FUNCTIONAL ROLE OF *CCND1* GENE POLYMORPHISM (G870A) IN MODIFYING SUSCEPTIBILITY TOWARDS LUNG CANCER RISK AND OVERALL SURVIVAL OF LUNG CANCER PATIENTS TREATED WITH DOUBLE PLATINUM THERAPY

A Dissertation
Submitted in partial fulfillment of the requirement
For the award of degree of
Masters in Technology

Under the guidance of
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DECLARATION

I, the under designed, hereby declare that the research work presented in the M.tech dissertation entitled **"Functional role of *CCND1* gene polymorphism (G870A) in modifying susceptibility towards lung cancer risk and overall survival of lung cancer patients treated with double platinum therapy"** has been carried out by me under the supervision and guidance of Shiddharth Sharma(Ph.D), Department of Biotechnology, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any university or exanmining body in India/elsewhere.

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This is to certify that dissertation entitled, "**Functional role of *CCND1* gene polymorphism (G870A) in modifying susceptibility towards lung cancer risk and overall survival of lung cancer patients treated with double platinum therapy** "submitted by Ms. Ankita Pandey in partial fulfilment of the requirements for the award of M.tech in Biotechnology at Thapar University, Patiala is an authentic work Carried out by her under our supervision and guidance.

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

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ABSTRACT

Title: Functional role of CCND1 gene polymorphism in modifying susceptibility towards lung cancer risk and overall survival of lung cancer patients treated with double platinum chemotherapy in North Indian population. Cyclin D1 is encoded by CCND1 gene which is present on 11q 13chromosome. Cyclin D1 is a key regulatory protein, playing an important role in the transition from the G1 phase to the S phase of the cell cycle. Cyclin D1 is essential for cell proliferation, differentiation and transcription. Objectives: To investigate the potential role of CCND1 gene G870A polymorphism in the likelihood of development of lung cancer and overall survival of lung cancer pateints in North Indian population. Material and methods: Study consisted of 353 lung cancer cases and 351 age and gender matched healthy controls. PCR-RFLP was done for CCND1 gene. Results: The GA genotype was associated with an increased risk for overall lung cancer (odds ratio OR 1.63; P=0.01). Combined variant genotype Showed a significant association for overall lung cancer (OR 1.50; P=0.03). In addition smokers with carrier genotype of CCND1 were found to have a significant (OR 1.57, P = 0.04) high risk for lung cancer. Kaplan meier survival analysis showed a trend for variant and combined variant genotype surviving more though no significant difference was seen. Conclusion: Here in our results suggest that polymorphic CCND1 may increase the risk of lung cancer in smokers from north India and it may be associated with the overall survival.

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ABBBREVIATION

ADCC	Adenocarcinoma
BSA	Bovine Serum Albumin
C.I	Confidence Interval
CCND1	Cyclin D1
DNA	Deoxyribonucleic Acid
ECOG	Eastern Cooperative Oncology Group
EDTA	Ethylene diamine tetracyclic acetic acid
KPS	Karnofsky Performance Status Scale
OR	Odds Ratio
PCR	Polymerase Chain Reaction
SCLC	Small Cell Lung Carcinoma
SQCC	Squamous Cell Carcinoma
TBE	Tris borate EDTA
TE	Tris Cl EDTA

CHAPTER- 1

INTRODUCTION

Epidemiological studies have contributed continually to the growing awareness of the importance of the genetic and acquired susceptibility factors which are governed to modulate the risk connected with the come across with the environmental contaminants. Knowledge, prevalence, distribution and understanding of these environmental contaminants in the population wherein an individual's weakness, which is predisposed in the form of his genetic makeup, differs and accounts for the danger of acquiring endangerments like Cancer. A connection towards understanding the mechanism of toxicity in order to assess the risk and alleviate the harm deemed to occur, is taken care of under the rubric of Molecular Epidemiology while taking into deliberation the sources of regular errors and random errors (confounding and bias).

Cancer is a multifactorial disease that results from the complex interactions between the environment and genetic factors. It has been seen that 80-90% of Lung Cancer incidence can be credited to cigarette smoking but only about 10% acquire Lung Cancer. This clearly demonstrates the role of susceptibility parameters in the host.

The WHO estimates that in 2030 the number of deaths attributable to the consumption of tobacco will be 100 million accompanied by an increased incidence of Lung Cancer. Differential susceptibilities amongst individuals are accounted by the complexity of experience to carcinogen, number of multiple alleles present for an enzyme to be encoded and most importantly the predisposed genes for the xenobiotic metabolism in the individual. Specific alterations in the genome or an abnormality in the pathways for detoxification and metabolism of environmental contaminants,glues together to form one of the important etiological factors for Lung Cancer.

Cyclin D1 is a key regulatory protein encoded by *CCND1* gene present on 11q 13 chromosomes (Suat Cakina et al; 2003). *CCND1* gene plays an important role in the transition of the cell cycle the G1 phase to S phase. Cyclin D1 protein plays an important role in cell cycle by interacting with Cyclin D Kinases, (CDK4/or CDK6) forming Cyclin-CDK complex. Cyclin D1 acts on a specific range of substrates where

Retinoblastoma tumor suppressor protein (RB) is the first substrate defined. Cell cycle progression comes to a halt with the activation of RB however activated cyclin/CDK complex phosphorylates RB, thereby discontinuing the anti-proliferative effect of RB (İLHAN YAYLIM-ERALTAN et al; 2010).

It has been reported m-RNA of *CCND1* gene is spliced in two variant transcripts $CCND^{traa}$ and $CCND^{trab.}$ It is so controlled due to G870A polymorphism at codon 242 in exon 4 site. This polymorphism does not cause any type of amino acid change. The *transcript a* has exon 1-5, where Thr 286 site of exon 5 is responsible for the expression of Crm1 (nuclear exporter) protein which translocates the *CCND1* protein from nucleus to the cytoplasm of the cell. The *transcript b* is formed by alternative splicing of 870A polymorphism which is encoded by the cyclin D1b. This transcript b does not have Thr 286 site of exon 5 due to which, it is not able to move out of the nucleus and start gathering in nucleus and induces malignant transformation in a normal cell. Further malignant cell continuous to grow and pass through G1/S transition phase accumulating genetic impairments (oliver et al; 2007).

Altered expression of *CCND1* gene is seen in various cancers that may be because of chromosomal translocation, excessive accumulation of protein or gene amplification. Amplification or protein accumulation of the *CCND1* gene is common in different types of cancer like lung, breast, colon, colorectal, cervical and ovarian cancer. Till date the studies done,have identified over 100 single nucleotide polymorphisms spanning cyclin D1 locus(K.E.Knudsen et al; 2006).

The previous studies of cyclin D1 G/A870 polymorphism have widely explored its susceptibility in various cancers, like bladder cancer, sarcoma, ovarian cancer, breast cancer, colorectal cancer, lung cancer etc(Turken Aytekin et al; 2014). Some studies have linked A- allele with increased risk of lung cancer whereas some have linked G – allele as a polymorphic risk factor.

The cyclin D1 G870A polymorphism is also studies in different cancer for its association with overall survival. Some of the studies on esophageal and lung carcinoma found no correlation with the overall survival rate. But the overall expression of the cyclin D1 may be associated in EAC with survival but not in non-small-cell carcinoma (NSCLC) *(Vanika K Gupta et al; 2008).* Another study on survival done in China on breast cancer found that *CCND1* A870G polymorphism

was inversely associated with overall and disease free survival (*Xiao. Ou Shu et al; 2006)*. Further one more study done on non-small cell carcinoma which suggested that A/G870 genotype had no significant difference with survival analysis (*Oliver Gautschi et al; 2006).*

So far, several researches focusing on the role of *CCND1* gene in lung cancer that was conflicted result will be found with G870A (*Changxi et al; 2013*). Dominant G870A polymorphism has been many studies of lung cancer in different populations. However the results are different or contradictory in different types of cancer. Overall survival with *CCND1* gene G870A polymorphism is not given with lung cancer patients with clinico- pathological data.

The aim of this study to investigate possible correlation between *CCND1* gene G870A single nucleotide polymorphism in risk of lung cancer and also investigate overall survival with lung cancer patients on north Indian population.

CHAPTER-2

REVIEW OF LITERATURE

➢ INRODUCTION OF CELL CYCLE

Cell cycle is an ordered series of events. Cell division consists of two consecutive processes, mainly characterized by DNA replication and segregation of replicated chromosomes into two separate cells. Originally, cell division was divided into two stages: mitosis (M), i.e. the process of nuclear division; and interphase, the interlude between two M phases (Fig. 1). Stages of mitosis include prophase, metaphase,anaphase and telophase. Under the microscope, interphase cells simply grow in size, but different techniques revealed that the interphase includes G1, S and G2 phases. Replication of DNA occurs in a specific part of the interphase called S phase. S phase is preceded by a gap called G1 during which the cell is preparing for DNA synthesis and is followed by a gap called G2 during which the cell prepares for mitosis. G1, S, G2 and M phases are the traditional subdivisions of the standard cell cycle (Fig. 1). Cells in G1 can, before commitment to DNA replication, enter a resting state called G0. Cells in G0 account for the major part of the non-growing, non-proliferating cells in the human body.

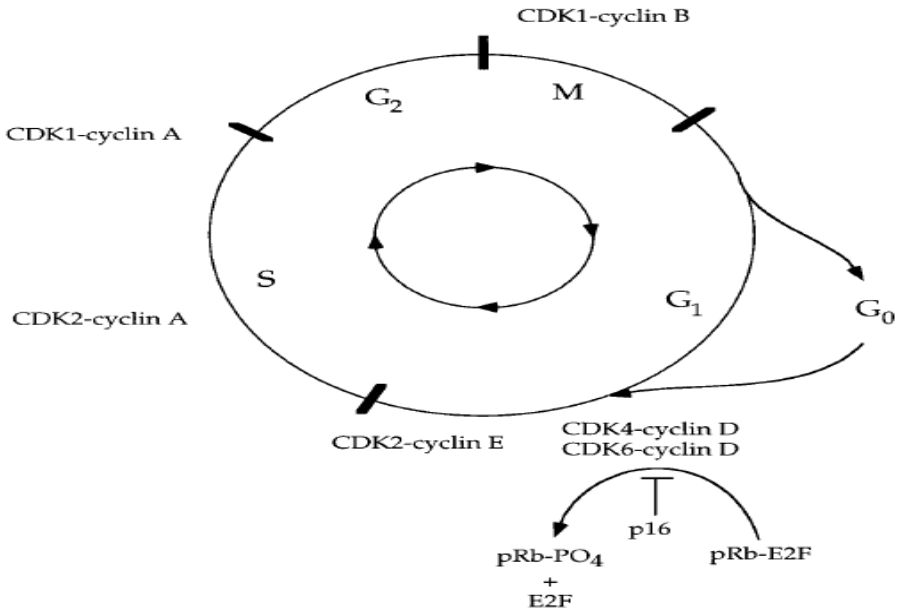


Figure 2.1- **The stages of the cell cycle.** The site of activity of regulatory CDK/cyclin complexes is also indicated *(KATHLEEN COLLINS et. at; 1997*)

The engines that derive progression from one step of the cell cycle to the next are of series of protein complexes composed of two subunits: a Cyclin and a Cyclin

Dependent Kinase(CDKs). Cyclin is regulatory component whereas CDK is catalytic and acts as protein kinase that is activated at specific points of the cell cycle. until now, nine CDKs have been identified and, of these, five are active during the cell cycle, i.e. during G1(CDK4,CKD6 and CDK2), S(CDK2), G2 and M (CKD1). Cyclin-dependent kinases also constitute a family of structurally and functionally related proteins. Kinases are enzymes that add phosphate groups to target substrats; for protein kinases such as CDKs, the substrats are proteins. CDKs are so named because their activites are regulated by cyclins and because they catalyse the phosphorylation of specific serine and threonine residues of specific target proteins. The target proteins for CDK phosphorylation are determined by the associated cyclin. Because different cyclins are present at different phases of the cell cycle so different phases of cell cycle are characterized by the phosphorylation of different target proteins. The phosphorylation events are transient and reversible. When the cyclin CDK complex disappears, the phosphorylated substrate proteins are rapidly dephosphorylated by protein phosphatises. There are three major classes of cylin-CDK complexes that controls passage through the cell cycle: the G1,S-phase, and mitotic cyclin-CDK complexes. Cyclins are so called because they undergo a cycle of synthesis and degradation in each division cycle of the cell. In every eukaryote, there is a family of structurally and functionally related cyclin proteins (Moon-Taek Park et al; 2003) .

In yeast cells, a single CDK protein binds all clases of cyclins and drives all cell-cycle events by changing cyclin partners at different stages of the cycle. In vertebrate cells, by contrast, there are more than one CDKs to interact with G1-cyclins, S-cyclins, and M-cyclin.

CDK	CYCLIN	CELL CYCLE PHASE ACTIVITY
CDK4	D1,D2,D3	G1 phase
CDK6	D1,D2,D3	G1 phase
CDK2	E	G1/S phase
CDK2	A	S phase
CDK1	A	G2/M phase
CDK1	B	Mitosis
CDK7	H	CAK, all cell cycle phases

Table no.2.1- cyclin-CDK complexes are activated at specific points of cell cycle

When, cells are stimulated to divide, G1 cyclin – CDK complexes are expressed first. These prepare the cell for the S phase by including enzymes synthesis required for DNA replication and S-phase cyclin and CDKs. In higher organisms, control of the cell cycle is achieved by regulating the synthesis and activity of G1-CDK complexes.

Now, sixteen cyclins have been identified so far but, like CDK, not all of them are cell- cycle related. Cyclin A and B contain a destruction box that bind with CDK1 and CDK2 and activated the S-phase and mitosis. Cyclins D (D1,D2,D3) contains a PEST sequence (segment rich in proline (P), glutamine acid (E), serine (S) and threonine (T) residuses) which bind with CDK and activate the G1- phase ; these are protein sequences required for effective ubiquitin- mediated cyclin proteolytic at the end of the cell cycle phase(Katrien Vermeulen et al; 2003).

- **Introduction of Cylin D1 and structure**

Cyclin D1 was identified in 1991 as a proto-oncogene; it is a key regulatory protein in the cell cycle. The cyclin D1 locus was initially identified based on its involvement in a chromosomal rearrangement of benign parathyroid tumors. Cyclin D1, a protein encoded by the *CCND1* gene located on the chromosome 11q13, which consist of 295 amino acids (fig 2).These amplicon core is a gene rich region containing at least 10 genes. It serves as a key sensor and integrator of extracellular signals of cells in early to mid-G1 phase. Cyclin D1 binds to CDKs and histone acetylase and histone deacetylase to modulate local chromatin structure of the genes that are involved in

regulation of cell proliferation and differentiation. It is the major involvement in the transition of cells from G1 to S phase. Cyclin D1 expression is induced as a delayed early response to many mitogenic singnals, its expression in normal cells is induced by growth signals involved Ras, Raf and mitogen- activated protein kinase (MAPK) (MAOFU FU, et al; 2004).

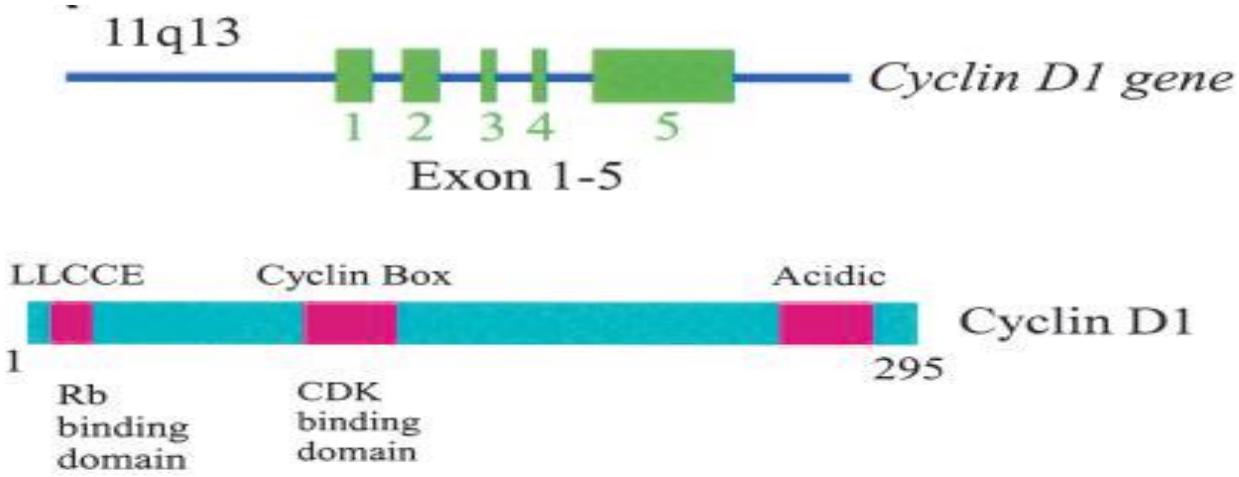
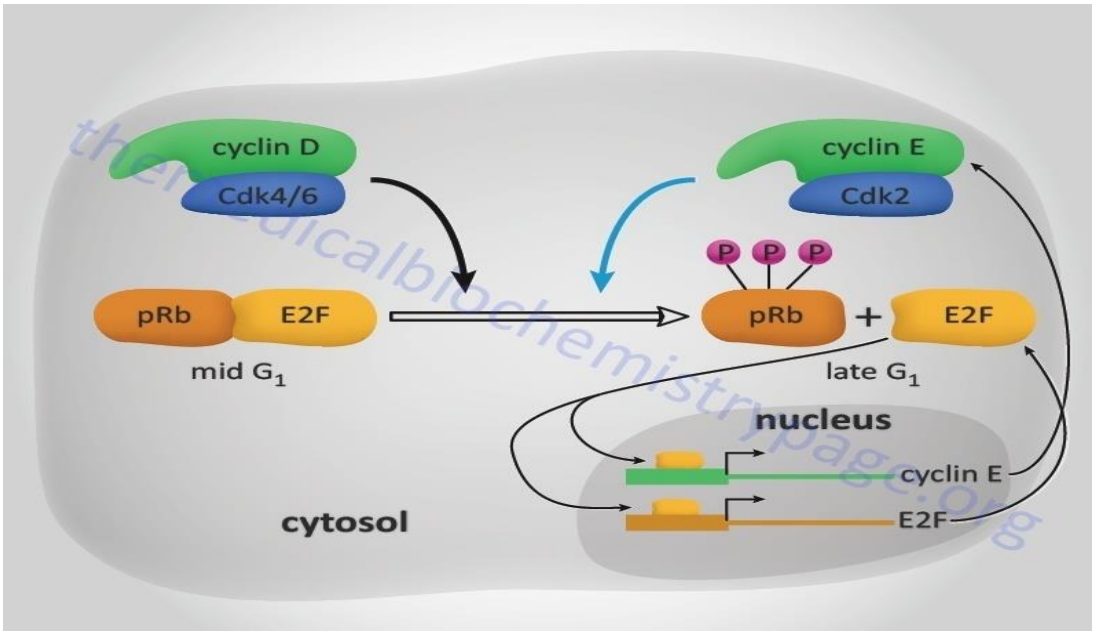


Figure 2.2- **Structure of cyclin D1**; Schematic representation of genomic structure of *CCND1* gene and fuctional domain of *CCND1* gene *(MAOFU FU, et al; 2004*)

- **Functional role of cyclin D1 (*CCND1)* gene**

Cyclin D1 regulates cell cycle progression by activating cyclin dependent kinase 4 (CDK4) and cyclin dependent kinase 6 (CDK6), which in turn phosphorylate the retinoblastoma (Rb) proteins. Retinoblastoma protein has been identified as a tumor suppressor protein, which is the product of retinoblastoma gene. Retinoblastoma protein remains throughout S, G2 and M phases. Phosphorylation of retinoblastoma protein releases the E2F transcriptional factor (E2 promotor-binding protein dimerization pattners) from the pRb/E2F complex. Released E2F transcriptional factor induces the transcription of cyclin E and other genes which are required for entry into S phase (MAOFU FU, et al; 2004).



13. **Figure 2.3**- Schematic representation of pRb phosphorylation by G1 phase cyclin (*http://themedicalbiochemistrypage.org/cell-cycle.php)*

- **Biochemical function**

- **CDK dependent**
  - Regulatory subunit CDK4,6, phosphorylation of pRb
  - Sequestraction of p27, indirectly activate cyclin E/CDK2
- **CDK independent**
  - Disassosiate HDAC1 from pRb repressor complex
  - Transcriptional coregulator
    - direct interaction with TFs
    - regulation of histone acetylase and deacetylase

- **Biological function**

- Sensor and integrator of mitogen signals, G1-S phase transition
- Promote cellular proliferation
- Block cellular differentiation
- Cellular apoptosis/survival
- Cellular migration
- Mitochondrial function, metabolism

- Neuronal regeneration

- **Transcriptional role**

Cyclin D1 has been regulate the transcription factors, including C/EBPβ (*Lamb et al 2003*); STAT3 (*Bienvenu et al., 2001*), DMP1 (*Inoue and Sherr et al, 1998*), Beta 2 /NeuroD (*Ratineau et al., 2002*). It also regulates the largest nuclear receptor super family like estrogen receptor (*Zwijsen et al., 1998; Lamb et al., 2000*), androgen receptor (*Knudsen et al., 1999*; *Ratineau et al., 2001; Petre et al., 2002; burd et al., 2005),* thyroid hormone receptor (Pibiri et al., 2001) and peroxisome proliferated activated receptor-γ (Qin et al., 2003) .

- **Overview of lung cancer**

  Cancer occurs when cells in the body undergo a mutation that causes them to produce quickly and wildly. Lung cancer forms when cancer cells invade and demolish healthy cells in the lung tissues and air passages. It can take several years to develop. It may begin as pre-cancerous changes in the lungs that neither cause symptoms nor show up on an X-ray.

Eventually cancer cells construct into a tumor. As the tumor grows, it impedes the ability of the lungs to function properly. Cancerous cells can split away from the original tumor, travel through the bloodstream, and form tumors in other parts of the body. This process is called metastasis.(Carbone et al; 1997)

- **Types of Lung Cancer**

There are two main types of lung cancer-**Small cell** and **Non-small cell**. The names refer to how the cells appear to pathologists under a microscope.

- **Small Cell Lung Cancer (SCLC)**

According to the National Cancer Institute (NCI), small cell lung cancer accounts for 15% of all lung cancers. Small cell lung cancer starts in neuroendocrine cells. These are the air tubes that lead to the lungs (the bronchi) and the cells in lung tissue. It grows very quickly. It produces large tumors that can travel through the blood and

spread quickly throughout the body. Small cell lung cancer mainly affects heavy or lifetime smokers.

There are two main types of small cell lung cancer. They are small cell carcinoma (oat cell cancer) and combined cell carcinoma. Oat cell cancer is the most common type of small cell lung cancer. (Carbone et al; 1997)

- **Non-Small Cell Lung Cancer (NSCLC)**

According to the National Institutes of Health, non-small cell lung cancer makes up about 85% of all lung cancer cases in the United States (Carbone et al; 1997). The cancer cells are larger, and the cancer is slower rising than small cell lung cancer. NSCLC consists of three subtypes:

- **Squamous cell carcinoma** (epidermoid carcinoma) accounts for 25 to 30% of all lung cancer. It begins in the cells that line the air passages. If not treated it may spread to the lymph nodes, bones, adrenal glands, liver, and brain. It's the most common type of lung cancer in men and is heavily linked to smoking.
- **Adenocarcinoma** makes up about 40% of all lung cancer. It forms in the mucus-producing (outer) part of the lungs. It develops slowly and is the most common type of lung cancer in women and nonsmokers.
- **Large-cell** (undifferentiated) carcinoma includes all non-small cell lung cancer that can't be classified as squamous or adenocarcinoma (about 10 to 15%). It sometimes forms near the surface, in the outer edges of the lungs, and grows rapidly(*Carbone et al; 1997*).

➢ **Epidemiological factors-**

- **Smoking tobacco**

Smoking tobacco, particularly cigarettes, is the main cause of lung cancer. Tobacco smoke contains many harmful chemicals that can cause cancer (are carcinogenisis). Other types of tobacco products such as low-tar and low-nicotine cigarettes, pipes, cigars, herbal cigarettes, hookahs and chewing tobacco also cause cancer and are not considered safe(Wynder et al; 1994).

- **Radon**

Radon is a colourless, odourless, tasteless gas that comes from the natural breakdown of uranium in rocks and soil. Radon exposure increases the risk of lung cancer. Radon is the leading cause of lung cancer in non-smokers and the second leading cause of lung cancer in smokers.

- **Asbestos**

Asbestos is group of minerals that occur naturally. Asbestos has been widely used in building materials and many industries. Exposure to asbestos fibres in the air that people breathe increases the risk of lung cancer.The risk of asbestos exposure is highest for people who work with asbestos, such as miners or those who work with it in manufacturing.

- **Occupational exposure to chemical carcinogens**

The risk of lung cancer is higher among people who work in certain industries or have certain occupations. In general, for many of these substances, the risk of developing lung cancer is even higher for people who smoke. *(http://www.cancer.ca/en/cancerinformation/cancertype/lung/risks/?region=on#ixzz3ek3mG5QZ*)

Occupational exposure to the following cancer-causing chemicals increases the risk of lung cancer:

- arsenic and inorganic arsenic compounds
- beryllium and beryllium compounds
- cadmium and cadmium compounds
- chemicals used in rubber manufacturing, iron and steel founding and painting
- chloromethyl ethers and bischloromethylether
- chromium (VI) compounds
- cobalt-tungsten carbide
- diesel engine exhaust
- mustard gas
- polycyclic aromatic hydrocarbons (PAHs)
- radioactive ores such as uranium and plutonium

- silica dust and crystalline silica
- some nickel compounds

Polycyclic aromatic hydrocarbons (PAHs) increase the risk of lung cancer. People can have occupational exposure to PAHs through chimney sweeping, coal gasification, coke production, using coal tar pitches (such as in roofing and paving) and aluminum production (Bergen et al; 1999)

- **Some signs and symptoms of lung cancer**
    - A prolonged cough that worsens over time
    - croakiness
    - Problem in breathing
    - Reduced weight
    - exhaustion
    - Shortness of breath
    - Coughing up blood

- **Diagnosis**

1. Imaging test like
    - X-ray
    - CT scan
    - MRI scan
    - PET scan ect
2. Cytology test
3. Microscope examination

- **Cyclin D1 alterations in cancer**

In cancer cell, fundamental alterations in the genetic controls of cell division, that resulting in the cell proliferation and modulating transcription. It is not surprising that deregulated in cancer. cyclin D1 is tumor type specific and it occurs through chromosomal translocation, gene amplification. Importantly, overexpression was often correlated with chromosomal translocation, a characteristic of 70—90% of mantle cell lymphomas (Bosch *et al*; 1994; Bigoni *et al*; 1996). Most notably, the

(11;14) (q13;q32) translocation was same found juxtapose the IgH heavy chain promoter with the *CCND1* gene (also referred to as B-cell leukaemia/lymphoma 1 gene, bcl-1), resulting in constitutive overexpression of cyclin D1/*CCND1*(*Bosch et al; 1994*;) . In breast cancer, increased cyclin D1 gene copy number has been reported. Esophageal cancer, laryngeal cancer and NSCLC were generally associated in such studies with *CCND1* overexpression. Overexpression of cyclin D1 was found to be associated with many cancers, forced expression of cyclin D1 alone did not to induce malignant transformation in rodent fibroblasts or lymphocytes (*Jiang et al; 1992; Buckley et al; 1993; Gillett et al; 1994*).

- **Cyclin D1 (*CCND1*) Polymorphism and Cancer risk**

Genetic polymorphisms are common variants in the genetic code. It is typically elucidated as a sample of interest comprising of a polymorphism seen in atleast 1% of the population. In this approach we assume that the low or medium penetrant genes account for the majority of the lung cancer susceptibility rather than those which are highly penetrating. A single nucleotide polymorphism (SNP) is an alteration in a single nucleotide present in the DNA sequence.

The molecular basis for enzyme polymorphism is backed by the occurrence of any of the three cases, ie.

Either we will see a nucleotide variation in the coding region of a gene therefore altering the enzyme activity due to the amino acid substitution.

There might be a deletion in the coding region of the gene leading to an inactive enzyme or even lack of protein synthesis.

Variation in the polyadenylation site causing post transcriptional repression hence affecting the quantity of the transcript formed. (Boffeta 2000)

Different approaches and models are recruited in order to study the polymorphism in a particular gene. Amongst them are the Candidate gene approaches, where the gene and the nucleotide polymorphism both are simultaneously selected and put to statistical and analytical studies. We too have followed the same approach for our study in the North India population.

- ***CCND1* (G870A) polymorphism mechanisms and splicing**

Cyclin D1 gene consists of 5 different exons, splicing of cyclin D1 was not measured a significant regulatory factor. Two transcripts of cyclin D1 can be detected in normal cells (Howe and Lynas, 2001), though regulation of splicing appears to be most related in cancer. Numerous groups have detected a cyclin D1 message that derives from alternative splicing (Betticher *et al*., 1995; Hosokawa *et al.,*1999; Bala and Peltomaki, 2001). This isoform was firstly defined following the identification of the G/A870 polymorphism, that occurs at the intron 4/exon 5 boundary.

As shown in Figure2. 4, the G870 allele creates a most favourable splice donor site, resulting in the well-described transcript for cyclin D1 ('transcript a'). By contrast, the A870 allele is predicted to hinder the splicing event, thus allowing for read-through into intron 4 and production of a variant splice product of cyclin D1, termed 'transcript b'. Constant with this hypothesis, Betticher *et al*. (1995) and Howe and Lynas (2001) confirmed that the A-allele of cyclin D1 was preferentially linked with transcript b production. However, individuals with the A/A genotype can still create transcript a (Bala and Peltomaki, 2001), thus representing that the 870-A allele is not completely go through for transcript b production, and individuals with the G/G genotype can produce transcript b.

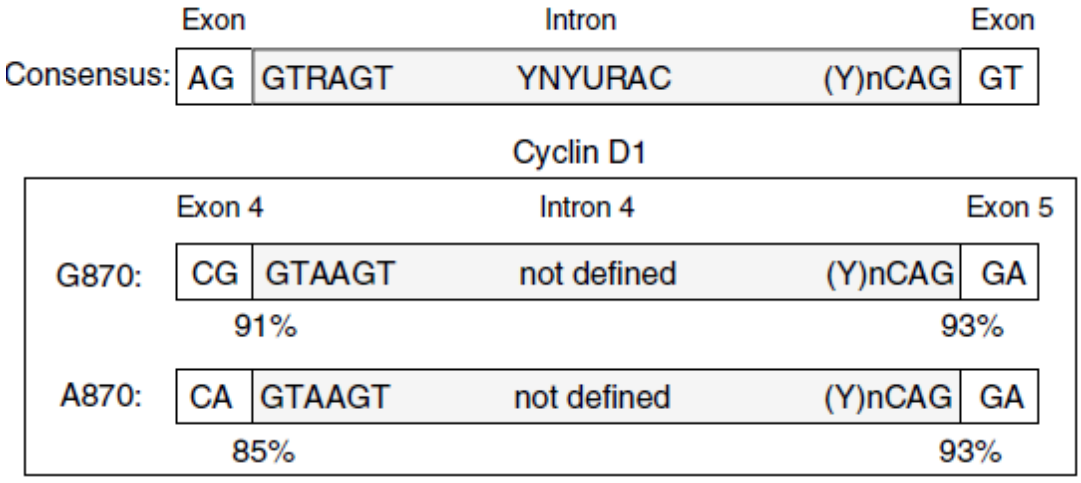


Figure 2.4- cyclin D1 polymorphism and splicing of exon 4 and exon 5. (*K E Knudsen et al; 2006)*

It should be noted that the presence of the A-nucleotide at the splice donor site does represent a change from the consensus (Figure 4), wherein the G-Allele represents 91% consensus using a weighted splice site identification matrix and the A-Allele is 85% (Shapiroand Senapathy *et al,* 1987).

Importantly, both sites would be predicted to be functional and mutations in splice donor sequence analgous to the A-allele are suitably spliced (Wieringa *et al*., 1983). Thus, modifiers in addition to the polymorphic 870-allele must influence the splicing event. In principle these could be additional polymorphisms within the cyclin D1 gene or trans-acting factors, which have yet to be defined.

Interpretation of epidemiological studies comparing the G/A870 polymorphism to cancer risk may be puzzled by the disjunction between the genotype and transcript b production. To date, no study has directly examined the relationship of the G/A870 polymorphism to transcript b production using quantitative/ mechanistic methodologies. However, individual assessment of the predictive values of the polymorphism and transcript b production in colon cancer revealed that while the polymorphism held no predictive value, alterations in transcript b production were of significance (Bala and Peltomaki, 2001). Therefore, possible sources of the heterogeneous influence of the cyclin D1 polymorphism in cancer are factors which influence transcript ‘a’ versus ‘b’ splicing independently of the polymorphic nucleotide. Thus, there is an urgent need to solidify the relationship of the polymorphism to transcript b production, and to respectively assess the impact of transcript b in human disease.

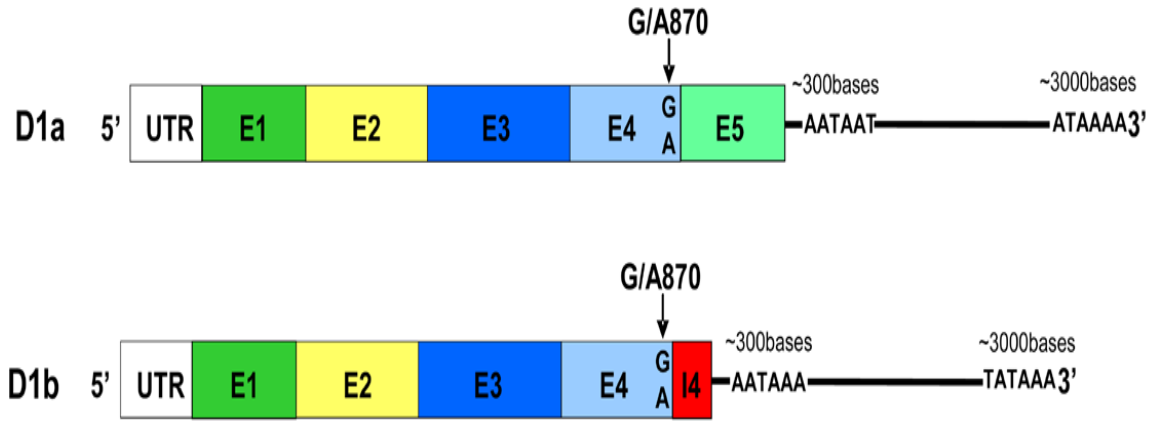


Figure2.5. Cyclin D1 splice variants Cyclin D1a encodes all 5 exons and a 3' UTR with 2 putative polyadenylation signals about 300 and 3000 bases downstream of the TGA stop codon. D1b encodes exons 1-4 and part of intron 4 with its 3'UTR found in intron 4. Similar to D1a, the cyclin D1b 3' UTR has 2 potential polyadenylation sites about 300 and 3000 bases downstream of the stop codon. The expression of D1a or D1b is hypothesized to be determined by the G/A polymorphism at nucleotide 870, the last nucleotide of exon 4, and recognition of the exon 4/intron 4 boundary (*Knudsen et al; 2006).*

Table2.2: Distribution of *CCND1* polymorphism among different cancer (*Knudsen et al; 2006)***

Tumor type	Effect polymorphism	Reference
Basal cell carcinoma	Increased risk with A/A	(Zhang et al., 2004)
Bladder cancer	Increased risk with A/A	Sanyal et al., 2004
Breast cancer	Increased risk with A/A	(Shu el al., 2005)
Cardiac cancer	Increased risk with A/A	(Wang et al., 2003)
Cervical cancer	Increased risk with G/G	(Catarino et al., 2011)
Colorectal cancer	Increased risk with A/A or A/G	(Lewis et al., 2003)
Esophageal cancer	Increased risk with A/A	(Wang et al., 2003)

Gastro intestinal	Increased risk with A/A	(Buch et al., 2005)
Gastro intestinal (adinocarcinoma of upper track)	No effect on polymorphism	(Geddert et al., 2005)
Head and neck(squamous cell carcinoma)	G/G assosiated with reduced disease free survival	(Matthias et al., 1998)
Head and neck	Increased risk with A/A or G/G	(Deng et al., 2002)
hemangioblastoma	Increased risk with G/A or G/G	(Zatyka et al., 2002)
Hemangioblastoma(VHL-assosiated)	No effect on the polymorphism	(Gijtenbeek et al., 2005)
leukemia	Increased risk with A/A	(Hau et al., 2005)
Lung cancer	Increased risk with A/A	(Qiuling et al., 1999)
Lung Cancer (Non-small cell)	A/A associated with reduced disease free survival	(Betticher et al., 1995)
Ovarian cancer	Increased risk with A/A	(Dhar et al., 1995)
Prosthetic adinocarcinoma	Increased risk with A/A	(Wang et al., 2003)
Squamous cell carcinoma of the uterine cervix	No effect of the polymorphism	(Jeon et al.,2005)

- **Overall survival in lung cancer**

The percentage of people in a study or treatment group who are still alive for a certain period of time after they were diagnosed with or started treatment for a disease, such as cancer. The overall survival rate is often stated as a five-year survival rate, which is the percentage of people in a study or treatment group who are alive five years after their diagnosis or the start of treatment. Also called as **survivalrate**. (*cancer.gov/publications/dictionaries/cancer*)

It was reported by **Cancer research UK cancer Survival group in 2010- 2011** that men were less surviving than the women. They said that 8% men were surviving for at least five years, and 12% women predicted to survive for at least five years.

It was also reported by **Lung Former Anglia Cancer Network, 2003-2006** One-year survival from lung cancer is strongly related to the stage of the disease at diagnosis. People presenting at stage I have the highest survival rates (71%). Survival is much lower for those diagnosed with stage IV disease (14%). Survival for those people with stage not known is similar to those with stage IV disease (17%).

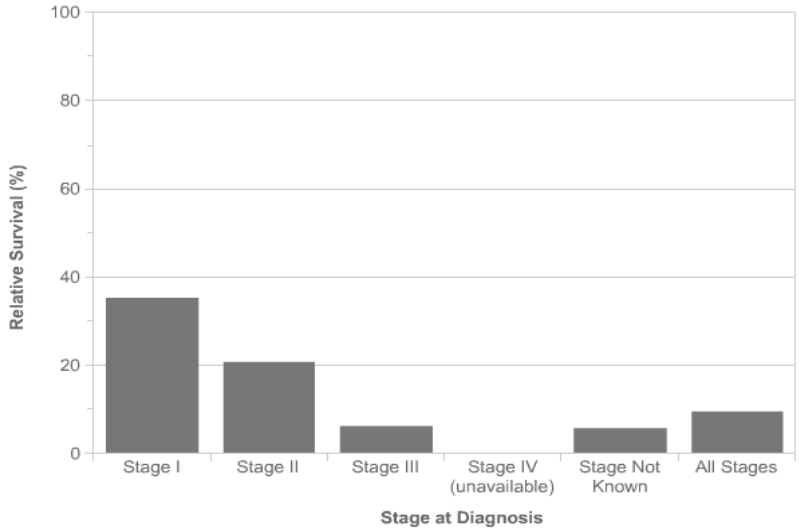


Figure 2.6 Five-Year Relative Survival Rates by Stages

(cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/lung-cancer/survival)

- **Chemo-therapeutic Treatment towards lung cancer**

Treatment of cancer different types of chemotherapy is used. Mainly platinum based and taxol based chemotherapy are used. These platinum based complexes are used as adjuvant therapy for cancer aiming to induce apoptosis.

- **Cisplatin**

Cisplatin is one of the most potent chemotherapy drugs widely used for cancer treatment. Cisplatin cis-[Pt(II)(NH(3))(2)Cl(2)] ([PtCl2(NH3)2] or CDDP), was mainly used for different types of cancer including: sarcoma cancers, cancers of soft tissue, bones, muscles, and blood vessels. Depending on cell type and concentration**,** Cisplatin cross links DNA in several different ways, interfering with cell division by mitosis. The damaged DNA extracts DNA repair mechanisms, which in turn activate apoptosis when repair shows unfeasible. Cisplatin might also lead to diverse side-effects such as neuro- and/or renal-toxicity or bone marrow-suppression. Moreover, the binding of cisplatin to proteins and enzymes may alter its biochemical mechanism of action.

While a combination-chemotherapy with cisplatin is a foundation stone for the treatment of multiple cancers, confront is that cancer cells could become cisplatin-resistant. (*Ana-Maria Florea et al* ; 2011)

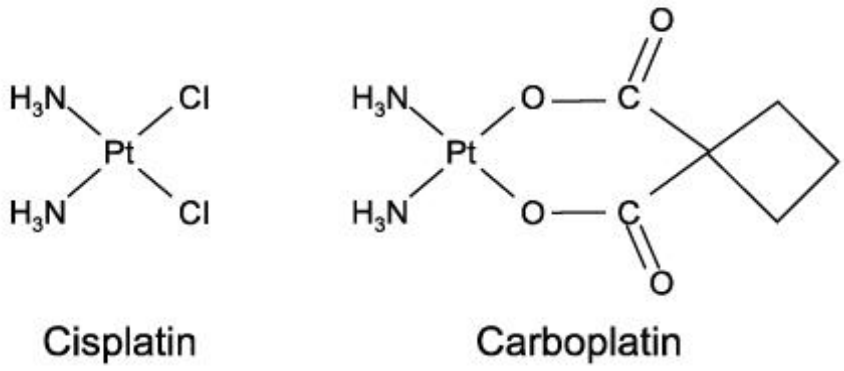


Figure-2.7 Structure of cisplatin and carboplatin

(*synapse.koreamed.org/DOIx.php?id=10.3802/jgo.2009.20.2.113&vmode=PUBREADER)*

- **Carboplatin**

Carboplatin **is** a platinum compound related to cisplatin. Carboplatin is formed by replacing the chloride leaving groups of cisplatin with 1, 1-cyclobutanedicarboxylato ligand, which increases the stability of the leaving groups. On a molar basis, carboplatin is 45 times less cytotoxic than cisplatin. Carboplatin has a more favorable adverse effect profile than cisplatin, which has led to the investigation of the replacement of cisplatin with carboplatin in many regimens. Carboplatin and cisplatin seem to be equally effective in ovarian, non-small cell and small cell lung cancers. Carboplatin cytotoxic activity is similar to cisplatin as it binds with DNA to form intrastrand crosslinks and adducts that cause changes in the conformation of the DNA and affect DNA replication.Visual changes or loss of vision has been reported with high doses of carboplatin and hypokalemia, hypomagnesemia, hyponatremia, hypophosphatemia, and/or hypocalcemia occur in about 30% of patients during therapy with carboplatin but are not as common or severe as with cisplatin.

- **Performance status**
- **KPS** Karnofsky Performance Status Scale (KPS) was designed to measure the level of patient activity and medical care requirements. It is a general measure of patient independence and has been widely used as a general assessment of patients with cancer. Although there is a long history of use **of** the KPS for judging cancer patients, its reliability and validity have been assumed without formal investigation. (*Yates et al; 1979)*

KARNOFSKY PERFORMANCE STATUS SCALE DEFINITIONS RATING (%) CRITERIA (*Yates et al; 1979)*

Able to carry on normal activity and to work; no special care needed.	100	Normal no complaints; no evidence of disease.
	90	Able to carry on normal activity; minor signs or symptoms of disease.
	80	Normal activity with effort; some signs or symptoms of disease.
Unable to work; able to live at home and care for most personal needs; varying amount of assistance needed.	70	Cares for self; unable to carry on normal activity or to do active work.
	60	Requires occasional assistance, but is

		able to care for most of his personal needs.
	50	Requires considerable assistance and frequent medical care.
Unable to care for self; requires equivalent of institutional or hospital care; disease may be progressing rapidly.	40	Disabled; requires special care and assistance.
	30	Severely disabled; hospital admission is indicated although death not imminent.
	20	Very sick; hospital admission necessary; active supportive treatment necessary.
	10	Moribund; fatal processes progressing rapidly.
	0	Dead

- **ECOG** The Eastern Cooperative Oncology Group (ECOG) is one of the largest clinical cancer research organizations in the United States, and conducts clinical trials in all types of adult cancers.The **ECOG performance status** is a scale used to assess how a patient's disease is progressing, assess how the disease affects the daily living abilities of the patient, and determine appropriate treatment and prognosis (*C. Gridelli et al; 2004).*

Eastern Cooperative Oncology Group (ECOG) performance status rang .

(Gridelli et al; 2004)

ECOG Performance Status Scale	
Grade	**Description**
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.
1	Symptoms but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (*e.g.*, light housework, office work).
2	In bed <50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead.

CHAPTER-3

AIM OF THE STUDY

1. To investigate the role of *CCND1* gene G870A polymorphism as a genetic modifier for risk towards lung cancer within a North Indian population.

2. To investigate whether the overall survival of polymorphism for *CCND1* gene are a risk factor for lung cancer development.

3. To investigate the role of smoking as a modifier for lung cancer.

4. To investigate the correlation with different clinico- pathological features for lung cancer.

5. To investigate the correlation with objective response and chemotherapeutic regimen for lung cancer.

6. To investigate the performance status (KPS, ECOG) of patients treated with above specific chemotherapeutic regimen for lung cancer.

CHAPTER-4

MATERIAL AND METHODS

- ***Study Subjects and Sample Collection***

Blood samples of 350 Lung cancer patients were recruited from the Department of Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India that was approved by the Institute ethics committee of PGIMER. This Informed written consent was obtained from all participants or their representatives. In brief, eligible cases included all patients who were newly diagnosed with primary lung cancer. All the recruited patients were histopathologically diagnosed as having NSCLC, ADCC, SQCC and SCLC. There were number of age, gender, smoking, histological, or TNM stage restrictions, but patients with a prior history of cancer were excluded from this study. During the same time, 350 unrelated individuals with no evidence of lung or other cancer who entered the hospital for health check-ups were enrolled as control group. Each control was pair-matched by sex, age (±10 years) and smoking parameters to a patient with lung cancer. These characteristics allowed us to obtain control population without any possible risk bias for lung cancer. A detailed questionnaire was filled for each case and control by a trained interviewer. The questionnaire included information on demographic and smoking characteristics. Smokers reported tobacco habits such as smoking of cigarette and/or beedi (a native cigarette like stick of coarse tobacco hand-rolled in a dry tembuhurni leaf). As an indication of cumulative smoking exposure, pack-years were calculated by the following formula: [(cigarettes or beedis per day/20) X years smoked]. While medical information of cases, including Histology, TMN classification, clinical staging, primary tumor size, involvement of lymph node and metastasis were obtained from medical records of the hospital. Approximately 3-5ml of venous blood was collected from each participant.

- ***Isolation of DNA from Blood***

Isolation of DNA from peripheral blood: Genomic DNA was isolated using standard Protein K digestion, phenol/chloroform extraction and ethanol precipitation method from whole blood samples of both cases and controls (Bartlett and White's method).

REQUIREMENTS:

- Washing buffer
- Lysis buffer
- Phenol:Chloroform:Isoamylalcohol (25:24:1)
- Chloroform:Isoamylalcohol (24:1)
- Isopropanol
- TE buffer

PROCEDURE:

Preparation of Buffers

Washing buffer, Lysis buffer and TE buffer were prepared as shown in tables below.

Table no.4.1 Preparation of washing buffer

Stock concentration	Working concentration
1M sucrose	320mM sucrose
100% Triton X-100	1% Triton X-100
100mM Magnesium Chloride	5mM magnesium Chloride
100mM Tris-HCl pH (8.0)	10mM Tris-HCl pH (8.0)

Table no.4.2 Preparation of lysis buffer

Stock concentration	Working concentration
1M Tris HCl pH (8.00)	400mM Tris HCl pH (8.00)
10% SDS	1% SDS
0.5M EDTA	60mM EDTA
5M NaCl	150mM NaCl
10mg/ml Proteinase-K	100µg/ml proteinase-K

Procedure of DNA Isolation

- Took 5ml of blood and 5ml of Washing Buffer was added and mixed thoroughly. Centrifuged it at 3500rpm for 5 minutes.

- Discarded the supernatant and added 5ml of Washing buffer (1.6ml 1M Sucrose,0.5 ml Triton X-100, 0.25ml MgCl2, 0.5 ml 100mM Tris HCl and 0.26ml of water) to the pellet and re suspended the pellet in the Buffer and centrifuged again (repeat this step thrice).

- Dissolved the pellet in 5ml of Lysis buffer (1 M Tris HCl 2ml,10% SDS 0.5ml ,0.5 M EDTA 0.6ml, 5M NaCl 0.15ml,10mg/ml Proteinase-K 0.05ml and water 1.7ml) and incubated at 44 °C overnight.

- Added an equal volume of Phenol: Chloroform: Isoamyl alcohol (PCI) 25:24:1(25ml Phenol, 2.4 ml Chloroform and 0.1ml isoamyl alcohol) and mixed the contents slowly.

- Centrifuged at 8000rpm for 10minutes at 4°C. Took the upper aqueous layer and again add PCI mix and centrifuged.

- Took the aqueous layer and added equal volume of Chloroform: Isoamyl alcohol (24:1).

- Centrifuged it at 6500 rpm for 5 minutes and took the upper layer.

- To the aqueous layer added equal volume of chilled Isopropanol or 2.5 times volume of absolute Ethanol and mixed it gently.

- Freeze it at -20°C for 1-2 hours.

- Centrifuged it at 12,000 rpm for 10 min at 4°C.The supernatant was discarded and the pellet of DNA was washed with chilled 70% Ethanol twice at 10,000 rpm for 5 minutes.

- Decant ethanol and air dry the pellet.

- Dissolved the pellet in 50µl-150µl Tris-EDTA buffer depending on the size of DNA pellet (Bartlett & White, 2003).

➢ **DNA Quantification**

The Thermo Scientific Nanodrop Spectrophotometer holds 1µl of sample without the need of traditional containment devices such as cuvettes and capillaries. Using fibre optic technology and surface tension, the sample is held in place between two optical surfaces that define the path length in vertical orientation. Removal of fixed containment devices from the system allows the path length to change in real time for a sample. This essentially eliminates the need to perform dilutions and hence less cumbersome.

Procedure

- Pipetted 1µl of Deionised water onto the lower optical surface of Nanodrop (Thermo Scientific) to clean it

- Opened the Nanodrop software and select Nucleic acid Module
- Performed a blank measurement by loading 1µl of TE and selecting “blank” from the screen
- Measured the Nucleic acid sample by loading 1µl of DNA sample and selecting “measure”
- Concentration and purity of DNA samples were calculated automatically

DNA concentration otherwise can be calculated as:

{DNA concentration (µg/ml) = O.D at 260nm X 50 X Dilution factor}

Where 50µg/ml of DNA is equal to 1 O.D

{Purity of DNA = O.D at 260nm/O.D at 280nm}

REMARK : A ratio of ~1.8 indicates purity of DNA; a ratio of ~2.0 is generally accepted as pure for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants.

- ***Resolution of DNA Fragments On Agarose Gels***

Requirements

- Electrophoresis buffer (TAE or TBE)
- Ethidium bromide solution
- Electrophoresis-grade Agarose
- 6X loading dye
- DNA molecular weight markers

- Horizontal gel electrophoresis apparatus
- Gel casting platform
- Gel combs (slot formers)
- DC power supply

Procedure

✓ ***Preparing 5X TBE (1000ml)***

- Tris base - 54 g
- Boric Acid - 27.5g
- EDTA (0.5M) - 20ml
- Make up final volume with water

✓ ***Preparing 6X Loading Dye (20ml)***

- 0.25% Bromophenol blue - 0.05gm
- 0.25% Xylene Cyanol - 0.05gm
- 40% Sucrose - 8gm
- Make up final volume with TE buffer

Preparation of the Agarose Gel for Electrophoresis

- Prepared an adequate volume of electrophoresis buffer.

- Added the desired amount of Electrophoresis-grade Agarose to a volume of Electrophoresis buffer sufficient for preparing the gel. For example, for genomic DNA 0.7% gel (0.7g agarose in 100ml 0.5X TBE) was prepared while for the PCR products 1.7% gel (1.7g agarose in 100ml 0.5X TBE buffer) was prepared.

- Melted agarose was cooled to 55°C in a water bath before pouring onto the gel platform to prevent warping of the gel apparatus.

- Before pouring Ethidium bromide solution was added to the melted well mixed agarose gel to a final concentration of 0.3µg/ml to facilitate visualization of DNA when seen under UV Transilluminator.

- Poured the melted Agarose onto the gel casting apparatus between 0.5 and 1 cm thick and with the gel comb inserted prior to pouring, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the surface of the agrose were removed before the setting of the gel.

Loading and running the gel

- After the gel got solidified, the gel comb was withdrawn with proper care without disrupting the sample wells.

- Placed the gel casting platform containing the set gel in the Electrophoresis tank. Added sufficient Electrophoresis buffer to cover the gel until the tops of the wells are submerged. Made sure no air pockets were trapped within the wells.

- DNA samples were prepared by mixing 5µl DNA with 2µl of 6X loading dye and 2ul water in case of genomic DNA or by mixing 5µl DNA with 2µl of 6X loading dye in case of PCR product.

- Samples were typically loaded into the wells with micropipette. Care was taken to prevent mixing of the samples between wells.

- Appropriate DNA molecular weight marker were also loaded in case of PCR and digestion products

- Connected the electrodes to a power pack and allowed the Electrophoresis apparatus to run at 60 V until the marker dyes migrated the desired distance.

- Turned off the electric power, disconnected the leads, and discarded the electrophoresis buffer from the reservoirs

- DNA was visualized by placing the gel on a UV transilluminator and then photographed using Gel Documentation.

POLYMERASE CHAIN REACTION (PCR) :AMPLIFICATION OF CYCLIN D1

Polymerase chain reaction, (PCR), a technique used to make numerous copies of a specific segment of DNA quickly and accurately. PCR was developed in 1983 by Kary B. Mullis, an American biochemist who won the Nobel Prize for Chemistry in 1993 for his invention.The PCR technique is based on the natural processes a cell uses to replicate a new DNA strand. Only a few biological ingredients are needed for PCR. The integral component is the template DNA—*i.e.,* the DNA that contains the region to be copied, such as a gene. As little as one DNA molecule can serve as a template. The only information needed for this fragment to be replicated is the sequence of two short regions of nucleotides (the subunits of DNA) at either end of the region of interest. These two short template sequences must be known so that two primers—short stretches of nucleotides that correspond to the template sequences—can be synthesized. The primers bind, or anneal, to the template at their complementary sites and serve as the starting point for copying. DNA synthesis at one primer is directed toward the other, resulting in replication of the desired intervening sequence. Also needed are free nucleotides used to build the new DNA strands and a DNA polymerase, an enzyme that does the building by sequentially adding on free nucleotides according to the instructions of the template.PCR is carried out in repeated cycles. PCR can also be utilized for rapid screening and/or sequencing of inserts directly from aliquots of individual phage plaques or bacterial colonies.

Requirements

- 10X PCR buffer
- BSA
- Forward Primer
- Reverse Primer
- dNTPs
- Taq DNA polymerase
- Water
- DNA sample

PCR Primers are short fragments of single stranded DNA (15-30 nucleotides in length) that are complementary to DNA sequences that flank the target region of interest. The purpose of PCR primers is to provide a “free” 3’-OH group to which the DNA polymerase can add dNTPs. Primer sequences need to be chosen uniquely. Specific region of DNA should be chosen and primer should be designed ubiquitously, avoiding the possibility of mis hybridization to a similar sequence nearby. Primers should not easily anneal with other primers in the mixture (either other copies of same or the reverse direction primer); this phenomenon can lead to the production of 'primer dimer' products contaminating the mixture. Primers should also not anneal strongly to themselves, as internal hairpins and loops could hinder the annealing with the template DNA. Given below are the primers used.

CCND1 PRIMERS;

Forward primer ; 5’ AGTTCATTTCCAATCCGCCC 3’

Reverse primer ; 5’ TTTCCGTGGCACTAGGTG 3’

Band size ; 212 b

Table no.4.3- Reaction mixture of PCR carried out for *CCND1* (20 reations)

Reagent	Stock concentration	Final Reaction concentration	Quantity Used
Additive 1 BSA	1000 µg/ml	100µg/ml	44µl
PCR Buffer (Mg Conc.)	10X 25mM	1X 1.5mM total	44µl
CCND1 Primer (forward)	10µM	0.5µl	22 µl
CCND1 Primer (reverse)	10µM	0.5µl	22 µl
Taq Polymerase	2.0U	1.5µl	22 µl
dNTPs	10mM each	0.2	8.8 µl
PCR Grade Water			189.2 µl
DNA Template	100ng/µl	300ng	4 µl

Table no.4.4- Cycling profile of PCR for *CCND1*

Se. No.	Steps	Temperature	Time
1.	Initial Denaturation	95ºC	5 mins
2.	Denaturation	94ºC	30 sec
3.	Annealing	55ºC	45 sec
4.	Polymerization	72ºC	45 sec
5.	Final Extension	72ºC	5 mins

4.5 Restriction Digestion of CCND1

This enzymatic technique can be used to cleave the DNA amplicons at specific sites ensuring that all the all DNA fragments that contain a particular sequence have the same size; furthermore, each fragment that contains the desired sequence has the sequence located at exactly the same position within the fragment. The enzyme used was:

Msp1*:** The source microorganism from which it has been isolated is *Moraxella* species (ATCC 49670). This enzyme was used for the digestion of amplicons obtained from the amplification of ***CCND1 rs 9344. It recognizes the G-→A site in the amplified stretch of DNA sequence.

Restriction site for the enzyme is

5'.....C CGG.....3'

3'.....GGC C.....5'

Procedure

The total reaction mixture of 20 µl consisted of 2.2 µl 10X Tango buffer, 0.35 µl (3U) of 10U/ml Msp1 enzyme (Thermo Fisher Scientific), and 10 µl of PCR product and 7.35 µl water. The buffer used for increase activity of enzyme in the reaction. All the samples are incubated at 37ºC in incubator for overnight. Next day take the sample and keeping it at -20ºC for stop the enzyme reaction. Made 2.5% polyacrylamide gel with ethidium bromide and gel poured into the caster. Samples were prepared by dye with water and loaded into the wells with micropipette. Turn on the power and allowed the electrophoresis run at 80 V until the marker dyes migrated the desired distance. The result was visualized by placing the gel on the UV transiluminator and then photographed by using Gel Doc (BIORAD).

Overall survival data collection

We called the all cases patient to check their survival status on the basis of alive or dead. We note down the dead date given by their family member and also note their last chemotherapy date. Survival time was measured from the date of sampling to date of death or last call (5th February 2015).

Statistical Analysis

Calculations were performed with Medcalc software version 12.1.2. We used the chi-square tests to analyze the demographic distribution of the *CCND1* genotypes in cases and controls. Chi- square test used for categorical data and student's t-test used for continuous variables. The Hardy-Weinberg equilibrium analysis were performed to use both cases and controls to calculate the genotypic frequency of *CCND1* gene G870A polymorphisms using chi-square test(df=1). The odd ratio (OR) and 95% confidence interval (95% CI) were calculated using logistic regression analysis with adjustment for age, sex, smoking status (heavy and light smoker) and also measure the association between *CCND1* genotype and lung cancer. Additionally, the cases were futher divided into various histological subclasses and clinico-pathological features like KPS, ECOG, clinical responses (CR, PR, SD, PD) and REGIMEN to identify any specific association with genotype. Kaplan-meier method and Cox proportional hazard analysis were used for analysis of overall survival. Multivarient Cox regression was used to evaluate the genotype association on survival. This regression was done to adjust for patient factors age, gender, smoking status; histological and clinic-pathological factor like KPS, ECOG. Kaplan-meier was used to evaluate the median and p-value. Probability criteria less than 0.05 were used as significance in two sided t tests

CHAPTER -5

RESULTS

DNA Isolation

Genomic DNA was isolated from the peripheral blood and these DNA samples were run on 0.7% of agarose gel. Total DNA collected from peripheral blood and it was preserved as a stock for further use. These DNA were diluted with TBE to a concentration of 100ng/µl. The diluted genomic DNA was used as a template in different volumes in the PCR as deduced by Biometra for the amplification reactions.

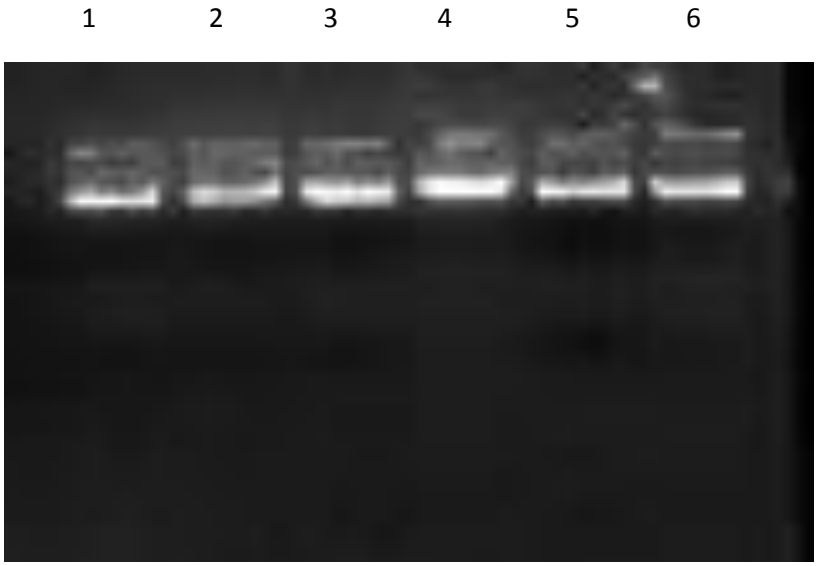


Figure 5.1: Representative example of the Agarose gel (0.7%) showing the presence of genomic DNA in the samples isolated using the White and Bartlet's method

Reverse and forward primers designed corresponding to the gene to be amplified was used for the amplification of the required region of the gene. The amplicons obtained after the amplification process were run on 1.7% of agarose gel. The agrose gel was stained with ethidium bromide (Etbr) which stacks between the basepairs of the DNA and is responsible for the illuminating when seen in the UV light. This acts as a test for whether the DNA has undergone amplification process or not.

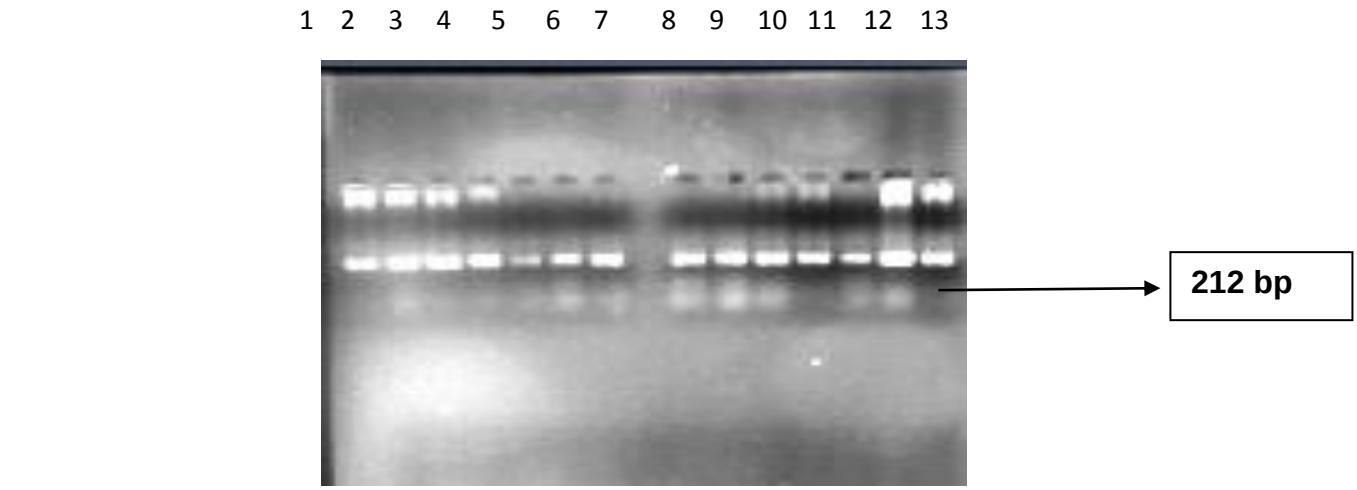


Figure 5.2: Representative example of the PCR product of *CCND1* rs 9344 with amplicon size 212bp.

The PCR products were then digested with their corresponding enzymes capable of excising the PCR product at the unique cleavage site which remains specific to a single nucleotide sequence and enzyme. The digestion reaction is again checked by running the sample in (2.5%) agarose gel for ***CCND1 rs9344.***

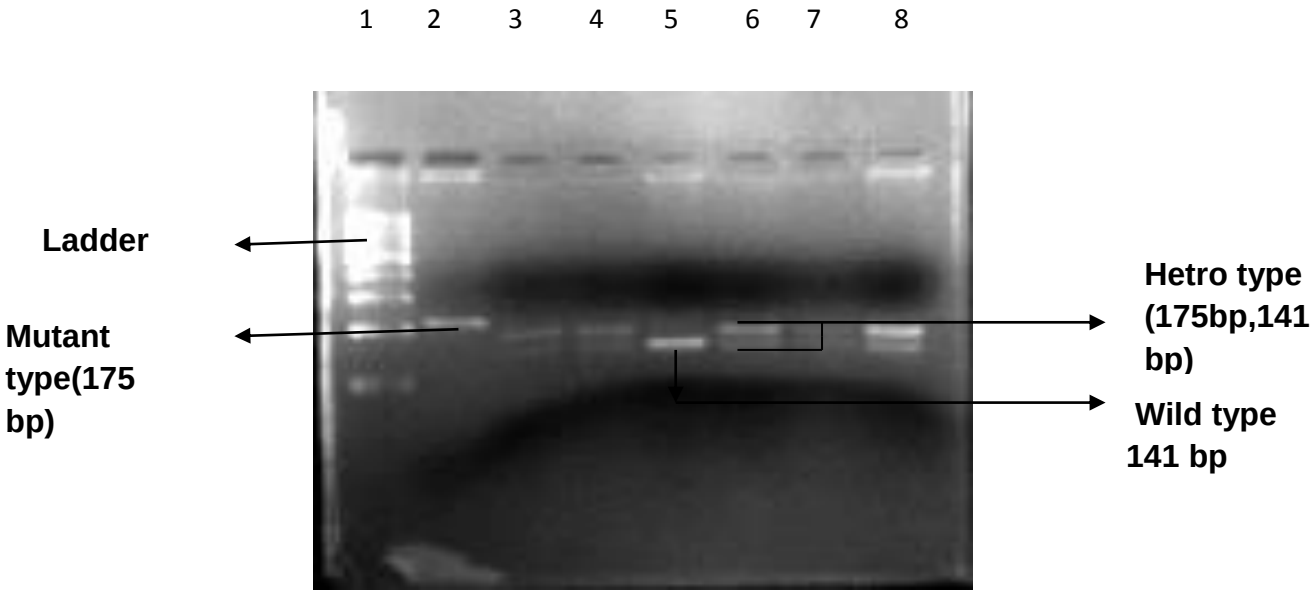


Figure 5.3- Representative example showing the restriction digestion of PCR product of *CCND1* rs 9344 showing the digested products (wild type: 141bp, 34 ; mutant type: 175bp,37bp; hetero type:175bp,141bp,37bp and 34bp) Lane 1:100 bp Ladder (G.Biosciences); Lane 5,: Wild type Lane 3,4,6,7,8:heterozygote type; Lane 2:Mutant type

Epidemiology for CCND1 rs9344

Table 5.1- distribution of demographic characteristics of cases and controls

`Variable	Total N	Cases, n (%)	Total N	Controls, n (%)	p-value
Age(years)	**353**		351		
Means± SD		57.49±10.70		53.14±10.38	<0.0001
Range		28-86		23-83	
Gender	**353**		351		
Male		305(86.40)		303(86.32)	0.00638
Female		48(13.59)		48(13.67)	
Smoking Status	**353**		351		
Smokers		279(79.03)		255(72.64)	0.0711
Non-Smokers		74(20.96)		96(27.35)	
Pack Years	**353**		351		
Mean± SD		34.89±34.013		24.23±18.90	<0.0001
Histologic Types	**353**				
ADCC		117 (33.14)			
SCLC		86 (24.36)			
SQCC		144 (40.79)			
Others		6 (1.69)			
TNM Staging	**353**				
I		3 (0.84)			
II		13 (3.68)			
III		163 (46.17)			
IV		147 (41.64)			
Unclassified		27(7.64)			
Tumor Size	**353**				
T1		16 (4.53)			
T2		43 (12.18)			
T3		89 (25.21)			
T4		161 (45.60)			

Tx Unknown		9 (2.54) 35 (9.91)			
Lymph Node Involvement N0 N1 N2 N3 N4 Unknown	**353**	 50 (14.16) 41 (11.61) 137 (38.81) 85 (24.07) 4 (1.13) 35 (9.91)			
Metastasis M0 M1 M Unknown	**353**	 178 (50.42) 138 (39.09) 2 (0.56) 35 (9.91)			
Objective response CR PR SD PD	**106**	 4(3.77) 47(44.33) 44(41.50) 11(10.37)			

SD= standard deviation

P<0.05 was considered statically significant

p-value were derived from pearson Chi-square test except age, student t-test was used for age. All p-value are two –sided

The characteristics of the study population are summarized in table no.1. Included in the study were 353 patients with lung cancer and 351 patients without lung cancer (control). Mean age with standard deviation for cancer patients 57.49±10.70 and 53.14±10.32 for controls (P<0.0001). Most of the study patients were male, 305(86%) in cases and 303(86.32%) in controls (P=0.00638) whereas female 48(13.59%) in cases and 48(13.67) in control were matched. Smoker showed high percentage among cases 279(79.03%) rather than the controls 255(72.64%). The

cancer patients had higher value of pack years 34±34.013 and control had lower pack years 24.23±18.90 (P<0.0001). According to the cell type SQCC was the most frequent tumor histology accounting for 144(40.78%) cases, while117 (33.14%) patients had Adenocarcinoma, 86 (24.36%) patients were reported small cell carcinoma, whereas only 6 (1.69%) patients had other type of tumor histology. Tumor staging according to UICC (Union for International Cancer control) were available for 353 patients; stage I, 3 (0.84%); stage II,13(3.68%); stage III, 163(46.17%); stage IV, 147(41.64%); 27(7.64%) patients had uncertain information on tumor stage. Tumor size T1, T2 and T3, had frequency of 16(4.53%) and 43(12.18%), 89(25.21%), respectively. T4 had high frequency of 161(45.60%) and one another Tx and unknown groups had very less frequency of 9(2.54%) and 35(9.91%) respectively. Lymph node involvement, N0, N1, N2, N3 and N4 had frequency of 50(14.16%), 41(11.61%), 137(38.81%), 85(24.07%),4(1.13%) respectively.35(9.91%) patients had uncertain information on lymph node involvement. No metastasis (M0) involvement had frequency of 178(50.42%) whereas distant metastasis (M1) involvement had frequency of 138(39.09%) another M and unknown groups had very low frequency 2(0.56%) and 35(9.91%) respectively.

Of the subjects, the objective responses are divided into 4 different categories i.e complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Here in further 4(3.77%) subject reported CR and 47(44.33%) PR we considered these two patients categories of good responders. Patients with 44(41.50%) SD and 11(10.37%) PD were classified as poor responders.

Table 5.2- distribution of demographic characteristics of overall survival patients.

Variables	**Total Numbers N=211(%)**
Overall Survival	**211**
Alive	67(31.75)
Dead	144(68.24)
Total KPS	**123**
Less than 70	23(10.90)
Greater than 70	100(47.39)
Unknown	88(41.70)
Total ECOG	**123**
0-1	56(26.54)
2	42(19.90)
3-4	25(11.84)
Unknown	88(41.70)
Total REGIMEN	**104**
1.Docetaxil-Cisplatin / Doccetaxil-Carboplatin	43(20.37)
2.Irinotecin-Cisplatin/ Irinotecin-Carboplatin	24(11.37)
3.Paclitaxin-Cisplatin/ paclitaxin-Carboplatin	8(3.79)
4.Pemetrexed-Cisplatin/Pemetrexed-Carboplatin	29(13.74)
Unknown	107(50.71)

For survival analysis, by the time of final data collection (Febuary 2015), 67(31.75%) patients were alive, and 144(68.24%) were dead out of all of enrolled patients. Clinical and pathological characteristics are also presented in table no.1 like objective response, Karnofsky Performance Status (KPS), Eastern Cooperative Oncology Group (ECOG) and chemotherapy regimen. Approximately 100 (81.30%) study subjects had ≥70 performance status and 23(18.69%) had ≤ 70 performance

status for KPS. Similarly 56(45.52%) had ECOG performance status of 0-1, 42(34.14%) had 2nd status and 25(20.32%) had 3-4 performance status.

As for the chemotherapy regimen cisplatin and carboplatin was combined with other drugs Docetaxi 43(41.3%) was combined with cisplatin and carboplatin. Further these Irinotecin 24(23.07%) and Paclitaxin 8(7.69) both were combined with above those platinum based chemotherapy. Lastly pemetrexed 28(26.92%) was also combined with cisplatin and carboplatin and others was given to 1(0.96%).

Table no.5.3a- Genotypic distribution and risk of lung cancer assosiated of lung cancer risk with overall genotype

	Cases N=353, n%	**Controls N=351, n%**	**OR[1] (95% of CI)**	**p-value**
GG(Reference)	62 (17.56)	84 (23.93)	1 (Reference)	
GA	241 (68.27)	206 (58.68)	1.63 (1.10-2.40)	**0.014**
AA	50 (14.17)	61 (17.37)	1.13 (0.68-1.88)	0.613
GA+AA	291 (82.24)	267 (76.06)	1.50 (1.03-2.19)	**0.032**
G **A**	182.5 172.5	187 164		
MAF	0.48	0.46		

OR and 95% CI were calculated by logistic regression analysis with *CCND1* GG genotype as the reference group and adjusted for age, smoking and gender.

CCND1 G870A gene polymorphism in patients and controls

The frequency distribution of *CCND1* G870A polymorphism is presented table no. 5.3a where the frequency of heterozygous genotype (GA) was higher in cases 241(68.27%) rather than the controls 206(58.68%). Homozygous mutant genotype (AA) was higher in control 60(17.37%) rather than the cases 50 (14.17%). Logistic regression analysis was conducted with adjustment for age, sex and smoking

parameter. To find the susceptibility towards lung cancer the heterozygous (GA) and combined heterozygous and mutant genotype (GA+AA) were associated with increased risk for lung cancer (OR=1.63; 95% of CI=1.10-2.40) and OR=1.50; 95% of CI=1.03-2.19) with significant p-values = 0.014 and 0.032 respectively for both genotypes.

Table 5.3b- Genotypic Distribution and association of *CCND1* gene rs9344 among the patients and controls with different histological forms of Lung Cancer

ADCC				
Genotype	**Cases N=117, n%**	**Controls N=351, N%**	**OR[1](95%of CI)**	**p-value**
GG	20(17.09)	84(23.93)	1 (Reference)	
GA	77(65.81)	206(58.68)	1.55(0.87-2.76)	0.13
AA	20(17.09)	61(17.37)	1.27(0.62-2.60)	0.50
GA + AA	97(82.90)	267(76.06)	1.47(0.84-2.58)	0.16
SCLC				
Genotype	**Cases N= 86, n%**	**Controls N=351, n%**	**OR[1] (95% of CI)**	**p- value**
GG	13(15.11)	84(23.93)	1 (Reference)	
GA	59(68.60)	206(58.68)	1.90(0.96-3.74)	0.06
AA	14(16.27)	61(17.37)	1.46(0.61-3.46)	0.38
GA + AA	73(84.88)	267(76.06)	1.77(0.91-3.41)	0.08
SQCC				
Genotype	**Cases N= 144, n%**	**Controls N=351, n%**	**OR[1] (95% of CI)**	**p- value**

GG	29(19.44)	84(23.93)	1 (Reference)	
GA	99(68.75)	206(58.68)	1.48(0.90-2.46)	0.12
AA	16(11.11)	61(17.37)	0.75(0.36-1.54)	0.43
GA +AA	115(79.86)	267(76.06)	1.30(0.80-2.13)	0.28

OR and 95% CI were calculated by logistic regression analysis with *CCND1* GG genotype as the reference group for Adenocarcinoma, SCLC and sqcc and adjusted for age, smoking and gender.

CCND1 gene polymorphism G870A in histological subtypes

Among the cases studied in the population , 33.14% of the cases were of those who were diagnosed with ADCC, (24.36%) had SCLC and (40.79%) were diagnosed with SQCC type of histology. On further stratification of histological groups of the population, on the basis of the genotypes it was found that 17.09%, 15.11%, and 119.44% of the individuals of ADCC, SCLC and SQCC respectively were of homozygous wild genotype whereas 65.81%, 68.60% and 68.75% had heterozygous genotype. The mutant homozygous genotype was shown by 17.09%, 16.27% and 11.11% of the population cases respectively. Due to low incidence of occurrence of the mutant genotype, we combined the homozygous mutant genotype with the heterozygous genotype under one single genotype. The count was found to be 82.90%, 84.88% and 79.86% respectively for ADCC, SCLC and SQCC, the different histological types of lung cancer.

The table 5.3b above shows the statistical values obtained for the association of G/A polymorphism in the *CCND1* gene with that of acquiring Lung Cancer. On stratifying the data on histologically different forms of Lung Cancer it was seen that individuals with heterozygote genotype having Adenocarcinoma did not show significant statistical value along with an approximate increase in the risk towards acquiring Lung Cancer (OR=1.55; 95%CI =10.87-2.76; p= 0.13).Individuals with heterozygote genotype having SQCC did not showcased a increase in the risk towards lung Cancer (OR= 1.48;95%CI=0.90-2.46;p=0.12).The SCLC subgroup amongst the population was partially competent in showcasing significant statistical values in the heterozygote genotypical subgroups (OR=1.90;95%CI = 0.96-3.76 ;p= 0.06) . The

joint combination of genotypes (mutants and heterozygotes) also did not show significant values in case of all the three subgroups of histological stratification. Among the all histological subtypes only in the small cell carcinoma (SCLC) heterozygous genotype GA show the higher risk of lung cancer (OR1.90, 95% CI 0.91-3.74) rather than the Adenocarcinoma and SQCC, but there was no significant p-value (0.06).

Table no.5.4a- Interaction of genotype and its association with lung cancer stratified according to smoking status

SMOKER				
Genotype	**Cases N= 279, n %**	**Controls N= 255, n%**	**OR[1] (95% of CI)**	**p- value**
GG	52 (18.63)	63 (24.70)	1(Reference)	
GA	190 (68.10)	150 (58.82)	1.57 (1.01-2.45)	**0.04**
AA	37 (13.26)	42 (16.47)	1.04 (0.58-1.87)	0.88
GA + AA	227 (81.36)	192 (75.29)	1.44 (0.94-2.21)	0.09
NON-SMOKER				
Genotype	**Cases N= 74 n%**	**Controls N= 96 n%**	**OR[1] (95% of CI)**	**p- value**
GG	10 (13.51)	21 (21.87)	1(Reference)	
GA	51 (68.91)	56 (58.33)	2.04 (0.84-4.97)	0.11
AA	13 (17.56)	19 (19.79)	1.49 (0.51-4.33)	0.45
GA + AA	64 (86.48)	75 (78.12)	1.93(0.81-4.50)	0.13

OR and 95% CI were calculated by logistic regression analysis with *CCND1* GG genotype as the reference group for smoker and non-smoker and adjusted for age and gender.

Table no.5.4b- Interaction of genotype and its association with lung cancer stratified according to pack years

LIGHT SMOKER P<25				
Genotype	**Cases N= 130 n%**	**Controls N= 152, n%**	**OR[1] (95% of CI)**	**p- value**
GG	29 (22.30)	43 (28.28)	1(Reference)	
GA	82 (63.07)	88 (57.89)	1.36 (0.76-2.41)	0.29
AA	19 (14.61)	21 (13.81)	1.25 (0.56-2.77)	0.57
GA + AA	101 (77.69)	109 (71.71)	1.34 (0.77-2.33)	0.29
HEAVY SMOKER P>=25				
Genotype	**Cases N= 149 n%**	**Controls N= 103, n%**	**OR[1] (95% of CI)**	**p- value**
GG	23 (15.43)	20 (19.41)	1(Reference)	
GA	108 (72.48)	62 (60.19)	1.73 (0.83-3.59)	0.13
AA	18 (12.08)	21 (20.38)	0.76 (0.31-1.86)	0.55
GA + AA	126 (84.56)	83 (80.58)	1.43 (0.71-2.87)	0.30

OR and 95% CI were calculated by logistic regression analysis with *CCND1* GG genotype as the reference group for heavy smoker and light smoker and adjusted for age and gender.

CCND1 gene polymorphism G870A in smoking status

Smoking is considered as one of the major contribution towards the development of lung cancer supported by various studies. In line with this *CCND1* Genotype was stratified according to smoking status as described in table 5.4a Total 534 smokers and 170 non-smoker included in case and control. The population recruited for the epidemiological study were categorized as Smokers and Non smokers to validate the association between cigarette smoking and the risk of developing Lung Cancer. 79.03% of the cases studied were smokers and 20.96% were non smokers. On the contrary 72.64% of the controls were smokers whereas 27.35% were non smokers. Smokers were further categorized as Heavy smokers and Light smokers on the basis of the pack years *i.e.* the no of cigarettes smoked in a day by an individual multiplied with the total number of years smoked. Heavy smokers were those with pack yrs > and equal to 20 and the light smokers were those for whom the values of pack yrs fell below 20.

Cigarette smoking showcases an additive effect on the polymorphism of *CCND1* in association with Lung Cancer .In line with the epidemiological studies; the above table5.4a displays statistically significant values for the Smokers in contrast to that of Non Smokers. Towards the heterozygous genotype GA in smoker presently significantly higher risk of lung cancer (OR=1.57, 95% CI=1.01-2.45, p-value 0.04) in compassion to other genotype. However It was found that heterozygous non-smokers has no significant effect on lung cancer(OR=2.04; 95%CI=0.84-4.97,p-value=0.11).

Now Smoker were further divided into heavy smoker and light smoker which was basically dependent on the pack years (table no.5.4b). Total 149 Heavy smokers having pack-years>=25 in cases and 103 in controls, whereas 130 light smokers having pack-years<25 in cases and 152 in controls. It was showed that heterozygous genotype(GA) heavy smokers associated with risk of lung cancer (OR=1.73, 95% CI=0.83-3.59) but it was not statically significant. There was no association between *CCND1* genotypes in light smoker.

Table no.5.5- *CCND1* Genotype and Clinical response to chemotherapy among lung cancer patients

Genotype	N=106 N (%)	Response of chemotherapy		OR (95% CI)	P - value
		CR+PR (%)	SD+PD (%)		
GG (reference)	17 (16.03)	7 (13.72)	10 (18.18)	1	
GA	73 (68.86)	38 (74.50)	35 (63.63)	0.64 (0.21-1.91)	0.71
AA	16 (15.09)	6 (11.76)	10 (18.18)	0.94 (0.21-4.13)	0.79
GA+AA	99 (93.39)	44 (86.27)	45 (81.81)	0.69 (0.23-2.01)	0.33

OR and 95% CI were calculated by logistic regression analysis with *CCND1* GG genotype as the reference group for responder and non-responder and adjusted for age,smoking and gender.

CCND1 gene polymorphism and chemotherapeutic response

The chemotherapeutic responses are divided into 4 different categories i.e complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). In the case 4(3.77%) patients shows complete response (CR) and 47(44.33%) showed partial response (PR). These two categories together were classified as good responders. Whereas patients with stable disease (SD) 44(41.50%) and progressive disease 11(10.37%) was classified as poor responders. Table no.5.5 shows the distribution of genotype according to response towards chemotherapy. As evidence from table none of the genotype could be classified in good responders . rather a trend would be inferred where mutant were better responder that heterozygous.

Table no.5.6 – Association analysis between clinical characteristics and overall survival of lung cancer patients after chemotherapy for univarient analysis

N=211		UNIVARIENT ANALYSIS		
	Genotype	**Median survival time**	**Log p-value**	**HR (95% of CI)**
OVERALL	**GG** **GA** **AA**	6.2 9.0 9.4	0.42	1 1.31(0.83-2.08) 1.25(0.68-2.29)
ADCC	**GG** **GA** **AA**	7.9 12.2 12.7	0.39	1 1.97(0.48-8.10) 2.17(0.43-10.90)
SCLC	**GG** **GA** **AA**	14.4 12.2 18.1	0.45	1 0.29(0.06-1.25) 0.33(0.05-2.20)
SQCC	**GG** **GA** **AA**	9.8 11.9 9.4	0.85	1 1.19(0.39-3.65) 0.90(0.17-4.68)

Overall survival and *CCND1* G870A gene polymorphism

The overall survival data were available for only 211 patients out of 351 patients. In the 211 patients 144 patients were dead and 67 patients were alive till 5 Feb 2015(table no.5). The frequency of heterozygous genotypes (GA) was higher 145(68.72%) rather than the homozygous genotype (AA) genotype 29(13.74%). The analysis for the overall survival was carried out using the Kaplan _maier analysis in (table no.5.6) order to find the correlation of the different genotypes of G/A870 SNP of *CCND1* gene, with the survival of 211 patients. However the median survival time for patients with rs9344 GG genotype had a longer overall survival rather than GA/AA genotype(median survival time,6.2months,9months and 9.4months, p-value=0.42).When stratified according to histological subtypes, there was no significant association seen between genotype and survival of ADCC, SQCC and SCLC patients(data shown in table).

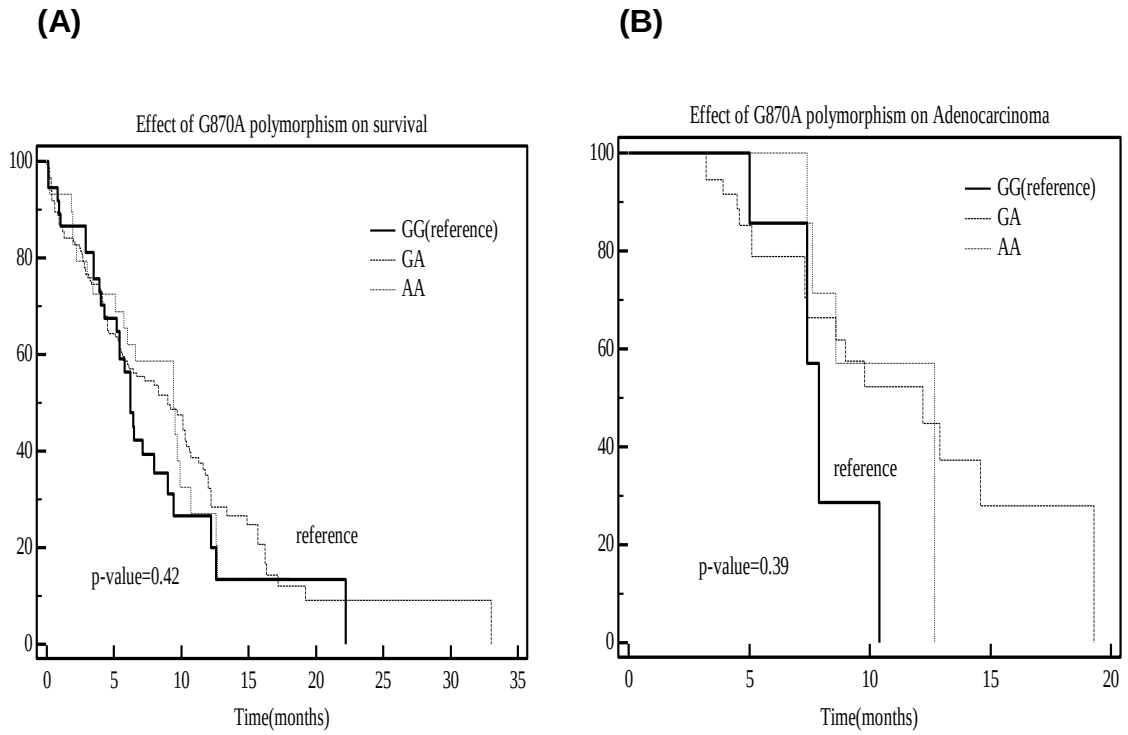


Figure 5.4 kaplan-meier survival curves of *CCND1* (rs 9344) for lung cancer patients from North Indian populationfor (A) overall genotype; (B) adenocarcinoma.

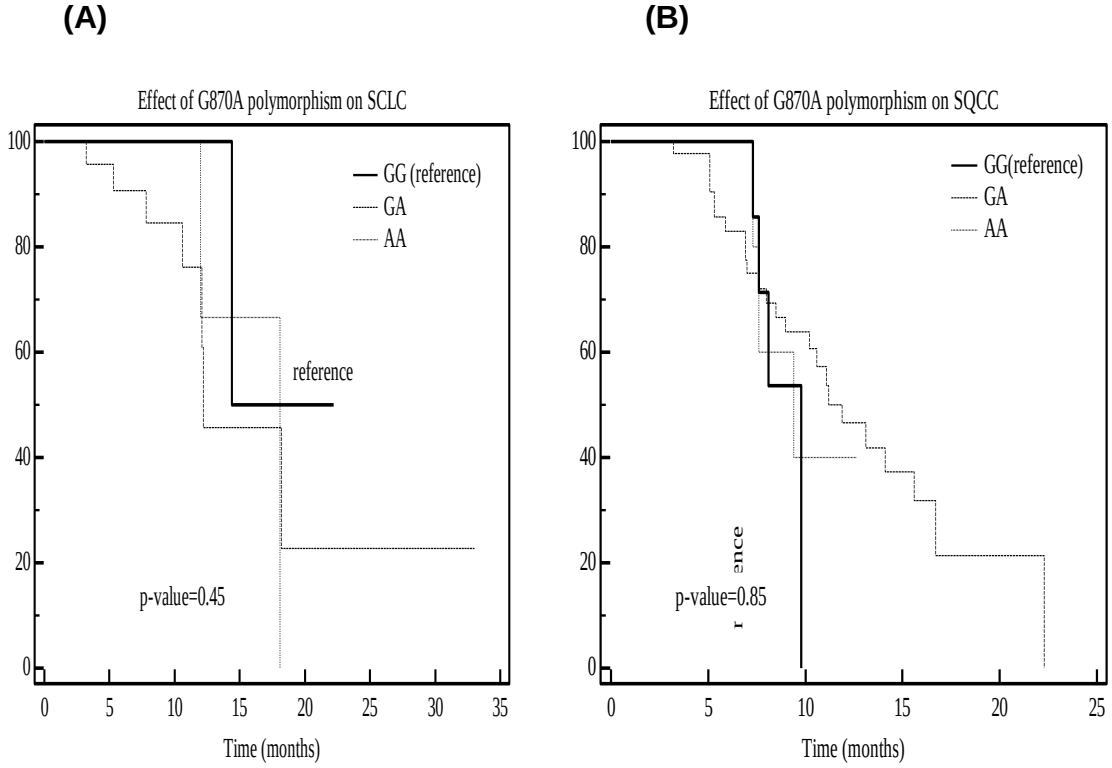


Figure 5.5 kaplan-meier survival curves for north Indian patients with lung cancer by *CCND1* rs 9344 for (A) SCLC; (B) SQCC.

TABLE NO.5.7- Association analysis between clinical characteristics (KPS, ECOG, REGIMEN) of lung cancer patients after chemotherapy for univarient analysis

		Median	P-value	HR(95% of CI)
KPS (0,1)	0(less than70)(reference) 1(greater than 70)	18.2 9.8	 0.14	- 0.47(0.15-1.13)
ECOG(0,1,2)	0 (0-1) (reference) 1(2) 2(3-4)	9.8 10.6 18.2	 0.28	- 1.16(0.58-2.32) 2.28(0.93-5.59)
REGIMEN (0,1,2,3,4)	0 (reference) 1 2 3 4	8 12.2 - 9.8 -	 **0.01**	- 2.46(1.07-5.67) 2.82(0.86-9.28) 1.93(0.85-4.41)

The median survival time in groups with a KPS of 0,1 were 18.2 and 9.8 months and ECOG of 0,1 and 2 were 9.8, 10.6 and 18.2 months respectively however, the log rank p values were not significant in both the cases. Median survival times in the groups with REGIMEN of 0, 1, 2, 3, 4 were 7.6, 12.2, and 9.8 months respectively with p- value 0.01. In this table only regimen was a significant independent predictor of survival (table no.5.7).

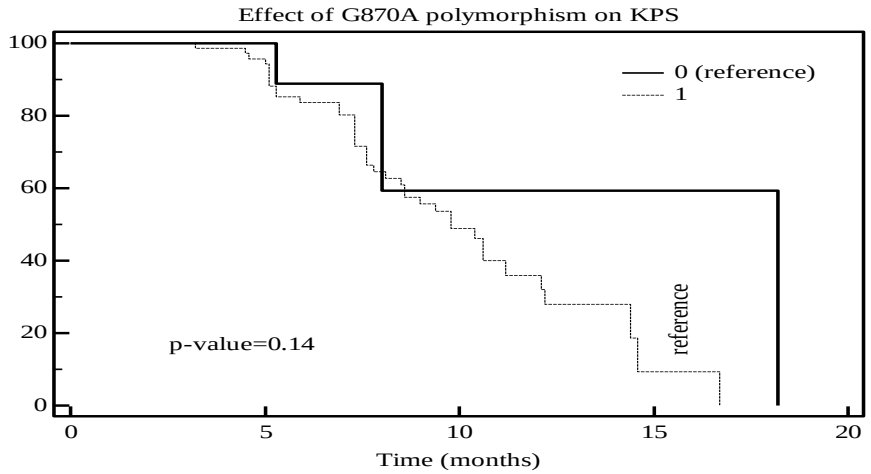


Figure 5.6- The relationship between the KPS (less than 70(0), greater than 70 (1)) in patients with inoperable lung cancer receiving platinum-based chemotherapy.

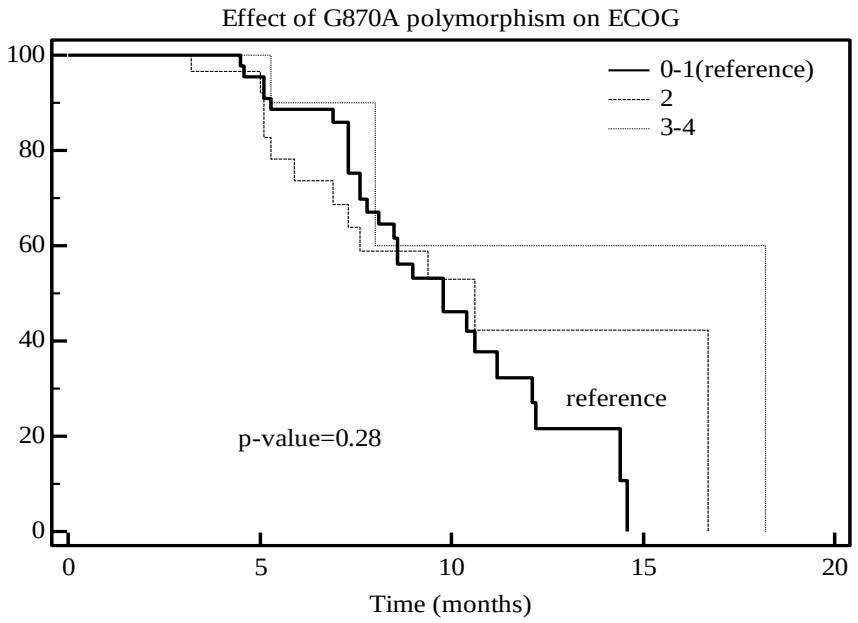


Figure 5.7-The relationship between ECOG performance status (0,1,2,3,4) in patients with inoperable lung cancer receiving platinum-based chemotherapy.

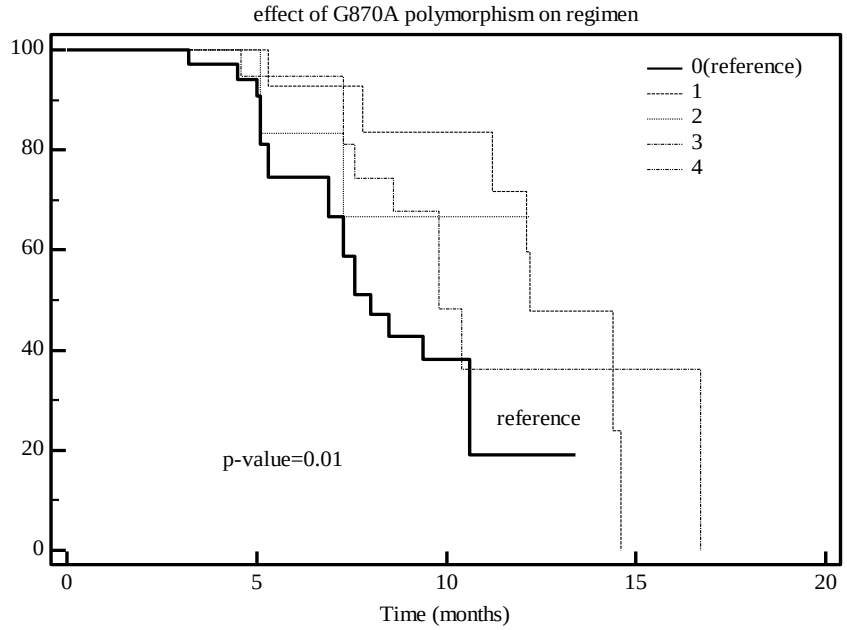


Figure 5.8-The relationship between REGIMEN (0,1,2,3,4) in patients with inoperable lung cancer receiving platinum-based chemotherapy

Table no.5.8- Association analysis between clinical characteristics and overall survival of lung cancer patients after chemotherapy for multivariate analysis on the basis of age, sex, smoking, stage,histology, kps, ecog and regimen

N=103	**MULTI VARIENT ANALYSIS(COX) (AGE, SEX, SMOKING, STAGE, HISTOLOGY, KPS, ECOG, REMINEN)**			
	N=103	**Genotype**	**HR(95% of CI)**	**P-value**
OVERALL	**103**	GG(reference) GA AA GA+AA	1 0.84(0.45 to 1.55) 1.07(0.63 to 1.82) 0.97 (0.64 to 1.46)	 **0.02** 0.52 **0.01**
MALE	**91**	GG(reference) GA AA GA+AA	1 0.94(0.46 to 1.92) 0.93(0.55 to 1.58) 0.99(0.64 to 1.51)	 0.06 0.34 0.05
FEMALE	**12**	GG(reference) GA AA GA+AA	1 0.00(0.00 to 10.1) - 2.37(0.04 to 119.64)	 0.005 - 0.14
SMOKER	**82**	GG(reference) GA AA GA+AA	1 0.88(0.41 to 1.91) 1.04(0.56 to 1.91) 1.06(0.63 to 1.62)	 0.10 0.44 0.11
NON-SMOKER	**21**	GG(reference) GA AA GA+AA	1 0.18(0.00 to 4.42) - 0.35(0.09to 1.34)	 0.06 - 0.10
ADCC	**35**	GG(reference) GA AA GA+AA	1 0.74(0.14to 3.85) 1.30(0.21 to 8.02) 0.82(0.34 to 2.19)	 0.12 0.54 0.10
SCLC	**23**	GG(reference) GA AA GA+AA	1 0.78(0.05 to 11.21) - 0.52(0.16to 1.70)	 **0.0016** - **0.01**
SQCC	**42**	GG(reference) GA AA GA+AA	1 0.38(0.12to 1.17) - 0.39(0.16 to 0.94)	 0.10 - 0.10
STAGE III	**56**	GG(reference) GA AA GA+AA	1 0.95(0.38 to 2.38) - 1.02(0.57 to 1.81)	 0.29 - 0.29
STAGE IV	**47**	GG(reference) GA AA GA+AA	1 1.22(0.38 to 3.85) 0.11(0.01 to 0.77) 0.98(0.52 to 1.86)	 **0.01** **0.01** **0.01**

KPS **Less than 70**	**13**	GG(reference) GA AA GA+AA	1 0.20(0.00to 13.66) - 1.08(0.15 to 7.50)	 0.14 0.11
KPS **Greater than 70**	**90**	GG(reference) GA AA GA+AA	1 0.89(0.43 to 1.86) 1.03(0.59 to 1.79) 1.02(0.65to 1.61)	 0.09 0.86 0.22
ECOG **(0 ,1)**	**51**	GG(reference) GA AA GA+AA	1 0.61(0.19 to 1.92) 1.15(0.60 to 2.20) 1.27(0.67 to 2.40)	 0.04 0.22 0.22
ECOG **2**	**37**	GG(reference) GA AA GA+AA	1 1.92(0.51 to 7.23) 17.69(0.00 to 10.1) 1.1561(0.50 to 2.64)	 0.59 0.004 0.64
ECOG **3 (3-4)**	**15**	GG(reference) GA AA GA+AA	1 0.37(0.03 to 3.90) - 0.56(0.14 to 2.21)	 **0.05** **-** **0.03**
REGIMEN Docetaxil- Cisplatin / Doccetaxil- Carboplatin	**42**	GG(reference) GA AA GA+AA	1 0.31(0.08 to 1.23) - 0.35(0.13 to 0.92)	 0.23 - 0.10
.Irinotecin- Cisplatin/ Irinotecin- Carboplatin	**24**	GG(reference) GA AA GA+AA	1 0.74(0.18 to 3.02) - 0.70(0.21 to 2.31)	 **0.0003** **-** **0.0023**
Paclitaxin- Cisplatin/ paclitaxin- Carboplatin	**7**	GG(reference) GA AA GA+AA	1 - - -	 - - -
PemetrexedCispl atin/Pemetrexed- Carboplatin/ others	**29**	GG(reference) GA AA GA+AA	1 2.57(0.116to 56.91) 9.57(0.77 to 129.74) 2.10(0.59 to 7.97)	 **0.07** **0.13** **0.04**

Adjusted for age, gender, smokingstage, performance status (KPS, ECOG), and REGIMEN, were treated as indicator variables

The prior section talks about the survival probability of different genotypes of *CCND1.* In line with this to be more precise we have also analysed the death rate. In multivariate Cox regression analysis (table no.5.8) was used to find the hazards ratio adjusted for various predictors such as age, sex, smoking, stage, histology,

KPS, ECOG, regimen in order to find out the effect of the various genotypes on the death probability of 103 patients which is given in terms of HR. Cox probability analysis reported that heterozygous, mutant and combined (GA, AA and GA+AA) genotype were not associated with survival compared with mutant (GG) genotype (HR=0.84, 95% of CI=0.45-1.55, P=0.02; HR 1.07, 95% of CI 0.63-1.82,P=0.52; HR 0.97, 95% of CI 0.64-1.46,P=0.01) so that rs9344 GA and GA+AA genotype were not a significant independent predictor of survival. When male patients, heterozygous and combined (GA and GA+AA) genotype were not showing association with survival compaired with mutant (GG) genotypes in cancer pateints (HR=0.94, 95% of CI=0.46-1.92, P=0.06; HR=0.99, 95% of CI=0.64-1.51, P=0.05). In the case of histological subdivision, only SCLC patients with GA genotype show the significant survival with less hazard value (HR=0.78, 95% of CI=0.05-11.21, p-value=0.001). For case of staging IV patients with heterozygous (GA) genotype is showed a marginal effect on their survival with significant value (HR 1.22, 95% of CI 0.38-3.85, p-value=0.01) but in case of stage III patients none of the genotype is showing any effect on their survival with significant value. Lung cancer patients undergoing chemotherapy show a response towards drugs give. This response is counted in medical in form of KPS and ECOG. Moving towards in our study we tried to find correlation of KPS(Karnofsky performance status) with survival but could not find any significant improved. We further performed an ECOG to determined the performance status of patients, ECOG (0 to 1) patients with heterozygous (GA) genotype were not associated with survival compared with mutant (GG) genotype (HR=0.61,95% of CI=0.19-1.92,p-value=0.04). ECOG 2 with heterozygous (GA) genotype was a significant independent predictor of survival with no significant value (HR=1.92, 95% CI 0.51-7.23, P0.59) but in ECOG range of 3-4 pateints with heterozygous (GA) and combined genotype (GA+AA) were a significant independent predictor of survival (HR 0.37, 95% CI 0.03-3.90, p-value=0.05; HR=0.56, 95% of CI=0.14-2.21, p-value=0.03). It was observed that the lung cancer patients with combined genotype (GG+AA) receiving the (PemetrexedCisplatin /Pemetrexed-Carboplatin) platinum based chemotherapy showed a significant death rate (HR=2.10, 95% of CI=0.55 - 7.97, p-value=0.04).

CHAPTER-6

DISCUSSION

The present data showed the association between *CCND1* G870A polymorphism and risk of lung cancer is based on the given information to detect significant differences. This study is based on 351 cases and 353 controls subjects, our results point towards there might be an overall increased risk between the *CCND1* G870A polymorphism and lung cancer risk. In the subgroup analysis like histology there was no association between the *CCND1* G870A polymorphism and the lung cancer risk. But in the smoking status there was increased risk between heterozygous genotype of *CCND1* and lung cancer with significant value.

Several studies said that lung cancer shows a multifunctional role in disease that was caused by gene to gene interaction, genetic manipulation, gene proliferation and apoptosis(*Pharoah PD et al;2004; Guan P et al; 2011)*many genetic manipulation factors played important role in cancer that will be identified by SNP searching tools. This SNP tools search the genetic variations in cancer gene and susceptibility (*Hoeijmakers JH*; 2001; *Wood RD et al; 2001*). It has been whispered that *CCND1* over expression promotes tumorigenesis through its role in proliferation. It is also involved in the cell cycle progression. Current research demonstrated that polymorphisms in cell cycle related genes are of great importance in lung cancer (*Buch SC et al; 2012*; *LiangYWet al; 2013*; Yue Wet al**;** *2011***;** *Camidge DR; 2010).*

CCND1 gene located on the 11q13 position chromosome and is encoded by 5 exons and 4 introns (*Ceschi M et al; 2005).* Approxe 100 SNPs are identified in this genomic region for *CCND1* gene. These SNPs are not affecting the any amino acid substitution. they only affect the *CCND1* expression because it lies at final introns and exons boundary and it altered the exon 4 splice donor site (*Knudsen et al; 2006*). Cyclin D1 lives into two isoforms cyclin D1a and cyclin D1b. Cyclin D1b grasps an altered C- terminus, characterized by 14 amino acids unchanging by a read through into intron 4. With the whole exons 5 prearranged sequences beging restored. Therefore cyclin D1b need reduced require for nuclear export. Accordingly cyclin D1b has been shown localization, with an increased transforming capability evaluated with the full length D1a and was supposed a nuclear oncoprotein (*Ceschi M et al; 2005*).

In our study we have observed a significant difference in distribution of the frequency of the hetero genotype for the *CCND1 gene* between the cases and controls. Furthermore, our data clearly demonstrates a significant association between the *CCND1* heterozygous genotype (GA) and combined genotype (GA+AA) genotype and lung cancer risk and this became more pronounced in SCLC with GA genotype. The present findings are consistent with previous studies conducted in various ethnic groups. Prior studies conducted in North Indian populations have shown that the increased risk of lung cancer is associated with heterozygous (AG) alleles and combined (AA+GA) genotypes was associated with significantly increased risk in smokers only. The presence of A allele might be associated with SCLC (*Sobti et.al; 2006*). Similarly *Quiling et al., 2003* reported that AA genotype was associated with a significant increased risk for lung cancer in Chinease population. Also previous studies conducted in different Asian populations such as South-West Asian (*McKay et al*), Ghanaian (*McKay et al),* Kenyan (*McKay et al)* and Chinese (*McKay et al*). All these studies reported that the AA allele was associated with cancer risk. The contradictory result has also shown with the *CCND1* polymorphism in different cancers like breast cancer *(Krippl et al ; 2003*), cardiac cancer (*Wang et al 2003b*), colorectal cancer (*McKay et al; 2000*), gastro intestinal cancer, head and neck cancer *(Wong et al; 2003*), hepatocellular cancer (*Zhang et al; 2002).*

Among various studies done on susceptibility of *CCND1* G870A polymorphism towards lung cancer there have been contradictory output with different ethinic population. Some in breast cancer studies have reported no association between cyclin D1 gene G870A polymorphism and breast cancer risk in different populations including Australian (Grieu et al; 2003), Malaysian (*Naidu et al ; 2008*) and German (*Justenhoven et al; 2009*) population. Whereas current meta analysis conducted by *lu et al (2003*) on the association between G870A polymorphism and the risk of breast cancer showed there was an increased risk of breast cancer for carriers of A allele in Caucasians but not in an Asian population. Whereas another contradictory result also shown by another meta analysis that showed A allele and AG genotype of cyclin D1 was associated with increased breast cancer risk.

Inline one more studies done with colorectal cancer in Caucasian population which reported that the A allele is associated with increased risk in colorectal carcinoma (CRC). However (GG) genotype was shown to be associated with higher risk in one of the study done on Singapore patients for CRC (*Yi hong et al; 2005*). Whereas other ethnic population like southern Chinese (*Dang et al; 2002*), Taiwan Chinese *(Zhang y et al; 2002*) and Whites (*Mc kay et al; 2000)* showed the same result that AA alleles is associated with the higher risk in CRC patients.

On the other hand *Deng et al, 2002* found that *CCND1* G870A polymorphism was associated with susceptibility to nasopharyngeal carcinoma, because the GG and AG genotypes in NPC patients were significantly higher than those in normal controls. But *Shih et al; 2012* reported that the G allele of *CCND1* G870A seemed to be a protective factor for NPC in Taiwan of China. *Catarino et al. 2006* also reported that individuals carrying the *CCND1* GG genotype had increased risk for the development of NPC. Therefore, it is worthy to make a meta-analysis to evaluate the interrelationship between cyclin D1 G870A polymorphism and NPC.

Same with USA patients AA alleles was associated with an increased risk of head and neck cancer in squamous cell carcinoma (*Catarino et al. 2006).*

In our studies the heterozygous GA of smoker was found to be significant with higher risk of lung cancer rather the other genotypes was not significant with no risk of cancer. It was also found that their no effect of non- smoker with *CCND1* genotypes. But it was different another study done by *Sobti et al 2006,* he said that AG and AG+GG both genotype of smoker was found to be significant with higher risk of lung cancer. One more study done on smoker habits that was responsible for non-small cell lung cancer patients.

Now Smoker was divided into heavy smoker and light smoker that was basically depend on the packing years. It was showed that heavy smokers had GA genotype associated with risk of lung cancer but it was not statically significant. There was no association between *CCND1* genotypes in light smoker. This subdivision is not done by another population with lung cancer patients.

One study on esophageal and lung carcinoma actually found that G/A870 genotype is not correlated with the overall survival rate. But the overall expression of the cyclin D1 may be associated in EAC with survival but not in non-small-cell carcinoma (NSCLC) *(Vanika K Gupta et al; 2008)*. Another study on survival done by china on

breast cancer it found that *CCND1* A870G polymorphism was inversely associated with overall and disease free survival. Further one more study done on non-small cell carcinoma which suggest that A/G870 genotype had no significant difference with survival analysis(*Oliver Gautschi et al; 2006).*

So far, several researches focusing on the role of *CCND1* gene in lung cancer that was conflicted result will be found with G870A and A870G polymorphism. Some studies said that AA genotype is a risk of lung cancer and some said GG genotype is a risk of lung cancer (*Changxi et al; 2013*). Dominant G870A polymorphism has been many studies of lung cancer in different populations. However the results are different or contradictory in different types of cancer. Overall survival with *CCND1* gene G870A polymorphism is not given with lung cancer patients with clinico-pathological data.

Chapter-7

CONCLUSION

In Cyclin D1 varients statistically significant values (High ORs validate the association of G870A SNPs with the risk of Lung cancer.

On subgrouping the population on the basis of histologically different forms of Lung cancer *CCND1 rs9344 did not* show any results. Patients from the ADCC subgroup also didn't show maximum risk towards Lung cancer. Trends changed for rs 9344 where patients with SCLC showed the highest association with the endangerment with partially significant.

The results validate the interrogation about Cigarette smoking association with Lung cancer. Smokers showcased a higher risk towards the advent of Lung cancer in case of rs9433, whereas non-smoker did not show any association with the lung cancer.

We report that the G870A polymorphism does not correlate with overall survival.

CHAPTER-8

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APPENDIX-1

1. 0.5M EDTA: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionised water, and then adjusted the pH to 8.0 by 1 M sodium hydroxide. Sterilized the solution by autoclaving.

2. 10% SDS: Dissolved 1g of SDS in 10ml of deionised water.

3. 100mM Tris-Cl (pH 8.0): Dissolved 0.32g of Tris-Cl in 10 ml of deionised water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilized the solution by autoclaving.

4. 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.

5. 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionised sterile water and kept at 4 C overnight.

6. 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionised water. Sterilized the solution by autoclaving.

7. 5X TBE buffer: Dissolved 54g of Tris base and 27.5g of boric acid in 980ml of double distilled water and then added 20ml of 0.5 EDTA. Sterilized the solution by autoclaving.

8. Ethidium Bromide (10mg/ml): Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.

9. Magnesium chloride (MgCl2) (100mM): Dissolved 0.41gms of MgCl2 in 20ml of deionised water and sterilized by autoclaving.

10. Sucrose (1M): Dissolved 3.41 g of sucrose in 10 ml of deionised water and sterilized by autoclaving.

11. TE buffer (pH 8.0): Added 1ml of 100mM Tris-Cl (pH 8.0) and 200 µl of 0.5M EDTA solution to 8.8 ml of deioinsed water. Sterilized the solution by autoclaving.

12. Triton X- 100 (10%): Took 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.