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COMPARATIVE STUDY OF EGFR MUTATION ANALYSIS WITH EGFR PROTEIN EXPRESSION IN PATIENTS WITH LUNG CANCER IN BASRAH

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ABSTRACT

Background: Lung cancer is a leading cause of cancer-related mortality worldwide, with most cases diagnosed at advanced stages and associated with poor prognosis. Non-small cell lung cancer accounts for the majority of cases, and cigarette smoking remains the main risk factor, alongside genetic and environmental influences. Advances in molecular profiling, including Epidermal Growth Factor Receptor (EGFR) and other driver mutations, together with immunohistochemical (IHC) analysis and improved imaging techniques, have enhanced diagnostic accuracy and enabled the use of targeted and immunotherapy-based treatments, leading to improved outcomes in selected patients. **Aim of the study:** To evaluate EGFR protein expression in different types of EGFR mutation in lung cancer. **Patient and method:** This was a prospective study, carried out in Basrah province. The data were collected incidentally from Al-Sader Teaching Hospital and private laboratories during the period from the First of October 2024 to the end of October 2025. All cases of lung cancer diagnosed during the period of study were included. Formalin fixed paraffin embedded blocks were collected. Three to Five micrometers thickness sections were obtained and stained with routine hematoxylin and eosin stains. The slides were examined to confirm the diagnosis and define the histological subtype. Then Additional sections using positively charged slides were provided for IHC staining for EGFR mutations. The piece of tumor tissue in appropriate size was taken from the wax block for DNA extraction for detection of EGFR mutation by using Real-time PCR. **Results:** Forty six cases of proved

lung cancer were included in this study. Thirty five cases (76.1%) were males and 11 cases (23.9 %) were females with mean age of 63.91 years (range 40-85 years). Thirty three cases (71.7%) diagnosed as adenocarcinoma, 9 cases (19.6%) diagnosed as squamous cell carcinoma and 4 cases (8.7%) diagnosed as metastatic adenocarcinoma. Using IHC, 10 cases (21.8%) were moderately positive (score +2) for EGFR protein expression, 2 cases (4.3%) were strongly positive (score +3), 23 cases (50.0%) were negative and 11 cases (23.9%) showed equivocal expression (score +1). Among the negative cases, 19 cases were males and 4 cases were females, while among the positive cases (scores +2 and +3), 8 cases were males and 4 cases were females. Using Real -Time PCR, 5 cases (10.9%) were positive for exon-19 mutation, 1 case (2.1%) positive for exon-18 mutation and 40 cases (87.0%) were negative for EGFR mutation. Among the positive cases, 5 cases were males and 1 case was female. The sensitivity of IHC in detecting EGFR mutation is (0%) with specificity (70%). The positive predictive value is (0%) and the negative predictive value is (82.4%). The ROC curve shows that the consistency between the IHC and RT-PCR is (31.3%). **Conclusion:** The findings from this study reveal a discrepancy between IHC and RT-PCR in identifying EGFR mutations. It is important to note that IHC should not be relied upon to evaluate different types of EGFR mutation. Depending solely on IHC may be unreliable due to its low sensitivity, which could potentially increase time, cost, and effort in the diagnostic process.

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TABLE OF ABBREVIATIONS

Abbreviation	Description
NSCLC	Non-Small Cell Lung Cancer
SCLC	Small Cell lung Cancer
ADC	Adenocarcinoma
LUAD	Lung Adenocarcinoma
SCC	Squamous Cell Carcinoma
LCC	Large Cell Carcinoma
EGFR	Epidermal Growth Factor Receptor
PDL1	Programmed Death-Ligand-1
ROS1	c-ros oncogene
ALK	Anaplastic Lymphoma Kinase
BRAF	B-type Rapidly Accelerated Fibrosarcoma
HER2	Human Epidermal Growth factor Receptor-2
MET	Mesenchymal-Epithelial Transition
RET	Rearranged during Transfection
NTRK	Neurotrophic Tropomyosin Receptor Kinase
TP53	Tumor Protein P53
RB1	Retinoblastoma-1
MYC	Myelocytomatosis Oncogene
TTF1	Thyroid Transcription Factor-1
AE1/AE3	Anti-Epithelial cytokeratin

INSM1	Insulinoma Associated Protein-1
NCAM	Neural Cell Adhesion Molecule
CK7	CytoKeratin-7
SOX2	SRY-related HMG-box gene-2
KRAS	Kirsten Rat Sarcoma
SMARCA4	SWI/SNF Related, Matrix Associated, Actin Dependent Regulation of Chromatin, Subfamily A, Member 4
PAX5	Paired box gene-5
RT-PCR	Real Time Polymerase Chain Reaction
TNM	Tumor Nodes Metastasis
WHO	World Health Organization
IHC	Immunohistochemistry
CB	Cell block
PTEN	Phosphatase and TENsin homolog
FNAC	Fine needle aspiration cytology
FFPE	Formalin fixed paraffin embedded
DPX	Dibutyl phthalate PolystyreneXylene
DNA	Deoxyribonucleic Acid

CHAPTER ONE INTRODUCTION

1.1 INTRODUCTION

Lung cancer (LC) ranks among the most common cancers globally and is associated with a high mortality rate, accounting for about 18% of all cancer-related deaths.[1] It ranks as the second most common cancer type by sex following prostate cancer in men and breast cancer in women.[2]

Although lung cancer remains a leading cause of death due to its frequent late-stage diagnosis and the limited curative potential of current treatments, recent progress in understanding tumor biology, the emergence of targeted therapies, and the adoption of low-dose computed tomography (LDCT) for screening have significantly improved survival outcomes, Use of LDCT was associated with a 20% decrease in lung cancer mortality and a 7% decrease in all-cause mortality compared with annual chest radiography.[3] It has demonstrated a significant decrease in lung cancer–related mortality, ranging from 20% to 39%, in individuals with a heavy smoking.[4]

Epidermal growth factor receptor (EGFR) is a transmembrane receptor protein made up of 486 amino acids, featuring four extracellular and three intracellular domains. The EGFR gene is located on chromosome 7p11.2 and contains 28 exons.[5] Approximately 10–30% of individuals with NSCLC carry mutations in the EGFR gene, most often within exons 18–21, which act as oncogenic drivers. Among these alterations, the exon 19 deletion (p.E746–

A750del) and the exon 21-point mutation (p.L858R) are the most prevalent, together representing over 85% of all EGFR mutations. In contrast, other variants of EGFR mutations are relatively rare.[6]

1.2 AIM OF STUDY

To evaluate EGFR protein expression in different types of EGFR mutation in lung cancer.

1.3 LITERATURE REVIEW

1.3.1 Lung Cancer

Lung cancer (LC) continues to be the primary cause of cancer-related deaths globally, with reported five-year survival rates between 10% and 20%. cigarette smoking, accounts for more than 80% of lung cancer cases. The disease is often difficult to detect at an early stage because of limitations in available screening approaches, leading to diagnosis in many patients only after the cancer has reached an advanced stages.[7]

1.3.2 Epidemiology

In 2022, lung cancer was the most common malignancy, causing about 2.5 million new cases (12.4% of all cancers) and 1.8 million deaths (18.7%) worldwide, according to International Agency for Research on Cancer (IARC). Non-small cell lung cancer (NSCLC) accounts for most cases, with AC being the most frequent subtype.[8] Lung cancer has the highest incidence and mortality of all cancers, Europe had the highest mortality rate (50.2 per 100,000), followed by North America (40.4), Oceania (28.6), Asia (24.6), Latin America (13.7), and Africa (3.2).[9] It ranks as the third most frequently diagnosed cancer in Iraq and is the most common malignancy among men. In 2023, a total of 3,020 new cases were reported. Of these, 2,129 cases (70.5%) occurred in males, while 891 cases (29.5%) were identified among females.[10] If current trends continue, population growth and aging could raise global cancer cases to over 35 million by 2050. In the U.S, incidence and mortality have declined since the 1990s in men and 2000s in women, likely due to improved treatments.[9]

1.3.3 Risk factors

Modifiable risk factors

1- Tobacco smoking

Cigarette smoking is a leading risk factor for lung cancer. Although smoking rates were historically higher in men, this difference has decreased, and evidence on sex-based differences in lung cancer risk among smokers remains inconsistent. Secondhand smoke may

increase lung cancer risk, but findings vary across studies.[11] Marijuana use has been found to cause precancerous histological changes in the bronchial epithelium that resemble those seen with tobacco smoking.[9]

2- Asbestos

Asbestos, a naturally occurring fire-resistant material commonly used in construction, can cause various respiratory diseases, including lung cancer. The combination of asbestos exposure and tobacco smoking significantly increases lung cancer risk, with smokers exposed to asbestos being up to 45 times more likely to develop the disease compared to the general population.[12]

3- Radon

Radon-222 is a common indoor source of ionizing radiation whose decay products can damage lung tissue and increase lung cancer risk especially squamous cell carcinoma. Evidence from miner studies suggests that residential radon exposure contributes to a proportion of lung cancer cases, although risk estimates are influenced by smoking and other confounding factors.[13]

4- Air pollution

Air pollution, especially fine particulate matter (PM2.5), increases the risk of lung cancer, including lung adenocarcinoma (LUAD), even among nonsmokers. Risk is higher with greater exposure and is amplified by smoking.

Individual susceptibility may also be influenced by genetic factors.[14]

5- Arsenic

High levels of arsenic exposure are strongly associated with lung cancer, as shown in studies from several countries, with elevated mortality persisting decades after exposure cessation. While many studies suggest that low to moderate arsenic exposure may also increase cancer risk, findings remain inconsistent and the dose–response relationship is unclear. Arsenic exposure has additionally been linked to other lung diseases, including pneumonia, bronchiectasis, and asthma.[15]

6- Infection

Infectious agents are unlikely to be direct causes of lung cancer but may promote chronic inflammation that supports tumor initiation, progression, and treatment response.[16]

Tuberculosis raises the risk 1.76-fold, while Human Immunodeficiency Virus (HIV) increases it up to 2.5 times, independent of smoking. With effective HIV treatments, lung cancer is now the leading cause of death in HIV-positive individuals. Long-term lung damage from COVID-19 may also elevate future risk.[17]

7- Chronic obstructive pulmonary disease (COPD)

Smoking is a risk factor for both COPD and lung cancer, but COPD independently increases lung cancer risk. Studies show that lung cancer incidence is higher in COPD patients, and greater disease severity further raises this risk. Inflammation, oxidative stress, and genetic factors linking COPD, emphysema, and lung cancer contribute to a carcinogenic lung environment.[18]

Non-modifiable risk factors

1- Age

The median age for lung cancer diagnosis is 71 years, with only 6% of cases occurring in individuals under 55 years. Lung cancer is rare in younger populations, affecting about 1.1% of patients under 45 years and 0.2% under 35 years.[19]

2- Sex

Globally, men are over twice as likely as women to develop and die from lung cancer, mainly due to higher smoking rates. In developed countries, male lung cancer rates are declining while female rates are rising. Non-smoking women are more prone than non-smoking men to EGFR positive adenocarcinoma and often carry genetic mutations (e.g., TP53, ALK, BRAF) that increase susceptibility. In addition, hormonal factors including estrogen receptor activity and reproductive history, also influence lung cancer risk.[17]

3- Race/ ethnicity

Lung cancer disproportionately affects racial and ethnic minorities, particularly Black Americans, who are more likely to be diagnosed at later stages and have lower survival rates. Other groups, including Asian and Hispanic populations, also face unique risks, such as higher incidence among never-smokers in certain subgroups. These disparities are linked to structural barriers, historical inequities, and underrepresentation in clinical trials, highlighting

the need for more inclusive screening and early detection strategies.[20]

4- Family history

A family history of lung cancer is associated with approximately a twofold increased risk of the disease. This association varies by geographic and sociodemographic factors and is stronger among Asians, younger individuals, smokers, and those with multiple affected relatives. No clear differences have been observed across histological subtypes, and the increased risk reflects a combination of genetic susceptibility, shared environmental exposures, and lifestyle factors.[21]

1.3.4 Sign and symptoms of lung cancer

Late-stage lung cancer is commonly diagnosed due to nonspecific or infrequent symptoms and delays in seeking medical care. Symptoms may be obvious or limited to general constitutional signs, with common manifestations including persistent cough, hemoptysis, chest pain, dyspnea, weight loss, fatigue, fever and lymphadenopathy.[22]

1.3.5 Morphology of lung cancer

Lung cancer primarily divided into non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). NSCLC makes up about 85% of all lung cancer cases and includes subtypes such as adenocarcinoma (ADC), squamous cell carcinoma(SCC), and large cell carcinoma (LCC). The remaining 15% consists of SCLC, which is marked by neuroendocrine differentiation.[22,23]

Non-small cell lung carcinoma

- **Adenocarcinoma:** It's typically originate in peripheral areas within a fibroblastic stroma. Some lung cancers arise in areas of inflammatory scarring; however, it is now recognized that AC elicit a desmoplastic stromal response, similar to that seen in breast and pancreatic adenocarcinomas, meaning they actively induce the surrounding stromal changes rather than originate within preexisting scar tissue.[24]

Its poorly defined, grayish-yellow lesion that can be single or multiple. When it produces large amounts of mucin, it appears gelatinous and glairy.[25]

Microscopically, ADC typically displays a glandular architecture and is composed of malignant cells, with growth patterns such as lepidic, acinar, papillary, micropapillary, or solid.[26]

- **Squamous cell carcinoma:** It arises centrally, near the hilar region.[27] and grossly these tumors typically appear gray-white to yellow-tan. SCC is the type most frequently associated with the formation of thick-walled, irregular cavities featuring central necrosis.[25]

Microscopically, it's marked by atypical squamous cell proliferation of keratinizing, non-keratinizing, or basaloid types according to WHO Classification.[28]

- **Large cell carcinoma:** These tumors are usually peripheral but can also arise centrally, and grossly they often present as large, necrotic masses.[29] It's diagnosed by excluding squamous or glandular differentiation under light microscopy. Histologically, it is composed of sheets or nests of large polygonal cells with vesicular nuclei and prominent nucleoli.[29]
- **Small cell carcinoma:** These tumors are visibly identifiable as soft, fleshy masses with an irregular shape, typically appearing white, grayish, or light tan in color, and often exhibiting areas of necrosis on cut surfaces. Most are located in the hilar or perihilar regions, with fewer than 5% occurring in peripheral areas. They commonly invade peribronchial tissues and lymph nodes, often spreading circumferentially along the bronchial submucosa.[30]

Microscopically, It consists of small round to spindle cells with scant cytoplasm, fine chromatin, and frequent crush artifacts. It shows high mitotic activity, necrosis, nuclear molding, and may display ribbons, rosettes, or palisading.[31]

1.3.6 Grading of lung cancer

Lung cancer histologic grading is standardized as[32]

-GX (grade cannot be assessed).

-G1 (well-differentiated).

-G2 (moderately differentiated).

-G3 (poorly differentiated).

-G4 (undifferentiated).

Adenocarcinomas and squamous cell carcinomas are graded G1–G3 based on the least favorable differentiation, while sarcomatoid, large cell, and small cell carcinomas are generally undifferentiated (G4).

1.3.7 Staging of lung cancer

The anatomical extent of cancer is defined using the TNM staging system, a standardized classification essential for prognosis assessment, treatment planning, and evaluation of therapeutic response. This system facilitates clinical communication and research and includes three components: the primary tumor (T), regional lymph node involvement (N), and distant metastasis (M). The recently introduced eighth edition of TNM staging for lung cancer is based on analyses from the International Association for the Study of Lung Cancer and has been adopted by the American Joint Committee on Cancer and the Union for International Cancer Control (**Appendix**).[33]

1.3.8 Diagnosis of lung cancer

Diagnosis of lung cancer typically involves several tests and procedures:

- Imaging is key for lung cancer diagnosis and staging, using X-ray, Computed Tomography (CT), Magnetic Resonance Imaging (MRI), and Positron Emission Tomography (PET scan). Ultrasound and CT-guided tissue sampling (minimally invasive procedures) help to confirm pulmonary lesion.[34]
- Cytological analysis of sputum, particularly when multiple samples are examined, aids in the detection of centrally located tumors arising from the major bronchi.[35]
- Lung tissue biopsy remains the definitive method for confirming a cancer Diagnosis.[35]

1.3.9 Immunohistochemistry

Histological type	IHC markers
Adenocarcinoma	TTF-1, napsin-A, CK7, beta catenin, AE1/AE3, PDL1.[36]
Squamous cell carcinoma	p40, CK5/6, TP63 (p63), PDL1.[37]
Small cell carcinoma	INSM1, chromogranin-A, NCAM/CD56, synaptophysin, CK7, TTF-1, p16, SOX2, PAX5, calretinin, CK8, Ki67.[26]

1.3.10 Molecular evaluation

Histological type	Molecular test
Adenocarcinoma	EGFR mutation**,** KRAS mutation, ALK rearrangement**,** ROS1gene fusion, BRAF mutation, NTRK mutation, RET fusion, HER-2 amplification, MET amplification,SMARCA4 loss of function.[38]
Squamous cell carcinoma	EGFR mutation, ALK rearrangement.[38]
Small cell carcinoma	P53 mutations, RB1 loss of function, PTEN mutation, MYC amplification, deletion of 3p, wild type c-KIT upregulation and telomerase Activation.[39]

1.3.11 Metastasis

Lung cancer is often diagnosed at stage IV with existing metastasis, spreading via lymphatic and blood vessels**.** Vascular invasion, even in early stages, predicts recurrence and poor survival. Hematogenous spread causes early distant metastases, commonly to the brain, bones, and adrenal glands**.** Metastatic patterns vary by subtype, liver metastases are typical of SCLC, chest wall invaded locally by SCC, while brain metastases occur in SCLC and adenocarcinoma.[40]

1.3.12 Prognosis of lung cancer

In non–small cell lung cancer (NSCLC), prognosis is affected by several factors, including the presence of lung-related symptoms, tumor size larger than 3 cm, non-squamous histological subtype, disease stage, spread to multiple lymph nodes, and vascular invasion. For patients who cannot undergo surgery, poor performance status and weight loss of more than 10% are linked to unfavorable outcomes.[41]

In small cell lung cancer (SCLC), key prognostic indicators include performance status, disease stage, and metastasis to the central nervous system or liver at the time of diagnosis.[41]

1.3.13 Treatment of lung cancer

1- Treatment of NSCLC depends on **stage, tumor characteristics, and patient health**.

- **Early stages (0–II):** Surgery is primary, sometimes with chemotherapy,
- radiation, or alternative local therapies.[42]
- **Stage III:** Combination therapy with surgery, chemo, radiation, and immunotherapy; targeted drugs for specific mutations.[42]
- **Stage IV:** Mainly palliative, using chemo, immunotherapy, targeted therapy, radiation, or local treatments**.** Gene testing guides targeted therapy and improves survival in advanced cases.[42]

2- Treatment of SCLC is well respond to chemotherapy and radiation but often relapses.

- **Limited stage-SCLC:** Concurrent chemo-radiation with early hyper
- fractionated Radiotherapy.[43]
- **Extensive-SCLC:** Chemotherapy alone.
- Prophylactic cranial irradiation is used for responders to reduce brain metastases.[43]

CHAPTER TWO PATIENTS AND METHOD

2. PATIENTS AND METHOD

This is a prospective study, carried out in Basrah. The data collected incidentally from Al-Sadr Teaching Hospital and private laboratories during the period from the first of October 2024 to the end of October 2025.

2.1 Patients and data collection

The patients included in the study were those diagnosed as lung cancer. Tissue biopsies from true-cut needle specimens were collected. Formalin fixed, paraffin embedded, 3-5µm thick sections were obtained. The following parameters were recorded

- Age and sex of patients.
- Histological types of lung cancer.
- Results of immunohistochemical staining (IHC) for EGFR protein expression.
- Results of Real time polymerase chain reaction (PCR) for EGFR mutation.

2.2 MATERIALS

The equipment used in processing and staining of the sections were displayed in the following box

Equipment & Instruments
BOND-MAX Fully Automated IHC & ISH Strainer (from Leica bio-systems)
Fully Automated real-time PCR
Super frosted microscopic slides
Microscopic slides (positively charged)
Cover slip
Slide holder
Microtome
Drying oven
Microwave
Water bath
Light microscope

Glass staining jar
Slide marker pen
Rubber gloves
Tissue paper
Timer

The Bond Polymer Refine Detection kit from Leica Biosystems, code number DS9800, was used in the study as Immunohistochemical specific detection kit, as shown in the following box

Immunohistochemical specific detection kit
Peroxidase Block: 3-4% (Leica Biosystems) Hydrogen peroxide
Post Primary: Rabbit anti mouse IgG in 10% animal serum in buffered saline 0.09% (prodin) / HRP conjugate
DAB substrate Buffer (polymer), buffered solution contain ≤ 0.1% hydrogen peroxide in the stabilizer solution
DAB chromogen 3.3 diaminobenzidine tetrahydrochloride hydrate in stabilizer solution
Hematoxylin < 0.1%.

The AmoyDX FFPE DNA Kit is specially designed for isolation and purification of DNA from formalin –fixed, paraffin –embedded tissue section. The content of the kit shown in the following box.

Content of DNA extraction KIT
DNA spin columns
Collection tubes (2ml)
Centrifugal tubes (1.5 ml)
Buffer DTL
Proteinase K solution
Buffer DES
Buffer DTB
Buffer DW1
Buffer DW2
Buffer DTE

The AmoyDX EGFR Mutations Detection kit is Real-time PCR assay for qualitative detection mutation in human genomic DNA. The content of detection kit shown in the following box:

Content of EGFR mutation detection kit
EGFR reaction mix (primers, probes, Mg2+, dNTPs)

EGFR enzyme mix (Taq DNA polymerase, Uracil-N-Glycosylase)
EGFR positive control (plasmid DNA)

2.3 METHODS

Tissue blocks that had been formalin-fixed and embedded in paraffin were collected. Thin sections, measuring 3 to 5 micrometers in thickness, were prepared and stained using standard hematoxylin and eosin (H&E) techniques. These stained slides were then reviewed to determine the tumor's histological type and grade, following the 2019 updated WHO tumor classification. Then additional sections using positively charged slides were provided for Immunohistochemical staining and EGFR mutations. Each staining run included an internal control to ensure accuracy.

Hematoxylin and eosin staining

- staining with hematoxylin solution for 5 to 10 minutes
- washing with tap water
- washed with ammonia water until stain turns blue
- washing with tap water
- counterstain with eosin 1% for 15-30 seconds
- washing with tap water
- dehydration with alcohol
- Extract the alcohol with xylene.
- Add one or two drops of mounting medium (DPX) and cover with a Coverslip.

Immunohistochemical staining

Staining for EGFR was performed by BOND-MAX fully automated IHC stainer, using Leica Bio systems detection kit. For EGFR staining, positive result was given for any percentage of definite membranous and/or cytoplasmic staining, weather it is focally or diffusely stained.

- The slides were incubated with hydrogen peroxide (3-4%) to block endogenous peroxidase activity.
- EGFR monoclonal antibody was applied as primary antibody that have been specifically optimized for use with Bond Polymer Refine Detection.
- Deparaffinization: is done by placing 2~5 sections of tumor tissue in a 1. 5mL centrifugal tube and addition of 1 ml xylene and centrifuging the tubes at 13000x g for 2 min at room temperature.

- Then addition of 1mL ethanol with concentration of (96 - 100%) and centrifuging the tubes at 13000xg for 2 min at room temperature. Then incubate the tubes at room temperature for 10 min, or at 37OC for 5 min.
- Rabbit anti mouse IgG reagent was applied as secondary antibody to localized the primary antibody.
- DAB chromogen (3.3 diaminobenzidine) visualized the complex as a brownish precipitate.
- Counterstaining with hematoxylin, Dehydrate, clear and mount with DPX. The stained sections were examined by two histopathologists to determine the result of EGFR mutation.

The intensity of EGFR reactivity in malignant cells was scored using a four-tier system 50 as follows:

- **0**: No reactivity.
- **1+:** Weak reactivity. Faint or light brown reactivity that was membranous, cytoplasmic, or both.
- **2+:** Moderate reactivity. Shades of brown staining of intermediate darkness (intensity). Membranous reactivity was usually, but not always, complete, producing a circular outline of the neoplastic cell.

Incomplete membrane reactivity of moderate intensity was also considered 2+.

- **3+:** Strong reactivity. Dark brown to black staining that was usually, but not always, in a complete membrane pattern, producing a thick outline of the cell.

Many cells with 3+ membrane reactivity also had moderately intense cytoplasmic reactivity.

The steps for DNA extraction and EGFR mutation detection using RT- PCR

Looking for EGFR mutation from extracted DNA was performed using real-time polymerase chain reaction (PCR), using the AmoyDX EGFR Mutations detection kit. The result was examined by histopathologists and geneticist.

Deparaffinization is done by

- Placing 2~5 sections of tumor tissue in a 1.5 mL centrifugal tube and addition of 1 ml xylene and centrifuging the tubes at 13000x g for 2 min at room temperature.

- Then addition of 1mL ethanol with concentration of (96 - 100%) and centrifuging the tubes at 13000xg for 2 min at room temperature.
- Then incubate the tubes at room temperature for 10 min, or at 37'C for 5 min.

DNA Extraction steps

- For lysis of sample DNA Add 180 µL extraction Buffer DTL and 20 µL Proteinase K Solution, mix by vertexing and incubation at 56 C for 1 hour.
- Add 10 µL Buffer DES, Transfer the centrifugal tube to heated orbital incubator and incubate at 90'C for 1 hour.
- Add 200 µL Buffer DTB and 200 µL ethanol (96~100%), mix by vertexing and briefly centrifuge for 5~10 seconds.
- Transfer the entire lysate to the DNA Spin Column (in a 2 ml collection tube) without wetting the rim, close the lid, and centrifuge at 10000xg for 1 minute.
- Discard the flow. through in collection tube.
- Add 600 µL Buffer DW1 to DNA Spin Column, centrifuge at 10000xg for 1 min.
- Discard the flow-through in collection tube.
- Add 600 µL Buffer DW2 to DNA Spin Column, centrifuge at 10000%g for 1 min.
- Place the DNA Spin Column in a clean 2 mL collection tube, centrifuge at 13000~g for 3 min.
- Discard the collection tube with flow-through.
- Place the DNA Spin Column in a clean 1.5 mL centrifugal tube.
- Apply 30-100 µL Buffer DTE to the center of the membrane.
- Incubate at room temperature for 1~5 min.
- 2.18 Centrifuge at 13000xg for 1 min.
- The eluted DNA is immediately ready for use or for storage under -20°C.

Mutation detection steps

- Centrifuge EGFR Enzyme Mix for 5~10 seconds prior to use.
- Prepare sufficient EGFR Master Mix containing EGFR Reaction Mix and EGFR Enzyme Mix in a separate sterile centrifuge tube. Mix EGFR Master Mix thoroughly by vortexing, and centrifuge for 5~10 seconds.
- Dispensing 35 µL EGFR Master Mix to each PCR tube.
- Take out the sample DNA and Add 5 µL of No template control (NTC) and 5 µL each sample DNA, 5 µL positive control (PC) to a PCR tube with 35 µL EGFR Master Mix

respectively, and cap the PCR tubes.

- Briefly centrifuge the PCR tubes to collect all liquid at the bottom of each PCR tube and Place the PCR tubes into the appropriate positions of the real-time PCR instrument.

2.4 Statistical analysis

Statistical analysis of the collected data was performed using SPSS software, version 26. Categorical (qualitative) variables were expressed as frequencies and percentages. The distribution of continuous (quantitative) variables was assessed for normality using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Quantitative results were presented as mean ± standard deviation. To examine associations between categorical variables, Fisher's Exact Test was applied. Differences in quantitative data across groups were evaluated using the One-Way ANOVA test. A p-value ≤ 0.05 was considered statistically significant.

CHAPTER THREE THE RESULTS

3. RESULTS

3.1 Demographic characteristics

The study included 46 patients. The mean age was 63.91 years (ranging from 40 to 85 years). Table-1 shows the distribution of patients according to the age groups. Thirty-Five (76.1%) were males and eleven (23.9%) were females (Table-2).

Table-1: Age distribution of the study sample.

Variable	Frequency	Percent
Age (Year):	63.91±10.91	
Mean ± SD	65 (40-85)	
Median (Min.-Max.)		
Younger than 51	7	15.2
From 51 to 60	8	17.4
From 61 to 70	17	37.0
Older than 70	14	30.4

Table 2: Sex distribution of study sample.

Sex	Frequency	Percentage
Male	35	76.1%
Female	11	23.9%
Total	46	100.0%

3.2 Histological types of lung cancer

The majority of cases were diagnosed as adenocarcinoma (33/46 cases, 71.7%) followed by Squamous cell carcinoma (9/46 cases, 19.6%) and metastatic adenocarcinoma (4/46

cases,8.7%), as shows in figure-1. Unfortunately, no case of small cell carcinoma was recorded during the period of the study.

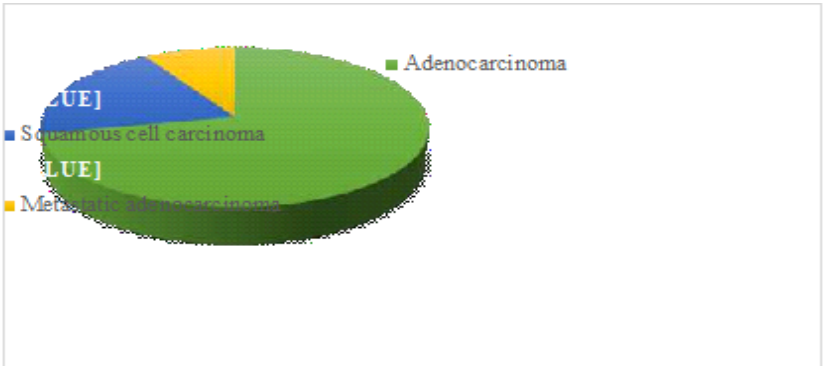

Figure-1: Distribution of histological types of lung cancer.

3.3 EGFR protein expression by IHC

EGFR protein expression was negative in 23/46 cases (50.0%), of which 19 cases were males and 4 cases were females. Equivocal expression in 11/46 cases (23.9%). Positive EGFR protein expression was seen in 12/46 cases (26.1%) including 8 cases males and 4 cases females; among the positive cases, 10/12 cases (21.8%) showed score +2 and 2/12 cases (4.3%) were score +3 (Table-3).

Table (3): Distribution of cases according to EGFR expression by Immunohistochemistry.

IHC	Frequency	Percentage
Negative	23	50.0%
Equivocal	11	23.9%
Score +2	10	21.8%
Score +3	2	4.3%
Total	46	100.0%

3.4 EGFR mutation by RT-PCR

Molecular analysis of EGFR mutations revealed that the majority of cases were negative (40/46 cases, 87.0%). Exon 19 mutations were detected in (5/46 cases, 10.9%), while Exon 18 mutation was identified in (1/46 case, 2.1%), among the EGFR-positive cases, 5 cases were males and 1 case was female (Table-4).

Table 4: Distribution of cases according to EGFR mutation by RT-PCR.

RT-PCR	Frequency	Percentage
Negative	40	87.0%
Positive Exon 19	5	10.9%
Positive Exon 18	1	2.1%
Total	46	100.0%

3.5 Immunohistochemistry versus RT-PCR in EGFR mutation

The association between the findings of IHC testing and mutation testing was investigated to determine sensitivity, specificity, predictive values, and total agreement and misclassification rates (Table-5).

Table-5: Validity and predictivity of IHC against RT-PCR.

		Real Time PCR		Predictive value (PV)
		Positive for EGFR	**Negative for EGFR**	
IHC	Positive	True positive 0	positive 12	Positive PV TP/(TP+FP) 0/(0+12) 0%
	Negative	False negative 6	True negative 28	Negative PV TN/ FN+TN) 28/(6+28) 82.4%
		Sensitivity TP/(TP+FN) 0/(0+6) 0%	Specificity TN/(FP+TN) 28/(12+28) 70%	

True Positive: test positive & mutation actually present. **False Positive**: test positive but mutation absent. **True Negative**: test negative& mutation absent. **False Negative**: test negative but mutation actually present.

-Total agreement rate= **60.9%.**

-Total misclassification rate= **39.1%.**

3.6 Diagnostic performance between IHC and RT-PCR

The curve shows that the Receiver Operating Characteristic (ROC) is 31.3% only (Figure-2).

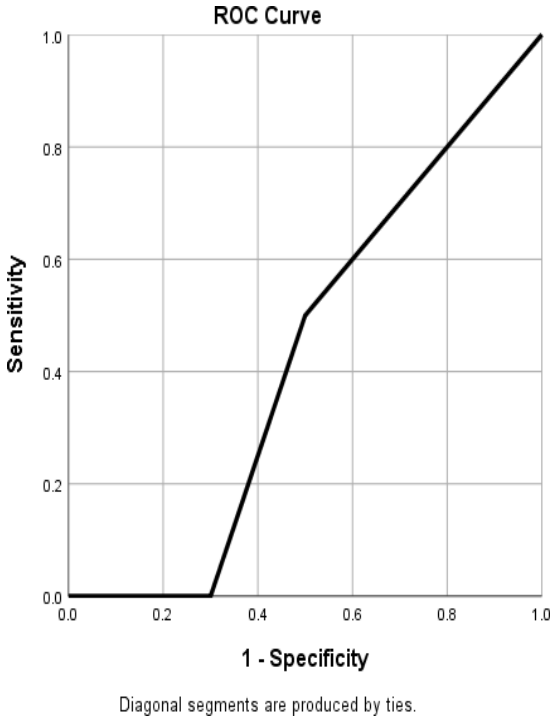

Figure-2: ROC curve of testing IHC results against RT-PCR results.

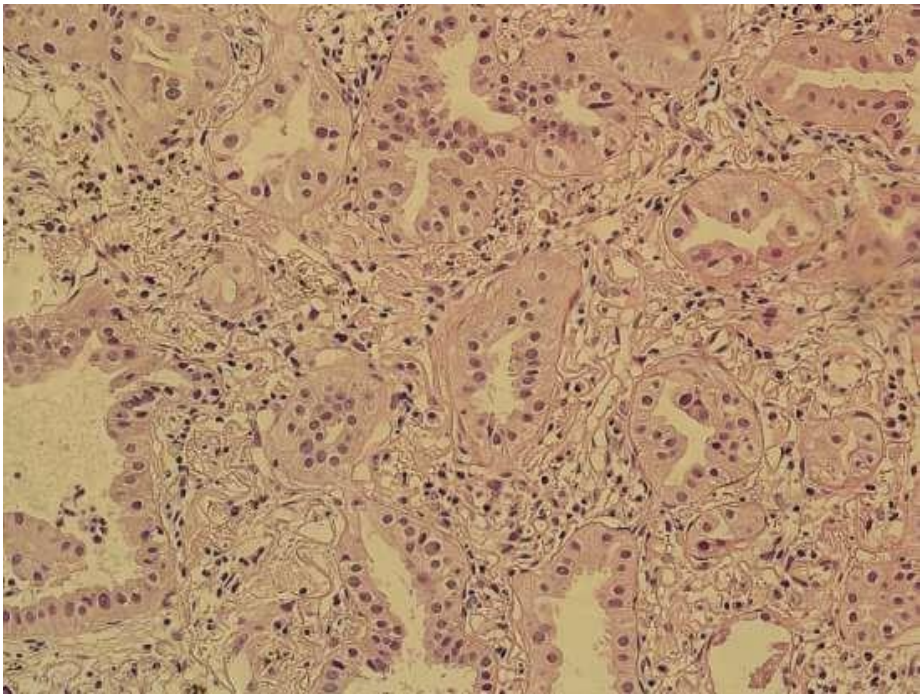

Figure-3: Invasive lung adenocarcinoma, predominant acinar type, grade-2 (H&E stain, 100x).

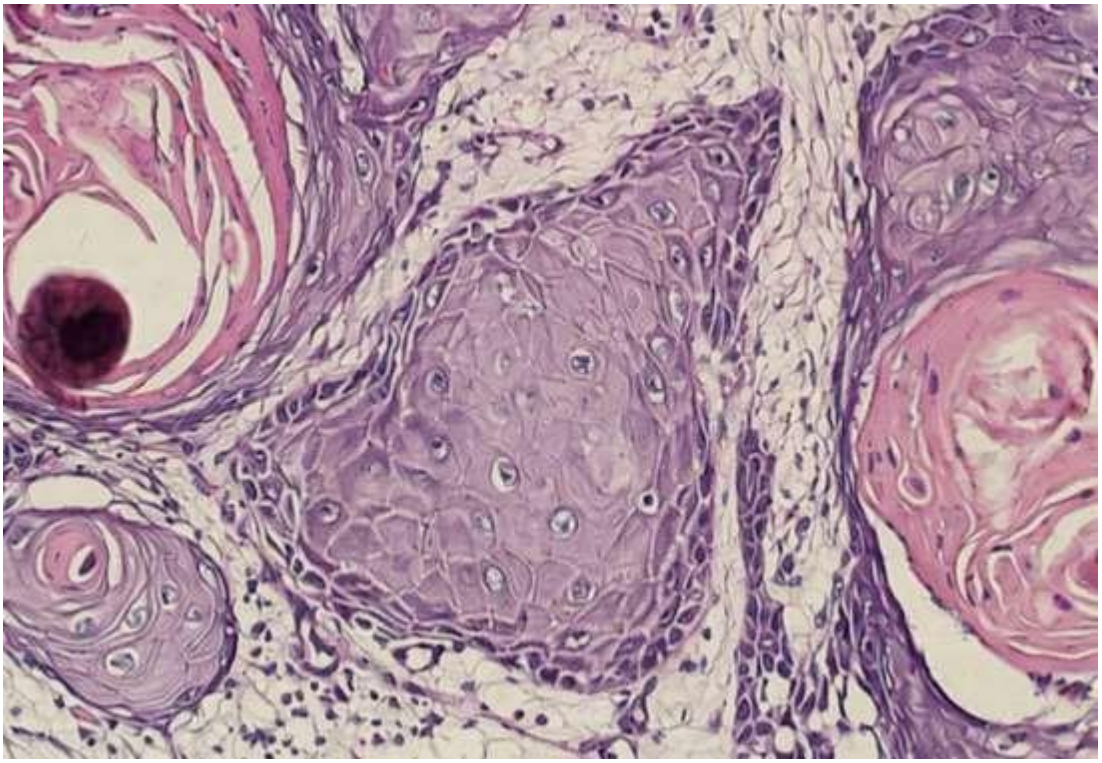

Figure-4: Invasive lung squamous cell carcinoma, grade (H&E stain, 400x).

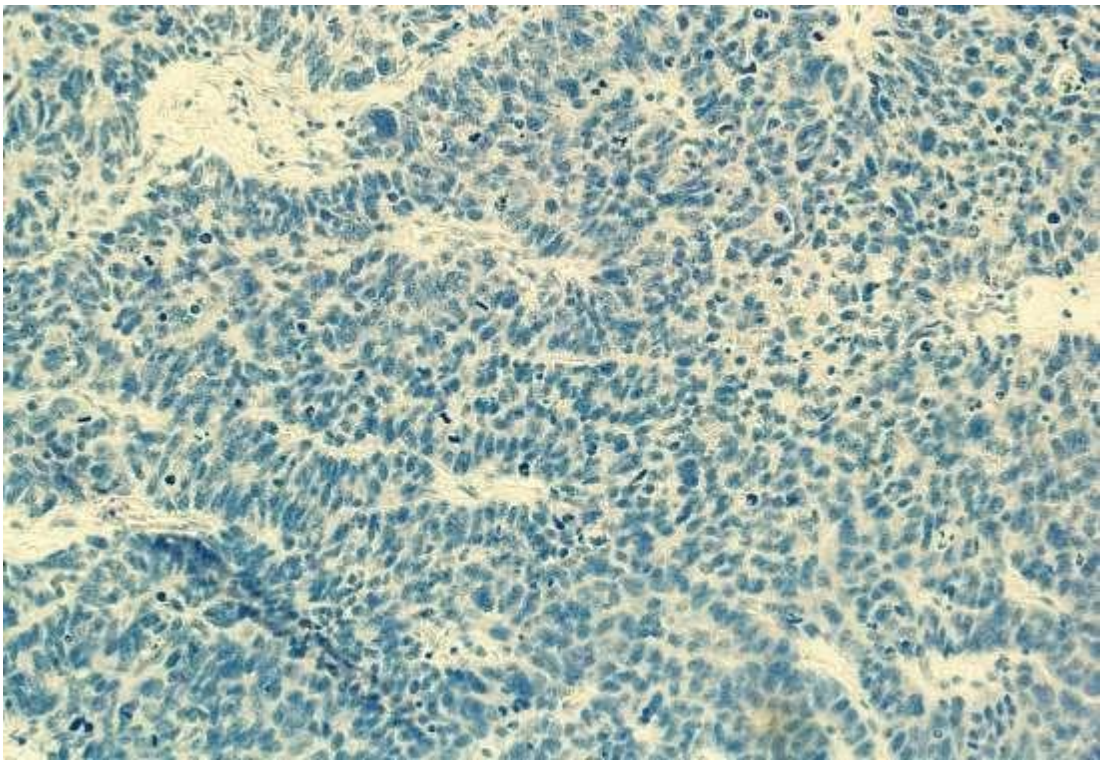

Figure-5: Lung adenocarcinoma, EGFR Negative. No cytoplasmic and/or membranous reactivity (IHC stain 100x).

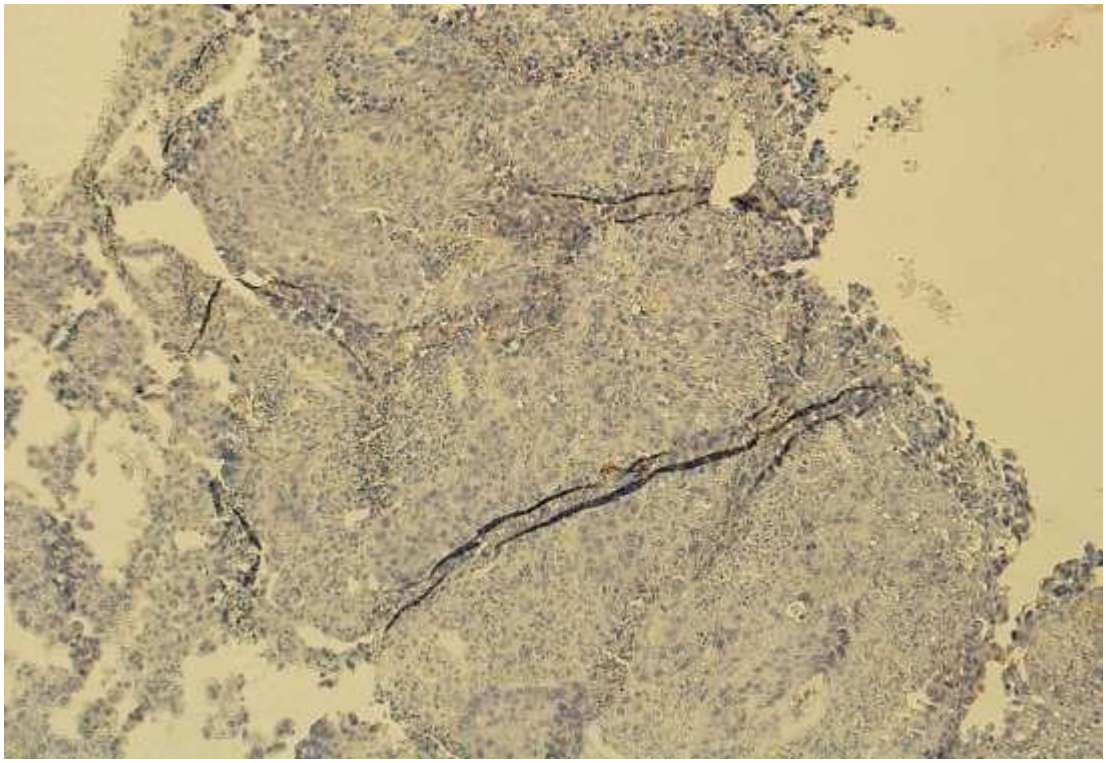

Figure-6: Lung squamous cell carcinoma, EGFR Negative. No cytoplasmic and/or membranous reactivity (IHC stain 100x).

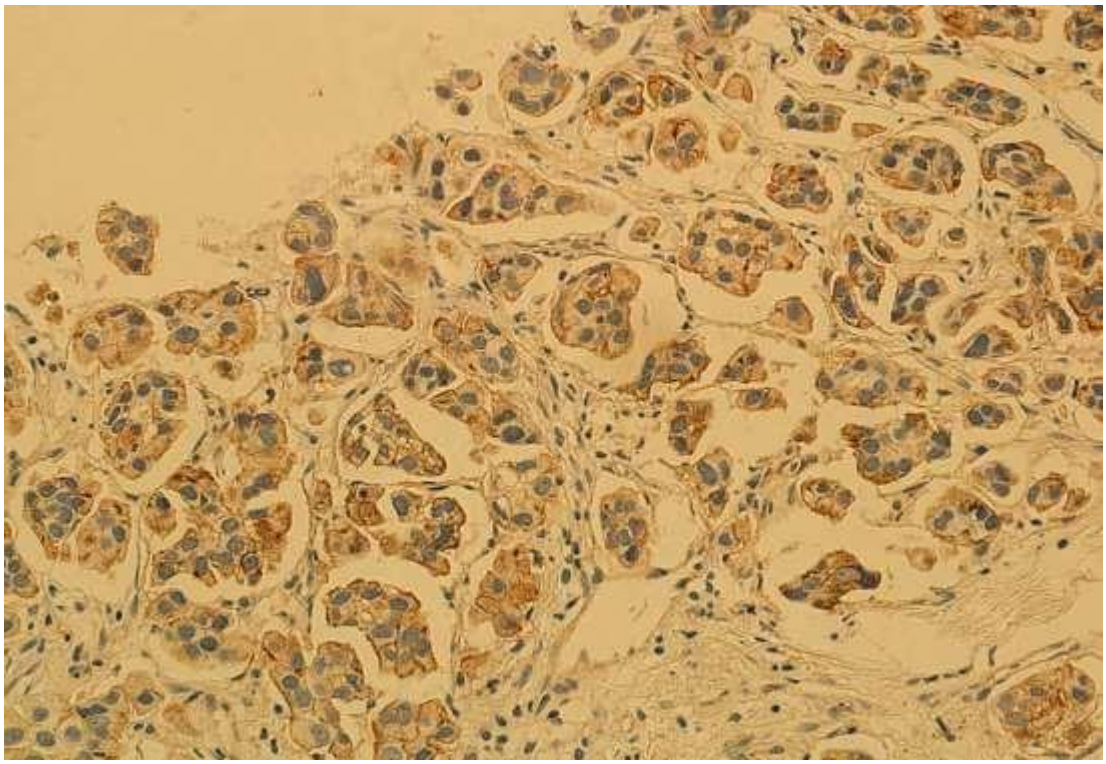

Figure-7: Lung adenocarcinoma, EGFR Equivocal (score+1). Weak cytoplasmic and membranous reactivity (IHC stain 100x).

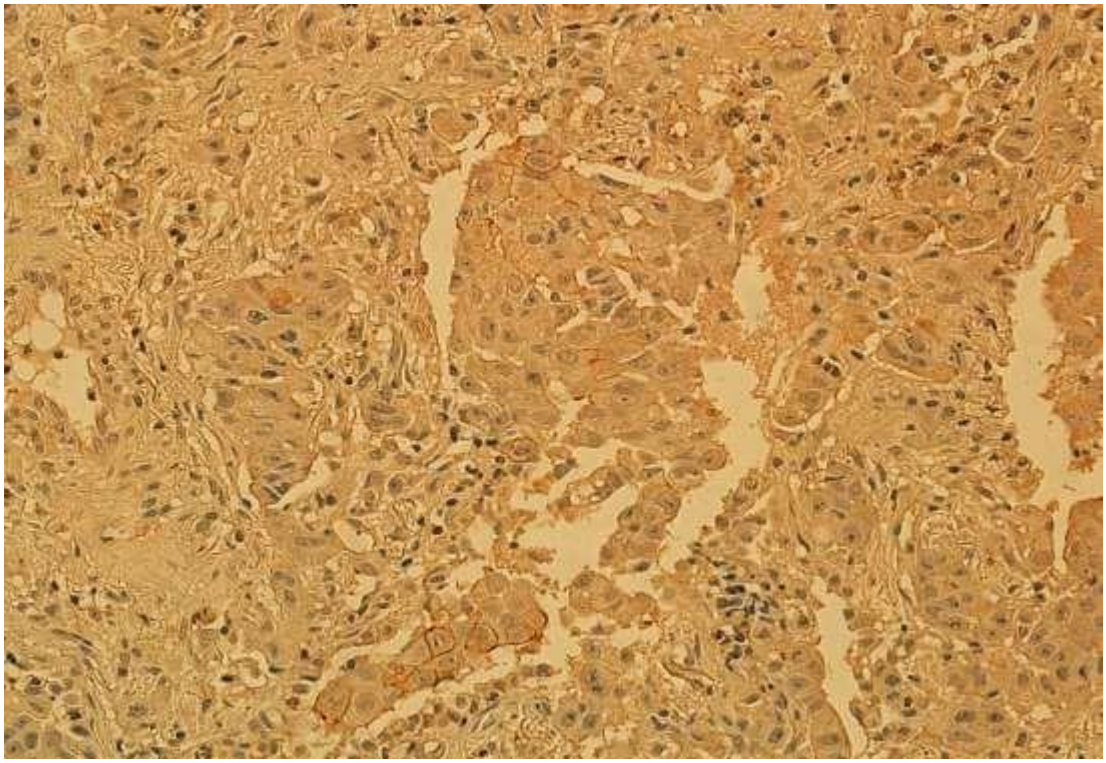

Figure-8: Lung squamous cell carcinoma, EGFR Equivocal (score+1).

Weak cytoplasmic and membranous reactivity (IHC stain 100x).

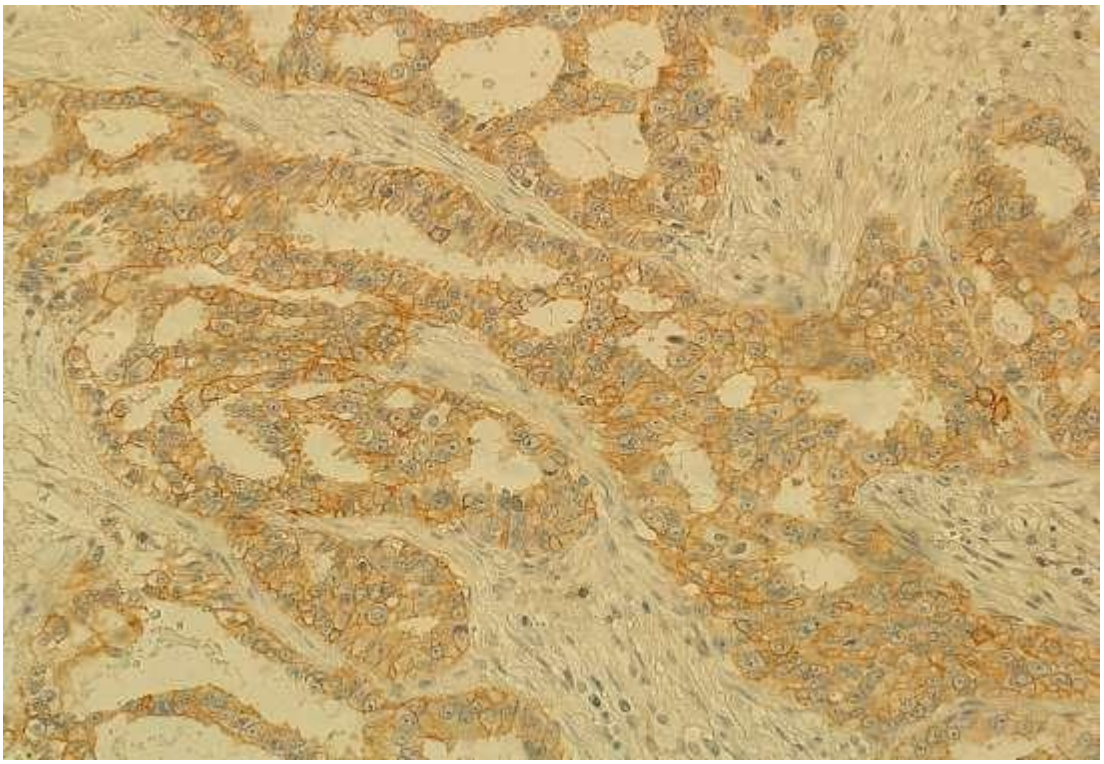

Figure-9: Lung adenocarcinoma, EGFR Positive (score+2).

Moderate membranous reactivity (IHC stain 100x).

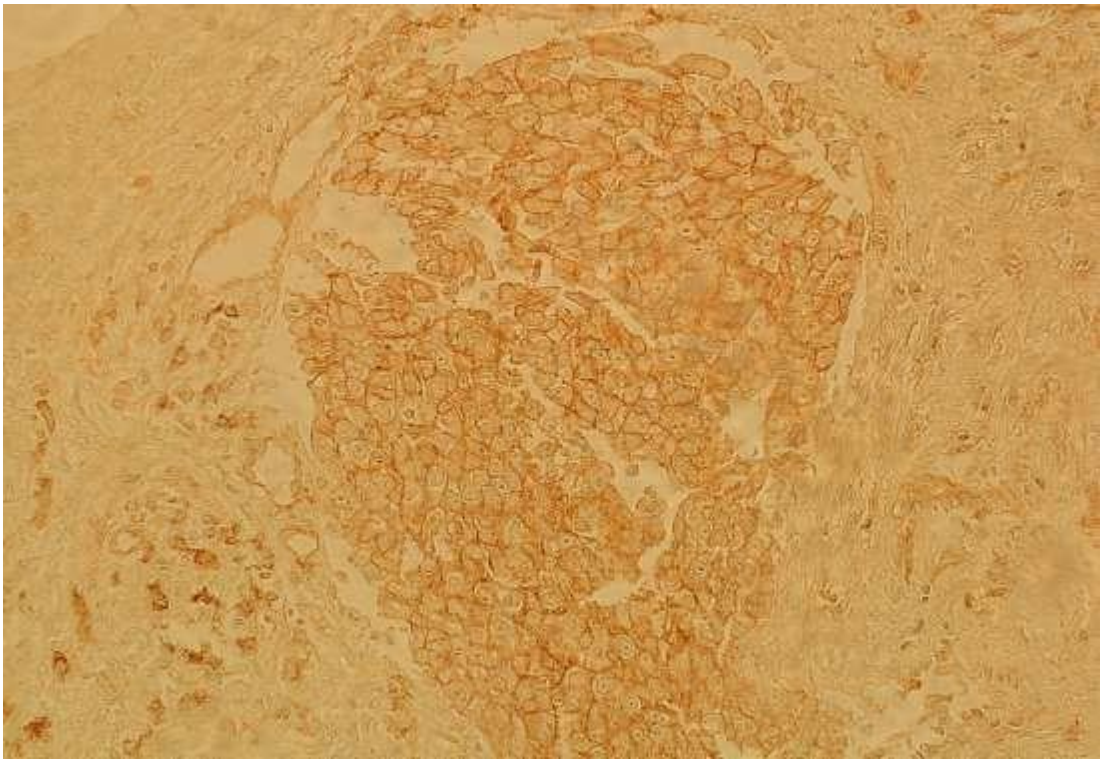

Figure-10: Lung squamous cell carcinoma, EGFR Positive (score+2). Moderate membranous reactivity (IHC stain 100x).

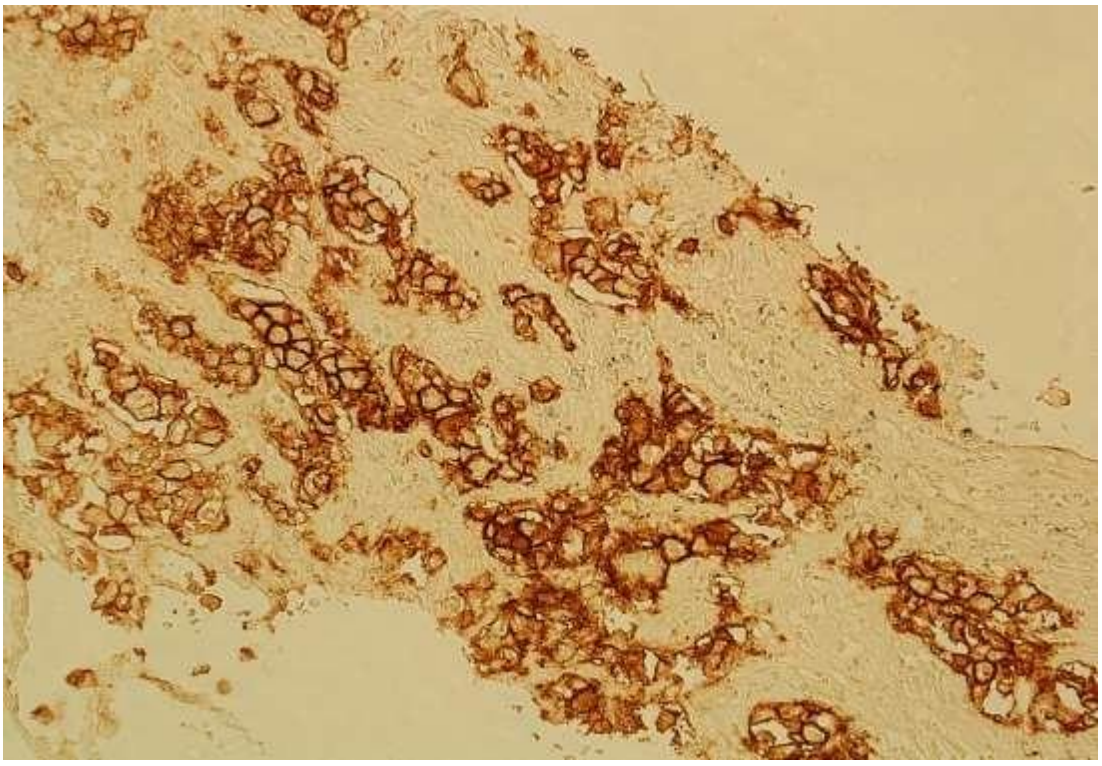

Figure-11: Lung adenocarcinoma, EGFR Positive (score+3). Strong membranous reactivity (IHC stain 100x).

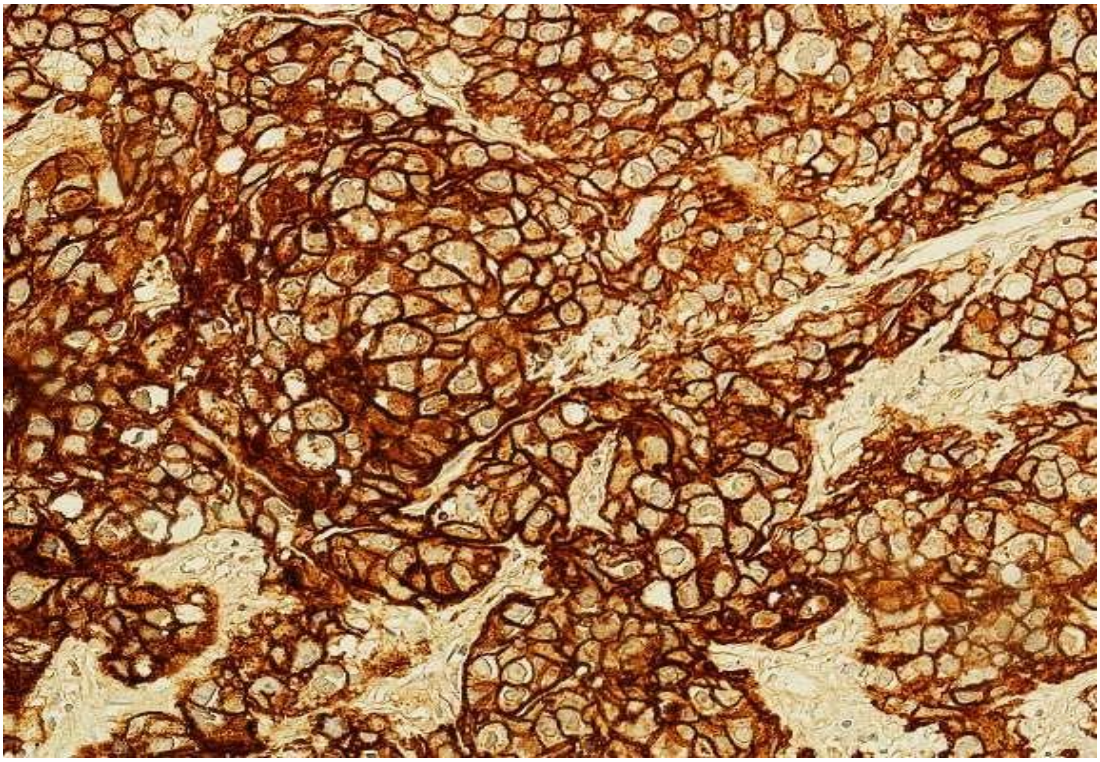

Figure-12: Lung squamous cell carcinoma, EGFR Positive (score+3). Strong membranous reactivity (IHC stain 400x).

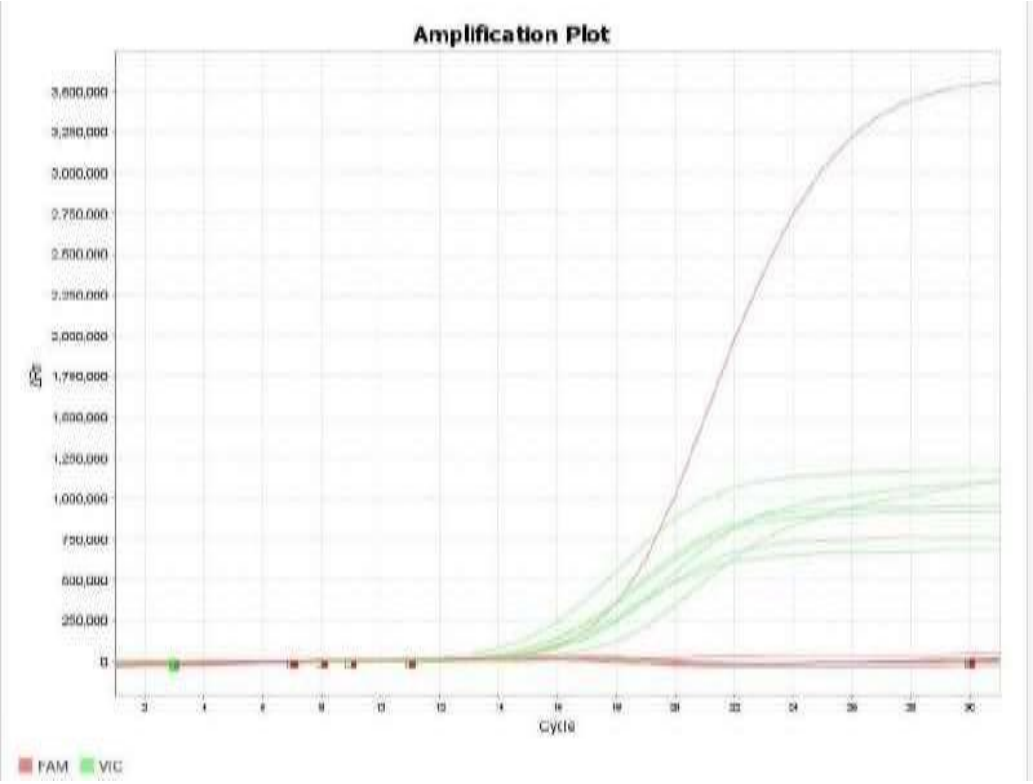

Figure-13: Lung carcinoma with positive EGFR mutation by using RT-PCR.

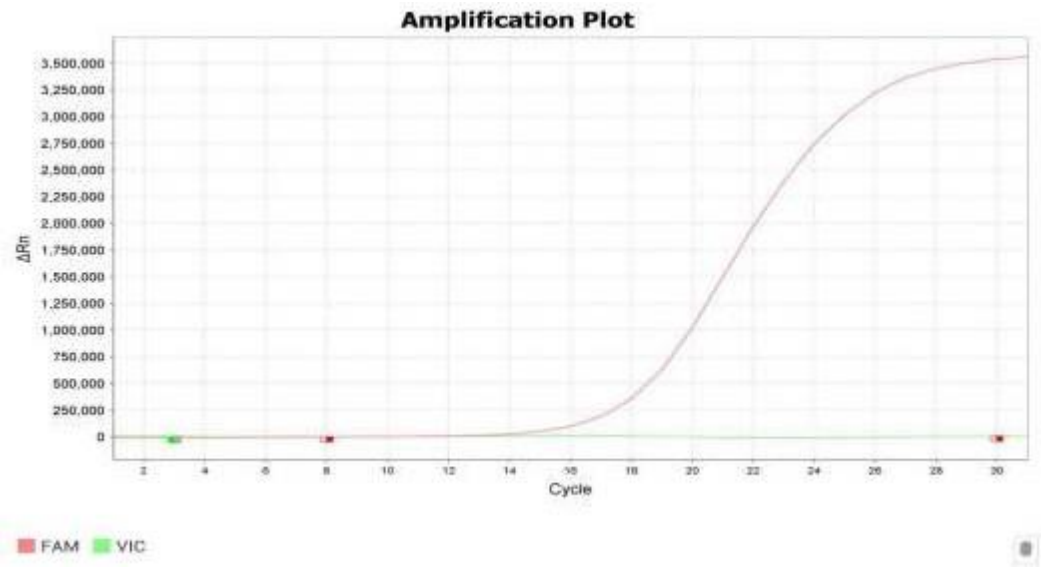

Figure-14: Lung carcinoma with negative EGFR mutation by using RT-PCR.

CHAPTER FOUR DISCUSSION

4. DISCUSSION

Lung cancer, particularly non-small cell lung cancer (NSCLC), constitutes a major health burden due to its high incidence and mortality. Although current modalities such as surgery, chemotherapy, radiotherapy, and targeted therapy are available, the overall prognosis remains poor. Recent advances in biotechnology have introduced promising directions for innovative treatment strategies.[6]

EGFR activating mutations most commonly occur in adenocarcinomas, non-smokers, females, and Asian patients.[44] Mutations in the EGFR gene, especially within the tyrosine kinase domain, trigger downstream signaling pathways that promote cancer development. Detecting these mutations has enabled the creation of targeted treatments, like tyrosine kinase inhibitors (TKIs), which have transformed NSCLC management, particularly for patients harboring activating EGFR mutations.[45] Standard PCR-based methods are used for detection with varying sensitivity.[45]

IHC has been extensively studied for EGFR mutation detection. It is a widely accessible technique routinely employed in solid tumor diagnostics across pathology laboratories, offering a cost-effective approach that requires only a small amount of tumor tissue for biomarker assessment.

In the present study, the mean age of patients was (63.91) years, which is nearly identical to

the previous Iraqi study (64 years).[46] and slightly higher than the Middle East and North Africa (MENA) study.[47] and the Pakistani study,[45] where the mean age were 61 years and 60.3 years respectively.

In the current study, a male predominance, consistent with other Iraqi.[47] and Pakistani studies.[45] In contrast, the MENA region study reported a female predominance.[47] These differences may reflect regional variations in smoking habits, occupational exposures, and cultural factors that influence sex-specific risks. Adenocarcinoma was the most common subtype (71.7%) in this study, which is in agreement with the MENA region (88.3%).[47] and the Pakistani studies.[45] (60%). However, this in contrast with another Iraqi study.[48] where squamous cell carcinoma was predominant during the period 2005-2019.

This difference highlights the potential influence of environmental, genetic and lifestyle factors on the histological profile of NSCLC across different populations.

By immunohistochemistry (IHC), this study showed that 26% of cases were positive for EGFR [score +2 (21.8%) and score +3 (4.3%)], including 8 male and 4 female cases. Half of the cases were negative (score 0), comprising 19 males and 4 females, while 23.9% were equivocal (score +1). Although the Pakistani study reported a higher EGFR positivity rate (44%) and 56% negative cases, both studies are in agreement in that EGFR positivity and negativity were more frequent in males than in females.[45]

In the present study, EGFR mutations were detected in 13% of patients, which is lower than the previously reported prevalence (27%) in Iraq by Ramadhan et al (2021).[49] In the broader MENA region,[47] the overall prevalence was 18%. Despite these differences in overall frequency, Exon-19 deletions were consistently the most common subtype across all studies. In the present study, EGFR-positive cases were predominantly male, which contrasts with findings from Chinese.[50] and Egyptian.[51] studies that reported a higher prevalence among females. This discrepancy may be attributed to differences in patient selection, tumor histology, smoking status, ethnic background, or technical variability in mutation detection methods.

The sensitivity of IHC in detecting EGFR mutation is 0% and specificity 70%. The positive predictive value of IHC, which refers to the probability that a person with a positive IHC result truly has positive EGFR mutation, was 0%. While the negative predictive value,

which refers to the probability that a person with a negative IHC result truly negative for EGFR mutation, was 82.4%. The total agreement rate was 60.9%, with a misclassification rate of 39.1%.

These results are lower than those reported in a Slovenian study.[52] which demonstrated a sensitivity of (84.8%) and specificity of (100%) and Korean study.[53] which demonstrated a sensitivity of 75.6% and specificity of 94.5%, with PPV of 85% and NPV of 90.4%. The low sensitivity of IHC in detecting EGFR mutation may be due to small sample size, differences in the type of antibodies used for IHC, technical and pre-analytical factors, scoring criteria and tumor heterogeneity.

The ROC curve showed the diagnostic performance between IHC and RT-PCR is only 31.3%, which is categorized as a very poor performance. This result is disagreement with Slovenian.[52] and Korean[53] studies that showed Immunohistochemistry can be useful without need for further molecular study.

CHAPTER FIVE CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSIONS

- Immunohistochemistry (IHC) demonstrated low sensitivity and limited concordance with RT- PCR in detecting EGFR mutations, indicating that IHC alone is insufficient to evaluate EGFR mutations, emphasizing that molecular testing remains essential for the diagnosis.
- The diagnostic performance between IHC and RT- PCR is only 31.3%, which is categorized as a very poor performance.
- The prevalence of EGFR mutations was 13%, lower than previously reported in Iraq and neighboring regions, though exon 19 deletions remained the most frequent subtype.
- Non-small cell lung cancer (NSCLC) is predominantly affects individuals in the sixth decade of life, with a male predominance.
- Adenocarcinoma as the most common histological subtype.

5.2 Recommendations

- Real-time PCR or other validated molecular methods should remain the gold standard for EGFR mutation detection in NSCLC, as IHC alone is insufficiently reliable.
- Future studies with larger patient cohorts are recommended to provide more robust estimates of EGFR mutation prevalence and to better evaluate the diagnostic utility of

IHC.

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54. APPENDICES

Appendix

Classification of TNM in lung cancer (UICC, 2025)

T – Primary Tumor
• TX: Tumor cannot be assessed.
• T0: No evidence of a primary tumor.
• Tis: Carcinoma in situ (very early cancer, not invasive).
• T1: Tumor ≤3 cm in greatest dimension, surrounded by lung or visceral pleura, without invasion more proximal than the lobar bronchus.
• T1a: ≤1 cm
• T1b: >1 cm but ≤2 cm
• T1c: >2 cm but ≤3 cm
• T2: Tumor >3 cm but ≤5 cm or has certain features like visceral pleura./central invasion.
• T2a: >3 cm but ≤4 cm
• T2b: >4 cm but ≤5 cm
• T3: Tumor >5 cm but ≤7 cm or directly invading nearby structures such as the chest wall, diaphragm, or pericardium or Separate tumor nodule(s) in the same lobe as the primary.
• T4: Tumor >7 cm or invading more critical structures:
((mediastinum - diaphragm - heart - large dishes - trachea - recurrent laryngeal nerve - esophagus - circles - main spur)) or with separate tumor nodules in a different lobe of the same lung or Separate tumor nodule(s) in a different ipsilateral lobe than that of the primary.
N – Regional Lymph Nodes
• NX: Regional lymph nodes cannot be assessed.
• N0: No regional lymph node metastasis.
• N1: Metastasis in ipsilateral (same side) peribronchial and/or hilar lymph nodes.
• N2: Metastasis in ipsilateral mediastinal and/or subcarinal lymph nodes.
• N2a: Single N2 station involvement.
• N2b: Multiple N2 station involvement.
• N3: Metastasis in contralateral mediastinal, hilar, or supraclavicular lymph nodes.
M – Distant Metastasis
• M0: No distant metastasis.
• M1: Distant metastasis present.
• M1a: Tumor with pleural or pericardial nodules or malignant pleural or pericardial effusions, a separate tumor nodule(s) in a contralateral lobe.
• M1b: Single extra thoracic metastasis in one organ.
• M1c: Multiple extra thoracic metastases.
• M1c1: Multiple extra thoracic metastases in one organ.
• M1c2: Multiple metastases in >1 organ systems.