Role of Genetic Variant in the promoter region of h*TERT* gene and its association towards Lung Cancer pathogenesis

A Thesis

Submitted in partial fulfilment of the requirements for the award of Degree of

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In

Biotechnology

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DECLARATION

I hereby declare that the work done in the seminar report entitled, "**Role of Genetic Variant in the promoter region of h*TERT* gene and its association towards Lung Cancer pathogenesis"** submitted towards partial fulfilment of the requirement for the award of **Master of Technology degree** in Biotechnology in the **Department of Biotechnology** at **Thapar Institute of Engineering and Technology, Patiala,** is an authentic record of work carried out by me under the supervision and guidance of Dr. Siddharth Sharma, Professor at the Department of Biotechnology in Thapar Institute of Engineering and Technology, Patiala.

I further declare that the contents of this report are a true representation of my independent work and research, and have not been submitted, either wholly or in part, to any other university or institute for the award of any degree or diploma.

Anjali

ANJALI

DATE:

This is to certify that the above declaration made by the student concerned is correct to the best of my knowledge and belief.

Siddha

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CERTIFICATE

This is to certify that the dissertation entitled, **"Role of Genetic Variant in the promoter region of h*TERT* gene and its association towards Lung Cancer pathogenesis"** submitted by **Anjali** in partial fulfilment of the requirements for the award of MSc in Biotechnology at Thapar Institute of Engineering and Technology, Patiala is an authentic work carried out by her under the supervision and guidance.

To the best of my knowledge, the matter embodied in the dissertation has not been submitted to any other university/institute for the award of any Degree or Diploma.

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ABSTRACT

Background: Cancer is fundamentally a genetic age-related disease that only appears when normal cells start to amass genomic instability and develop the ability to proliferate indefinitely. In genomic rearrangements that might cause chromosomal instability and cancer, telomere attrition during subsequent cell divisions is essential. Cancer cells need telomeres, which are repetitive (TTAGGG) DNA–protein complexes located at the ends of chromosomes, to survive. Primary cells have a finite number of divisions possible. This restriction is also known as the Hayflick restriction. In most eukaryotes, the ribonucleoprotein complex telomerase is required for the upkeep of linear chromosomes. It consists of a long non-coding RNA that contains the reverse transcriptase catalytic subunit and the template sequence needed to generate telomeres. The two main components of telomerase are human telomerase reverse transcriptase (hTERT), a catalytic subunit with reverse transcriptase activity, and human telomerase RNA component (hTR), an RNA component that serves as a template for telomere lengthening.

Purpose: We aim to investigate the Role of Genetic Variants in the promoter region of h*TERT* gene and its association towards Lung Cancer pathogenesis in the North Indian Population.

Experimental Design: The study was performed on 387 cases and 384 controls, all receiving platinum-based chemotherapy, and the hTERT polymorphism is being investigated by PCR for potential links to lung cancer risk. Following conducting this association analysis, 95% confidence intervals (CIs) and the adjusted odds ratio (AOR) were obtained using logistic regression. The adjusted hazard ratio was determined by Cox regression analysis, and the univariate Kaplan-Meier approach was utilised to assess the overall survival of lung cancer patients. A p-value of < 0.5 was considered statistically significant in all analyses.

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CHAPTER 1

INTRODUCTION

Lung cancer is the most common cancer and is the leading cause of cancer death worldwide. An uncontrollably developing tumour is the outcome of specific lung cells being malignant. This is known as lung cancer. 10 million deaths worldwide are expected to be caused by cancer in 2020 (WHO, 2021). Cancer is the top cause of mortality globally. Lung cancer accounts for 18.0% of all cancer-related deaths. Colorectal (9.4%), liver (8.3%), stomach (7.7%), and breast cancer (6.9%) are the following most common causes of cancer-related deaths. The hallmark of cancer is unchecked cell proliferation. (Jang *et al*.,2021). India is the second-biggest consumer and producer of tobacco in the world. Approximately 267 million individuals in India use tobacco products, accounting for 28.6% of the nation's total population (42.4% of men and 14.2% of women), according to the Global Adult Tobacco Survey-2 [GATS]-2 2016–2017. Tobacco usage has a significant detrimental impact on health in India, especially concerning cancer (Singh N et al., 2012). Considering the population and number of cancer patients in India, the country's overall infrastructure for cancer care is still insufficient. If these issues are not resolved, India would see higher death rates and lower cancer patient survival rates than wealthier countries. Lung cancer is a significant cause of morbidity and mortality in India, especially for men, and it is expected that the disease will become more common in the years to come. The disease's range has expanded over time, impacting a higher percentage of younger age groups, light or nonsmokers, and females (with adenocarcinoma predominating). Previously, older adults who smoked tobacco and developed squamous or small cell carcinoma were the primary victims of the disease (Singh N et al.,2012).

Cigarette smoking is directly to blame for around 80% of cases of lung cancer. Tobacco smoking is considered the world's leading preventable cause of death due to the increased risk of lung cancer (as well as neoplasms of the bladder, colorectum, and other organs) (Bray F et al., 2018). The most addictive ingredient in tobacco smoke, nicotine, binds to the nicotinic acetylcholine receptors in the brain, altering gene and receptor expression and neurotransmitter levels to induce dependence. Lung cancer propensity is associated with risk factors such as smoking and exposure to certain carcinogenic chemicals. For those who do not smoke, lung cancer may even be brought on by exposure to tobacco smoke or other environmental toxins (Kiyohara *et al*.,2002). About one in ten lifetimes, smokers acquire lung cancer, suggesting that the host factor may affect a person's vulnerability to tobacco smoke. (Kiyohara *et al*.,2002).

Small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) are the two types of lung cancer. Large cell carcinoma (LCC), squamous cell carcinoma (SQCC), and adenocarcinoma (ADCC) are all types of NSCLC, which make up around 85% of all cases of lung cancer. Meanwhile, 15% of instances of lung cancer are still due to SCLC. Compared to NSCLC kinds, SCLC metastasizes to other organs considerably more quickly, and in most cases, if treatment is not received within a few weeks, SCLC can be fatal. (Travis, 2002).

Globally, cancer is one of the most critical health problems in both developed and developing countries. Any cancer treatment aims to destroy or remove the cancerous cells while leaving healthy cells alone. The conventional therapeutic modalities that are most frequently employed are radiation therapy, chemotherapy, and surgery. You can use these separately or in conjunction with each other. Receiving systemic chemotherapy with platinum-based combinations improves survival and quality of life for patients with advanced non-small cell lung cancer (NSCLC) by a moderate amount (Belani CP, 2005). There are variations in survival among patients with similar diseases, even though the disease's stage is one of the significant prognostic factors for lung cancer.

At the ends of eukaryotic chromosomes are preserved, repeating sections called telomeres that protect the integrity of genomic DNA (Akincilar et al.,2016). Cancer is essentially a genetic disease associated with ageing that manifests only when normal cells accumulate genomic instability and acquire the potential to replicate immortally. Telomere attrition during successive cell divisions is crucial in genomic rearrangements that can result in cancer and produce chromosomal instability. Telomeres are repeating (TTAGGG) DNA–protein complexes found at the ends of chromosomes that are essential to the survival of cancer cells. There is a limit to how many times primary cells can divide. Another name for this limit is the Hayflick Limit. Telomere lengthening is a vital process in healthy cells. It can worsen genomic instability and perhaps cause cancer to begin in its early stages when combined with other carcinogenic changes. According to Jafri et al. (2016), telomere length shrinks in human somatic cells grown at 50–150 bp with each cell division. The end replication problem theory states that the terminals of linear DNA cannot be appropriately replicated by lagging strand DNA synthesis (Ohki et al., 2001).

Telomerase is a ribonucleoprotein complex necessary for the maintenance of linear chromosomes in most eukaryotes. It comprises a lengthy non-coding RNA that carries the template sequence for telomere formation and the catalytic subunit of the reverse transcriptase

enzyme (Yadav PS *et al*.,2019).Human telomerase reverse transcriptase (hTERT), a catalytic subunit with reverse transcriptase activity, and the RNA component known as human telomerase RNA component (hTR), which acts as a template for telomere extension, are the two primary components of telomerase. For in vivo telomerase activity, additional components that bind to hTERT and hTR to form the holoenzyme are required (Blackburn et al.,2011).The 42 kb hTert gene has 16 exons and is found on chromosome 5. The exons 5-8-6-7-8-9 code for the reverse transcriptase domain. There is a suggestion that the 16-exon telomerase transcript can be spliced into 22 different isoforms (Hrdlickova R*et al*.,2012); however, the reverse transcriptase activity required for elongating telomeres is only present in full-length Tert transcripts (Akincilar*et al*.,2016).

The polymorphic variant of the hTERT gene under study is TERT rs2735940. This functional polymorphism is located in the promoter region of the hTERT gene. This functional polymorphism involves T>C transition at position -1327 bp upstream of the transcription start site. The polymorphic variant rs2735940 T > C polymorphism increases the transcriptional efficiency of hTERT. Their biochemical investigations discovered that the T allele of the rs2735940 T > C polymorphism had a promoter effectiveness that was almost 25% higher than the C allele (Bayram *et al*.,2016). Increased risk of cancer is linked to the single nucleotide polymorphism (SNP) at the rs2735940 location in the human telomerase reverse transcriptase (hTERT) gene (Wang *etal*.,2017). The hTERT gene's rs2735940 polymorphism has been the focus of extensive research since it has been linked in several studies to an increased and decreased cancer risk.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Cancer

The uncontrolled proliferation of cells with damaged DNA expression characterizes cancer. These cancerous cells continuously divide, displacing normal tissue. Cancer fundamentally arises from disruptions in tissue growth regulation. For a normal cell to transform into a cancerous one, alterations must occur in the genes that govern cell growth and differentiation. (Gilbert, 2011) The accumulation of alterations in the function of critical regulatory genes is associated with the initiation and progression of human solid tumours. The proper functioning of the gene can be disrupted by various factors, such as changes in its copy number or structure. It is widely agreed that the genes of importance for tumour development can be associated with particular recurrent genetic anomalies. (Tsafrir*et al*., 2006)

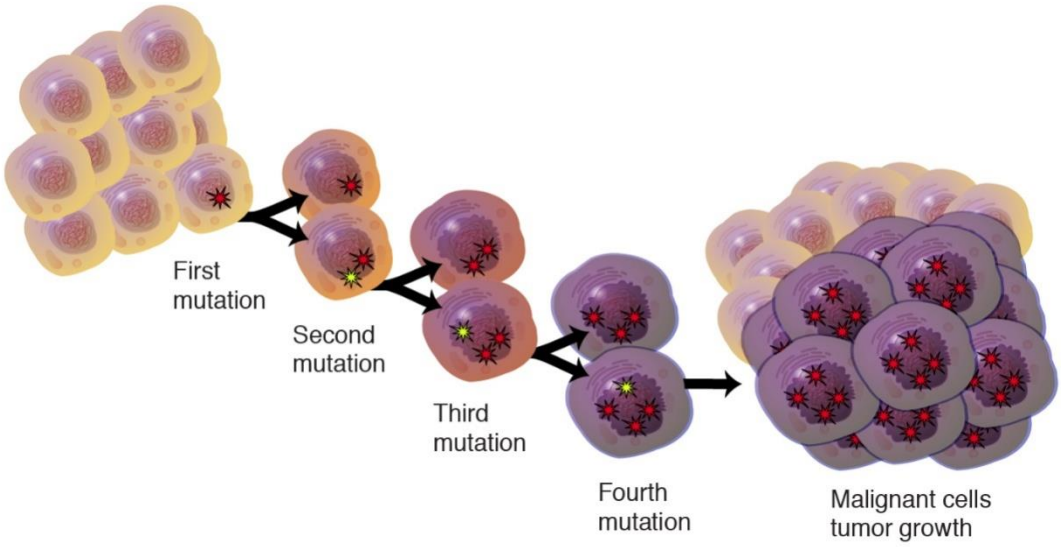


Figure 2.1: **Schematic representation of the neoplastic transformation of a normal cell with DNA damage, lack of repair, and the accumulation of further mutations**. **(Source:** National Human Genome Research Institute (NHGRI)

Note: The neoplastic transformation of a normal cell illustrates that the cell undergoes a loss of identity, structure, and function due to mutations in several genes. This

transformed cell subsequently exhibits uncontrolled and unlimited proliferation, ultimately developing malignant tumours.

2.1.1. Molecular Origins of Cancer

The process by which healthy cells gradually turn into malignant cells involves the sequential acquisition of mutations in the genomic DNA.(John SBertram,2001). Errors in DNA replication or damage to DNA caused by exposure to the environment, e.g., cigarette smoke or UV radiation, may lead to these mutations. Neoplasms, commonly referred to as cancer, can be classified as either benign or malignant. Benign neoplasms remain confined to their tissue of origin, whereas malignant cancers can potentially spread to other organs. Uncontrolled cell division and the development of malignant tumours result from genetic and epigenetic changes within individual cells, giving rise to the intricate and multidimensional disease known as cancer. Developing secondary growths, or metastases, poses a significant complication in treating malignant cancers. A tumour refers to any abnormal mass of cells, which may be benign or malignant. (Gilbert, 2011). Numerous genetic abnormalities, such as point mutations, deletions, gene fusions, and chromosomal rearrangements, are frequently seen in cancer cells. These abnormalities can activate oncogenes or inactivate tumour suppressor genes (Macaluso *et al*.,2003; Podlaha*et al*.,2012; Khanna & Berek, 2012).

- **Genetic changes:** Critical cellular functions, including apoptosis, DNA repair, and cell cycle regulation, can all be interfered with by genetic changes, giving the afflicted cells an edge in development (Floor et al.,2012). Different causes, including errors in DNA replication, exposure to carcinogens, or inherited deficiencies in DNA repair pathways, might result in these genetic alterations (Macaluso *et al*.,2003). Transforming a normal cell into a cancer cell is usually not driven by a single mutation but rather by accumulating many genetic abnormalities over time (Kumar, 2022).
- **Mutations and Genetic Alterations:** Errors in DNA replication or repair pathways can lead to spontaneous mutations in oncogenes and tumour suppressor genes. These mistakes could occur spontaneously as cells divide over time. On the other hand, outside causes can significantly increase the chance of mutations. Toxins present in the environment, like those in cigarette smoke and industrial pollutants, can directly harm DNA or disrupt cellular functions, which can result in mutations. DNA strands can

break or change due to radiation, including ultraviolet (UV) light from the sun, ionising radiation from medical imaging, and radioactive exposure.

- Furthermore, some viral infections can introduce their genetic material into host cells, which can cause mutations and interfere with regular gene activity. These genetic changes can interfere with how cells divide and expand usually, which can accelerate the development of cancer (Marco A,2017). Changes in DNA, such as chromosomal translocations, point mutations, and gene amplifications or deletions, are frequently the first signs of cancer.
- **Role of Oncogenes in Carcinogenesis:** Proto-oncogenes are essential regulators of biological processes found in normal cells. Proto-oncogenes can be nuclear transcription factors, growth factors, or transducers of cellular signals (Kontomanolis*et al*.,2020). Mutations affecting these genes' ability to regulate their behaviour or the structure of the proteins they encode can manifest as activated oncogenes in cancerous cells. Once these oncogenes are created, they promote cell division and play a crucial part in the aetiology of cancer. Two categories of physical mutations can be identified that result in the activation of proto-oncogenes: those that alter the structure of the encoded protein and those that disrupt the production of the protein (Jan et al.,2020; Abel et al.,2010).
- **Gain of Function in Protooncogenes-** Protooncogenes are activated through structural changes in the proteins that their mutations encode. The mutant protein's unchecked, continuous activity is frequently the result of these changes, which typically affect important protein regulatory areas. Various changes, including nucleotide substitutions, deletions, and insertions, can activate proto-oncogenes. Point mutations are commonly found in the protooncogenes K-ras, H-ras, and N-ras that belong to the ras family (Marco A *et al*.,2003)

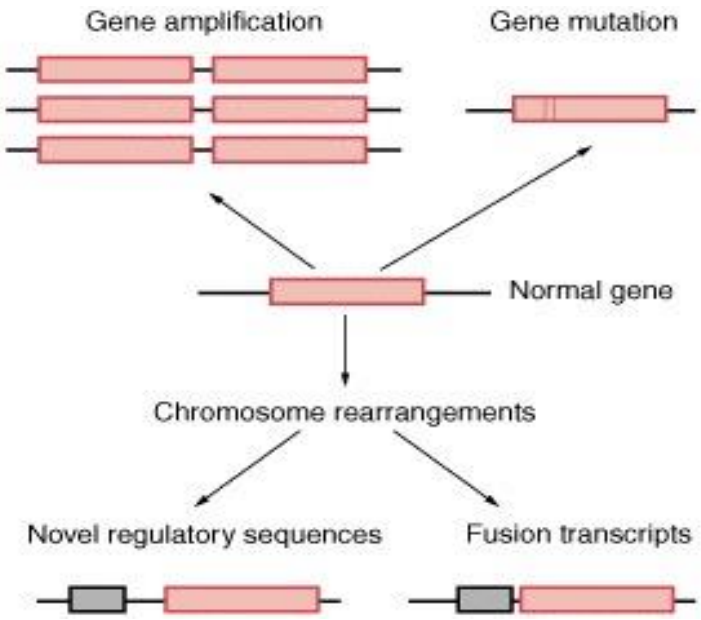


Figure 2.2: Schematic representation of the primary processes involved in oncogene activation (from protooncogenes to oncogenes). (Source: Holland-Frei Cancer Medicine,2003)

Note: The transcribed section (rectangle) of a normal gene (protooncogene) is shown. Amplified Genes can be duplicated 100 times, producing an overabundance of normal protein. A similar circumstance might arise when unique regulatory sequences from another gene now control a gene's transcription due to chromosomal rearrangements like translocation. Single amino acid substitutions in point mutation cases might change the biochemical characteristics of the gene product, leading, for example, to constitutive enzymatic activation. Fusion transcripts produced by chromosomal rearrangements like translocation and inversion can subsequently result in chimeric oncogenic proteins.

- **Tumor-suppressive Genes and Carcinogenesis Blocking:** Most tumour suppressor genes encode proteins that impede cell survival or proliferation, as opposed to proto-oncogene and oncogene proteins. Tumour formation consequently results from the inactivation of tumour suppressor genes, which do this by removing harmful regulatory proteins. Tumour suppressor proteins often block the exact cell regulatory mechanisms that oncogene products stimulate (Cooper GM,2000). **Loss of function in tumour-suppressive genes**-One prevalent mechanism that contributes to the development of cancer is TSG inactivation (Wang *et al*.,2019). According to molecular research, cytogenetically undetectable microdeletions that were found by exhibiting LOH of

polymorphic markers that map within or close to tumour suppressor loci are frequently linked to TSG inactivation (Tomlinson IP *et al*.,2002)

Gene	Familial cancer syndrome	Function	Chromosomal location
TP53	Li-Fraumeni syndrome	Cell cycle regulation, apoptosis	17p13.1
RB1	Familial retinoblastoma	Cell cycle regulation	13q14.1-q14.2
p16(INK4a)	Familial melanoma	Cell cycle regulation	9p21
p14(ARF)	Familial melanoma	Mdm2 antagonist	9p21
CHK 1/2	Li-Fraumeni syndrome	Protein kinase (G1 control)	22q12.1
KLF6	Unknown	Transcriptional regulation	10q21-q22
NF1	Neurofibromatosis type I	Catalysis of RAS inactivation	17q11.2
APC	Familial adenomatous polyposis	Inhibition of signal transduction	5q21-q22
TSC1	Tuberous sclerosis 1	Interaction with tuberin	9q34
DCC	Deleted in colorectal carcinoma	Transmembrane receptor	18q21.3
BRCA1	Familial breast cancer	Cell cycle, DNA repair	17q21
MSH2	HNPCC1	DNA mismatch repair	2p22-p21
MLH1	HNPCC2	DNA mismatch repair	3p21.3
PTEN	Cowden syndrome	PI-3 kinase signal transduction	10q23.3
LKB1	Peutz-Jeghers syndrome	Phosphorylation and activation of AMPK	19q13.3
CDH1	Familial diffuse gastric cancer	Cell-cell adhesion protein	16q22.1
TGF-R I	Unknown	Growth inhibition	9q22.33-q31.1
TGF-R II	Unknown	Growth inhibition	3p24.1
SMAD4	Familial juvenile polyposis syndrome	Regulation of TGF-β/BMP signaling	18q21.1
SMAD2	Juvenile polyposis	TGF-β signal transduction	18q21.1

Figure 2.3: Table illustrating the distribution and function of tumour suppressor genes(**Source:** Wang *et al*.,2019)

2.1.2. Tumor cell heterogeneity to the metastatic potential of cancer cells

Clinically, human cancer has been shown to progress step-by-step. Before ultimately malignant invasive tumours manifest, a variety of pre-malignant abnormalities, including dysplasia and hyperplasia, can be found in many organs. Genetic changes that lead the cells to increase monoclonally or environmental variables like viral infection that cause the cells to proliferate polyclonally are the causes of the pre-malignant lesions. One (or a few) of the pre-malignant cells then accumulate genetic changes, transforming them into malignant cells of clonal origin that give rise to a primary tumour. The cells are not invasive or metastatic at this early stage of the primary tumour progression. After that, further genetic changes accumulate in the cells, giving rise to new invasive clones capable of spreading. Malignant cells are invasive and metastatic; however, only a small percentage of cells inside a single tumour are regarded as highly metastatic. That is to say, the various genes mutated in each cancer cell result in the phenotypic and biological heterogeneity of cells within a primary tumour. Because of this, highly metastatic cells frequently undergo more gene modifications than non-metastatic cells and different genes are expressed differently in metastatic and non-metastatic cells for diverse reasons (Yokota, 2000).

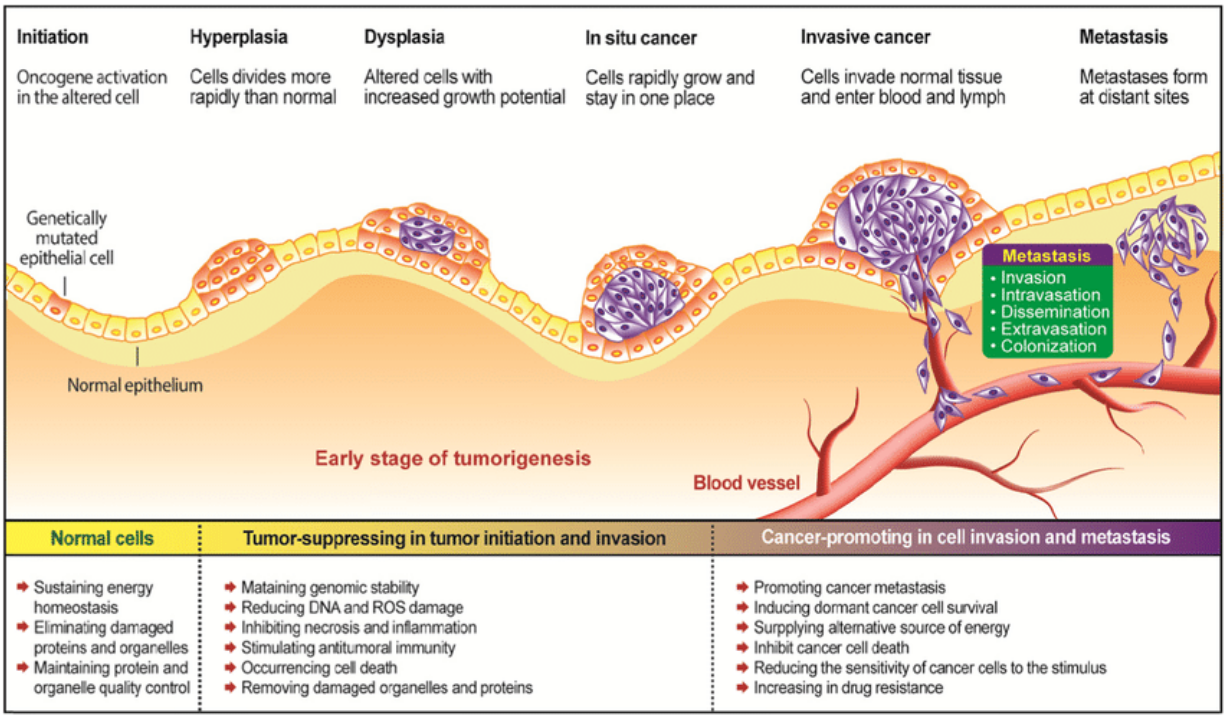


Figure 2.4: Schematic representation of tumour development stages and metastatic mechanisms (Source: Li *et al*.,2020)

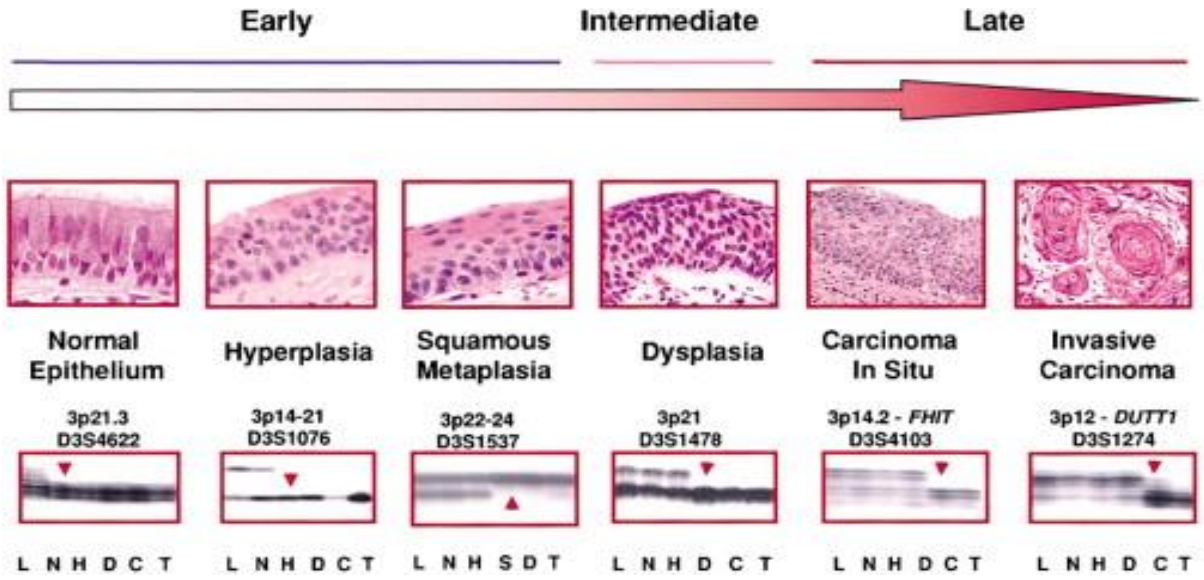


Figure 2.5: Squamous cell lung carcinomas evolve in multiple stages, each characterised by progressive morphological and molecular alterations. (Source: Wistuba*et al*.,1999)

Note: These show the progressive allelic losses at chromosome 3 (3p)'s short arm. Every panel depicts a squamous cell carcinoma that has been removed. With various polymorphic markers, each preinvasive lesion can be detected, meticulously micro dissected, and examined for loss at several 3p loci. According to Wistuba*et al*. (1999), losses happen early at some sites (like 3p21) and late at other sites (like 3p12). The lesion with the first allelic loss is determined for every marker in every case. N=histologically normal epithelium; H=hyperplasia; D=dysplasia; C=carcinoma in situ; T=invasive tumour; L=lymphocytes (a source of constitutional DNA). We appreciate Ivan Wistuba's help in getting the figurine ready.

2.2. Anatomy of the Lung

The process of gas exchange, in which oxygen is taken in, and carbon dioxide is released from the body, is carried out by the extraordinarily sophisticated and intricate human lung. The two main parts of the lung are the lower respiratory tract, which contains the trachea, bronchi, bronchioles, and alveoli, and the upper respiratory tract, which includes the nose, throat, and larynx (McLafferty *et al*., 2013; Hakim & Usmani, 2014; Richards & Davies, 1977). The anatomy of the lung is primarily discussed in the lower respiratory tract. The primary airway, the trachea, is a cartilaginous and membranous tube that transports air to and from the lungs. The lobar bronchi, segmental bronchi, terminal bronchioles, and the two significant bronchi

split out near the base of the trachea. Ultimately, the alveoli—tiny air sacs in charge of gas exchange—are reached through the bronchioles. The extensive blood capillary network that envelops the alveoli makes it possible for oxygen and carbon dioxide to exchange efficiently (Hakim et al.,2014).

Borders and Surfaces: The anterior, inferior, and posterior borders are the boundaries of the lungs. Convergence of the costal and mediastinal surfaces forms the anterior boundary. The anterior, inferior, and posterior borders are the boundaries of the lungs. Convergence of the costal and mediastinal surfaces forms the anterior boundary (Walker,2024)

Pleura: The mesothelial structures that line the inside of the thoracic cavity and the lungs are called the visceral and parietal pleural membranes, respectively. These layers define the pleural space; it is a sterile, safe environment with lymphatics that are intended to recycle the regular pleural fluid. During breathing, the fluid permits the lung surface to slide against the thoracic wall (Agaliot*et al*.,2015)

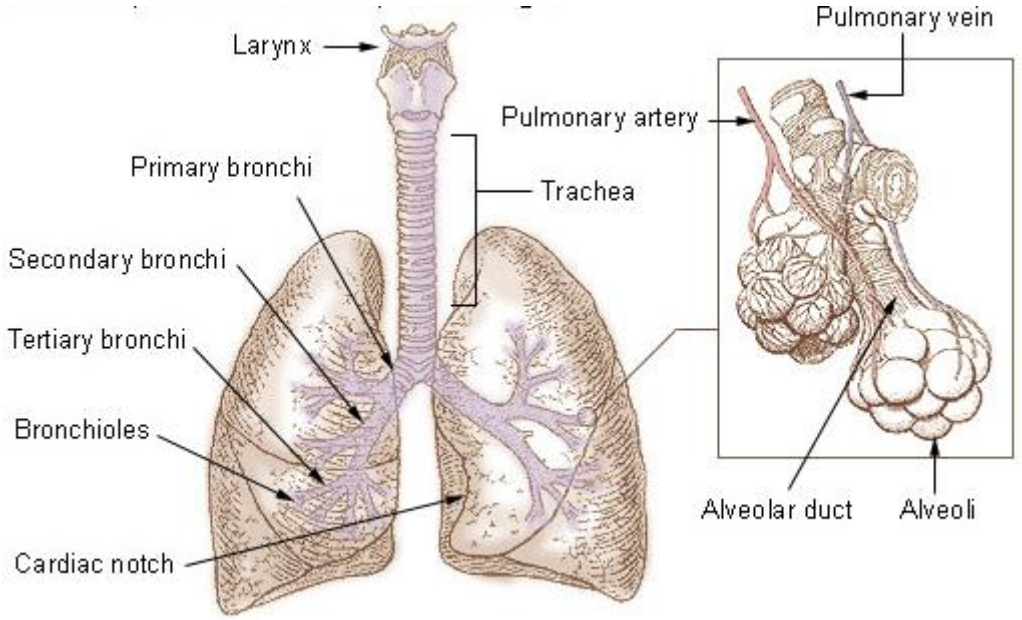


Figure 2.6: A labelled representation of the human lung (Source: Physiopedia Lung Anatomy)

2.3. Lung Cancer

Tumours that begin in the bronchi or lung parenchyma are called lung cancer or bronchogenic carcinoma (Siddiqui F et al.,2024). With an estimated 1.2 million new cases in 2000, lung cancer is the most frequent type of cancer worldwide (12.3% of all malignancies) (Parkin *et al*.,2001). Lung cancers are mainly caused by tobacco use, with smokers accounting for 80% to 90% of cases. Exposed individuals to additional carcinogens, such as asbestos, are in far greater danger. Cellular and molecular subtypes are the foundation for the histopathological classification of lung malignancies, which is crucial for diagnosis and treatment. While there are other subtypes of lung cancer, the most significant differential in the past has been between small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) (Travis WD,2004). The primary source of squamous and SCLC is the central airways, whereas adenocarcinomas, which include bronchioloalveolar cancer, are found in the periphery. The other non-small cell lung cancer (NSCLC) types are less distinct than large cell lung cancer. The average lung lacks squamous epithelium, and smoking-related metaplastic alterations are the source of these tumours. Adenocarcinoma in situ and atypical adenomatous hyperplasia (AAH) are two. AAH, a preinvasive lesion associated with lung cancer, is typically 5 mm or more minor. Adenocarcinoma in situ is typically a localised lesion measuring less than or equal to 3 cm. It can be mucinous or nonmucinous. According to the definition of "lepidic" growth, it exhibits growth-restricted growth along the alveolar structures. The alveolar setae are still intact and non-invasive (Siddiqui F et al.,2024). Adenocarcinomas originate from cells that produce mucin, such as Type II pneumocytes or Clara cells, which are progenitor cells of the bronchioles or alveoli. The most prevalent type of lung cancer in the world today is adenocarcinoma, and its incidence is rising quickly (Minna,2000).

Lung tumours containing more than 10% glandular and squamous components are adenosquamous carcinomas. Due to the significant likelihood of brain metastasis and recurrence with this subtype, current recommendations suggest adjuvant chemotherapy even in cases of Stage I severely resected lung tumours receiving whole-brain postoperative preventive radiation therapy. This is an unusual and aggressive lung tumour (Filosso PL et al.,2011). Intercellular desmosomes and keratin identify the pathophysiology of squamous cells on cytology or by desmoglein, p40, p63, CK5, CK5/6, or immunohistochemical (IHC) evidence. Squamous cell carcinoma comes in nonkeratinizing, keratinizing, and basaloid subtypes. Large-scale central necrosis with ensuing cavitation is seen in squamous cell

carcinomas. Hypercalcemia and Pancoast tumours are two possible symptoms of squamous cell carcinomas. A Pancoast tumour is a lung tumour located in the superior sulcus.

When Pancoast tumours reappear after surgery, the brain is the most often affected area (Siddiqui F et al.,2024). "large cell carcinoma" (LCC) refers to a malignant epithelial tumour lacking the cytologic characteristics of neuroendocrine, squamous, or glandular malignancies. They often lack the cytologic characteristics of small cell carcinoma and do not express TTF-1 and p40 on immunohistochemistry. Round to polygonal cells with noticeable nucleoli are typically found in LCCs. The enormous, featureless cells lack distinguishing characteristics and are filled with cytoplasm. The diagnosis of exclusion is LCC (Rajdev K et al.,2018). The cells that make up small cell carcinoma (SCLC) are round, oval, or angulated, have a tiny amount of cytoplasm, and resemble resting lymphocytes in size. There are no visible nucleoli. SCLCs exhibit widespread necrosis. They often have positive chromogranin or synaptophysin staining. Oat cell, intermediate cell, and mixed cell (SCLC with NSCLC component, squamous or adenocarcinoma) were the three cell subtypes that the WHO previously identified. However, research has indicated that there is no clinical importance or predictive utility to this classification (Aisner SC et al.,1990)

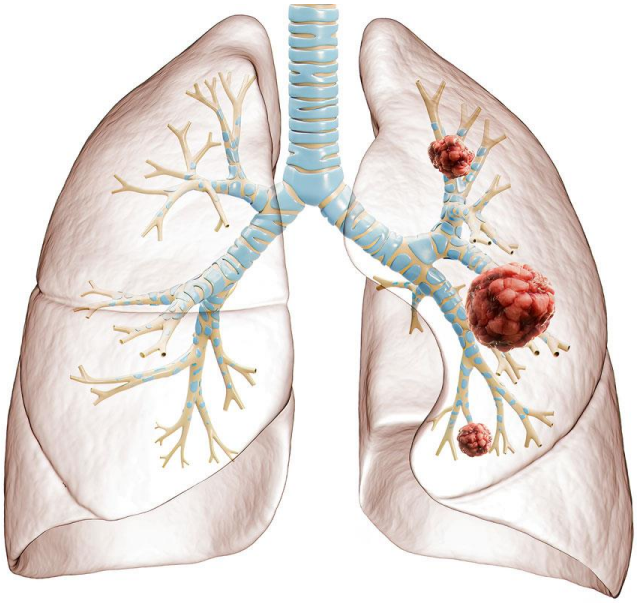


Figure 2.7: Image illustrating Lung carcinoma (Source: Washington University School of Medicine in St. Louis)

2.4. Global Epidemiology of Lung cancer

Globally, lung cancer is the most prevalent cancer to be diagnosed and the primary cause of cancer-related mortality. As per the GLOBOCAN 2020 report, there were approximately 1.8 million lung cancer deaths (18.0%) and 2.2 million new cases of lung cancer (11.4%) in 2020.The incidence of lung cancer differs across nations primarily due to varying risk factors, including the amount of smoking, pollution in the environment, and even dietary practices. Because of strong tobacco control laws and regulations, the incidence of lung cancer has been progressively declining in the United States of America (USA) since 1990[3]. On the other hand, China and other developing nations have lower lung cancer incidence but higher lung cancer fatality rates. These trends can be attributed to inadequate early cancer screening, unfavourable health outcomes, and difficulties in implementing tobacco control laws and programmes (Li C *et al*.,2023). According to estimates, there will be 234,030 new instances of lung cancer identified in the US in 2018—121,680 new cases for men and 112,350 new cases for women—or 641 cases per day.

After breast cancer in women and prostate cancer in men, lung carcinoma is the second most prevalent cancer diagnosis by gender. Lung cancer makes up 13% of new cancer cases in women and 14% of new cancer cases in men in the United States in 2018 (Siegel RL *et al*.,2018). Pancreatic, liver, and lung cancers have some of the lowest survival rates. The 5-year relative survival rate for lung malignancies diagnosed between 1975 and 1977 was 12% for all stages combined. New cancer diagnoses between 2003 and 2009 are at 18% (SEER cancer static review; Torre LA *et al*.,2016). In comparison to white Americans, black Americans are much more likely not to receive a lung cancer diagnosis until the disease has progressed to an advanced stage. The prognosis for advanced lung cancer is terrible, with only 5% of patients surviving for five years (Wu *et al*.,2018).

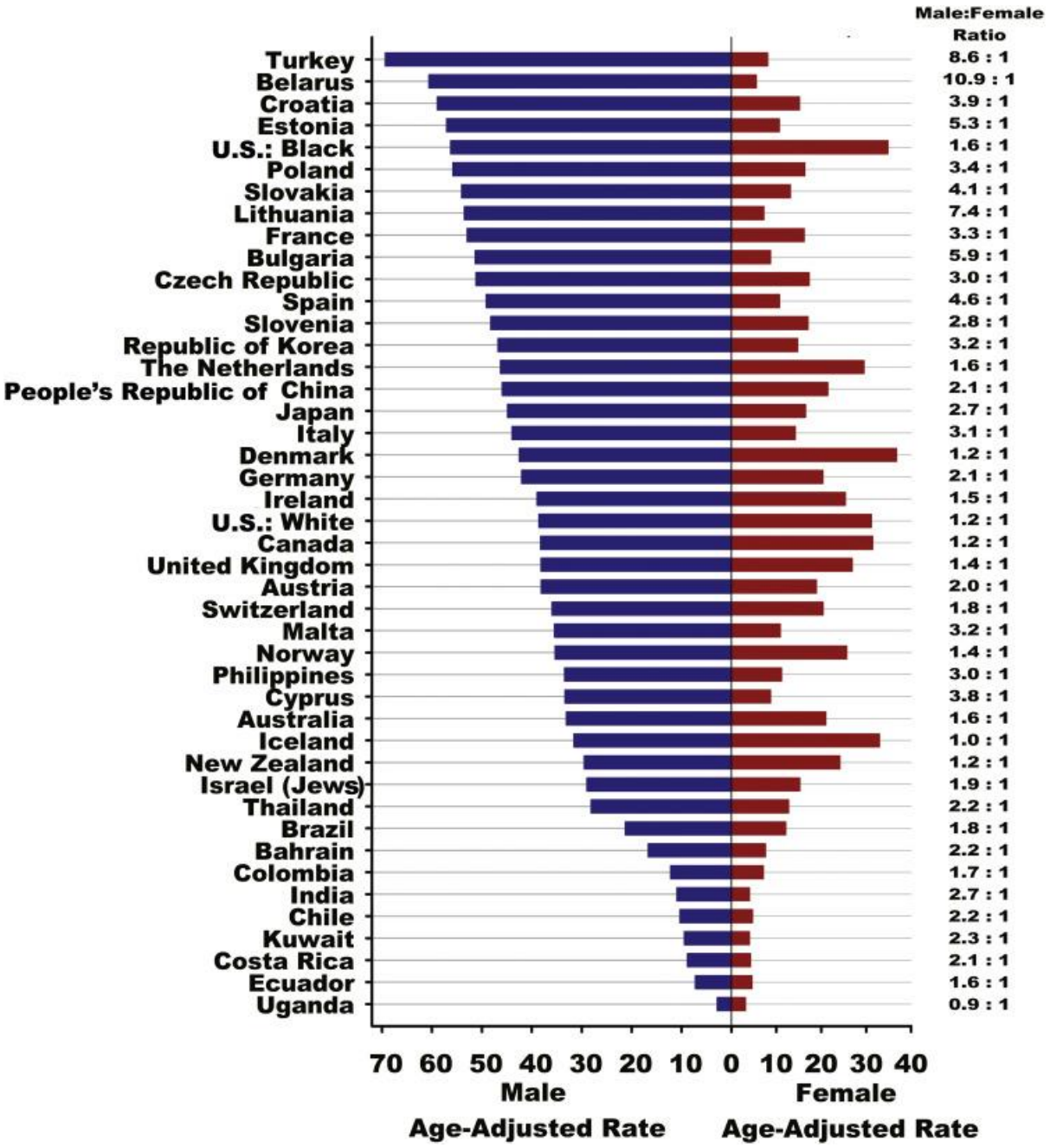


Figure 2.8. Global Lung Cancer Incidence Trends and Patterns among Men and Women in 43 nations globally between 2008 and 2012. Age-adjusted rates are based on the world population standard (Zhang *et al*.,2021)

As to the most recent statistics from GLOBOCAN, 2,094,000 new cases of lung cancer were detected worldwide in 2018, making it the most common cancer incidence in the globe. After prostate cancer, lung cancer is the second most frequent cancer in males (estimated at 1,369,000 cases) and the second most common cancer in women (estimated at 725,000 cases). Men have an age-standardized cumulative lifetime risk of 3.8%, while women have a chance of 1.77% of receiving a lung cancer diagnosis. With a variance in incidence of over 20 times between

places, lung cancer is most common in developing countries where cigarette smoking is most common. Lung cancer is the most prevalent cancer among males in 37 countries, including China, Russia, and much of Eastern Europe, the Middle East, and Southeast Asia, whereas prostate cancer is the most common disease among men in 104 countries (Thandra*et al*.,2021). In one country, North Korea, lung cancer is the most frequent malignancy among women. With 52.2/100,000 instances among men, Micronesia/Polynesia has the highest incidence of lung cancer globally; Hungary has the highest incidence among men, at 77.4/100,000. North America and Northern and Western Europe have the most significant incidence globally among women. The lowest incidence is in males and women in Western, Central, and Eastern Africa (Bray F *et al*.,2018).

2.5. Indian Epidemiology of Lung Cancer

India is the world's third-largest producer and second-largest user of tobacco. According to the Global Adult Tobacco Survey-2 [GATS]-2 2016–2017, around 267 million people in India use tobacco products, making up 28.6% of the country's total population (42.4% of men and 14.2% of women).In India, tobacco use has a significant negative influence on health, particularly concerning cancer (Singh N et al.,2012). India's lung cancer epidemiology has changed over time, moving from a period where the two histologic kinds most strongly linked to tobacco use—small cell and squamous—to one where adenocarcinoma became equivalent and, finally, the dominant histologic type (Mohan A et al.,2020, Singh N et al.,2012). India reports about 63,000 new cases of lung cancer annually. Tobacco use is the primary risk factor for lung cancer, which is frequently perceived as a disease exclusive to smokers.

Nonetheless, a sizable portion of lung cancer patients have never smoked. 31.5% of male patients and 68.5% of female patients, or 32.4% of all lung cancer patients, were never smokers in a study of 975 patients from Singapore who had non-small-cell lung cancer (NSCLC).Lung cancer among never-smokers shows a clear gender bias worldwide, with women experiencing it more commonly. Specifically, a large percentage of Asian women who receive a lung cancer diagnosis have never smoked. While smoking-related carcinogens affect the proximal and distal airways, causing all main types of lung cancer, malignancies that arise in non-smokers primarily affect the distal airways, favouring the histology of adenocarcinomas (Norohna V et al.,2012). This histologic profile transformation has primarily happened in the last ten years and has, in this regard, lagged behind the transition seen in industrialised nations. According

to theories, the "time lag" may be caused by the fact that, in India, "bidi"—handmade tobacco smoking products, primarily based on cottage industries—has been and remains the most popular kind of smoking product, in contrast to the more regulated and mechanised manufacturing of cigarettes, with slight variation in the former's manufacturing process over time, unlike the latter, where low nicotine content and filtered cigarettes have been marketed for a substantial period) (Singh N et al.,2012).

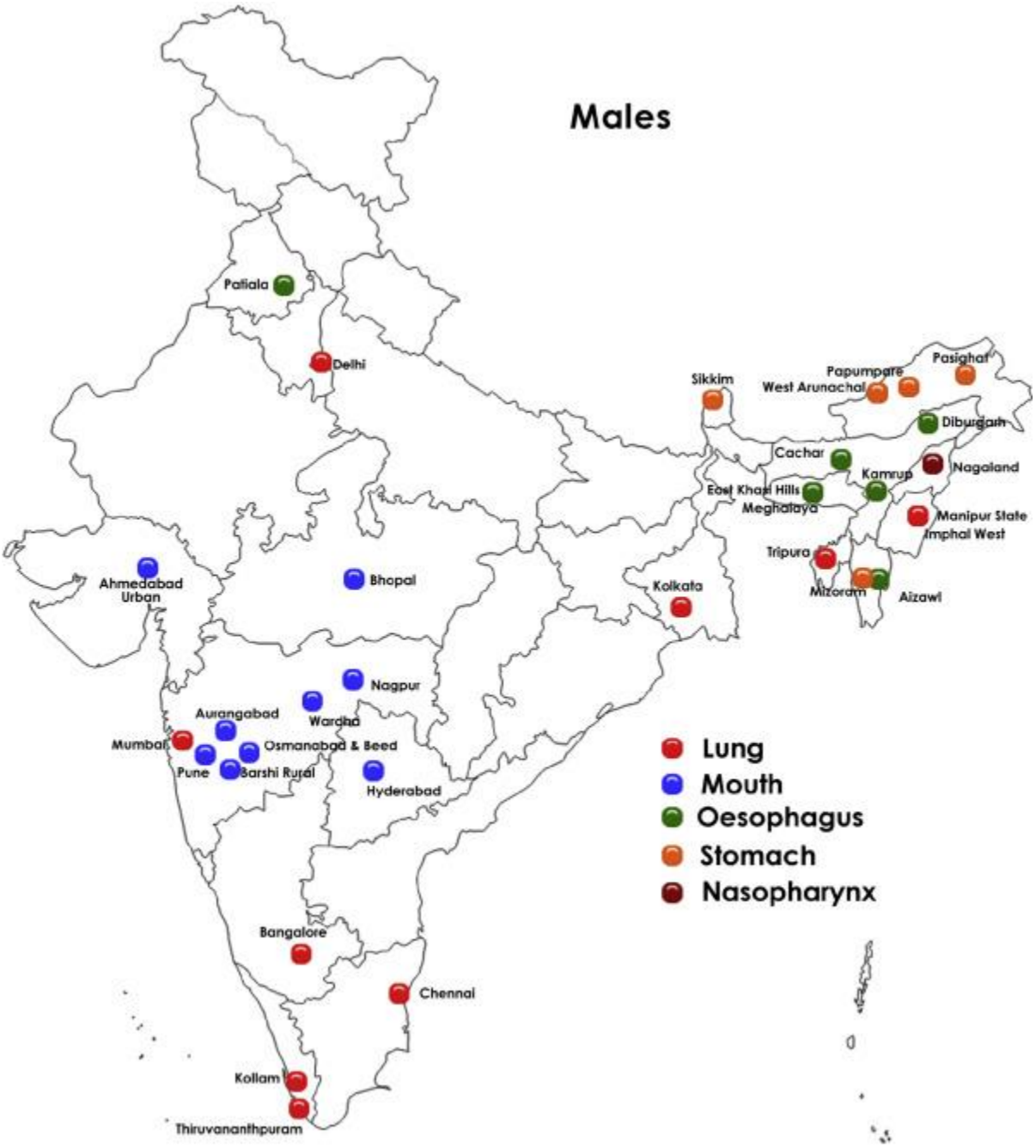


Figure 2.9. Many of the NCRP's population-based cancer registries, which are run and managed by the ICMR-NCDIR, show that lung cancer is the most common cancer among men.

India's overall infrastructure for cancer care is still inadequate, given the country's population and cancer patient load. In contrast to wealthy nations, India may have lower cancer patient survival rates and higher death rates as a result of these shortcomings if they are not addressed. The cost of therapy and the financial burden of receiving it are significantly higher when

seeking care in the private health sector. In addition, most patients who cannot pay for private medical facilities typically seek care at institutions supported by the government. Large hospitals and major academic institutions are still typically the only places with the knowledge and resources needed to treat cancer.

In conclusion, lung cancer is a significant source of morbidity and death in India, particularly for men, and the incidence of this illness is predicted to increase in the coming years. Over the years, the disease's spectrum has changed, affecting a more significant proportion of younger age groups, light or nonsmokers, and female individuals (with adenocarcinoma predominating). Previously, the disease primarily affected elderly tobacco-smoking men with squamous or small cell carcinoma (Singh N et al.,2012).

2.6. TNM staging

The internationally recognised tumour, node, and metastasis (TNM) staging classification for lung cancer is used to describe the disease's progression. The TNM system combines the tumour's characteristics to create stage groups associated with therapy recommendations and corresponding with prognosis. The design criteria for stage classifications consider each tumour's unique features while also considering external priorities for implementation and usability across global health systems. In particular, simplicity, clarity, and applicability to a wide range of patients and geographical areas are requirements for stage classification (Thomas *et al*.,2024). The TNM staging system provides anatomical information on the degree of cancer. For prognosis prediction, treatment selection and optimisation, and response evaluation, such uniform, repeatable classification is necessary. This standardised system makes communication and clinical research easier. The characteristics and extent of the primary tumour (T), the involvement of one or more lymph nodes locally (N), and distant metastases (M) are the three elements of TNM staging. The eighth edition of TNM staging and classification for lung cancer was released recently. The International Association for the Study of Lung Cancer recommended changes to this edition, which were subsequently embraced by the Union for International Cancer Control and the American Joint Committee on Cancer (Lababede et al.,2018).

Table 2.1. TNM Staging System (8th edition) (Source: Goldstraw P et al.,2016)

Stage	Tx	N0	M0
Occult Carcinoma	Tx	N0	M0
Stage 0	Tis	N0	M0
Stage IA1	T1a (ml)	N0	M0
	T1a	N0	M0
Stage IA2	T1b	N0	M0
Stage IA3	T1c	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T2b	N0	M0
Stage IIB	T1a-c	N1	M0
	T2a	N1	M0
	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1a-c	N2	M0
	T2a-b	N2	M0
	T3	N1	M0
	T4	N0	M0
	T4	N1	M0
Stage IIIB	T1a-c	N3	M0
	T2a-b	N3	M0
	T3	N2	M0
	T4	N2	M0
Stage IIIC	T3	N3	M0
	T4	N3	M0
Stage IVA	Any T	Any N	M1a
	Any T	Any N	M1b
Stage IVB	Any T	Any N	M1c

Table 2.2. Understanding Tumour Stage, Lymph Node Invasion and Metastasis (Source: Lababede et al.,2018)

Primary Tumour (T)	
Tx	A tumour that is histopathologically confirmed but cannot be evaluated by bronchoscopic or radiographic methods
T0	No signs of a tumour
Tis	In situ carcinoma: In situ squamous cell carcinoma (SQCC) In situ adenocarcinoma (ADCC): (pure lepidic pattern with a maximum dimension of ≤ 3cm)
T1	A tumour that is≤ 3cm in size Location of the airway: within or outside the lobular bronchus No local invasion
T1a(mi)	Minimally invasive adenocarcinoma
T1a	A tumour whose most significant dimension is 1 cm or less
T1b	Tumours more noticeable than 1 cm, however,≤ 2 cm in the most significant dimension
T1c	Tumours that are more noticeable than 2 cm, however,≤ 2 cm in the most significant dimension
T2	Tumours size: > 3cm but ≤ 5cm Location of the airway: obstruction, atelectasis, or invasion of the significant bronchus (independent of the distance to the carina). Pneumonitis affects the hilar area. Regional incursion: Pleura Viscera
T2a	Tumours larger than 3 cm that are, nonetheless, ≤ 4 cm in the largest dimension or else may not be determined
T2b	More than 4 cm in prominence, however, ≤ 5 cm in the most significant dimension
T3	Tumours size: > 5cm but ≤ 7cm Location of the airway: parietal pericardium, phrenic nerve, or direct invasion of the chest wall Distinct tumour nodules within the original tumour's lobe

T4	Tumour size > 7cm Location of the airway: Invasion of the Trachea or Carina Local invasions include Diaphragm, mediastinum, heart, major vessels, oesophagus, vertebral body, and recurrent laryngeal nerve. Distinct cancer nodules in the original tumour's ipsilateral, distinct lobe.
Lymph nodes (N)	
Nx	Evaluation of regional lymph nodes is not possible.
N0	Absence of involvement of regional lymph nodes
N1	Involvement of the ipsilateral hilar lymph nodes and peribronchial lymph nodes
N2	Involvement of the subcarinal and mediastinal lymph nodes on the ipsilateral side
N3	Involvement of the contralateral hilar, ipsilateral or contralateral scalene, contralateral hair, or supraclavicular nodes lymph node groupings
Distant Metastasis (M)	
M0	No distant metastasis
M1	Presence of distant metastasis
M1a	Distinct tumour nodules in a lobe opposite the primary tumour or tumours with pericardial, pleural, or malignant infusion nodules
M1b	Solitary metastasis outside the chest
M1c	multiple organ metastases from the extra thoracic region

2.7. Lung Cancer Histopathological Classification

Since most occurrences of lung cancer are asymptomatic in the early stages and frequently go undetected until they have spread, early detection is essential yet complex, complicating treatment and drastically lowering patient prognosis (Patorino,2001). The primary cause of cancer-related mortality is lung cancer (LC). Histology-based subtypes of lung cancer account

for 15 and 85% of all instances, respectively, and are categorised as small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC).Three other forms of non-small cell lung cancer (NSCLC) are squamous-cell carcinoma, adenocarcinoma, and large-cell carcinoma. About 25–30% of cases with LC have squamous-cell carcinoma. The source of it is primitive squamous cells found in the airway epithelial cells of the bronchial tubes in the middle of the lungs. Adenocarcinoma (ADC), accounting for approximately 40% of all LC cases, is the most prevalent kind of LC. Lung ADCs originate from type II alveolar cells, tiny airway epithelial cells that release mucus and other materials. Around 5–10% of LC cases are large-cell (undifferentiated) carcinoma. Often diagnosed by default when alternative possibilities are ruled out, this kind of cancer lacks evidence of squamous or glandular maturation (Denisenko et al.,2018). Lung cancer's histopathological classification was recently updated and released as the WHO classification for 2015 (Inamura,2017).

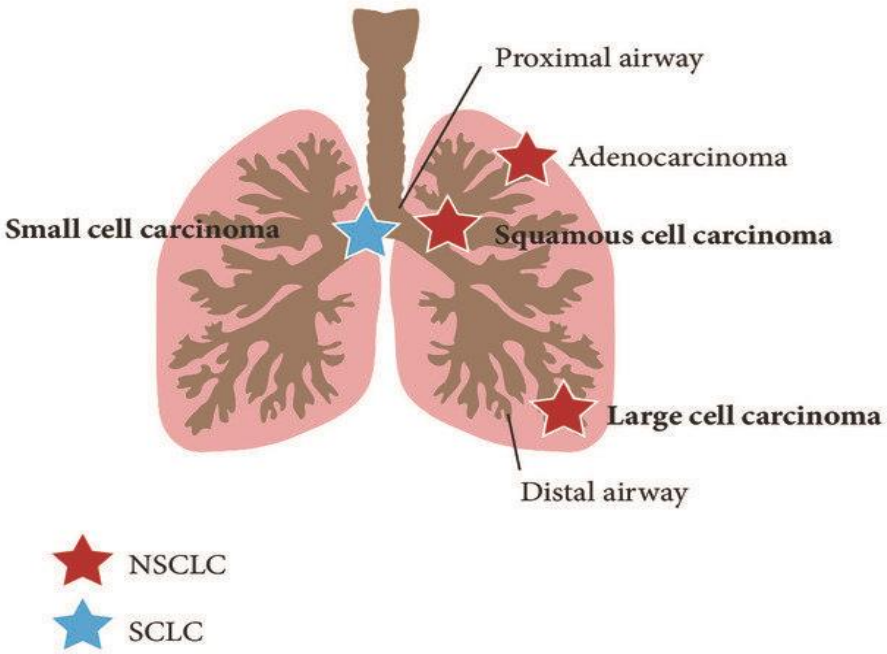


Figure 2.10.Small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) are the two types of lung cancer, which is the most common cause of cancer-related death globally(Abe *et al*.,2016)

2.7.1. Large cell carcinoma (LCC)

The term "large cell carcinoma" refers to undifferentiated malignant epithelial cells; nonetheless, there are currently five forms of the disease. LCNEC and basaloid carcinoma are recognised variations with bleak prognoses (Brambilla *et al*., 2001). A small percentage of NSCC cases have LCCs, characterised by morphology and immunohistochemistry free of any indication of adenocarcinoma, SCC, or neuroendocrine carcinoma (null immunophenotype). These cases also lack lineage-specific differentiation. In most cases, LCC is massive, necrotic-looking, and peripherally situated. Vesicular and pleomorphic nuclei characterise the giant, polygonal tumour cells. Pattern less solid sheets or nests are formed by the tumour cells. According to the following explanation, lung neuroendocrine tumours (NETs) include large-cell neuroendocrine carcinomas (LCNEC). Approximately 3% of lung cancer cases are LCCs (Zheng,2016). Recent data indicates that tumours presently categorised as LCC and have a null immunophenotype have molecular characteristics in common with solid adenocarcinomas (Hwang DH *et al*.,2014). This group of tumours is not invariably exempt from molecular testing. In the appropriate clinical setting, metastatic tumours ought to be ruled out. Most LCC cases result in adverse outcomes, particularly for those with null immunophenotypes (Rekhtman N *et al*.,2013).

2.7.2. Adenocarcinoma (ADCC)

The most prevalent form of lung cancer is adenocarcinoma, which accounts for about 40% of lung cancer diagnoses, 60% of NSCC cases, and more than 70% of cases that require surgical resection (Travis WD *et al*.,2015; Lewis *et al*.,2014). In the last few decades, there has been a steady increase in the incidence of adenocarcinoma. A tumour with pleural puckering and central fibrosis usually found in the periphery is caused by lung adenocarcinomas. Other gross appearances it may present include pleural thickening, widespread lobar consolidation, a centrally positioned mass, and bilateral multi-nodular distribution. An epithelial malignancy with glandular differentiation or mucus production is what lung adenocarcinoma is. The tumour can be diagnosed as an adenocarcinoma even with small biopsy specimens when such morphologic traits are identified. Most lung cancer cells display markers associated with pneumocytic cells. Based on ancillary tests like immunohistochemistry (IHC), a tiny biopsy specimen is classified as an "NSCC, favour adenocarcinoma" tumour. A more thorough subclassification is made possible by resection specimens. Adenocarcinoma categorization has

substantially improved in recent years due to better pathologic and clinical correlations (Zheng,2016).

Lung cancer classification by the World Health Organisation

Adenocarcinoma

Adenocarcinoma lepidic

Acinar cancer in situ

Collagenous adenocarcinoma

Adenocarcinoma micropapillary

Adenocarcinoma solidum

Mucinous invasive adenocarcinoma

Adenocarcinoma colloid

Adenocarcinoma fetalis

Adenocarcinoma enteric

minimally invasive carcinoma of the mouth

2.7.3. Squamous cell carcinoma (SQCC)

The most common histologic subtypes of non-small cell lung cancer (NSCLC) are adenocarcinoma and squamous cell carcinoma (SCC), which represent 50% and 30% of cases, respectively. Changes in tobacco smoking habits have led to a decrease in the incidence of lung SCC, yet SCC is still a serious health concern. The most common characteristics of SCCs are keratinization and intercellular bridges; these tumours originate from bronchial epithelial cells through squamous metaplasia/dysplasia. However, on resected specimens, a diagnosis of SCC is only possible if at least 10% of the tumour bulk exhibits these differentiating traits. When there is little differentiated squamous component, poorly differentiated SCC is the diagnosis. This suggests that since large-cell carcinoma is rarely recognised as a diagnosis in tiny specimens, many small biopsy specimens can appear to be NSCLC (Moreno *et al*.,2012). Four types of SCC are recognised by the 2004 World Health Organisation classification: basaloid, papillary, small cell, and clear cell. The latter two are worthy of inclusion in the following

classifications since they have a particular clinicopathologic significance. The papillary variant has invasive development that obstructs the endobronchi, often with modest intraepithelial dissemination, and may be challenging to evaluate. The basaloid variation has limited squamous differentiation and a primarily basaloid morphology, with basal bronchial stem cell growth (Brambilla *et al*.,1992). One of the primary processes in cancer is the acquisition of somatic genetic mutations. It is generally acknowledged that this is typically a multi-stage process caused by a gradual accumulation of mutations and aberrant epigenetics. Lung SCC is currently the most common histologic subtype and the most common in under developed nations despite the rising incidence of adenocarcinoma. Sufferers with adenocarcinomas have benefited from encouraging new treatments, but sadly, this is not the case with SCC sufferers. The most effective initial treatment for advanced lung SCC is still chemotherapy. A single-phase III trial utilising targeted medicines has not yet found benefits in this group; furthermore, some trials demonstrated increased toxicity compared to the nonsquamous disease population (Moreno *et al*.,2012).

2.7.4. Small cell lung carcinoma (SCLC)

The aggressive tumour known as small cell lung cancer (SCLC) is distinguished by a quick time to doubling and a strong inclination towards the early onset of widespread illness. The majority of patients with restricted stage and almost all patients with metastatic illness eventually experience tumour progression for which there are few treatment choices, even though most patients react to initial therapy with a platinum doublet (Waqar *et al*.,2017). Only one-third of patients have earlier-stage cancer that is responsive to possibly curative multimodality therapy; the majority had metastatic disease at the time of diagnosis. Genomic analysis of small cell lung cancer (SCLC) typically identifies a high mutation burden and several chromosomal rearrangements, nearly always with functional inactivation of the tumour suppressor genes TP53 and RB1 (Rudin *et al*.,2021). Typically, SCLC is found centrally in the central airway. Due to its unique morphologic characteristics, SCLC can be accurately diagnosed when the routine hematoxylin and eosin (H&E)-stained section is carefully examined. Compared to other forms of lung cancer, the tumour cells are often less than the diameter of three mature lymphocytes. There are no noticeable nucleoli in the coarsely granular chromatin. There is little cytoplasm, and the boundaries of the cells are barely noticeable. More than 10 mitoses per 2 mm2 often indicate a mitotic solid rate. In addition, there's often a high

rate of apoptosis and widespread tumour necrosis. Crush artefact is commonly observed in bronchial biopsy samples. Comparing cytology to tiny biopsies that include few intact, viable tumour cells, cytology can produce a greater diagnosis yield and be far more valuable. According to WHO categorization, there are two subtypes of SCLC: combined SCLC, which includes an NSCC component and pure SCLC (Zheng,2016).

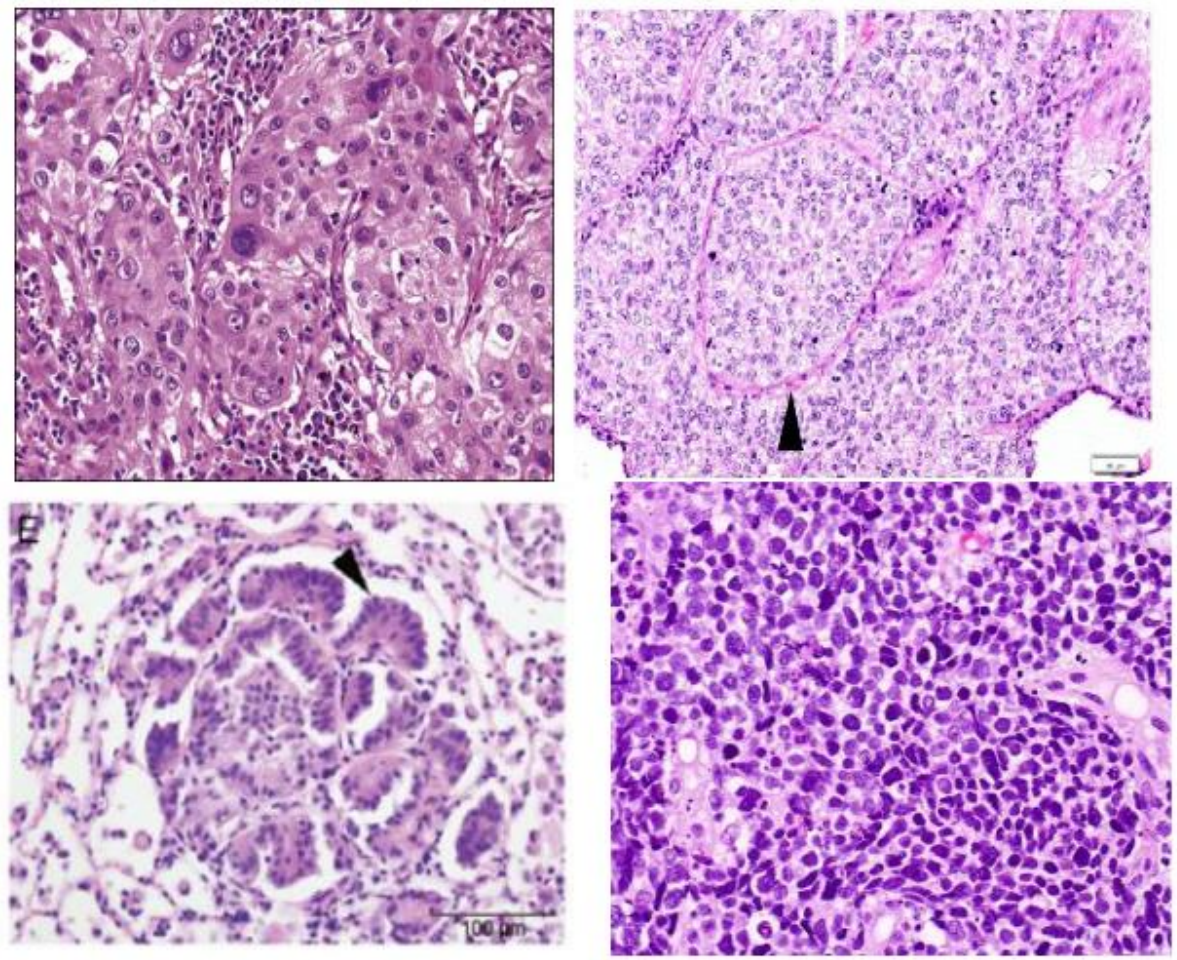


Figure 2.11. Microscopic images of different histological subtypes of lung cancer; a) large cell carcinoma (Source: Zander DS *et al.*,2018) b) Squamous cell carcinoma (Source:Hayashi*et al*.,2013) c) Adenocarcinoma (Source: Yousse*et al*.,2015) d) small cell lung carcinoma (Source:Singh P *et al*.,2015)

2.8. Key Risk factors for lung cancer

2.8.1. Tobacco Smoking

Cigarette smoking is directly responsible for about 80% of lung cancer incidences in the West. Because of the elevated risk of lung cancer (as well as neoplasms of the bladder, colorectum, and other organs), tobacco smoking is regarded as the world's most significant preventable cause of death (Bray F *et al*.,2018). Nicotine, the most addictive component of tobacco smoke, attaches itself to the brain's nicotinic acetylcholine receptors, changing the expression of genes and receptors as well as the levels of neurotransmitters to promote dependency. More than 60 recognised carcinogens, such as N-nitrosamines and polycyclic aromatic hydrocarbons (PAH), are released when tobacco is burned. Decades after usage, these substances cause DNA damage and mutations that raise the risk of carcinogenesis in all organ systems (Thandra*et al*.,2021). With efforts since the 1960s, the percentage of US people who smoke has decreased from 42.4% in 1965 to 13.7% in 2018 (Jamal *et al*.,2018). In Western countries, women's proportional smoking prevalence reductions have not kept up with men's, which has led to women's lung cancer rates climbing at a disproportionate rate. In the West, prevention initiatives that target healthcare disparities and tobacco use among disadvantaged groups are thought to be the most successful ways to lower the incidence of lung cancer (Thandra*et al*.,2021).

2.8.2. Tobacco smoke carcinogens

Lung cancer is affected by smoking in a variety of ways. In both human and animal models of lung carcinogenesis, tobacco use is still the most reliable causal agent, but during the last ten or so years, it has also become a predictive and prognostic clinical feature (Furrukh,2013). In an aflame cigarette, just 20 of the approximately 3,500 compounds that could cause cancer have been found. Most well-known are the polycyclic aromatic hydrocarbons (PAH) such as benzo(a) pyrenes and the N-nitrosamine 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) that is specific to tobacco. Other compounds include Dibenz(a,h)acridine, Asz-arenes, and inorganic compounds such as arsenic, cadmium, nickel, chromium, and radioactive polonium (Po210), as well as organic compounds such as butadiene. When you smoke tobacco, its nitrates are converted to NH2− and NH3. Higher amounts of aromatic amines, such as the bladder cancer-causing substances 4-aminobiphenyl and β2-naphthylamine, are found in tobacco that has been air-cured than in tobacco that has been flue-cured (Furrukh,2013). Cigarette smoke contains significant concentrations of chemicals that are known to be

carcinogenic to the ciliated lining of the lungs, including formaldehyde, acetaldehyde, nitrogen oxides, and phenols. These compounds may also indirectly cause lung cancer in both people and animals (WHO,2012).

2.8.3. Cannabis smoking

In the US, 33 states have approved the medical use of cannabis, sometimes known as marijuana, and 11 states have legalised its recreational use. As a result of the long and ongoing history of marijuana prohibition, reports of marijuana usage have increased by 4% in the US between 2002 and 2014, especially among low-income individuals (Hasin DS,2018). Similarly, due to the drug's illicit status, little research has been done on how smoking marijuana affects lung health. Carcinogenic compounds are known to be produced during the combustion of marijuana, with some of these compounds—like tar and polyaromatic hydrocarbons—producing higher levels of cancer than tobacco does. In the bronchial epithelium, marijuana use has been demonstrated to cause premalignant histological alterations that are comparable to those caused by tobacco smoking. Men who regularly smoked marijuana had a 2.4-fold higher risk of lung cancer, according to case-control studies from North Africa, even after accounting for occupational exposure and tobacco smoke (Berthiller J *et al*.,2008). Compared to tobacco users, marijuana consumers consume significantly less marijuana daily, and it is thought to be less addictive than nicotine (Hall W *et al*.,2015).

2.8.4. Passive smoking

The risk of lung cancer has also been found to be dose-dependently related to second-hand smoke exposure. Because of the filters on cigarettes, several carcinogens are breathed in higher concentrations when second-hand smoke is present than when a smoker smokes; for example, benzopyrene exposure is four times higher. Exposure to ambient tobacco smoke is causally associated with an increased risk of lung cancer, as supported by data from toxicological, epidemiological, and studies of biochemical markers of exposure. There are carcinogens in tobacco smoke that nonsmokers can inhale and metabolise. In nonsmokers exposed to ambient tobacco smoke, there is an increase in cotinine and nicotine, two further markers of inhalation (Hackshaw *et al*.,1998). There is a 20–30% higher risk of lung cancer for nonsmoking spouses of smokers. Certain chronic illnesses like asthma, SIDS, and developmental delays are linked to exposure in pregnant women and children (Thandra*et al*.,2021).

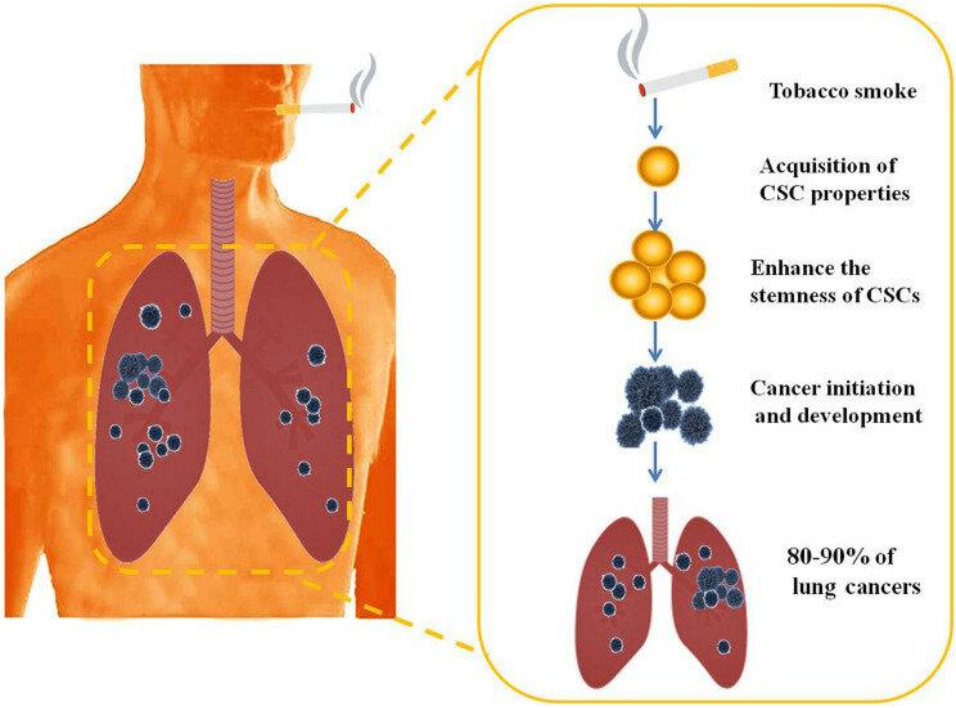


Figure 2.12. An illustration depicting the risk factors associated with causing lung cancer

2.8.5. Alcohol and diet

Alcohol may increase the carcinogenic effects of cigarette smoke on tissues by activating procarcinogens found in alcoholic beverages and cytochrome P-450 enzymes. Excessive alcohol consumption seemed to be associated with an increased risk of lung cancer in smokers, according to extensive Italian population-based case-control research. This finding may indicate a combined effect of alcohol and tobacco, underscoring the need for better methods to assist individuals in cutting back on alcohol intake and quitting smoking, even though residual confounding by tobacco use can never be ruled out (Bagnardi*et al*.,2009).

Arsenic in drinking water: A greater risk of lung cancer has been identified in studies involving individuals living in regions of South America and Southeast Asia where drinking water contains high amounts of arsenic. Most of these investigations showed that the amounts of arsenic in the water were far higher than usually observed in the US, even in places where arsenic levels are above average. Drinking water is not a significant source of arsenic for most Americans who use public water systems.

2.8.6. Occupational Hazard

2.8.6.1. Asbestos

Five to ten per cent of instances of lung cancer worldwide are thought to be caused by occupational exposure to carcinogens, with asbestos being the most common cause (Devesa,2005). A standard commercial material used in thermal and acoustical insulation is asbestos, a naturally occurring fibrous silicate. The chrysotile and amphibole groups, which include the fibres of amosite, crocidolite, anthophyllite, actinolite, and tremolite, can be separated out. There is a risk of lung cancer and mesothelioma from all forms of asbestos. Compared to chrysotile, amphiboles appear to have more biological effects on the peritoneum and pleura (Shankar *et al*.,2019). Asbestos is a naturally occurring mineral employed in buildings due to its flame-retardant qualities. In addition to being known to deposit fibres in the lungs, asbestos has also been linked to several lung diseases, such as pneumoconiosis, bronchogenic lung cancer, and mesothelioma, an uncommon pleural tumour of the lung (Thandra*et al*.,2021). In a North American cohort of insulators who frequently worked with asbestos, the prevalence of lung cancer among nonsmokers increased by 3.5 times. The synergistic effect of asbestos and tobacco smoking on lung cancer is explained by the fact that asbestos fibres are known to trap tobacco particulates, with a cohort study revealing a 14.4-fold greater risk (Markowitz *et al*.,2013). All types of asbestos, including chrysotile, crocidolite, amosite, tremolite, actinolite, and anthophyllite, are considered carcinogenic to humans by the IARC (group 1). The Environmental Protection Agency (EPA) also categorised asbestos as a Group A human carcinogen (Shankar *et al*.,2019).

2.8.6.2. Radon gas exposure

Radon, a naturally occurring gas with carcinogenic qualities, is created when uranium decays in the earth. Radon exposure is linked to time spent underground, such as in mines or basements, particularly in areas with high uranium concentrations. There is evidence that underground miners of uranium or metals have a significantly higher chance of developing squamous cell carcinoma in their lungs and other organs (Roscoe *et al*.,1995; Lubin et al.,1995). With an estimated 10% of cases, residential radon exposure is the second most significant risk factor for lung cancer in the Western world. Radon exposure is linked to up to 30% of non-smoking individuals' incidences of lung cancer (Krewski*et al*.,2006). Radon exposure has been linked to up to 30% of nonsmoker incidences of lung cancer. The amount of radon found below and in residential buildings is restricted in the US. All underground

homes in high-risk areas, which cover a large portion of the Northeast, are advised to have air circulation systems. When combined with tobacco use, radon exposure increases the risk of lung cancer (Krewski*et al*.,2006).

2.8.6.3. Metals and Other Occupation Exposures

Arsenic is a lung carcinogen found in the environment and the workplace. It is most frequently found as arsenide and arsenate. Most occupational exposures happen to workers near lead, gold, and copper ore miners and smelters who breathe in dust. The IARC concluded that breathing in arsenic raises the risk of lung cancer (Lubin *et al*.,2008). Additionally, vascular diseases like stroke and ischemic heart disease have been linked to arsenic (Palma *et al*.,2020). To shield employees against excessive beryllium exposure and beryllium disease, the Department of Energy (DOE) launched the Chronic Beryllium Disease Prevention Programme. According to the National Emission Standards for Hazardous Air Pollutants established by the Clean Air Act and the EPA, beryllium compounds are classified as hazardous air pollutants (National Toxicology Program,2016). As set by the US EPA, the maximum contamination level for total chromium in drinking water is 0.1 mg/L. According to FDA inspections, the amount of chromium in bottled water shouldn't be more than 0.1 mg/L (National Toxicology Program,2016). The U.S. EPA lists nickel compounds as mobile-source air toxics, meaning that restrictions must be created for them. According to the National Emission Standards for Hazardous Air Pollutants, Nickel and its compounds are included in the list of hazardous air pollutants. According to the Urban Air Toxics Strategy, nickel compounds are among the 33 dangerous air pollutants that pose the most considerable risk to human health in urban environments (National Toxicology Program,2016).

2.8.6.4. Air Pollution

Outdoor Air Pollution: Numerous sectors, including mining, municipal waste facilities, and insufficient home incineration, discharge air pollutants into the atmosphere (Shankar *etal*.,2019). Lung cancer risk factors include specific materials suspended in the air and carcinogens released while burning fossil fuels. While all residents of industrial districts absorb air levels of carcinogens like PAH, workers with lengthy occupational exposure are more vulnerable. Due to their ongoing exposure to vehicle emissions, truck drivers are 50% more likely to develop lung cancer (Bade *et al*.,2020). Similarly, there is increased risk for many

industrial workers, particularly in developing countries where safety regulations are not as strictly designed and implemented. In a US survey of major cities, the six cities with the highest airborne particulate matter concentrations had a 40% higher risk of lung cancer. Nonsmokers had the strongest correlation with cancer. According to the US Environmental Protection Agency, particulate matter is strictly regulated in industrial and automotive production. It is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) (Alberg *et al*.,2013).

Indoor Air Pollution: When coal is burned indoors for cooking or heating, it releases particulate matter and gas pollutants that can include formaldehyde, benzene, carbon monoxide, and polycyclic aromatic hydrocarbons (PAHs), among other carcinogens. Humans are carcinogenic from indoor air pollution caused by domestic coal combustion (IARC). In developing countries and rural areas, where wood and charcoal are frequently used for cooking and heating, indoor air pollution from combustion products is especially concerning. According to studies, ventilation in these cooking facilities can reduce lung cancer risk by up to 50% (Alberg *et al*.,2013).

2.9. Molecular Pathogenesis of Lung Cancer

Lung malignancies can be categorised into multiple histological subtypes according to their molecular profiles and genetic changes that can be targeted. The disease exhibits heterogeneity in terms of biological behaviour and clinical characteristics. The molecular alterations in various lung cancer subtypes are reflected in the heterogeneity of the molecular process (Gandhi S *et al*.,2023). The detection of genetic changes in human lung cancer has been made possible by the advancing molecular biology field (Vaporciyan AA *et al*.,2003). Genetic defects linked to cancer development include growth factor expression and its receptors, gain-of-function mutations of oncogenes such as EGFR, BRAF, KRAS, AKL, MET, and HER-2, and loss-of-function mutations of tumour suppressor genes such as TP53, PTEN, and STK11. Because targeted therapies heavily rely on molecular pathology, a complete understanding of the molecular mechanisms underlying the various forms of lung cancer is essential to comprehending the disease's biological behaviour and forecasting how it will react to targeted treatment. It is possible to ascertain the biological behaviour of lung cancer by identifying the genetic mutations linked to a specific type of cancer. This information is crucial for developing diagnostic and treatment plans targeting molecular abnormalities (Gandhi S *et al*.,2023). The

pathophysiology of lung cancer has been widely linked to molecular abnormalities in oncogenes, proto-oncogenes, and tumour suppressor genes. Adenocarcinoma and small-cell carcinoma are associated with gain-of-function mutations of genes encoding tyrosine kinase receptors. In contrast, gain-of-function mutations of oncogenes and tumour suppressor genes lead to the accumulation of multiple genetic abnormalities over time that act by causing cellular alterations such as loss of function mutation of TP53 and Rb genes (Gandhi S *et al*.,2023).

2.9.1. Proto-oncogene Activation

Protooncogenes, the normal homologues of oncogenes, are involved in transcription and signal transduction, two essential cell processes. Malignant transformation requires one mutated allele. Point mutation, amplification, translocation, and rearrangement are the principal changes in the dominant oncogenes that provide gain of transforming function. Tumour suppressor genes and dominant oncogenes interact dynamically inside the cell to limit the growth of individual cells (Vaporciyan AA *et al*.,2003).

2.9.1.1. Ras Activation

The three prominent members of the ras oncogene family, similar to the rat sarcoma virus, are H-ras, K-ras, and N-ras. These members are among the most often activated oncogenes detected in human cancer. The GTPase-active protein p21, found on the inner surface of the plasma membrane and may be involved in signal transduction, is encoded by the ras genes. Point nucleotide mutations that change the amino acid sequence of p21 activate the ras oncogenes (Vaporciyan AA *et al*.,2003). Point mutations in lung cancer cell lines activate the K-ras oncogene.Ras mutations can affect how tumour cells differentiate. For instance, the Harvey murine sarcoma virus infection of SCLC cell lines modifies the phenotypic of variant cells but not classic cells. The mutant SCLC cell line exhibited characteristics of a giant cell undifferentiated lung carcinoma after infection, such as elevated keratin and carcinoembryonic antigen expression. Research supports the theory that lung cancer advances because of ras activation. About one-third of adenocarcinomas that develop in persons with a history of heavy smoking appear to activate Ras. Unlike colon adenocarcinoma, premalignant lung lesions have not been investigated to see if these mutations exist at the precancerous stage (Rodenuhuis S *et al*.,1991).About 25–40% of instances of lung adenocarcinoma include an activating KRAS mutation, the most prevalent oncogenic change. HRAS and NRAS mutations are highly uncommon. Males and smokers are more likely to have KRAS mutations. KRAS mutation was found in 0–15% of adenocarcinoma cases among nonsmokers. KRAS mutation is exceptionally

uncommon in small-cell or squamous-cell carcinomas. Most KRAS mutations happen at codon 12, sometimes at codon 13, and exceptionally infrequently at codon 61. G to T transversions (~84%) are smokers' most prevalent mutations in KRAS, whereas G to A transitions are found in non-smokers(Gandhi S *et al*.,2023).

2.9.1.2. EGFR Mutations

The receptor tyrosine kinases known as the erbB family, which also contains erbB1 (often referred to as EGFR), erbB2 (HER2), erbB3, and erbB4, includes EGFR. EGFR possesses three distinct domains: an intracellular tyrosine kinase and regulatory domain, a transmembrane segment, and an external ligand binding domain. A conformational shift and phosphorylation of the intracellular domain happen upon binding a particular ligand (e.g., epidermal growth factor). This phosphorylation triggers downstream signal transduction by several pathways, such as the RAS/RAF/MAPK, JAK/STAT, and PI3K/AKT/mTOR signalling pathways. Depending on the approach selected, the ultimate goal is either cell maintenance by apoptosis suppression or cell multiplication. DNA mutations in EGFR can affect the protein's intracellular or extracellular regions. Overexpression of EGFR or mutations in intracellular EGFR have been found in 43–89% of non-small-cell lung cancer cases. The EGFR tyrosine kinase domain is mutated in around 25% of non-small cell lung cancers (NSCLCs), and in 75% of these cases, the mutation is linked to elevated receptor expression. Regardless of the presence of ligands at the extracellular site, the most familiar domain mutations in EGFR tyrosine kinase are exon 19 frame deletions and exon 21-point mutations, which cause leucine to replace arginine at codon 858 and activate signal transduction pathways that promote cell proliferation and inhibit apoptosis. All EGFR mutations are seen in lung adenocarcinomas, while they are also present in adenosquamous carcinomas. While there are occasional exceptions, young female patients without a history of smoking are more likely to have EGFR mutations. Pure lung squamous cell carcinoma seldom exhibits EGFR alterations. Secondary mutations in EGFR are seen in patients who become resistant to EGFR TKIs; the most prevalent of these is the T790M activating point mutation in exon 20 (Ding L *et al*.,2008; Yip PY et al.,2013; Inamura *et al*.,2010; DA et al.,2008; Liu P *et al*.,2012).

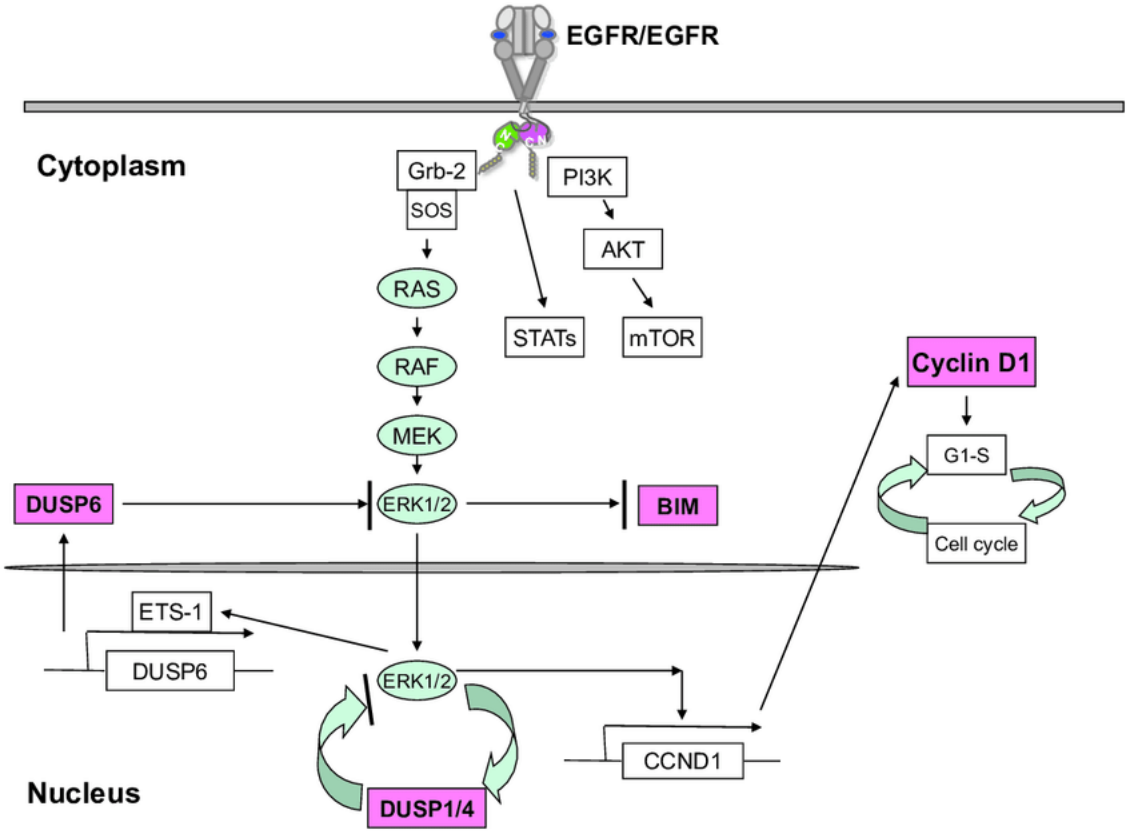


Figure 2.13. An illustration of EGFR-mutated lung cancer—a molecular oncology paradigm (Source: Zhang et al.,2010)

2.9.1.3. BRAF Alterations

A member of the serine-threonine kinase family, BRAF is one of three members of the RAF kinase family (A-RAF, BRAF, and C-RAF). It is essential to the pathways of mitogen-activated protein kinase (MAPK") (Davies *et al*.,2002; Pendharkar D *et al*.,2013). BRAF mutations have been detected in a variety of cancer types, most commonly melanoma, papillary thyroid carcinoma, and metastatic colorectal cancer. Approximately 50%, 9%, and 45% of all mutations have occurred, respectively (Chen D *et al*.,2014). As a downstream effector protein of RAS, BRAF is a proto-oncogene that encodes a serine/threonine protein kinase. This protein transduces signals via the mitogen-activated protein kinase pathway, promoting cell survival and proliferation. After BRAF is activated, downstream mediators MEK1 and MEK2 are phosphorylated. ERK1 and ERK2 are involved in regulating growth-regulating proteins such as c-JUN and ELK1. Increases in kinase activity that show transforming activity in vitro are caused by BRAF-activating mutations. BRAF mutations are most frequently found in cases of

melanoma, although they can also be found in approximately 3% of NSCLC cases. Different mutations are observed in NSCLC compared to melanoma and colorectal carcinoma. The most frequent BRAF mutations in lung adenocarcinoma are V600E mutations in exon 15, which account for 50% of cases. G469A mutations follow these in exon 11 and D594G mutations in exon 15. BRAF mutations in non-small cell lung cancer (NSCLC) can be found in the kinase domain (V600E, D594G, and L596R) or the G-loop of the activation domain of the gene (G465V and G468A). Mutations in EGFR and BRAF are exclusive. While V600E mutations are more prevalent in female non-smokers, non-V600E BRAF mutations are seen in current or past smokers. Mutations in BRAF act as an essential therapeutic target in NSCLC (Gandhi S *et al*.,2023).

2.9.1.4. MYC overexpression

The transcription factors that comprise the Myc gene family, which comprises the genes c-, N-, and L-Myc, are involved in cell division, apoptosis, and the growth of human tumours (Zajac,2001). Myc family members are overexpressed and amplified in lung cancer cells and tumour tissues (Richardson et al.,1993). A subset of SCLC cell lines showed amplification of the c-myc oncogene, one of the specific genetic alterations initially linked to lung cancer (Vaporciyan AA *et al*.,2003). Myc has been suggested to play a part in growth control and cell cycle progression by activating and repressing the expression of critical cell cycle regulators, even though the exact mechanism of Myc activity is still unclear. The function of Myc in inducing the G1/S transition of the cell cycle is regulated by controlling the expression and function of cyclins, pRb-binding transcription factor E2F, cyclin-dependent kinases (CDK), and CDK-inhibitors. It is suggested that by activating cyclin/cdk2 complexes in lung cancer cells, both the overexpression of Myc and the dysregulation of the pRB/E2F pathway enhance the G1 to S transition in parallel (Zajac-Kaye, M.,2001).

2.9.2. Loss of function of tumour suppressor genes

A tumour suppressor gene called TP53 (Tumour Protein 53) is found on chromosome 17p13. It encodes a protein with domains for transcriptional activation, DNA binding, and oligomerization. The encoded protein controls the expression of specific target genes related to metabolic modifications, DNA repair, and cell cycle arrest. It also controls the expression of genes involved in triggering cell death in response to genotoxic stress or growth arrest during

the G1 phase. P53 is hence referred to as "**the genome's guardian**." The injured cells are shielded from mitosis by P53. When cells reach the G2 phase, p53 prevents them from continuing by blocking the cyclin-dependent kinase necessary to begin mitosis. Eighty to one hundred percent of small-cell lung carcinomas have been linked to TP53 missense mutations. It has been established that accumulation of NSCLC proteins or mutations occurs more frequently in SCC than in ADC. In addition, a Cancer Genome Atlas (TCGA) meta-analysis revealed that TP53 mutations were present in at least 81% of SCCs. The variable character of the mutation spectrum has also been linked to tobacco exposure and smoking since smoking-associated malignancies are more likely to have G to T transversions than G to C transversions because of the polycarbonates found in tobacco smoke (Gandhi S *et al*.,2023).

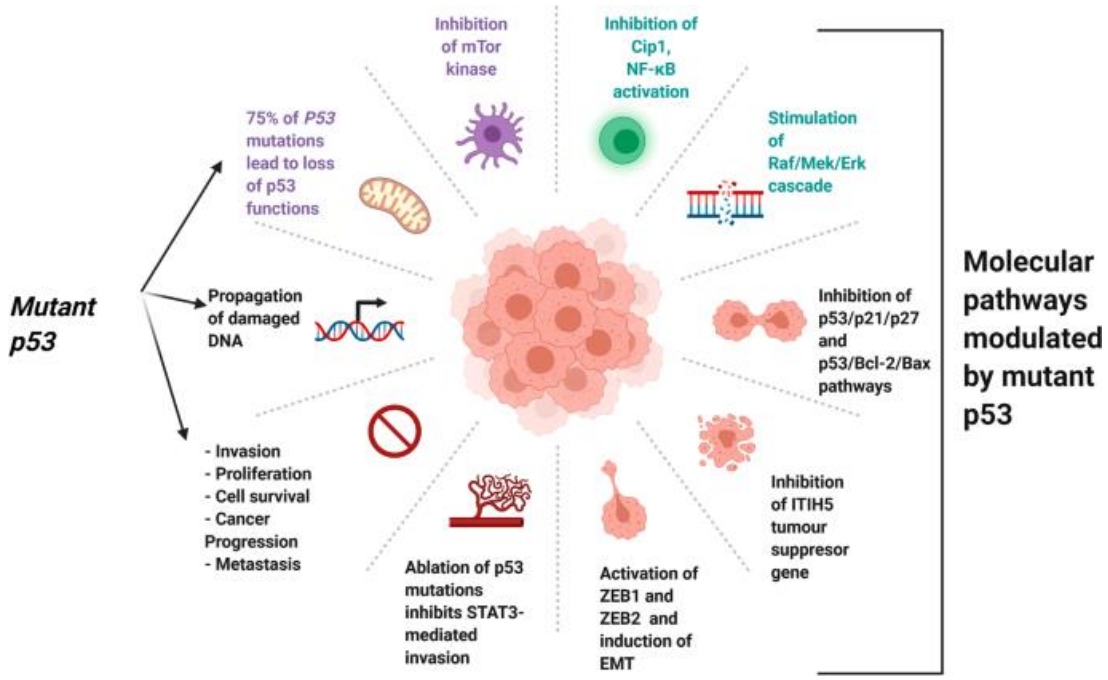


Figure 2.14. Various p53 mutations and their possible impacts on the oncogenic activity and functionality of the protein (Source: Marei *et al*.,2021).

2.10. Lung Cancer Diagnosis

The leading cause of cancer-related mortality in North America and other affluent nations is lung cancer. As per the 2020 special report on lung cancer, it is the most often diagnosed cancer and the primary cause of cancer-related deaths in Canada. Lung cancer typically goes undetected until it has progressed to a point where it kills people. Lung cancer therapy is made

more effective by a thorough pathophysiology, efficient early detection, and appropriate medications. Consequently, it is imperative to find new biomarkers as soon as possible to screen high-risk populations (smokers, fume exposers, those who work in oil fields, and other hazardous environments). Early detection of lung cancer is, therefore, critical. For each patient with lung cancer, the best course of treatment depends on a precise diagnosis. For early diagnosis, finding Histological confirmation of the diagnosis, staging (an assessment of the tumour's extent of dissemination), and examining the patient's functional state to potential treatments are all required components of the diagnostic workup for lung cancer. The patient's overall condition and the potential prognosis must always guide the scope of the workup. For example, in a patient with confirmed significant distant metastases, one would not perform complex invasive diagnostic procedures for accurate N-staging. Specifically for non-small cell lung cancer, precise staging and functional status assessment are critical treatment components. For this reason, the sections on assessing the disease's extent and functional status are relevant to non-small cell lung cancer (Hammerschmidt et al.,2009).

2.10.1. Bronchoscopy: In a bronchoscopy, a tube known as a bronchoscope is inserted into the patient's mouth or nose, down the trachea (windpipe), and into the lungs, where the suspicious lesion is situated, to perform a biopsy. The patient is then given a needle from the bronchoscope to remove tissue they do not feel. General anaesthesia or sedation will be needed for the procedure, depending on whether a flexible or rigid bronchoscope is utilised. One benefit of a bronchoscopy is that it allows the surgeon to assess the airways simultaneously. Surgeons at Hopkins can do navigational or ultrasound-guided bronchoscopies. Electromagnetic technology is used in navigational bronchoscopy to guide the bronchoscope.

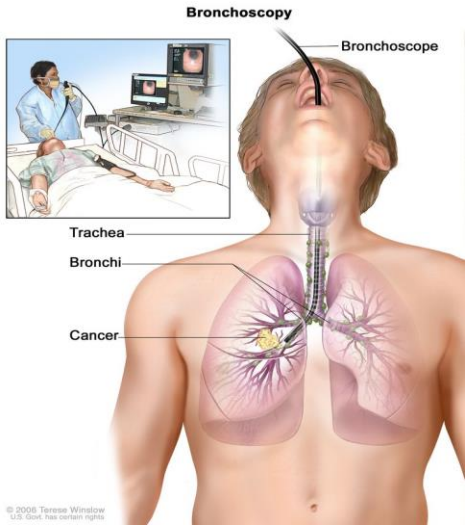


Figure 2.15. Illustration of Bronchoscopy

2.10.2. Lung cancer oncopanels

Worldwide, lung cancer is the primary cause of cancer-related fatalities. Effective treatment and better patient outcomes depend on early and precise diagnosis. Lung cancer oncopanels are comprehensive genetic tests that can identify multiple genetic abnormalities related to the disease, thanks to recent advances in molecular biology and genomic technologies. Lung cancer has significant heterogeneity, encompassing several subtypes and unique genetic profiles (Chen *et al*.,2014). Treatment responses are frequently transient despite the introduction of specific medicines, underscoring the need for a greater comprehension of the molecular pathways underlying the disease. Since radiographic interpretation can be difficult because of the complex anatomical structures seen in chest radiographs, computer-aided diagnosis tools have been developed to help radiologists detect lung nodules at an early stage. Using next-generation sequencing (NGS), oncopanels for lung cancer diagnosis simultaneously examine a panel of genes linked to the disease. These panels can identify various genetic changes, including fusions, amplifications, rearrangements, and mutations, which might influence the choice of targeted therapy and provide prognostic information (Chen *et al*.,2014).

2.11. Treatment of Lung Cancer

Worldwide, cancer continues to rank among the most severe health issues in both industrialised and developing nations. Any cancer treatment aims to eradicate or eliminate the malignant cells while sparing healthy cells. Surgery, radiation therapy, and chemotherapy are the most often utilised conventional therapeutic modalities. These can be employed singly or in combination with one another. Patients with stage-I lung cancer may not be able to have surgery if their health is not well. Although patients unable to undergo surgery have found relief with radiotherapy, the rate of cure is significantly lower than with surgical excision. It is commonly recognised that radiation therapy damages the cells in the surrounding area, which significantly reduces the ability of the lungs to operate. Therefore, patients with a seriously impaired pulmonary system might not fit this technique well. Therefore, in contrast to radiation and surgery, there is a pressing need to create new and more sophisticated therapeutic methods that can efficiently eradicate tumour cells while being less intrusive and harmless to healthy cells. Photodynamic therapy, or PDT, is one of the most recently described methods for curing lung cancer. Nevertheless, This method is applied to early-stage lung malignancies that impact the

central airways, particularly the tissues exposed to light and photosensitivity (Sharma *et al*.,2019).

2.11.1 Platinum-based chemotherapy

2.11.1.1. Docetaxel + cisplatin/carboplatin

Patients with advanced non-small cell lung cancer (NSCLC) experience moderate increases in survival and quality of life while receiving systemic chemotherapy with platinum-based combinations (Belani CP,2005). The anticancer action of carboplatin and docetaxel is broad-spectrum. In a phase I study, we investigated the efficacy of carboplatin and docetaxel as second-line chemotherapy for non-small-cell lung cancer (NSCLC) that had already received treatment (Oka M *et al*.,2005). Docetaxel is a semi synthesized toxoid isolated from the European yew's needles, Taxus baccata, and is generated from a noncytotoxic precursor called 10-deacetyl baccatin III. By attaching itself to free tubulin and encouraging the formation of stable microtubules that result in G2-M phase arrest, docetaxel demonstrates anticancer efficacy. Docetaxel demonstrates a noteworthy clinical efficacy in treating head and neck, ovarian, breast, and non-small cell lung malignancies. In chemotherapy-naive patients with non-small-cell lung cancer, phase II trials using docetaxel alone at a dose of 100 mg/m2 have shown notable efficacy, with response rates of 23–38% and median survivals of 25–47 weeks (Ando *et al*.,2005). When used with cisplatin, docetaxel exhibits a noteworthy impact on non-small-cell lung cancer, with response rates varying between 35% and 45% and a 12-month median overall survival (Millward *et al*.,1997). The clinical use of cisplatin is occasionally hindered by its severe toxicity, including nephrotoxicity, neurotoxicity, and gastrointestinal toxicity, which includes nausea and vomiting, despite its strong anticancer efficacy. Compared to its parent chemical, cisplatin, carboplatin—a second-generation platinum derivative—has significantly lower nephrotoxicity, neurotoxicity, and emetogenic potential. In a randomised Eastern Cooperative Oncology Group investigation, carboplatin as a monotherapy demonstrated the greatest 1-year survival rate with the least toxicity (Bonomi *et al*.,1989).

Mechanism of action

Cisplatin stops DNA replication, which stops cancer-causing cells from proliferating. The aqua-complex cis-[PtCl(NH3)2(H2O)]+ is produced via aquation, which is the displacement of one of the two chloride ligands by water. The aqua ligandin cis-[PtCl(NH3)2(H2O)]+ is displaced by the N-heterocyclic bases on DNA. After guanine attaches preferentially,

[PtCl(guanine-DNA)(NH3)2]+ is formed, which causes DNA cross-linking and disrupts mitosis. Damaged DNA activates the DNA repair mechanism, which results in apoptosis when the damage is irreparable. Despite being called an alkylating agent, cisplatin cannot perform alkylating reactions since it does not include an alkyl group (Yvon *et al*., 1999). For the treatment of many malignancies, cisplatin is now a standard when paired with another chemotherapy medication. The majority of cancer patients eventually relapse because of cisplatin resistance, even though their initial response to platinum is excellent. Numerous processes, such as enhanced drug uptake and efflux, improved drug detoxification, prevention of apoptosis, and accelerated DNA repair, contribute to the manifestation of this resistance.

Williamson et al. (2005) state that docetaxel prevents microtubule depolymerisation and stabilises microtubule assembly when GTP is absent. This promotes and stabilises microtubule assembly. Inhibiting mitotic cell division between metaphase and anaphase and preventing the offspring of cancer cells results in a decrease in the number of free tubules required for microtubule synthesis. (Yvon *et al*., 1999).

2.11.1.2. Irinotecan + cisplatin/carboplatin

The active component of irinotecan (Camposter), a molecule derived from campothecin, SN.38, forms a DNA stabilising complex with topoisomerase-1. Irinotecan is distinct from other chemotherapeutic drugs due to its unique cytotoxic processes and multidrug-resistant cell lines that retain irinotecan sensitivity. In the US, it is a medication that is approved for the treatment of metastatic colon cancer. For the treatment of colon cancer, it is either injected alone or in conjunction with fluorouracil. It is also used in combination with cisplatin to treat small-cell lung cancer. In NSCLC, the response rate might vary from 15–34% when one agent is employed (Pillot *et al*., 2006).

Mechanism of action

Irinotecan is a prodrug that is converted into SN-38, the active form, once it is within the cell. Human carboxylesterases may transport SN-38 and irinotecan via multidrug transporters like ABCB1. Nevertheless, CYP3A4 and CYP3A5 can also convert it to inactive metabolites, and UGT1A1 can inactivate SN-38 by glucuronidating it to SN-38G (Artac*et al*.,2010). This inactivation results in the inhibition of transcription as well as DNA replication. According to Fukuda *et al*. (2004), the best evidence supporting pharmacogenetics in predicting irinotecan toxicity comes from the UGT1A1*28 allele.

2.11.1.3. Pemetrexed + cisplatin/carboplatin

Anti-metabolite inhibitor pemetrexed inhibits TS and other enzymes that are dependent on folate. NSCLC and pleura mesothelioma are its principal uses (Metro *et al*., 2011). Corticosteroids, vitamin B12, and folic acid treatment reduce its toxicity. The most frequent adverse effects include rash, fatigue, nausea, vomiting, and mucositis. For NSCL, pemetrexed is authorised as a second-line treatment (Tomasini *et al*., 2016). Remembrane with carboplatin, a platinum-based medication, is commonly used in patients with non-small cell lung cancer (NSCLC).

Mechanism of action

Chemotherapy medication pemetrexed shares the same chemical makeup as folic acid. It is a member of the group of medications used in chemotherapy called folate antimetabolites. Pemetrexed inhibits thymidylate synthase (TS), glycinamide ribonucleotide formyl transferase (GRAFT), and dihydrofolate reductase (DHFR), three enzymes known to be involved in the production of purines and pyrimidines. According to Metro*et al*. (2011), pemetrexed prevents the synthesis of purine and pyrimidine nucleotides, usually required to survive both normal and malignant cells. This prevents the synthesis of DNA and RNA.

2.11.2 Targeted therapy and immunotherapy

Lung cancer immunotherapy has garnered much attention recently since it helps the immune system recognise and destroy cancer cells. How immune and tumour cells interact inside the tumour microenvironment (TME) determines the anticancer response. TME, comprised of immune cells, extracellular matrix, mesenchymal cells, stromal fibroblasts, endothelial cells, and adipocytes, is a complex signal interaction area surrounding the tumour. Over the last ten years, much research has been conducted on the makeup and pathogenic importance of the tumour immune microenvironment (TIME). An unusual development in the history of immunotherapy has been the identification of immunological checkpoints (ICP), proteins produced by specific immune cells (such as T cells) and cancer cells. The ICPs bind with their complementary companion proteins (receptor-ligand interaction), initiate inhibitory signals, deactivate the T-cell response and under normal physiological conditions (Lahiri *et al*.,2023). With a high specificity and distinct side effect profiles from chemotherapy, targeted therapy has shown to be an essential component of cancer treatment. Targeted medications are

primarily categorised as small molecules or monoclonal antibodies based on their action, including actions on growth factors, angiogenesis receptors, signal pathways, etc (Li *et al.,2023).*Vascular endothelial growth factor is the cause of angiogenesis in tumours (VEGF). VEGF both promotes cell survival and prevents apoptosis. It is a growth factor that regulates the permeability of vascular endothelial cells. Cancer cells produce the protein VEGF. Anti-angiogenic substances may improve the effectiveness of treatment or reinstate normal blood flow within the tumour. (Cabanero *et al*.,2017). It was recently licenced to treat advanced non-small cell lung cancer in combination with carboplatin/paclitaxel, showing a two-month increase in overall survival compared to chemotherapy alone. (Wu *et al*.,2018).

Immune checkpoint inhibitors

In immuno-oncology, discovering immune checkpoint inhibitors (ICIs) represents a significant turning point. Through various strategies, such as activating immunological checkpoint pathways that stifle antitumor immune responses, tumour cells avoid immunosurveillance and advance. By blocking co-inhibitory signalling pathways, ICIs stimulate the immune system to fight cancer by facilitating the immune system's removal of tumour cells. The first immune checkpoint inhibitor to be licenced for treating metastatic melanoma patients is Ipilimumab, which targets cytotoxic T-lymphocyte antigen-4 (CTLA-4). This antibody stimulates effector T-cell activation and proliferation while blocking T-cell inhibition. After ipilimumab was approved, researchers looked at additional antibodies that target immunological checkpoints. (Darvin *et al*.,2018). In the past, patients with advanced non-small cell lung cancer (NSCLC) had poor treatment outcomes, with a median survival of 4-5 months and a 10% 1-year survival rate. More recently, agents like pemetrexed and bevacizumab combined with platinum-based therapies for four to six cycles and single-agent or combination maintenance until disease progression have further improved survival (Aguiar et al.,2016). Nivolumab is a monoclonal antibody that is entirely human and targets PD-1. It was the first immune checkpoint inhibitor authorised in NSCLC second-line therapy. In the phase, Ib clinical trial CheckMate-003, 129 strongly pretreated patients with advanced non-small cell lung cancer (NSCLC), independent of the histology of their tumours, were given nivolumab at 1, 3, or 10 mg/kg every two weeks (Aguiar et al.,2016). For targeting PD-1, nivolumab is a monoclonal antibody made entirely of human tissue. Initially approved for use in NSCLC second-line therapy, it was the first immune checkpoint inhibitor. Nivolumab at 1, 3, or 10 mg/kg every two weeks was administered to 129 heavily pretreated patients with advanced non-small cell lung cancer (NSCLC) in the phase Ib clinical study CheckMate-003, regardless of the histology of their tumours (Garon *et al*.,2015).

2.12. Role of Telomeres in Cancer

Telomeres are conserved, repeated regions that guard the integrity of genomic DNA at the ends of eukaryotic chromosomes (Akincilar*et al*.,2016). Cancer is primarily an ageing-related genetic disorder that only becomes apparent when healthy cells gradually amass genomic instability and develop the capacity for replicative immortality. In addition to causing chromosomal instability, telomere attrition during subsequent cell divisions is a significant factor in genomic rearrangements that can lead to cancer. Cancer cells depend on telomeres, repeating (TTAGGG) DNA–protein complexes at the ends of chromosomes, to survive. Telomeres shield chromosome ends from fusion and recognition as potential sites of DNA damage. When telomeres in normal somatic cells critically shorten after successive cell divisions, it results in dysfunctional telomeres. This causes DNA damage responses (DDRs), which cause cellular senescence. Until several critically shortened telomeres trigger a crisis (a time of total replicative senescence, chromosome end-to-end fusions, and massive death), cells that acquire oncogenic alterations avoid senescence and continue to reproduce (extended lifespan period). This causes breaking, fusion, and bridge formation cycles, in which two sister chromatids without telomeres unite to form a bridge connected by a chromatin. Due to movement towards opposite poles, sister chromatids are pulled apart during anaphase, creating uneven offshoot chromosomes and genomic instability. There is widespread cell death throughout the crisis. However, occasionally, rare cells manage to avoid crises and continue to proliferate by maintaining stable, albeit typically reduced, telomere lengths. Eventually, these cells develop malignant phenotypes (Jafri *et al*.,2016). Chromosome ends are not the only locations for telomeric repeat sequences. In many animals, the interstitial regions of chromosomes have repeat arrays that resemble telomeres. Some mechanisms, including telomere-telomere fusions and aberrant DNA double-strand break (DSB) repair, are probably the source of these (Azzalin*et al*.,2001). These interstitial telomeric repeats are hotspots for chromosome breakage or seed sequences for developing a new telomere, which can affect the stability and structure of chromosomes in at least some situations. Broken DNA ends, such as those resulting from double-strand breaks, are more severe DNA damage than telomeric ends. They are also a significant cause of many, if not most, chromosomal rearrangements. Nonhomologous end joining or homologous recombination are the two natural repair processes for DNA double-strand breaks. Telomerase does not ordinarily add to these breaks (Aylon Y *et al*.,2004). Thus, telomeres play a crucial role in maintaining chromosomal integrity.

Telomeres protect the integrity of the genome by preventing homologous recombination or nonhomologous end joining of chromosomal ends, especially in and around sub telomeric areas.

2.12.1. Telomeres: Structure and Function

A distinct and genetically stable structure known as a telomere makes up the ends of linear genomes. Comprising tandem repeats (TTAGGG) that terminate in a 3' single-stranded G-rich overhang, mammalian telomeres play a crucial role in maintaining a wide range of telomeric activities. A lariat-like structure known as the telomeric loop, or T-loop, is formed when the G-rich overhang, which has between 30 and 500 nucleotides, folds back and invades the double-stranded telomeric helix. A smaller displacement loop, the D-loop, is created when the overhang pairs with the opposing strand. The shelter complex stabilises this entire secondary structure. The G-rich nature of the single-stranded overhang at telomere 3' additionally stabilises the T-loop. The 3' overhang acquires a secondary G-quadruplex structure that results from the hydrogen bonding of guanine residues in tetrad forms. This structure prevents telomeric DNA linearization, which prevents telomerase from physically accessing telomeres. The ability of telomeres to perform homologous recombination and their length are regulated by their heterochromatic state, which is crucial for healthy telomere function. There are two fundamental issues with linear genomes that telomeres address. To begin with, they avoid undesirable DNA damage signalling and genomic instability by differentiating between chromosomal ends and DNA double-strand breaks due to their unique shape. Secondly, they stop vital genetic information from being lost.

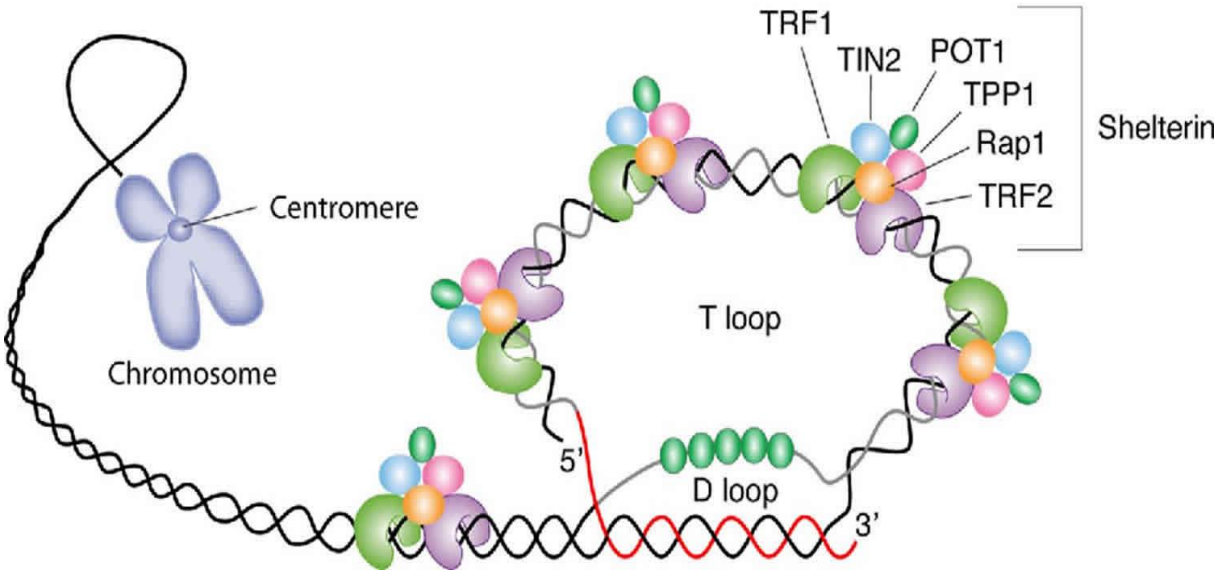


Figure 2.16. Diagrammatic depiction of the telomere structure (Source: Calado and Young,2008)

Note: The single-stranded, G-rich overhang at the 3′ end of the telomere is where it ends (shown in red). The shelterin protein complex, which caps telomeres, physically shields the DNA.

2.12.2. Shelterin-Mediated Telomere Length Regulation

Telomeres are linked to a particular protein complex comprising six components called shelterin. The shelterin complex-associated telomeres are crucial for controlling telomere length and protecting chromosomes (Mir *et al*.,2020). Telomeric DNA has a variable number of G-rich, non-coding tandem repeats of double-stranded DNA sequence, 5′-(TTAGGG) n -3′, which are 10–15 kilobases (kb) long in humans at birth. These repeats are followed by a terminal 3′ G-rich single-stranded overhang, which is 150–200 nucleotides long. The homologous double-stranded TTAGGG region is invaded by the 3′ G-rich overhang, which helps telomeric DNA form a higher-order structure. This forms a telomeric loop (T-loop), which protects the 3′ end by preventing it from being recognised by the DDR machinery (Doksani*et al*.,2013). The three major shelterin subunits, TRF1 and TRF2, which directly recognise and bind duplex TTAGGG repeats, and POT1, which recognises and binds single-stranded TTAGGG overhangs, make up the protein complex known as the shelterin complex, which is linked to telomeres. Three other shelterin proteins, TIN2, TPP1, and RAP1, link these three proteins to produce a complex that allows DDR surveillance equipment to discriminate

between genomic DNA damage sites and telomere DNA. Telomere stability is maintained by the crucial and unique functions carried out by the shelterin complex. TRF2, for instance, is necessary for creating T-loops, sustaining ATM-mediated DDR suppression, and repressing non-homologous end joining (Arnoult *et al*.,2015). TRF1 regulates telomeric DNA replication (Zimmermann M*et al*.,2014). However, POT1 binds the single-stranded 3′ overhang in conjunction with TPP1 to inhibit ATR-mediated DDR by blocking the recruitment of replication protein A (RPA) (Denchi*et al*.,2007). Since TIN2 connects TRF1 and TRF2 to the TPP1/POT1 heterodimer and maintains TRF1 and TRF2 interactions with telomeric DNA, it is critical to the shelterin complex's overall stability. TRF2 and RAP1 interact to enhance TRF2's ability to bind to telomeric DNA selectively (Jafri *et al*.,2016). In addition to protecting DNA ends, telomeres carry out additional crucial tasks, including controlling the expression of genes by transcriptionally silencing genes near the telomeres, or TPE (Pedram M *et al*.,2006) or situated far away from telomeres; this is known as TPE over long distances (TPE-OLD) (Robin *et al*.,2014). The minimum length of telomeric repeats and the efficiency of the corresponding shelterin protein complexes are critical for the strictly controlled function of telomeres. Furthermore, it is believed that appropriate telomere function is aided by higher-order DNA conformations like the G-quadruplexes, which are G-rich four-stranded non-helical structures, and the T-loop (Jafri *et al*.,2016).

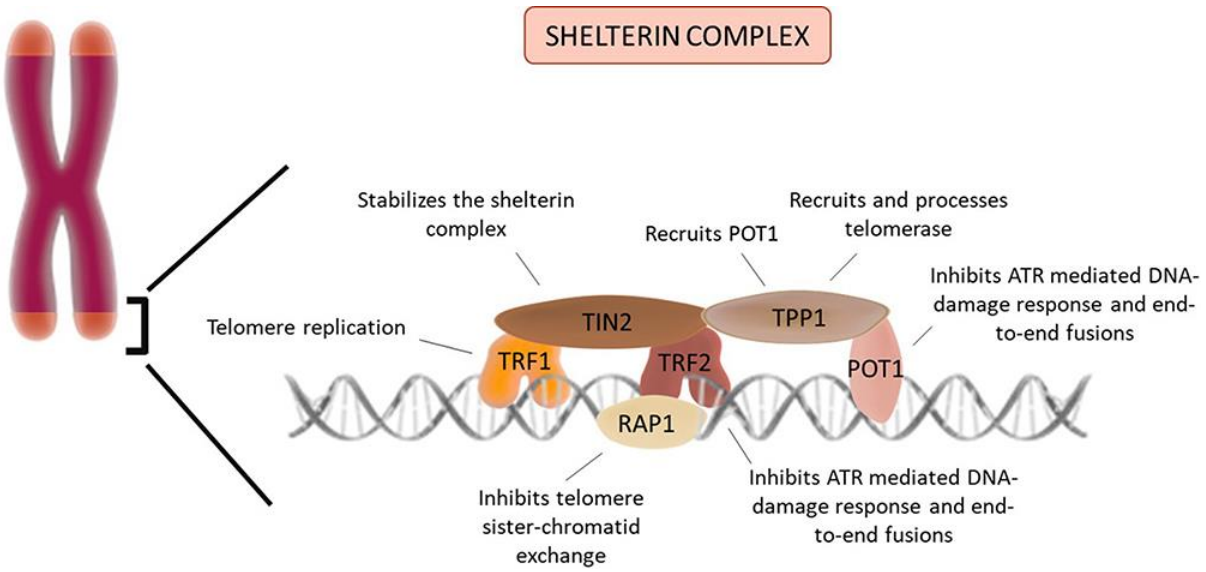


Figure 2.17. Schematic representation of Telomeres and the intricate structure of Shelterin complex (Source: Morais M *et al*.,2020)

2.12.3. Telomeres: Role in Cellular ageing and Replication

The End Replication Problem

Primary cells can potentially divide up to a limited number of times. This limit is also known as the **Hayflick Limit**. In healthy cells, telomere lengthening plays a crucial role. When paired with other carcinogenic alterations, it can exacerbate genomic instability and perhaps trigger the onset of cancer in its early stages. In humans, there is variability in the distribution of telomere length among the various chromosomal arms. Telomere length decreases in human somatic cells cultured at 50–150 bp for each cell division (Jafri *et al*.,2016). According to the end replication problem theory, lagging strand DNA synthesis cannot fully replicate the ends of linear DNA (Ohki *et al*.,2001). Short RNA primers are produced by RNA primase for lagging-strand DNA replication. DNA polymerase then extends these to create Okazaki fragments. No lagging-strand sequence complementary to the little region at the end of the chromosome (at least as large as an RNA primer) can be synthesised once these RNA primers are deleted. Thus, the ends of linear chromosomes lose sequence as cell division proceeds. This strategy produces some blunt-ended daughter molecules regardless of whether the initial terminal has a 3' extension or is blunt-ended (Kipling,1997).

Each type of cell or tissue has a unique rate of telomere shortening, which determines when a chromosomal end will uncap. Because it might be the only factor contributing to the senescence onset signal, the shortest telomere is vital for cell viability and chromosomal stability (Bourgeron T *et al*.,2015). Two significant obstacles must be overcome to stop cell immortalization and, eventually, malignant transformation: Crisis and replicative senescence (Wright *et al*.,1989). Cellular proliferation is inhibited during the cellular senescence phase, sometimes referred to as mortality stage 1 (M1), most likely due to one or more shorter telomeres uncapping. M1 can be omitted when cancer-initiating alterations are present, allowing for a longer time for cell division. However, more telomeres shorten during this phase, and these "marked" telomeres lead to a new dysfunctional condition known as crisis (or M2 crisis). During the M2 phase, signals for cells to divide continuously and to go through replicative senescence are balanced. Chromosome end-to-end fusions and widespread cell death (apoptosis) are the final results (Hayashi *et al*.,2015).

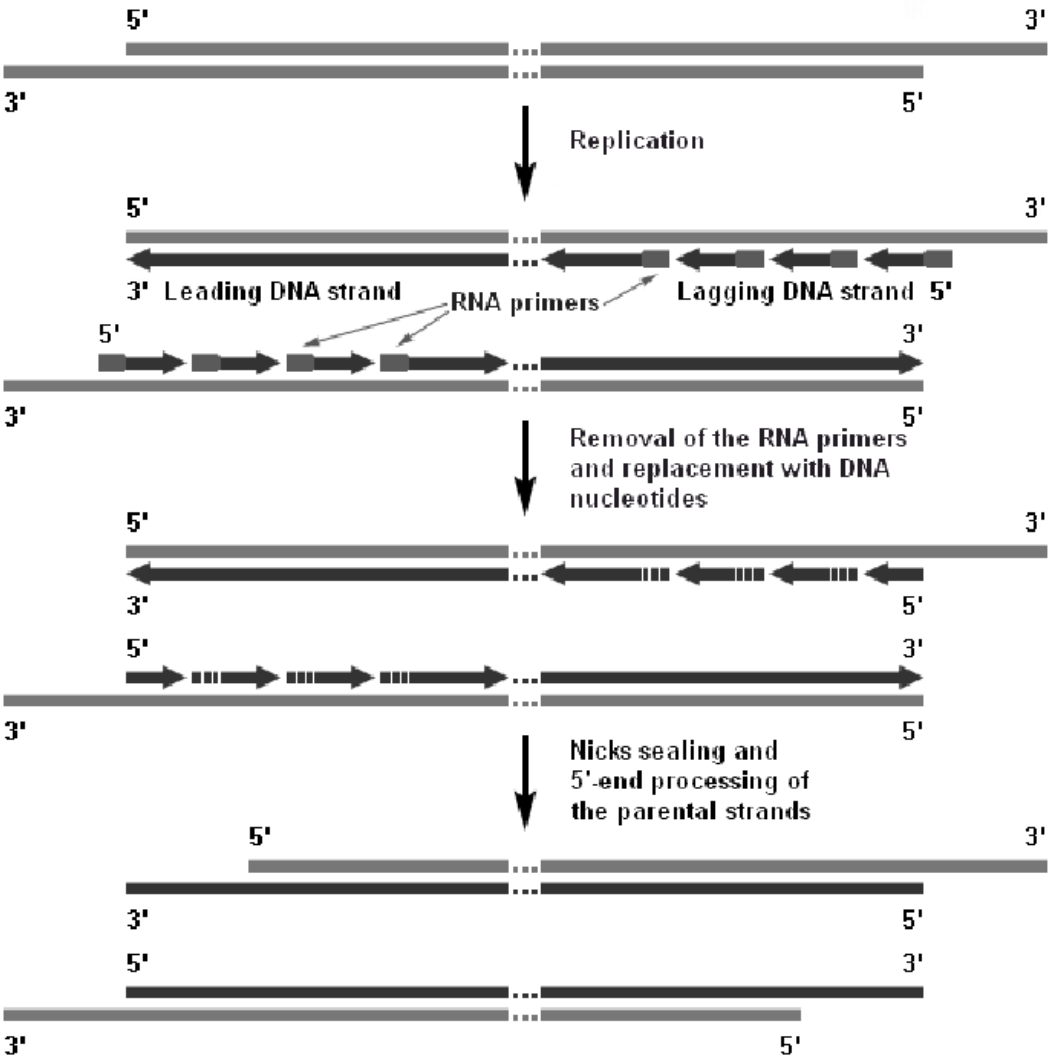


Figure 2.18. Schematic representation of End Replication Problem in Eukaryotes

(Source: Grach, Andrew; 2013)

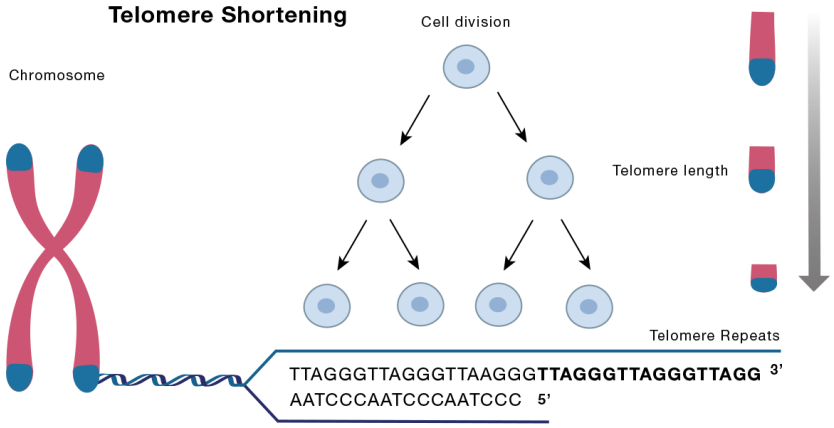


Figure 2.19. Illustration showing Telomere shortening and cellular ageing

2.13. Telomerase: The Enzyme of Immortality

Telomerase is a ribonucleoprotein complex necessary for the maintenance of linear chromosomes in most eukaryotes. It comprises a lengthy non-coding RNA that carries the template sequence for telomere formation and the catalytic subunit of the reverse transcriptase enzyme (Yadav PS *et al*.,2019). To restore telomere length for the following generation, the enzyme telomerase is active in germ cells and during the early stages of embryogenesis. But if an old somatic cell with short telomeres is used for cloning, the progeny may begin with a reduced capacity for cell replication and, after that age, or at least approach senescence, more quickly (Burgstaller JP *et al*.,2017). Since malignant tumours must reactivate telomerase or adopt an alternate method for lengthening telomeres to obtain unlimited proliferation potential, replicative senescence is a crucial barrier in the growth of tumours (Liu Y *et al*.,2002). While telomere elongation is the primary function of telomerase, there is growing evidence that telomerase also has telomere-independent effects, such as increased survival, resistance to chemotherapy, invasion, and metastasis of malignant cells. The enzyme telomerase uses telomeres as substrates to add DNA to the ends of chromosomes, maintaining chromosome length. At the ends of each chromosome, telomerase adds a tandem array of simple-sequence repeats to counteract the DNA erosion that comes with genetic stability. The RNA subunit of the ribonucleoprotein complex, which also includes extra telomerase holoenzyme protein

components within cells that build the active ribonucleoprotein and support its function at telomeres, serves as the template for telomerase reverse transcriptase. Telomerase differs from other polymerases in that it reuses an internal template repeatedly. Like many other polymerases, telomerase catalyses the formation of a product-template duplex by adding nucleotides to the 3′ hydroxyl group of primers (Yadav PS *et al*.,2019).

2.13.1. Telomerase: Structural Organisation

The two main parts of telomerase are the RNA component known as human telomerase RNA component (hTR), which serves as a template for telomere lengthening, and the catalytic subunit known as human telomerase reverse transcriptase (hTERT), which has reverse transcriptase activity. Additional elements that bind to hTERT and hTR to create the holoenzyme are necessary for in vivo telomerase activity (Blackburn *et al*.,2011). Except for certain immune cell types with vital proliferative requirements, germ cells, and stem cells, somatic cells do not exhibit detectable telomerase activity. But in these cells, telomerase activity is only enough to postpone, not entirely reverse, telomere shortening (Vaziri *et al*.,1994). The RNA component known as TERC is necessary for the synthesis of telomeres; it acts as a template for the elongation of the telomeric G-rich strand's 3′ overhang and indicates the repeat sequence that should be added. Three highly conserved structural domains comprise the TERC in vertebrates: the short Cajal-body RNA domain, the CR4-CR5 domain, and the template pseudoknot domain. The template area for telomeric DNA synthesis and a conserved pseudoknot structure essential for telomerase activity is found in the template pseudoknot domain (Chen J et al.,2005). The size and nature of the RNA component differ significantly amongst eukaryotes. TERT is a protein component that functions as a reverse transcriptase and has catalytic domains. In humans, telomerase activity requires TERT and TERC in an in vitro setting; however, in an in vivo setting, some proteins are linked to the holoenzyme complex and are also necessary for the catalytic activity of the telomerase enzyme (Harrington L,2003).

The best classification for proteins linked to telomerase activity has been found in eukaryotes. The telomerase-specific component of the Ciliate Telomerase RNPs complex conforms to telomerase RNA so that the TERT component can recognise it (Collins K,2006). Proteins with this kind of function have not been described in humans; nevertheless, dyskerin (the H/ACA-motif RNA binding proteins), NHP2, NOP10, and GAR1 are known interaction partners of human telomerase RNA (hTR). Together with vast families of H/ACA-motif small nucleolar (sno) and small Cajal body (sca) RNAs, these four proteins combine with hTR. It was proposed

by proteomics of highly pure active human telomerase that only hTERT and dyskerin are connected to Htr (Cohen SB *et al*.,2007).

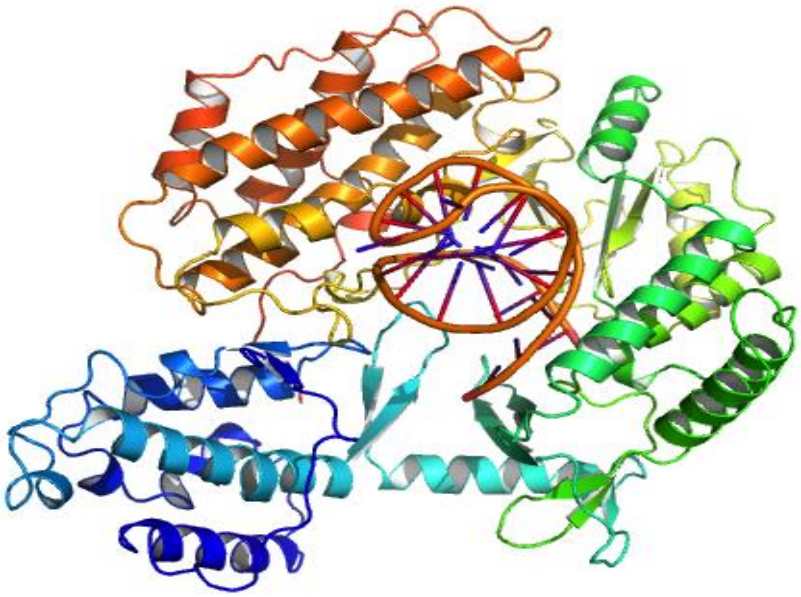


Figure 2.20. Structure of telomerase catalytic subunit, TERT, bound to putative RNA template and telomeric DNA

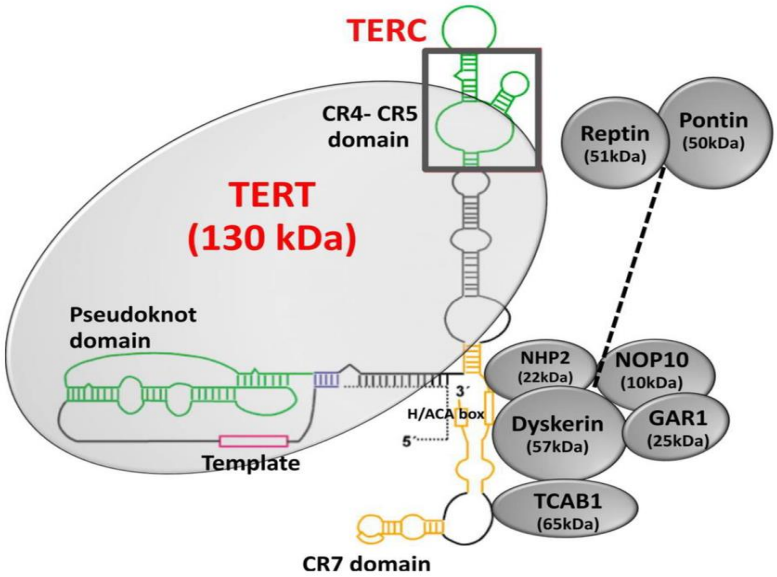


Figure 2.21. Illustration of Telomerase enzyme (Source: Srinivas *et al*.,2020)

2.14. Human Telomerase Reverse Transcriptase (hTERT)

Due to its critical function in preserving genomic integrity, telomerase activity is extensively regulated. Multicellular organisms have developmental, tissue-specific, and stress-reaction methods for inhibiting telomerase (MW et al.,2003; Schaetzlein S*et al*.,2005). The level of hTERT is thought to be the limiting factor for telomerase activity (Liu *et al*.,2000). The fact that hTERT expression is transcriptionally shutoff in somatic cells but that the other telomerase-associated components are constitutively expressed in the majority of mammalian cell types, that hTERT mRNA expression in somatic cells is sufficient to reconstitute telomerase activity and that there is a strong correlation between hTERT expression levels and telomerase activity all support this theory (Meyerson *et al*.,1997; Vaziri *et al*.,1998). The transcriptional activity of the hTERT gene promoter is the primary determinant of hTERT expression. Instead of the usual transcription regulatory elements in TATA and CAAT boxes, the hTERT promoter has multiple binding sites for significant transcription factors. These transcription factors integrate the hTERT transcriptional responses with numerous critical pathways that are dysregulated in different tumours (Akincilar*etal*.,2016). Positive stimulation of the hTERT promoter expression can be achieved by transcription factors and signalling pathways that are often activated in tumour cells, such as c-Myc, specific protein 1 (SP1), upstream transcription factor 1 (USF1), signal transducer and activator of transcription 3 (STAT3), phosphoinositide 3-kinase (PI3K), and nuclear factor of activated T-cells (NFAT).On the other hand, hTERT promoter expression is negatively regulated by Mad, histone deacetylases, E2F1, transforming growth factor-β-activated kinase 1 (TAK1), Wilms tumour 1 (WT1), p53, mothers against decapentaplegic homolog (Smad3), and Menin signalling (Yaswen*et al*.,2015). Despite their abundance, none of the transcription factors that regulate telomerase expression can satisfactorily explain why hTERT expression is particular to cancer (Gladych*et al*.,2011). Because of its GC-rich promoter, the hTERT gene may be regulated by epigenetic mechanisms (Guillere*tet al*.,2004; Cong *et al*.,1999). Histone acetyltransferase (HAT) activity is recruited when DNA hypomethylation or histone methylation surrounding the transcription start site of the hTERT promoter occurs, enabling hTERT transcription (Kyo *et al*.,2008). Additionally, it has been reported that telomerase expression is caused by hypermethylation at particular CpG sites upstream of the hTERT transcription start site in childhood brain tumours (Castelo-Branco *et al*.,2013). Additionally, the following external variables affect hTERT transactivation: It is known that several viruses or virus proteins that interact with telomerase contribute to the tumorigenesis of tissues that are

infected (Dolcetti *et al*.,2012). Understanding the process of telomerase reactivation is crucial for creating diagnostic and therapeutic applications, as telomerase activity is a defining feature of the immortal cell phenotype (Shay *et al*.,2011).

2.14.1. Structure of TERT gene

The 42 kb hTert gene has 16 exons and is found on chromosome 5. The exons 5-8-6-7-8-9 code for the reverse transcriptase domain. There is a suggestion that the 16-exon telomerase transcript can be spliced into 22 different isoforms (Hrdlickova R*et al*.,2012); however, the reverse transcriptase activity required for elongating telomeres is only present in full-length Tert transcripts (Akincilar*et al*.,2016). The isoforms most frequently examined in cancer cell lines are those found in exons 6–9, which partially encode the reverse transcriptase domain (Yi X et al.,2000). This area contains alternatively spliced isoforms that result in isoforms known as minus alpha, minus beta, and alpha/beta. A 36-bp acceptor site is where the minus alpha isoform is spliced into exon 6, resulting in a dominant-negative protein devoid of reverse transcriptase function (Yi X et al.,2000). Cell death or senescence is caused by suppressing telomerase caused by overexpression of the minus alpha form (Colgin *et al*.,2000). Because of a frameshift that occurs after skipping exons 7 and 8, the minus beta isoform has a stop codon in exon 10. The most prevalent type among cancer cell lines is this isoform. It has been demonstrated that the transcripts of minus beta Tert can be translated into proteins, even though its premature stop codon causes nonsensical mediated mRNA degradation (Wong MS *et al*.,2013).

According to Listerman et al., endogenous telomerase activity and overexpression of the minus beta version competed for hTR. Listerman et al. found that overexpression of the minus beta form gave breast cancer cells a growth advantage by shielding them from apoptosis caused by cisplatin (Listerman I*etal*.,2013). Why cancer cells preferentially express the catalytically inactive minus beta-spliced form of Tert is still unknown. In a given cell, TERT isoforms can be produced simultaneously; however, the ratios between distinct cell types may vary in response to external stimuli (Wong MS *et al*.,2013). Shorter versions of the TERT protein are also produced by deletions or insertions outside the RT domain; these versions require meticulous characterization as their roles are yet unclear.

2.14.2. TERT Promoter

The transcription factors c-myc and SP1 have consensus binding sites called E-boxes and GC-boxes, respectively, in the Tert promoter region. Numerous biological processes, including chromatin remodelling, apoptosis, and cell proliferation, are known to be regulated by these molecules. Tert expression is cooperatively regulated in a cell type-specific manner by Sp1 and c-myc (Kyo *et al*.,2000). The Tert promoter stands out for having significant GC-rich content without the usual TATA or CCAAT boxes. The proximal Tert promoter has two E-boxes (CACGTG) in the 165 and +44 regions that are targetable by c-myc and max proteins through their helix–loop–helix leucine zipper domains and five GC-boxes that function as SP1 binding ports (Kyo *et al*.,2000). According to luciferase reporter tests, deleting the E-box at position -165 leads to a 60% decrease in transcriptional activity in C33A and ME180 cells but not in SiHa cells. Remarkably, only in ME180 cells did a mutation at position +44 in the other E-box domain result in a 60% reduction. The sex hormone estrogen's expression and Tert transcription are highly correlated. Tert transcription was significantly decreased by mutations in the oestrogen receptor (ER) binding regions of the Tert promoter.

Furthermore, it is known that ER and a few other growth factors target c-myc. Kyo et al. showed that altering the E-boxes in the proximal Tert promoter eliminated the estrogenic effect. Additionally, in all cell lines, altering the GC-box, which is −32 from TSS, reduced transcription by 20–40%, and when all SP1 binding sites were modified, a 90% reduction was seen (Kyo *et al*.,2000). Thus, we can conclude that E-boxes can accelerate transcription in a cell-type-specific way and that Sp1 binding sites (GC-boxes) are necessary for Tert transcriptional activity. E2F transcription factors play a role in DNA synthesis and cell cycle regulation. In cancer cells, ectopic expression of E2F1 inhibited SP1 binding to DNA, repressing Tert promoter activity.

On the other hand, normal human fibroblasts overexpressing E2F1 saw an increase in Tert promoter activity via a non-canonical E2F1 site between –51 and –88 (Won J *et al*.,2002). These findings point to a dual role for E2F1 in controlling Tert expression, which needs more research. The transcription start site of the Tert promoter is upstream of the two binding sites of the tumour suppressor gene p53, which are −1240 and −1877. When p53 is overexpressed in conjunction with SP1, Tert expression is suppressed. Conversely, p53 siRNA silencing postpones senescence but is insufficient to induce cell immortality. Additionally, overexpression of Tert is insufficient to propel cells towards immortality. Suppression of p53

may increase telomerase activity and TERT expression, but both processes are necessary for immortalising primary human ovarian surface epithelial cells (Kanaya *et al*.,2000; Yang G *et al*.,2007).

2.14.3. TERT Promoter Methylation

There are three CpG islands in the GC-rich promoter of the Tert gene. Although it is commonly understood that methylation of gene promoters represses transcription, several studies have shown that the active/inactive Tert promoter exhibits complicated methylation patterns. Positions −1100 to +150 on the CpG island in the Tert promoter region are primarily hypermethylated in cancer cells due to specific DNA methyltransferases (DNMTs) (Renaud S *et al*.,2007). The Tert promoter represents the regular gene expression pattern between -150 and +150. In this particular area, constitutive expression is caused by the absence of methylation; partial hypomethylation of the core promoter is necessary for Tert transcription, while hypermethylation of the 5′ Tert promoter inhibits the binding of the methylation-sensitive CTCF repressor to the first exon. As a result, Tert promoter methylation offers a distinct transcription model in which suppression of Tert production results from hypermethylation of cytosine islands, with variations in this model across various cell types (Devereux TR et al.,1999; Renaud S *et al*.,2007). In B-cell chronic lymphocytic leukaemia, hypermethylation of the Tert core promoter lowers telomerase activity and increases survival.

Conversely, some studies have shown that hypomethylation in normal tissues and hypermethylation in telomerase-active cancer cells may prevent repressor elements from binding to the Tert promoter region and increase transcriptional activity. Renaud et al. conducted a thorough investigation that showed most cancer cell lines' Tert promoters are substantially methylated between 500 and 600 bp upstream of TSS. However, they typically have partial methylation in the TSS area (Renaud S *et al*.,2007). Histone acetyltransferases (HATs) are brought to the promoter regions by the Myc/Max proteins to bind through their consensus sequences (McMahon SB et al.,2000). Increased acetylation of H3 and H4 histones produces a more open chromatin structure, which improves the accessibility of other transcription regulatory elements.

On the other hand, Mad proteins may attach to the same E-boxes and form heterodimers with Max proteins, which work against each other to prevent transcription (Ge Z *et al*.,2010). The

promoter region's methylation pattern was complex and unusual in the Tert promoter-specific methylation analysis. This suggests that the methylation patterns of the proximal and distal promoter regions differ and can be partially altered due to the activation or inactivation of the Tert gene. These findings emphasise the significance of chromatin modifiers, mainly the functions of histone methyl transferases and demethylases in the transcription dynamics.

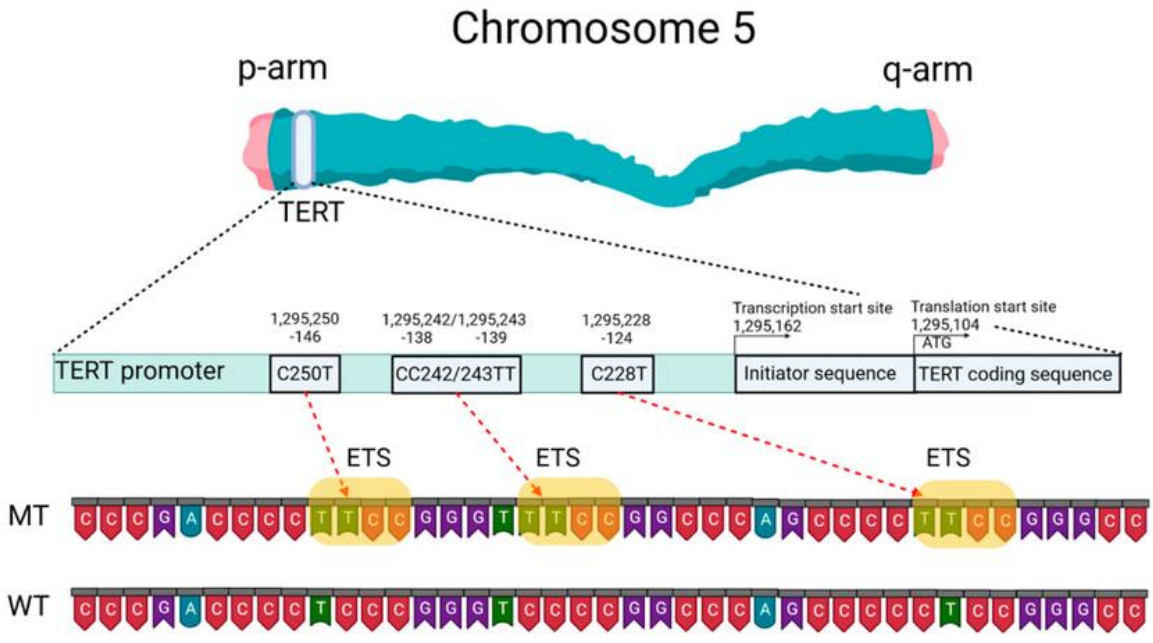


Figure 2.22. The TERT gene's promoter structure and schematic representation at chromosome 5p (Source: Hasanau et al.,2022).

2.14.4. TERT Promoter Mutations

Point mutations in the Tert promoter are widespread in cancer cases, such as thyroid, glioma, melanoma, bladder, urothelial, and hepatocellular carcinomas (Killela PJ *etal*.,2013; Borah S *et al*.,2015). These mutations are linked to increased Tert mRNA and typically result in novel consensus motifs for transcriptional regulators such as ETS/TCF factors. Consequently, a unique pathway for Tert reactivation in cancer cells may be revealed by point mutations in the Tert promoter. Chr.5:1,295,228(C>T) or (CC>TT), Chr.5:1,295,250 C>T, and Chr.5:1,295,242_1,295,243 CC>TT mutations are recurrent in 19% of cancer cases. When telomerase activity is absent, a recombination-based mechanism called alternative lengthening of telomeres (ALT) is triggered. Double-strand DNA break signals trigger ALT recombination

and meiotic HOP2-MND1 heterodimer causes RAD51 and DMC1-mediated recombination. Sarcomas (25–60%), brain tumours (10–25%), and pancreatic neuroendocrine tumours (40%) are the conditions most commonly associated with ALT observations; colon, breast, lung, prostate, and pancreas malignancies are less common. Compared to tumours with telomerase activity, those with ALT typically have a worse prognosis. Glioblastomas with ALT, however, indicate a two- to three-fold greater survival rate. Additionally, it was recently demonstrated that mutations in the ATRX or DAXX genes enhance the expression of the non-coding RNA TERRA, which is known to impede the action of telomerase. Moreover, TERRA can cause ALT by creating R-loops in telomeres. ATRX and DAXX alter telomeric chromatin dynamics that function as a chromatin remodeler and histone chaperone, respectively. Therefore, cells having these mutations depend on ALT for telomere maintenance. Interestingly, ATRX/DAXX genes and *Tert* promoter mutations are mutually exclusive. Additionally, some subpopulations exhibit both ALT and telomerase mechanisms for maintaining telomeres. Depending on the cellular context and other signalling milieu, this could be a brief or transient stage before a permanent commitment to ALT and/or telomerase as the primary mechanism. However, nothing is known about how cells pick between ALT and telomerase as their preferred telomere maintenance method or how they might use both systems simultaneously. The reactivation of telomerase is a primary cancer marker and has been proposed as a way to maintain telomeres. As previously mentioned, the cell has adequate numbers of other telomerase complex components. Therefore, telomerase reactivation mostly depends on the amount of TERT present. Thus, TERT increases could be compensated for by upregulating the number of copies of a gene, as in the case of HeLa cells (five copies), overexpressing oncogenes such as c-Myc that can bind its promoter for transcription, or employing alternative splicing to produce catalytically active or inactive proteins (Akincilar*et al*.,2016).

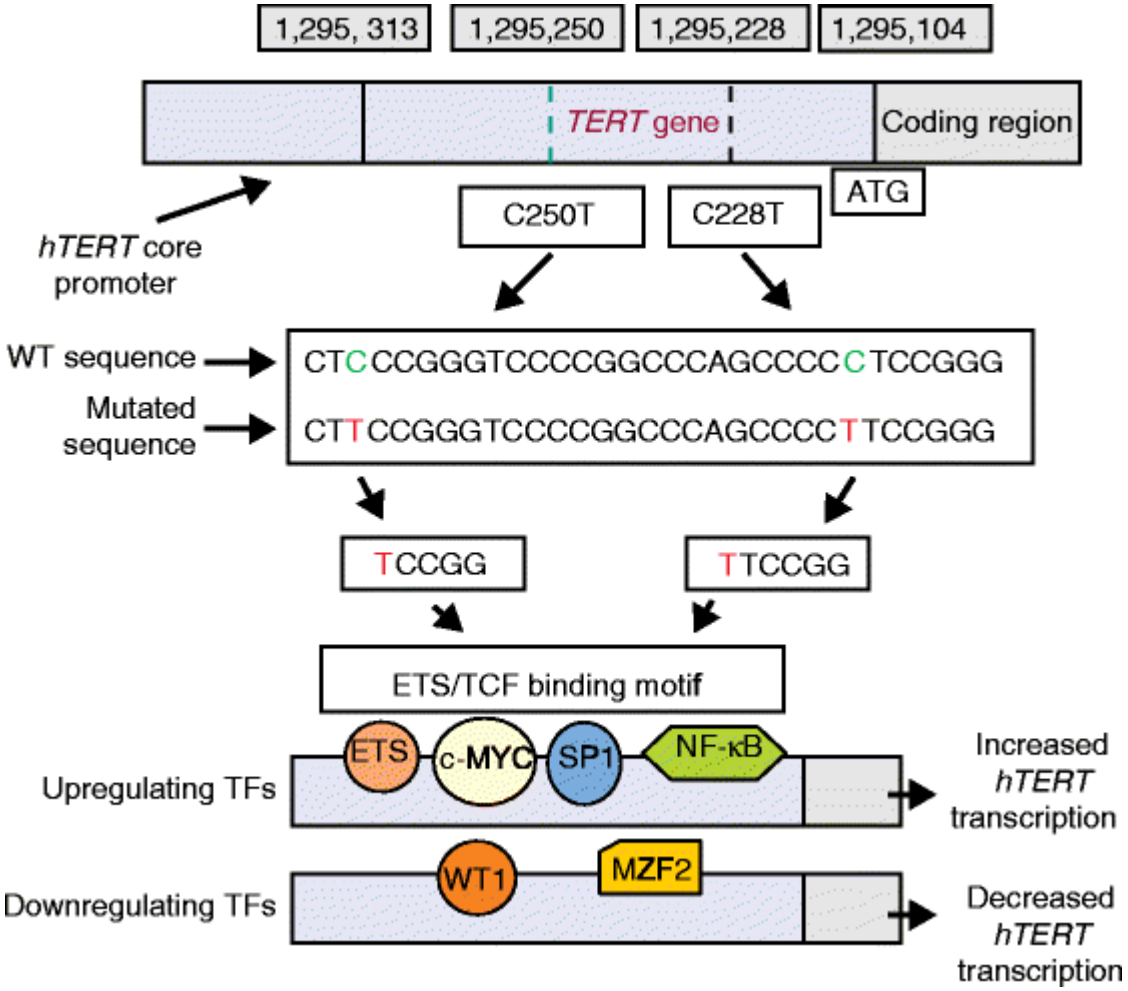


Figure 2.23. Schematic representation of TERT Promoter mutations (Source: Jafri *et al*.,2016).

Note: Mutations in the promoter of hTERT and transcription. In practically all normal cells and tissues, the hTERT gene is tightly suppressed. Specific hTERT promoter mutations that result in elevated hTERT transcription are a part of the course of cancer. Many transcription factors (TFs) are involved in regulating hTERT transcription. hTERT promoter alterations result in binding motifs for ETS and TCF. Every mutation produces a new ETS/TCF binding site. Upregulating TFs can stimulate hTERT transcription by binding to their specific locations, including NF-kB, c-MYC, SP1, ETS, and c-MYC. A favourable chromatin microenvironment is necessary for hTERT transcription, even if TF binding is also necessary. Reduction of transcription occurs when downregulating transcription factors bind. MZF2 myeloid zinc finger protein 2, WT wild type, and WT1 Wilms tumour protein

2.15. Impact of hTERT Polymorphism on Cancer

The repetitive sequences known as telomeres, found at the end of linear chromosomes, prevent genetic information from being lost during cellular division. Repeated cell cycles shorten telomeres, put cells in a state of senescence, and ultimately produce programmed cell death I, which may shield cells against carcinogenesis and genetic instability. Telomeres may, therefore, be seen as a crucial component of cellular genomic maintenance and a possible candidate for carcinogenesis (Hashemi *et al*.,2014). The hTERT locus, which has downstream introns and a promoter, contains single nucleotide variations (SNVs), which could represent an extra or different mechanism affecting the expression and activity of the enzyme). In addition to influencing telomerase function and telomere length, genetic variability in the hTERT genomic area may also influence the course of chemotherapy and cancer development. Specific TERT promoter polymorphisms have been linked to an increased risk of haematological disorders and have even been proposed as survival prognostic indicators (Ropio J *et al*.,2016). The preservation of telomere length is closely related to cellular ageing and the emergence of different malignancies, and it is dependent primarily on the human telomerase reverse transcriptase (hTERT) gene.

2.16. hTERT Promoter Polymorphism rs2735940

After a certain degree of telomere shortening, senescence is thought to be induced, serving as a control mechanism that limits the ability of cells to replicate. Therefore, the activation of a telomere maintenance mechanism is necessary for the continuing growth of cancer cells. Eleven percent of human cancer cells use the Alternative Lengthening of Telomeres (ALT) mechanism, whereas the remaining fifteen per cent use the telomerase enzyme, which appends telomeric repeats to chromosome ends. Various variables influence telomerase activity, but in many tumours, the expression of hTERT (human Telomerase Reverse Transcriptase), the enzyme's catalytic component, limits the enzyme's function. Since hTERT expression determines cell immortalization and resistance to senescence and apoptosis, it is also linked to tumour formation and progression and its effect on telomere elongation. These non-canonical telomerase actions are regarded as telomere-independent. As a result, the hTERT complex is tightly regulated in cancer cells by various processes, including transcriptional and post-transcriptional pathways (Ropio J *et al*.,2022). Specific TERT promoter polymorphisms have been linked to an increased risk of haematological disorders and have even been proposed as

survival prognostic indicators. Regarding hTERT transcription activation, childhood ALL risk was linked to the TERT promoter region SNP rs2735940. TERT mRNA levels were higher in the rs2735940 T than in the C allele (Ropio J *et al*.,2016).

2.17. Impact of hTERT polymorphism rs2735940 on Cancer Progression

TERT, the telomerase reverse transcriptase component, is essential for maintaining telomeres, chromosome stability, and cancer prevention (Ding D *et al*.,2013). Besides telomere extension, numerous biological activities of TERT have been demonstrated to be connected to the development and spread of tumours. Tandem nucleotide repeats of the (TTAGGG)n sequence make up human telomeres. These repeats are bound by a multiprotein complex called "shelterin" or the telosome, which functions fundamentally in arranging telomerase activity (Zhang *et al*.,2012).

The T → C transition located 1327 base pairs upstream of the transcription start site, hTERT rs2735940 T > C polymorphism, affects the transcriptional efficiency of hTERT. Their biochemical investigations discovered that the T allele of the rs2735940 T > C polymorphism had a promoter effectiveness that was almost 25% higher than the C allele. Furthermore, compared to the TT and TC genotypes of hTERT rs2735940 G > A polymorphism, people with the homozygous CC genotype of hTERT rs2735940 G > A polymorphism had shorter telomere length in their peripheral blood leukocytes (Bayram *et al*.,2016). Increased risk of cancer is linked to the single nucleotide polymorphism (SNP) at the rs2735940 location in the human telomerase reverse transcriptase (hTERT) gene (Wang *etal*.,2017). The rs2735940 variant in the human telomerase reverse transcriptase (*hTERT*) promoter is a functional polymorphism that affects the transcriptional activity of the hTERT gene. However, studies have shown that this variant is not significantly associated with telomere length. For example, a study on breast cancer patients found no significant differences in telomere length between patients with different rs2735940 genotypes (CC, CT, and TT) (Rampazzo *et al*.,2020). The hTERT gene's rs2735940 polymorphism has been the focus of extensive research since it has been linked in several studies to an increased and decreased cancer risk. It is controversial to conclude that the hTERT rs2735940C > T polymorphism increases the risk of developing cancer. According to studies by Pande et al. and Walsh et al., individuals who identify as African Americans, non-Hispanic white people, and ethnic Koreans may be much more likely to get lung cancer if they have the T allele or TT genotype of the hTERT rs2735940C > T polymorphism (Pande et

al.,2011; Walsh et al.,2013). Furthermore, in the Chinese population, the TT genotype was associated with a higher incidence of infantile acute lymphoblastic leukaemia. Furthermore, in codominant, dominant, and log-additive inheritance models, carriers of the TT genotype and/or T allele of hTERT rs2735940C > T polymorphism are at increased risk of developing gastric cancer, according to Bayram et al. Based on the previously provided information, a meta-analysis involving 19,385 cancer patients and 17,558 healthy people showed that the hTERT rs2735940 polymorphism's T allele and TT genotype significantly enhanced cancer risk (El Feky *et al*.,2019).

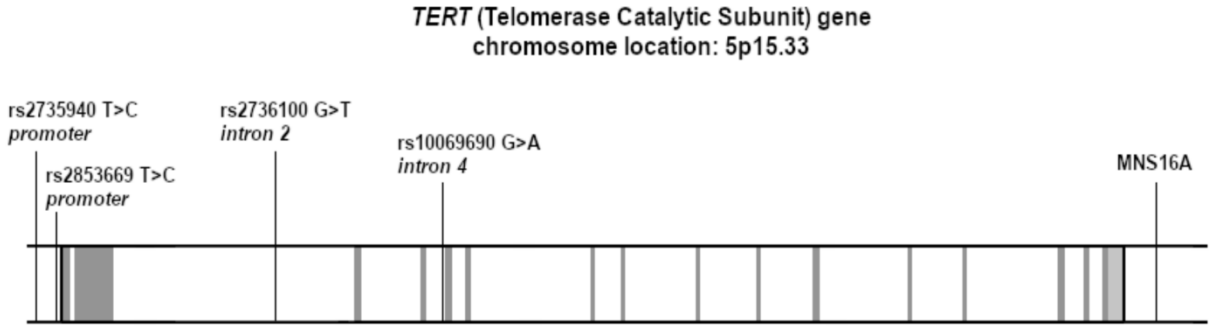


Figure 2.24. Figure representing the location of the SNP under study rs2735940 T>C and the genomic organisation of the human telomerase TERT gene (Source: Dratwa*et al*.,2022).

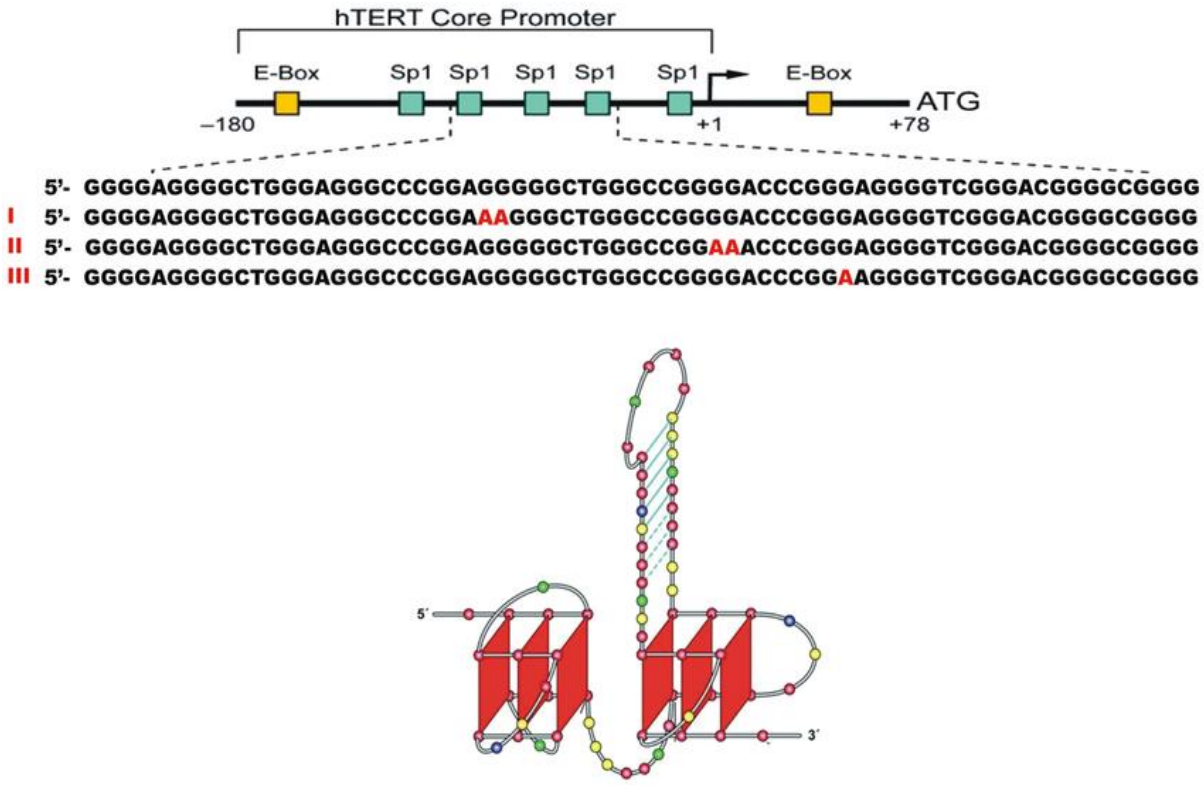


Figure 2.25. Figure representing the sequence and the proposed structure of the hTERT promoter (Source: Chaires *et al*.,2014)

CHAPTER 3

GAPS IN STUDY AND OBJECTIVES

3.1 Gaps in the study

The hTERT gene polymorphism is a crucial factor in cancer susceptibility. Lung cancer cases and genetic variants are highly prevalent in India. In addition, Indians use local cigarettes more often, which contain significantly more carcinogenic ingredients. As a result, there is a good chance that the North Indian population is more likely to acquire lung cancer. On the other hand, no studies in the North Indian Population have examined the association of the hTERT rs2735940 polymorphic variant with lung cancer. Hence, it is necessary to study the association of the hTERT rs2735940 polymorphic variant in the North Indian population.

Furthermore, no research has been done in the Indian population on the function of the SNP rs2735940 in the hTERT gene and its correlation with the chemotherapy results of patients with lung cancer receiving platinum-based chemotherapy. Thus, finding hTERT gene polymorphisms and comprehending their relationship to lung cancer patient survival and susceptibility to lung cancer would explain how this polymorphic variation affects chemotherapy outcomes negatively or positively in North Indians. The choice of chemotherapy regimen may enhance the patient's reaction and, consequently, their overall prognosis. The response rate and survival of lung cancer patients may be enhanced by individualized treatment based on their molecular markers. In addition, the SNP has the potential to serve as a biomarker, aid in the creation of genotype-based customised chemotherapy regimens, and ascertain the relationship between this polymorphism and clinical outcomes, such as patient OS and objective response rates (ORRs) measured by RECIST.

3.2 Objectives of the study

- To evaluate the role of polymorphic variant rs2735940 of the hTERT gene towards lung cancer susceptibility.
- To evaluate the overall survival and clinicopathological features of the hTERT gene polymorphism.
- To evaluate the toxicity profiles of lung cancer patients undergoing platinum-based chemotherapy.

CHAPTER 4

MATERIALS AND METHODS

4.1 Design of the study

A case-control study design was chosen to assess the north Indian population's propensity for lung cancer and its relationship to the hTERT polymorphic variant rs2735940. Three hundred eighty-seven lung cancer patients were recruited to study the hTERT gene, and the study also included 384 healthy controls. Blood samples of lung cancer patients were collected in this research study from the Department of Pulmonary Medicine, Postgraduate Institute of Medical Education and Research (PGIMER) Chandigarh, India. The Institute Ethics Committee of PGIMER reviewed and authorized this study (Approval No. IEC-04/2018-8840 was conducted in agreement with the ethical standards outlined in the 1964 Declaration of Helsinki and its subsequent amendments. The ethical clearance letter is attached in Appendix I. All participants or their representatives provided informed written consent, Appendix II includes. Patients' information was also obtained from participants or their representatives, as attached in Appendix III.

Patients newly diagnosed with primary lung cancer were included in the current study. The enrolled patients had NSCLC and SCLC subtypes identified histopathologically. There was no age, smoking, gender, histology, or TNM stage limitations.

Patients meeting all of the prerequisites will be qualified for enrolment.

- Histology or cytology is utilized to accurately diagnose non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC).
- Clinical stage I, II, III, and IV disease ().
- No limitations on age, gender, smoking status, histology, or staging were applied.
- Untreated to undergo definitive chemotherapeutic treatment (administered with platinum-based agents, i.e., cisplatin/carboplatin as the first or second line).
- As to the Eastern Cooperative Oncology Group (ECOG), the performance status (PS) is 0-2.
- At least one bi-dimensionally quantifiable lesion, according to the Response Evaluation Criteria in Solid Tumour Group (RECIST) criteria.

- Proficiency in organ function, as demonstrated by platelet count >100,000/L, absolute neutrophil count >1500/L, creatinine levels, and liver enzymes such as alanine aminotransferase (ALT) levels less than two times the upper limits of normal (ULN).
- Written informed consent was acquired.

The exclusion criteria included the following:

- Patients had a prior history of any other malignancy.
- Patients with immunosuppressive conditions or active infection.
- Patients were undertaking any systemic steroids.
- Patients are experiencing a chronic form of diarrhoea due to any cause.
- Patients who are not receiving chemotherapy.

However, physiological tests were performed before the patient's recruitment, including a complete blood count (CBC), which evaluates neutrophil count, and biochemical tests such as liver and renal function tests. In addition, the patient had a chest X-ray and a contrast-enhanced computed tomography (CT) scan of the thorax and upper abdomen region.

In addition, 384 unrelated individuals with no history of lung or any other cancer who regularly visited PGIMER for health check-ups were enlisted as a control group. An attempt was made to match controls concerning age (+- ten years), gender, and smoking parameters concerning cases (i.e. to include people from all age groups, both genders and with less as well as high pack-years). A trained interviewer filled out a detailed questionnaire for each case and control. The questionnaire included crucial demographic information such as age, gender, the patient's region, smoking, and drinking habits. Smokers reported tobacco habits such as cigarette and beedi smoking (a native cheaper than a cigarette-like stick of unprocessed tobacco that is hand-rolled in a dried temburini leaf).

The following formula was used to compute pack years as a measure of cumulative smoking exposure:

Pack years= [(Number of beedis or cigarette per day/20) * Number of years smoked]

The hospital's medical record contained all of the pertinent case details, including histology, TNM classification, clinical staging, performance status (KPS and ECOG), chemotherapeutic regimen, and objective response measured by the Response Evaluation Criteria in Solid Tumour Group (RECIST) criteria.

4.1.1 Chemotherapeutic Regimen

All lung cancer enrolled in this study were administered a double platinum-based chemotherapy regimen. This combination contained a platinum-based medication, such as cisplatin or carboplatin, and many medications, including docetaxel, paclitaxel, irinotecan, and pemetrexed. The drugs, as mentioned above, were given intravenously at a fixed dosage based on the body's surface area. A one-hour infusion of docetaxel (75 mg/m2), pemetrexed (500 mg/m2), or 100 mg/m2 irinotecan was given as part of the combination chemotherapy regimen. An intravenous infusion *iv*. of 70 mg/m2 cisplatin was then administered for more than three hours. Supplements of standard folate and Vitamin B_{12} formulations were administered to each patient. Certain drugs, such as dexamethasone, granisetron, and ranitidine, were also given as a part of the premedication routine. As per standard PGIMER protocol, four rounds of chemotherapy were administered before performing the tumour response assessment. The tumour response was examined, and, if necessary, chemotherapy was halted if there was intolerable toxicity or clinic-radiological signs of cancer progression before the completion of four cycles. Patients who demonstrated an objective response to therapy received two additional cycles (*i.e*., a maximum of six).

4.1.2 Follow-up and Tumour Response Assessment

All subjects were contacted for follow-up telephone conversations after every two months until the end of the study period. The survival time was calculated from the date of diagnosis of lung cancer till the last date of follow-up or death due to uncertain reasons. The primary endpoint of the research was overall survival, and the secondary endpoint was to evaluate the association of genetic polymorphism with treatment response and adverse events (AEs). Based on RECIST criteria, responses are broadly classified as "responders" who exhibit complete response (CR) or partial response (PR) and "non-responders" who exhibit stable disease (SD) and progression disease (PD).

- CR: Complete eradication of the targeted lesions
- PR: At least a 30% reduction in the sum of target lesions diameters, using the baseline sum diameter as a reference.

- PD: At a minimum of 20% increase in the total diameter of target lesions, taking the smallest sum in the study (this includes the baseline sum if that is the smallest in the study). In addition to the relative increase of 20%, the sum must demonstrate an absolute increase of at least 5 min. (Note: the emergence of one or more new lesions is also considered progression).
- SD: Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking the minor sum diameters as a reference while on study (Eisenhauer *et al*., 2009)

The standard toxicity criteria (CTC) version 3.0 was used to record and categorize adverse events (AEs). Toxicity levels were evaluated for patients receiving three or more cycles of chemotherapy as well as for all the patients who received at least one chemotherapy cycle. PGIMER's standard protocols were used to handle AEs. In cases where outpatient therapy was either impractical or unsuccessful, patients with febrile neutropenia or grade 3 or higher gastrointestinal adverse events (AEs) were admitted to the hospital. In addition, any other AEs caused by chemotherapy were recorded.

4.2 DNA Extraction

4.2.1 Genomic DNA Extraction

Genomic DNA was isolated from peripheral blood samples of both cases and controls according to the protocol of Bartlett and White (2003) with slight modifications.

- Firstly, 3ml of whole blood was treated with an equal volume of washing buffer containing 320 mM sucrose, 1.5% Triton X-100, 20 mM Tris-HCl (pH 8.0), and five mM $MgCl_2$ separating red blood cells (RBCs) from the blood lymphocytes.There were three iterations of this procedure. The samples were centrifuged at 3,500 rpm for 10 minutes at four °C each time.
- Further, the RBC-free pellet was lysed at 45°C overnight using an equivalent volume of WBC lysis buffer that contained 400 mM Tris-HCl (pH 8.0), 150 mM NaCl, 60mM EDTA, 1% SDS, and 100 ug/ml proteinase-K.

- An equal volume of phenol/chloroform/isoamyl alcohol (P:C: I=25:24:1) solution was utilized to remove proteins. After gentle mixing, the solution was centrifuged at 8000 rpm for 7 minutes at four °C, resulting in two distinct layers. The upper aqueous layer was transferred into a new vial, and this procedure was repeated.

- An equal volume of chloroform/isoamyl alcohol (C: I=24:1) solution was added to the aqueous layer. To separate the aqueous layer into another vial, the mixture was carefully mixed and centrifuged at 6500rpm for 5 minutes at 4°C.

- The DNA precipitation was done using 2.5 times the ethanol or an equal volume of chilled isopropanol. The solution was maintained at -20°C for 1-2 hours, followed by centrifugation at 10,000 rpm for 5 min.

- After centrifugation, the retrieved pellet was washed with ice-cold 70% ethanol by centrifugation at 10,000 rpm for 5 min. The pellet obtained was then allowed to be air-dried.

- The DNA pellet was then discovered in 200ul of sterile Tris-EDTA buffer.

4.2.2 Qualitative and quantitative estimation of genomic DNA

The agarose gel electrophoresis method was used to assess the quality of the extracted DNA. The visualization of extracted DNA samples was examined using a 0.8% agarose gel with 0.5ug/ml ethidium bromide (EtBr) immersed in 0.5X TBE. The samples were mixed with 6X loading dye and diluted with sterile water before loading in the wells. A UV transilluminator was used to determine whether DNA was present in the gel (Johansson, 1972).

The quantitative estimation of extracted DNA was done using spectrophotometric analysis by Nanodrop (Nanodrop ND-100 spectrophotometer. Fifty nanograms per litre of double-stranded DNA (50ug/l) equals one absorbance unit (1OD) at 260 nanometres. The sample purity was determined using the absorbance ratio of 260 nm to absorbance at 280 nm. The ratio for a pure DNA sample is supposed to be 1.8. Protein contamination may be present in the DNA sample if the ratio is less than 1.8, while RNA or phenol contaminants are indicated by a ratio greater

than 1.8. (Desjardins and Conklin, 2010). Following a thorough analysis of purity and concentration, DNA was diluted to 100 ng/l and kept at -20°C to be used as a template later on.

4.3 Genotyping of hTERT polymorphic variant rs2735940

4.3.1 Genotyping of hTERT polymorphism by PCR-RFLP

PCR-RFLP analysis was used for genotyping analysis. Since its introduction in 1988 (Wang *et al*., (2017), the PCR-RFLP approach has demonstrated its efficacy as a sensitive and quick way to discover gene polymorphisms. (Table 4.1). A standard PCR was employed for the amplification of the polymorphic site. The amplification was carried out in a 15ul mixture, which consisted of 1.5X PCR buffer, 0.75uM of Forward and Reverse primer, 0.3uM dNTPs, 1.5X bovine serum albumin (BSA), and 0.2U Taq polymerase (DNAzyme, Thermo Scientific) and 0.5ul DNA template. PCR conditions utilized were 94°C for 5 min and followed by 30 cycles of 94°C for 30 seconds (Denaturation), an annealing step at an annealing temperature of the specific primer for 45s (Annealing), followed by primer extension at 72°C (Extension) for the 29 cycles as well as the final extension step at 72°C for 5 mins.

Restriction Fragment Length Polymorphism

After the successful PCR amplification, the PCR product was treated with a suitable restriction enzyme procured from New England Biolabs, Ipswich, MA, USA. 10ul of each PCR product was treated with five units of enzyme in the presence of IX respective NEB buffer, resulting in a restriction volume of 20ul. The reaction mixture was then incubated at 37°C for 12-16 hours at the desired temperature. The amplification of the SNP post-PCR was confirmed using 2.2% agarose gel electrophoresis. A 100 bp molecular weight marker was utilized to verify successful amplification.

4.3.2 Agarose Gel Electrophoresis

Agarose is the supporting medium in the electrophoretic process known as agarose gel electrophoresis. Agarose can be shaped into various sizes, shapes, and porosities, and it has a network structure in agarose gel. It is frequently employed for nucleic acid analysis and detection, serving as a "molecular sieve" and an "electrophoresis." Regular agarose gel has a broad separation range and is simple to make. Agarose gel concentrations can be adjusted to separate DNA fragments ranging from 200 bp to 50 kb. (Miao *et al*., 2020). A predetermined quantity of agarose powder is dissolved in gel buffer (refer to Table 5.1) by heating the combination in a microwave oven to create a gel.

After cooling to around 60°C, the agarose gel solution is put onto a sample preparation tray, and the sample comb(s) is/are inserted (Fig. 5.1). Because there are more bubbles in higher percentage gels, like 3%, special care must be taken to puncture the molten gel's bubbles with clean pipet tips to prevent distorted nucleic acid migration during electrophoresis. Sample comb(s) can be gently removed to leave well-formed sample wells once a gel has fully solidified. Before loading, nucleic acid specimens must be combined with a loading buffer containing glycerol (sucrose or Ficoll can also be added to increase sample density) and one or more tracking dyes, such as xylene cyanol FF or bromophenol blue. Apart from marking the correct loading of a nucleic acid sample into a sample well, the dyes also function as crude trackers to track the electrophoresis process. (Sun *et al*.,2010)

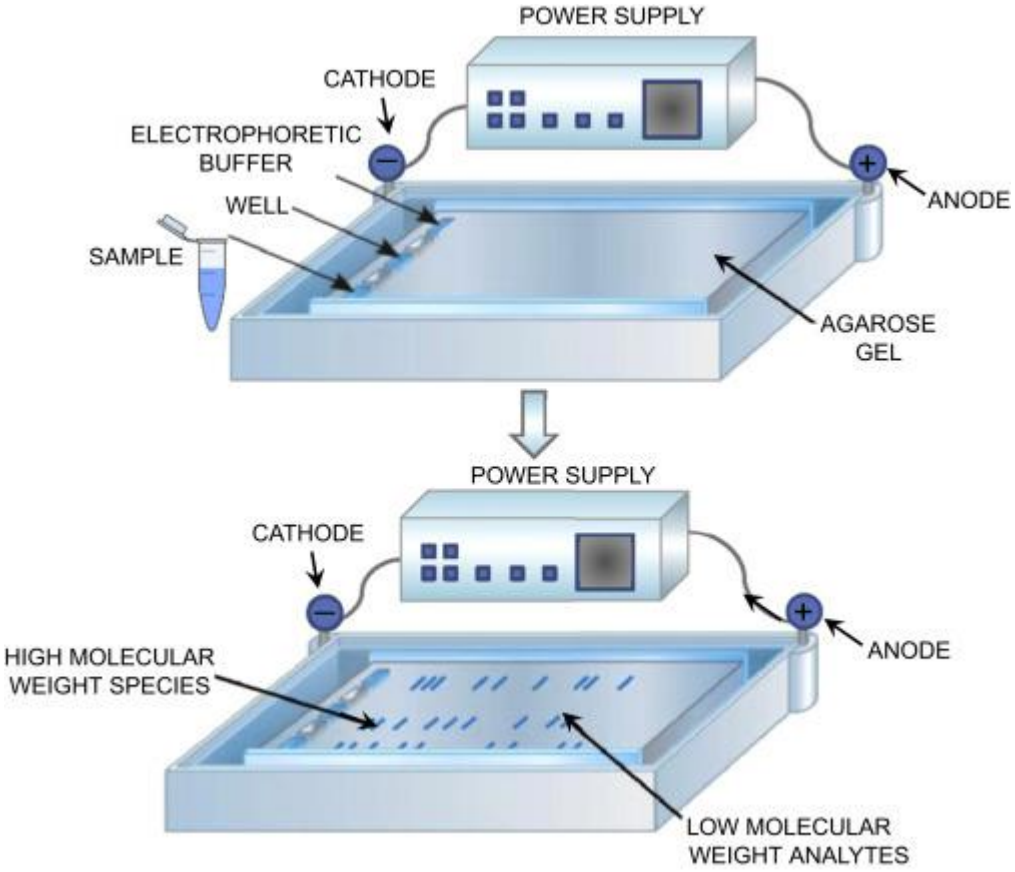


Figure 4.1 A labelled representation of the Agarose Electrophoresis system (Drabik *et al*., 2016)

4.3.3 Ethidium bromide (EtBr) staining

Ethidium bromide (EtBr) is added to the agarose gel solution just before pouring, to the tank buffer, or to both to enable nucleic acid specimens to be visualized by UV transillumination. Since EtBr is a recognised mutagen and strong DNA intercalator, gloves must always be worn when handling gels or buffers containing it.

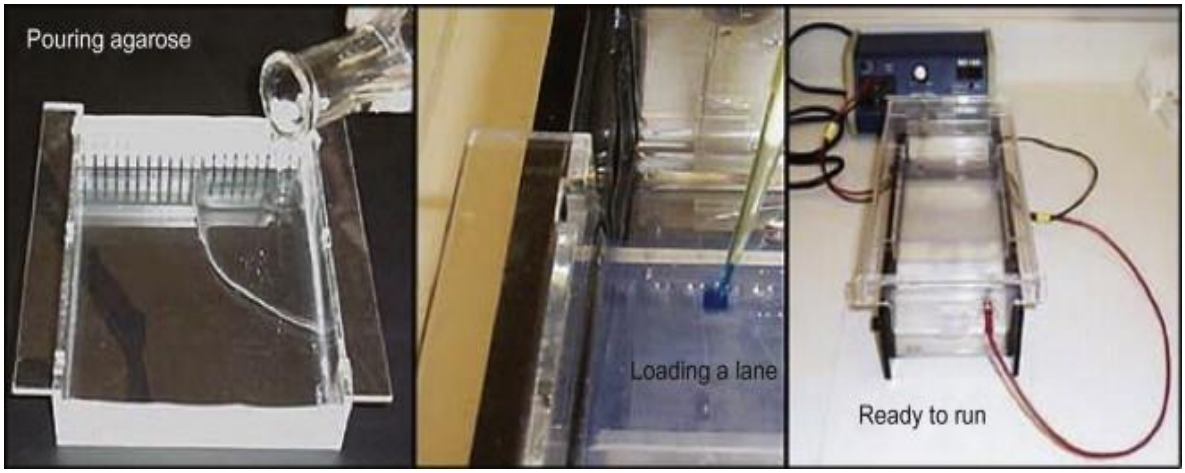


Figure 4.2 Separate images depict three crucial steps in the agarose gel electrophoresis preparation process: a) Agarose pouring, b) lane loading, and c) power supply connection are the three steps. (Sun et al.,2010)

Table 4.1: List of primer and restriction enzymes used for the hTERT genetic variant

Gene	**SNP ID**	**Primer sequence (forward and reverse)**	**Annealing Temperature**	**Restriction enzyme**	**Restriction Recognition site**	**Restriction pattern**
TERT	rs273590 (T>C)	FP:5'ATCTTCTGCTTCC ATTTCTTCTC-3' RP:3'TCGTCTTGTAAAT ACTTAGGATTCC-5'	55°C	Msp1	5'…C↓CGG …3'	T/T: 235 T/C:235, 211, 24 C/C: 211, 24

4.4 Statistical Analysis

4.4.1 Demographic analysis

Demographic factors such as age, gender, smoking status, and packyears were classified into categorical and continuous variables. The chi-square test was employed to compare the cases and controls for the chosen demographic characteristics, such as gender and smoking status, which are the categorial variables. A t-test was used to compare groups about continuous variables such as age and smoking.

4.4.2 Genotypic and allelic Frequency analysis

The Hardy-Weinberg equilibrium theory ($p^2+2pq+q^2=1$,where p represents the frequency of the wild-type allele and q represents the frequency of the variant allele) was applied to compute the genotype frequencies of the SNP under study using the χ^2 test in both cases and controls. Pearson's χ^2 test showed a statistically significant difference between cases and controls in allelic and genotypic frequencies. Additionally, the minor allele frequency for polymorphic position was computed.

4.4.3 Association analysis

To calculate the polymorphisms and lung cancer risk, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. The study employed logistic regression to generate adjusted odds ratios (AORs) to examine further plausible risk factors, including smoking, age, and gender. All the variables used in the study were dichotomous except for continuous variables like age. In the logistic regression study, genotype was selected as the independent variable that can predict a person's susceptibility to lung cancer, and the occurrence of lung cancer was selected as the dependent variable. In addition to the thorough association investigation, a stratified analysis was done to calculate the risk of different subgroups according to tumour histology and smoking status. The above statistical analysis was performed with Medcalc version 16.8.1 (Medcalc Software, Ostend, Belgium).

4.4.4 Overall survival (OS) and Hazard rate analysis

The association between overall survival and genetic polymorphism was investigated using the Kaplan Meier and Log-rank tests. A statistical procedure known as a log-rank test was employed as a univariate study to compare the patient's median survival time (MST) according to the genotype of that specific variant. The hazard ratio (HR) was calculated using the Cox proportional hazard model, which also examined the effect of genetic polymorphism on overall survival after controlling for variables such as age, gender, smoking, tumour stage, histology, regimen, and performance status. The Cox proportional hazards models obtained the hazard ratios and 95% confidence intervals (CI). Like a multiple regression model, Cox's proportional hazards model allows comparisons between patient survival times across many groups to be assessed while adjusting for other variables. (Mahimkar*et al*., 2012). The hazard ratio was utilized to compute the death rate. The risk is that a patient may experience a relapse or pass away at a particular point in time, assuming the subject has lived to that point without encountering the incident. Additionally, the Kaplan-Meier and Cox regression technique was used in several subgroups stratified according to performance status, gender, smoking behaviours, histology, and chemotherapy regimen. In all analyses, a p-value of <0.05 was considered significant. Medcalc version 16.8.1 (Medcalc Software, Ostend, Belgium) was used for all statistical analysis.

4.4.5. Stratified analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using stratified analysis to assess risk for subgroups based on various factors, including tumour stage, lymph node invasion, primary tumour extension, metastasis, and response rate. The adjusted odds ratio (AOR) and 95% Confidence Intervals associated with each genotype and different genotypic combinations were found using unconditional multivariate logistic regression. Due to the possibility of confounding factors, the odds ratio was modified for age, gender, and smoking. Except for the continuous variable, such as age, every variable in the study was dichotomous. All p-values were two-sided, and a p-value of <0.05 was considered statistically significant (Begg and Zhang,1994). Medcalc version 16.8.1 (Medcalc Software, Ostend, Belgium) was used for all statistical analysis.

4.4.5 Adverse events (AEs) analysis

Toxicity in lung cancer patients was assessed according to standard National Cancer Institution Criteria 3.0. The hepatotoxicities (SGPT, SGOT, and ALP) and nephrotoxicity (neutropenia, anaemia, leukopenia, and thrombocytopenia) received the most attention. Grades 3 or 4 toxicity constituted severe toxicity or a serious incident. Due to significant hematologic toxicity, the next course of treatment was delayed until either grade 1 or grade 0 recovery was achieved. Platinum was lowered by 25% if creatinine clearance fell to 59–41 ml min–1. Platinum was discontinued if creatinine clearance fell to less than 40 ml min-1. During treatment, clinical data have been symmetrically documented. The 95% confidence intervals and odds ratio were computed for the analysis. The adjusted odds ratio (AOR) and 95% Confidence Intervals were computed concerning each genotype and different genotypic combinations using multivariate logistic regression. To account for potential confounding variables, the odds ratio was modified for age, gender, smoking, and chemotherapy treatment. In all analyses, a p-value of <0.05 was considered significant. Medcalc version 16.8.1 (Medcalc Software, Ostend, Belgium) was used for all statistical analysis.

Table 4.2: Summarizing adverse events grading for haematological, nephrotoxicity and hepatological toxicity

Toxicity	Gradation of Adverse events	Toxicity profiles			
		Grade 1	**Grade 2**	**Grade 3**	**Grade 4**
Haematological toxicity	**Anaemia**	Hb<12.0-10.0 gm/dl	Hb<10.0-8.0 gm/dL	Hb<8.0-6.5 gm/dL	Hb<6.5gm/dL
	Leukopenia	TLC<4500-3000/uL	TLC<3000-2000/uL	TLC<2000-1000/uL	TLC<1000/uL
	Thrombocytopenia	Plt<1,50,000-75,000/uL	Plt<75,000-50,000/uL	Plt<50,000-25,000/uL	Plt<25,000/uL
	Neutropenia	*ANC<2000-1500/uL	ANC<1500-1000/uL	ANC<1000-500/uL	ANC<500/uL
Nephrotoxicity	**Chronic kidney disease**	*GFR>90ML/MIM/1.73^2	GFR=89-60 mL/min/1.73^2	GFR=59-30 mL/min/1.73^2	GFR=29-15/mL/min/1.73^2
Hepatotoxicity	**SGOT**	*AST=1.25-2.5	AST>2.5-5.0	AST>5.0-10	AST>10
	SGPT	*ALT=1.25-2.5	ALT>2.5-5.0	ALT>5.0-10	ALT>10
	Alkaline Phosphatase	*ALP=1.25-2.5	ALP>2.5-5.0	ALP>5.0-10	ALP>10

Note: Hb- Haemoglobin; ANC- Absolute neutrophil; Plt- Platelets; GFR; Glomerular filtration rate (GFR); SGOT; Serum glutamic oxaloacetic transaminase also known as AST; Aspartate aminotransferase; SGPT; Serum glutamic pyruvic transaminase also known as ALT; Alanine aminotransferase

Absolute neutrophil count (ANC)= $\frac{Total\ leucocyte\ count\ (TLC) * Neutrophils}{100}$ Glomerular filtration rate (GFR)= (140-Age)*Weight (Kg)/72*serum creatinine

CHAPTER 5

Results

5.1 Genomic DNA isolation

Genomic DNA was extracted and analysed for both quality and quantity. The quality of the isolated genomic DNA was assessed by running the DNA samples on a 0.8% agarose gel electrophoresis, as illustrated in Figure 5.1, which shows intact genomic DNA isolated from blood lymphocytes collected from lung cancer patients. The integrity of the bands was verified. The concentration of the isolated DNA samples was determined by measuring the absorbance at 260 nm. The purity of the nucleic acid was evaluated by calculating the absorbance ratio at 260 nm to 280 nm.

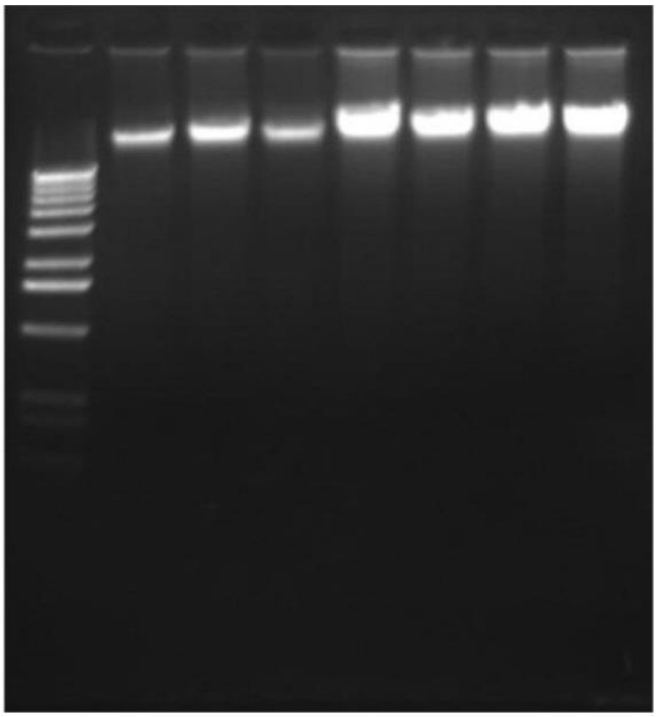


Figure 5.1: A 0.8% agarose gel depicting genomic DNA isolated from blood samples of patients with lung cancer (Lane 1: 1 kb DNA ladder, Lane 2-5: genomic DNA from blood samples of cases, Lane 6-8: genomic DNA isolated from healthy subjects.)

5.2 PCR Amplification

PCR was employed to amplify a specific gene. The amplification of the SNP post-PCR was confirmed using 2.2% agarose gel electrophoresis. A 100 bp molecular weight marker was utilized to verify successful amplification. Figure 5.2 illustrates the amplification of genomic DNA, highlighting the TERT rs2735940 polymorphism. An amplified PCR product of 235 bp was obtained and run alongside the 100 bp ladder.

5.3 Genotyping of Single Nucleotide Polymorphic Variant by RFLP

5.3.1 Genotyping of hTERT genetic variant

The polymorphic variant of the hTERT gene under study is TERT (rs2735940). This functional polymorphism is located within the promoter region, involving a T to C transition at position -1327 bp upstream of the transcription start site. Following amplification, the full-length PCR product of 235 bp was digested with 1.5 U of the *MspI* restriction enzyme (Thermo Fisher Scientific Inc.) at 37°C. The digested samples were then subjected to electrophoresis on a 2.2% agarose gel and visualized under a UV trans-illuminator.

Upon digestion with*MspI*, the wild-type homozygous genotype (*TT*) yielded a single uncut band of 235 bp. In contrast, the homozygous variant genotype (*CC*) produced a single band of 211 bp, and the heterozygous genotype (*TC*) resulted in two bands, one at 235 bp and the other at 211 bp.

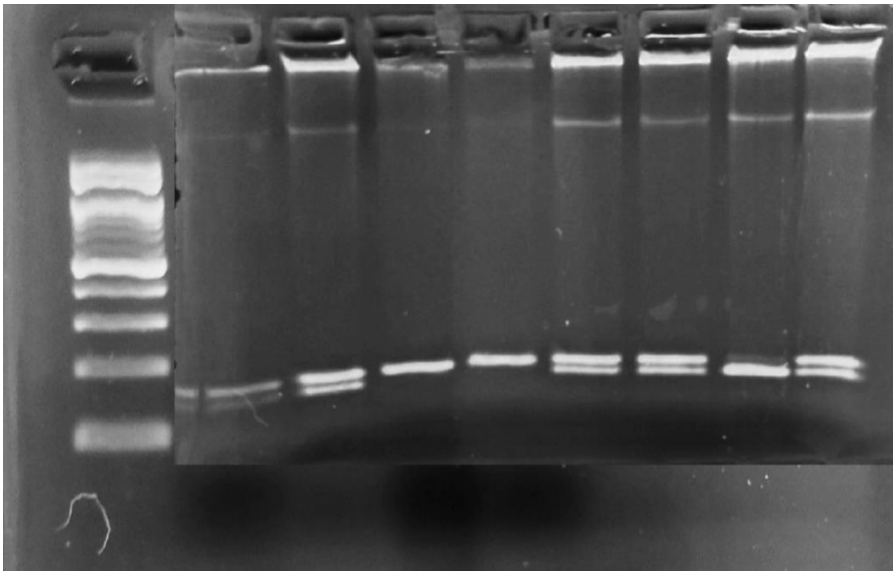


Figure 5.2: Restriction digestion pattern for the detection of hTERT polymorphism

5.4 Epidemiological statistics of the North Indian population under study were analysed.

5.4.1 Regional distribution of patients with lung carcinoma

Blood samples were collected from patients with lung cancer who visited the PGIMER Department of Pulmonary Medicine. Patients from different parts of North India are referred to PGIMER, Chandigarh, a government hospital with multiple specialties. A total of 387 patients were enrolled in the current study, with Himachal Pradesh (116, 29.97%), Punjab (76, 19.63%), and Haryana (65, 16.79%) having the highest proportion of patients, as indicated in Figure. The remaining lung cancer patients recruited in the study were from states and Union territories like Uttar Pradesh (35, 9.04%), Chandigarh (23, 5.94%), Jammu & Kashmir (18, 4.65%), and Uttarakhand (10, 2.58%). The most minor lung cancer patients were from Mohali (5, 1.29%), Saharanpur (3, 0.77%), Una (2, 0.51%), Shimla (2, 0.51%), Paonta sahib (2, 0.51%), Karnal (2, 0.51%), Hamirpur (2, 0.51%), Ambala (2, 0.51%), and only one patient was from few other states.

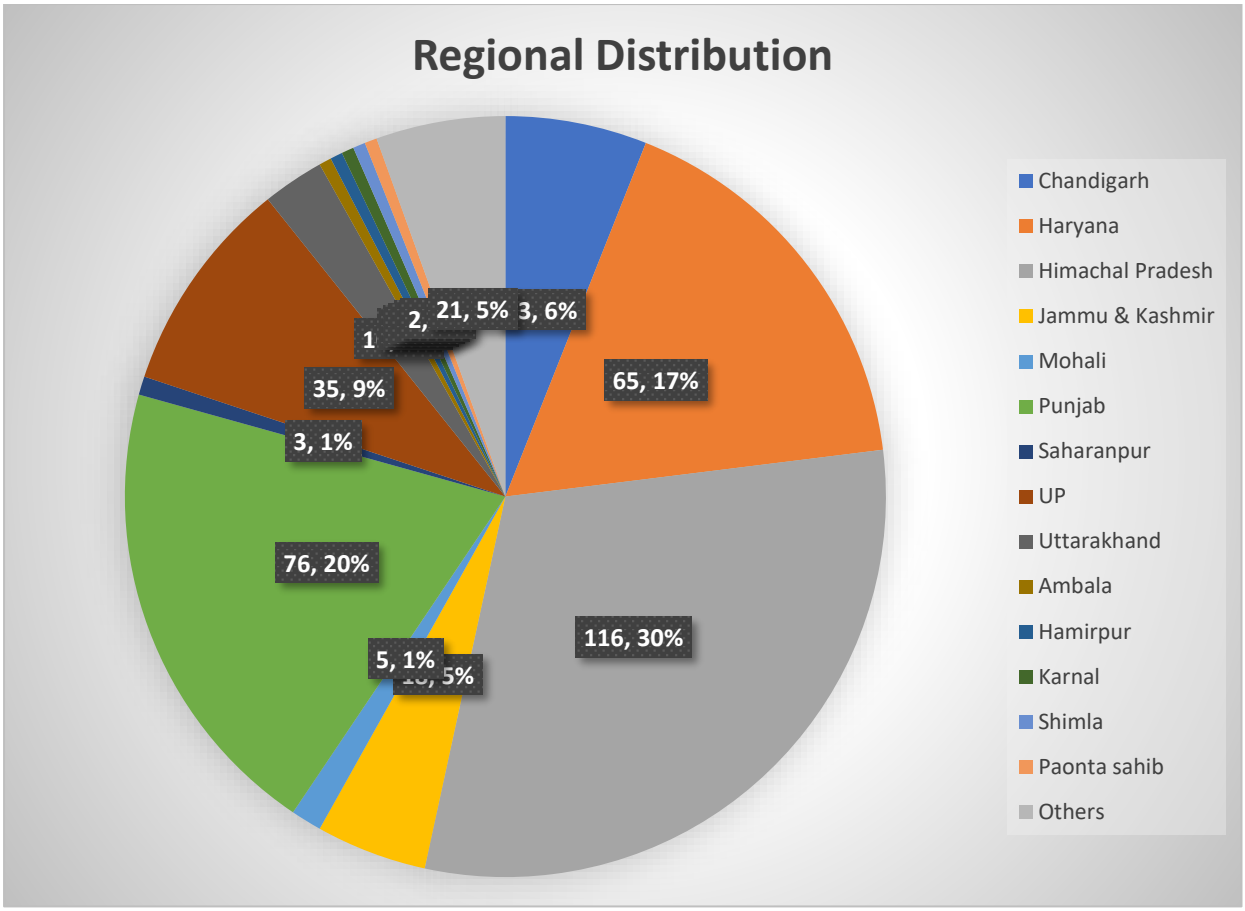


Figure 5.3: The regional distribution of North Indian lung cancer patients

The subjects were also analysed for their regional distribution based on gender. In 387 cases, the study population comprises 312 (80.62%) males and 75 (19.37%) females. Out of 384 control subjects, 338 (88.02%) were recorded as males and 46 (11.97%) as females. The diagrammatic representation of regional distributional of males and females study population is shown in figure 5.5.

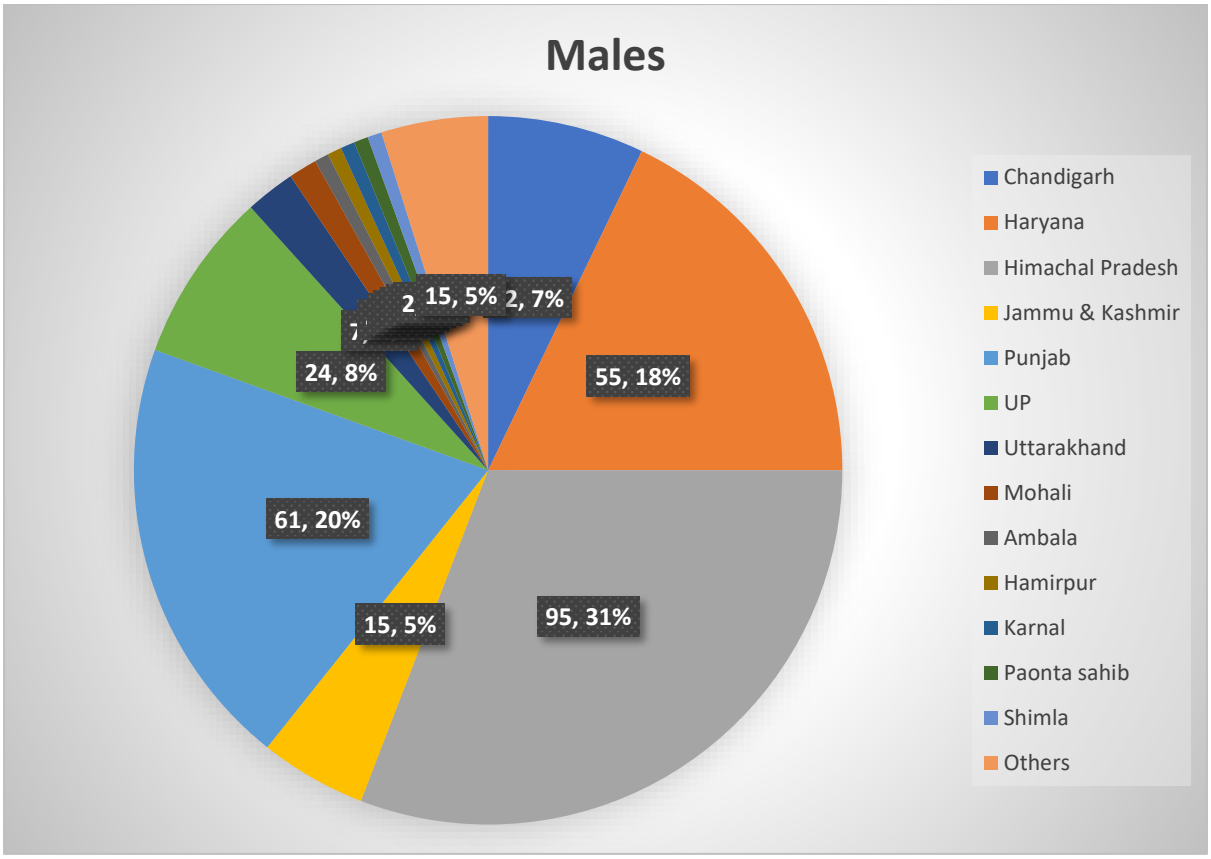


Figure 5.4. a): Pie chart illustrating the geographic distribution of male lung cancer patients in North India

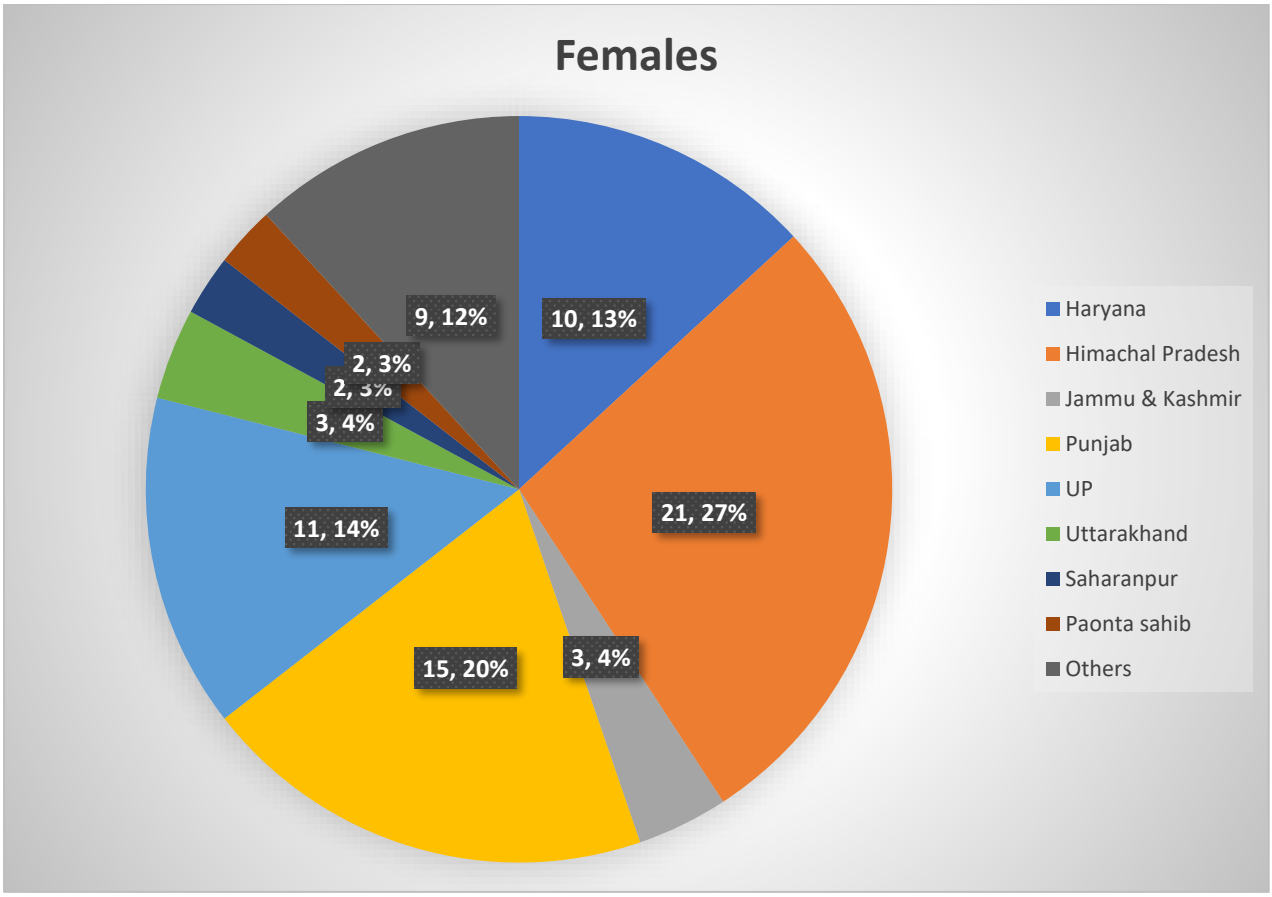
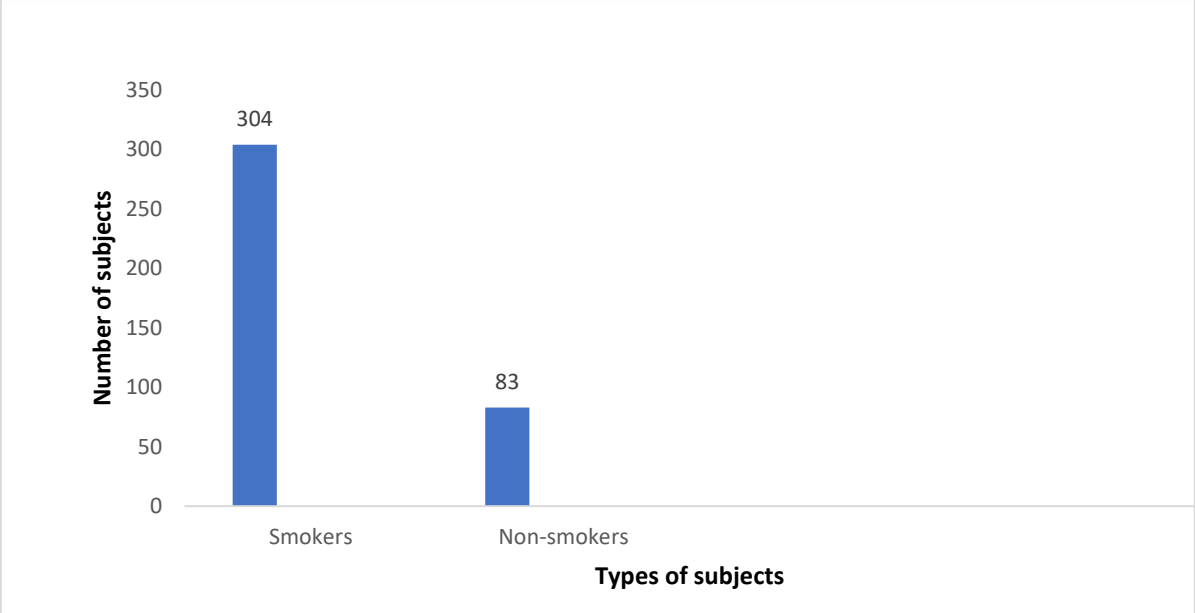


Figure 5.5. b): Pie chart illustrating the regional distribution of female lung cancer patients in North India

5.4.2. Distribution of study subjects based on smoking

One of the most important epidemiological data that was gathered was the smoking status of all patients and controls. Based on their smoking behaviours, the study population was divided into smokers and non-smokers. Since lung cancer is known to be caused by smoking, there were more smokers among cases than non-smokers. In a similar vein, an effort was made to remove sample bias by matching the controls with respect to smoking status. The percentage of smokers among cases and controls was 78.55% and 72.91%, respectively, as shown in figure 5.7.



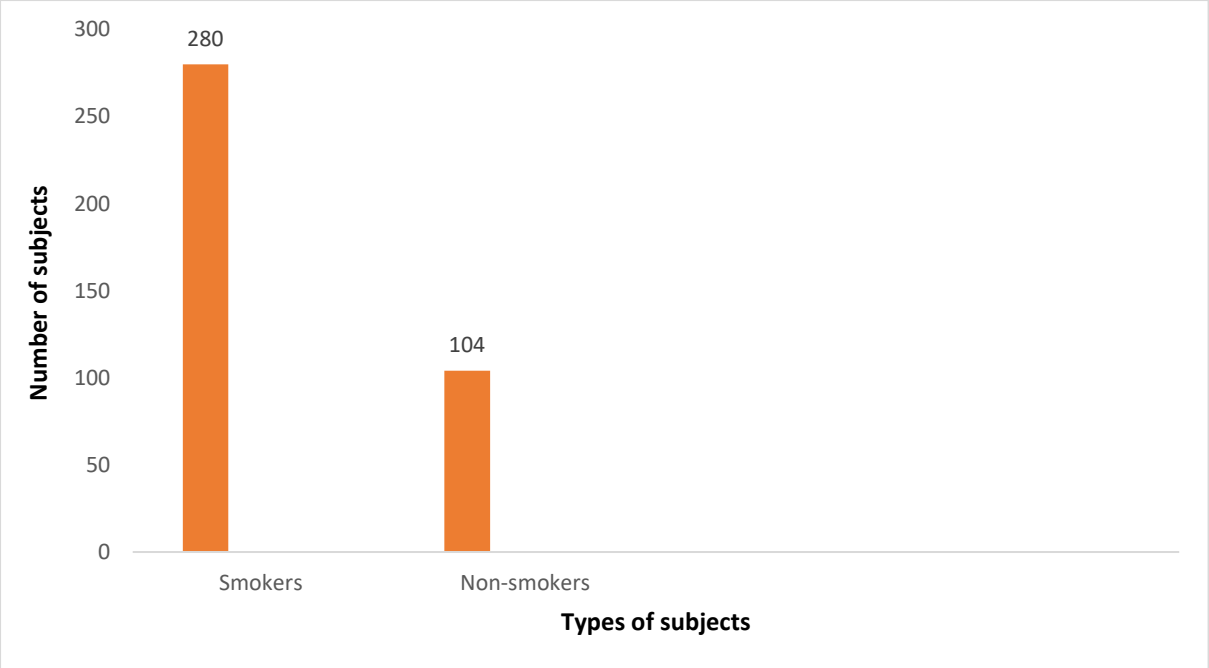


Figure 5.6: Graph representing the distribution of study subjects based on smoking status a): Among cases b): Among controls

Furthermore, detailed data about the kind of smoking was documented. This data indicates that the study participants smoked cigarettes, beedi, and a mixture of cigarettes and beedi. Based on the subjects' gender, the smoking habits of the research were also examined. The percentage of smokers among males in cases 283 (93.09%), is lower than those in the control group 272 (97.14%) as shown in figure 5.8.

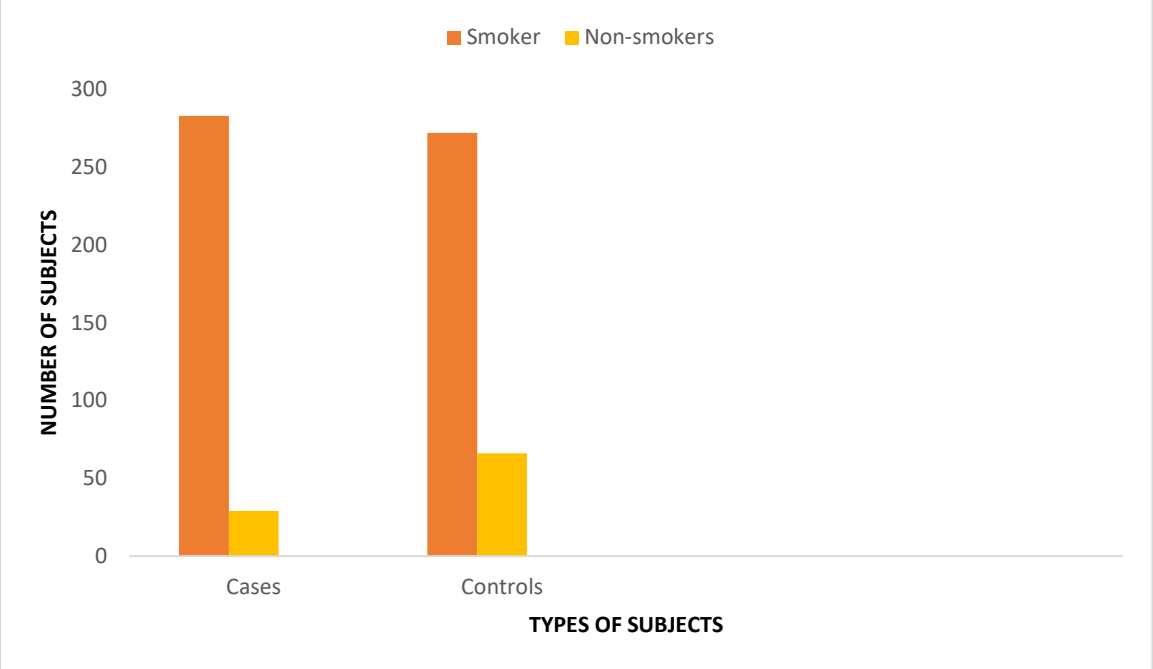


Figure 5.7: Graph representing the distribution of male study subjects based on smoking status

5.4.3. Distribution of study subjects based on age

Age is another crucial demographic factor that has been linked to the onset of many diseases. The age of all subjects included in the study, both cases and controls, were recorded. The mean age of the cases was estimated to be 63.5 years. A sincere attempt was made to match the age of cases and controls with (±5 years) difference. The mean age calculated for control subjects was 65 years. The median age of both cases and controls was found to be 63.5 years.However, for the age factor, none of the cases and controls matched one another exactly, therefore this was taken into account as a covariate for additional analysis. The diagrammatic representation of the distribution of study subjects based on their gender is shown in figure 5.9.

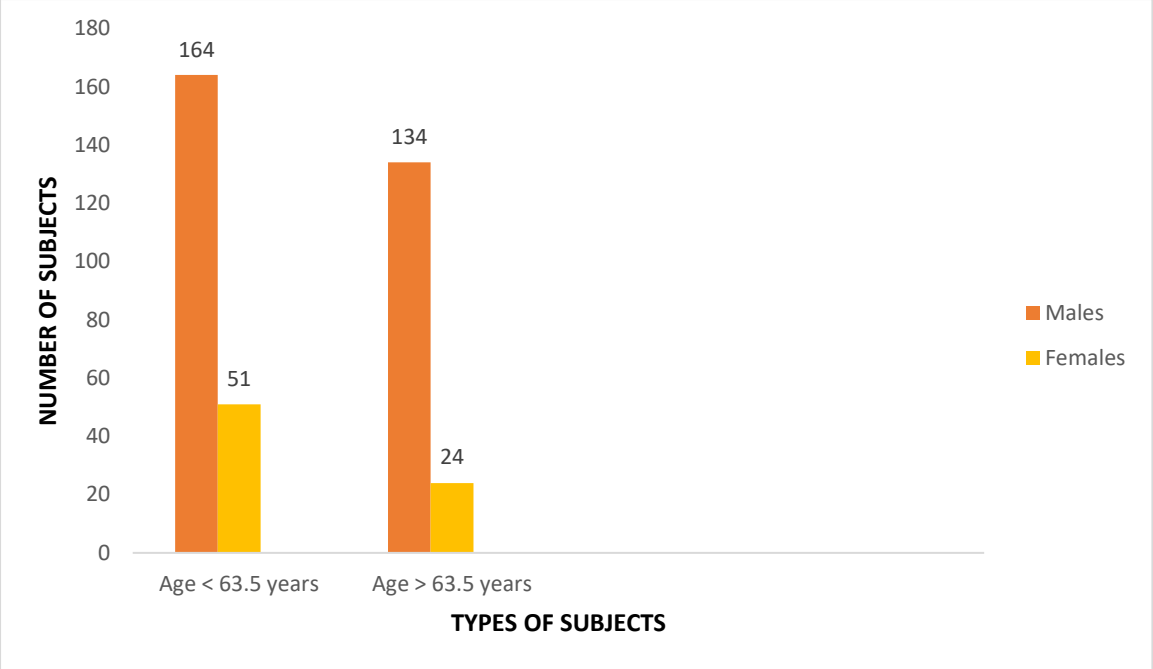


Figure 5.8: Graph representing the age-wise distribution of lung cancer based on gender

A slightly higher percentage of males were reported to have age < 63.5 years than those more than 63.5 years among lung cancer patients.

5.4.4. Distribution of lung cancer patients based on the stage of tumor and TNM

The clinic-pathological parameters of all the lung cancer subjects included in the study were recorded from the hospital's medical records. The patients were classified based on the clinical stage of the tumor into stage I, stage II, stage III, and stage IV. The proportion of stage I and stage II patients were deficient compared to stage III and stage IV, as shown in figure. TNM stage data [stage I: 5 (1.29%), II: 16 (4.13%), III: 147 (37.98%), IV: 209 (54%)]. Further, the patients were also stratified based on the various parameters of TNM, such as primary tumor extension (T), lymph node involvement (N), and metastasis (M). The detailed distribution of patients based on tumor size is as follows: Tumor size T1 and T2 had a very low frequencies of 6.71% and 9.04%, whereas T3 and T4 had frequencies 17.57% and 61.75%, respectively. For lymph-node involvement, N0 had 13.17% whereas N1, N2, N3, and N4 had frequencies of 0.25, 7.49, 19.89, and 33.07%, respectively. Among all the cases for which TNM data were

available, 16.79% had no metastasis involvement (M0), whereas 55.81% had distant metastasis (M1).

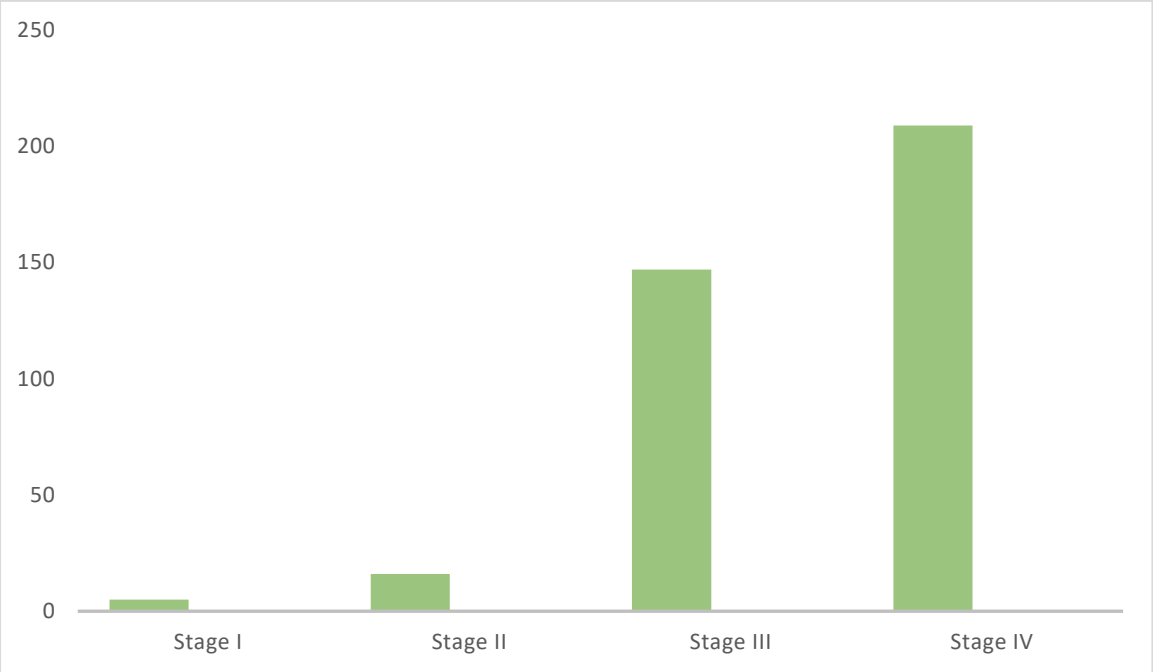


Figure 5.9: Distribution of the study subjects according to stages

5.4.5. Distribution of lung cancer patients based on histology of the tumor

SQCC, ADCC, and SCLC are the three significant classifications of lung cancer-based histology. The patients in this study were all stratified in the same way. In the present study, the prevalence of SQCC patients (44.96%) was highest among all the other histological subtypes, followed by ADCC (40.56%) and SCLC (11.36%), as illustrated in figure 5.11. The remaining patients (1.55%) were diagnosed with miscellaneous histological subtypes such as large cell carcinoma, mesothelial, and NSCLC undifferentiated, and 1.80% subjects was those whose histology was not clear. Therefore, it is evident that SQCC is the most frequently occurring type of lung cancer in North Indians.

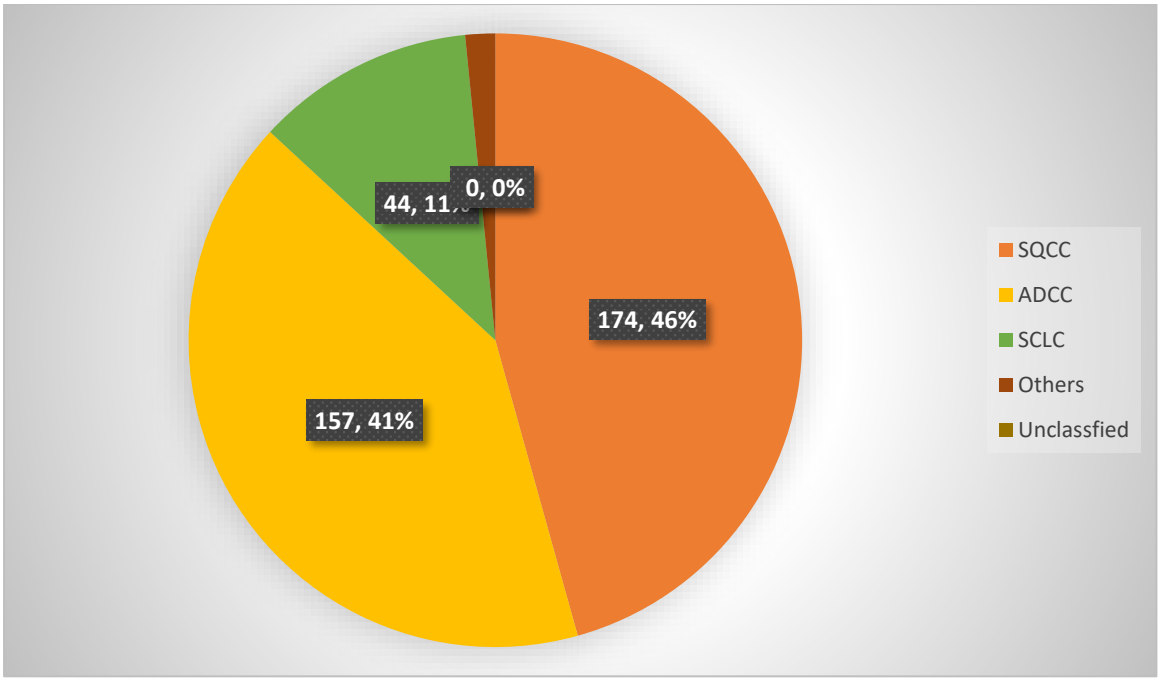


Figure 5.10: Pie chart illustrating the distribution of lung cancer patients in North India according to histological subtypes

5.4.6. Distribution of lung cancer patients based on performance status

Patients are classified using two standard ways to evaluate their performance throughout therapy and estimate their disease's prognosis: Eastern Cooperative Oncology Group-performance status (ECOG-PS). These methods aid in determining the functional status of patients. 4.90% patients had ECOG status (0), 39.79% had ECOG status (I), 38.50% had ECOG status (2), and 16.27% had ECOG status (3-4) as shown in figure 5.12.

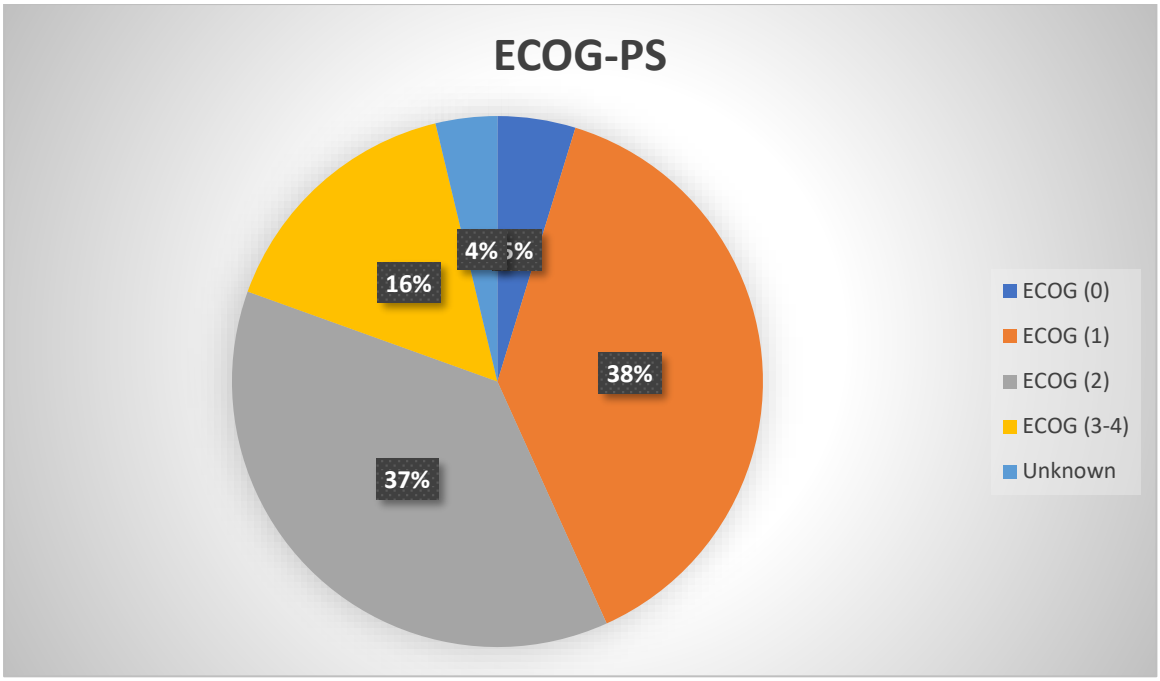


Figure 5.11: Pie chart illustrating the Eastern Cooperative Oncology Group-Performance status (ECOG-PS) distribution among North Indian lung cancer patients

5.4.7. Distribution of lung cancer patients based on double platinum-based chemotherapeutic regimen

The current study used a second-line chemotherapeutic drug and platinum-based doublet chemotherapy to treat lung cancer patients, taking into account the patients' basic clinical parameters and the histological subtype of the disease. Cisplatin and carboplatin are examples of platinum-based medications that were coupled with other chemotherapeutic medicines such irinotecan, docetaxel, paclitaxel, and pemetrexed. The chemotherapeutic regimen data was available for 229 subjects. The highest proportions (37.99%) of patients were treated with pemetrexed and cisplatin/carboplatin regimen followed by paclitaxel with cisplatin/carboplatin (33.18%) and docetaxel with cisplatin/carboplatin (16.59%). The regimen irinotecan and cisplatin/carboplatin were administered to 12.22% of the subjects, shown in figure 5.13.

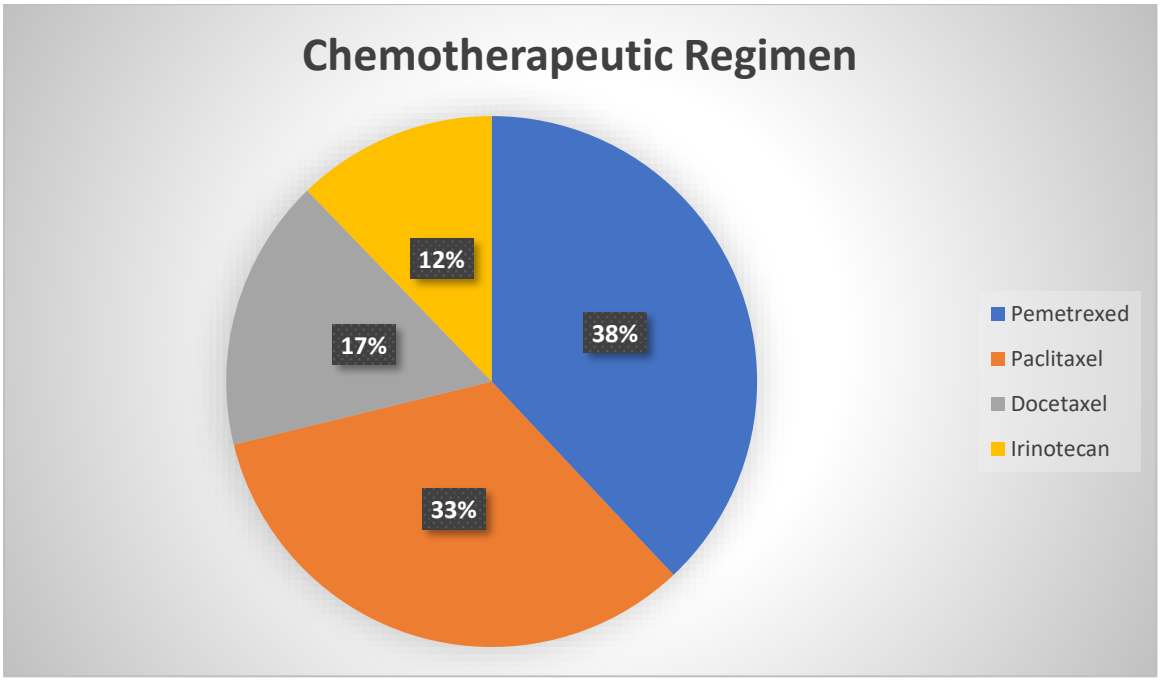


Figure 5.12: Pie chart illustrating the distribution of lung cancer patients in North India according to the Doublet platinum-based chemotherapy regimen

5.5 Statistical analysis of the hTERT gene

5.5.1 Demographic characteristics of the study subject for the h*TERT* gene

The sample population in the present study has been characterized by a range of epidemiological and demographic parameters, including age, gender, smoking status, and the clinical specifications of the cases, as outlined in Table 5.1. The mean age at diagnosis was calculated to be 63.5 ± 9.46 years for cases and 65 ± 13.72 years for controls, respectively, and hence there was no significant difference between the mean ages of both cases and controls (p=0.077). The study was performed on 387 cases and 384 controls, including 312 (80.62%) males in cases and 338 (88.02%) males in controls, whereas 75 (19.37%) and 46 (11.19%) were females both in cases and control groups, respectively. However, there was a significant difference observed between the distribution of males and females in cases and controls (p=0.0048). The number of smokers and non-smokers was compared between the two study groups. The case group comprised 78.55% of smokers, whereas controls had 72.91%; no significant difference was observed in the proportion of smokers and non-smokers (p=0.068). An attempt was made to adequately match the number of smokers and non-smokers in our study. Smoking was quantified in terms of pack years [(cigarettes or beedis per day/20) X number of years a person has smoked], and a significant difference was observed (**p<0.0001**).

Further, the different clinical specifications such as histology, clinical stage, tumour size extensions, lymph node invasion, and distant metastasis were recorded from medical reports. Out of 387 cases, 174 (44.96%) were histologically identified as squamous cell carcinoma (SQCC) patients, 157 (40.56%) were detected with adenocarcinoma (ADCC), and 44 (11.36%) had small-cell lung cancer (SCLC). The patients were classified based on TNM staging: 1.29% of patients were diagnosed at stage I, 4.13% at stage II, 37.98% at stage III, and 54.00% at stage IV lung cancer.

Table 5.1: Demographic characteristics among cases and control				
Variable	**Total (N)**	**Cases, n (%)**	**Controls, n (%)**	***p*-value**
Age (years)		387	384	0.077
Mean ± SD		63.5±9.46	65±13.72	
Range				
Gender		387	384	
Male		312 (80.62)	338 (88.02)	**p <0.0048**
Female		75 (19.37)	46 (11.19)	
Smoking status		387	384	
Smokers		304 (78.55)	280 (72.91)	0.068
Non-Smokers		83 (21.44)	104 (0.36)	
Pack years		387	384	
Mean ± SD		14.85 ± 0.21	14.43 ± 11.91	**p <0.0001**
Histological Types	387	387		
SQCC		174 (44.96)		
ADCC		157 (40.56)		
SCLC		44 (11.36)		
Others		6 (1.55)		
Unknown		7 (1.80)		
Overall Survival	387	387		
Dead		318 (82.17)		
Alive		69 (17.82)		
TNM staging	387			
I		5 (1.29)		
II		16 (4.13)		
III		147 (37.98)		
IV		209 (54.00)		
Other		23 (5.94)		
Tumour Size	387			
Tx		15 (3.87)		
T1		26 (6.71)		
T2		35 (9.04)		
T3		68 (17.57)		
T4		239 (61.75)		

Unknown		17 (4.39)		
Lymph Node	387			
Nx		0		
N0		51 (13.17)		
N1		1 (0.25)		
N2		29 (7.49)		
N3		77 (19.89)		
N4		128 (33.07)		
Unknown		114 (29.45)		
Metastasis	387			
Mx		1 (0.25)		
M0		65 (16.79)		
M1		216 (55.81)		
M2		1 (0.25)		
Unknown		117 (30.23)		

Abbreviations: SD= Standard Deviation, n= total number of lung cancer cases or control subjects, a) p-values were derived from Pearson Chi-square test except for age and pack-years; Student t-test was used for age and pack-years. All p-values are two-sided. $p < 0.05$ was considered statistically significant

5.6 Polymorphism in human *telomerase reverse transcriptase* (h*TERT*) gene and susceptibility towards lung cancer in a North Indian population

5.6.1 Potential Influence of hTERT Polymorphism on Lung Cancer Susceptibility

5.6.1.1. Genotypic and allelic frequencies of the hTERT polymorphic variant in cases and controls and their potential impact on lung cancer susceptibility

Table 5.2 demonstrates the allelic and genotypic frequency distribution of the hTERT polymorphic variant h*TERT* rs2735940 T > C polymorphism. The genotypic frequencies of hTERT rs27354940 T > C exhibit a significant difference between cases and controls ($\chi 2$= 13.959; df = 2; p= **0.0009**) as shown in table 5.2.

The wild-type genotype (*TT*) for the hTERT gene was less prevalent in the lung cancer subjects than in the controls (65.11% vs 76.82%). However, as shown in Table 5.2, the mutant genotype (*CC*) frequency for the hTERT gene was overrepresented by 6.20% in the cases compared to the controls (2.86%).

When the odds ratio was calculated using the homozygous *TT* (wild) genotype as a reference, the association analysis revealed that subjects with mutant genotype (*CC*) demonstrated a 2.40-fold higher risk for lung cancer development (AOR=2.40; 95% C.I.= 1.08-5.34, p=**0.031**) and were statistically significant. Similarly, in the dominant model, the combined genotype (*TC+CC*) also exhibited an increased risk towards susceptibility with an adjusted odd ratio of (AOR=1.67; 95% C.I.= 1.21-2.31, p=**0.0016**). Further, the analysis used the recessive model wherein heterozygous genotype was used as a reference. It was observed that subjects carrying the two copies of the mutant allele (*CC*) exhibited a significant association toward risk for lung cancer (AOR=2.17; 95% C.I.=1.03-4.5, p=**0.039**) (Table 5.2).

Table 5.2: Genotyping and allelic distribution of the hTERT genetic variant and its association with the risk of lung cancer				
Genotype rs2735940 T/C	**Controls (384) N (%)**	**Cases (387) N (%)**	**AOR (95 % C.I.)a**	**p**
Codominant model				
TT	295 (76.82)	252 (65.11)	1.0 (Reference)	
TC	78 (20.31)	111 (28.68)	1.50 (1.02-2.19)	0.03
CC	11 (2.86)	24 (6.20)	2.40 (1.08-5.34)	**0.031**
Dominant model				
TT	295 (76.82)	252 (65.11)	1.0 (Reference)	
TC + CC	89 (23.17)	135 (34.88)	1.67 (1.21-2.31)	**0.0016**
Recessive model				
TT + TC	373 (97.13)	363 (93.79)	1.0 (Reference)	
CC	11 (2.86)	24 (6.20)	2.17 (1.03-4.57)	**0.039**
T (Allele)	668	615		
C (Allele)	100	159		
MAF	0.129	0.20		
$\chi2$= 13.959, df = 2				0.0009

a)- Adjusted Odds ratios, 95% confidence intervals, and corresponding p-values were calculated by logistic regression analysis after adjusting for age, gender, and smoking status. (b)-Two-sided $\chi2$ test for either genotype distribution or allelic frequencies between the cases and controls. The number in bold indicates the significant values in the Table

5.6.1.2. Genetic and allelic distribution of hTERT based on histological subtypes

The study group was divided into histological subgroups to evaluate the severity of the risk towards the disease based on lung cancer histology. As shown in Table 5.4, the frequency of the mutant (*CC*) genotype was overrepresented in SCLC (15.90%) compared to both ADCC (4.79%) and SQCC (4%) respectively. Three alternative genetic models (co-dominant, dominant, and recessive) were assessed to investigate further associations between hTERT polymorphism and lung cancer risk among histological subtypes. After applying statistics, subjects diagnosed with ADCC did not show any significant association with lung cancer susceptibility. It was observed that in the co-dominant model, 6.8 times higher odds of developing SCLC were observed for lung cancer patients carrying mutant genotype (AOR=6.80; 95% C.I.= 2.33-19.78**; p=0.0004**), and also a highly significant association between the genetic variant and SCLC was observed. In the dominant model, 2.38-fold high risk was observed for lung cancer patients (AOR=2.38; 95% C.I.= 1.24-4.54; **p=0.0085**), and are highly significant. Further, in the recessive model, 5.89 times higher risk odds were observed having (*TT+TC*) as a reference (AOR=5.89; 95% C.I.=2.09-16.51; **p=0.0008**).

Similarly, the SQCC patients carrying heterozygous genotype (*TC*) exhibited an increased risk of acquiring squamous cell carcinoma, with an adjusted odd ratio of (AOR=1.62, 95% C.I.=1.06-2.48; **p=0.023**), when wild-type genotype was taken as a reference. Furthermore, the combined genotype (*TC+CC*) had a trend, exhibiting a strong association with an increased risk of developing squamous cell carcinoma, in case the dominant model (AOR=1.57 95%C.I.=1.05-2.35**; p=0.02**) as depicted in Table 5.4.

Table 5.3: Genotypic and allelic distribution of the hTERT genetic variant and its association with risk of lung carcinoma on histology

ADCC				
Genotype rs2735940 T/C	**Controls (384) N (%)**	**Cases (157) N (%)**	**AOR[a] (95 % C.I.)a**	**p[b]**
Codominant model				

TT	295 (76.82)	111 (70.05)	1.0 (Reference)	
TC	78 (20.31)	38 (25.14)	1.19 (0.75-1.91)	0.14
CC	11 (2.86)	8 (4.79)	2.08 (0.78-5.52)	0.16
Dominant model				
TT	295 (76.82)	111 (70.05)	1.0 (Reference)	
TC + CC	89 (23.17)	46 (29.94)	1.30 (0.84-2.03)	0.22
Recessive model				
TT + TC	373 (97.13)	149 (95.20)	1.0 (Reference)	
CC	11 (2.86)	8 (4.79)	2.12 (0.80-5.62)	0.12
		SCLC		
Genotype Rs2735940 T/C	**Controls (384) N (%)**	**Cases (44) N (%)**	**AOR[a] (95 % C.I.)a**	**p[b]**
Codominant model				
TT	295 (76.82)	24 (55.55)	1.0 (Reference)	
TC	78 (20.31)	13 (29.54)	1.74 (0.83-3.61)	0.13
CC	11 (2.86)	7 (15.90)	6.80 (2.33-19.78)	**0.0004**
Dominant model				
TT	295 (76.82)	24 (54.54)	1.0 (Reference	
TC + CC	89 (23.17)	20 (45.45)	2.38 (1.24-4.54)	**0.0085**
Recessive model				

TT + TC	373 (97.13)	37 (84.09)	1.0 (Reference	
CC	11 (2.86)	7 (15.90)	5.89 (2.09-16.51)	**0.0008**

SQCC				
Genotype **rs2735940 T/C**	**Controls (384)** **N (%)**	**Cases (174)** **N (%)**	**AOR[a]** **(95 % C.I.)**	**P[b]**
Codominant model				
TT	295 (76.82)	111 (64)	1.0 (Reference)	
TC	78 (20.31)	56 (32)	1.6287 (1.06 - 2.48)	**0.023**
CC	11 (2.86)	7 (4)	1.220 (0.45-3.24)	0.69
Dominant model				
TT	295 (76.82)	111 (64)	1.0 (Reference)	
TC + CC	89 (23.17)	63 (36)	1.57 (1.05-2.35)	**0.02**
Recessive model				
TT + TC	373 (97.13)	167 (96)	1.0 (Reference)	
CC	11 (2.86)	7 (4)	1.07 (0.40-2.82)	0.89

a)- Adjusted Odds ratios, 95% confidence intervals, and corresponding p-values were calculated by logistic regression analysis after adjusting for age, gender, and smoking status. (b)-Two-sided $\chi 2$ test for either genotype distribution or allelic frequencies between the cases and controls. The number in bold indicates the significant values in the Table

5.6.1.3. Interactive effect of smoking on the h*TERT* polymorphism and their association with lung cancer risk

Interaction between smoking and the hTERT polymorphism and its correlation with the risk of lung cancer

To evaluate the role of smoking in modulating the association between hTERT gene polymorphism and lung cancer risk, the subjects were classified based on their smoking status into smokers and non-smokers (Table 5.5). Our data showed that controls who were smokers and carrying heterozygous genotype (*TC*) or mutant genotype (*CC*) for hTERT polymorphism were less represented in controls as compared to cases (22.5% vs. 28.47% and 3.62% vs. 6.07%, respectively). Smokers harbouring wild genotypes were more prevalent in controls than in cases (73.57% vs. 65.23%). Lung cancer subjects who were smokers and carrying the combined (*TC+CC*) genotype in the dominant model for the hTERT gene exhibited a 1.47-fold increased risk of developing lung cancer (AOR=1.47; 95% C.I.=1.02-2.10; **p=0.03**) as compared to smokers with the reference genotype (*TT*).

In the case of non-smokers, subjects harbouring heterozygous (*TC*) genotype depicted a 2.20-fold elevated risk of acquiring lung cancer (AOR=2.20; 95% C.I.=1.03-4.72; **p=0.04**) in the co-dominant model. In the dominant model, non-smoker subjects carrying combined (*TC+CC*) genotype indicate a very high association, suggesting a higher risk of developing lung cancerwith AOR of 2.73 (95% C.I.=1.30-5.71; **p=0.007**) when compared with the wild reference genotype (*TT*).

Table 5.4: Relationship of different hTERT genotypes with the smoking status of cases and controls

SMOKER				
Genotype rs2735940 T/C	**Controls (384) 280, N (%)**	**Cases (387) 304, N (%)**	**AOR[a] (95 % C.I.)a**	**p[b]**
Codominant model				
TT	206 (73.57)	199 (65.23)	1.0 (Reference)	
TC	63 (22.5)	86 (28.47)	1.40 (0.95-2.05)	0.08
CC	11 (3.92)	19 (6.07)	1.83 (0.85-3.97)	0.12
Dominant model				
TT	206 (73.57)	199 (65.23)	1.0 (Reference)	
TC + CC	74	105	1.47	**0.03**

	(26.42)	(34.76)	(1.02-2.10)	
Recessive model				
TT + TC	269 (96.07)	285 (93.70)	1.0 (Reference)	
CC	11 (3.92)	19 (6.29)	1.70 (0.79-3.65)	0.17

NON-SMOKER				
Genotype **rs2735940 T/C**	**Controls (384)** **104, N (%)**	**Cases (387)** **83, N (%)**	**AOR[a]** **(95 % C.I.)a**	**p[b]**
Codominant model				
TT	89 (85.57)	53 (63.85)	1.0 (Reference)	
TC	15 (14.42)	25 (30.12)	2.20 (1.03-4.72)	**0.04**
CC	0	5 (6.02)	-	0.99
Dominant model				
TT	89 (85.57)	53 (63.85)	1.0 (Reference)	
TC + CC	15 (14.42)	30 (36.14)	2.73 (1.30-5.71)	**0.007**
Recessive model				
TT + TC	104 (100)	78 (93.97)	1.0 (Reference)	
CC	0	5 (6.02)	-	0.99

a) Adjusted odds ratios, 95% confidence intervals, and corresponding p-values were calculated using logistic regression analysis after adjusting for age and gender. (b)-Two-sided $\chi 2$ test for either genotype distribution or allelic frequencies between the cases and controls. The number in bold indicates the significant values in the Table

5.6.1.4. Stratification analysis of the hTERT polymorphic variants with lung cancer risk based on gender

The stratification analysis was based on gender for lung cancer cases and healthy controls, as tabulated in Table 5.6. The frequency distribution of heterozygous genotype (*TC*) and mutant genotype (*CC*) were less represented in controls as compared to cases of males (19.23% vs 28.75% and 3.25% vs. 6.56% respectively). Males with wild type genotype (*TT*) were over-represented in controls than in cases (77.51% vs. 64.68%). Further, when the co-dominant model was used, it was discovered that male lung cancer patients bearing heterozygous (*TC*) genotype or mutant (*CC*) genotype had a higher risk of lung cancer when compared to the reference (*TT*) genotype (AOR=1.60; 95% C.I.=1.10-2.33; **p=0.013**) and (AOR=2.23; 95% C.I.=1.04-4.78; **p=0.038**) respectively. In addition, when we applied the dominant model, wherein the hTERT allele genotypes (*TC+CC*) were combined and compared to the (*TT*) genotype, it was pronounced that male lung cancer subjects carrying the variant allele showed a significantly increased genetic predisposition towards lung cancer (AOR=1.71; 95% C.I.=1.20-2.43; **p=0.0026**) (Table 5.5). Similarly, a significant association was observed in the recessive model towards lung cancer risk (AOR=2.03; 95% C.I.=0.95-4.31; **p=0.064**). In females, the hTERT polymorphic variant did not reveal an association towards risk for lung cancer in any of the models applied.

Table 5.5: Relationship of different hTERT polymorphism based on gender

MALES				
Genotype rs2735940 T/C	**Controls (384) 338, N (%)**	**Cases (387) 312, N (%)**	**AOR[a] (95 % C.I.)a**	**p[b]**
Codominant model				
TT	262 (77.51)	204 (64.68)	1.0 (Reference)	
TC	65 (19.23)	87 (28.75)	1.60 (1.10-2.33)	**0.013**
CC	11 (3.25)	21 (6.56)	2.23 (1.04-4.78)	**0.038**
Dominant model				
TT	262 (77.51)	204 (64.68)	1.0 (Reference)	
TC + CC	76	108	1.71	**0.0026**

	(22.48)	(35.31)	(1.20-2.43)	
Recessive model				
TT + TC	327 (96.74)	291 (93.43)	1.0 (Reference)	
CC	11 (3.25)	21 (6.56)	2.03 (0.95-4.31)	**0.064**

FEMALES				
Genotype **rs2735940 T/C**	**Controls (384)** **46, N (%)**	**Cases (387)** **75, N (%)**	**AOR[a]** **(95 % C.I.)a**	**p[b]**
Codominant model				
TT	33 (71.73)	48 (64)	1.0 (Reference)	
TC	13 (28.26)	24 (32)	1.11 (0.48-2.57)	0.79
CC	0	3 (4)	-	0.99
Dominant model				
TT	33 (71.73)	48 (64)	1.0 (Reference)	
TC + CC	13 (28.26)	27 (36)	1.30 (0.57-2.96)	0.52
Recessive model				
TT + TC	46 (100)	72 (96)	1.0 (Reference)	
CC	0	3 (4)	-	0.99

a)- Adjusted Odds ratios, 95% confidence intervals, and corresponding p-values were calculated using logistic regression analysis after adjusting for age smoking. (b)-Two-sided $\chi 2$ test for either genotype distribution or allelic frequencies between the cases and controls. The number in bold indicates the significant values in the Table

5.6.1.5. Relationship between the hTERT Genotypic Variants and Clinicopathological Characteristics

In the current study, we have evaluated the influence of hTERT variant SNP on several clinic-pathological features; the subjects were segregated based on the stage of cancer (III vs. IV), direct tumour extension (T3 vs. T4), lymph node involvement (Nx+N0+N1 vs. N2+N3+N4), and metastatic status (M0 vs. M1), and the data are reported in Table 5.7.

To study the relationship of different genotypes of hTERT polymorphic variants, the genotypic frequencies were calculated for each polymorphic site for stage III and stage IV patients, as shown in Table 5.7. The genotypic frequencies were compared to determine whether any significant difference lies in genotypic frequencies among the two subgroups based on the stage of the tumour. In the case of hTERT polymorphism, the wild-type genotype was more represented in stage III (67.34%) than in stage IV (63.15%). The heterozygous patients (*TC*) were found at a slightly higher frequency among stage III cases (29.25%) than in stage IV patients (28.70%), as shown in Table 5.7. For the homozygous variant type, the prevalence of subjects having (*CC*) genotype was higher among the stage IV (8.13%) subgroup than the stage III (3.40%) subgroup—none of the polymorphic sites depicted any significant difference in the genotypic frequencies among the two subgroups. Thus, the statistical association between these genotypes and the tumour stage was lacking. No significant AOR was observed for both heterozygous (AOR=0.53, 95% C.I.=0.17-1.70) and mutant (AOR=0.21; 95% C.I.=0.015-2.90) genotypes for hTERT polymorphism. Further, hTERT genetic variants were evaluated, and no association with tumour stage can be established.

The following parameter, which was evaluated for the hTERT polymorphism, was the primary tumour extension. The patients were stratified into two groups based on tumour size, T3 and T4, as shown in Table5.7. A higher proportion of lung cancer patients were reported in the T4 category (239), whereas the T3 subgroup comprises 68 patients. The genotypic frequencies were estimated for each polymorphic site, and it was observed that hTERT (T>C) heterozygous genotype (*TC*) was represented (36.76%) of patients with T3 tumour as compared to 29.28% of patients with a T4 tumour. It was also observed that the frequency of mutant genotype (*CC*) in subjects with T3 extension was almost similar to the lung cancer cases with T4 extension (4.41% vs. 5.85%), indicating no correlation with tumour extension. None of the genotypes of hTERT polymorphism correlated with the risk of lung cancer and lacked statistical significance.

Table 5.7 demonstrates the relationship between the different genotypes and the patient's metastatic status. These were further classified based on metastatic status into M0 and M1 groups. Patients showing positive distant metastasis (M1) were compared with those lacking metastasis (M0), also regarded as a reference group. Among the co-dominant model, homozygous genetic variant (*CC*), 6.91% of patients showed metastasis, while 3.37% did not show any metastasis. The frequency of subjects harbouring wild genotype (*TT*) was 64.51% for distant metastasis and 66.66% for subjects lacking metastasis. The combined variants (*TC+CC*) illustrated a higher frequency for the metastatic group than the non-metastatic group (35.48% vs. 33.33%). The patients with the mutant genotype for hTERT polymorphism (*CC*) exhibited a 4.02-fold risk of metastasis towards lung cancer as compared to the subjects who were homozygous for the wild type genotype (*TT*) (co-dominant model: AOR=4.02; 95% C.I.=1.06-15.1; p=**0.03**). Similarly, the recessive model subjects showed a substantially increased effect of lung cancer metastasis to other organs when patients who did not show metastasis were considered reference (recessive model: AOR=3.88; 95% C.I.=1.03-14.50; p=**0.04**).

Further, Table 5.7 demonstrates the relationship between these different genotypes after stratifying patients based on lymph node invasion. These were classified into Nx+N0+N1 and N2+N3+N4 groups. Patients in the Nx+N0+N1 tumour group were compared with the N2+ N3+N4 group, also regarded as the reference group. For the hTERT polymorphism, the subjects, the subjects carrying heterozygous genotype (*TC*) were represented more amongst the N2+N3+N4 tumour group than patients with the Nx+N0+N1 tumour group (30.56% vs. 22.61%). Similarly, the variant genotype (*CC*) also exhibits a higher percentage in the N2+N3+N4 group than the Nx+N0+N1 tumour group (6.64% vs. 2.38%). No significant association was observed between the hTERT polymorphism and the lymph node invasion in all three models evaluated, as shown in Table 5.7.

Table 5.6. Relationship between the hTERT Genotypic Variants and Clinicopathological Characteristics				
CLINICAL STAGE				
Genotype **rs2735940 T/C**	**III** **147, N(%)**	**IV** **209, N(%)**	**AOR** **(95 % C.I.)a**	**p**
Codominant model				
TT	99 (67.34)	132 (63.15)	1.0 (Reference)	
TC	43 (29.25)	60 (28.70)	0.53 (0.17-1.70)	0.29
CC	5 (3.40)	17 (8.13)	0.21 (0.015-2.90)	0.24
Dominant model				
TT	99 (67.34)	132 (63.15)	1.0 (Reference)	
TC + CC	48 (32.65)	77 (36.84)	0.50 (0.16-1.51)	0.21
Recessive model				
TT + TC	142 (96.59)	192 (91.86)	1.0 (Reference)	
CC	5 (3.40)	17 (8.13)	0.29 (0.02-3.86)	0.35

PRIMARY TUMOR EXTENSION				
Genotype **rs2735940 T/C**	**T3** **68, N (%)**	**T4** **239, N (%)**	**AOR** **(95 % C.I.)a**	**p**
Codominant model				
TT	40 (58.82)	155 (64.85)	1.0 (Reference)	
TC	25 (36.76)	70 (29.28)	0.53 (0.27-1.05)	0.07
CC	3 (4.41)	14 (5.85)	0.89 (0.23-3.47)	0.87
Dominant model				
TT	40	155	1.0	

	(58.82)	(64.85)	(Reference)	
TC + CC	28 (41.17)	84 (35.14)	0.57 (0.30-1.09)	0.09
Recessive model				
TT + TC	65 (95.58)	225 (94.14)	1.0 (Reference)	
CC	3 (4.41)	14 (5.85)	1.07 (0.28-4.05)	0.91

METASTASIS				
Genotype rs2735940 T/C	**NO 168, N (%)**	**YES 217, N (%)**	**AOR (95 % C.I.)a**	**p**
Codominant model				
TT	112 (66.66)	140 (64.51)	1.0 (Reference)	
TC	50 (29.76)	62 (28.57)	1.12 (0.63-1.96)	0.68
CC	6 (3.37)	15 (6.91)	4.02 (1.06-15.1)	**0.03**
Dominant model				
TT	112 (66.66)	140 (64.51)	1.0 (Reference)	
TC + CC	56 (33.33)	77 (35.48)	1.35 (0.79-2.30)	0.26
Recessive model				
TT + TC	162 (96.42)	202 (93.08)	1.0 (Reference)	
CC	6 (3.57)	15 (6.91)	3.88 (1.03-14.50)	**0.04**

LYMPH NODE INVASION				
Genotype rs2735940 T/C	**Nx+N0+N1 84, N (%)**	**N2+ N3 + N4 301, N (%)**	**AOR (95 % C.I.)a**	**p**
Codominant model				
TT	63 (75)	189 (62.79)	1.0 (Reference)	
TC	19 (22.61)	92 (30.56)	1.38 (0.70-2.71)	0.34
CC	2 (2.38)	20 (6.64)	2.41 (0.52-11.08)	0.25
Dominant model				
TT	63 (75)	189 (62.79)	1.0 (Reference)	

TC + CC	21 (25)	112 (37.20)	1.49 (0.78-2.84)	0.21
Recessive model				
TT + TC	82 (97.61)	281 (93.35)	1.0 (Reference)	
CC	2 (2.38)	20 (6.64)	2.13 (0.47-9.68)	0.32

a)- Adjusted Odds ratios, 95% confidence intervals, and corresponding p-values were calculated using logistic regression analysis after adjusting for age smoking. (b)-Two-sided $\chi 2$ test for either genotype distribution or allelic frequencies between the cases and controls. The number in bold indicates the significant values in the Table

5.6.1.6. Stratification analysis of the hTERT polymorphic variant with lung cancer risk based on age

The association between age and hTERT polymorphism in lung cancer patients was investigated for both cases and controls, as shown in Table 5.8. The average age of 63.5 was calculated for both cases and controls. To study the association of different genotypes of the hTERT polymorphic variant, the genotypic frequencies were calculated for lung cancer subjects younger than the mean age (63.5) and those older than the average age (63.5). Our data showed that lung cancer cases who were younger (63.5 age) and carrying heterozygous genotype (*TC*) or mutant genotype (*CC*) for hTERT polymorphism were overrepresented in cases as compared to controls (20.35% vs. 30.69% and 1.76% vs. 4.65%, respectively). As depicted in Table 5.8, the lung cancer subjects carrying heterozygous (*TC*) genotype with age younger than (63.5) exhibited an increased risk of acquiring lung cancer. The analysis showed an adjusted odds ratio of 1.62 with (AOR=1.62, 95% C.I.=1.04-2.53, **p=0.03**), compared with subjects carrying wild-type genotype (*TT*). Similarly, in the dominant model, lung cancer subjects carrying the combined genotype (*TC+CC*) exhibited a 1.72-fold higher risk of lung cancer development with (AOR=1.72, 95% C.I.=1.12-2.64, p=**0.01**). However, no significant association was observed between lung cancer subjects older than (63.5) and the hTERT polymorphic variant in all three models, as depicted in Table 5.8.

Table 5.7. Association between hTERT polymorphic variant and an average age of 63.5				
Age <63.5				
Genotype **rs2735940 T/C**	**Controls (384)** **226, N (%)**	**Cases (387)** **215, N (%)**	**AOR** **(95 % C.I.)a**	**p**
Codominant model				
TT	176 (77.87)	139 (64.65)	1.0 (Reference)	
TC	46 (20.35)	66 (30.69)	1.62 (1.04-2.53)	**0.03**
CC	4 (1.76)	10 (4.65)	2.70 (0.81-9.04)	0.10
Dominant model				
TT	176 (77.87)	139 (64.65)	1.0 (Reference)	
TC + CC	50 (22.12)	76 (35.34)	1.72 (1.12-2.64)	**0.01**
Recessive model				
TT + TC	222 (98.23)	205 (95.34)	1.0 (Reference)	
CC	4 (1.76)	10 (4.65)	2.40 (0.73-7.89)	0.14

Age >63.5				
Genotype rs2735940 T/C	**Controls (384) 158, N (%)**	**Cases (387) 172, N (%)**	**AOR (95 % C.I.)a**	**p**
Codominant model				
TT	119 (77.87)	113 (64.65)	1.0 (Reference)	
TC	32 (20.35)	45 (30.69)	1.47 (0.85-2.53)	0.16
CC	7 (1.76)	14 (4.65)	1.62 (0.60-4.36)	0.33
Dominant model				
TT	119 (77.87)	113 (64.65)	1.0 (Reference)	
TC + CC	39 (22.12)	59 (35.34)	1.52 (0.92-2.52)	0.09
Recessive model				
TT + TC	151 (98.23)	158 (95.34)	1.0 (Reference)	
CC	7 (1.76)	14 (4.65)	1.58 (0.60-4.18)	0.35

a)- Adjusted Odds ratios, 95% confidence intervals, and corresponding p-values were calculated using logistic regression analysis after adjusting for age smoking. (b)-Two-sided $\chi 2$ test for either genotype distribution or allelic frequencies between the cases and controls. The number in bold indicates the significant values in the Table

5.7. Clinical features of lung cancer patients assessed for detecting the prognostic relevance of the hTERT gene

Table 5.9 details the clinical characteristics and demography of the study conducted for the overall survival of lung cancer patients with the variant hTERT. Of 387 subjects, 80.62% were males, and 19.37% were females. Since smoking is an established factor in lung cancer, most of the sample group (80.62%) were smokers, while (21.44%) were non-smokers. Table 5.9 also includes clinical parameters, such as histology, TNM stage, performance status, and chemotherapeutic regimen.

According to the histological classification, there was a total of 40.56% were adenocarcinomas (ADCC), 44.96% squamous cell carcinomas (SQCC), 11.36% small cell lung carcinomas (SCLC), and 3.10% of patients with unclassified histology. The TNM staging classified the study subjects as follows: 5.42% stage I and staged II patients, 37.98% stage III patients, 54.00% stage IV patients, and the remaining 2.58% were unclassified. Additionally, the cases were analyzed for Karnofsky's performance status (KPS) and the Eastern Cooperative Oncology Group (ECOG). The classification according to the performance status of ECOG divided the study subjects into 42.63% of subjects with an ECOG score of 0-1, 37.72% with an ECOG score of 2, 16.02% with an ECOG score of 3-4, and the remaining 3.61% with unclassified ECOG score. Further, the KPS score classified the study subjects into 23.51% subjects with KPS scores between 90-100, 53.48% subjects with KPS scores in the range of 70-80, 18.08% with KPS score of below 60, and the remaining 4.90% with unclassified KPS score. Among the case group, 256 subjects were given cisplatin/carboplatin-based therapy. The maximum number of patients were administered pemetrexed with cisplatin/carboplatin (33.98%), followed by (29.68%) who received paclitaxel and cisplatin/carboplatin. Furthermore, (14.84%) patients were administered with docetaxeland cisplatin/carboplatin, (10.93%) patients received irinotecan and cisplatin/carboplatin. Further, (10.15%) of patients were given gemcitabine, and only one subject was given ceritinib (0.39%).

Table 5.8: Patient characteristics and clinical features with overall survival

Patient's characteristics	No. of Patients, n(%) N=387
Age (years)	
Mean±SD (Range)	63.5±2.12
Gender	
Male	312 (80.62)
Female	75 (19.37)
Smoking status	
Smokers	304 (78.55)
Non-Smokers	83 (21.44)
Histology	
ADCC	157 (40.56)
SQCC	174 (44.96)
SCLC	44 (11.36)
Others	12 (3.10)

TNM Stage	
I/II	21 (5.42)
III	147 (37.98)
IV	209 (54.00)
Unclassified	10 (2.58)
ECOG performance status	
0-1	165 (42.63)
2	146 (37.72)
3-4	62 (16.02)
Unclassified	14 (3.61)
KPS performance status	
90-100	91 (23.51)
70-80	207 (53.48)
60 & below	70 (18.08)
Unclassified	19 (4.90)
Median Follow-up (months)	
Events, Deaths	
Chemotherapy Regimen	N=256
Pemetrexed + cis/ carboplatin	87 (33.98)
Irinotecan + cis/ carboplatin	28 (10.93)
Docetaxel + cis/ carboplatin	38 (14.84)
Paclitaxel + cis/ carboplatin	76 (29.68)
Gemcitabine + cis/ carboplatin	26 (10.15)
Gefitinib	-
Etoposide	-
Ceritinib	1 (0.39)
Others	-

Abbreviations: SD= Standard Deviation, n= Total number of case-patients of lung cancer

5.8. The association of single nucleotide polymorphism (SNP) in the TERT gene in patients with lung cancer

5.8.1 Association of functional polymorphic variant of the hTERT gene towards lung cancer pathogenesis

5.8.1.1 Elucidation of Polymorphic hTERT Genetic Variant with overall survival of lung cancer patients

Survival analysis for 387 lung cancer subjects and its association with the hTERT polymorphism has been studied. Table 5.10 depicts the results of univariate and multivariate analysis of hTERT polymorphism. Kaplan-Meier statistics define univariate analysis offering median survival time (MST) in months and survival graphs for each genotype. The log-rank p test in Kaplan-Meier statistics analyses the MST for different genotypes using the wild genotype as a reference. Kaplan-Meier statistics analyses unadjusted hazard ratios. The adjusted hazard ratios are obtained by using Cox regression. The hazard ratio was adjusted using the Cox-regression model for co-variates such as age, gender, smoking, histology, ECOG, clinical stage, and KPS. All 387 patients were followed from the day of diagnosis of lung cancer, and we found that 69 (17.82%) patients were alive and 318 (82.17%) patients died due to lung cancer.

For hTERT polymorphism, the homozygous (*TT*) genotype was taken as a reference genotype. Although the median survival time (MST) of patients with the mutant (*CC*) genotype was slightly higher in comparison with wild genotype (*TT*) (7.97 months vs. 7.80 months, log-rank p=0.51), however the difference in the MST was not found to be significant. A better hazard rate was observed for subjects carrying the heterozygous alleles both in univariate (HR=1.14, 95% C.I.=0.88-1.46, Log-rank p=0.29) and multivariate (HR=1.15, 95% C.I.=0.83-1.57, Log-rank p=0.38) analysis but lacks statistical significance.

Table 5.9.Univariate and Multivariate analysis of hTERT SNP (rs 2735940) in lung cancer cases								
Genotype rs2735940 T/C	**CASES n (%) N=387**	**DEAD n (%) N=318**	**ALIVE n (%) N=69**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR[a]**	**Adjusted HR[b] (95% C.I.)**	**p**
TT	252 (65.11)	202 (63.5)	50 (72.46)	7.80		1.0 (reference)		
TC	111 (28.68)	97 (30.50)	14 (20.28)	7.37	0.29	1.14 (0.88-1.46)	1.15 (0.83-1.57)	0.38
CC	24 (6.20)	19 (5.97)	5 (7.24)	7.97	0.51	1.19 (0.69-2.06)	0.66 (0.34-1.27)	0.21
TC + CC	135 (34.88)	116 (36.47)	19 (27.53)	7.57	0.39	1.10 (0.87-1.39)	1.03 (0.76-1.38)	0.83
TT + TC	363 (93.79)	299 (94.02)	64 (92.75)	7.97	0.78	1.06 (0.67-1.67)	0.67 (0.36-1.24)	0.20

[a]Unadjusted Hazards ratio for Kaplan-Meier analysis, [b]hazards ratio for age, sex, smoking, stage, histology, KPS, ECOG

5.8.1.2 Impact of hTERT Polymorphism on Overall Survival by Histological Subtype in Lung Cancer Patients

The relationship between hTERT polymorphism and overall survival varies according to the histological subtype in lung cancer patients. The stratified data was tabulated in Table 5.11. The percentage of heterozygous patients was 24.20%, 32.18%, and 29.54%, harbouring ADCC, SQCC, and SCLC subtypes, respectively. Similarly, the rate of patients harbouring mutant genotype was 5.09%, 4.02%, and 15.90%, respectively, among ADCC, SQCC and SCLC subtypes. In the present study, ADCC patients carrying the mutant genotype (*CC*) showed poor survival outcomes as compared to the patients carrying the wild genotype *(TT*) for the hTERT polymorphic variant (3.90 vs 8.20 months, Log-rank p=0.21). Both the Univariate (HR=1.83, 95% C.I.=0.70-4.76, p=0.21) and Multivariate cox analysis (HR'=1.75, 95% C.I.=0.72-4.24, p=0.21) did not show any significant association. Similarly, the combined genotype (*TT+TC*) in the case of ADCC patients also showed reduced survival outcomes as compared to the wild genotype (*TT*) (3.90 vs 8.20 months, Log-rank p=0.26)

On the contrary, patients having SQCC and hTERT polymorphism and carrying the mutant genotype (*CC*) showed a better OS (overall survival) as compared to the wild genotype (*TT*) (7.97 vs. 6.93 months, log-rank p=0.93). However, the results were not statistically significant even after the Univariate and multivariate Cox analysis (HR=1.03, 95% C.I.=0.45-2.33, log-rank p=0.93) and (HR'=0.88, 95% C.I.=0.26-2.98, log-rank p=0.83) respectively. Similarly, the combined (*TT+TC*) genotype in the case of SQCC subjects also showed a better survival rate than the wild genotype (*TT*) (7.97 vs. 6.93 months, log-rank p=0.92). However, the results lack the statistical significance.

Moreover, an analysis of the survival trend in SCLC patients having the hTERT polymorphic variant indicates that patients carrying mutant genotype (*CC*) showed a better survival rate as compared to wild genotype (*TT*) (11.57 vs. 9.33 months, Log-rank p=0.25). Both the univariate (HR=1.73, 95% C.I.=0.75-3.98, Log-rank p=0.25) and multivariate (HR'=0.22, 95% C.I.=0.03-1.51, Log-rank p=0.12) did not show any significant association. The combined genotype (*TT+TC*) also showed a better OS as compared to the wild-type genotype (*TT*) (12.17 vs. 9.33 months). After univariate and multivariate Cox analysis, the HR was 1.93 (95% C.I.=0.91-4.09, Log-rank p=0.15) and (HR'=0.36, 95% C.I.=0.08-1.49, Log-rank p=0.16) did not show any significant association. However, this polymorphism had no significant bearing on the prognosis for SCLC patients. Therefore, as demonstrated in Table 5.11, these results indicate that none of the histological parameters, such as ADCC, SQCC, and SCLC, were associated with predicting any prognostic significance for the hTERT polymorphism.

Table 5.10. Association of hTERT SNP (rs2735940) on overall survival in lung cancer cases according to histology subtypes

ADCC								
Genotype rs2735940 T/C	**CASES n (%) N=157**	**DEAD n (%) N=121**	**ALIVE n (%) N=36**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR[a]**	**Adjusted HR[b] (95% C.I.)**	**p**
TT	111 (70.70)	83 (68.59)	28 (77.77)	8.20		1.0 (reference)		
TC	38 (24.20)	31 (25.61)	7 (19.44)	7.57	0.54	1.14 (0.74-1.74)	1.41 (0.84-2.35)	0.18
CC	8 (5.09)	7 (5.78)	1 (2.77)	3.90	0.21	1.83 (0.70-4.76)	1.75 (0.72-4.24)	0.21
TC + CC	46 (29.29)	38 (31.40)	8 (22.22)	7.57	0.35	1.20 (0.81-1.80)	1.46 (0.91-2.35)	0.11
TT + TC	149 (94.90)	114 (94.21)	35 (97.22)	3.90	0.26	1.70 (0.67-4.31)	1.53 (0.64-3.63)	0.33

SQCC								
Genotype rs2735940 T/C	**CASES n (%) N=174**	**DEAD n (%) N=148**	**ALIVE n (%) N=26**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR[a]**	**Adjusted HR[b] (95% C.I.)**	**p**
TT	111 (63.79)	93 (62.83)	18 (69.23)	6.93		1.0 (reference)		
TC	56 (32.18)	49 (33.10)	7 (26.92)	7.27	0.97	1.00 (0.71-1.42)	1.06 (0.66-1.69)	0.79
CC	7 (4.02)	6 (4.05)	1 (3.84)	7.97	0.93	1.03 (0.45-2.33)	0.88 (0.26-2.98)	0.83
TC + CC	63 (36.20)	55 (37.16)	8 (30.76)	7.33	0.98	1.00 (0.71-1.40)	1.04 (0.67-1.63)	0.84
TT + TC	167 (95.97)	142 (95.94)	25 (96.15)	7.97	0.92	1.03 (0.46-2.32)	0.84 (0.25-2.75)	0.77

SCLC								
Genotype rs2735940 T/C	**CASES n (%) N=44**	**DEAD n (%) N=41**	**ALIVE n (%) N=3**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR**	**Adjusted HR[b] (95% C.I.)**	**p**
TT	24 (54.54)	23 (56.09)	1 (33.33)	9.33		1.0 (reference)		
TC	13 (29.54)	13 (31.70)	0 (0)	7.23	0.57	1.21 (0.60-2.44)	1.02 (0.42-2.45)	0.95
CC	7 (15.90)	5 (12.19)	2 (66.66)	11.57	0.25	1.73 (0.75-3.98)	0.22 (0.03-1.51)	0.12
TC + CC	20 (45.45)	18 (43.90)	2 (66.66)	7.60	0.78	1.09 (0.59-2.01)	0.84 (0.35-2.03)	0.70
TT + TC	37 (84.09)	36 (87.80)	1 (33.33)	12.17	0.15	1.93 (0.91-4.09)	0.36 (0.08-1.49)	0.16

[a]Unadjusted Hazards ratio for Kaplan-Meier analysis, [b]Hazards ratio for age, gender, smoking, KPS, ECOG, and Regimen

5.8.1.3. Relationship between hTERT polymorphism and overall survival in lung cancer patients based on gender

In lung cancer patients, 82.69% of males and 80% of females were dead at the time of statistical analysis. Table 5.12 presents stratified data for overall survival and hTERT polymorphism. Our results revealed that the males harbouring both copies of mutant alleles (*CC*) for hTERT polymorphism had a higher survival rate as compared to the subjects carrying wild genotype (*TT*) (MST=7.97 vs. 7.17 months, p=0.94) respectively. The statistical significance could not be achieved after a multivariate analysis (HR'=0.58, 95% C.I.=0.29-1.15, p=0.12). Similarly, the combined variants (*TC+CC*) showed an increased survival as compared to patients carrying wild genotype (*TT*) (MST=7.57 vs. 7.17 months, p=0.54). However, a higher death rate was

obtained after applying multivariate Cox regression. However, statistical significance could not be achieved (HR'=0.88, 95% C.I.=0.63-1.24, p=0.48).

However, the female subjects carrying the mutant genotype (CC) showed lower survival as compared to subjects carrying the wild genotype (*TT*) (MST=8.23 vs. 10.40 months, p=0.82). Also, the multivariate analysis revealed a higher death ratio than the wild-type genotype (HR'=1.17, 95% C.I.=0.20-6.57, p=0.85), but no significant association was achieved. Similarly, the combined variants (*TC+CC*) and (*TT+TC*) showed a lower survival rate when compared to the wild-type genotype (*TT*). However, no significant association was observed. (Table 5.12). Based on the analysis results, there appears to be no significant association between hTERT polymorphism and the overall survival of both males and females.

Table 5.11. Relationship between hTERT SNP and overall survival according to gender

MALE

Genotype rs2735940 T/C	**CASES n (%) N=312**	**DEAD n (%) N=258**	**ALIVE n (%) N=54**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR[a]**	**Adjusted HR[b] (95% C.I.)**	**p**
TT	204 (65.38)	164 (63.56)	40 (74.07)	7.17		1.0 (reference)		
TC	87 (27.88)	77 (29.84)	10 (18.51)	7.57	0.45	1.11 (0.84-1.46)	0.94 (0.66-1.35)	0.77
CC	21 (6.73)	17 (6.58)	4 (7.40)	7.97	0.94	1.01 (0.61-1.67)	0.58 (0.29-1.15)	0.12
TC + CC	108 (34.61)	94 (36.43)	14 (25.92)	7.57	0.54	1.08 (0.83-1.40)	0.88 (0.63-1.24)	0.48
TT + TC	291 (93.26)	241 (93.41)	50 (92.59)	7.97	0.86	1.04 (0.64-1.69)	0.62 (0.32-1.20)	0.16

FEMALE								
Genotype rs2735940 T/C	**CASES n (%) N=75**	**DEAD n (%) N=60**	**ALIVE n (%) N=15**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR[a]**	**Adjusted HR[b] (95% C.I.)**	**p**
TT	48 (64)	38 (63.33)	10 (66.66)	10.40		1.0 (reference)		
TC	24 (32)	20 (33.33)	4 (26.66)	7.27	0.34	1.31 (0.74-2.32)	1.63 (0.71-3.73)	0.24
CC	3 (4)	2 (3.33)	1 (6.66)	8.23	0.82	1.15 (0.30-4.38)	1.17 (0.20-6.57)	0.85
TC + CC	27 (36)	22 (36.66)	5 (33.33)	7.37	0.43	1.24 (0.72-2.14)	1.69 (0.77-3.69)	0.18
TT + TC	72 (96)	58 (96.66)	14 (93.33)	8.23	0.70	1.27 (0.36-4.43)	0.85 (0.18-3.95)	0.84

[a]Unadjusted Hazards ratio for Kaplan Meier analysis, [b]hazards ratio for age, smoking, stage, regimen, histology, KPS, ECOG

5.8.1.4. Association of the hTERT Polymorphism with Overall Survival Concerning Smoking Status

To examine the interaction between genetic factors and environmental influences, the case group was divided into subgroups based on their smoking habits, *i.e.*, smokers and non-smokers. Among lung cancer patients, 84.86% of patient smokers were dead, while 15.13 of smokers were alive, as shown in Table 5.13. For hTERT polymorphism, in the case of smokers, our study revealed a better survival of both the heterozygous (*TC*) genotype and combined variant (*TC+CC*) when the wild-type (*TT*) genotype was taken as a reference (MST=7.33 vs. 7.17 months, p=0.46 and MST=7.33 vs. 7.17 months, p=0.47). However, no significant association was observed (HR'=1.05, 95% C.I.=0.73-1.50, p=0.76) and (HR'=0.98, 95% C.I.=0.70-1.38, p=0.95) respectively. On the other hand, smokers carrying the mutant (CC) genotype and combined genotype (*TT+TC*) showed a decrease in overall survival when

compared to subjects carrying wild genotype (*TT*) (MST=6.90 vs.7.17 months, p=0.80 and MST=6.90 vs. 7.17 months, p=0.86) respectively. After applying multivariate analysis, no significant association was observed (HR'=0.62, 95% C.I.=0.31-1.25, p=0.18) and (HR'=0.66, 95% C.I.=0.34-1.30, p=0.23), respectively.

When assessing the impact of hTERT polymorphism on the prognosis of non-smokers, it was found that 72.28% of non-smokers had died, while 27.71% remained alive after the follow-up period. In the case of non-smokers, patients carrying the hTERT polymorphism and heterozygous genotype (*TC*) revealed a very drastic decrease in overall survival when compared to the wild-type genotype (*TT*) (MST=7.37 vs. 13.03 months, p=0.29), but the association was insignificant (HR'=1.32, 95% C.I.=0.66-2.63, p=0.42). In the case of the combined variant (*TT+TC*), a marginal decrease in overall survival was observed (MST=12.17 vs 13.03 months, p=0.41), but the outcomes were insignificant (HR'=0.80, 95% C.I.=0.22-2.90, p=0.74). Consequently, the hTERT polymorphism gene was not associated with prognosis in lung cancer patients in smokers and non-smokers.

Table 5.12. Association of hTERT SNP on overall survival according to smoking status

Smoker								
Genotype rs2735940 T/C	**CASES n (%) N=304**	**DEAD n (%) N=258**	**ALIVE n (%) N=46**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR[a]**	**Adjusted HR[b] (95% C.I.)**	**p**
TT	199 (65.46)	165 (63.95)	34 (73.91)	7.17		1.0 (reference)		
TC	86 (28.28)	77 (29.84)	9 (19.56)	7.33	0.46	1.10 (0.84-1.46)	1.05 (0.73-1.50)	0.76
CC	19 (6.25)	16 (6.20)	3 (6.52)	6.90	0.80	1.06 (0.63-1.81)	0.62 (0.31-1.25)	0.18
TC + CC	105 (34.53)	93 (36.04)	12 (26.08)	7.33	0.47	1.09 (0.84-1.42)	0.98 (0.70-1.38)	0.95
TT + TC	285 (93.75)	242 (93.79)	43 (93.47)	6.90	0.86	1.04 (0.62-1.75)	0.66 (0.34-1.30)	0.23

Non-Smoker								
Genotype rs2735940 T/C	**CASES n (%) N=83**	**DEAD n (%) N=60**	**ALIVE n (%) N=23**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR[a]**	**Adjusted HR[b] (95% C.I.)**	**p**
TT	53 (63.85)	37 (61.66)	16 (69.56)	13.03		1.0 (reference)		
TC	25 (30.12)	20 (33.33)	5 (21.73)	7.37	0.29	1.35 (0.76-2.41)	1.32 (0.66-2.63)	0.42
CC	5 (6.02)	3 (5)	2 (8.69)	12.17	0.51	1.39 (0.50-3.81)	1.06 (0.26-4.22)	0.92
TC + CC	30 (36.14)	23 (38.33)	7 (30.43)	7.80	0.52	1.18 (0.69-2.02)	1.23 (0.64-2.36)	0.52
TT + TC	78 (93.97)	57 (95)	21 (91.30)	12.17	0.41	1.48 (0.57-3.81)	0.80 (0.22-2.90)	0.74

[a]Unadjusted Hazards ratio for Kaplan Meier analysis, [b]hazards ratio for age, gender, stage, regimen, histology, KPS, ECOG

5.8.1.5. Impact of hTERT Polymorphism on Overall Survival Relative to Performance Status

The ECOG Performance Status (ECOG-PS) is the standard criteria for measuring how the disease impacts a patient's daily living abilities (walking, working), and it is known to physicians and researchers as a patient's performance status. To evaluate the influence of ECOG-PS in conjunction with hTERT polymorphism to predict survival in lung cancer patients, the individuals were categorized based on an ECOG-PS score of 0,1 (good/restricted performance status), ECOG-PS score of 2 (limited performance status), and ECOG-PS score of 3,4 (bad performance status), as depicted in Table 5.14. For ECOG-PS 0,1, in the case of hTERT polymorphism, subjects carrying heterozygous genotype (*TC*) showed reduced survival as compared to wild-type genotype (*TT*) (MST=11.47 vs. 12.63 months, p=0.25). After applying multivariate analysis, no significant association was observed in the heterozygous

genotype (HR'=1.10, 95% C.I.=0.69-1.76, p=0.67). Similarly, the subjects carrying the combined genotype (*TC+CC*) revealed a reduced survival time when compared to subjects carrying the wild-type genotype (*TT*) (MST=12.20 vs. 12.63 months, p=0.59); however, no significant outcomes were observed. For the ECOG-PS 2 score, the patients carrying both homozygous mutant alleles (*CC*) and combined genotype (*TC+CC*) showed a better survival rate when the wild-type genotype was taken as a reference (MST=8.23 vs 6.80 months, p=0.60) and (MST=7.00 vs. 6.80 months, p=0.78) respectively, but lacks statistical significance.

However, patients with ECOG-PS 3,4 having heterozygous genotype (*TC*) and hTERT polymorphism showed a better survival rate when compared to subjects having wild-type genotype (*TT*) (3.67 vs.2.80 months, p=0.80). After adjusting with multiple covariates, the hazard ratio decreased to 0.79 times in the heterozygous genotype, and a significant association was observed (HR'=0.79, 95% C.I.=0.25-2.52, p=**0.002**). On the other hand, patients carrying the combined genotype (*TT+TC*) showed reduced survival when the wild-type genotype was taken as a reference (MST=0.53 vs. 2.80 months, p=0.11). After applying multivariate analysis, the death ratio increased to 18.85 times in the combined genotype and significant association was observed (HR'=18.85, 95% C.I.=3.07-115.5, p=**0.0015**).

Table 5.13. Association of hTERT polymorphism with overall survival based on ECOG-PS

ECOG 0,1								
Genotype rs2735940 T/C	**CASES n (%) N=166**	**DEAD n (%) N=117**	**ALIVE n (%) N=49**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR**	**Adjusted HR (95% C.I.)**	**p**
TT	110 (66.26)	74 (63.24)	36 (73.46)	12.63	0.09	1.0 (reference)		
TC	51 (30.72)	42 (35.89)	9 (18.36)	11.47	0.25	1.26 (0.84-1.87)	1.10 (0.69-1.76)	0.67
CC	5 (3.01)	1 (0.85)	4 (8.16)	-	0.08	2.29 (0.88-5.99)	0.17 (0.02-1.33)	0.09
TC + CC	56 (33.73)	43 (36.75)	13 (26.53)	12.20	0.59	1.11 (0.75-1.62)	0.94 (0.59-1.49)	0.79
TT + TC	161	116	45	-	0.06	2.33	0.18	0.09

	(96.98)	(99.14)	(91.83)			(0.95-5.70)	(0.02-1.32)	

ECOG 2

Genotype rs2735940 T/C	**CASES n (%) N=145**	**DEAD n (%) N=133**	**ALIVE n (%) N=12**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR**	**Adjusted HR (95% C.I.)**	**p**
TT	92 (63.44)	84 (63.15)	8 (66.66)	6.80		1.0 (reference)		
TC	39 (26.89)	36 (27.06)	3 (25)	4.63	0.49	1.15 (0.76-1.72)	1.33 (0.81-2.18)	0.25
CC	14 (9.65)	13 (9.77)	1 (8.33)	8.23	0.60	1.16 (0.67-2.03)	0.51 (0.20-1.25)	0.14
TC + CC	53 (36.55)	49 (36.84)	4 (33.33)	7.00	0.78	1.05 (0.73-1.50)	1.18 (0.73-1.89)	0.48
TT + TC	131 (90.34)	120 (90.22)	11 (91.66)	8.23	0.48	1.20 (0.71-2.05)	0.50 (0.21-1.14)	0.10

ECOG 3,4

Genotype rs2735940 T/C	**CASES n (%) N=62**	**DEAD n (%) N=57**	**ALIVE n (%) N=5**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR**	**Adjusted HR (95% C.I.)**	**p**
TT	39 (62.90)	36 (63.15)	3 (60)	2.80		1.0 (reference)		
TC	19 (30.64)	17 (29.82)	2 (40)	3.67	0.80	1.07 (0.60-1.90)	0.79 (0.25-2.52)	**0.002**
CC	4 (6.45)	4 (7.01)	0	0.53	0.13	2.99 (0.70-12.78)	51.89 (3.96-679.5)	0.69
TC + CC	23 (37.09)	21 (36.84)	2 (40)	3.67	0.87	1.04 (0.60-1.80)	1.16 (0.41-3.23)	0.77

TT + TC	58 (93.54)	53 (92.98)	5 (100)	0.53	0.11	3.27 (0.75-14.21)	18.85 (3.07-115.5)	**0.0015**

[a]Unadjusted Hazards ratio for Kaplan Meier analysis, [b]hazards ratio for age, gender, smoking, stage, histology, regimen

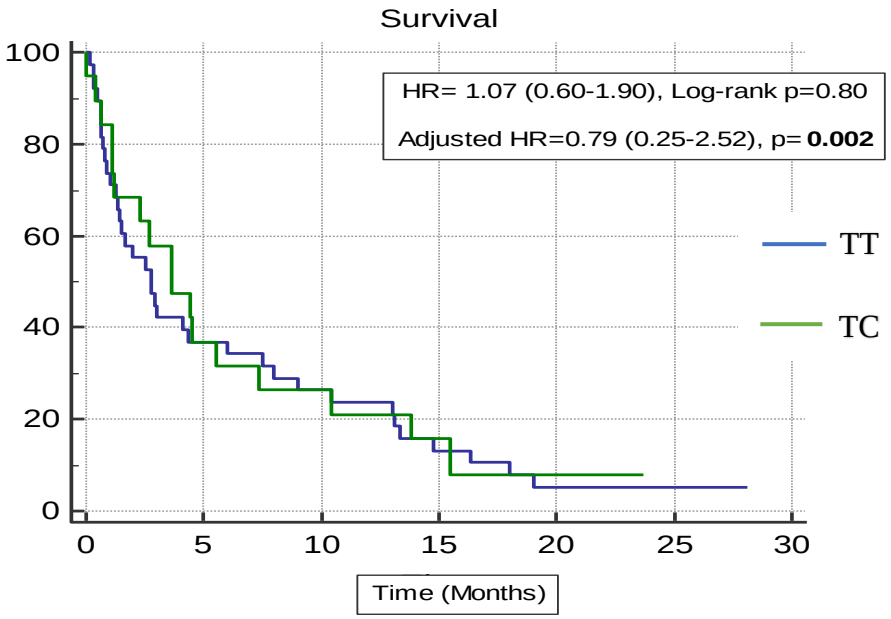


Figure 5.13:Kaplan Meier curves illustrating the association between overall survival in lung cancer patients with heterozygous genotype (*TC*) of hTERT polymorphism based on ECOG-PS score 3,4 (Note: 0 represents wild-type genotype (*TT*), 1 represents heterozygous genotype (*TC*)

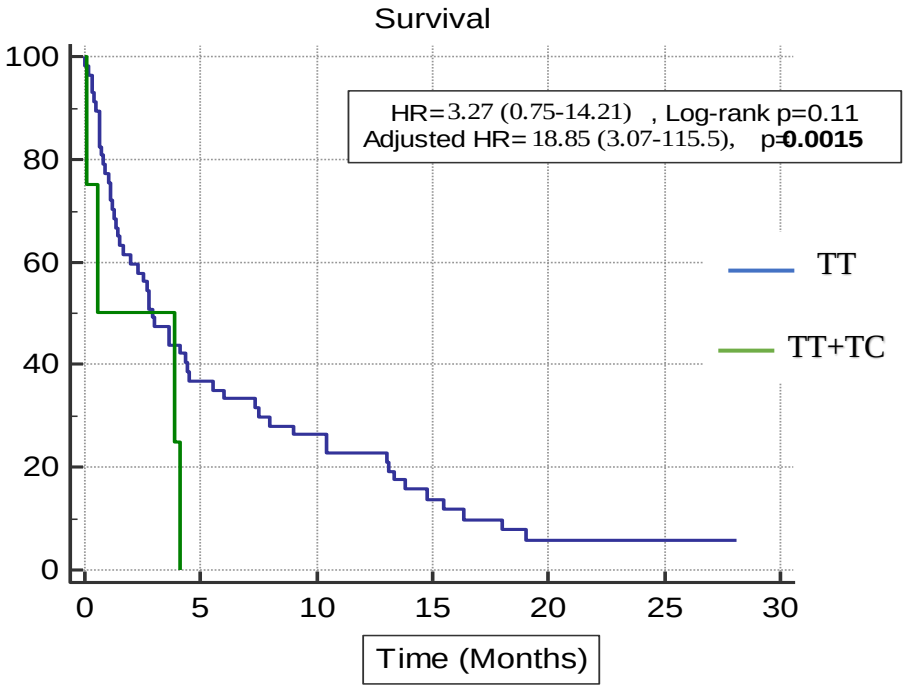


Figure 5.14:Kaplan Meier curve illustrating the association between overall survival in lung cancer patients with combined (*TT*+*TC*) genotype of hTERT polymorphism based on ECOG-PS score 3,4 (Note: 0 represents wild-type genotype (*TT*), 1 represents combined (*TT*+*TC*) genotype)

5.8.1.6. Prognosis and Overall Survival of Lung Cancer Patients Undergoing Different Chemotherapeutic Regimens

The lung cancer patients in this study received platinum-based chemotherapy in combination with docetaxel, paclitaxel, pemetrexed, and irinotecan. Therefore, we investigated the modulating effects of the hTERT polymorphism and its correlation to chemotherapy treatment and overall patient survival. The survival of lung cancer cases treated with varying chemotherapeutic regimens and its correlation with hTERT polymorphism has been studied and tabulated in Table 5.12. In this study, various chemotherapeutic regimens were administered to treat lung cancer patients, but the analysis focused on those used to treat the maximum number of patients. Lung cancer patients were cyclically administered chemotherapy medications. Patients underwent examinations to ascertain whether medications had an independent impact on survival. The Overall Survival of patients treated with four regimens *viz*, docetaxel + cis/carboplatin, irinotecan + cis/carboplatin, pemetrexed + cis/carboplatin, and paclitaxel + cis/carboplatin and their significant association with hTERT polymorphism has been tabulated in Table 5.12.

The lung cancer patients who were administered cisplatin/carboplatin along with docetaxel and carrying the hTERT mutant type alleles (*CC*) showed better survival when compared to patients harbouring wild-type genotype (*TT*) (22.40 vs. 9.13 months, Log-rank p=0.09). After applying the univariate and multivariate Cox regression analysis, adjusting for various covariates, a significant but lower hazard ratio was obtained (HR=2.12, 95% C.I.=0.87-5.13, Log-rank p=0.09) and (HR'=0.0009, 95% C.I.=0.0007-0.10, Log-rank p=**0.0002**). Similarly, the lung cancer subjects carrying the combined genotype (*TC+CC*) showed a marginal survival time difference when compared with subjects carrying the wild genotype (*TT*) as a reference (9.77 vs. 9.13 months). After applying multivariate Cox analysis, a significant association was found with a lower hazard ratio (HR=0.22, 95% C.I.=0.07-0.68, Log-rank p=**0.008).** Likewise, after applying multivariate Cox analysis, lung cancer patients carrying the combined genotype (*TT+TC*) showed a significant association and lower hazard ratio (HR'=0.03, 95% C.I.=0.005-0.20, Log-rank p=**0.0002**). Thus, as shown in Table 5.12, lung cancer patients treated with cisplatin/carboplatin combined with docetaxel, who also had the hTERT polymorphism, exhibited a protective effect and improved survival rates.

Furthermore, the patients who received cisplatin/carboplatin combined with a paclitaxel regimen carrying the heterozygous genotype (*TC*) showed a better MST as compared to the patients harbouring wild genotype (TT) (6.90 vs. 5.67 months, Log-rank p=0.46). Both the univariate (HR=1.23, 95% C.I.=0.70-2.17, Log-rank p=0.46) and multivariate Cox analysis (HR'=1.60, 95% C.I.=0.90-2.85, Log-rank p=0.10) depicted no significant association between the subjects with heterozygous (*TC*) genotype for hTERT polymorphism. On the other hand, patients carrying the mutant (*CC*) genotype showed poor survival as compared to subjects carrying the wild genotype (*TT*) (0.07 vs. 5.67 months). Both the univariate (HR=3.95, 95% C.I.=0.46-33.9, Log-rank p=0.21) and multivariate Cox analysis (HR'=3.34, 95% C.I.=0.59-18.7, Log-rank p=0.16) showed a higher death rate but lacked statistical significance. Similarly, the patients carrying the combined genetic models (*TC+CC*) and (*TT+TC*) depict a higher hazard ratio but lack statistical significance.

The patients administered with pemetrexed and cisplatin/carboplatin carrying the mutant (*CC*) genotype showed poor MST as compared to patients harbouring wild (*TT*) genotype (3.90 vs. 7.23 months, log-rank p=0.55). Both the univariate (HR=1.36, 95% C.I.=0.48-3.84, Log-rank p=0.55) and multivariate (HR'=1.60, 95% C.I.=0.59-4.29, Log-rank p=0.34) analysis depicts the higher hazard ratio but lacks statistical significance. The patients carrying the combined genotype (*TC+CC*) revealed a higher survival time as compared to the patients carrying the wild genotype (*TT*) (7.57 vs. 7.23 months, p=0.30). The univariate (HR=0.77, 95% C.I.=0.47-1.26, Log-rank p=0.30) and multivariate (HR'=1.51, 95% C.I.=0.89-2.54, Log-rank p=0.11) data depicts higher hazard ratio but lacks statistical significance.

As shown in Table 5.12, the lung cancer patients who were administered cisplatin/carboplatin combined with Irinotecan exhibited a higher survival of 10.57 months, especially in those subjects who were carrying heterozygous genotype (*TC*) as compared to subjects harbouring the wild genotype (*TT*) (10.57 vs. 7.67 months, Log-rank p=0.85). A higher hazard ratio was obtained after applying the multivariate Cox analysis (HR'=1.19, 95% C.I.=0.45-3.18, Log-rank p=0.71) but lacked statistical significance. On the contrary, patients carrying mutant genotype (*CC*) exhibit poor survival rates as compared to the patients carrying wild genotype (*TT*) (2.60 vs. 7.67 months, log-rank p=0.18). Both in univariate (HR=12.51, 95% C.I.=0.30-513, Log-rank p=0.18) and multivariate analysis (HR'=2.78, 95% C.I.=0.25-29.98, Log-rank p=0.39) revealed a higher hazards ratio but lacked statistical significance. Similarly, the patients carrying the combined model (TC+CC)). Furthermore (TT+TC) exhibitsa higher death

rate but lacks statistical significance. Thus, the results indicate that lung cancer patients treated with a chemotherapeutic regimen of cisplatin or carboplatin in combination with docetaxel exhibit a significant association with overall survival (OS) and hTERT polymorphism, suggesting a protective effect. On the other hand, patients who received alternative chemotherapeutic regimens did not demonstrate any association between overall survival and the hTERT polymorphic variant.

Table 5.14. Association of hTERT SNP (rs2735940) on overall survival according to chemotherapeutic regimen

Docetaxel + cis/ carboplatin								
Genotype rs2735940 T/C	**CASES n (%) N=38**	**DEAD n (%) N=31**	**ALIVE n (%) N=7**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR**	**Adjusted HR (95% C.I.)**	**p**
TT	`20 (52.63)	18 (58.06)	2 (28.57)	9.13		1.0 (reference)		
TC	11 (28.94)	9 (29.03)	2 (28.57)	8.50	0.67	1.19 (0.51-2.79)	0.67 (0.22-2.06)	0.49
CC	7 (18.42)	4 (12.90)	3 (42.85)	22.40	0.09	2.12 (0.87-5.13)	0.009 (0.0007-0.10)	**0.0002**
TC + CC	18 (47.36)	13 (41.93)	5 (71.42)	9.77	0.40	0.74 (0.36-1.50)	0.22 (0.07-0.68)	**0.008**
TT + TC	31 (81.57)	27 (87.09)	4 (57.14)	22.40	0.05	0.46 (0.21-1.02)	0.03 (0.005-0.20)	**0.0002**

Paclitaxel + cis/ carboplatin								
Genotype rs2735940 T/C	**CASES n (%) N=76**	**DEAD n (%) N=64**	**ALIVE n (%) N=12**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR**	**Adjusted HR (95% C.I.)**	**p**
TT	53 (69.73)	43 (67.18)	10 (83.33)	5.67		1.0 (reference)		
TC	21 (27.63)	19 (29.68)	2 (16.66)	6.90	0.46	1.23 (0.70-2.17)	1.60 (0.90-2.85)	0.10
CC	2 (2.63)	2 (3.125)	0	0.07	0.21	3.95 (0.46-33.9)	3.34 (0.59-18.7)	0.16
TC + CC	23 (30.26)	21 (32.81)	2 (16.66)	6.90	0.34	1.30 (0.75-2.25)	1.62 (0.93-2.82)	0.08
TT + TC	74 (97.36)	62 (96.87)	12 (100)	0.07	0.21	3.93 (0.46-33.5	1.90 (0.39-9.24)	0.42

Pemetrexed + cis/ carboplatin								
Genotype rs2735940 T/C	**CASES n (%) N=87**	**DEAD n (%) N=73**	**ALIVE n (%) N=14**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR**	**Adjusted HR (95% C.I.)**	**p**
TT	56 (64.36)	45 (61.64)	11 (78.57)	7.23		1.0 (reference)		
TC	25 (28.73)	23 (31.50)	2 (14.28)	7.57	0.33	1.30 (0.76-2.21)	1.44 (0.81-2.57)	0.20
CC	6 (6.89)	5 (6.84)	1 (7.14)	3.90	0.55	1.36 (0.48-3.84)	1.60 (0.59-4.29)	0.34
TC + CC	31 (35.63)	28 (38.35)	3 (21.42)	7.57	0.30	0.77 (0.47-1.26)	1.51 (0.89-2.54)	0.11
TT + TC	81 (93.10)	68 (93.15)	13 (92.85)	3.90	0.70	1.21 (0.45-3.24)	1.31 (0.50-3.41)	0.57

Irinotecan + cis/ carboplatin								
Genotype rs2735940 T/C	**CASES n (%) N=28**	**DEAD n (%) N=26**	**ALIVE n (%) N=2**	**Univariate analysis**			**Multivariate analysis**	
				MST (months)	**Log-rank p**	**Unadjusted HR**	**Adjusted HR (95% C.I.)**	**p**
TT	18 (64.28)	16 (61.53)	2 (100)	7.67		1.0 (reference)		
TC	9 (32.14)	9 (34.61)	0	10.57	0.85	1.08 (0.47-2.47)	1.19 (0.45-3.18)	0.71
CC	1 (3.57)	1 (3.84)	0	2.60	0.18	12.51 (0.30-513)	2.78 (0.25-29.98)	0.39
TC + CC	10 (35.71)	10 (38.46)	0	7.23	0.70	1.16 (0.52-2.62)	1.29 (0.49-3.36)	0.59
TT + TC	27 (96.42)	25 (96.15)	2 (100)	2.60	0.10	28.90 (0.47-1768)	4.26 (0.45-40.17)	0.20

[a]Unadjusted Hazards ratio for Kaplan Meier analysis, [b]hazards ratio for age, gender, smoking, histology, stage, KPS, and ECOG

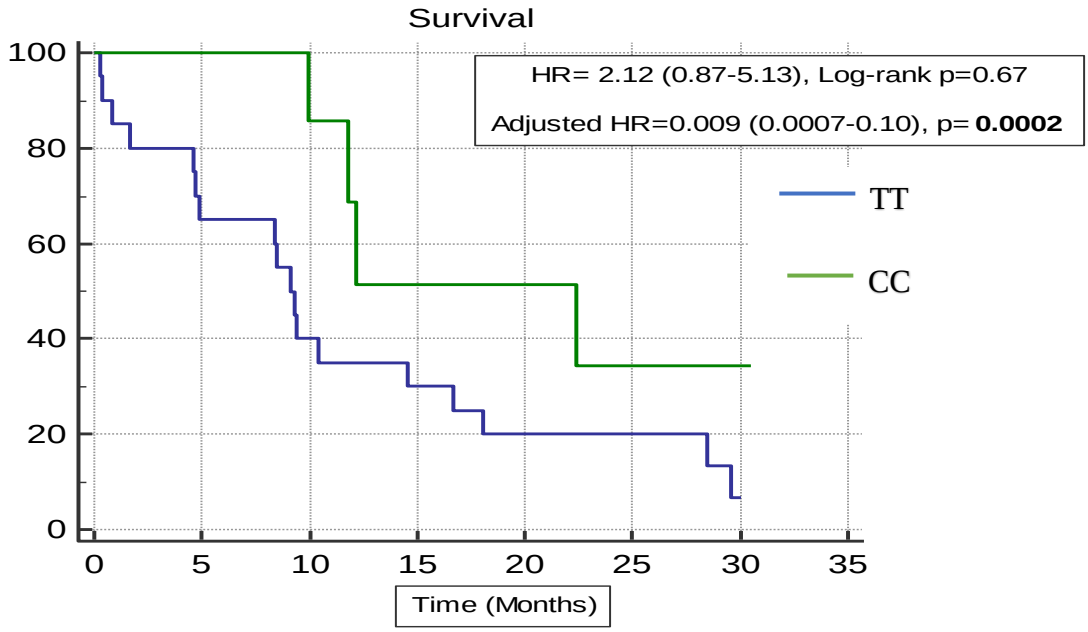


Figure5.15: Kaplan Meier curves illustrating the association between overall survival in lung cancer patients with mutant (*CC*) genotype for patients treated with cisplatin/carboplatin combined with docetaxel depicting hTERT polymorphism (Note: 0 represents wild-type genotype (*TT*), 1 represents homozygous mutant genotype (*CC*)

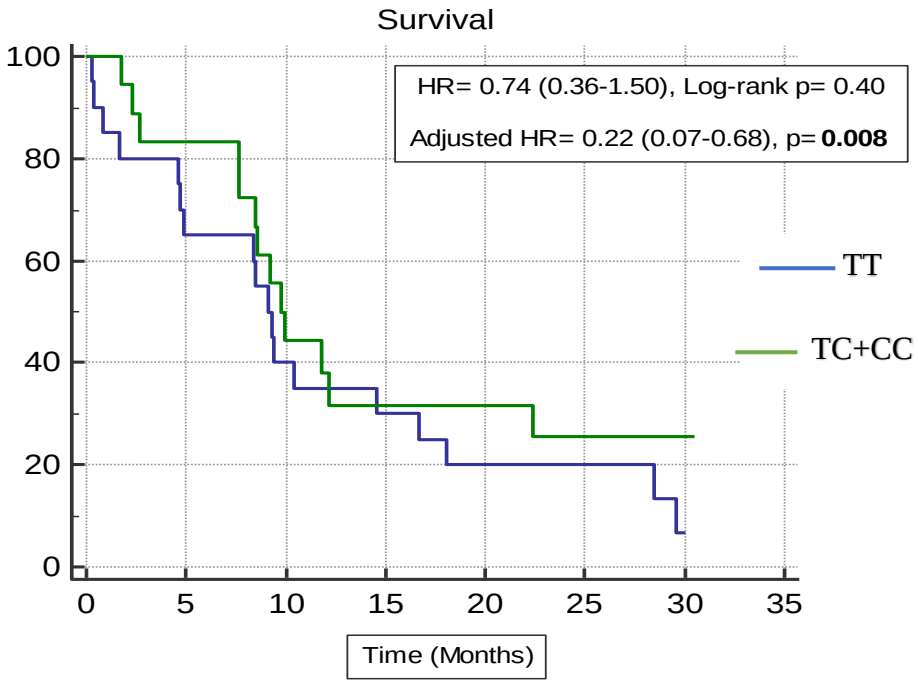


Figure 5.16:Kaplan Meier curve illustrating the association between overall survival in lung cancer patients with combined (*TC+CC*) genotype treated with cisplatin/carboplatin along with docetaxel (Note: 0 represents wild-type genotype (*TT*), 1 represents combined (*TC+CC*) genotype)

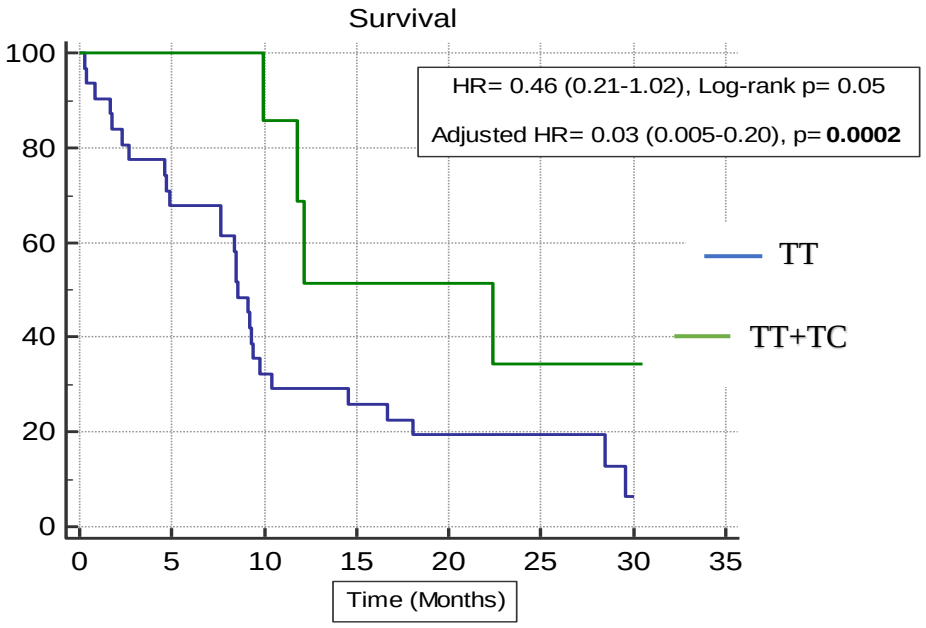


Figure 5.17: Kaplan Meier curve illustrating the association between overall survival in lung cancer patients with combined (*TT+TC*) genotype treated with cisplatin/carboplatin along with docetaxel (Note: 0 represents wild-type genotype (*TT*), 1 represents combined (*TT+TC*) genotype)

5.8.1.7 Association of hTERT (T>C) Polymorphism with Treatment Response to Chemotherapy and its Correlation with Objective Response

The patients were evaluated to respond to the treatment outcomes for the hTERT polymorphism. The patients were categorized as good or poor responders according to the RECIST criteria. Patients who achieved complete remission (CR) or partial remission (PR) were considered good responders. In contrast, those with stable disease (SD) and progressive disease (PD) were classified as poor responders, as tabulated in Table 5.13. The percentage of respondents who were sound (CR+PR) and poor (SD+PD) responders was almost similar. The adjusted odds ratio for the hTERT polymorphic site was determined using the wild genotype as the reference while controlling for confounding factors such as age, gender, smoking status, cancer stage, histology, KPS, ECOG score, and chemotherapeutic regimen. The objective response outcome data were available for a total of 194 patients. In the case of the mutant genotype (*CC*), the good responders had a higher frequency (6.34) representation than

inadequate responders (1.52) with (AOR=0.38, 95% C.I.=0.05-2.47, p=0.31) but lacked statistical significance. Similarly, in the dominant model, a combined genotype (*TC+CC*), the good responders (CR+PR) represent higher frequency (33.33) as compared to inadequate responders (SD+PD) (30.53), when the wild genotype (*TT*) was taken as a reference (AOR=0.82, 95% C.I.=0.41-1.62, p=0.57) but no significant difference was observed. According to logistic regression analysis (Table 5.13), the hTERT polymorphism showed no significant association towards lung cancer risk in subjects receiving platinum-based therapy.

Table 5.15. Association between genotypic distribution and clinic-pathological parameters based on response to chemotherapy

Response of chemotherapy				
Genotype **rs2735940 T/C**	**CR+PR** **63, N (%)**	**SD+PD** **131, N (%)**	**AOR[a]** **(95 % C.I.)**	**p[b]**
Codominant model				
TT	42 (66.66)	91 (69.46)	1.0 (Reference)	
TC	17 (26.98)	38 (29.00)	0.90 (0.44-1.84)	0.77
CC	4 (6.34)	2 (1.52)	0.38 (0.05-2.47)	0.31
Dominant model				
TT	42 (66.66)	91 (69.46)	1.0 (Reference)	
TC + CC	21 (33.33)	40 (30.53)	0.82 (0.41-1.62)	0.57
Recessive model				
TT + TC	59 (93.65)	129 (98.47)	1.0 (Reference)	
CC	4 (6.34)	2 (1.52)	0.36 (0.05-2.33)	0.28

a)-Adjusted Odds ratios, 95% confidence intervals, and corresponding p-values were calculated by logistic regression analysis after adjusting for age, gender, regimen, and smoking. (b)-Two-sided $\chi 2$ test for either genotype distribution or allelic frequencies between responders and non-responders. The number in bold indicates the significant values in the table.

5.8.1.8. Relationship Between the hTERT Polymorphic Variant (rs2735940) and Toxicity

The goal of this research was to investigate the occurrence of toxicity in lung cancer patients treated with platinum-based doublet chemotherapy combined with paclitaxel, pemetrexed, docetaxel, and irinotecan. To assess the risk of developing various toxicity grades following therapy in patients with each genotype, the odds ratio OR and 95% C.I. were calculated using the odd ratio test. Additionally, we employed the multiple regression model to calculate the adjusted odds ratio with the toxicity grades as the dependent variable. In this model, the covariates included were gender, age, performance status, and polymorphism, which were evaluated for possible interaction or confounding effects. We employed two distinct analytical methods to check the impact of hTERT polymorphism on different toxicity levels. In the initial assessment, we compared subjects with any toxicity grade (1-4) to those with no toxicity (grade 0), and in the second, we focused on comparing patients with intermediate/ severe toxicity (grade 3-4) to determine if there was a broader correlation between hTERT polymorphism and severe toxicities. Therefore, the patients were divided into two groups based on toxicity, and all patients were concurrently included in the analysis. Following each treatment cycle, we documented the chemotherapy-related side effects experienced by the patients.

5.8.1.9 Relationship between hTERT polymorphism and haematological toxicity

All the side effects associated with anti-cancer chemotherapy were recorded after each treatment cycle. Regarding haematological toxicity, we evaluated leukopenia, anaemia, thrombocytopenia, and absolute neutropenia. Upon assessing the effect of hTERT polymorphism in leukopenia toxicity, our results revealed that lung cancer patients harbouring heterozygous genotype (*TC*) with intermediate/severe toxicity (grade 2-4) when compared with low/no toxicity (grade 0-1) had a 12.56-fold increased risk of developing leukopenia when compared with patients carrying wild-type genotype (*TT*), (OR=12.56, 95% C.I.=1.44-109.33, p=**0.02**) and a significant association was observed (Table 5.18). Similarly, after adjusting with different covariates such as age, gender, chemotherapeutic regimen, and performance status,

14 times higher odds of developing leukopenia toxicity were observed in heterozygous genotype (*TC*) when compared with wild-type (*TT*) genotype, with the result being statistically significant (AOR=14.01, 95% C.I.=1.48-132.58, p=**0.02**). Furthermore, in the dominant model, lung cancer patients harbouring the combined genotype (*TC+CC*) revealed an 11.93-fold elevated risk of developing leukopenia toxicity when compared with wild-type genotype (*TT*) (OR=11.93, 95% C.I.=1.41-100.57, p=**0.02**). Even after adjusting with multiple covariates, the risk remained 9.22 times in the combined (*TC+CC*) genotype, and a significant association was observed (AOR=9.22, 95% C.I.=1.01-83.78, p=**0.04**). In the case of anaemia, lung cancer patients carrying heterozygous genotype (*TC*) with low/intermediate/severe toxicity (grade1-4) compared with no toxicity grade (grade 0) odd ratio observed was (OR=1.26, 95% C.I.=0.70-2.29, p=0.70) but no significant association was observed. Similarly, in the case of thrombocytopenia, no significant association was observed between hTERT polymorphism and the risk of developing thrombocytopenia toxicity.

Table 5.16. Haematological and nephrotoxicity with CTC grades in lung cancer patients

Toxicity	**Adverse events**	**Toxicity profiles**			
		Grade 0, n(%)	**Grade 0-1, n(%)**	**Grade 1-4, n(%)**	**Grade 2-4, n(%)**
Haematological toxicity	**Anaemia**	84	199	212	97
	Leukopenia	268	287	26	7
	Thrombocytopenia	254	287	34	1
	Neutropenia	248	258	16	6
Nephrotoxicity	**GFR**	223	126	53	160

In absolute neutrophil count grading (ANC), we evaluated the presence of low/intermediate/severe toxicity (grade 1-4) against no toxicity (grade 0), as depicted in Table

5.18. Lung cancer patients with heterozygous (*TC*) genotype showed a 3.23-fold increase in the odds of developing neutropenia (OR=3.23, 95% C.I.=1.12-9.31, p=**0.02**) when compared with the subjects carrying wild-type genotype (*TT*). After adjusting the multiple confounding factors such as age, gender, chemotherapeutic regimen, and performance status, 4.65 times elevated risk of developing absolute neutropenia was observed when compared with wild-type genotype (*TT*) (AOR=4.65, 95% C.I.=1.12-9.31, p=**0.02**). Furthermore, the combined genotype (*TC+CC*) in the dominant model for hTERT polymorphism also showed an increased risk of developing neutropenia in lung cancer patients when compared with the wild-type genotype (*TT*) (OR=2.80, 95% C.I.=1.00-7.79, p=**0.04**), also, after adjusting for multiple confounding factors like age, gender, chemotherapeutic regimen, and performance status, a 3.88-fold risk of developing absolute neutropenia toxicity was observed (AOR=3.88, 95% C.I.=1.01-14.85,

Table 5.17 a). Haematological Toxicity (grades 0 vs 1-4: grades 0-1 vs 2-4) associated with the hTERT polymorphism in multivariate analysis

Leukopenia						
Genotype rs2735940 T/C	**Grade of toxicity 0, n(%) N=268**	**Grade of toxicity 1-4, n(%) N=26**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	179 (66.79)	13 (50)	1.00 (Reference)		1.00 (Reference)	
TC	70 (26.11)	11 (42.30)	2.16 (0.92-5.05)	0.07	1.97 (0.77-4.99)	0.15
CC	19 (7.08)	2 (7.69)	1.44 (0.30-6.91)	0.64	0.74 (0.08-6.86)	0.79
Dominant model						
TT	179 (66.79)	13 (50)	1.00 (Reference)		1.00 (Reference)	
TC + CC	89 (33.20)	13 (50)	2.01 (0.89-4.51)	0.09	1.73 (0.70-4.28)	0.23
Recessive model						
TT + TC	249 (92.91)	24 (92.30)	1.00 (Reference)		1.00 (Reference)	
CC	19	2	1.09	0.90	0.58	0.62

	(7.08)	(7.69)	(0.23-4.97)		(0.07-4.90)	

p=**0.04**) as tabulated in Table 5.18 a.

Leukopenia						
Genotype rs2735940 T/C	**Grade of toxicity 0-1, n(%) N=287**	**Grade of toxicity 2-4, n(%) N=7**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	191 (66.55)	1 (14.28)	1.00 (Reference)		1.00 (Reference)	
TC	76 (26.48)	5 (71.42)	12.56 (1.44-109.33)	**0.02**	14.01 (1.48-132.58)	**0.02**
CC	20 (6.96)	1 (14.28)	9.55 (0.57-158.60)	0.11	-	-
Dominant model						
TT	191 (66.55)	1 (14.28)	1.00 (Reference)		1.00 (Reference)	
TC + CC	96 (33.44)	6 (85.71)	11.93 (1.41-100.57)	**0.02**	9.22 (1.01-83.78)	**0.04**
Recessive model						
TT + TC	267 (93.03)	6 (85.71)	1.00 (Reference)		1.00 (Reference)	
CC	20 (6.96)	1 (14.28)	2.22 (0.25-19.39)	0.46	-	0.99

ANAEMIA						
Genotype rs2735940 T/C	**Grade of toxicity 0-1, n(%) N=199**	**Grade of toxicity 2-4, n(%) N=97**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	134 (67.33)	60 (61.85)	1.00 (Reference)			
TC	51 (25.62)	30 (30.92)	1.31 (0.76-2.26)	0.32	1.28 (0.67-2.45)	0.44
CC	14 (7.03)	7 (7.21)	1.11 (0.42-2.90)	0.82	0.60 (0.17-2.10)	0.43
Dominant model						
TT	134 (67.33)	60 (61.85)	1.00 (Reference)		1.00 (Reference)	
TC + CC	65 (32.66)	37 (38.14)	1.27 (0.76-2.10)	0.35	1.13 (0.62-2.09)	0.67
Recessive model						
TT + TC	185 (92.96)	90 (92.78)	1.00 (Reference)		1.00 (Reference)	
CC	14 (7.03)	7 (7.21)	1.02 (0.40-2.63)	0.95	0.67 (0.21-2.18)	0.51

ANAEMIA						
Genotype rs2735940 T/C	**Grade of toxicity 0-1, n(%) N=199**	**Grade of toxicity 2-4, n(%) N=97**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	134 (67.33)	60 (61.85)	1.00 (Reference)			
TC	51 (25.62)	30 (30.92)	1.31 (0.76-2.26)	0.32	1.28 (0.67-2.45)	0.44
CC	14 (7.03)	7 (7.21)	1.11 (0.42-2.90)	0.82	0.60 (0.17-2.10)	0.43
Dominant model						
TT	134 (67.33)	60 (61.85)	1.00 (Reference)		1.00 (Reference)	
TC + CC	65 (32.66)	37 (38.14)	1.27 (0.76-2.10)	0.35	1.13 (0.62-2.09)	0.67
Recessive model						
TT + TC	185 (92.96)	90 (92.78)	1.00 (Reference)		1.00 (Reference)	
CC	14 (7.03)	7 (7.21)	1.02 (0.40-2.63)	0.95	0.67 (0.21-2.18)	0.51

Thrombocytopenia						
Genotype rs2735940 T/C	**Grade of toxicity 0, n(%) N=254**	**Grade of toxicity 1-4, n(%) N=34**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	169 (66.53)	20 (58.82)	1.00 (Reference)		1.00 (Reference)	
TC	68 (26.77)	12 (35.29)	1.49 (0.69-3.21)	0.30	1.10 (0.42-2.87)	0.83
CC	17 (6.69)	2 (5.88)	0.99 (0.21-4.62)	0.99	0.44 (0.04-4.18)	0.47
Dominant model						
TT	169 (66.53)	20 (58.82)	1.00 (Reference)		1.00 (Reference)	
TC + CC	85 (33.46)	14 (41.17)	1.39 (0.67-2.89)	0.37	0.99 (0.39-2.49)	0.99
Recessive model						
TT + TC	237 (93.30)	32 (94.11)	1.00 (Reference)		1.00 (Reference)	
CC	17 (6.69)	2 (5.88)	0.87 (0.19-3.94)	0.83	0.55 (0.06-4.78)	0.59

Thrombocytopenia						
Genotype rs2735940 T/C	**Grade of toxicity 0-1, n(%) N=287**	**Grade of toxicity 2-4, n(%) N=1**	**OR (95% C.I.)**	**P**	**AOR (95% C.I.)**	**P**
Codominant model						
TT	188 (65.50)	1 (100)	1.00 (Reference)		1.00 (Reference)	
TC	80 (27.87)	0	0.00	0.99	-	0.99
CC	19 (6.62)	0	0.00	0.99	-	0.99
Dominant model						
TT	188 (65.50)	1 (100)	1.00 (Reference)		1.00 (Reference)	
TC + CC	99 (34.49)	0	0.00	0.99	-	0.99
Recessive model						
TT + TC	268 (93.37)	1 (100)	1.00 (Reference)		1.00 (Reference)	
CC	19 (6.62)	0	0.00	0.99	-	0.99

ANC						
Genotype rs2735940 T/C	**Grade of toxicity 0, n(%) N=248**	**Grade of toxicity 1-4, n(%) N=16**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	170 (68.54)	7 (43.75)	1.00 (Reference)		1.00 (Reference)	
TC	60 (24.19)	8 (50)	3.23 (1.12-9.31)	**0.02**	4.65 (1.22-17.70)	**0.02**
CC	18 (7.25)	1 (6.25)	1.34 (0.15-11.59)	0.78	0.00	0.99
Dominant model						
TT	170 (68.54)	7 (43.75)	1.00 (Reference)		1.00 (Reference)	
TC + CC	78 (31.45)	9 (56.25)	2.80 (1.00-7.79)	**0.04**	3.88 (1.01-14.85)	**0.04**
Recessive model						
TT + TC	230 (92.74)	15 (93.75)	1.00 (Reference)		1.00 (Reference)	
CC	18 (7.25)	1 (6.25)	0.85 (0.10-6.82)	0.87	1.07 (0.12-8.95)	0.94

ANC						
Genotype rs2735940 T/C	**Grade of toxicity 0-1, n(%) N=258**	**Grade of toxicity 2-4, n(%) N=6**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	175 (67.82)	2 (33.33)	1.00 (Reference)			
TC	65 (25.19)	3 (50)	4.03 (0.65-24.71)	0.13	8.41 (0.84-84.18	0.06
CC	18 (6.97)	1 (16.66)	4.86 (0.41-56.27)	0.20	-	0.99
Dominant model						
TT	175 (67.82)	2 (33.33)	1.00 (Reference)		1.00 (Reference)	
TC + CC	83 (32.17)	4 (66.660	4.21 (0.75-23.48)	0.10	7.03 (0.69-71.11)	0.09
Recessive model						
TT + TC	240 (93.02)	5 (83.33)	1.00 (Reference)		1.00 (Reference)	
CC	18 (6.97)	1 (16.66)	2.66 (0.29-24.06)	0.38	-	0.99

OR[1] Crude odds ratio, 95% confidence interval, and AOR[2] Adjusted odds ratio data were calculated by unconditional logistic regression and adjusted for age, gender, chemotherapeutic regimen, and performance status. Ap-value < 0.05 was considered statistically significant.

5.8.10. Relationship between hTERT polymorphism and nephrological toxicity

Chemotherapeutic drugs, which are excreted through the kidneys, can cause nephrotoxicity; therefore, renal functions are closely monitored while the patients are receiving chemotherapy. We have evaluated chemotherapy-induced nephrotoxicity by comparing low/intermediate/severe toxicity (grades 2-5) vs. no grade (grades 1) toxicity. We compared intermediate/severe toxicity (grades 3-5) vs. no/low grade (grades 1-2) toxicity in chemotherapy-induced nephrotoxicity for hTERT polymorphism. As depicted in Table 5.18 b, lung cancer patients with mutant genotype (*CC*) showed reduced nephrological toxicity when compared with type wild-type genotype (*TT*), but lacks statistical significance (OR=0.62, 95%

C.I.=0.19-2.67, p=0.62). Thus, no significant association was observed between hTERT polymorphism and nephrotoxicity in lung cancer patients receiving chemotherapy.

Table 5.17.b) Nephrological Toxicity (grades one vs 2-5: grades 1-2 vs 3-4) associated with hTERT polymorphism in multivariate analysis

GFR						
Genotype rs2735940 T/C	**Grade of toxicity 1, n(%) N=223**	**Grade of toxicity 2-5, n(%) N=53**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	144 (51.12)	32 (60.37)	1.00 (Reference)			
TC	62 (27.80)	16 (30.18)	0.93 (0.40-2.15)	0.87	0.58 (0.17-1.94)	0.38
CC	17 (7.62)	5 (9.43)	0.72 (0.19-2.67)	0.62	0.36 (0.06-2.02)	0.24
Dominant model						
TT	144 (51.12)	32 (60.37)	1.00 (Reference)		1.00 (Reference)	
TC + CC	79 (35.42)	21 (39.62)	0.88 (0.41-1.89)	0.74	0.49 (0.16-1.47)	0.20
Recessive model						
TT + TC	206 (92.37)	48 (90.56)	1.00 (Reference)		1.00 (Reference)	
CC	17 (7.62)	5 (9.43)	0.73 (0.20-2.65)	0.64	0.47 (0.09-2.32)	0.35

GFR						
Genotype rs2735940 T/C	**Grade of toxicity 1-2, n(%) N=126**	**Grade of toxicity 3-4, n(%) N=160**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	76 (60.31)	110 (68.75)	1.00 (Reference)		1.00 (Reference)	

TC	40 (31.74)	39 (24.37)	0.67 (0.39-1.14)	0.14	0.61 (0.30-1.24)	0.17
CC	10 (7.93)	11 (6.87)	0.76 (0.30-1.87)	0.55	0.67 (0.19-2.35)	0.54
Dominant model						
TT	76 (60.31)	110 (68.75)	1.00 (Reference)		1.00 (Reference)	
TC + CC	50 (39.68)	50 (31.25)	0.69 (0.42-1.12)	0.13	0.32 (0.32-1.20)	0.16
Recessive model						
TT + TC	116 (92.06)	149 (93.12)	1.00 (Reference)		1.00 (Reference)	
CC	10 (7.93)	11 (6.87)	0.85 (0.35-2.08)	0.73	0.82 (0.25-2.65)	0.75

OR[1] Crude odds ratio, 95% confidence interval, and AOR[2] Adjusted odds ratio data were calculated by unconditional logistic regression and adjusted for age, gender, chemotherapeutic regimen, and performance status. Ap-value < 0.05 was considered statistically significant.

5.8.11. Relationship between hTERT polymorphism and hepatotoxicity

An unforeseen reaction usually causes chemotherapy-induced hepatotoxicity. The liver function test (LFT) measures the enzyme levels of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALP). Upon evaluating patients for hTERT polymorphism based on SGOT levels, 223 lung cancer patients were classified in (grade 1), 274 patients in (grades 1-2) toxicity, 53 patients in (grades 2-4), while only two patients in grade (3-4). However, the analysis of SGOT for hTERT polymorphism showed no significant statistical association.

Table 5.18. Hepatotoxicity with CTC grades in lung cancer patients					
Toxicity	**Adverse events**	**Toxicity profiles**			
		Grade 1-1,n(%)	**Grade 1-2,n(%)**	**Grade 2-4,n(%)**	**Grade 3-4,n(%)**
Hepatotoxicity	**SGOT**	223	274	53	2
	SGPT	231	264	45	12
	ALP	159	213	108	54

Upon assessing the patients with SGOT levels, our study did not find any significant correlation between liver injury (SGOT) adverse events in lung cancer patients and hTERT polymorphism. On categorizing lung cancer patients based on ALP levels, our study demonstrated a decreased risk of liver dysfunction in patients harbouring a combined genotype of (*TC+CC*) in the dominant model when compared with patients carrying wild-type genotype (*TT*) (OR=0.61, 95% C.I.=0.31-1.18, p=0.14). After adjusting with multiple factors like age, gender, chemotherapeutic regimen, and performance status, the combined genotype (*TC+CC*) showed a lower incidence of liver toxicity when compared with wild-type genotype (*TT*) (AOR=0.39, 95% C.I.=0.17-0.96, p=**0.02**) as shown in Table 5.20.

Table 5.19. Hepatotoxicity (grades one vs 2-4: grades 1-2 vs 3-4) associated with hTERT polymorphism in multivariate analysis

SGOT						
Genotype rs2735940 T/C	**Grade of toxicity 1, n(%) N=223**	**Grade of toxicity 2-4, n(%) N=53**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	144 (64.57)	32 (60.37)	1.00 (Reference)		1.00 (Reference)	
TC	62 (27.80)	16 (30.18)	1.16 (0.59-2.26)	0.66	1.11 (0.51-2.38)	0.78
CC	17 (7.62)	5 (9.43)	1.32 (0.45-3.85)	0.60	0.94 (0.25-3.56)	0.93
Dominant model						
TT	144 (64.57)	32 (60.37)	1.00 (Reference)		1.00 (Reference)	
TC + CC	79 (35.42)	21 (39.62)	1.19 (0.64-2.21)	0.56	1.06 (0.52-2.16)	0.83
Recessive model						
TT + TC	206 (92.37)	48 (90.56)	1.00 (Reference)		1.00 (Reference)	
CC	17 (7.62)	5 (9.43)	1.26 (0.44-3.59)	0.66	0.90 (0.25-3.24)	0.87

SGOT						
Genotype rs2735940 T/C	**Grade of toxicity 1-2, n(%) N=274**	**Grade of toxicity 3-4, n(%) N=2**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	176 (64.23)	0	1.00 (Reference)		1.00 (Reference)	
TC	78 (28.46)	0	-	-	-	-
CC	20 (7.29)	2 (100)	0	0.99	0	0.99
Dominant model						
TT	176 (64.23)	0	1.00 (Reference)		1.00 (Reference)	
TC + CC	98 (35.76)	2 (100)	0	0.99	0	0.99
Recessive model						
TT + TC	254 (92.70)	0	1.00 (Reference)		1.00 (Reference)	
CC	20 (7.29)	2 (100)	0	0.99	0	0.99

SGPT						
Genotype rs2735940 T/C	**Grade of toxicity 1, n(%) N=231**	**Grade of toxicity 2-4, n(%) N=45**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	147 (63.63)	29 (64.44)	1.00 (Reference)		1.00 (Reference)	
TC	65 (28.13)	13 (28.88)	1.01 (0.49-2.07)	0.97	1.09 (0.49-2.39)	0.82
CC	19 (8.22)	3 (6.66)	0.80 (0.22-2.88)	0.73	0.59 (0.11-3.01)	0.82
Dominant model						
TT	147 (63.63)	29 (64.44)	1.00 (Reference)		1.00 (Reference)	
TC + CC	84 (36.36)	16 (35.55)	0.96 (0.49-1.88)	0.91	0.95 (0.44-2.02)	0.90
Recessive model						
TT + TC	212 (91.77)	42 (93.33)	1.00 (Reference)		1.00 (Reference)	
CC	19 (8.22)	3 (6.66)	0.79 (0.22-2.81)	0.72	0.51 (0.10-2.51)	0.41

SGPT						
Genotype rs2735940 T/C	**Grade of toxicity 1-2, n(%) N=264**	**Grade of toxicity 3-4, n(%) N=12**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	170 (64.39)	6 (50)	1.00 (Reference)		1.00 (Reference)	
TC	74 (28.03)	4 (33.33)	1.53 (0.41-5.58)	0.51	1.45 (0.29-7.12)	0.64
CC	20 (7.57)	2 (16.66)	2.83 (0.53-14.99)	0.22	2.07 (0.24-17.42)	0.50
Dominant model						
TT	170 (64.39)	6 (50)	1.00 (Reference)		1.00 (Reference)	
TC + CC	94 (35.60)	6 (50)	1.80 (0.56-5.76)	0.31	1.74 (0.42-7.17)	0.43
Recessive model						
TT + TC	244 (92.42)	10 (83.33)	1.00 (Reference)		1.00 (Reference)	
CC	20 (7.57)	2 (16.66)	2.44 (0.50-11.90)	0.27	2.03 (0.31-13.26)	0.45

ALP						
Genotype rs2735940 T/C	**Grade of toxicity 1, n(%) N=159**	**Grade of toxicity 2-4, n(%) N=108**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	97 (61.00)	73 (67.59)	1.00 (Reference)			
TC	49 (30.81)	28 (25.92)	0.75 (0.43-1.32)	0.33	0.88 (0.46-1.68)	0.71
CC	13 (8.17)	7 (6.48)	0.71 (0.27-1.88)	0.49	0.66 (0.19-2.23)	0.50
Dominant model						
TT	97 (61.00)	73 (67.59)	1.00 (Reference)		1.00 (Reference)	
TC + CC	62 (38.99)	35 (32.40)	0.75 (0.44-1.25)	0.27	0.84 (0.46-1.55)	0.59
Recessive model						
TT + TC	146 (91.82)	101 (93.51)	1.00 (Reference)		1.00 (Reference)	
CC	13 (8.17)	7 (6.48)	0.77 (0.30-2.01)	0.60	0.71 (0.22-2.29)	0.56

ALP						
Genotype rs2735940 T/C	**Grade of toxicity 1-2, n(%) N=213**	**Grade of toxicity 3-4, n(%) N=54**	**OR[1] (95% C.I.)**	**P**	**AOR[2] (95% C.I.)**	**P**
Codominant model						
TT	131 (61.50)	39 (72.22)	1.00 (Reference)		1.00 (Reference)	
TC	65 (30.51)	12 (22.22)	0.62 (0.30-1.26)	0.18	0.42 (0.17-1.01)	0.05
CC	17 (7.98)	3 (5.55)	0.59 (0.16-2.12)	0.42	0.33 (0.06-1.78)	0.26
Dominant model						
TT	131	39	1.00		1.00	

	(61.50)	(72.22)	(Reference)		(Reference)	
TC + CC	82 (38.49)	15 (27.77)	0.61 (0.31-1.18)	0.14	0.39 (0.17-0.96)	**0.02**
Recessive model						
TT + TC	196 (92.01)	51 (94.44)	1.00 (Reference)		1.00 (Reference)	
CC	17 (7.98)	3 (5.55)	0.67 (0.19-2.40)	0.54	0.45 (0.08-2.31	0.34

OR[1] Crude odds ratio, 95% confidence interval, and AOR[2] Adjusted odds ratio data were calculated by unconditional logistic regression and adjusted for age, gender, chemotherapeutic regimen, and performance status. Ap-value < 0.05 was considered statistically significant.

CHAPTER 6

DISCUSSION

6.1. Genetic Variation of Telomerase (hTERT) promoter

This dissertation aimed to evaluate the role of genetic variants in the promoter region of the hTERT gene and its association with lung cancer pathogenesis. To maintain genome stability and integrity in mammals, faithful replication of genetic material and defence against indications of DNA damage is necessary for maintaining the ends of the linear chromosomes, or telomeres. Telomerase holoenzyme is responsible for performing these functions. Primary cells can potentially divide up to a limited amount of time. This limit is known as the Hayflick limit or the End Replication problem. The end replication problem results from improper telomere maintenance, which causes telomere length to shorten with each cycle of DNA replication (WeisiLu *et al*., 2013). The catalytic subunit of the reverse transcriptase enzyme (hTERT) and a lengthy non-coding RNA that carries the template sequence for telomere synthesis (TERC) make up the ribonucleoprotein complex known as telomerase (Armstrong *et al*., 2017). The polymorphic variant of the hTERT gene under study is TERT (rs2735940). The T → C transition located 1327 base pairs upstream of the transcription start site, hTERT rs2735940 (T > C) polymorphism, affects the transcriptional efficiency of hTERT (Bayram *et al*., 2016).

6.1.1. Polymorphism in human *telomerase reverse transcriptase* (h*TERT*) gene and susceptibility towards lung cancer in a North Indian population

This study assessed the association between hTERT polymorphism (rs2735940) and risk towards lung cancer pathogenesis. When the odds ratio was calculated using the homozygous *TT* (wild) genotype as a reference, the association analysis revealed that subjects with mutant genotype (*CC*) demonstrated a 2.40-fold higher risk for lung cancer development (p=0.031). Our data support the findings of Yang *et al*. (2015), who found a significant association between cancer risk and TERT rs2735940 polymorphism. Our study also revealed that, in the dominant model, the combined genotype (*TC+CC*) also exhibited an increased risk towards susceptibility with an adjusted odd ratio (p=0.0016). Further, the analysis used the recessive model wherein heterozygous genotype was used as a reference. It was observed that subjects carrying the two copies of the mutant allele (*CC*) exhibited a significant association toward risk for lung cancer (p=0.039), similar to the results revealed by Eskandari and Hashemi *et al*. (2018). A study by Yang et al. (2015) found a marginal association among Caucasians in the subgroup analysis by ethnicity. Also, in the subgroup analysis, rs2735940 was significantly associated with lung cancer risk. Our data here also supports Sheng et al.'s(2013) case-control study, where SNP in the *TERT* gene was investigated concerning the association with risk of childhood ALL in a Chinese population. They found that the *TERT* promoter region SNP (rs2735940) was associated with the risk of childhood ALL in the Chinese population.

Our study also looked at the role of the TERT rs2735940 variant in different histological subtypes of lung cancer. Our study revealed that lung cancer patients carrying the mutant genotype (*CC*) were significantly associated with an increased lung cancer risk in SCLC subjects (p=0.0004). The dominant and recessive models also exhibited a significant association between rs2735940 and the risk of developing lung cancer. The findings of our study are consistent with earlier studies of (Lawi *et al*. (2023). Their study rs2735940 showed significant associations with the development of NSCLC in the investigated Iraqi subjects.

Furthermore, our results revealed that the SQCC patients carrying heterozygous genotype (*TC*) exhibited an increased risk of acquiring squamous cell carcinoma (p=0.023). Furthermore, the combined genotype (*TC+CC*) had a trend, exhibiting a strong association with an increased risk of developing squamous cell carcinoma in the case of the dominant model (p=0.02). After applying statistics, subjects diagnosed with ADCC did not show any significant association with lung cancer susceptibility in our study. On the contrary, the studies of Choi *et al*. (2009) state that there was no statistically significant difference in the impact of the combined genotypes of TERT rs2735940 on the risk of lung cancer across the three main histologic categories ADCC, SQCC, and SCLC.

Since tobacco smoking is another well-known risk factor for lung cancer, we stratified our data based on smoking status to investigate the potential synergistic effect of smoking and hTERT polymorphism toward lung cancer susceptibility. The subjects were classified based on their smoking status into smokers and non-smokers. Our data indicate that the hTERT rs2735940 polymorphic variant was found to be the risk factor specifically for the patients who were smokers and carrying the combined (*TC+CC*) genotype in the dominant model (p=0.03). The previous studies of Choi et al. (2009) exhibited a significant interaction of the hTERT rs2735940 polymorphic variant with tobacco smoking towards lung cancer pathogenesis. However, in the study conducted by Zhang *et al*. (2017), the authors did not find any significant association between the hTERT rs2735940 polymorphic variant and smoking with susceptibility to gastric cancer in the Chinese Han population. Furthermore, the study by Kalpouzos et al. (2016), which investigated the relationship between TERT rs2735940 and two Swedish cohorts of older adults, also found no significant association.

In the case of non-smokers, our results revealed that subjects harbouring heterozygous (*TC*) genotype depicted a 2.20-fold elevated risk of acquiring lung cancer (p=0.04). In the dominant model, non-smoker subjects carrying combined (*TC+CC*) genotypes indicate a very high association, suggesting a higher risk of developing lung cancer (p=0.007). However, in the previous studies of (Zhang *et al*. (2017) and Kalpouzos*et al*. (2016), the authors did not find any significant association between hTERT polymorphic variant rs2735940 and non-smoker subjects.

Another factor under consideration was gender and its role in modulating the risk for lung cancer when stratified based on hTERT polymorphism. In our study, male subjects harbouring all the genotypes exhibited a significant association between the hTERT rs2735940 polymorphic variant and showed a significantly increased genetic predisposition towards lung cancer ($p<0.05$). On the other hand, females did not show any significant effect on lung cancer susceptibility. However, no significant association was observed in the previous studies by Zhang *et al*. (2017) between gender and hTERT rs2735940 polymorphic variant in the Chinese Hand population towards gastric cancer. Similarly, the study conducted by Choi et al. (2009) reported no significant association between gender and the rs2735940 polymorphic variant.

Furthermore, we investigated the association between average age and hTERT polymorphic variant rs2735940 for both cases and controls. The mean age diagnosed was 63.5. Our data revealed that the lung cancer subjects carrying the heterozygous (*TC*) genotype with age younger than (63.5) exhibited an increased risk of acquiring lung cancer. The analysis showed an adjusted odds ratio of 1.62 with (AOR=1.62, 95% C.I.=1.04-2.53, p=0.03). Similarly, in the dominant model, lung cancer subjects carrying the combined genotype (*TC+CC*) exhibited a 1.72-fold higher risk of lung cancer development with (AOR=1.72, 95% C.I.=1.12-2.64, p=0.01). However, all three models observed no significant association between lung cancer subjects older than (63.5) and the hTERT polymorphic variant. This suggests that genetic variant hTERT rs2735940 studies may not significantly affect lung cancer risk for individuals older than 63.5 years.

On the contrary, other studies by Catarino et al. (2010) have reported no significant association between average age (64 years) and the TERT polymorphic variant with the risk of developing lung cancer. While it is challenging to account for the discrepancies, they may be partially attributed to the varying ages of the subjects. In previous studies done by Lizuka*et al*. (2013) on the hTERT rs2735940 polymorphic variant with the risk of epithelial cancer, the mean age of subjects was 80.3 years, while it was 61.3 years in Choi *et al*.(2009)study.

6.1.2.Relationship between hTERT rs2735940 polymorphic Variant and Clinicopathological Characteristics and prognosis

We also looked into the significance of hTERT rs2735940 polymorphism in clinical-pathological features such as stage, tumour size, invasion by lymph nodes, and metastasis. Based on the tumour stage, lung cancer patients were divided into two subgroups: stage III and stage IV patients. However, no significant association was found with lung cancer susceptibility among the two subgroups. The findings of our study are consistent with earlier studies by Catarino et al. (2010), where they did not find any significant association between TERT rs2735940 and clinical stages. The following parameter, which was evaluated for the hTERT polymorphism, was the primary tumour extension. The patients were stratified into two groups based on tumour size, namely T3 and T4. However, our data showed no correlation with tumour extension. None of the genotypes of hTERT polymorphism correlated with the risk of lung cancer and lacked statistical significance. Our data supports the findings by Zhang et al. (2017), who found no significant association between the hTERT rs2735940 polymorphic variant and tumour size.

Further, we evaluated the relationship of different genotypes with the metastatic status of lung cancer patients. Patients showing positive distant metastasis (M1) were compared with those lacking metastasis (M0), also regarded as a reference group. The patients with the mutant genotype for hTERT polymorphism (CC) exhibited a 4.02-fold risk of metastasis towards lung cancer (AOR=4.02; 95% C.I.=1.06-15.1; **p=0.03).**

Similarly, the recessive model subjects showed a substantially increased effect of lung cancer metastasis to other organs when patients who did not show metastasis were considered reference (recessive model: AOR=3.88; 95% C.I.=1.03-14.50; **p=0.04**). On the other hand, Lizuka et al. (2013), studies reported that the mutant genotype exhibited a lower risk of lung cancer malignancy.

Similarly, in the recessive model, subjects showed a lower risk of lung cancer. After stratifying patients based on lymph node invasion, we also investigated the relationship between these different genotypes. These were classified into Nx+N0+N1 and N2+N3+N4 groups. However, no significant association between hTERT polymorphism and lymph node invasion was observed in all three models. Surprisingly, few studies have described the level of hTERT subcellular intensity in tumour tissues and how it relates to clinicopathological features.

This study also evaluated the prognostic effect of the functional polymorphism in the hTERT gene in lung cancer patients treated with platinum-based doublet chemotherapy. Our results revealed that the lung cancer patients who were administered cisplatin/carboplatin along with docetaxel and carrying the hTERT mutant type alleles (*CC*) showed better survival when compared to patients harbouring wild-type genotype (*TT*) (22.40 vs. 9.13 months, Log-rank p=0.09). After applying the univariate and multivariate Cox regression analysis, adjusting for various covariates, a significant but lower hazard ratio was obtained (HR=2.12, 95% C.I.=0.87-5.13, Log-rank **p=0.09**) and (HR'=0.0009, 95% C.I.=0.0007-0.10, Log-rank **p=0.0002**). Similarly, the lung cancer subjects carrying the combined genotype (*TC+CC*) showed a significant association with a lower hazard ratio (p=0.008). Likewise, after applying multivariate Cox analysis, lung cancer patients carrying the combined genotype (*TT+TC*) showed a significant association and lower hazard ratio (HR'=0.03, 95% C.I.=0.005-0.20, Log-rank **p=0.0002**). Thus, lung cancer patients treated with cisplatin/carboplatin combined with docetaxel, who also had the hTERT polymorphism, exhibited a protective effect and improved survival rates. On the other hand, patients who received alternative chemotherapeutic regimens did not demonstrate any association between overall survival and the hTERT polymorphic variant. Matsubara et al. (2006) and Nordfjall et al. (2007) reported the role of 1327 T/C hTERT genetic variations in cancer prognosis and clinical outcomes of NSCLC patients for the first time in their study. Thus far, this genetic variation has been linked to the development of coronary artery disease, telomerase activity, and telomere length. In the previous studies of Catarino *et al*. (2010), the patients also received the first-line chemotherapeutic protocol consisting of platin-based doublet chemotherapy in combination with a third-generation cytotoxic compound such as paclitaxel, gemcitabine, or docetaxel.

6.1.3. Elucidation of Polymorphic hTERT genetic variant rs2735940 with overall survival of lung cancer patients

Our study studied survival analysis for 387 lung cancer subjects and its association with the hTERT polymorphism. Our data revealed that a higher hazard rate was observed for subjects carrying the heterozygous alleles both in univariate (HR=1.14, 95% C.I.=0.88-1.46, Log-rank p=0.29) and multivariate (HR=1.15, 95% C.I.=0.83-1.57, Log-rank p=0.38) analysis but lacks statistical significance. In the previous research done by Tee et al., the authors found that the

OS rate in early cervical cancer was not observed to be significantly predicted by hTERT activation (HR = 0.28, 95% CI: 0.02–3.32). However, the study of Wang et al. revealed that a thorough and systematic analysis determined that low survival rates in human solid tumours were linked to the overexpression of hTERT. For prognosis, hTERT might be a useful predictive biomarker.

6.1.4. Impact of hTERT Polymorphism on Overall Survival by Histological Subtype in Lung Cancer Patients

We have also evaluated the role of hTERT polymorphism in predicting lung cancer patient's overall survival based on histological subtypes. Our data revealed no significant association between the hTERT rs2735940 polymorphic variant and overall survival. We observed a trend of poor survival in ADCC patients with hTERT polymorphism harbouring mutant genotype (*CC*). However, patients having SQCC and hTERT polymorphism and carrying the mutant genotype (*CC*) showed a better OS (overall survival) than the wild genotype (*TT*). Moreover, an analysis trend of SCLC patients with hTERT polymorphism showed a better survival but lacked significant association. In the previous study by Catarino et al. (2010), authors found better survival in NSCLC patients, and the difference reached the threshold significance. According to Lawi *et al*. (2023), TERT-rs2735940 exhibited a significant association with an increased risk of NSCLC in lung cancer patients. Therefore, as demonstrated, these results indicate that none of the histological parameters, such as ADCC, SQCC, and SCLC, were associated with predicting any prognostic significance for the hTERT polymorphism.

6.1.5 hTERT polymorphism and its relationship with overall survival of lung cancer patients and toxicity

Among oncologic patients in Western countries, lung cancer is the primary cause of mortality due to its meagre survival rate. The two primary telomerase components are the RNA template (hTR) and the catalytic subunit (hTERT). A functional polymorphism in the hTERT gene was found in the promoter region (−1327T/C) rs2735940 (Catarino et al., (2010). Treatment with platinum-based chemotherapy for non-small cell lung cancer (NSCLC) improves the absolute cure rates for stage II/IIIA patients and the survival of patients in stage IIIB/IV NSCLC by minor amounts Kachuri*et al*., (2016). All the side effects associated with anti-cancer chemotherapy were recorded after each treatment cycle. Regarding haematological toxicity,

we evaluated leukopenia, anaemia, thrombocytopenia, and absolute neutropenia. Upon assessing the effect of hTERT polymorphism in leukopenia toxicity, our results revealed that lung cancer patients harbouring heterozygous genotype (*TC*) with intermediate/severe toxicity (grade 2-4) when compared with low/no toxicity (grade 0-1) had a 12.56-fold increased risk of developing leukopenia (OR=12.56, 95% C.I.=1.44-109.33, **p=0.02**). Similarly, after adjusting with different covariates such as age, gender, chemotherapeutic regimen, and performance status, 14 times higher odds of developing leukopenia toxicity were observed in heterozygous genotype (*TC*) when compared with wild-type (*TT*) genotype, with the result being statistically significant (AOR=14.01, 95% C.I.=1.48-132.58, p=**0.02**). Even after adjusting with multiple covariates, the risk remained 9.22 times in the combined (*TC+CC*) genotype, and a significant association was observed (AOR=9.22, 95% C.I.=1.01-83.78, p=**0.04**). The study done by Morais et al. (2020) stated that the single nucleotide polymorphism hTERT-1327 rs2735940 was linked to shorter leukocyte telomere length (LTL) in healthy persons and was also associated with the time to disease development, making it a potentially useful prognostic biomarker in the future. However, no significant association was observed between the genotypes and renal cell carcinoma. In the case of anaemia and thrombocytopenia toxicity, no significant association was observed between hTERT polymorphism and the risk towards lung cancer susceptibility.

In absolute neutrophil count grading (ANC), we evaluated the presence of low/intermediate/severe toxicity (grade 1-4) against no toxicity (grade 0). Lung cancer patients with heterozygous (*TC*) genotype showed a 4.65 times elevated risk of developing absolute neutropenia was observed when compared with wild-type genotype (*TT*) (AOR=4.65, 95% C.I.=1.12-9.31, p=**0.02**). Furthermore, in the combined genotype (*TC+CC*) in the dominant model for hTERT polymorphism, a 3.88-fold risk of developing absolute neutropenia toxicity was observed (AOR=3.88, 95% C.I.=1.01-14.85, p=**0.04**).

This study also assessed the relationship between the hTERT polymorphic variant rs2735940 and nephrological toxicity. Chemotherapeutic drugs, which are excreted through the kidneys, can cause nephrotoxicity; therefore, renal functions are closely monitored while the patients are receiving chemotherapy. Our data revealed that lung cancer patients with mutant genotype (*CC*) showed reduced nephrological toxicity when compared with type wild-type genotype (*TT*) (OR=0.62, 95% C.I.=0.19-2.67, p=0.62). However, no significant association was

observed between hTERT polymorphism and nephrotoxicity in lung cancer patients receiving chemotherapy.

Our study also looked at the relevance of hTERT polymorphism in chemotherapy-induced hepatotoxicity. The liver function test (LFT) measures the enzyme levels of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), and alkaline phosphatase (ALP). Upon assessing the patients with SGOT levels, our study did not find any significant correlation between liver injury (SGOT) adverse events in lung cancer patients and hTERT polymorphism. On categorizing lung cancer patients based on ALP levels, our study demonstrated a decreased risk of liver dysfunction in patients harbouring a combined genotype of (*TC+CC*) in the dominant model (**p=0.02**). To our best knowledge, toxicities due to anticancer chemotherapy in lung cancer patients to this SNP rs2735940 have not been reported in earlier studies.

6.2 Strengths of the study

- The current research has been investigated at a large sample size compared to the previous studies done in the North Indian population, which could potentially enhance the reliability of the results.
- Three outcomes (OS, chemotherapeutic response, and clinic-pathological characteristics related to the hTERT rs2735940 polymorphic variant) were analysed in this study.
- All the patients receiving platinum-based chemotherapy were enrolled, and all the patients were treated in the PGIMER hospital.
- The clinical parameter data collection and patient enrolment were done independently, without the knowledge of the polymorphism under investigation.
- The stratified analysis was also performed for smoking and histology to identify high-risk-posing subgroups.
- Our study is the first attempt to evaluate the association of hTERT rs2735940 SNP with chemotherapeutic-related toxicities.

6.3 Limitations of the study

- The limitations of this study include the fact that more population-based studies in different populations of India need to be carried out to minimise the extent of selection bias. Our research has so far been confined to the North Indian population.

- Various ethnic populations should be included, as genetic polymorphism often varies throughout ethnic groups.

- Since cancer is frequently discovered at a late stage, our study only includes advanced stage III and IV lung cancer patients.This might result from public awareness campaigns or the disease's resemblance to other illnesses like tuberculosis.

- The scope of study on chemotherapy response is weakened by the fact that many patients receiving chemotherapy at advanced stages III and IV of cancer are unable to meet the treatment's endpoint.

- Most patients receiving treatment at the PGIMER clinic come from rural parts of northern India. Many patients in remote places did not routinely attend the hospital since it was difficult for them to travel from their area.

- Progression-free survival (PFS) analysis on the current trial group was impossible due to a lack of clinical data.

- The majority of individuals in our study exhibited intermediate or low-grade toxicity (grade 1-2), with only a small number having grade 3–4 toxicity. Significant side effects that some research participants reported led to treatment delays or discontinuations. Therefore, it is essential to conduct multi-centre research, including numerous instances.

CHAPTER 7

CONCLUSION

Salient findings from the present study

- The study was performed on 387 cases and 384 controls, including 312 (80.62%) males in cases and 338 (88.02%) males in controls, whereas 75 (19.37%) and 46 (11.19%) were females both in cases and control groups, respectively.

- The subjects carrying the mutant genotype (*CC*) for hTERT polymorphism demonstrated a 2.40-fold higher risk for lung cancer development (AOR=2.40; 95% C.I.= 1.08-5.34, p=**0.031**) and were statistically significant.

- The subjects carrying the combined genotype (*TC+CC*) in the dominant model also exhibited an increased risk towards susceptibility with an adjusted odd ratio of (AOR=1.67; 95% C.I.= 1.21-2.31, p=**0.0016**). It was also observed that subjects carrying the two copies of the mutant allele (*CC*) exhibited a significant association toward risk for lung cancer (AOR=2.17; 95% C.I.=1.03-4.5, p=**0.039**).

- Subjects diagnosed with ADCC did not show any significant association with lung cancer susceptibility. In the co-dominant model, 6.8 times higher odds of developing SCLC were observed for lung cancer patients carrying mutant genotype (AOR=6.80; 95% C.I.= 2.33-19.78**; p=0.0004**), and also a highly significant association between the genetic variant and SCLC was observed. Also, a significant association and a risk of developing SCLC was observed in the dominant and recessive models.

- The subjects diagnosed with SQCC and carrying heterozygous genotype (*TC*) exhibited an increased risk of acquiring squamous cell carcinoma, with an adjusted odd ratio of (AOR=1.62, 95% C.I.=1.06-2.48; **p=0.023**). Furthermore, the combined genotype (*TC+CC*) had a trend, exhibiting a strong association with an increased risk of

developing squamous cell carcinoma, in case the dominant model (AOR=1.57 95%C.I.=1.05-2.35**; p=0.02**)

- Lung cancer subjects who were smokers and carrying the combined (TC+CC) genotype in the dominant model for the hTERT gene exhibited a 1.47-fold increased risk of developing lung cancer **p=0.03.**

- In the case of non-smokers, subjects harbouring heterozygous (*TC*) genotype depicted a 2.20-fold elevated risk of acquiring lung cancer (AOR=2.20; 95% C.I.=1.03-4.72; **p=0.04**) in the co-dominant model. In the dominant model, non-smoker subjects carrying combined (*TC+CC*) genotypes indicate a very high association, suggesting a higher risk of developing lung cancer.

- Further, male lung cancer patients bearing the heterozygous (*TC*) genotype or mutant (*CC*) genotype had a higher risk of lung cancer when compared to the reference (*TT*) genotype ,**p=0.013**and **p=0.038,**respectively. In addition, it was pronounced that male lung cancer subjects carrying the variant allele showed a significantly increased genetic predisposition towards lung cancer; **p=0.0026**. Similarly, a significant association was observed in the recessive model towards lung cancer risk **p=0.064**.

- Based on clinicopathological characteristics, the patients with the mutant genotype for hTERT polymorphism (*CC*) exhibited a 4.02-fold risk of metastasis towards lung cancer p=**0.03.** Similarly, the recessive model subjects showed a substantially increased effect of lung cancer metastasis to other organs when patients who did not show metastasis were considered reference p=**0.04.**

- The lung cancer subjects carrying heterozygous (*TC*) genotypes with an age younger than (63.5) exhibited an increased risk of acquiring lung cancer. The analysis showed an adjusted odds ratio of 1.62 with **p=0.03.** Similarly, in the dominant model, lung cancer subjects carrying the combined genotype (*TC+CC*) exhibited a 1.72-fold higher risk of lung cancer development with p=**0.01**. No significant association between lung cancer subjects older than (63.5) and the hTERT polymorphic variant rs2735940 was observed.

- Based on overall survival relative to performance status, patients with ECOG-PS 3,4 having heterozygous genotype (*TC*) and hTERT polymorphism showed a better survival rate.). After adjusting with multiple covariates, the hazard ratio decreased to 0.79 times in the heterozygous genotype, and a significant association was observed. On the other hand, patients carrying the combined genotype (*TT+TC*) showed reduced survival. After applying multivariate analysis, the death ratio increased to 18.85 times in the combined genotype, and a significant association was observed p=**0.0015.**

- Based on the chemotherapeutic regimen, lung cancer patients treated with cisplatin/carboplatin combined with docetaxel, who also had the hTERT polymorphism, exhibited a protective effect and improved survival rates.

- Based on toxicity profiling, we observed that lung cancer patients harbouring heterozygous genotype (*TC*) with intermediate/severe toxicity (grade 2-4) had 14 times higher odds of developing leukopenia toxicity **p=0.02.** Furthermore, in the dominant model, lung cancer patients harbouring the combined genotype (*TC+CC*), the 9.22 times risk of developing leukopenia toxicity was observed **p=0.04**.

- In absolute neutrophil count grading (ANC), we observed a 4.65 times elevated risk of developing absolute neutropenia **p=0.04.** Furthermore, the combined genotype (*TC+CC*) in the dominant model for hTERT polymorphism also showed an increased risk of developing neutropenia in lung cancer patients.

- On categorizing lung cancer patients based on ALP levels, the subjects carrying the combined genotype (*TC+CC*) showed a lower incidence of liver toxicity, **p=0.02.**

In conclusion, this study represents a novel analysis of the hTERT polymorphic variant rs2735940 and its association with chemotherapy-induced toxicities in lung cancer patients from the North Indian region. The present findings, which included a larger sample size for the overall and stratified group, might aid in the development of biomarkers both for diagnosis of lung cancer and the prognosis.

Role of Genetic Variant in the promoter region of h*TERT* gene and its association towards Lung Cancer pathogenesis

Plagiarism Report

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	Word count
Identical	107
Minor Changes	203
Paraphrased	1622
Omitted	0

	Word count
Identical	23
Minor Changes	0
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Omitted	0

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Sr. No.	Course No.	Title of the Course	Grade
1.	PBT110	Microbiology	A-
2.	PBT113	Biochemistry	A-
3.	PBT114	Molecular Biology	A-
4.	PBT115	Biotechniques	B
5.	PHU003	Professional Communication	A-
6.	PM101	Basic Mathematics	B
7.	PBT202	Bioprocess Technology	A-
8.	PBT204	Genetic and Metabolic Engineering	B
9.	PBT205	Immunology	B
10.	PBT206	Microbial Technology	B
11.	PBT209	Food Processing	B
12.	PBT211	Biostatistics	A-
13.	PBT301	Animal Biotechnology	A
14.	PBT303	Bioinformatics	A-

Topic of the Dissertation / Thesis: Role of Genetic Variant in the Promoter region of TERT gene and its association in lung Cancer.

Name of the Supervisor: Dr. Siddharth Sharma

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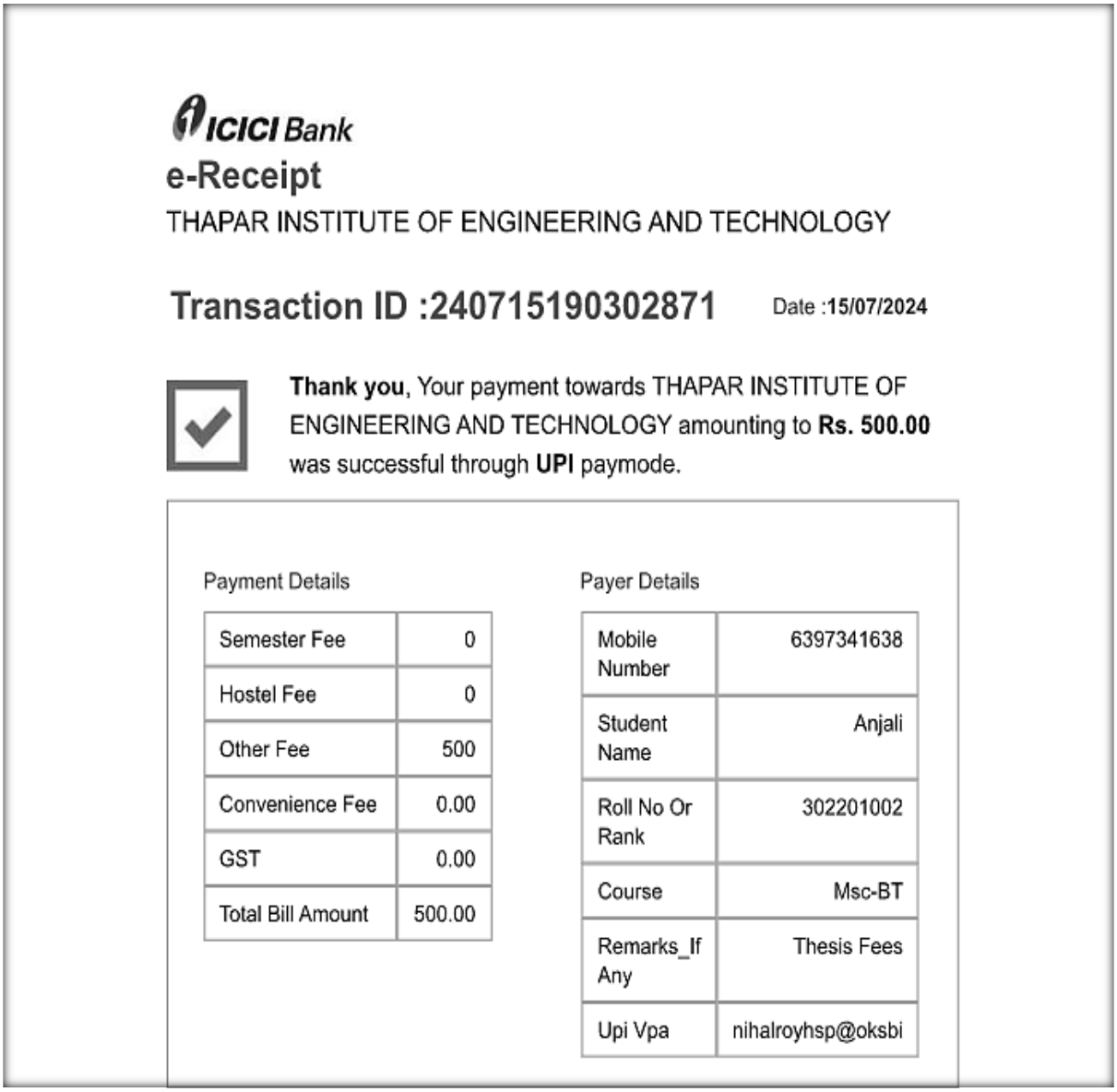

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CHAPTER 8

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