Prognostic role of p53 codon 72 polymorphism in North Indian lung cancer patients treated with platinum based chemotherapy

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

Under the guidance of
Dr. Siddharth Sharma
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Submitted by

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DECLARATION

I, the under designed, hereby declare that the research work presented in the M.Tech dissertation entitled **"Prognostic role of p53 codon 72 polymorphism in North Indian lung cancer patients treated with platinum based chemotherapy"** has been carried out by me under the supervision and guidance of Dr. Siddharth Sharma, 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 examining body in India/elsewhere.

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CERTIFICATE

This is to certify that dissertation entitled, **"Prognostic role of p53 codon 72 polymorphism in North Indian lung cancer patients treated with platinum based chemotherapy"** submitted by Ms. Ankita Kumari in partial fulfillment 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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Date: 15th July, 2016

Place: Patiala Ankita Kumari

ABSTRACT

Title: Prognostic role of p53 codon 72 polymorphism in North Indian lung cancer patients treated with platinum based chemotherapy. *p53* gene is located at chromosome 17p13.1. p53 protein plays an important role in maintaining genomic stability by undergoing cellular arrest during DNA damage. Depending on the extent of DNA damage it either performs DNA repair or cellular apoptosis. Objectives: To investigate the association of $p53Arg^{72}Pro$ with susceptibility and improved survival status in lung cancer patients. Results: lung cancer patients with mutant (CC) genotype found to have significant association with overall survival (HR=0.65; 95% CI=0.45-.0.95; p=0.03) and on adjustment made with Cox regression model for different parameters association was nearly significant (HR=0.68; 95% CI=0.63-1.16; p=0.06). Female lung cancer patients with any (GG, GC (HR=0.08; 95% CI=0.02; p=0.0005) and CC (HR=0.21; 95% CI=0.06-0.67; p=0.0085)) genotype showed significant improve in overall survival showing $p53Arg^{72}Pro$ genotype. Conclusion: The present study found no significant association of $p53Arg^{72}Pro$ genotype and other epidemiological and clinic-pathological parameters towards increased susceptibility and pathological development of lung cancer in different provinces of North Indian population. However nearly significant association was found for overall survival of lung cancer patients with mutant (CC) genotype. Females lung cancer patients with any (GG, GC and CC) genotype showed significant improve in overall survival showing $p53Arg^{72}Pro$ genotype to be the independent prognostic factor for females in North Indian population.

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Abbreviations

1. MDM2- Mouse double minute 2
2. BAX- Bcl2-Associated X Protein
3. PCR- Polymerase Chain Reaction
4. RFLP- Restriction Fragment Length Polymorphism
5. BSA- Bovine Serum Albumin
6. OR- Odds Ratio
7. CI- Confidence Interval
8. KPS- Karnofsky Performance Status
9. ECOG- Eastern Cooperative Oncology Group
10. OS- Overall Survival
11. CR- Complete Response
12. PR- Partial Response
13. SD- Stable Disease
14. PD- Progressive Disease
15. SQCC- Squamous Cell Carcinoma
16. SCLC- Small Cell Lung Cancer
17. ADCC- Adenocarcinoma
18. NSCLC- Non-small Cell Lung Cancer
19. TNF- Tumor Necrosis Factor
20. NES- Nuclear Export Sequence
21. CDK- Cyclin Dependent Kinase
22. DISC- Death Inducing Signaling Complex
23. APAF- Apoptotic Protease Activating Factor
24. TRAIL- TNF-related apoptosis inducing ligand
25. SNP- Single Nucleotide Polymorphism
26. LC- Lung Cancer

INTRODUCTION

Lung cancer is one of the deadliest and most common malignant neoplasms all over the world. It has reached epidemic proportion in both women as well as men. Smoking is accepted as major cause for lung cancer with relative risk of 2.64 for beedi and 2.3 for cigarette smokers (overall 2.45) (Behra *et al*., 2015). Along with smoking and environmental factors, occupational hazards pose a higher risk of lung cancer. Cytogenetics study reveal that chromosomal structure aberrations including mutations leads to the activation of protooncogenes as *ras/myc* or inactivation of tumor suppressor gene as *p53* leads to cancer. So, to understand the mechanism of cancer and its diagnosis, genes involved in signaling pathways need to analyze. The most promising study was made on tumor suppressor gene named *p53* (George *et al*., 2011).

p53 is a nuclear transcription factor located at chromosome 17p13.1 which undergoes activation during DNA damage or abnormal cell proliferation. It consists of five functional domains which include N-terminal domain for transcription activity, Proline rich domain for Mdm2 binding, central core domain for DNA binding, Oligomerization domain for NLR (Nuclear Localization Signals) and C-terminal domain for its own negative regulation (Sakiyama *et al*., 2005). Under normal condition low level of *p53* is maintained in cytoplasmic region however if the level increases mdm2 (E3 ubiquitin protein ligase) mediates the degradation of the p53 protein. Mdm2 is a negative regulator for *p53* and is over expressed in many cancers and is mainly reported in non-small cell lung carcinoma (Ozaki *et al*., 2011, Han *et al*., 2008). It restricts aberrant cell proliferation during DNA damage, oncogenic activation and hypoxia by arresting cell cycle at G1/G2 phase. It also eliminates damage or infected cell by apoptosis. So, overall p53 maintains the integrity of whole genome. Under normal condition it is expressed at extremely low level in cytoplasmic region. When DNA damage occurs it accumulates in nucleus through post translational modification like phosphorylation by *ATM/chk2* and acetylation by P300/PCAF which leads to its activation. Nucleotide sequence which binds to its promoter region reduces its binding affinity for promoter region of *p21* which is cell cycle regulating gene and Gadd45 (DNA repair associated gene) senescence signals through Mdm2 binding activates p53 along with $p21^{waf1/cip1}$ leads to *p53* dependent senescence (George *et al*., 2011).

p53 consists of 20kb of DNA with 11 exons which on translation gives 3kb mRNA with 1179 bp ORF producing 53kDa protein with five functional domain. Among the five functional domain the mutational site for *p53* inactivation during lung cancer is proline rich domain (102 aa-292 aa). It binds DNA as homotetramer with 1 Zn per subunit. However, proline residue at polymorphic site induces higher level of cellular arrest. Furthermore considering proline rich domain which is the site of polymorphism related to our study we found that it induces apoptotic activity by enhancing the transcription of pro-apoptotic genes like BAX (Hamp *et al*., 2003).

Inactivation of *p53* activity due to chromosomal aberration like mutation has been reported in different form of cancer like breast, colorectal, cervix and lung (Vijayaraman *et al*., 2012, Katkoori *et al*., 2009, Fan *et al*., 2000). *p53* regulates carcinogenic compound named benzo-[a]-pyrene present in tobacco smoke by formation of PAH *o*-quinones (Yu *et al*., 2002, Shen *et al*., 2006).

Along with environmental factors and occupational hazards polymorphism of DNA damage response gene is associated with lung cancer susceptibility and among them most common is polymorphism found in p53 at codon 72 of exon 4 with two allele encoding for either Arg (GCG) to Pro (CCC). Such polymorphism found in proline rich domain of *p53* which is required for its growth suppression and apoptotic activity (Figueras *et al*., 1996, Ozeki *et al*., 2000, Dumont *et al*., 2003).

Several Studies revealed that the presence of arginine residue at the polymorphic codon 72 of p53 gene is responsible for apoptosis as it induces mitochondrial localization of p53 and activates oncogenes to prevent malignancy in cells. It also shows more resistance toward Mdm2 mediated degradation with significantly longer half-life (Hampt *et al*., 2008).

Thus, polymorphism observed at $p53Arg^{72}Pro$ abolishes its functions and leads to malignancy which is mainly due to alteration in *p53* primary structure. Mutant type allele (*Pro/Pro*) was found to have risk of lung cancer particularly squamous cell carcinoma in different studies (Vijayaram *et al*., 2012). It has been reported that patients with proline allele were more susceptible towards lung cancer due to up regulation of Mdm2 (Katkoori *et al*., 2009).

Several studies have been evaluated the association of polymorphism in *p53* gene with lung cancer predisposition, but the findings have been contradictory. It has been reported that patients with proline allele were more susceptible towards lung cancer due to up regulation of

Mdm2 (Ozeki *et al*., 2000). Several studies suggested *p53Arg^{72}Pro* polymorphism is associated with lung cancer susceptibility in Indian population (Tilak *et al*., 2003, Sreeza *et al*., 2008, Gupta *et al*., 2001).

Some studies have established a positive role of the mutant (*Pro/Pro*) form of the *p53Arg^{72}Pro* polymorphism towards increasing risk for lung cancer (Popanda *et al*., 2007) (Murata *et al*., 1996, Wang *et al*.,1999, Hiraki *et al*.,2003, Zhang *et al*., 2006, Jung *et al*., 2008, Chua *et al*., 2010). Similarly a few studies have implicated the role of the heterozygous (*Arg/Pro*) cancer (Popanda *et al*., 2007) (Murata *et al*., 1996, Wang *et al*.,1999, Hiraki *et al*.,2003, Zhang *et al*., 2006, Jung *et al*., 2008, Chua *et al*., 2010) genotype of *p53* gene towards modulating the risk of lung cancer. On the other hand, various studies have contradicted the above findings (Pierce *et al*., 2000, Piao *et al*., 2011, Liu *et al*., 2013).

It has been also observed that *p53Arg^{72}Pro* variant can play a role in predicting the prognosis of lung cancer patients undergoing chemotherapeutic treatment, toxicity and survival (Steels *et al*., 2001). SNPs variants are an emerging tool for predicting prognosis and survival of lung cancer patients rather than m-RNA profiling. SNP genotyping is a non-invasive technique unlike m-RNA analysis it does not requires any tissue biopsy which is slightly complicated in case of lung tumors (Ryo *et al*., 2004). *p53Arg^{72}Pro* polymorphism has been studied extensively to establish it as a prognostic biomarker for lung cancer. However, the findings have not been consistent where in some studies have found a positive correlation of variant genotype of *p53Arg^{72}Pro* with the overall survival of lung cancer patients (Mitsudomi *et al*., 2000, Mahesh *et al*., 2012). On the other hand few reports contradict the same (Tagawa *et al*., 1998, Hu *et al*., 2005, Matakidou *et al*., 2007).

So, the aim of the present study is to understand the relevance of *p53Arg^{72}Pro* polymorphism for lung cancer susceptibility and analyze its role as predictive biomarker among North Indian Population.

REVIEW OF LITERATURE

2.1. *p53* gene

p53 is a nuclear transcription factor locating on human chromosome 17p13.1 p53 gene which consists of 20kb of DNA with 11 exons which on transcription gives 3kb mRNA. Furthermore mRNA encompasses 1179bp ORF producing 53KDa protein named p53.

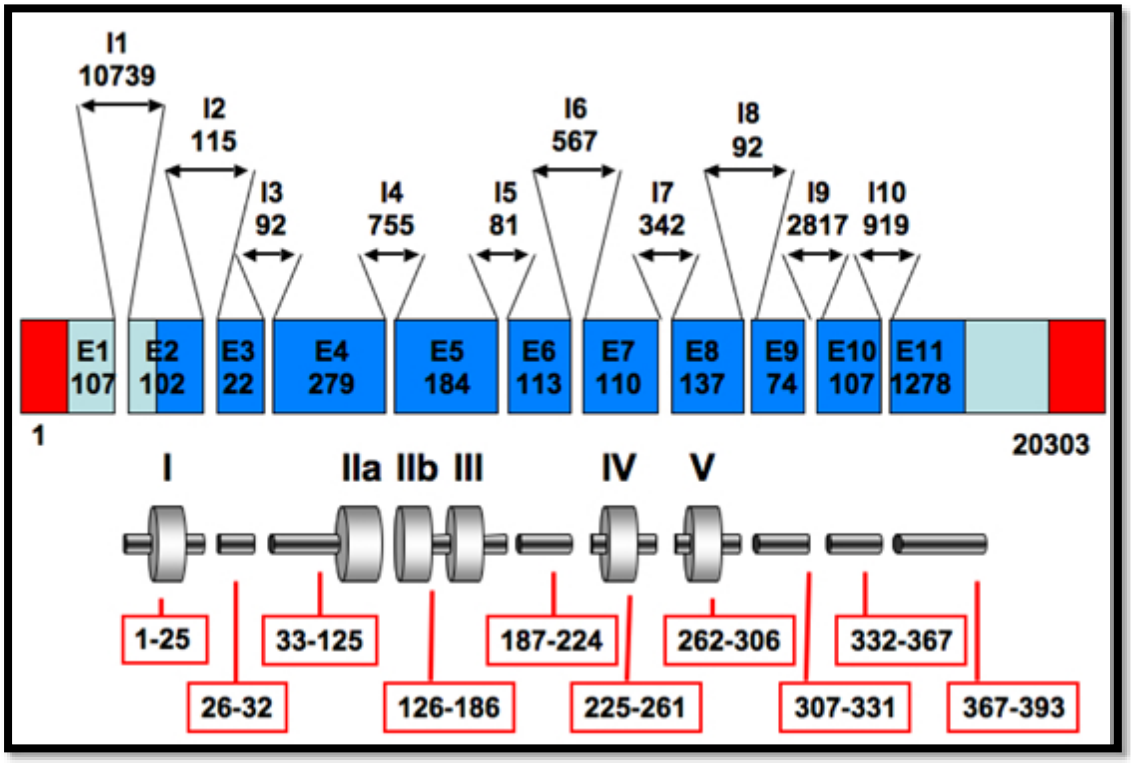


Fig.2.1. Showing structure of *p53* gene (George *et al.*)

2.2. p53 protein

It is 393 aa consisting polypeptide with 5 functional domains which are as:

1. N-terminal domain (1-43)aa - involved in transcriptional activity

2. Proline rich domain (63-97)aa - involved in Mdm2 binding and having multiple copies of PXXP sequence (X is other aa)
3. Central core domain (100-300)aa - plays important role in sequence-specific DNA binding.
4. Oligomerization domain (320-360)aa - involved in p53 oligomerization and nuclear localization as consists of NLR (Nuclear Localization Signals)
5. C-terminal domain (364-393)aa - regulate the ability of core DNA to block the binding domain by its latent conformational changes.

When the interaction between C-terminal and core DNA domain is disrupted by post-translational modification like phosphorylation/acetylation, binding domain become active and induces transcriptional activity (George *et al*., 2011).

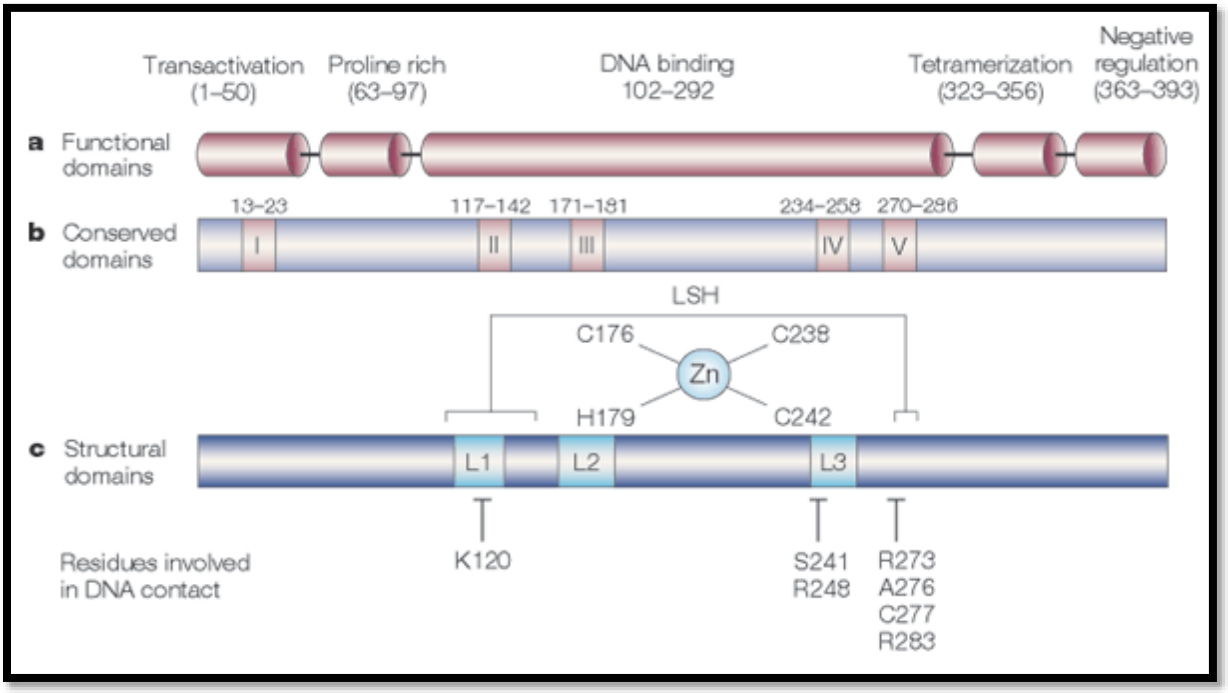


Fig.2.2. Showing structure of p53 protein (George *et al.*)

2.3. Functions of p53 protein

1. It undergoes cellular arrest during DNA damage and undergoes DNA repair to prevent transmission of damaged genetic material which requires factor named E2F (binds to promoter region of proto-oncogenes c-myc and c-fos required for mitosis).
2. It performs cellular apoptosis if damage is severe and works as an emergency brake.

3. It acts as a tumor suppressor factor which control uncontrolled growth of cells and prevent tumorogenesis thus maintaining the overall stability and integrity of human genome.
4. It is a potent transcription factor which after activation activates other factors like *p21* involved in cell cycle growth.

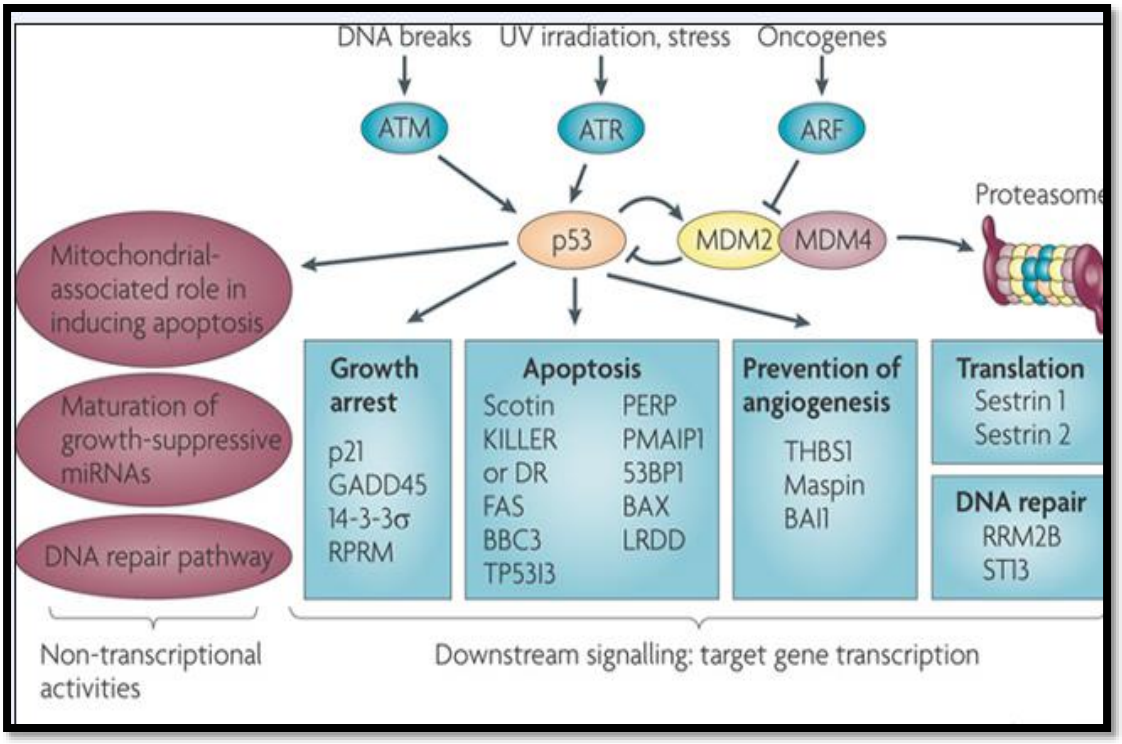


Fig.2.3. Showing different functions of p53 protein (pinterest.com pin/531002612290744199)

2.4.Signaling pathways of p53

Under normal condition p53 is latent (do not interfere with cell progression and survival) and is activated by stresses like hypoxia, heat shock, exposure to nitric acid etc. Its activity induced by oncogenic protein (Myc, Ras, beta-catenin).When activated p53 get transported to nucleus or else remain inactivated in cytoplasm.

2.4.1. p53 –Mdm2 loop:-

Mmd2 is an oncogene which regulates *p53* activity. Mdm2 inactivates *p53* by binding to its transactivation domain while *p53* binds to Mdm2 gene and activates its transcription. Mdm2

expression increases during abnormal growth like carcinogenesis which inactivates p53 to cause excessive cell proliferation. DNA damage activate protein kinase (*ATM*, *CHK2*) to phosphorylate at either of three places (Ser15, Thr18, Ser20).When *p53* binds to Mdm2 these sites remain unphosphorylated (Normal cells).

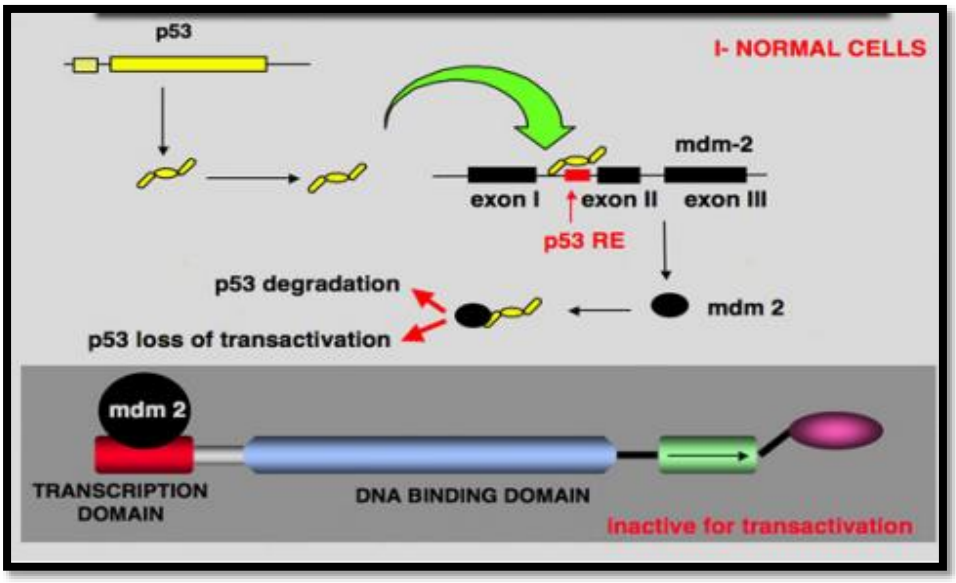


Fig.2.4. Showing interaction of *p53* and Mdm2 (George *et al.*)

2.4.2. *p53*-Cell cycle arrest

E2F1–3 via conserved domain in the N-terminus binds with p53. Cyclin and p53 and compete for binding to E2F1–3. E2F and *p53* interaction is important in regulating cell cycle as E2F1–3 binding a region in *p53* overlapping its C-terminal nuclear export sequence (NES) due to which *p53* retained in the nucleus. Ser315-phosphorylated *p53* in the nucleus destabilizes the *p53* tetramer which activates NES and increases export of *p53* into the cytoplasm. Therefore, the binding of E2F1–3 to *p53* could mask the NES, preventing nuclear export (Braithwaite *et al*., 2006).

E2F more effectly regulates *p53* at the G/S transition when E2F1–3 levels. Similar consequences observed when *p21* $^{waf1/cip1}$ is required to perform its G1 checkpoint function which on excess cause cell cycle arrest. The expression of *p21*$^{Waf1/Cip1}$ is highest at the G1/S transition and lowest in S phase. *p21* $^{waf1/cip1}$ is a common mediator of p53-dependent G1 cell cycle arrest

during any cellular damage. It induces arrest by blocking cyclin E mediated phosphorylation of Rb and release of E2F.

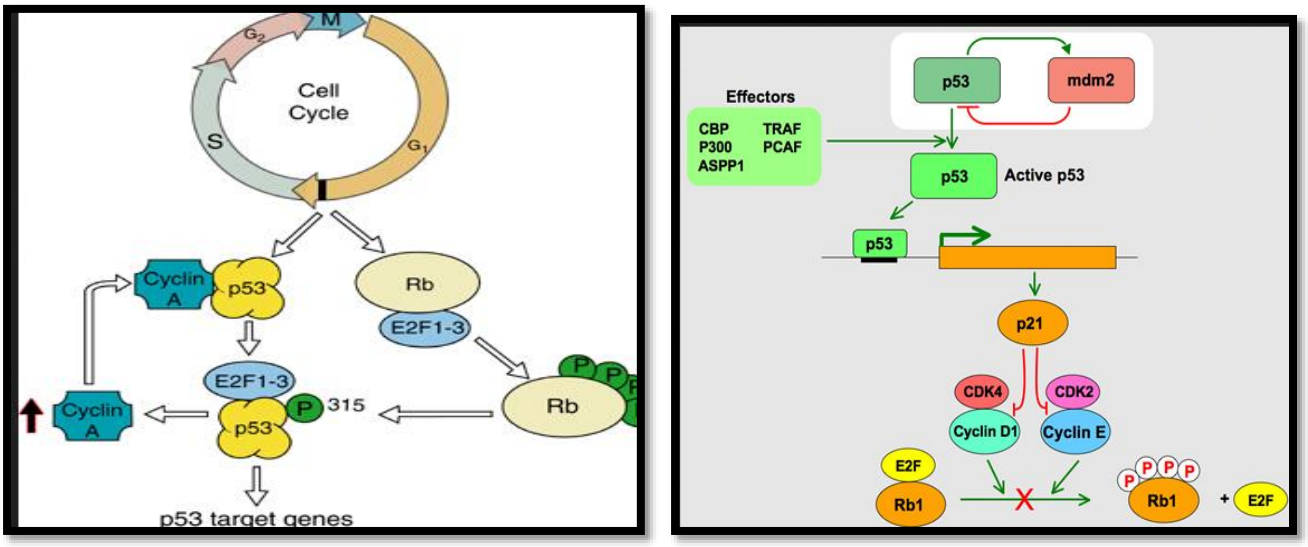


Fig.2.5. Showing involvement of *p53* in cellular arrest during abnormal cellular growth (PubMed/15735718)

2.4.3. ***p53*-Apoptosis**

p53 is involved both the apoptotic signaling pathways activating aspartate-specific cysteine proteases (caspases) mediating apoptosis. The extrinsic pathway involves `death' receptors through the formation of the death-inducing-signaling-complex (DISC), leading to a cascade activation of caspases (caspase-8 and caspase-3) and induces apoptosis. It is helpful in mitochondrial depolarization and release of cytochrome c from the mitochondrial inter-membrane space into the cytoplasm (Hampt *et al*., 2008).

a) Extrinsic Pathway

p53 activates transmembrane proteins named : Fas, DR5 and PERP where Fas is a transmembrane receptor and member of the TNF. It is activated by binding of its ligand, FasL, *DR5/KILLER*, the death-domain-containing receptor for TNF-related apoptosis-inducing ligand (TRAIL) is induced by p53 in response to DNA damage and promotes cell death.

b) Intrinsic Pathway

p53 target genes includes *Bax*, *Noxa*, *PUMA* (Bcl-2 family). In response to stress activation, Bax forms a homodimer and releases cytochrome c from the mitochondria which results in caspase-9 activation.

I. Apoptosome activation by *p53*

The release of cytochrome is promoted by *p53* via induction of BH3-only proteins. It induces *APAF-1* expression through a response element. *p53* activation is achieved by E2F-1, which induces *APAF-1* expression and activates p53 in an ARF-dependent manner .

II. Caspase activation

p53 leads to the activation of the caspase cascade by both transcription-dependent and -independent mechanism. In response to DNA damage, p53 directly induces *caspase-6* expression through a response element within the third intron of the gene.

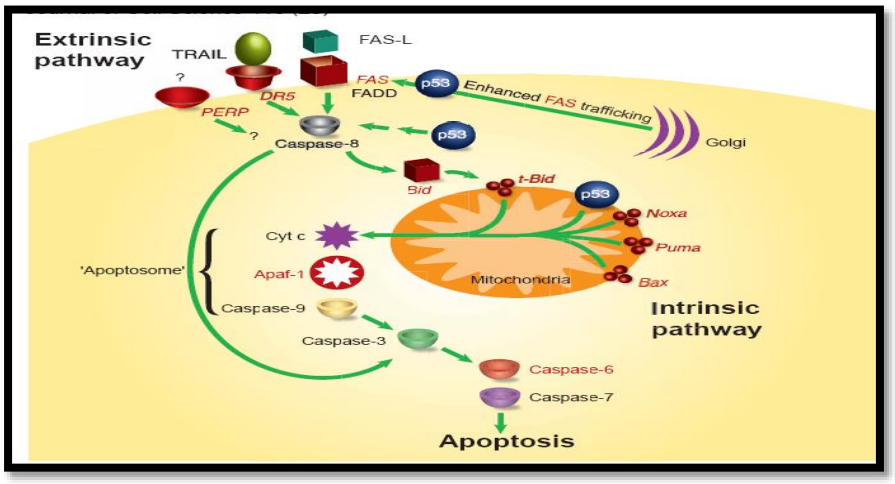


Fig.2.6. Showing involvement of *p53* in cellular apoptosis during cellular damage (Haupt *et al.*)

2.5.Overview of lung cancer

Cancer occurs when cells in the body undergo a mutation that causes them to produce quickly and wildly. In lung cancer malignant cells invade and demolish healthy cells in the lung tissues and air passages. During initial stage it neither causes any symptoms nor shows on an X-ray and

begins as pre-cancerous changes. Eventually cancer cells leads to a tumor. And as it grows, it impedes the proper functioning of the lungs cells. Cancerous cells after splitting from original tumour travel through the bloodstream, and form get locates in other parts of the body. This process is called metastasis.

2.5.1. Types of Lung Cancer

There are two main types of lung cancer on the basis of its morphology appears under a microscope.

A. Small Cell Lung Cancer (SCLC)

Small cell lung cancer starts in neuroendocrine cells which forms the air tubes that lead to the lungs (the bronchi) and the cells in lung tissue. It grows very quickly and produces large tumors which may leads to metastasis and accounts for 15% of all lung cancers. Small cell lung cancer is mainly seen in 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.

B. Non-Small Cell Lung Cancer (NSCLC)

The cancer cells are larger, and the cancer is slower rising than small cell lung cancer and accounts for 85% of all lung cancer cases. NSCLC consists of three subtypes:

I. **Squamous cell carcinoma** (epidermoid carcinoma) accounts for 25 to 30% of all lung cancer and 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.

II. **Adenocarcinoma** It forms in the mucus-producing (outer) part of the lungs and accounts for about 40%. It develops slowly and is the most common type of lung cancer in women and nonsmokers.

III. **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.

2.5.2. Epidemiological factors

1. **Smoking tobacco**

Smoking tobacco (cigarettes and beedis) is the main cause of lung cancer. Tobacco smoke contains many harmful chemicals that can cause cancer. 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.

2. **Occupational exposure to chemical carcinogens**

In general the risk of developing lung cancer is even higher for people who smoke. Occupational exposure like chemicals increases the risk of lung cancer:

- arsenic and inorganic arsenic compounds
- polycyclic aromatic hydrocarbons (PAHs)
- silica dust and crystalline silica

Polycyclic aromatic hydrocarbons (PAHs) increase the risk of lung cancer. People can have occupational exposure to PAHs through different sources like chimney sweeping, coal gasification, coke and aluminium production.

2.5.3. Signs and symptoms of lung cancer

- A prolonged cough that worsens over time
- Problem in breathing
- Reduced weight
- Coughing up blood

2.5.4. Diagnosis of lung cancer

a) X-ray
b) CT scan

c) MRI scan

I. Cytology test

2.6. *p53Arg^{72}Pro* Polymorphism and lung cancer risk

Genetic polymorphism is elucidated as a sample of interest comprising of a polymorphism observed in at least 1% of the population whereas a single nucleotide polymorphism (SNP) is an alteration in a single nucleotide present in the DNA sequence. The nucleotide variation in the coding region of a gene alters protein activity due to the amino acid substitution. For understanding the effect of polymorphism an approach is made, where the gene and the nucleotide polymorphism both are simultaneously selected and put to statistical and analytical studies for analyzing lung cancer susceptibility in the North India population.

p53 is a master regulator of several transcription factors as it binds to large network of transcription factors via its transactivation domain. The genes involved with *p53* networking having mainly perform DNA repair to cause either cellular arrest or apoptosis during DNA damage which associate it with mechanism of carcinogenesis. Cellular arrests cause activation of p53 protein by kinases and acetylase whereas down regulated by mdm2 to maintain its proportion.

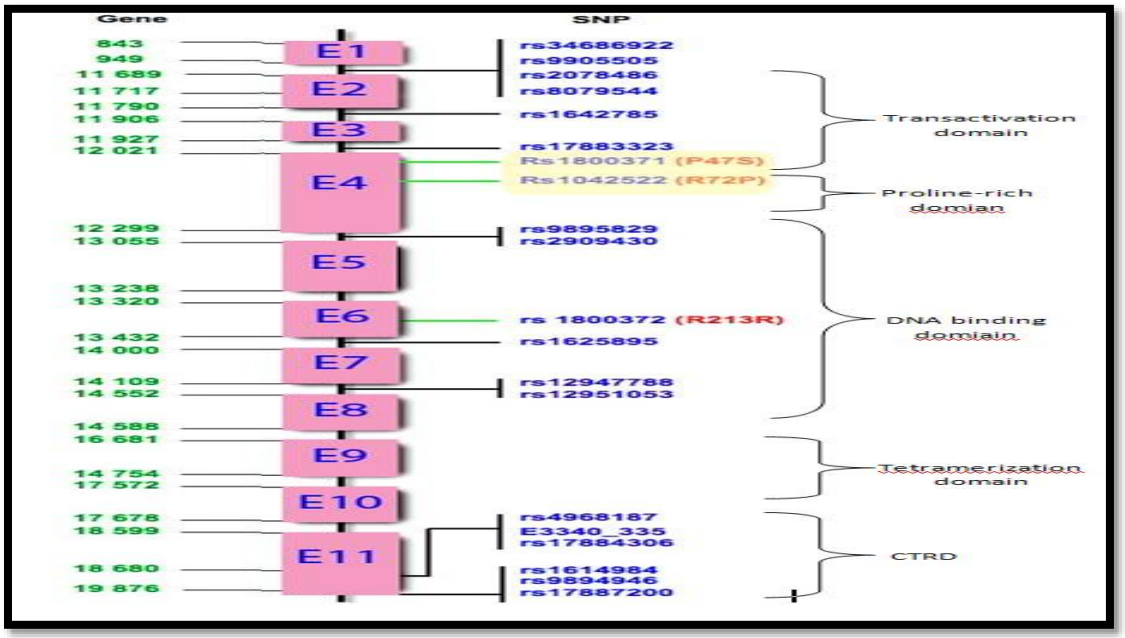


Fig.2.7. Showing different (Single Nucleotide Polymorphism) SNPs found at different domains of *p53* (ssmedicalresearch)

2.7. *p53Arg^{72}Pro* polymorphism mechanisms and splicing

Among all the polymorphism most common is at exon 4 encodes either Arginine (CGC) or Proline (CCC) residue which effect primary structure as well as biological function of *p53*. It is located in exon 4 and occurs in proline rich domain of p53 hence plays important role in inducing apoptosis to inhibit tumorogenesis. Most of the tumor associated mutation located in this region. Thus Arg residue at codon 72 is more efficient in G1 cell cycle arrest. Both amino acids at this region serve different biological activity in p53 protein (George *et al*., 2011, Sakiyama *et al*., 2005, Ozaki *et al*., 2011).

1. Proline (CCC) residue is found to be strongly associated with p53 interaction with TFIID factors TFII32 and TFII70 so having increased transcriptional capacity. It also induces G_1/G_2 cellular arrest and activates cell cycle checkpoints.
2. Arginine (GCG) residue was found to be strongly associated with translocation of p53 to mitochondria for apoptotic activity. It shows better interaction with mitochondrial protein like GPRF and CRMI for nuclear export.

Different studies were made in different regions towards risk of lung cancer associated with *p53Arg72Pro* genotype where some studies in consistent while some contradicts the fact that *p53Arg72Pro* polymorphism associated with risk towards lung cancer as given in Table 2.1.

Table 2.1. Representing studies on risk of lung cancer along with its associated genotype in different ethnicities.

Area	Parameters	Types of cancer	Risk associated genotype	Results	References
New Zealand (320 women and 348 men)	Smoking and Sex	Lung Cancer	*GG*	*GG* genotype more susceptible to smoking induced lung impairement *CC* genotype more resistant to lung damage by cellular arrest and DNA repair.	Hancox *et.al.* 2012

Baltimore (103 women and 206 men)	Smoking and Sex	Lung Cancer	*CC*	*GG* and *CC* genotype showed higher risk of smoking related LC with pack years 0-50 yrs.	Mechanic *et.al*.,2005
China (764 ADCC patients)	Smoking and Histology	Lung Cancer	*CC*	*GG* and *CC* genotype showed higher risk of smoking related ADCC.	Yang-Wu Ren *et.al*.,2013
Asian (7,929 cases) (Meta-analysis)	Smoking, Age and Gender	Lung Cancer	*GC*	*GC* shows significant risk towards LC in dominant model. No significant risk associated to smoking, Histology and Sex.	Wang *et.al.*,2013
China (Meta-analysis)	Smoking, Age and Gender	Lung Cancer	*GC* and *CC*	No significant risk associated to smoking, Histology and Sex.	Wang *et.al*., 2013
Europe (Meta-analysis)	Smoking, Age and Gender	Lung Cancer	*CC*	*CC* shows strong association with SQCC.	Hung *et.al*., 2008
China (Meta-analysis)	Age, Sex and Histology	Lung Cancer	*CC*	*CC* shows strong association with LC specially ADCC.	Yan Li *et al*., 2008
Asian and American (Meta-analysis)	Age, Sex and Histology	Lung Cancer	*CC*	*CC* shows strong association with LC specially ADCC and SQCC.	Hung *et al*., 2008
Taiwan (194 cases)	Age, Sex and Smoking	Lung Cancer	*CC*	Higher risk of LC in female patients having *CC* genotype Increased OR at earlier age.	Wang *et al.*, 1999
Brazil (144 men and 56 women)	Age, Sex and Smoking	Lung Cancer	-	Frequency of smokers having NSCLC is higher in *CC* genotype with no significant association.	Homa *et al.*, 2008
China (250 men and 110 female)	Age, Sex and Smoking	Lung Cancer	*CC*	*CC* genotype significantly associated with risk for smokers and ADCC subtype.	Lin *et al.*, 2012

Iran (114 men and 27 women)	Age, Sex and Smoking	Lung Cancer	*GG* and *CC*	ADCC more prevalent in Females with CC and SCLC in male smokers having GG genotype.	Nadji *et al*., 2006
North Poland (188 men and 52 women)	Age, Sex and Smoking	Lung Cancer	*CC* and *GC*	Increased risk of NSCLC in *GC* and *CC* genotype.	Amelia *et al*., 2005
North Western Mediterranean (136 men and 11 women)	Age, Sex and Smoking	Lung Cancer	-	No significant risk associated after adjustment made for all parameters and *CC* genotype overrepresented.	Figuerus *et al.*, 1996
Chile (111 cases)	Age, Sex and Smoking	Lung Cancer	*GC* and *CC*	Significant protective effect showed by *GC* genotype. *GC* and *CC* genotype having higher risk of lung cancer.	Dante *et al*., 2009
Massacusettes (264 men and 217 women)	Age, Sex, Histology and Smoking	Lung Cancer	*GC* and *CC*	Strongly associated risk showed by *CC* and *GC* genotype for ADCC.	Fan *et al.*, 2000
Europe (314 men and 91 women)	Age, Sex, Histology and Smoking	Lung Cancer	*GC* and *CC*	Strongly associated risk showed by *CC* genotype. *GC* and *CC* genotype having higher risk of SQCC.	Odilia *et al.*, 2007
North Spain (272 men and 36 women)	Age, Sex, Histology and Smoking	Lung Cancer	CC	*CC* genotype having higher risk of SCLC and stage-IV NSCLC.	Rubio *et al*., 2008
Massacusettes (576 men and 680 women)	Age, Sex, Histology, Tumor (grade and stage) and Smoking	Lung Cancer	CC	*CC* genotype having higher risk of ADCC and no association was found between polymorphism and tumor grade and stage.	Liu *et al*., 2001
North Spain (272 men and 36 women)	Age, Sex, Histology and Smoking	Lung Cancer	CC	CC showed significant risk of LC in heavy smokers.	Rubio *et al*., 2008

Similarly investigations were performed to analyze the association of different *p53Arg*^{72}*Pro* genotypes towards cancer susceptibility with various parameters as summarized in Table 2.2.

Table 2.2. Representing studies on risk of different form of cancer along with its associated genotype in different ethnicities.

Area	Parameters	Types of cancer	Risk /Protective genotype	Results	References
South West Iran (145 cases)	Age and Sex	Colorectal cancer	GC	*GC* genotype found to have higher risk.	Doosti *et al.*, 2011
Isfahan (180 cases)	Age and Sex	Colorectal cancer	GC	Frequency of *GC* genotype found to have higher but no significant risk.	Dastjerdi *et al.*, 2008
Japan (157 cases)	Age and Sex	Breast cancer	CC	*CC* genotype showed poor disease free survival	Toyama *et al*., 2007
Massachusetts, Hampshire and Wisconsin (1,490 cases)	Age and Sex	Breast Cancer	-	No risk associated with breast cancer.	Spragne *et al*., 2007
North Carolina (264 cases)	Age and Sex	Ovarian cancer	-	No significant risk association.	Joellen *et al.*, 2009
Bangkok (2,000 cases)	Age and Sex	Cervical cancer	*GG*	*GG* genotype was associated with risk of cervical cancer in HPV16 infected women.	Jira *et al*., 2013
Poland (152 cases)	Age and Sex	Endometrial cancer	*CC*	Patients with *CC* genotype showed reduced cancer risk.	Zajac *et al*., 2013
China Meta-analysis (3,704 cases)	Age and Sex	Hepatocellular carcinoma	*GC*	Female patients with *GC* genotype were more susceptible to cancer.	Surong *et al*., 2013

Different studies found association of *p53Arg*^{72}*Pro* genotype with increased overall survival in lung cancer patients while some studies predicted increased mortality related to *p53Arg*^{72}*Pro* genotype as mentioned in Table 2.3.

Table 2.3. Representing studies on different forms of cancer along with its survival associated genotype in different ethnicities.

Area	Parameters	Types of cancer	Overall survival and prognosis associated genotype	Results	References
India (422 cases)	Age, Sex, Histology and Smoking	Lung cancer	*GC*	*GC* was found to be overall independent prognostic factor	Sreeja *et al*., 2008
Denmark (9,219 cases)	Age, Sex, Histology and Smoking	Lung cancer	*GC*	Increase survival of *GC* genotype	Orsted *et al*., 2008
China (126 cases)	Age, Sex, Histology and Smoking	Lung cancer	-	No significant association was found	Chen *et al*., 2010
Europe (619 female cases)	Age, Sex, Histology and Smoking	Lung cancer	-	No significant association was found	Matakidon *et al*., 2007
Taiwan (114 cases)	Age, Sex, Histology and Smoking	Lung cancer	*GC* and *CC*	*GC* showed high MST. *CC* genotype showed poor prognosis and short survival period in early stage patients.	Wang *et al*., 1999
Japan (178 cases)	Age, Sex, Histology and Smoking	Lung cancer	-	No significant association was found	Taqawa *et al*., 1998
Taiwan (266 cases)	Age, Sex, Histology and Smoking	Lung cancer	*CC*	Stage I patients showed increased hazard ratio	Chun *et al.*, 2009
Japan (Meta-analysis)	Age, Sex, Histology and Smoking	Lung cancer	*GC* and *CC*	Variant genotype showed good prognosis for patients with ADCC	Mitsudomi *et al*,. 2000
India (170 cases)	Age, Sex, Histology and Smoking	Lung cancer	-	No significant association was found and patients of SQCC subtype showed least survival median.	Mahesh *et al*., 2012
France (Meta-analysis)	Age, Sex, Histology and Smoking	Lung cancer	*CC*	Higher death risk for NSCLC patients with stage III and IV	Steels *et al.*, 2001
Turkey (50 female cases)	Age, Sex, Histology and Smoking	Lung cancer	*CC*	ADCC subtype was positive prognostic factor	Babacan *et al.*, 2014

AIM OF STUDY

The present study focused on the following aspects which correlates its association towards lung cancer susceptibility.

1. To study various epidemiological and clinic-pathological parameters which were associated with lung cancer risk in different provinces of North Indian population.

2. To find out the genotypic distribution of *p53Arg^{72}Pro* genotypes in lung cancer cases and controls.

3. To analyze the association between *p53Arg^{72}Pro* polymorphism and different epidemiological and clinic-pathological parameters towards lung cancer risk.

4. To evaluate the correlation between *p53Arg^{72}Pro* polymorphism and different epidemiological and clinic-pathological parameters to find its effect on overall survival of lung cancer patients.

MATERIALS AND METHODS

4.1. Study Subjects and sample collection

420 lung cancer cases and 420 controls were recruited in our study. The samples were collected from Department of Pulmonary Medicine, PGIMER Chandigarh with written consent obtained from each patient. A questionnaire was filled to obtain various details about subjects like age, gender, histology and smoking status. It also includes details about smoking status like cigarette/beedi with indication of cumulative smoking exposure, pack years etc. Pack year was calculated by following formula:-[cigarette or beedis/20] × years smoked. Medical records include histology, TNM classification, Clinical staging, Primary tumor size, Lymph node involvement and metastasis. Ethical clearance was also made from PGIMER to precede our study. 5 ml of blood was collected from each subject. 420 cases included in our study were newly diagnosed with lung cancer. All the patients were histopathologically diagnosed as NSCLC, ADCC, SQCC, SCLC and LC. 420 unrelated individual with no evidence of lung cancer were included and were enrolled for health check up. Each control was pair matched by sex, age and smoking parameters with that of cases (lung cancer patients).

4.2.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) (Sodhi *et al*., 2013).

- **Requirements**

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

- Isopropanol
- TE buffer

➢ **Preparation of Buffers**

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

Table 4.1. Representing requirements for preparation of washing buffer	
Stock Concentration	**Working Concentration**
1 M Sucrose	320 mM Sucrose
100% Triton X-100	1% Triton X-100
100 mM Magnesium chloride	5 mM Magnesium chloride
100 mM Tris-HCl (pH-8.0)	10 mM Tris-HCl (pH-8.0)

Table 4.2. Representing requirements for preparation of lysis buffer	
Stock Concentration	**Working Concentration**
1 M Tris HCl (pH-8.0)	400mM Tris HCl (pH-8.0)
10 % SDS	1 % SDS
0.5 M EDTA	60 mM EDTA
5 M Nacl	150 mM Nacl
10 mg/ml Proteinase-K	100 µg/ml Proteinase-K

➢ **Procedure of DNA isolation**

1. 5 ml of venous blood was collected from each patients and 5 ml of Washing Buffer (1.6 ml of 1M Sucrose, 0.5 ml of Triton X- 100, 0.25ml of $Mgcl_2$, 0.5 ml of 100 Mm of Tris-HCl and 0.26ml of water) was added. Centrifuged it at 3500 rpm for 5 minutes.
2. Supernatant was discarded and equal volume of washing buffer was added to pellet and centrifuged again. This step was repeated thrice.
3. Pellet obtained was dissolved in Lysis Buffer (2 ml of 1 M Tris HCl, 0.5 ml of 10% SDS, 6 ml of 0.5 M EDTA, 0.15 ml of 5 M Nacl, 0.05 ml of 10mg/ml of Proteinase-K and 1.7 ml of water) and incubated at 47^{o}C overnight.

4. Equal volume of Phenol: Chloroform: Isoamyl alcohol (PCI) in the ratio 25:24:1(25ml of Phenol, 24ml of Chloroform and 1ml of Isoamyl alcohol) was added and mixed properly.
5. It was centrifuged for 10 min at 4^{o}C at 8000 rpm. Upper aqueous layer was taken and PCI was added in fixed proportion and centrifuged.
6. Aqueous layer obtained was taken and equal volume of Chloroform: Isoamyl alcohol (24:1) was added.
7. It was centrifuged for 5 min at 6500 rpm and upper aqueous layer was taken.
8. Equal volume of chilled Isopropanol was added to aqueous layer and was stored at -80^{o}C.
9. It was centrifuged at 12,000 rpm for 10 min at 4^{o}C.The supernatant was discarded and the pellet was washed with 70% ethanol twice.
10. It was centrifuged at 10,000 rpm for 5 min. Ethanol was decanted and was dried.
11. Later pellet was dissolved in 50µl-150 µl Tris-EDTA buffer depending on the size of pellet.

4.3. 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.

1. 1µl of deionized water was pipetted onto the lower optical surface of Nanodrop (Thermo Scientific) to clean it.
2. Nanodrop software was opened and Nucleic acid Module was selected.
3. A blank measurement was performed by loading 1µl of TE and selecting “blank” from the screen.
4. Nucleic acid sample was measured by loading 1µl of DNA sample and select ”measure” which calculate the purity of DNA.
    - DNA concentration can be calculated as

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

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

- **Purity of DNA = O.D at 260 nm/O.D at 280 nm**

  A ratio of 1.8 indicates purity of DNA; a ratio of 2.0 indicates purity of RNA and in other case it indicates contamination or presence of protein and phenol.

4.4. Resolution of DNA fragments on Agarose Gels

Requirements

- Electrophoresis buffer (TAE or TBE)
- Electrophoresis-grade Agarose
- Ethidium bromide Solution
- 6X loading dye
- DNA molecular weight markers
- Electrophoresis apparatus (horizontal)
- Gel Casting Platform
- Gel combs
- DC power supply

Procedure

1. **Preparation of 5X TBE (1000 ml)**
   - Tris base-54 g
   - Boric Acid-27.5 g
   - EDTA (0.5M)-20 ml
   - Make up final volume with water
2. **Preparation of 6X Loading Dye (20ml)**
   - 0.25% Bromophenol blue- 0.05 g
   - 0.25% Xylene Cyanol- 0.05 g
   - 40% Sucrose-8 g
   - Make up final volume with TE buffer
3. **Preparation of Agarose gel for electrophoresis**

- An adequate volume of electrophoresis buffer was prepared.
- Desired amount of electrophoresis grade agarose was added to electrophoresis buffer (for genomic DNA 0.7% gel was prepared which consist of 0.7 g agarose in 100ml of 0.5X TBE and for PCR products 1.7% gel was prepared which contains 1.7 g agarose in 100ml of 0.5X TBE) .
- Agarose was melted and was cooled to 55^{o}C and was later poured into gel platform.
- Before pouring gel into casting apparatus Ethidium Bromide was added to a final concentration of 0.3µg/ml which facilitates proper visualization of DNA under UV Transilluminator.
- Once gel was poured for about thickness of 1cm gel combs were inserted while bubbles formation should be avoided.

4. Loading and running the gel

- The gel combs were withdrawn as the gel got solidified.
- The gel casting platform containing the set gel was placed in electrophoresis tank and sufficient buffer was added so that well get submerged in it.
- DNA samples were prepared by mixing 5µg DNA with 2µl of 6X loading dye and 2µl water in case of PCR product.
- Samples were loaded into the wells with micropipette.

4.5. PCR (Polymerase Chain Reaction)

It is a technique used to make numerous copies of a specific segment of DNA quickly and accurately. It was developed by Kary B. Mullis in 1983.PCR is based upon natural process of cell replication. Main component is template DNA which is a region of DNA need to be copied and requires sequence of two short of DNA named primers. The primers bind to template at their complementary site and serve as starting point of amplification. One primer is directed toward the other resulting in replication initiation point. Free nucleotides are needed along with enzyme for binding. PCR primers provide 3'OH-group to which DNA polymerase adds dNTPs. So primer sequence must be unique. One primer anneal with other in the mixture to form "primer dimer" products.

Requirements

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

4.6. PCR amplification of *p53Arg^{72}Pro*

Genotypic analysis of *p53Arg^{72}Pro* was performed using PCR-RFLP techniques by using suitable primers and restriction enzyme previously as described by (Lakshmi *et al.*, 2012) . The two primers were 5'-TTCACCCATCTACAGTCC -3' and 5'-CTCAGGGCAACTGACCGT-3'. Each PCR mixture consists of 25µl which included 1X PCR buffer, 1.5mM $Mgcl_2$, 0.5µM forward primer, 0.5µM reverse primer, 200µM dNTPs, 100µg/ml of bovine Serum albumin (BSA) and 2U Taq polymerase (Finzymes) and 200ng of DNA approximately. PCR conditions used for reaction mixture were 95^{o}C for 5 min and 94^{o}C for 30 sec (denaturation), 58^{o}C for 45 sec (annealing) followed by 72^{o}C (extension) for 45 sec and 5 min final extension for 30 cycles to obtain product size of 309 bp.

Forward primer:- 5'- TTCACCCATCTACAGTCC -3'

Reverse primer:- 5'- CTCAGGGCAACTGACCGT-3'.

Band size:- 309 bp

Table 4.3. Representing requirements for Polymerase Chain Reaction (PCR)			
Reagents	**Stock Concentration**	**Working Concentration**	**Volume Used**
Additive 1 BSA	1000µg/ml	100 µg/ml	44 µl
PCR Buffer (Mg concentration)	10X (15mM)	1X (1.5mM)	44 µl
p53Arg^{72}Pro Primer (Forward)	10 µM	0.5 µM	22 µl
p53Arg^{72}Pro Primer (Reverse)	10 µM	0.5 µM	22 µl
Taq Polymerase	2.0 U	1.0 U	4.4 µl
dNTPs	10 mM	0.2 mM	8.8 µl
PCR Grade Water			100 µl
DNA template	100 ng/ µl	300 ng	4 µl

Table 4.4. Representing steps of PCR along with their specified temperature			
S. No.	**Steps**	**Temperature**	**Time**
1.	Initial Denaturation	95^{o}C	5 min
2.	Denaturation	94^{o}C	30 sec
3.	Annealing	58^{o}C	45 sec
4.	Polymerization	72^{o}C	45 sec
5.	Final Extension	72^{o}C	5 min

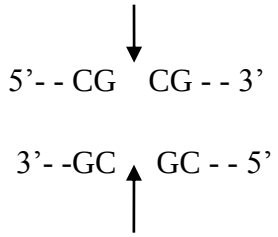
*4.7.*Restriction Digestion of *p53Arg^{72}Pro*

This enzymatic technique can be used to cleave DNA amplicons at specific site so to produce DNA fragments of same size. Each DNA fragments contain desired sequence located at same position within the fragment enzyme located at same position within the fragments enzyme used for restriction digestion was Bsh12361 (*BstU1*).

BstU1

It was isolated from *Bacillus Stearothermophillus* U458. It is used to digest amplicons obtained from amplification of *p53Arg^{72}Pro*. It recognizes G→C site in amplified portion of DNA sequence.

Restriction site for the enzyme is



Procedure:-

The total reaction mixture of 20µl consist of 2.2 µl of 10X Tango buffer, 0.2 µl (2 U) of 10 U/ µl *BstU1* enzyme (Thermo Fisher Scientific) and 10 µl of PCR product and 7.6 µl of water. The buffer was added to maintain the enzymatic activity. All the samples were incubated at 37^{o}C in incubator for overnight. Next day samples were kept at -20^{o}C to stop enzymatic reaction. 2.5% agarose gel was prepared with ethidium bromide and was poured into the caster. Samples were loaded in wells with required concentration of dye and water. Electrophoresis unit was allowed to rum at 80 V till marker dye migrated to desired distance. The results were visualized by placing gel on UV transiluminator and gel pictures were captured using Gel Doc (BIORAD).

Amplified products were digested with 2U of *BstU1* at 57^{o}C (NEB) restriction enzyme. The digested samples were run in 2.5% agarose gel stained with Ethidium bromide, the wild homozygous genotype (*GG*) did not have a site for *BstU1* (309) whereas the mutant genotype (*CC*) gave two fragments of (171/154) bp and heterozygous homozygous genotype (*GC*) gave three fragments of (309, 171 and 154) bp respectively.

Cutting Pattern of *p53Arg^{72}Pro* with *BstU1*

Wild genotype (*GG*) – 309 bp

Mutant genotype (*CC*) – 171 bp / 154 bp

Heterozygous genotype (*GC*) – 309 bp, 171 bp and 154 bp

4.8. Overall Survival data collection

All Lung cancer patients were called to check their survival status on the basis of dead or alive. We note down the date of death given by their family members along with the date when they got their last chemotherapy. Survival time was measured from the date of enrollment to date of death (for dead patients) and to date of last follow up i.e 23rd March, 2016 (for alive patients).

4.9.Statistical Analysis

All the statistical analysis was performed using Medcalc Software version 12.1.2. For demographic studies variables were categorized into continuous (gender, sex, smoking status) and categorical variables (age and pack years).Paired t-test and chi-square test was preformed for each category respectively. The Hardy-Weinberg equilibrium analysis ($p^2+2pq+q^2=1$; where p and q is the frequency of wild and mutant type respectively) was performed to find significant difference between two groups. The association analysis between p53 codon72 genotype and susceptibility towards lung cancer was carried out using logistic regression. It finds OR (odds ratio) along with its confidence intervals (CI) with adjustment of Age, gender and smoking status, clinic-pathological features (KPS, ECOG and tumor stage) and clinical responses to find specific association with p53 codon 72 towards lung cancer susceptibility.

Kaplan-Meir and Cox proportional hazard analysis was performed to study overall survival. Kaplan-Meir was used to evaluate overall survival time using median OS time and *p*-value. Multivariate Cox regression analysis was used to perform secondary analysis which evaluates the independent risk factor for death for each parameter. Probability criteria less than 0.05 was significant for all the statistical analysis.

RESULTS

5.1.DNA Isolation

Genomic DNA was isolated from peripheral blood and was diluted with TE to a concentration of 100ng/µl. They were visualized under trans-UV-illuminator using 0.7% of agarose gel. These diluted genomic DNA were used as template for Polymerase Chain Reaction (PCR) for amplification.

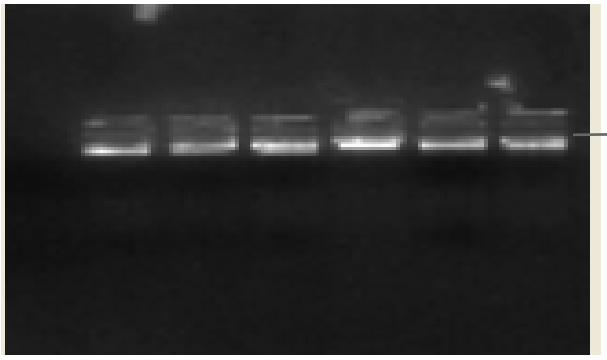


Fig.5.1. Showing genomic DNA of lung cancer patients

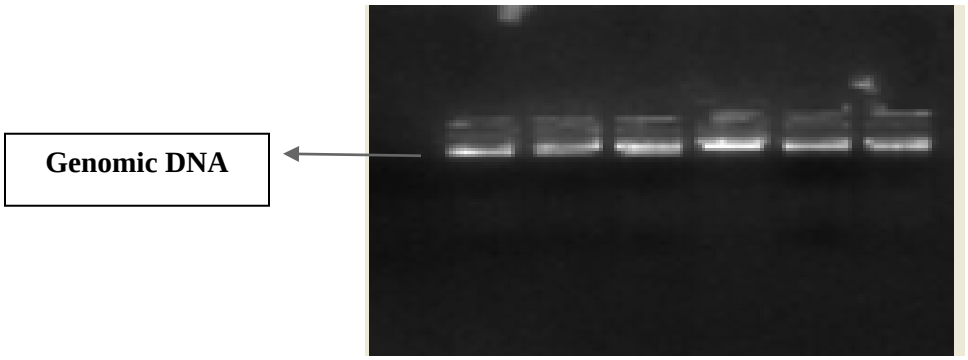


Fig.5.2. Showing genomic DNA of controls

5.2.Restriction Fragment Length Polymorphism of *p53Arg^{72}Pro*

Reverse and forward primers were designed for the required region of gene which is codon 72 of exon 4 for *p53* for the amplification reaction. Amplicons obtained were analyzed using 1.7% agarose gel to obtain amplicon size of 309 bp as shown in Fig.5.1 and Fig.5.2.

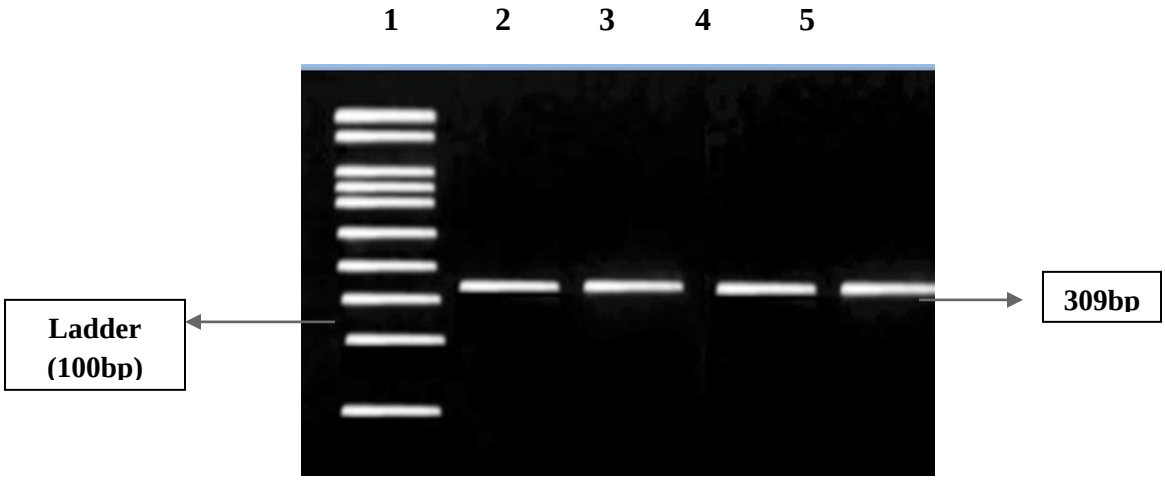


Fig.5.3. Showing PCR products of *p53Arg*^{72}*Pro* with amplicon size of 309 bp for lung cancer patients

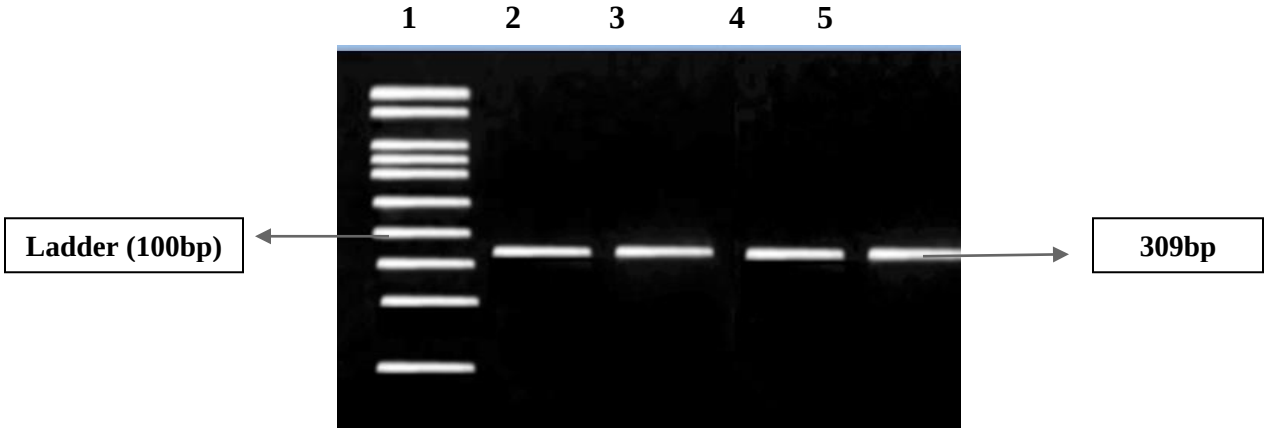


Fig.5.4. Showing PCR products of *p53Arg*^{72}*Pro* with amplicon size of 309 bp for controls.

*5.3.*Restriction digestion of *p53Arg*^{72}*Pro* with restriction enzyme *BstU1*

The PCR products were further digested with the *BstU1* restriction enzyme which cleaves the PCR product at specific site and is checked by 2.5% agarose gel to obtain cutting pattern for different genotypes (wild-309 bp and mutant-154 and 171 bp) as shown in Fig.5.3 and Fig.5.4.

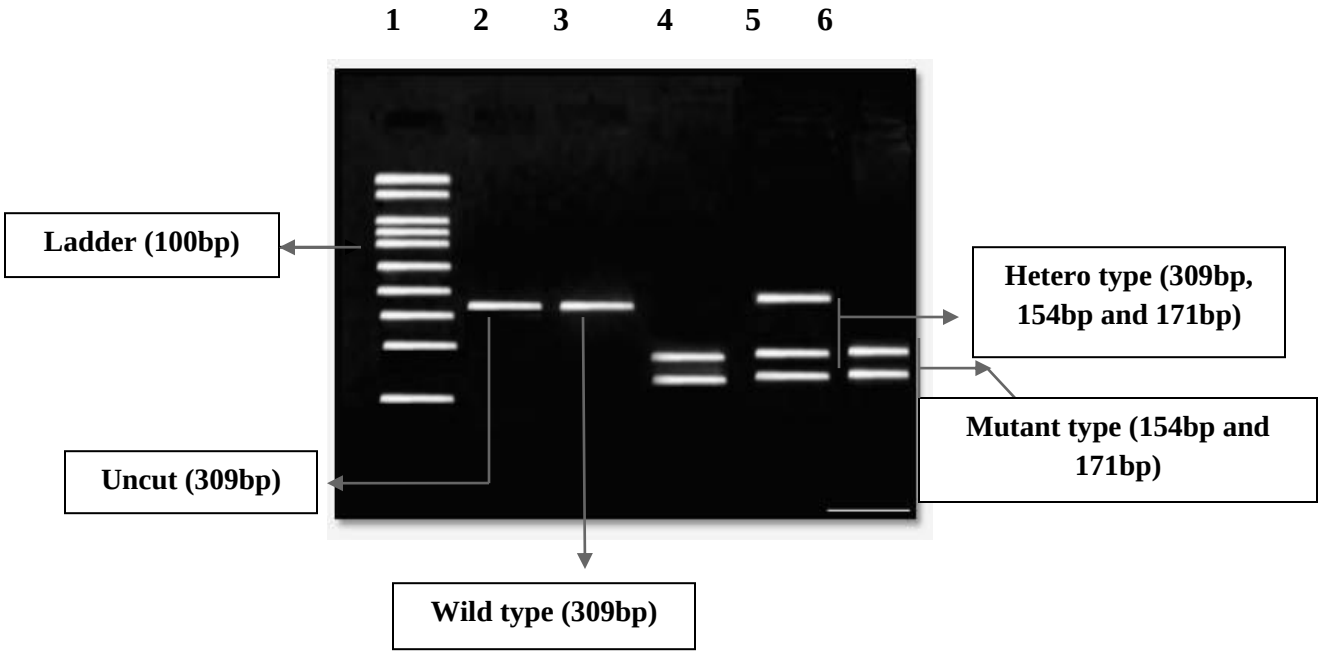


Fig.5.5 Showing restriction digestion of PCR product of *p53Arg*^{72}*Pro* for lung cancer patients (Uncut: 309bp, Wild type: 309bp, Mutant type: 154bp and 171 bp, hetero type: 309bp, 154bp and 171bp) Lane1: 100bp ladder (G. Bioscience); Lane2: Uncut; Lane3: Wild type; Lane4: Mutant type; Lane5; Hetero type; Lane6: Mutant type.

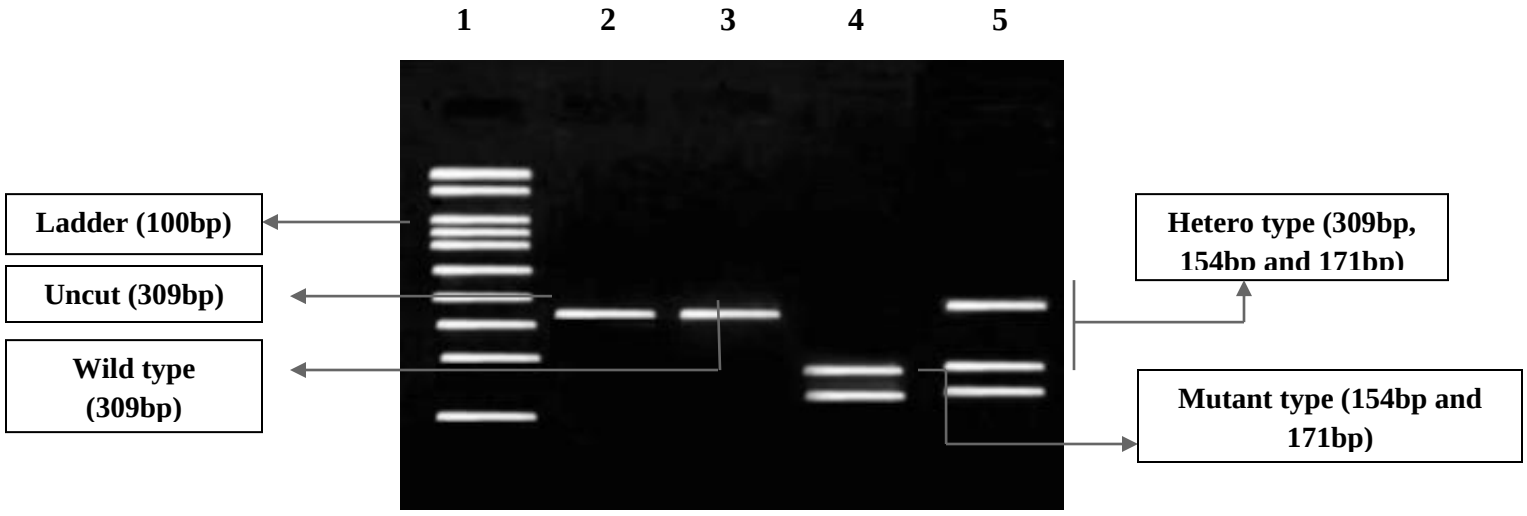


Fig.5.6.Showing restriction digestion of PCR product of *p53Arg*^{72}*Pro* for controls (Uncut: 309bp, Wild type: 309bp, Mutant type: 154bp and 171 bp, hetero type: 309bp, 154bp and 171bp) Lane1: 100bp ladder (G. Bioscience); Lane2: Uncut; Lane3: Wild type; Lane4: Mutant type; Lane5; Hetero type.

Table 5.1.Representing distribution of demographic characteristics of cases and controls					
Variables	**Total(N)**	**Cases n (%)**	**Total(N)**	**Control n (%)**	***p***
Age(years)					
Mean ± SD	420	57.87 ± 10.39	420	52.14 ± 10.95	**<0.0001**
Range		28-86		19-83	
Gender					
Male	420	359 (85.48)	420	353 (84.04)	0.56
Female		61 (14.52)		67 (15.95)	
Smoking Status					
Smokers	420	345 (82.14)	420	303 (72.14)	**0.0006**
Non-smokers		75 (17.86)		117 (27.86)	
Pack-Years					
Mean ± SD	345	33.89 ± 33.80	303	23.55 ± 18.58	**<0.0001**
Histology					
ADCC	420	125 (29.76)			
SCLC		134 (31.90)			
SQCC		152 (36.19)			
Others		9 (2.14)			
TNM Staging					
I	402	3 (0.74)			
II		11 (2.73)			
III		204 (50.75)			
IV		184 (45.77)			
Tumor Size					
T1	386	18(4.66)			
T2		46 (11.92)			
T3		95 (24.61)			
T4		214 (55.44)			
Tx		13 (3.37)			
Lymph node involvement					
N0	386	52 (13.47)			
N1		39 (10.10)			
N2		173 (44.82)			
N3		117 (30.31)			
N4		4 (1.04)			
Nx		1(0.25)			
Metastasis					
M0	386	212 (54.92)			
M1		174 (45.08)			
Objective Response					
CR	213	10 (4.69)			
PR		104 (48.83)			
SD		20 (9.39)			
PD		79 (37.09)			

Performance Status				
KPS				
(80-100)	296	189(63.85)		**<0.0001**
(60-70)		93(31.42)		
(< 60)		14(4.73)		
ECOG				
(0 and 1)	296	162(54.73)		**<0.0001**
2		93(31.42)		
(3 and 4)		41(13.85)		

Abbreviations: SD=Standard Deviation, n=total number of case patients or controls subjects .[a] *p*-values were derived from Pearson Chi-square test except age; Student t-test was used for age. All *p*-values are two-sided. $p < 0.05$ was considered statistically significant.

5.4. Demographic distribution of lung cancer patients and controls

The demographic distribution for parameters in cases and controls are summarized in Table5.1. The parameters include age, sex, smoking status, pack years for cases and controls. The clinical parameters of lung cancer patients included histology, tumor stage, tumor size extension, lymph node extension, metastasis along with clinical response parameter (complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD)) for cases. Our study recruited total of 420 cases and 420 controls having mean age of 57.87 (±10.39) and 52.14(±10.95). Men were over presented than females in both cases (85.48% vs 14.52%) and controls (84.04% vs 15.95%) without any significant level of distribution within population (p=0.56) suggesting non-bias sampling. The number of smokers was overrepresented in cases than controls (82.14% vs 72.14%) which shows smoking as a significant parameter for occurrence lung cancer as mentioned in other studies also. Pack years was calculated as [((cigarettes or beedis) / 20) × No. of years smoked]. A significant difference was observed for mean pack years when a comparison was made between cases and controls **(p<0.0001).** Among the cases SQCC histological alteration was found to be the most abundant type of cancer (36.19%) with higher prevalence in heavy smokers (pack years >25 years).TNM data was available for 402 patients among which Stage III and IV were highly proportionated (96.51%). During metastasis study of 386 cases proximal tumors (M_1) were found in large fraction (45.07%). Out of 420 patients 213 were assessable for clinical response among which large number of patients showed progressive response (PR) (48.83%) toward chemotherapy treatment.

Table 5.2. Representing relationship of different genotypes different histological subtypes

	Overall				ADCC			SCLC			SQCC		
Genotype	**Controls (420) n(%)**	**Cases (420) n,(%)**	**[a] AOR (95% CI) [b]**	***p***	**Cases (125) n,(%)**	**[a] AOR (95%CI) [b]**	***p***	**Cases (134) n,(%)**	**[a]AOR (95%CI) [b]**	***p***	**Cases (152) n,(%)**	**[a] AOR (95%CI) [b]**	***p***
GG	148 (35.23)	142 (33.80)	1	**Reference**	48 **(38.40)**	1	**Reference**	39 (29.10)	1	**Reference**	45 (29.60)	1	**Reference**
GC	189 (45.00)	196 (46.67)	1.04 (0.75-1.42)	0.82	53 **(42.40)**	0.93 **(0.58-1.49)**	0.77	72 (53.73)	1.30 (0.80-2.10)	0.28	65 (42.76)	0.87 (0.56-1.35)	0.53
CC	83 (19.76)	82 (19.52)	0.99 (0.66-1.48)	0.96	24 **(19.20)**	0.90 **(0.50-1.63)**	0.73	23 (17.16)	0.98 (0.52-1.83)	0.94	33 (21.71)	1.04 (0.60-1.80)	0.90
(GC+CC)	272 (64.76)	278 (66.19)	1.02 (0.75-1.37)	0.92	77 **(61.60)**	0.92 **(0.80-1.40)**	0.69	95 (70.89)	1.20 (0.77-1.88)	0.42	98 (64.47)	0.90 (0.60-1.36)	0.63
G	485	480											
C	355	360											
MAF	0.42	0.43											

[a] Adjusted Odds ratios, [b] 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.
Two-sided χ^2 test for either genotype distribution or allelic frequencies between the cases and controls. Abbreviations: ADCC, Adenocarcinoma; SQCC, Squamous cell carcinoma; SCLC, Small cell lung carcinoma.

Table 5.3. Representing relationship of different genotypes with the smoking status of lung cancer patients

	Smokers					Non-smokers			
Genotype	**Controls (303) n (%)**	**Cases (345) n (%)**	**[b] AOR (95% CI) [b]**	***p***		**Controls (117) n (%)**	**Cases (75) n (%)**	**[b] AOR (95% CI) [b]**	***p***
GG	105(34.65)	113(32.75)	1	**Reference**	**GG**	43(36.75)	29(38.67)	1	**Reference**
GC	144(47.52)	164(47.54)	0.98(0.69-1.41)	0.93	**GC**	45(38.46)	32(42.67)	1.30(0.63-2.69)	0.47
CC	54(17.82)	68(19.71)	1.05(0.66-1.66)	0.84	**CC**	29(24.79)	14(18.67)	0.92(0.37-2.26)	0.85
(GC+CC)	198(65.35)	232(67.25)	1.00(0.71-1.40)	0.99	**(GC+CC)**	74(63.25)	46(61.33)	1.14(0.59-2.20)	0.69
	Light Smokers					**Heavy Smokers**			
Genotype	**Controls (195) n (%)**	**Cases (174) n (%)**	**[b] AOR (95% CI) [b]**	***p***		**Controls (108) n (%)**	**Cases (171) n (%)**	**[b] AOR (95% CI) [b]**	***p***
GG	70(35.90)	65(37.36)	1	**Reference**	**GG**	35(32.41)	48(28.07)	1	**Reference**
GC	86(44.10)	78(44.82)	0.94(0.59-1.51)	0.80	**GC**	58(53.70)	86(50.29)	0.97(0.55-1.71)	0.91
CC	39(20.00)	31(17.82)	0.76(0.41-1.38)	0.37	**CC**	15(13.89)	37(21.64)	1.52(0.71-3.28)	0.28
(GC+CC)	125(64.10)	109(62.64)	0.88(0.57-1.37)	0.58	**(GC+CC)**	73(67.60)	123(71.93)	1.09(0.63-1.87)	0.76

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.

ECOG and KPS were found to be an independent predictor of survival in the present study. A significant log Rank *p*-value was observed in both KPS **(*p*<0.0001)** and ECOG **(*p*<0.0001)** patients having different grades of ECOG (0, 1 and 2) and KPS also.

5.5.Genotypic distribution of *p53Arg^{72}Pro* among cases and controls and its association with lung cancer susceptibility

Table5.2. elaborates the genotypic frequencies for *p53Arg^{72}Pro* in cases and controls with no significant difference of distribution between cases and controls (χ^2=0.516; df=2; *p*=0.77). The frequency of heterozygous genotype (*GC*) (45% and 46.67%) was found to be higher than wild (*GG*) (35.23% and 38.80%) and mutant (*CC*) (19.76% and 19.52%) genotype for both cases and controls. Minor allele frequency was found to be 0.42 and 0.43 in controls and cases respectively. Allelic frequency for variant allele (C) was found to nearly similar in cases (42.86%) and controls (42.26%). Samples from both lung cancer patients (χ^2=0.93; df=1; *p*= 0.33) and controls (χ^2=2.55, df=1; *p*=0.11) were in accordance to Hardy-Weinberg equilibrium. Using wild allele (*GG*) as reference genotype it was observed that mutant variant (*CC*) showed no the association towards lung cancer susceptibility (OR=0.99; 95% CI=0.66-1.48; *p*=0.96). Similarly heterozygous variant (*GC*) showed no association (OR=1.04; 95% CI=0.75-1.42; *p*=0.82).

5.6.Genotypic distribution of *p53Arg^{72}Pro* among cases and their role in altering lung cancer susceptibility in association with different histological subtypes

The patients were then stratified on the basis of different histological subtypes so as to analyze the risk towards lung cancer. On stratification it was observed that the frequency of heterozygote variant (*GC*) genotype was found to be overrepresented in SCLC patients as compared to controls (53.73% *vs* 45%). Both the variant genotype (*CC+GC*) showed no significant correlation with different histological subtypes (ADCC, SCLC and SQCC). However a slight trend towards SCLC (OR=1.30; 95% CI=0.80-2.10; *p*=0.28) was observed for heterozygous (*GC*) variant. Significant decrease in frequency of variant genotype (*GC*) was observed in SCLC and SQCC when compared to controls (29.10%, 29.60% *vs* 35.23%).

5.7.Association between *p53Arg^{72}Pro* and lung cancer risk based on smoking status of lung cancer patients:

Table5.3. summarizes the correlation between smoking status and lung cancer susceptibility for *p53Arg^{72}Pro* through multivariate regression analysis taking wild allele (*GG*) as reference. Patients were categorized as smokers and non-smokers. Among smokers further categorization was made for heavy and light smokers on the basis of pack years. Patients with pack years >25 years and with pack years < 25 years were categorized as heavy and light smokers respectively. On classification we found a slight increase in mutant variant (*CC*) frequency in patients who are smokers than controls (19.71% *vs* 17.82%) while for non-smokers frequency decreases (18.67% *vs* 24.79%) although statistically not significant (p=0.84). Among heavy smokers mutant variant (*CC*) shows slight trend toward susceptibility but no significance was observed. When light smokers were analyzed no significant risk was observed. Among non-smokers the frequency of heterozygote variant decreases as compared to controls (38.46% *vs* 42.67%) with no risk associated to lung cancer susceptibility (OR=1.30; 95% CI=0.63-2.69; p=0.47). Overall large frequency of smokers were observed among cases than controls and in addition heavy smokers had higher OR than light smokers (0.97 and 1.52 *vs* 0.94 and 0.76) which indicated smoking as a risk factor associated in our study population but was not significantly associated with lung cancer risk.

Table 5.4. Representing relationship of different genotypes with gender

	Male				**Female**			
Genotype	**Controls (353) n (%)**	**Cases (359) n (%)**	**[b]AOR (95% CI)[b]**	***p***	**Controls (67) n (%)**	**Cases (61) n (%)**	**[b]AOR (95% CI)[b]**	***p***
GG	127 **(35.98)**	123 **(34.26)**	1	**Reference**	21 **(31.34)**	19 **(31.15)**	1	**Reference**
GC	165 **(46.74)**	70 **(19.50)**	1.00 **(0.72-1.41)**	0.60	24 **(35.82)**	26 **(42.62)**	1.32 **(0.47-3.74)**	0.60
CC	61 **(17.28)**	66 **(18.38)**	1.07 **(0.69-1.66)**	0.39	22 **(32.83)**	16 **(26.23)**	0.61 **(0.20-1.86)**	0.39
(GC+CC)	226 **(64.02)**	136 **(37.88)**	1.02 **(0.75-1.41)**	0.91	46 **(68.66)**	42 **(68.85)**	0.95 **(0.39-2.34)**	0.91

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.

5.8. Association between *p53Arg^{72}Pro* and lung cancer risk based on gender of lung cancer patients:

Further stratification was done on the basis of gender so as to analyze the risk towards lung cancer as mentioned in table 5.4. Men were over represented in both cases (85.47%) and controls (84.04%). No significant association was found for any genotype (*GG*, *GC* and *CC*) in male as well as female patients and controls towards lung cancer risk however, proportion of heterozygous genotype (*GC*) (46.4% and 19.50%) (35.28% and 42.62%) was higher among patients of the both gender when compared to mutant (*CC*) and wild (*GG*) genotype in both cases and controls.

5.9. Association of *p53Arg^{72}Pro* genotypic distribution with clinic-pathological parameters:

Table5.5. summarized data for regression analysis showing association of clinic-pathological parameters and risk of lung cancer. While considering histological grade of 386 patients, 77(19.95%) patients were found to have moderately differentiated (T_1+T_2+T_x) and 309 (80.05%) were characterized by well differentiated (T_3+T_4) tumors. No significant correlation was observed toward lung cancer susceptibility in any of the *p53Arg^{72}Pro* genotypes. However heterozygote (GC) genotype was found to be predominant in well differentiated (47.72%) and moderately differentiated (57.14%) tumors than that of wild (35.27% and 28.57%) and mutant (22.01% and 14.28%) genotypes.

Based on TNM stage criteria, out of 388 lung cancer patients, 204 (52.28%) were categorized as stage III and 184 (47.42%) as stage IV. Among both the stages (III and IV), heterozygote (*GC*) genotype was found to have highest frequency (45.10% and 47.83%) with no significant risk associated to lung cancer. Furthermore subjects carrying mutant (*CC*) genotype showed marginally significant lower frequency in stage IV category with (OR=0.58; 95% CI=0.32-1.04; *p*=0.07). Among 386 patients, 54.92% were characterized with absence of metastasis whereas 45.92% showed distant metastasis. The *p53* polymorphism was not found to have a role in progression of lung cancer as no significant difference was found in the frequency of patients with variant genotype in both metastatic (M_0+M_1) subgroups.

5.10. Association of *p53Arg^{72}Pro* genotypic distribution with lung cancer susceptibility on the basis of chemotherapy response:

Association of *p53Arg^{72}Pro* polymorphism with chemotherapy response was summarized in Table 5.6. Out of 213 patients, 104 (48.83%) patients showed partial response (PR) while 10 (4.70%) patients showed complete response (CR). However 20 (9.39%) were exhibiting progressive (PD) and 79 (37.09%) exhibiting stable disease (SD) after chemotherapy treatment. Patients categorized as good responders (CR+PR) towards chemotherapy tend to have higher genotypic frequency of heterozygous (*GC*) than mutant (*CC*) and wild (*GG*) genotypes (50% *vs* 20.17% and 28.28%). Similar result was predicted for bad responders (SD+PD) (49.49% *vs* 22.22% and 28.28%) however significant correlation was not found for any of the genotype (*GC* and *CC*) towards lung cancer susceptibility in patients undergoing chemotherapy.

5.11. Association of *p53Arg^{72}Pro* genotypic distribution with overall survival of lung cancer patients:

Overall survival analysis was performed for 296 lung cancer patients and result are summarized in Table5.7. Evaluation was made first using univariate Kaplan-meir analysis then by multivariate Cox hazard proportional analysis providing adjusted and unadjusted hazard ratio (HR), log rank test with 95% confidence interval and Kaplan-meir curves. It correlates overall survival time (months) and various prognostic factors (age, histology, gender, smoking status, stage and performance status) considering genotype as an independent factor.

On evaluating patients from the day of diagnosis to the last follow-up date, we found that 86 (29.05%) patients were alive while 210 (70.94%) were found to be dead due to the disease. Considering wild genotype as reference the median survival time (MST) of patients with mutant (*CC*) genotype was found to be higher than wild (*GG*) and heterozygote (*GC)* genotype (10 *vs* 7.33 and 5.93 months) as shown in Fig 2A. Using univariate analysis mutant (*CC*) variant was found to have a significantly good prognostic effect as compared to other two genotypes (HR=0.65; 95% CI=0.45-0.95; *p*=0.03). However, on further adjusting with different covariates marginally significant HR was observed (HR=0.68; 95% CI=0.63-1.16; *p*=0.06) in patients with better survival.

Table 5.5. Representing relationship of different genotypes with the clinic-pathological parameters

	Clinical Stage		[b] AOR (95% CI) [b]	*p*	Primary Tumor Extension		[b] AOR (95% CI) [b]	*p*	Lymph node Invasion		[b] AOR (95% CI) [b]	*p*	Metastasis		[b] AOR (95% CI) [b]	*p*
	III (204) n (%)	IV (184) n (%)			Tx+T1+T2 (77) n (%)	T3+T4 (309) n (%)			N0 (52) n (%)	N1+N2+N3 (334) n (%)			No (212) n (%)	Yes (174) n (%)		
Genotype																
GG (Reference)	64 (31.37)	67 (36.41)	-	-	22 (28.57)	109 (35.27)	-	-	14 (26.92)	117 (35.03)	-	-	69 (32.55)	62 (35.63)	-	-
GC	92 (45.10)	88 (47.83)	0.93 (0.58-1.48)	0.76	44 (57.14)	132 (42.72)	0.57 (0.41-1.24)	0.23	27 (51.92)	149 (44.61)	0.66 (0.33-1.31)	0.23	92 (43.40)	84 (48.27)	1.07 (0.67-0.57)	0.77
CC	48 (23.53)	29 (15.76)	0.58 (0.32-1.04)	0.07	11 (14.28)	68 (22.01)	0.18 (0.53-2.62)	0.68	11 (21.15)	68 (20.36)	0.74 (0.31-1.73)	0.48	51 (24.06)	28 (16.09)	0.61 (0.34-1.10)	0.10
GC+CC	140 (68.63)	117 (63.59)	0.80 (0.52-1.23)	0.31	55 (71.43)	200 (64.72)	0.71 (0.41-1.24)	0.23	38 (73.08)	217 (64.97)	0.67 (0.35-1.30)	0.24	143 (67.45)	112 (64.37)	0.89 (0.58-1.38)	0.61

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.

Table 5.6. Representing relationship of different genotypes with the chemotherapy response

	N(213) n (%)	Response of chemotherapy		[b]AOR (95% CI)[b]	*p*
Genotype		CR+PR (114) n (%)	SD+PD(99) n (%)		
GG	62 (29.11)	34 (29.82)	28 (28.28)	-	**Reference**
GC	106 (49.76)	57 (50.00)	49 (49.49)	0.98 (0.52-1.85)	0.94
CC	45 (21.13)	23 (20.17)	22 (22.22)	0.76 (0.34-1.70)	0.51
(GC+CC)	151 (70.89)	80 (70.17)	71 (71.71)	0.92 (0.50-1.67)	0.78

[b] Adjusted Odds ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender and smoking.

Heterozygote variant (*GC*) exhibited no significant association with overall survival (OS) (HR=0.80; 95% CI=0.59-1.09; *p*=0.15) even after adjusting with different covariates(HR=0.86; 95% CI=0.63-1.16; *p*=0.32).However combined variant genotype (*GC+CC*) with MST of 8.27 months showed nearly significant association with survival (HR=0.75; 95% CI=0.56-1.02; *p*=0.05) using multivariate analysis as shown in Fig.2E.

5.12. **Association of *p53Arg^{72}Pro* genotypic distribution with overall survival of lung cancer patients on the basis of histological subtypes:**

When association between genotype and histological subtypes was analyzed for significant prognostic effect no significant correlation was found. Longer survival period was observed for patients with mutant (*CC*) genotype in each histological subgroup (ADCC, SQCC and SCLC) (13.4, 7.57 and 9.87 months) when compared to wild (*GG*) and heterozygote (*GC*) genotype as shown in Fig.2B, 2C and 2D. Furthermore, ADCC patients with combined variant genotype (*GC+CC*) showed highest MST (9.43 months) as compared to SQCC (7.33 months) and SCLC (8.27 months) patients with combined variant genotypes shown in Fig.2F, 2G and 2H. However, the difference was not found to be statistically significant.

5.13. **Association of *p53Arg^{72}Pro* genotypic distribution with overall survival of lung cancer patients on the basis of gender:**

Further classifying patients on the basis of their gender, decreased mortality was seen in male patients with mutant (*CC*) genotype (10 months) which was not statistically significant. However, among females lower death risk was reported as females having heterozygote (*GC*) variant showed better survival among all the genotypes (13.70 *vs* 9.4 and 4.13 months) as shown in Fig.2J. Females having mutant (*CC*) genotype showed non-significant association with hazard ratio (HR=0.51; 95% CI=0.20-1.27; *p*=0.13).

However, after adjusting with different cofactors statistically significant trend was observed for decreased mortality in females with mutant genotype due to lung cancer (HR=0.21; 95% CI=0.06-0.67; ***p*=0.01**).Also, significantly lower probability of death was predicted in female patients with heterozygote (*GC*) genotype (HR=0.08; 95% CI=0.02-0.34; ***p*=0.005**).

Similarly, combined variant genotype (*GC+CC*) also showed overall survival of 12.07 months with significantly good prognostic effect in females (HR=0.17; 95% CI=0.06-0.41; ***p*=0.005**) as shown in Fig.2I. So, overall female patients with mutant (*CC*) and heterozygote (*GC*) variant showed good prognosis towards lung cancer.

5.14. Association of *p53Arg^{72}Pro* genotypic distribution with overall survival of lung cancer patients on the basis of clinic-pathological parameters:

Further data was stratified on the basis of clinic-pathological parameters. Patients were classified on the basis of clinical stage III and IV. Patients in both the stages (III and IV) having CC genotype exhibited longer survival time than *GG* and *GC* genotype (9.47 *vs* 7.57 and 7.23) (10.67 *vs* 6.73 and 4.93) months respectively. While comparing MST of patients diagnosed with stage III and IV mutant (*CC*) genotype was found to have higher MST in patients with stage IV than stage III (10.67 *vs* 9.47) months suggesting increased survival among patients diagnosed with stage IV. None of the genotype showed significant association with survival however stage IV patients with mutant (*CC*) genotype were found to have a significantly higher survival probability (HR=0.52; 95% CI=0.38-1.15; ***p*=0.03**). However, on adjusting, the hazard ratio showed no significant prognosis for lung cancer patients (HR=0.61; 95% CI=0.38-1.15; *p*=0.13).

5.15. Association of *p53Arg^{72}Pro* genotypic distribution with overall survival of lung cancer patients on the basis of their performance status after receiving chemotherapy:

Considering performance status of patients (ECOG and KPS) it has been observed that MST was nearly same for all genotypes in subjects with KPS (80-100) (10.27, 10.67 and 10.67) months and also for KPS (60-70) (5.87, 5.93 and 5.87) months. Similarly patients with ECOG 2, 3 and 4 showed higher survival period among subjects with mutant (*CC*) genotype than *GG* and *GC* genotype (6.30 vs 5.87) (6.77 vs 3.43) months but within the subjects categorized as ECOG 0 and 1, the heterozygote (*GC*) variant showed reduced death probability as compared to *GG* and *CC* variant (11.83 vs 10.13) suggesting a trend of good improvement among patients with *GC* genotype after chemotherapy treatment but no significant association with overall survival was observed.

Table.5.7. Representing relationship of *p53Arg^{72}Pro* genotype with overall survival of lung cancer patients

	Overall								**ADCC**						
Genotype	**Dead (210) n (%)**	**Alive (86) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Genotype**	**Dead (68) n (%)**	**Alive (27) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
GG	76 (36.19)	21 (24.42)	5.93	1	-	-	**Reference**	**GG**	26 (38.23)	8 (29.63)	7.60	1	-	-	**Reference**
GC	98 (46.67)	38 (44.19)	7.33	0.80 (0.59-1.09)	0.15	0.86 (0.63-1.16)	0.32	**GC**	32 (47.06)	12 (44.44)	7.57	0.97 (0.58-1.62)	0.90	0.79 (0.46-1.37)	0.41
CC	36 (17.14)	27 (31.40)	10.00	0.65 (0.45-0.95)	**0.03**	0.68 (0.63-1.16)	0.06	**CC**	10 (14.70)	7 (25.92)	13.43	0.60 (0.30-1.17)	0.16	0.58 (0.25-1.34)	0.20
(GC+CC)	134 (63.81)	65 (75.58)	8.27	0.75 (0.56-1.02)	0.05	0.80 (0.60-1.07)	0.13	**(GC+CC)**	42 (61.76)	19 (70.37)	9.43	0.85 (0.51-1.40)	0.51	0.72 (0.43-1.20)	0.21
	SQCC								**SCLC**						
Genotype	**Dead (84) n (%)**	**Alive (28) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Genotype**	**Dead (56) n (%)**	**Alive (28) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
GG	32 (38.09)	7 (25.00)	5.37	1	-	-	**Reference**	**GG**	18 (32.14)	6 (21.43)	4.50	1	-	-	**Reference**
GC	36 (42.86)	10 (35.71)	7.27	0.80 (0.49-1.29)	0.34	0.89 (0.53-1.48)	0.65	**GC**	29 (51.78)	14 (50.00)	8.27	0.68 (0.36-1.27)	0.19	0.77 (0.40-1.49)	0.44
CC	16 (19.05)	11 (39.28)	7.57	0.65 (0.37-1.15)	0.15	0.66 (0.34-1.26)	0.21	**CC**	9 (16.07)	8 (28.57)	9.87	0.77 (0.33-1.53)	0.39	0.83 (0.32-2.17)	0.71
(GC+CC)	52 (61.90)	21 (75.00)	7.33	0.74 (0.47-1.75)	0.18	0.80 (0.50-1.27)	0.34	**(GC+CC)**	38 (67.86)	22 (78.57)	8.27	0.68 (0.37-1.25)	0.17	0.75 (0.41-1.37)	0.35
	Male								**Female**						
Genotype	**Dead (180) n (%)**	**Alive (76) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Genotype**	**Dead (30) n (%)**	**Alive (10) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
GG	65 (36.11)	20 (26.31)	7.13	1	-	-	**Reference**	**GG**	11 (36.67)	1 (10.00)	4.13	1	-	-	**Reference**
GC	87 (48.33)	32 (42.10)	6.87	0.88 (0.64-1.22)	0.45	0.95 (0.68-1.32)	0.77	**GC**	11 (36.67)	6 (60.00)	13.70	0.42 (0.17-1.0)	**0.03**	0.08 (0.02-0.34)	**0.0005**
CC	28 (15.55)	24 (31.58)	10.00	0.67 (0.44-1.01)	0.07	0.72 (0.46-1.14)	0.16	**CC**	8 (26.67)	3 (30.00)	9.47	0.51 (0.20-1.27)	0.13	0.21 (0.06-0.67)	**0.0085**
(GC+CC)	115 (63.89)	56 (73.68)	8.20	0.82 (0.60-1.12)	0.19	0.87 (0.64-1.19)	0.38	**(GC+CC)**	19 (63.33)	9 (90.00)	12.07	0.44 (0.18-1.07)	**0.02**	0.17 (0.06-0.47)	**0.0005**
	Tumor stage-III								**Tumor stage-IV**						
Genotype	**Dead (103) n (%)**	**Alive (51) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Genotype**	**Dead (101) n (%)**	**Alive (32) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
GG	36 (34.95)	9 (17.65)	7.23	1	-	-	**Reference**	**GG**	40 (39.60)	11 (34.37)	4.93	1	-	-	**Reference**
GC	47 (45.63)	26 (50.98)	7.57	0.75 (0.48-1-17)	0.18	0.73 (0.47-1.14)	0.16	**GC**	47 (46.53)	12 (37.50)	6.73	0.91 (0.59-1.39)	0.65	0.93 (0.61-1.44)	0.76

CC	20 (19.42)	16 (31.37)	9.47	0.94 (0.55-1.62)	0.83	0.89 (0.49-1.63)	0.72	**CC**	14 (13.86)	9 (28.12)	10.67	0.52 (0.30-0.90)	**0.03**	0.61 (0.38-1.15)	0.13
(GC+CC)	67 (65.05)	42 (82.35)	7.57	0.78 (0.51-1.19)	0.23	0.77 (0.51-1.71)	0.22	**(GC+CC)**	61 (60.40)	21 (65.62)	8.27	0.77 (0.51-1.17)	0.20	0.81 (0.54-1.22)	0.32
	KPS (80-100)								**KPS (60-70)**						
Genotype	**Dead (120) n (%)**	**Alive (69) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Genotype**	**Dead (76) n (%)**	**Alive (17) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
GG	45 (37.50)	16 (23.19)	8.40	1	-	-	**Reference**	**GG**	27 (35.53)	5 (29.41)	4.10	1	-	-	**Reference**
GC	52 (43.33)	31 (44.93)	10.27	0.77 (0.51-1.16)	0.20	0.85 (0.56-1.28)	0.43	**GC**	38 (50.00)	7 (41.18)	5.87	0.77 (0.46-1.27)	0.28	0.89 (0.53-1.48)	0.65
CC	23 (19.17)	22 (31.88)	10.67	0.68 (0.42-1.10)	0.13	0.70 (0.42-1.78)	0.18	**CC**	11 (14.47)	5 (29.41)	5.93	0.62 (0.32-1.19)	0.18	0.66 (3.17-1.38)	0.27
(GC+CC)	75 (62.50)	53 (76.81)	10.67	0.74 (0.50-1.09)	0.11	0.79 (0.54-1.15)	0.22	**(GC+CC)**	49 (64.47)	12 (70.59)	5.87	0.71 (0.43-1.17)	0.15	0.81 (0.50-1.32)	0.41
	ECOG (0,1)								**ECOG (2)**						
Genotype	**Dead (100) n (%)**	**Alive (62) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***	**Genotype**	**Dead (74) n (%)**	**Alive (19) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
GG	34 (34.00)	15 (24.19)	9.00	1	-	-	**Reference**	**GG**	28 (37.84)	6 (31.58)	4.57	1	-	-	**Reference**
GC	45 (45.00)	27 (43.55)	11.83	0.75 (0.47-1.18)	0.19	0.80 (0.50-1.27)	0.35	**GC**	36 (48.65)	8 (42.10)	5.87	0.86 (0.52-1.41)	0.54	0.81 (0.48-1.35)	0.41
CC	21 (21.00)	20 (32.26)	10.13	0.75 (0.44-1.28)	0.30	0.77 (0.44-1.34)	0.35	**CC**	10 (13.51)	13 (68.42)	6.30	0.82 (0.51-1.34)	0.42	0.56 (0.26-1.23)	0.15
(GC+CC)	66 (66.00)	47 (75.81)	10.67	0.75 (0.48-1.16)	0.17	0.78 (0.51-1.93)	0.25	**(GC+CC)**	46 (62.16)	5 (26.31)	7.57	0.72 (0.36-1.41)	0.36	0.74 (0.45-1.20)	0.22
	ECOG (3,4)														
Genotype	**Dead (36) n (%)**	**Alive (5) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***								
GG	14 (38.89)	0 (0.00)	3.43	1	-	-	**Reference**								
GC	17 (47.22)	3 (60.00)	3.43	0.10 (0.49-2.02)	0.99	0.96 (0.45-2.08)	0.92								
CC	5 (13.89)	2 (40.00)	6.77	0.53 (0.21-1.33)	0.20	0.58 (0.16-2.09)	0.40								
(GC+CC)	22 (61.11)	5 (100.00)	5.17	0.84 (0.42-1.67)	0.59	0.85 (0.42-1.74)	0.66								

[b] Adjusted Hazard ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, stage, KPS, ECOG and smoking.

Table.5.8. Representing relationship of *p53Arg^{72}Pro* genotype with overall survival of lung cancer patients on the basis of smoking status

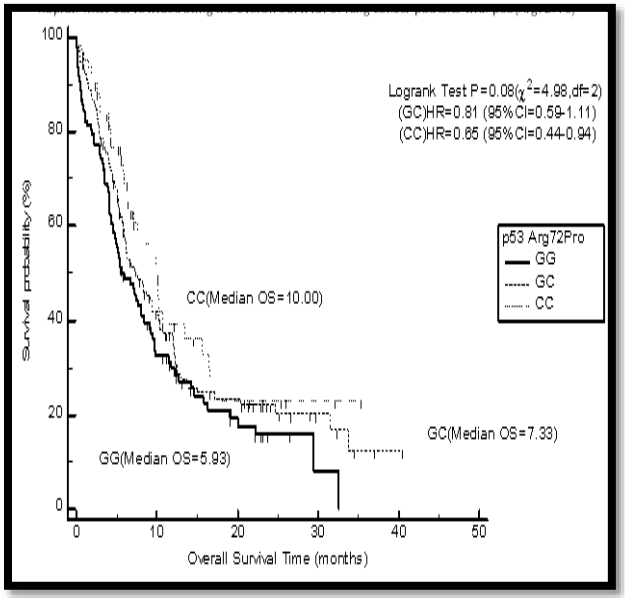
Smokers							
Genotype	**Dead (170) n (%)**	**Alive (73) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR[b] (95% CI)[b]**	***p***
GG	61 (35.88)	18 (24.66)	6.77	1	-	-	**Reference**
GC	82 (48.23)	30 (41.10)	6.30	0.90 (0.65-1.26)	0.55	0.91 (0.64-1.28)	0.59
CC	27 (15.88)	25 (34.25)	10.10	0.65 (0.42-0.99)	0.06	0.64 (0.40-1.03)	0.06
(GC+CC)	109 (64.12)	55 (75.34)	7.57	0.82 (0.59-1.14)	0.22	0.83 (0.60-1.15)	0.26
Non-smokers							
Genotype	**Dead (40) n (%)**	**Alive (13) n (%)**	**Median OS (months)**	**HR (95% CI)**	**Log *p***	**HR' (95% CI)**	***p***
GG	15 (37.50)	3 (23.08)	4.17	1	-	-	**Reference**
GC	16 (40.00)	8 (61.54)	12.07	0.48 (0.23-1.04)	**0.03**	0.61 (0.27-1.37)	0.23
CC	9 (22.50)	2 (15.39)	9.47	0.69 (0.31-1.53)	0.36	0.60 (0.22-1.60)	0.30
(GC+CC)	25 (62.50)	10 (76.92)	12.07	0.54 (2.61-1.11)	0.05	0.57 (0.28-1.16)	0.12

[b] Adjusted Hazard ratios, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, stage, KPS, ECOG and smoking.

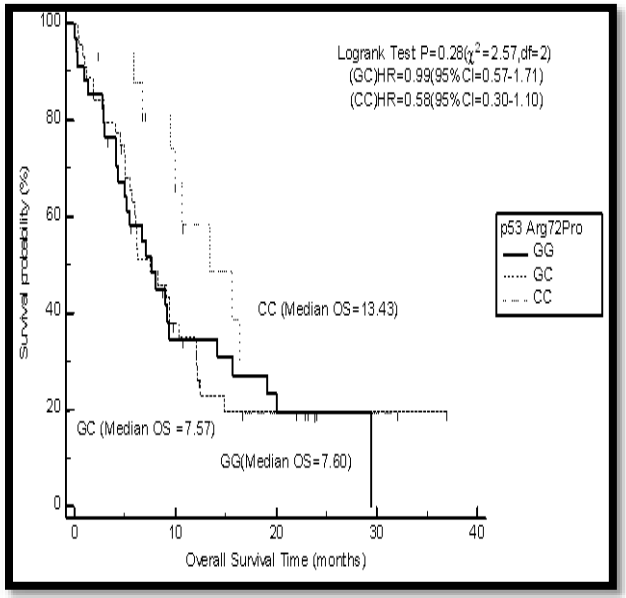
5.16. Association of *p53Arg^{72}Pro* genotypic distribution with overall survival of lung cancer patients on the basis of their smoking status:

On stratifying patients on the basis of smoking status no significant association was observed. However, nearly significant improved in survival was observed for patients with mutant (*CC*) genotype who were smokers (HR=0.64; 95% CI=0.40-1.03; ***p*=0.06).** In case on non-smokers patients with heterozygous (*GC*) genotype showed improved survival (HR=0.48; 95% CI=0.23-1.04; ***p*=0.03)** while performing univariate analysis however, no such correlation was observed after adjustment made by different parameters (HR=0.61; 95% CI=0.22-1.60; *p*=0.30). Furthermore smokers with mutant (*CC*) genotype having overall survival time (10.10) months whereas for non-smokers MST was higher for patients with heterozygous (*GC*) genotype (12.07) months. Frequency of dead patients was higher for *GC* genotype for both smokers (48.23) and non-smokers (40.00).

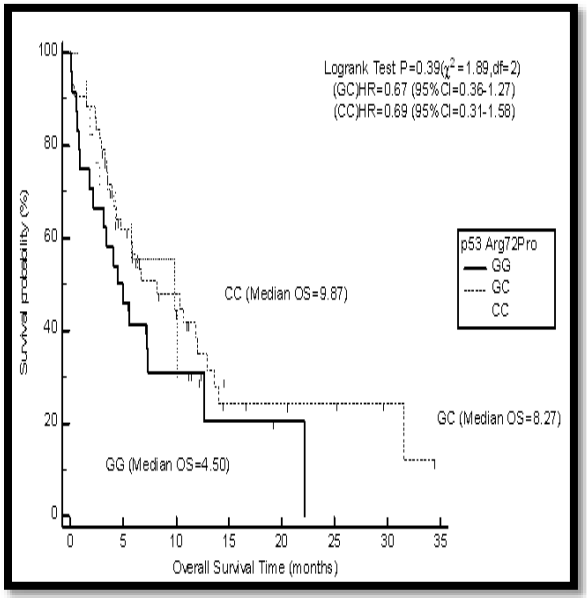
A


Logrank Test P=0.08(χ^2=4.98,df=2)
(GC)HR=0.81 (95%CI=0.59-1.11)
(CC)HR=0.65 (95%CI=0.44-0.94)
p53 Arg72Pro
GG
GC
CC
CC(Median OS=10.00)
GC(Median OS=7.33)
GG(Median OS=5.93)
Survival probability (%)
Overall Survival Time (months)

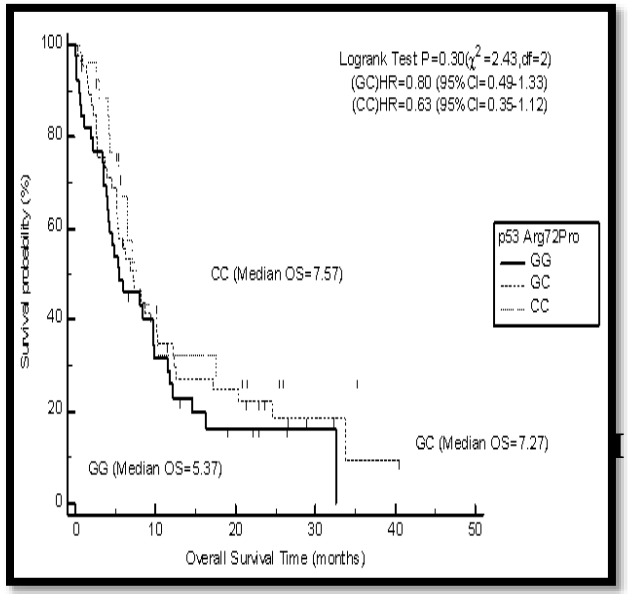
B


Logrank Test P=0.28(χ^2=2.57,df=2)
(GC)HR=0.99(95%CI=0.57-1.71)
(CC)HR=0.58(95%CI=0.30-1.10)
p53 Arg72Pro
GG
GC
CC
CC (Median OS=13.43)
GC (Median OS =7.57)
GG(Median OS=7.60)
Survival probability (%)
Overall Survival Time (months)

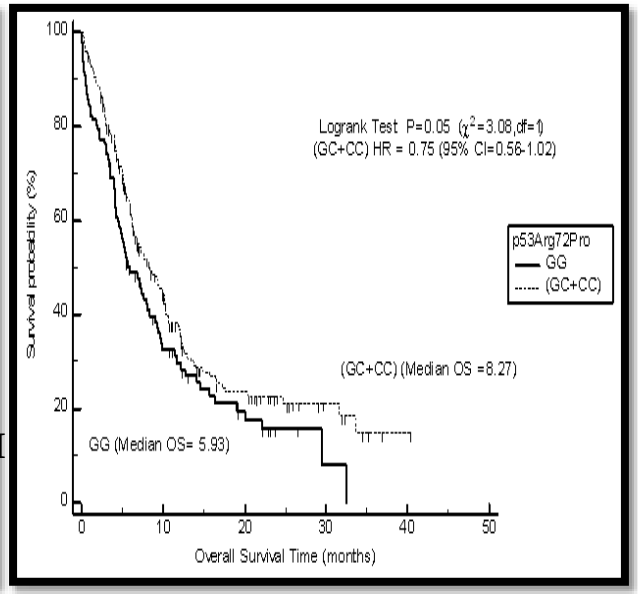
C


Logrank Test P=0.39(χ^2=1.89,df=2)
(GC)HR=0.67 (95%CI=0.36-1.27)
(CC)HR=0.69 (95%CI=0.31-1.58)
p53 Arg72Pro
GG
GC
CC
CC (Median OS=9.87)
GC (Median OS=8.27)
GG (Median OS=4.50)
Survival probability (%)
Overall Survival Time (months)

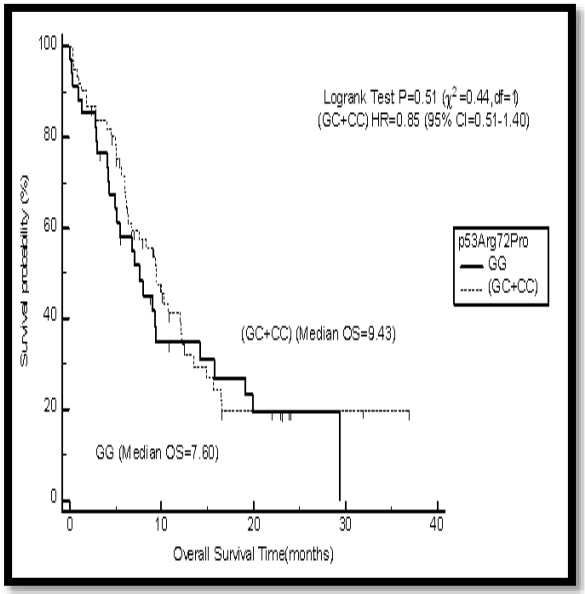
D


Logrank Test P=0.30(χ^2=2.43,df=2)
(GC)HR=0.80 (95%CI=0.49-1.33)
(CC)HR=0.63 (95%CI=0.35-1.12)
p53 Arg72Pro
GG
GC
CC
CC (Median OS=7.57)
GC (Median OS=7.27)
GG (Median OS=5.37)
Survival probability (%)
Overall Survival Time (months)

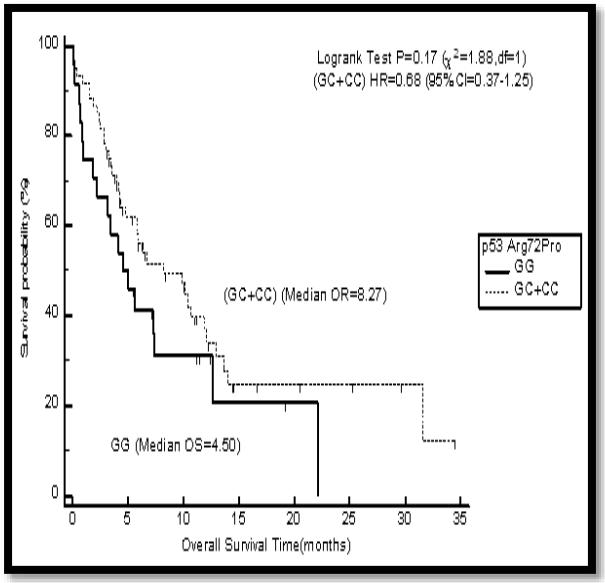
E

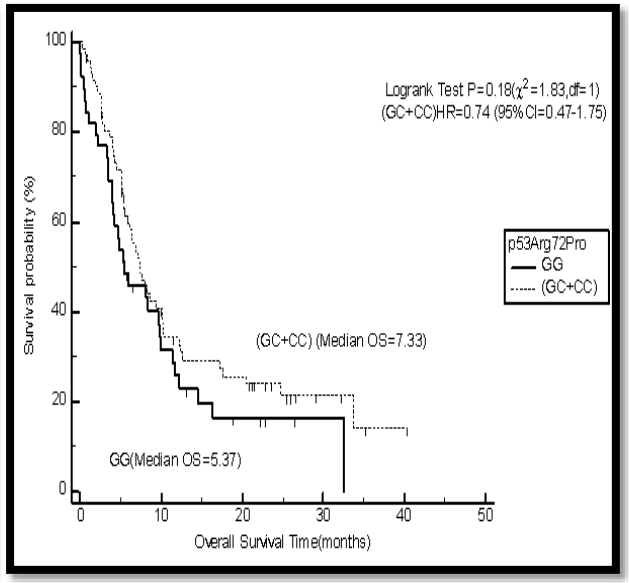

Logrank Test P=0.05 (χ^2=3.08,df=1)
(GC+CC) HR = 0.75 (95% CI=0.56-1.02)
p53Arg72Pro
GG
(GC+CC)
(GC+CC) (Median OS =8.27)
GG (Median OS= 5.93)
Survival probability (%)
Overall Survival Time (months)

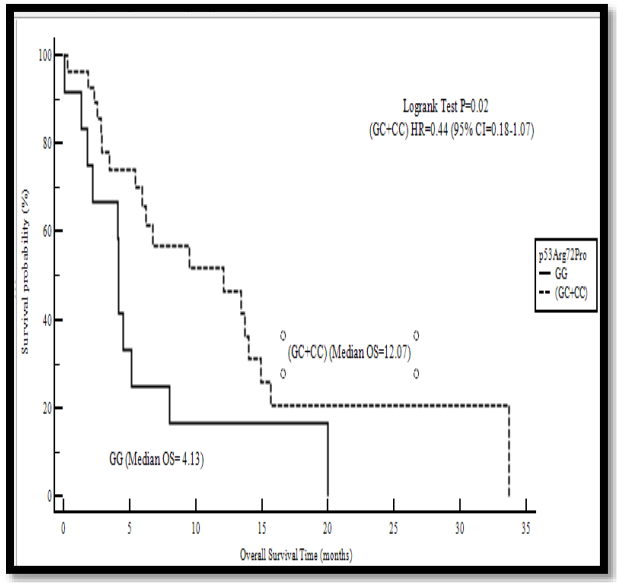
F


Logrank Test P=0.51 (χ^2=0.44,df=1)
(GC+CC) HR=0.85 (95% CI=0.51-1.40)
p53Arg72Pro
GG
(GC+CC)
(GC+CC) (Median OS=9.43)
GG (Median OS=7.60)
Survival probability (%)
Overall Survival Time(months)

G **H** **I**







J

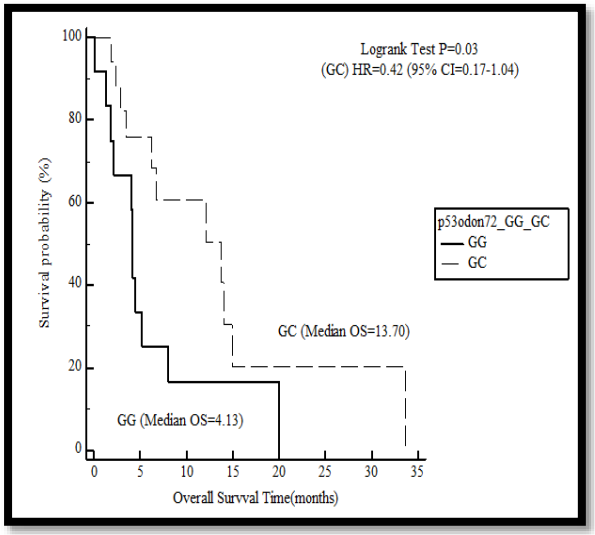


Fig.5.7. Kaplan-Meir curves of *p53Arg*^{72}*Pro* polymorphism. **A**: Survival of patients having (*GG*, *GC* and *CC*) genotype, **B**: Survival of ADCC patients having (*GG*, *GC* and *CC*) genotype, **C**: Survival of SCLC patients having (*GG*, *GC* and *CC*) genotype, **D**: Survival of SQCC patients having (*GG*, *GC* and *CC*) genotype, **E**: Survival of patients having (*GG* and (*GC+CC*)) genotype **F:** Survival of ADCC patients having (*GG* and (*GC+CC*)) genotype, **G**: Survival of SCLC patients having (*GG* and (*GC+CC*)) genotype, **H**: Survival of SQCC patients having (*GG* and (*GC+CC*)) genotype, **I**: Survival of female patients having (*GG* and (*GC+CC*)) genotype and **J**: Survival of female patients having (*GG* and *GC*) genotype.

DISCUSSION

The history of cancer cells revealed several genetic disruptions which effect the normal functioning of cells along with surrounding tissue to cause malignancy. In order to understand the complex mechanism involved in malignancy of cells, the signaling pathways of disrupted regulators need to be targeted and analyzed. *p53* is one of the competent cell cycle regulator which shows potential involvement in the study of different cancers.

p53 pathway helps to prevent abnormal cellular proliferation by either activating cellular apoptosis or activating genes involved in DNA repair system. Among all the polymorphism most common is at exon 4 encodes either Arginine (CGC) or Proline (CCC) residue where Proline (CCC) residue is found to be strongly associated with p53 interaction with TFIID factors and enhanced transcriptional capacity. It also induces G_1/G_2 cellular arrest and activates cell cycle checkpoints while arginine (GCG) residue was found to be strongly associated with translocation of *p53* to mitochondria for apoptotic activity as it shows better interaction with mitochondrial protein like GPRF and CRMI for nuclear export. Dysregulation and genetic aberration (mutation) of pathway involving p53 has been reported in several human cancers (Li *et al*., 2015, Lasaki *et al*., 2008, Chikako *et al*., 2004, Dastjerdi *et al*., 2008, Jira *et al*., 2013, Wu *et al*., 2014), among them *p53Arg*^{72}*Pro* at exon 4 is found to be correlated with lung cancer risk (George *et al*., 2011, Sakiyama *et al*., 2005, Ozaki *et al*., 2011, Han *et al*., 2008). Therefore, present study was framed to analyze *p53Arg*^{72}*Pro* polymorphism which impairs its normal biological function of maintaining cellular balance and genomic stability and to confer its association with lung cancer susceptibility in North Indian population. We also investigated different factors to analyze the prognostic effect and its relationship with overall survival in lung cancer patients.

Different epidemiological studies revealed that Arg and Pro alleles may increase susceptibility of different types of cancer (Li *et al*., 2015, Lasaki *et al*., 2008, Chikako *et al*., 2004, Dastjerdi *et al*., 2008, Jira *et al*., 2013, Wu *et al*., 2014). A study suggested higher risk of cervical cancer in patients having *Arg/Arg* and *Arg/Pro* genotype in Asian Population (Wu *et al*., 2014). Similarly, in Japanese population homozygous Pro allele was found to be an independent prognostic marker in breast cancer (Chikako *et al*., 2004). Significant risk of breast cancer was also reported in females in Iran with *Arg/Arg* genotype (Lasaki *et al*., 2015).

In the current study, patients with *Arg/Arg* and *Arg/Pro* genotypes were found to have a higher frequency than that of CC genotype with no statistically significant correlation associated with lung cancer risk as described by study done in Brazilian population also (Helen *et al*., 2007). An investigation done on Chinese population reported higher adjusted OR for variant genotype (*Pro/Pro* + *Arg/Pro*) than wild genotype (GG) which was concordant with our study (Liu *et al*., 2013). On the other hand another finding reported in female patients of Taiwan population found significant association of *Pro/Pro* genotype towards lung cancer susceptibility showing no consistency with the present study (Wang *et al*., 1999).

Furthermore, on investigating association of lung cancer risk and genotype on the basis of histology no significant correlation was observed in our data. On the contrary, Wang *et al*., 1999 have reported a risk for ADCC in patients with *Pro/Pro* genotype in Taiwanese population. On the other hand studies done in Polish and German populations have indicated that the mutant genotype (*Pro/Pro*) was found to be associated with SQCC development (Amelia *et al*., 2006, Popanda *et al*., 2007). Similar results were also observed in North American population (Fan *et al*., 2000). However a report in Asian subjects has suggested no significant association of the *Pro/Pro* genotype towards risk for NSCLC and SCLC (Wang *et al*., 2013).

Investigations in the present study showed no correlation of smoking and lung cancer susceptibility which was supported by study done on lung cancer patients of Mediterranean population (Figueras *et al*., 1996). In contrast a strong correlation of combined genotype (*Arg/Pro* + *Pro/Pro*) and lung cancer risk for smokers was reported in Brazilian lung cancer subjects (Helen *et al*., 2007). Odelia *et al.* have reported significant risk for lung cancer for individuals who were heavy smokers and carrying mutant (*Pro/Pro*) genotype (Popanda *et al*., 2007). Smoking was found to be associated with lung cancer susceptibility in *p53Arg*^{72}*Pro* polymorphism as mentioned in studies done by Aida *et al.* and Goeffrey *et al.* in Spanish and North American population (Rubio *et al*., 2008, Liu *et al*., 2001). Case-control studies in Chinese population suggested a significant association of mutant genotype (*Pro/Pro*) with lung cancer risk in heavy smokers and who were diagnosed with SQCC (Liu *et al*., 2013). On the contrary it was observed that *Arg/Pro* genotype was conferring a protective effect towards lung cancer development in Chile population (Dante *et al*., 2009). Another report stated an increase risk of

SQCC in heavy smokers for mutant genotype (*Pro/Pro*) in European population (Popanda *et al*., 2007). However investigation made by Wang *et al.*, 2013 found no elevated or reduced risk of lung cancer when stratification was made on the basis of histology and smoking.

The association of *p53* with lung cancer prognosis and overall survival of patients was also examined. Taking into consideration other epidemiological and clinic-pathological parameters in the present study no significant association was found for *Arg/Pro* genotype while *Pro/Pro* genotype showed a nearly significant effect for good prognosis towards lung cancer with higher overall survival. Study done by Chang *et al.*,1999 suggested poor prognosis for *Pro/Pro* genotype when compared to *Arg/Pro* with reduced overall survival in Taiwanese population. Furthermore findings showed non-significantly higher death risk for patients with *Pro/Pro* genotype in north Poland Amelia *et al*., 2006.

On stratifying data based on histology, ADCC patients have a higher survival among *Pro/Pro* genotype but was not significantly associated with any of the genotype of *p53Arg* ^{72}*Pro.* The study done in Japanese population suggested strong prognostic impact of *p53* alterations in ADCC patients and non significant survival impact in SQCC patients (Mitsudomi *et al*., 2000). Similarly our study conferred the SQCC subjects with most favored prognosis for *Pro/Pro* genotype. Patients having SQCC showed the minimum median survival time when compared to ADCC and SCLC patients and is supported by study done in Indian population (Mahesh *et al*., 2012).

Considering smoking as a cofactor for prognosis, heterozygous genotype showed a significant relation for lung cancer prognosis. However in the same study smokers with *Pro/Pro* genotype showed significant lower death risk. Similar observations were depicted by Orested *et al.,* 2007 in Danish population for smokers. On the contrary, our analysis was not consistent with the investigation made in Indian population where hazard ratio of non-smokers was found to increase with short duration of symptoms (Mahesh *et al*., 2012).

When effect of *p53Arg*^{72}*Pro* genotype was correlated with gender towards prognosis of lung cancer patients, females in all the three genotypic categories (*Arg/Arg*, *Arg/Pro* and *Pro/Pro*) showed a significant better prognosis for lung cancer as compared to males. Females with heterozygous (*Arg/Pro*) variant showed significantly lower mortality rate in comparison to

mutant (*Pro/Pro*) variant. No such correlation was found in males. Our study was consistent with the investigation made in Japanese population where lower hazard ratio was predicted for female patients with mutant (*Pro/Pro*) genotype (Mitsudomi *et al*., 1993).

Further investigation was carried out in the present study on the basis of tumor staging. Our data suggests better prognosis for stage IV patients with mutant (*Pro/Pro*) genotype as noted in Taiwan population (Wang *et al*., 1999). However patients with late tumor stage showed significantly good prognosis for lung cancer in univariate analysis which was not consistent with analysis made by Chang *et al.* where patients possessing mutant genotype (*Pro/Pro*) with early stage showed nearly significant trend for short survival with poor prognosis in Taiwanese population (Wang *et al*., 1999). Further survival rate was lower for patients with *Arg/Arg* genotype which did not confer with observation made in another study of Taiwan population which suggested a lower survival rate for stage III or stage IV patients with combined variant genotype (*Pro/Pro + Arg/Pro*) (Mitsudomi *et al*., 1993). No correlation was seen between death risk and performance status (KPS and ECOG) of patients undergoing platinum based chemotherapy. However, in a study done in Korean population mutant variant (*Pro/Pro*) were found to be more resistant to first line chemotherapy of regimen (irinotecan plus cisplatin) (Lin *et al*., 2008).

Therefore overall *p53Arg*^{72}*Pro* showed lower risk for mutant genotype and good prognosis for females and non-smokers with reduced mortality rate. Codon 72 is a hotspot of polymorphism and encodes the p53 protein which interacts to play a major role in apoptotic pathway (mechanism to control malignancy in normal cells). However single SNP of *p53* alone may not influence death risk in patients diagnosed with lung cancer.

Therefore, overall *p53Arg*^{72}*Pro* showed lower risk for mutant genotype and good prognosis for females and non-smokers with reduced mortality rate. Codon 72 is a hotspot of polymorphism and encodes the p53 protein which interacts to play a major role in apoptotic pathway (mechanism to control malignancy in normal cells). However single SNP of *p53* alone may not influence death risk in patients diagnosed with lung cancer.

In conclusion, the present study help us to analyze the genetic variation in *p53* gene and its role in predicting lung cancer risk in North Indian population with sample size of 420 subjects. We

also investigated the role of clinic-pathological parameters in lung cancer susceptibility and prognosis, but no such significant correlation was noted. Genes are the potent markers for analyzing

the molecular mechanism involved in carcinogenesis. Hence the present study will analyze the affect of genetic variants in *p53* signaling pathway which plays a major role in maintain the integrity of human genome and further studies with larger sample size in different ethnicities will help in validating the present conclusions and also have a path for using *p53* as a prognostic marker.

CONCLUSION

We conclude that the present study found no significant association of *p53Arg^{72}Pro* genotype and other epidemiological and clinic-pathological parameters towards increased susceptibility and pathological development of lung cancer in different provinces of North Indian population.

However nearly significant association was found for overall survival of lung cancer patients with mutant (*CC*) genotype.

On the other hand females lung cancer patients with any (*GG*, *GC* and *CC*) genotype showed significant improve in overall survival showing *p53Arg^{72}Pro* genotype to be the independent prognostic factor for females in North Indian population.

Hence, the present study analyzed the affect of genetic variants in p53 signaling pathway which validate the present conclusions and also have a path for using *p53* as a prognostic marker for lung cancer.

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APPENDIX

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 4^0C overnight.

6. 5M Sodium chloride (NaCl): Disso olved 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 ($MgCl_2$) (100mM): Dissolved 0.41gms of $MgCl_2$ in 20ml of deionised water and sterilized by autoclaving.

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

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

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