest type of primary lung carcinoma. The mean age of patients was 60.5 (range 51 to 70years) years. About 29% of the cases were between the above mentioned age group. Only about 7% of cases were below 40Years of age, the youngest being 40years.Most western studies have constantly proved a low incidence of lung cancer in young individuals (< 40yrs). One study correlates with the above study are we got only 7% (2 cases) below the age of 40years.[77] The mean age of adenocarcinoma in the present study was 60.5years. Which shows that carcinoma lung in specific adenocarcinoma is a disease of old age (51-70years). In the study of Wager et al, the age ranged between 37 to 82 years. Which is in match with one study. This average age is also comparable to various Indian studied 64% (18cases) were males and 36%

(10cases) were females in our study. The age group was mixed among the males and females, no particular range of age was noted in male or female. Similarly, the sex ratio reported in various Indian studies ranged from 4.5:1 to 8.2: 4. A study from USA showed male: female ratios were 5:1. The sex ratio in our study was 1.8:1 which means there is a male preponderance, and the entity matches with that of the above mentioned studies. This can be explained by the history of smoking which is more in the males that females in our Indian set up comparison to the west. Among the adenocarcinoma cases (28cases), 61% (17cases) were nonsmokers and 39% (11 cases) were smokers. Then non-smokers (17cases, 61%) ,(10 cases) 59% were females. The rest 2% (7 cases) were male non-smokers and all smokers i.e. (11 cases) 39% were all males. The overall smoker: Non-smokers ratio is 0.64:1.Our study showed 39% were male smokers and only 2% were nonsmokers. This again is comparable to the WHO statistics which states that smoking (tobacco use) in the major etiology for cancer deaths. But in our study, in all the adenocarcinoma proven cases, the number of non-smokers were more which again correlates with the WHO 2015 studies in which they have mentioned that adenocarcinoma's arise in non-smokers [(5)]*(Table 4)*

A mass was the most common radiological findings (in all cases diagnosed as adenocarcinoma) which accounts to 100%. Majority, i.e. (18 cases) 64% of the cases had involvement of the right side. The upper zone

was predominantly involved. And (15 cases) 54% of the cases presented as peripheral lesion on CT scan.

Coming to the clinical features, the main symptoms or complaint the patient came up with was cough, then breathlessness and weight loss. Various other studies also had cough to be the major symptom at the time of presentation which is similar to this study, in which almost all 90-99% of the adenocarcinoma's process cases presented with cough[78,80]. Radiographic evaluation of patients with adenocarcinoma in various published studies showed a preponderance of right lung involvement with upper lobe being affected the most. Similarly, in this study 64% of adenocarcinoma has affected the right upper lobe. A study by Rawat et al proved that most of the adenocarcinoma's present as peripheral lesion which has been followed even in our study, where 54% of the adenocarcinoma cases were peripheral lesions. This also follows the WHO 2015 data about the radiological correlation of lung adenocarcinoma. [5]*(Table 4)*

In this study all the 28 cases were pure tumor with one evident pattern. Among the architectural patterns acinar/glandular was the most common in our study, 68% of cases (19cases) show this pattern. Followed by lepidic 21% (6 cases), then papillary 4% (2 cases) and we had just one case with solid pattern (4%) and no case with micropapillary pattern. One of study, they have also noted the association of the pattern and other factors like tumor necrosis, smoking status and age. They noted that tumors with

solid pattern were associated with more tumor necrosis, was more common in younger patient's and they were all current smokers. Whereas tumor with other patterns has fewer tumors necrosis was noted in older patients and new smokers. So with the help of identifying the pattern, an approximate prognosis can also be thought off, solid pattern, poorer prognosis, and other non-solid pattern better prognosis. [79]

MORPHOLOGY AND IMMUNOHISTOCHEMISTRY:

For adenocarcinoma, only cells which showed nuclear expression of TTF-I and cytoplasmic expression for Napsin were considered positive. This positivity was semi quantitatively evaluated by using a combination of proportion scoring and intensity scoring. The proportion score indicated the percentage of tumor cells which took up the marker, this was evaluates 0-0% cells, 1-1% to 10%; 2-11 to 30%, 3-31% to 50%, 4-51% to 70% whereas the intensity score assessed the intensity of staining and was graded into four semi quantitative categories, i.e. 0-None, 1+- weak, 2+- moderate and 3+ as strong intensity. [74,75]

Few studies have used AB/PAS stain to detect Mucin in cases of adenocarcinoma Mucin was readily detected on a few well to moderately differentiated H&E sections because of which mucin stains were not used in our study.

Of the overall 28 adenocarcinoma cases, 24 cases showed TTF-I positivity (86%). 25 cases showed Napsin positivity (89%), 3 TTF-1and NAPSIN positive cases showed P63 positivity (focal and weak positivity) (11%).whereas staining for TTF-1 and Napsin was diffuse.

Out of these above mentioned data in the 28 cases, 20cases (71%) showed strong (proportion score – 5, intensity score -3) for dual markers (TTF-I and Napsin). 3 cases (11%) showed strong TTF-I positivity only(TTF-1 positive adenocarcinoma). Another 3 cases (11%) showed moderate positivity for Napsin only(NAPSIN A positive adenocarcinoma) . These two categories have been considered as adenocarcinoma with only TTF-I or only Napsin positivity. We also had 2 cases (7%) which showed weak (proportion score 2 and intensity score of 1+) for P63 along with strong TTF-I positivity. These 2 cases have also been considered adenocarcinoma with weak P63 activity.*(Table 3)*

According to our study, TTF-I & Napsin are specific markers for adenocarcinoma. Through they have their over sensitivity and specificity. Other studies done by Sanjay Mukhopadhyay and Anna-Luise A. Katzenstein[(76)] in the Indian population similar to ours have suggested TTF-I to as a more specific markers contrary to our study which showed the percentage of Napsin positivity to be more (89%) than TTF-I (86%). Napsin A is considered a promising marker and is highly useful in differentiating primary lung carcinoma from squamous cell carcinoma.

Other studies have shown low sensitivity for napsin A(33% TO 69%) but our study has shown (89%) positivity. In this study 11% cases have shown P63 positivity which correlates with the other study mentioned above which states that P63 although sensitive for squamous cell carcinoma is not entirely specific as it can also be expressed in adenocarcinomas. There was 0% positivity for the marker CK 5/6.

Majority of the adenocarcinoma cases (dual markers) had its intensity of 3+ (20cases, around 71%) and (15 cases,54%) showed a proportion score of 4 (51 to 20%), rest were around 2 & 3+ (around 11-50%).

It is always better to have two markers for each subtype in a panel. Because in case of adenocarcinoma, inclusion of Napsin A may be helpful when TTF-I staining is equivocal, especially because it is a cytoplasmic markers than nuclear stain. And useful in rare cases of TTF-I negative but Napsin positive pulmonary adenocarcinoma. To prove the above statement, even in our study, 3 cases, were only Napsin positive (strong) after which they were diagnosed as adenocarcinoma. If only one marker was used it would have been difficult to diagnose such cases.

SQUAMOUS CELL CARCINOMA :(n:22)

CLINICAL FEATURES:

The age, gender and smoking history of the IHC proven squamous cell carcinoma's were similar to adenocarcinomas. These tumors few also

noted in the older age groups. In this study about 41% (9cases / 22 cases) of the cases were between the age group of 61years to 70years with a median age of 65.5 years. This again, like adenocarcinoma correlates with the Wagner et al study which showed that carcinoma lung is a disease of the oldage. We hardly had any case of squamous cell carcinoma below 40years (0 cases).[79,80]

Just like adenocarcinoma, even in for squamous cell carcinoma, our study showed a male preponderance. We got 91% of males (i.e. 20 cases) and remaining 9% (2 cases) were females. The male: female ratio is 10:1, which process the male majority. The dominance of males with squamous cell carcinoma can again be attributed to the smoking habits in more than women in an Indian set up. The sex and age group showed no correlation. In our study, 77% (17cases) well smokers and 23% (3 cases) were nonsmokers. All the smokers were males i.e. 100% of smokers in our study were all males and only 2 males were non-smokers. This correlation between smoking and development of squamous cell carcinoma has already been noted in various other studies WHO. And smokers being predominantly males could be explained due to the low incidence of female smokers in India as compared to the west. [80]*(Table 6)*

Like other studies from India and abovecough was the common symptom is about 90% of the patients in our study followed by breathlessness, weight loss, hemoptysis etc.

Radiographic analysis revealed the right lung being affected more than the left (may be due to its anatomically make up, i.e. the right bronchi being shorter, wider and more vertical) and it correlates with various other studies. In this study (12 cases) 56% of the lesions were in the right lung. Our study correlates with various other studies which have also proved the right lung being involved more.[78,79]

In contrast to studies majority of our squamous cell carcinomas cases were peripheral lesions. Few studies have also mentioned that squamous cell carcinoma's are central lesions. Studies done in the mayo clinic proved 53% of the squamous cell carcinoma to be central lesions various Indian studies by Gupta et al and Sharma et al have also proved about 94.9% and 83.6% of squamous cell carcinoma presented as central lesions. But in this study, we found 50% of the cases to be peripheral lesions, 32% (7cases) to be central lesions and for about four cases we could not retrieve the radiological findings from the PSG IMSR archives.

MORPHOLOGY AND IMMUNOHISTOCHEMISTRY:

For squamous cell carcinoma of the 22 cases, all 22 showed P63 positivity (i.e. 100%). 21 cases (95%) showed CK5\6 positivity and 1 case showed weak TTF-I positivity. From this data, P63 seems to be more specific for the diagnosis, but as mentioned above P63 can also can also be expressed in adenocarcinoma 11% of our cases have also expressed P63. 20

cases (around 91%) showed strong dual marker positivity, whereas 1 case (5%) show only P63 positivity(P63 positive squamous cell carcinoma) 1 another case showed dual markers positivity (CK5/6 and P63) and in addition showed weak TTF-I positivity. Study by Sanjay Mukhopadhyay and Anna-Luise A. Katzenstein[76] also showed few cases(3% to 21%) of squamous cell carcinoma with weak TTF 1 positivity but they attributed it to either the entrapped lung epithelium which has taken up TTF-1 within the squamous cell carcinoma. Some studies have considered weak staining or <10% staining as positive. P63 in the above mentioned study shows 100% sensitivity which correlates with our study and the literature. But is not completely specific as mentioned above due to its positivity in adenocarcinoma.

In squamous cell carcinoma, majority of the cases (86%) showed an intensity score of 3+ (strong) and a proportion score of 5-more than 70% in 67% of cases for both markers approximately. Literature quotes CK5/6 as a specific marker in comparison to p63,even these can be expressed in adenocarcinoma but to a very amount. Studies show cases with positive p63 negative CK5/6, NAPSIN,TTF-1being diagnosed as squamous cell carcinoma.*(Table 5)*

EPIDERMAL GROWTH FACTOR IN LUNG CANCER:

Molecular testing of patients with adenocarcinoma of lung for selection of specific targeted therapy is the standard of care in clinical practice and determination of mutation status is now an integral part of the pathological evaluation. However there are many barriers to the widespread implementation of molecular testing due to the cost limitations in our Indian set up, tissue availability, turn over time for the test, results availability and due to the need for specialized skills to perform and evaluate the test results. The other alternative to molecular testing is the use of immunohistochemistry which crosses all the barriers of molecular testing.[5]

Especially in reference to our study, identification of the EGFR mutational status has been very significant. Exon 19 and Exon21 are the most common EGFR mutations noted in adenocarcinoma lung. And are known to be predictive for response to EGFR TKI such as Gefitinib or Erlotinib. Whichever method we use the priority is to give rapid results without depleting the samples and should be cost friendly and patient friendly. The accessible method employed in our department is immunohistochemistry.

As mentioned earlier, in our study, the EGFR marker which we used was a monoclonal marker (EP22) which identified EGFR mutations in

common and it did not individualize the mutation as either exon19 or exon 21. So the positivity just indicated presence of EGFR mutation.

Of the available blocks i.e. 21 adenocarcinoma cases, 17 cases showed EGFR positivity. This positivity was given based on the intensity score. According to another similar study, the intensity of 2+ and 3+ were considered positive, whereas 0 and 1+are considered negative. Of the 21 cases in our study we got 14% (3 cases) of 2+ positivity and 67% (14cases) of 3+ positivity and 19% (4cases) of 0 and 1+ positivity. So, 81% of the cases showed EGFR mutation positivity in our study. The above mentioned study approximately 65% to 75% EGFR mutation was detected.*(Table 7&8)*

In our study the majority of EGFR mutation positive cases were males with a mean age of 60.5 years, 61% were males and 59% were nonsmokers. Most of these lesions were peripherally located and predominantly showed glandular pattern.*(Table 9)*

Overall from our study, we can conclude saying that EGFR mutation in our sample cases has been of a significant number. The identification is so important more than these patients (the 81% of cases) can be referred for tyrosine kinas inhibitor therapy. Various studies have analyzed the 5years survival among patients with EGFR mutant lung adenocarcinoma treated with an EGFR- TKI in contrast to those unselected patients with a diagnosis of NSCLC.

Since the EGFR antibody used was not mutation specific, we plan to obtain a mutation specific antibody with more accurate statistics in our subsequent study.

With regard to these clinical / patients characteristics we have our usual limitations, those that are usually associated with any retrospective study or analysis which is usually registry based data involving patients who are not treated uniformly. Detailed analysis was not possible for further detailed correlation and study.

SUMMARY AND CONCLUSION

This study was done on 50 lung biopsy specimens, which were diagnosed as either NSCLC , adenocarcinomas or squamous cell carcinoma in the department of pathology PSGIMSR.

We evaluated the most commonly used panel of four immunohistochemical markers which included TTF-1, NAPSIN,P63 and CK5/6 on 50 cases. This IHC panel helped in sub typing the NSCLC into adenocarcinomas and squamous cell carcinoma which was difficult on H and E sections of small biopsy samples. Apart from the immunohistochemical sub typing, all 50 cases were thoroughly studied for their clinical characteristics such as age, sex, smoking status and radiological features such as side and site of the lesion. From the 50 cases studied we derived a total of 28 adenocarcinoma and 22 squamous cell carcinomas. On these IHC proven adenocarcinomas, EGFR mutational status analysis was undertaken. The method employed to detect the mutation was immunohistochemistry. 21 cases were studied and we found 17 to be mutation positive.

The specific IHC markers for adenocarcinomas are TTF-1, NAPSIN. Although TTF-1 is highly specific for adenocarcinoma a few cases of squamous cell carcinomas in our study also showed weak TTF-1 positivity

which is sometimes attributed to the entrapped lung epithelium within the tumor.

NAPSIN showed a better positivity in comparison to TTF-1 . Napsin A is an antibody used for the diagnosis of adenocarcinomas which has recently emerged. In our hands NAPSIN A was more sensitive and TTF-1,but equally specific. We recommend inclusion of NAPSIN A in any immunohistochemical panel because its positive staining is helpful in the diagnosis of adenocarcinomas when TTF-1 staining is equivocal. In addition rare cases of TTF-1 negative but NAPSIN A positive adenocarcinomas have also been noted in our study.

The diagnosis of adenocarcinoma should be made if the cases show strong positivity for both TTF-1 and NAPSIN or for either TTF-1 or NAPSIN, with or without focal p63 positivity.

With regard to squamous cell carcinomas, CK5/6 and p63 were sensitive markers and showed strong positivity in majority of the cases. Though p63 showed 100% positivity, it was not entirely specific because it was also expressed in a few cases of adenocarcinomas in our study. Recognition of this fact is important because the expression of TTF-1 and NAPSIN A allows poorly differentiated NSCLC'S to be diagnosed as adenocarcinomas despite the p63 expression. Of the two markers though p63

is less specific it is considered the first line marker in various other studies and in our study may be due to its 100% sensitivity.

The diagnosis of squamous cell carcinoma should be made if the cases show strong positivity for both P63and CK5/6 or for either P63 or CK 5/6 with or without focal TTF-1 positivity.

IHC using monoclonal antibodies demonstrated to be a reliable test for detecting EGFR mutation in adenocarcinomas of the lung in our study. Even though our study lacked a mutation-specific antibody it still helped in the diagnosis of an overall common presence of mutation. EGFR mutation IHC could be used as a prescreening test for selecting EGFR-TKI patients. The mutation positive cases can be selected for EGFR-TKI therapy, while the negative cases can be referred for DNA analysis. Furthermore , it may be possible to use IHC as a substitute when the quantity of sample id not sufficient for molecular methods.

In conclusion, with regard to small lung biopsies diagnosed as NSCLC'S, sub typing can be accurate if an algorithmic approach utilizing a panel of IHC markers are used which includes TTF-1,NAPSIN A,CK5/6 AND p63.A pattern based approach with prioritization of markers and helps in better utilization of the resources and the tissue samples in order to conserve tissue samples for further molecular testing if required. This sub classification approach has the potential benefit to improve the IHC

diagnostic utilization. Similarly for the identification of EGFR mutation through a basic immunohistochemical methodology helps in guiding the patients for early TK-I therapy which plays a key role in the prognosis of patients diagnosed as lung adenocarcinoma.

Although it is cumbersome, a more complete and thorough examination and sub typing of poorly differentiated NSCLC's is possible by combining H and E, IHC study and a mutation specific IHC marker study in IHC proven adenocarcinoma cases of small lung biopsy specimens is therapeutically significant.

HISTOPATHOLOGY
(H&E)

LEPIDIC PATTERN

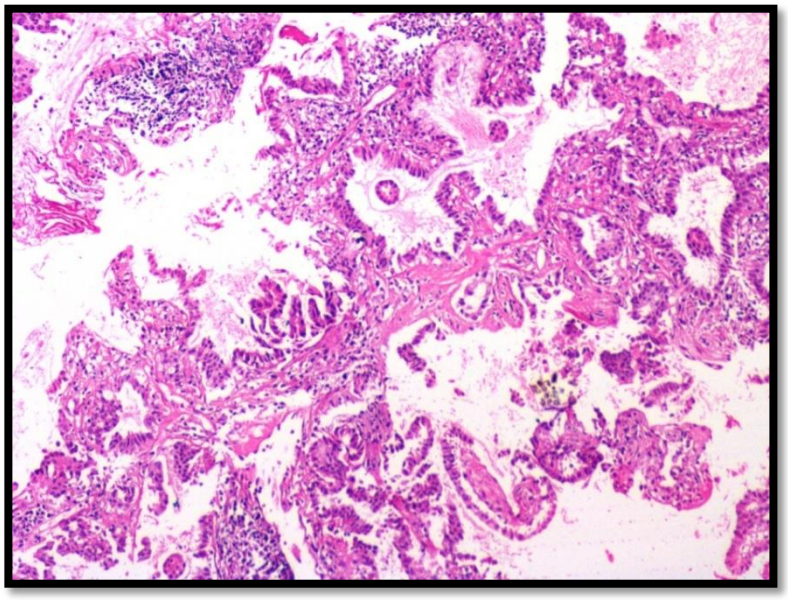


Fig 1(10x)-Lung biopsy of adenocarcinoma with "LEPIDIC GROWTH PATTERN" growth of atypical cells are seen lining the alveolar spaces.

GLANDULAR PATTERN

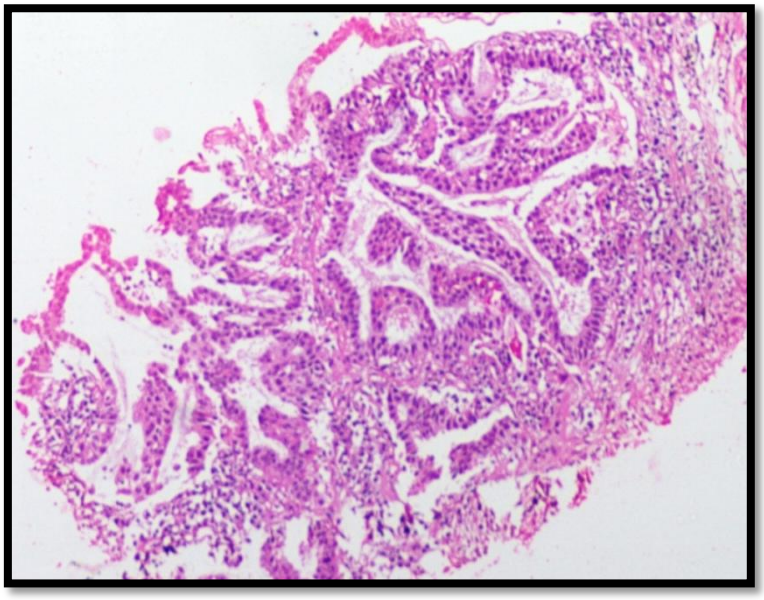


Fig 2(10x)-Lung biopsy of moderately differentiated adenocarcinoma with regular evenly distributed glands separated by desmoplastic stroma exhibiting "GLANDULAR GROWTH PATTERN"

PAPILLARY PATTERN

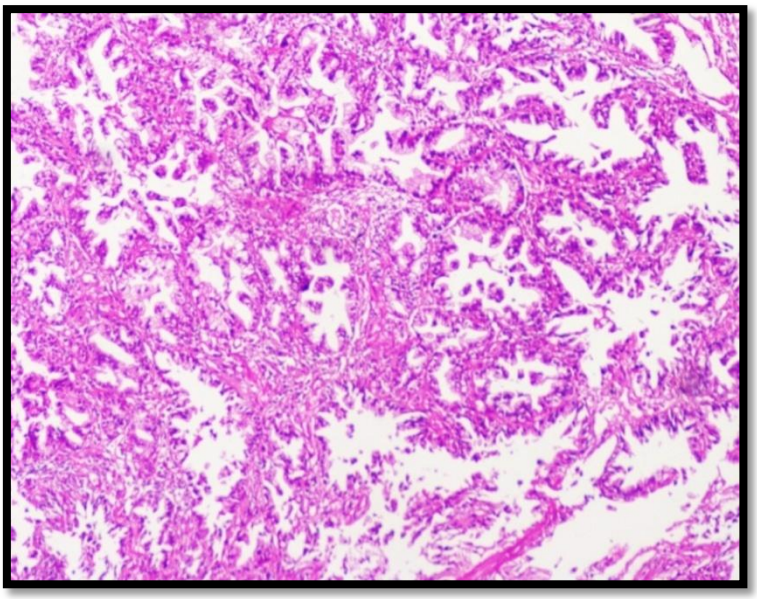


Fig 3(10x)-“PAPILLARY ADENOCARCINOMA” of the lung shows complex branching of papillary structures lined by cells with marked cytological atypia

MUCINOUS VARIANT

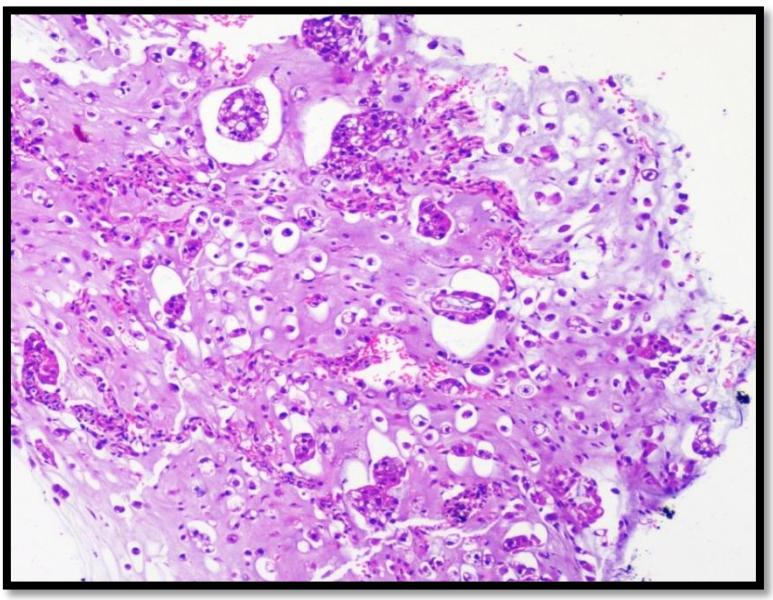


Fig 4(10x)-“MUCINOUS ADENOCARCINOMA” showing atypical cells lining the alveoli with intracellular mucin, surrounding alveolar spaces also show mucin extravasation

NON SMALL CELL LUNG CARCINOMA

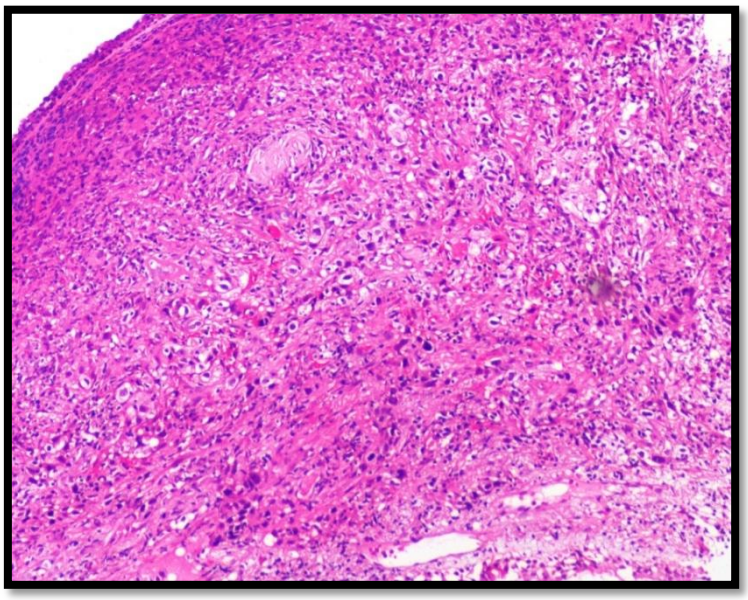


Fig 4(10x)-Poorly differentiated malignancies, which on Morphology (H & E) were unclassifiable and labeled “NSCLC”

IMMUNOHISTOCHEMISTRY

INTENSITY SCORING

(Majority of the cases in this study showed either moderate or strong intensity scoring/positivity)

TTF 1

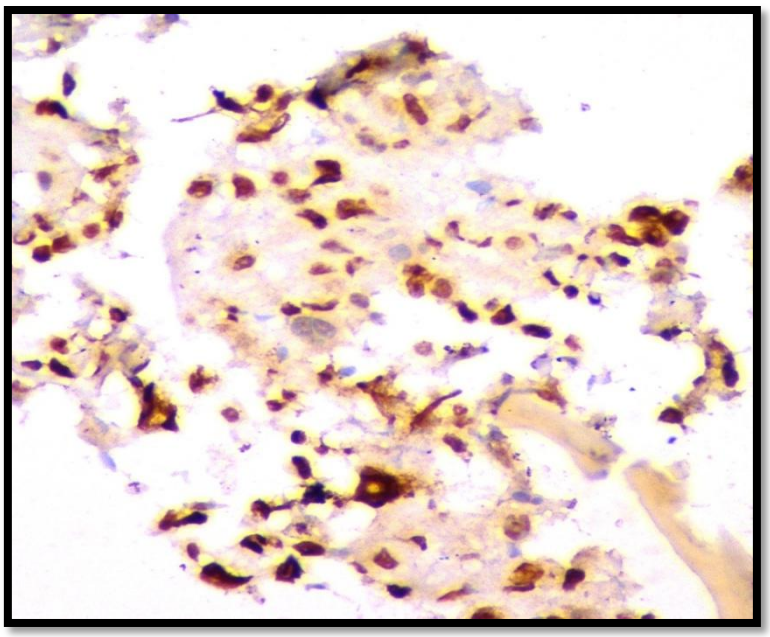


Fig 5(40X)-Moderate (2+) Intensity
(Nuclear Positivity)

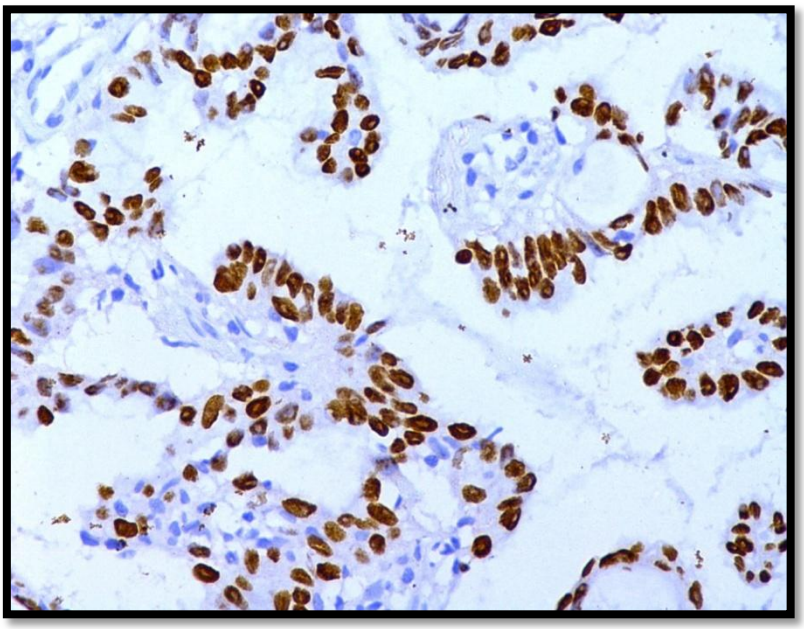


Fig 6(40X)-Strong (3+) Intensity
(Nuclear Positivity)

NAPSIN

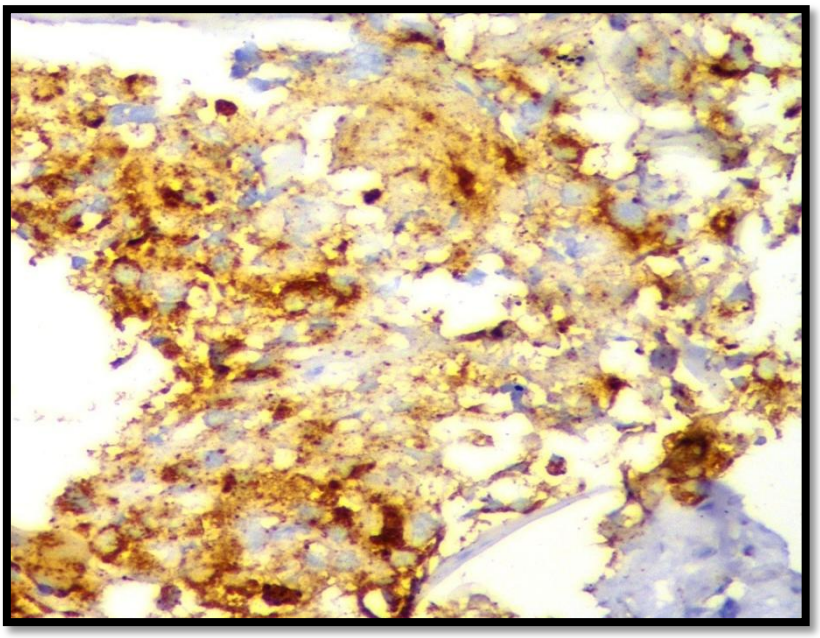


Fig 7(40X)-Moderate (2+) Intensity
(Cytoplasmic Positivity)

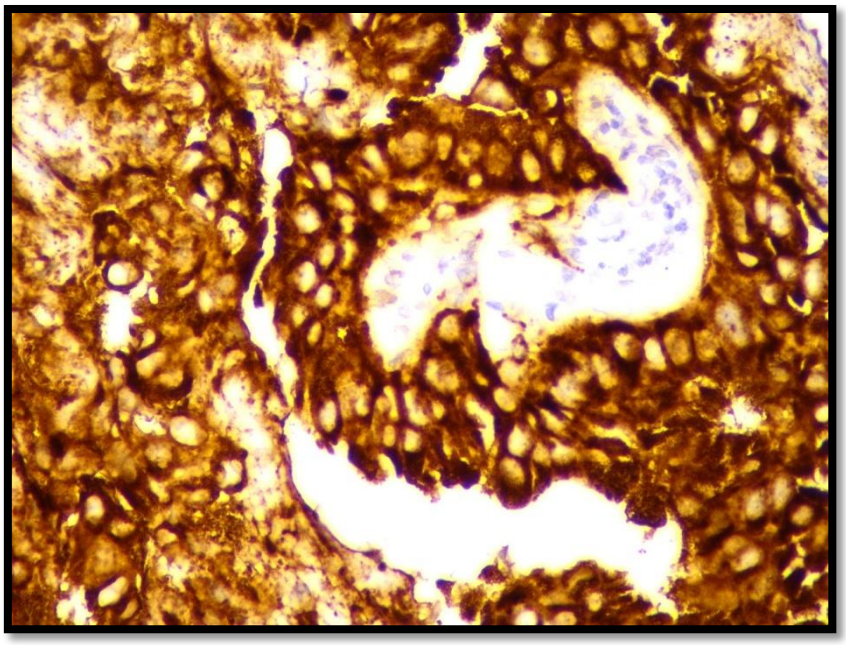


Fig 8(40X)-Strong (3+) Intensity
(Cytoplasmic Positivity)

P63

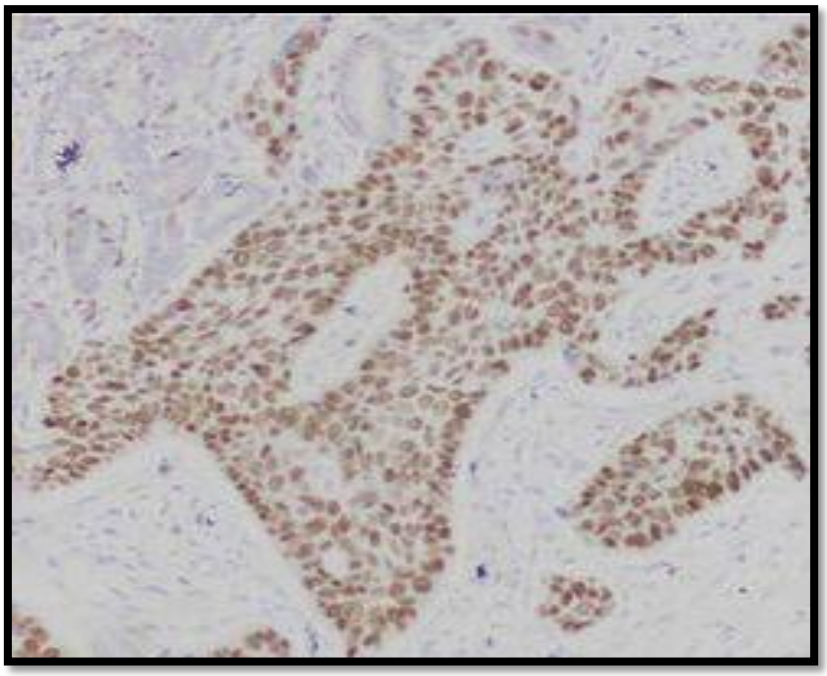


Fig 9(10X)-Moderate (2+) Intensity

(Nuclear Positivity)

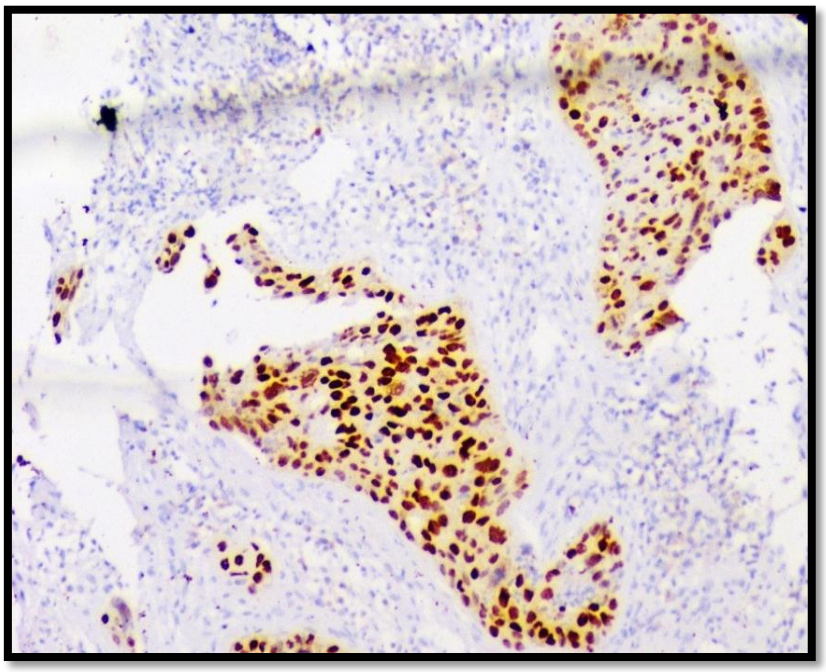


Fig 10(10x)-Strong (3+) Intensity

(Nuclear Positivity)

CK 5/6

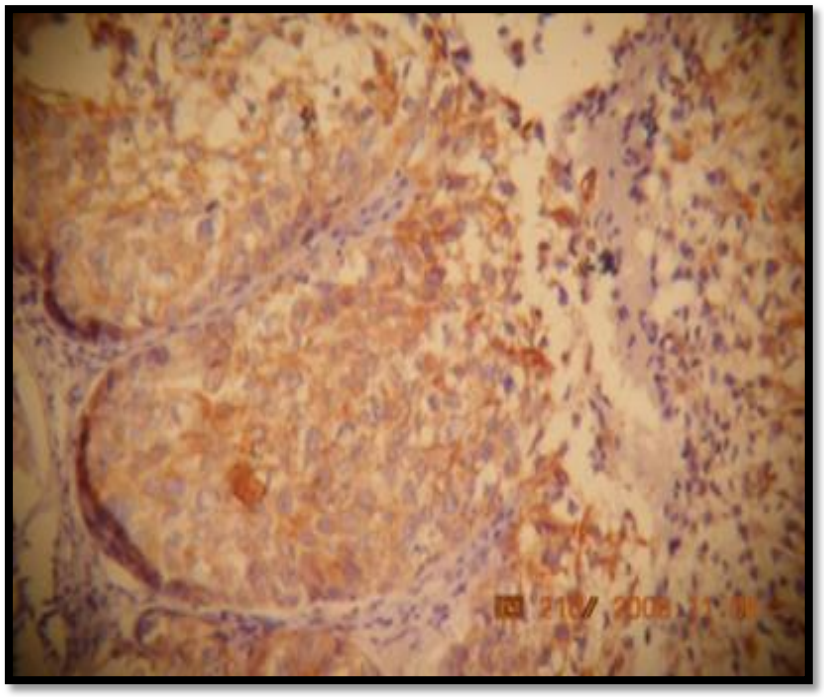


Fig 11(40x)-Moderate (2+) Intensity

(Cytoplasmic Positivity)

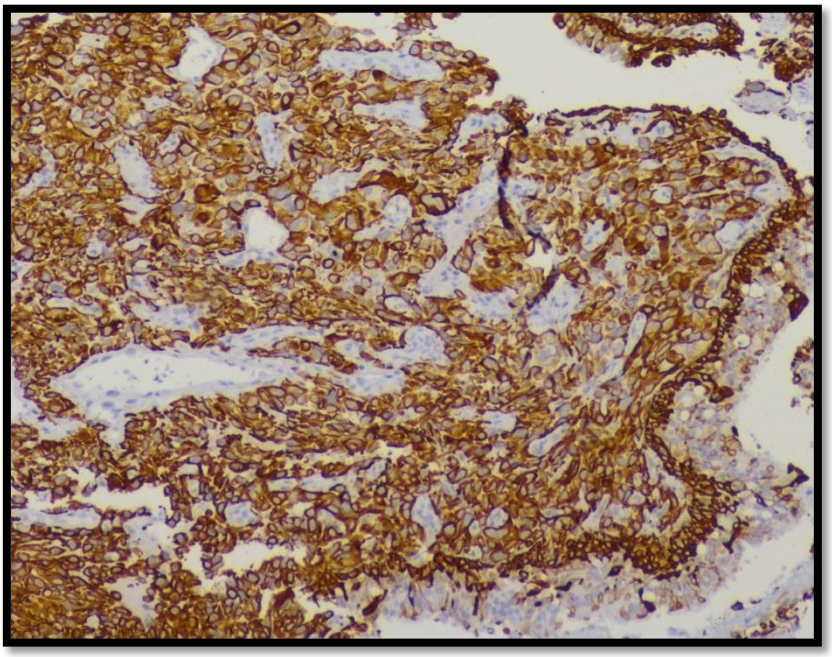


Fig 12(40x)-Strong (3+) Intensity

(Cytoplasmic Positivity)

HISTOPATHOLOGICAL & IMMUNOHISTOCHEMICAL SUBTYPING OF LUNG MALIGNANCIES

ADENOCARCINOMA

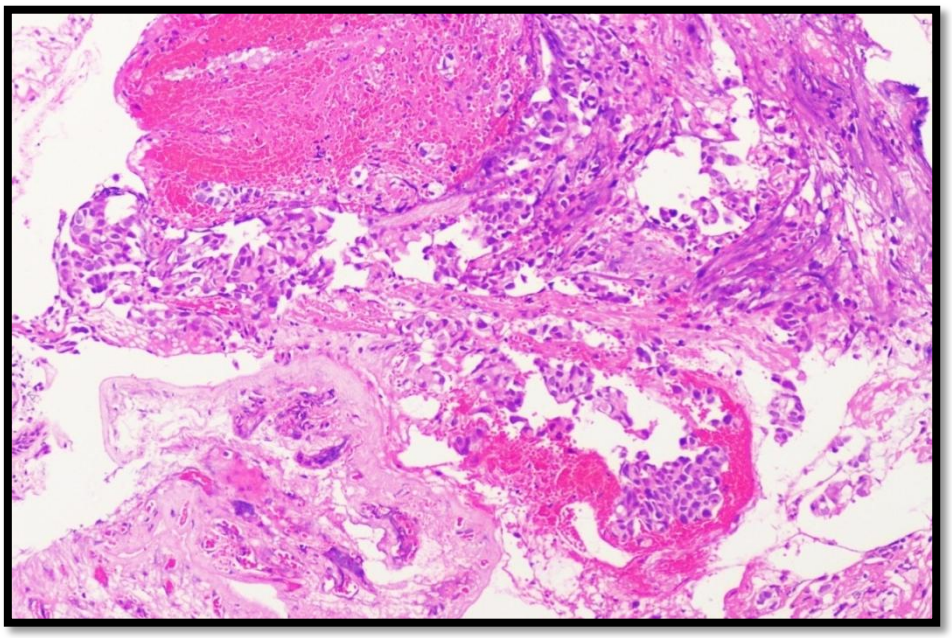


Fig 13(10x)- H & E Section Of Lung Adenocarcinoma

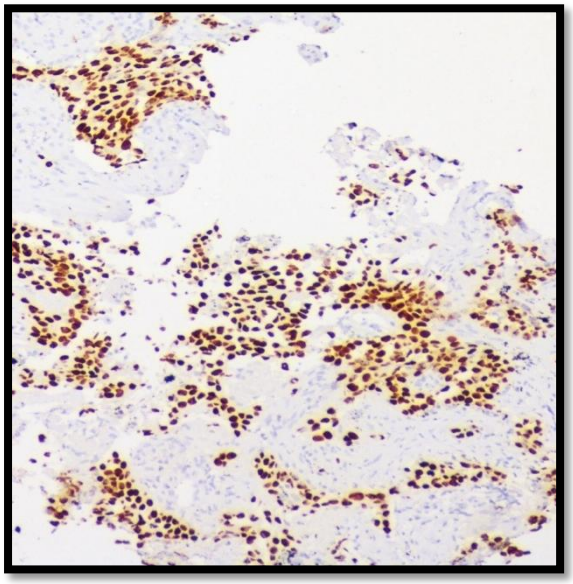


Fig 14(10x)-Exhibiting strong TTF-1 positivity

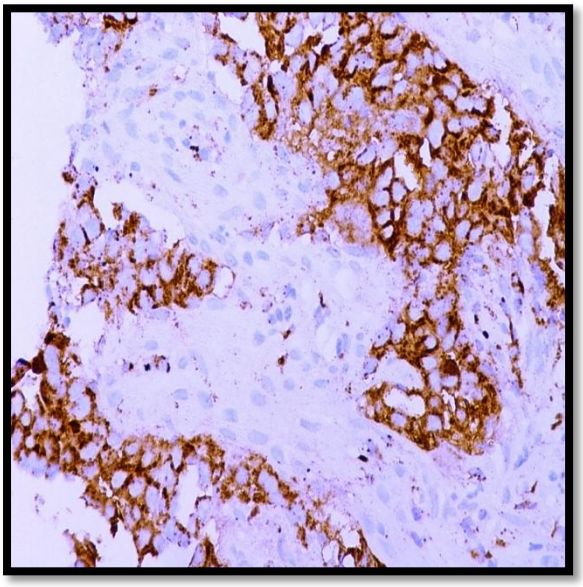
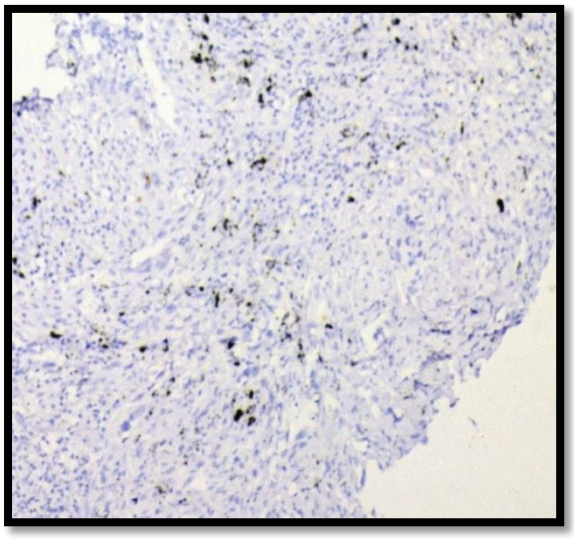
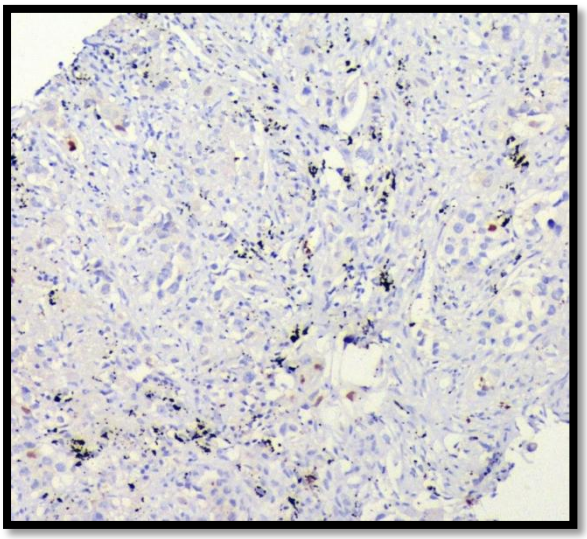


Fig 15(10X)-Exhibiting strong NAPSIN A positivity





Figs 16 & 17(10X)-Markers are negative for CK5/6 AND p63

SQUAMOUS CELL CARCINOMA

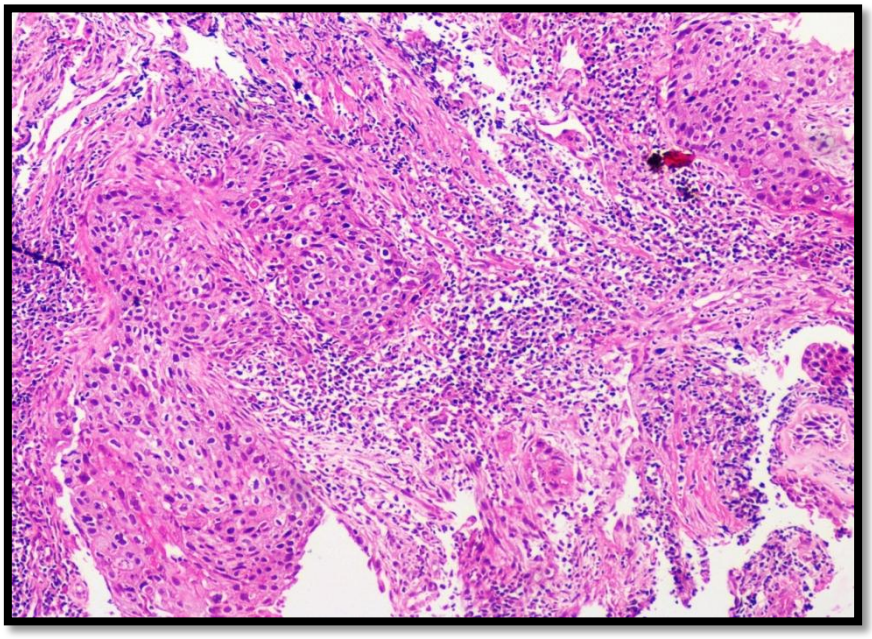


Fig 18(10X)-Shows H & E sections of squamous cell carcinoma

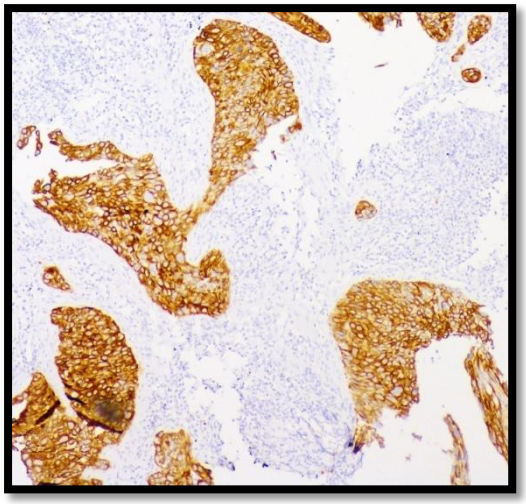


Fig 19(10X)-exhibiting strong CK5/6 positivity

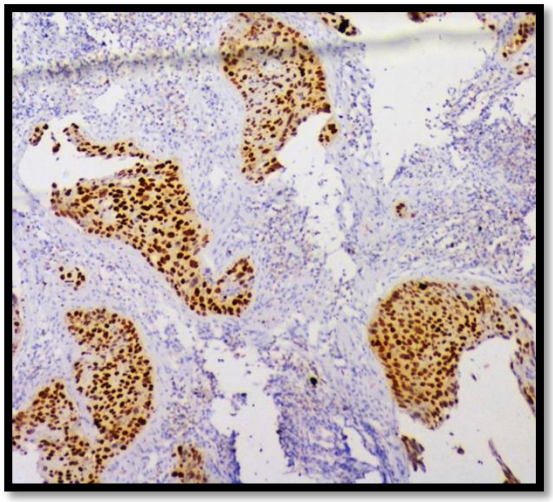
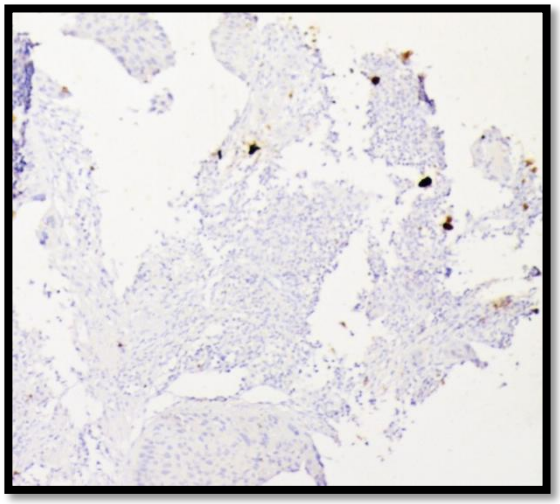
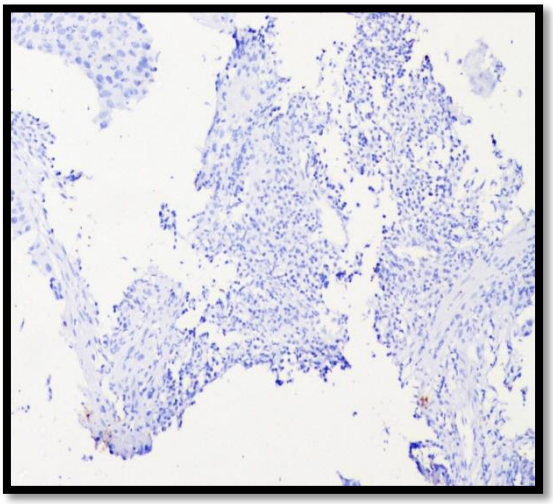
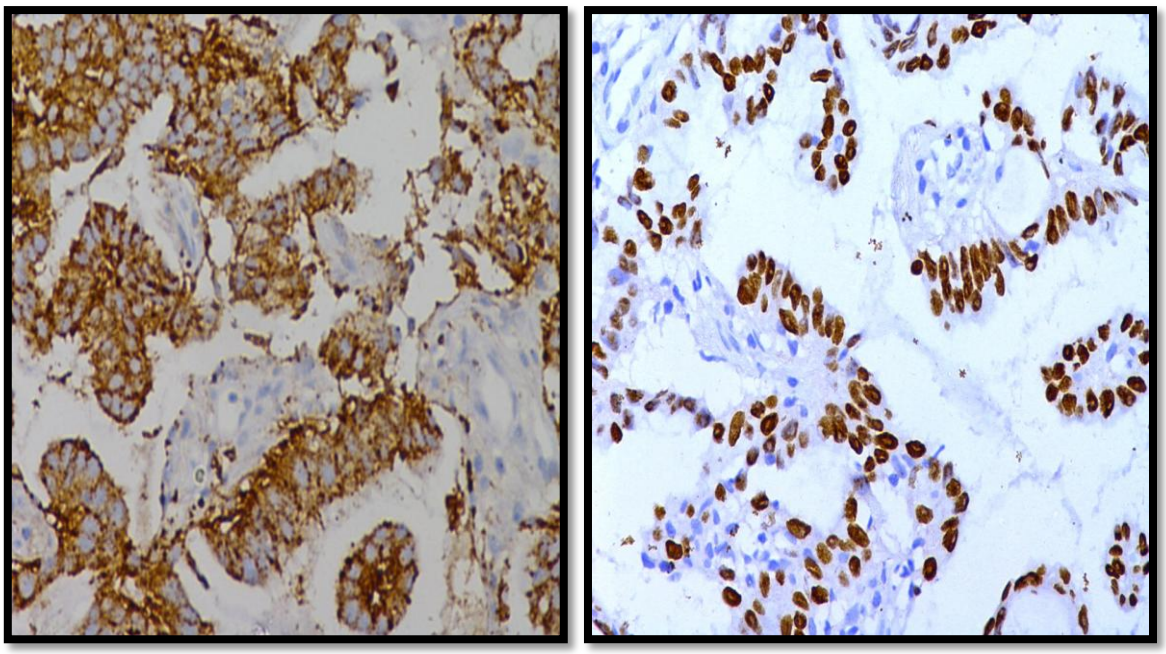


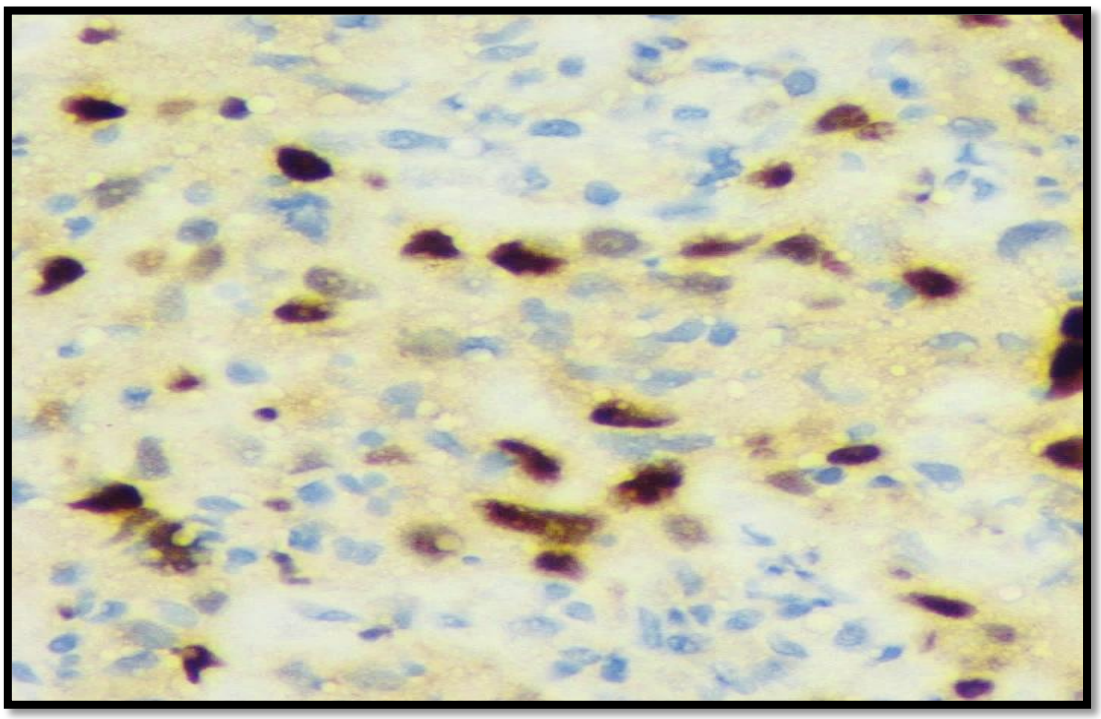
Fig 20(10X)-exhibiting strong p63 positivity





Figs 21 & 22(10X)-Markers are negative for TTF-1 AND NAPSIN A





Figs. 23 to 25 denotes a case of adenocarcinoma
(strong TTF-1 & NAPSIN A positive) which also shows focal “P63 positivity”

EGFR

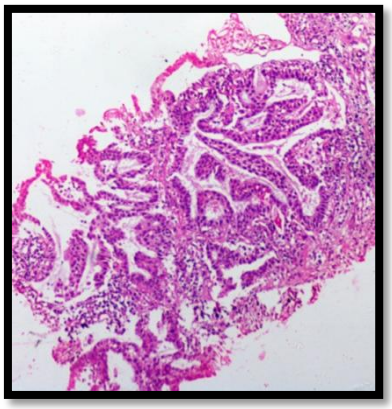
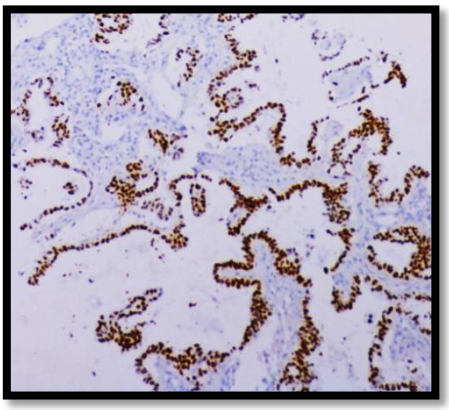
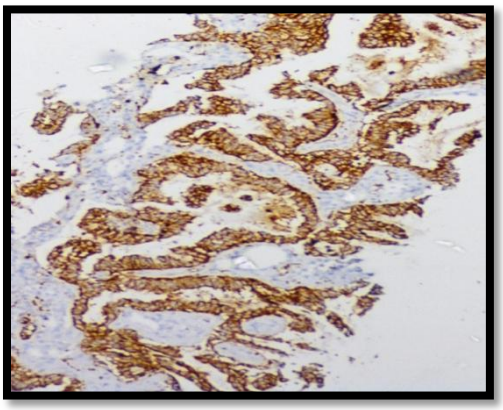


Fig 26(10X)-H & E section of an adenocarcinoma case





Figs 27 & 28(10X)-markers (TTF 1 & NAPSIN A)
In adenocarcinoma show strong positivity

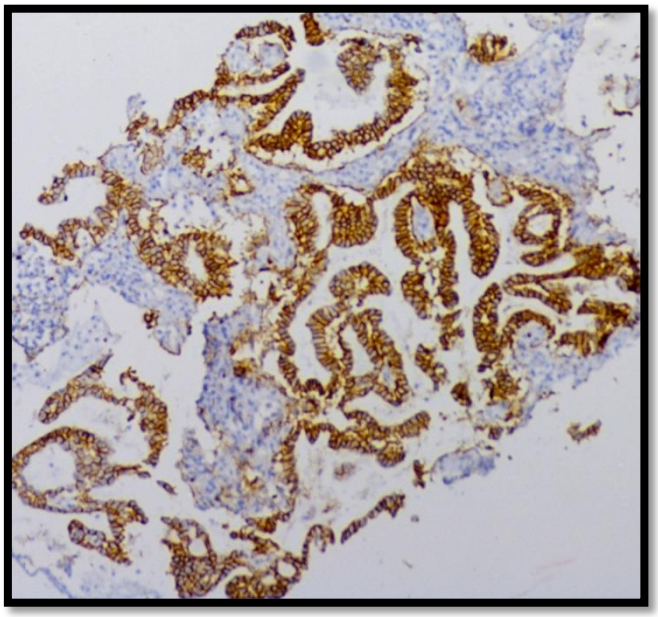


Fig 29(10X)- section shows EGFR strong positivity,
which denotes the presence of mutation

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SL NO	AGE	SEX	SMOKING STATUS	RADIOLOGICAL LOCATION	HP DIAGNOSIS	TTF 1 (PS)	TTF1 (IS)	NAPSIN(PS)	NAPSIN(IS)	CK5/6(PS)	CK5/6(IS)	p63(ps)	p63(IS)	FINAL DIAGNOSIS	PATTERN IN ADENO	EGFR
1	55	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	ADENOCARCINOMA	4	3	4	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3
2	45	F	NON SMOKER	LT UPPER LOBE PARAHILAR(CENTRAL)	ADENOCARCINOMA	3	3	4	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	NB
3	49	F	NON SMOKER	RT LOWER LOBE(PERIPHERAL)	ADENOCARCINOMA	5	3	5	3	0	0	2	1	ADENOCARCINOMA	LEPIDIC	3
4	80	M	NON SMOKER	RT LOWER LOBE(PERIPHERAL)	ADENOCARCINOMA	4	3	0	0	0	0	0	0	ADENOCARCINOMA	GLANDULAR	NB
5	58	M	SMOKER	LT LOWER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
6	70	M	NON SMOKER	LT ENDOBRONCHIAL(CENTRAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	1	3	5	3	SQUAMOUS CELL CARCINOMA		
7	65	F	NON SMOKER	RT LOWER LOBE(PERIPHERAL)	ADENOCARCINOMA	4	3	5	3	0	0	1	2	ADENOCARCINOMA	GLANDULAR	2
8	68	F	NON SMOKER	RT LUNG (CENTRAL)	ADENOCARCINOMA	3	2	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	0
9	88	M	SMOKER	RT LOWER LOBE(PERIPHERAL)	NSCLC	0	0	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
10	48	M	SMOKER	RT MIDDLE LOBE (CENTRAL)	SQUAMOUS CELL CARCINOMA	5	3	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	NB
11	40	M	NON SMOKER	RT MAIN BRONCHUS(CENTRAL)	NSCLC	3	2	3	3	0	0	0	0	ADENOCARCINOMA	LEPIDIC	1
12	55	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	ADENOCARCINOMA	5	3	5	3	0	0	0	0	ADENOCARCINOMA	PAPILLARY	3
13	73	M	NON SMOKER	RT UPPER LOBE(PERIPHERAL)	NSCLC	5	3	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3
14	60	M	NON SMOKER		ADENOCARCINOMA	5	2	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3
15	43	M	SMOKER	CENTRILOBULAR LESION(CENTRAL)	ADENOCARCINOMA	0	0	5	3	0	0	0	0	ADENOCARCINOMA	LEPIDIC	1
16	66	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	NSCLC	0	0	0	0	1	3	4	3	SQUAMOUS CELL CARCINOMA		
17	78	M	SMOKER	CENTRILOBULAR LESION(CENTRAL)	NSCLC	0	0	0	0	1	3	5	3	SQUAMOUS CELL CARCINOMA		
18	73	F	NON SMOKER	RT UPPER LOBE(PERIPHERAL)	ADENOCARCINOMA	5	3	5	3	0	0	0	0	ADENOCARCINOMA	PAPILLARY	3
19	65	M	SMOKER	RT ENDOBRONCHIAL MASS (CENTRAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
20	66	M	SMOKER	RT LOWER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	4	2	SQUAMOUS CELL CARCINOMA		
21	71	M	SMOKER	RT LOWER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	3	3	5	3	SQUAMOUS CELL CARCINOMA		
22	45	F	NON SMOKER	LT HILUM(CENTRAL)	ADENOCARCINOMA	3	2	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3
23	63	M	SMOKER	LT UPPER LOBE(PERIPHERAL)	NSCLC	0	0	2	3	0	0	0	0	ADENOCARCINOMA	LEPIDIC	3
24	45	M	NON SMOKER	RT UPPER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	0	0	5	3	SQUAMOUS CELL CARCINOMA		
25	68	M	SMOKER		SQUAMOUS CELL CARCINOMA	0	0	0	0	1	1	5	3	SQUAMOUS CELL CARCINOMA		
26	65	M	SMOKER	RT PERIHILAR MASS(CENTRAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
27	80	M	SMOKER	LT MAIN BRONCUS (CENTRAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
28	57	M	NON SMOKER		NSCLC	5	3	0	0	0	0	3	2	ADENOCARCINOMA	GLANDULAR	NB
29	57	M	NON SMOKER	RT UPPER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	4	3	SQUAMOUS CELL CARCINOMA		
30	45	M	SMOKER	LT LOWER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
31	55	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	4	2	SQUAMOUS CELL CARCINOMA		
32	69	M	SMOKER		SQUAMOUS CELL CARCINOMA	2	1	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
33	54	F	NON SMOKER	RT MIDDLE LOBE (CENTRAL)	ADENOCARCINOMA	3	2	4	2	0	0	0	0	ADENOCARCINOMA	GLANDULAR	2
34	75	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	4	2	SQUAMOUS CELL CARCINOMA		
35	45	M	SMOKER	LT HILUM(CENTRAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	4	3	5	2	SQUAMOUS CELL CARCINOMA		
36	60	F	NON SMOKER		SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	3	2	SQUAMOUS CELL CARCINOMA		
37	55	M	SMOKER		NSCLC	4	3	4	3	0	0	0	0	ADENOCARCINOMA	LEPIDIC	NB
38	48	M	NON SMOKER		NSCLC	4	3	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	NB
39	54	M	SMOKER	RT LOWER LOBE(PERIPHERAL)	SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
40	65	F	NON SMOKER	LT HILUM(CENTRAL)	NSCLC	0	0	2	1	0	0	0	0	ADENOCARCINOMA	LEPIDIC	1
41	66	F	NON SMOKER		ADENOCARCINOMA	5	3	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3
42	40	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	NSCLC	5	3	0	0	0	0	0	0	ADENOCARCINOMA	SOLID	3
43	67	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	ADENOCARCINOMA	5	3	4	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3
44	58	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	ADENOCARCINOMA	5	3	3	2	0	0	0	0	ADENOCARCINOMA	GLANDULAR	2
45	80	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	ADENOCARCINOMA	5	3	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	NB
46	70	F	NON SMOKER	LT MAIN BRONCUS (CENTRAL)	NSCLC	0	0	0	0	4	3	5	3	SQUAMOUS CELL CARCINOMA		
47	64	M	SMOKER		SQUAMOUS CELL CARCINOMA	0	0	0	0	5	3	5	3	SQUAMOUS CELL CARCINOMA		
48	65	M	SMOKER	RT UPPER LOBE(PERIPHERAL)	INVASIVE MUCINIOUS ADENOCA	5	3	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3
49	66	F	NON SMOKER	RT UPPER LOBE(PERIPHERAL)	NSCLC	5	3	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3
50	51	F	NON SMOKER	RT UPPER LOBE(PERIPHERAL)	ADENOCARCINOMA	5	3	5	3	0	0	0	0	ADENOCARCINOMA	GLANDULAR	3

ABBREVIATIONS

LC	**-**	**LUNG CARCINOMA**
NSCLC	**-**	**NON SMALL CELL LUNG CARCINOMA**
SCLC	**-**	**SMALL CELL LUNG CARCINOMA**
LCC	**-**	**LARGE CELL CARCINOMA**
NET	**-**	**NEUROENDOCRINE TUMOR**
ADC	**-**	**ADENOCARCINOMA**
SQ C C	**-**	**SQUAMOUS CELL CARCINOMA**
TTF-1	**-**	**THYROID TRANSCRIPTION FACTOR 1**
CK5/6	**-**	**CYTOKERATIN 5/6**
EGFR	**-**	**EPIDERMAL GROWTH FACTOR RECEPTOR**
IP	**-**	**INTENSITY SCORE**
PP	**-**	**PROPORTION SCORE**
(n)	**-**	**NUMBER OF CASES**