Synergistic association of *CYP1A1* and *CHRNA5* $D^{398}N$ genetic variants towards increased susceptibility for Lung Cancer in North Indian Population

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Masters in Technology

Under the guidance of
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DECLARATION

I, the under designed, hereby declare that the research work presented in the M.tech dissertation entitled **"Synergistic association of CYP1A1 and CHRNA5 D398N genetic variants towards increased susceptibility for Lung Cancer in North Indian Population"** has been carried out by me under the supervision and guidance of Dr. Shiddharth 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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i | Page

CERTIFICATE

This is to certify that dissertation entitled, **"Synergistic association of CYP1A1 and CHRNA5 D398N genetic variants towards increased susceptibility for Lung Cancer in North Indian Population"** submitted by Ms. Yashila in partial fulfilment of the requirements for the award of M.tech in Biotechnology at Thapar University, Patiala is an authentic work Carried out by her under our supervision and guidance.

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

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iii | Page

ABSTRACT

Number of studies done so far in different populations has shown that polymorphism within the *CYP1A1*and *CHRNA5*gene play an important role in determining individual susceptibility towards lung cancer; however data so far obtained has been contradictory within the same or different populations. Few studies have focused on the synergistic effect of the *CYP1A1*and *CHRNA5*polymorphisms towards susceptibility for lung cancer and also for different histological sub-types along with the impact of smoking. Objectives: Our aim is to investigate the role of *CYP1A1*and *CHRNA5*polymorphisms as a genetic modifier of risk for lung cancer and histological subtypes with larger sample size in a North-Indian population. Methods: A total of 704 subjects (353 lung cancer and 351 controls) were evaluated for the *CYP1A1* polymorphism and total of 513 subjects (206 lung cancer and 307 controls) were evaluated for *CHRNA5* polymorphism. Genotyping for the *CYP1A1*and *CHRNA5*gene was done by using a PCR-RFLP method. Results: *CYP1A1* mutant genotype was found to be significantly associated with lung cancer (OR=3.15; 95%CI=1.75-5.71; p=0.0001) and this risk was four-fold higher in case of SQCC. The *CYP1A1m2* was also found to be associated with risk towards lung cancer however when stratified for histological subtypes a significant association was observed for SQCC and ADCC. The combined 'at risk' genotypes of *CYP1A1m1* and *m2*geneswere found to be associated for lung cancer risk and this risk was higher in case of SQCC (OR=2.0; 95%CI=1.97-3.81; p=0.028).There has been no significant association have been found in relationship between *CHRNA5* polymorphic gene and risk of lung cancer (OR= 1.22; 95%CI=0.56-2.6; p= 0.60). However, the study has also evaluated the genetic variants in *CHRNA5* polymorphism with respect to different histology of lung cancer but no significant OR has been found in this relation to increase risk of lung cancer (OR=1.23; 95%CI= 0.4-3.6; p=0.70). Further, study subjects stratified according to smoking status and pack year but similarly it has been seen that in North Indian population smoking has not significant effects on the $D^{398}N$ polymorphism in α*5* subunit of nAChRs coded by *CHRNA5* gene having G>A mutation in North Indian population (OR= 1.46; 95%CI= 0.6-3.3; p= 0.37).Conclusions: The polymorphism in the *CYP1A1* gene seem thus to be important risk modifiers for lung cancer and related histological subtypes, along with smoking. But on the other hand, the polymorphism in the *CHRNA5* gene did not seem to be important risk modifiers for lung cancer and related histological subtypes, along with smoking North Indian population.

Table of Contents

List of Tables

List of Figures

Abbreviation

ACh	Acetylcholine
ADCC	Adenocarcinoma
AHH	Aryl hydrocarbon hydroxylase
CI	Confidence Interval
CYP	Cytochrome P450
DFP	Diisopropylfluorophosphate
DNA	Deoxyribo nucleic acid
dNTPs	Deoxynucleotide
DRE	Dioxin Responsive Elements
EDTA	EDTA Ethylene diamine tetracyclic acetic acid
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide
FMO3	Flavin- containing monooxygenase- 3
HAT	Histone Acetyl transferase
HWE	Hardy Wienberg Equilibrium
MAF	Minor Allelic Frequency
MAPK	Mitogen-activated protein kinases
nAChRs	Nicotinic Acetylcholine Receptors
NAD(P)	Nicotinamide adenine dinucleotide phosphate
NcoA2	Nuclear co activator 2
NNK	Nicotine-derived nitrosamine ketone
NSCLC	Non-Small Cell Lung Carcinoma
ORs	Odds Ratios
PAGE	Poly Acrylamide Gel Electrophoresis
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PY	Pack Years
SCLC	Small Cell Lung Carcinoma
SD	Standard Deviation

SDS	Sodium Dodicyl sulphate
SQCC	Squamous Cell Carcinoma
SRC-1	Steroid receptor co-activator
TBE	Tris Borate EDTA
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TE	Tris EDTA
TNM	Tumor Node Metastasis
XRE	Xenobiotic Responsive Elements

CHAPTER 1

INTRODUCTION

1. Introduction

Lung cancer is the leading cause of cancer related deaths worldwide and the incidence of lung cancer is increasing in developing countries (Siegel *et al*., 2014). Approximately 63,000 lung cancer patients are reported each year in India (Noronha *et al*., 2012). It is postulated that tobacco smoke is the major contributor and risk factor for lung cancer and 90% of all cases is due to smoking (Behera, 2012).However, not all smokers develop lung cancer and it has been estimated that fewer than 20% of smokers develop the disease (Carlsten *et al*., 2008). Tobacco smoke contains abundant of both known and unknown chemicals which produce highly carcinogenic compounds when activated (Shukla *et al*., 2014). Nicotine, a pale yellow colour natural occurring liquid alkaloid is well known chemical carcinogen found in tobacco plants. Along with this there are various other chemical carcinogens like PAH; NNK etc are present in tobacco smoke. The process of carcinogen metabolism is mediated by Phase I enzymes called as Cytochrome P450 (CYP). Cytochrome P450 is heme containing enzymes present in both hepatic and extra hepatic tissues (Androutsopoulos etal., 2009). Cytochrome P450 enzymes are involved in catalyzing the first step of phase I metabolic biotransformation reactions like C-, N-, S-oxidation and dealkylation of xenobiotic compounds, pro-carcinogens, toxins, *etc*.(Oyama *et al*., 2004).Cytochrome P450 enzymes are coded by *CYP* multigene family which includes approximately 57 functional genes.Among the various CYP genes, *CYP1A1*located on chromosome 15q22-q24 (Hildebrand *et al*., 1985) encodes for the enzyme aryl hydrocarbon hydroxylase (AHH) which is responsible for the activation of Polycyclic Aromatic Hydrocarbons (PAHs) such as benzo(a)pyrene (Gonzalez and Nebert, 1990). Once activated PAH metabolites act as powerful carcinogens which then bind to DNA to form adducts and produce somatic mutations in either tumor suppressor or oncogenes and trigger the process of carcinogenesis and hence cancer initiation. Moreover, the excessive use nicotine containing tobacco products also promotes the lung cancer by activating signalling pathways that leads to cell proliferation, cell survival, angiogenesis and metastasis (Roman *et al*., 2009). The activation of these cancer signalling pathways by nicotine is mediated by its cognate receptor Nicotinic Acetycholine Receptors (nAChRs). The nAChRs expressed in lung epithelial cells, are activated by binding of nicotine and additionally NNK leads to opening of ion channels and increase in calcium influx in the cell. Calcium acts as second messenger and activates the cancer signalling pathways along with the secretion of mitogenic factors. This process is considered as hallmark for lung cancer (Improgo *et al*., 2011).

The *CYP1A1* gene is polymorphic and has been shown to have 15 different allelic variants (Androutsopoulos *et al*., 2009). Among the different polymorphic variants of the *CYP1A1* gene four of these variants designated as *m1*, *m2*, *m3* and *m4* have been widely studied in Asian and Caucasian populations as a risk factor for lung carcinogenesis. The *m1* and *m2* polymorphism are found to have involvement in lung carcinogenesis (Shah *et al*., 2008; Xu *et al*., 1996). The *CYP1A1m1*(rs4646903)polymorphism has been associated with elevated enzyme activity due to a single base point mutation at nucleotide position 3801 which results in transition of thymine to cytosine in the 3' untranslated region. The *CYP1A1m2*polymorphism (rs1048943) involves a base substitution from adenine (A) to guanine (G) at codon 462 in exon 7, resulting in substitution of isoleucine with valine ($Ile^{462}Val$) which results in the increase in enzyme activity (Oyama el al., 1994).

On the other hand, structurally, nAChRs are composed of 9α (α1-α7, α9, α10) and 4β (β1-β4) transmembrane subunits encoded by *CHRNA1-CHRNA7*, *CHRNA9*, *CHRNA10* and *CHRNB1- CHRNB4* genes respectively (Lam *et al*., 2007). Multiple genome wide association studies have revealed that *CHRNB4*/A3/A5 on 15q24 chromosome locus has significant contribution in nicotine dependence and lung cancer (Hung *et al*., 2008). *CHRNA5* involves in the modifying calcium permeability of receptors and its affinity towards agonist. The non-synonymous SNP that lies in the fifth exon of *CHRNA5* results in the change from aspartic acid to aspargine residue at amino acid position 398 (D398N) and decreased maximal response to agonist indicating altered receptor function (Improgo *et al*., 2013).

Thus the variation in the genotype of *CYP1A1m1*, *m2* and *CHRNA5* may associate with the risk of lung cancer in relation to smoking. To find out the association between the risk of lung cancer and genotype of *CYP1A1m1*, *m2* and *CHRNA5* polymorphism several studies have been conducted on Caucasian population, Asian population particularly Chinese, Japanese, Korean and Indian populations; however results obtained were inconsistent (Chen *et al*., 2011; Hansen *et al*., 2010; Ji *et al*., 2012; Lee *et al*., 2008; Shiraishi *et al*., 2009; Wu *et al*., 2009.). Furthermore this risk was compounded in case of SQCC and tobacco smokers. When evaluated in Caucasians the above findings were not confirmed because the prevalence of both *m1* and *m2* alleles is low (Bouchardy *et al*., 1997; Hung *et al*., 2003). However, similar studies conducted in larger sample size of mixed American populations did show a positive correlation between *CYP1A1m1* polymorphism and lung cancer risk (Le Marchand*et al*., 2003). Meta-analysis study done in Asian and Caucasian populations has suggested that the *m2* variant allele in exon 7 of the *CYP1A1* gene is associated with an increased risk for

lung cancer; especially for SQCC individuals (Zhan *et al*., 2011). These finding indicated that both polymorphisms in the *CYP1A1*gene might be an important risk factor in the lung cancer development (Ji *et al*., 2012). In context to studies done in Indian populations contradictory results were observed. Studies done in Kashmiri population of North India and North-Central India observed a significant association between the *CYP1A1m1* and *m2* polymorphism and elevated risk of lung cancer, especially SQCC (Shaffi *et al*., 2002; Shah *et al*.,2008). On the contrary, a similar study in North Indian population has shown that the *CYP1A1m1* variant allele is not associated with lung cancer risk; however the *m2* variant allele was found to besignificantly associated with risk for lung cancer (Sobti *et al*., 2004). Similarly, in case of *CHRNA5* polymorphism only few studies have been conducted in Asian population (Sa M *et al*., 2012) and Caucasian population (Saccone *et al*., 2009). A study on Japanese population has shown the significant association of *CHRNA5* SNP genotype with increase risk of lung cancer whereas the no significance has been shown by Bangladeshi population (Sa, M., *et al*., 2012; Shiraish, *et al*., 2009). Likewise Caucasian population has shown no significance association between *CHRNA5* genotype and occurrence of disease (Hassen *et al*., 2010). There is no studies have been conducted on Indian population till date except a study on Gujrati Indians in Houston (GII), Texas, USA (Sa M *et al*., 2012).

Most of the studies done so far in Indian population have been inconclusive. Therefore, the present study was undertaken for couple of reasons. Firstly, to provide with substantial evidence of the association between *CYP1A1* and *CHRNA5* polymorphism and lung cancer risk with a much larger sample size. Secondly very few studies have been conducted so far which have looked at the combined effect of both the polymorphic variants of the *CYP1A1* gene in conjunction with role of smoking and its synergistic interaction with the *CYP1A1*. Therefore, the present aim of this work is to further investigate the role of *CYP1A1m1* (rs4646903),*m2* (rs1048943), *CHRNA5* (rs16969968) polymorphic variants as a genetic modifier of risk for lung cancer and also for histological sub-types. Furthermore we also planned to set an objective to study the relationship of the *CYP1A1*and *CHRNA5*allele variants in relation to tobacco smoking with larger sample size in a North-Indian population. We performed association analysis in this case control study for the two genetic variants of the *CYP1A1* gene and *CHRNA5* gene individually and also in different combinations in order to evaluate the cumulative role of the same in modifying the susceptibility towards lung cancer

CHAPTER 2

STUDY FIELD

2. Literature Review

2.1 Biotransformation of Xenobiotics

With the advancement of industries and technology individuals exposed constantly and unavoidable various harmful environmental chemicals or *Xenobiotics*, which includes manmade chemicals, alkaloids, plant secondary metabolites, smoke from cigarette/ Tobacco smoking, industrial chemical, pesticides etc. All these chemicals have physical properties of getting easily absorbed by passive diffusion through skin, lungs and gastrointestinal track as they are lipophilic in nature. Due to their lipophilicity they are easily get reabsorbed in body and difficult to remove. Thus these chemicals have slow and lethal effects on the human body if not eliminated. The human body has tendency to convert liphophilic compounds into water soluble compounds by the process of *Biotransformation*. After the absorption, the xenobiotic compounds undergo series of Biotransformation reactions catalyzed by enzymes in the liver and other tissues and make them favourable for the excretion (Oyama *et al*., 1994).

Xenobiotic compounds exert a variety of effects on biological system dependent on physicochemical properties of the compounds. The effects may be harmful in case poisons chemicals or carcinogens or it may be beneficial in case pharmaceutical drugs. It may also possible that the biotransformation reaction may change the biological activity of the substance. The importance of this change involves in metabolic fate of the xenobiotic compounds having an important bearing on the toxic potential, the disposition of the compound in the body and the excretion of the compound. Thus according to this principle of toxicology, the xenobiotics must undergo biotranformation to exert their characteristic toxic or tumorigenic effect (i.e., many chemicalswould be considerably less toxic or tumorigenic if they were notconverted to reactive metabolites by xenobiotic-biotransformingenzymes).In most cases, however, biotransformation terminatesthe pharmacologic effects of a drug and lessens the toxicity of xenobiotics. Xenobiotic biotransformation reaction is accomplished by a vast variety of enzymes with substrate specificities which determine the intensity and duration of action of drugs and play a key role in chemical toxicity and chemical tumorigenesis (Anders,1985; Jakoby, 1980; Jakoby *et al*., 1982; Kato *et al*., 1989).The synthesis of some of these enzymes is triggered by the xenobiotic (by the process of enzyme induction), but in most cases the enzymes are expressed constitutively (i.e., they are synthesized in the absence of a discernible external stimulus).

The chemical conversion of Xenobiotics can be occur in two ways- Detoxification which produce less toxic compounds and ultimately excreted out from the body and bioactivation which on biotransformation of procarcinogens/ prodrugs yields more toxic compounds which ultimately leads to carcinogenesis. In the bioactivation process, the biotransformation covert some inert chemical or inactive compounds into reactive intermediated like epoxides, radicals, carbonium ions and nitrenium ions and these intermediates have capability of damaging the cells and tissues of the body (Hinson *et al* 1994). The generated reactive intermediates may react with the DNA and form adducts which, if they escape from repair mechanism can cause carcinogenic mutations. Therefore, the toxicity or carcinogenicity of these chemical is linked to the metabolism of the compounds.

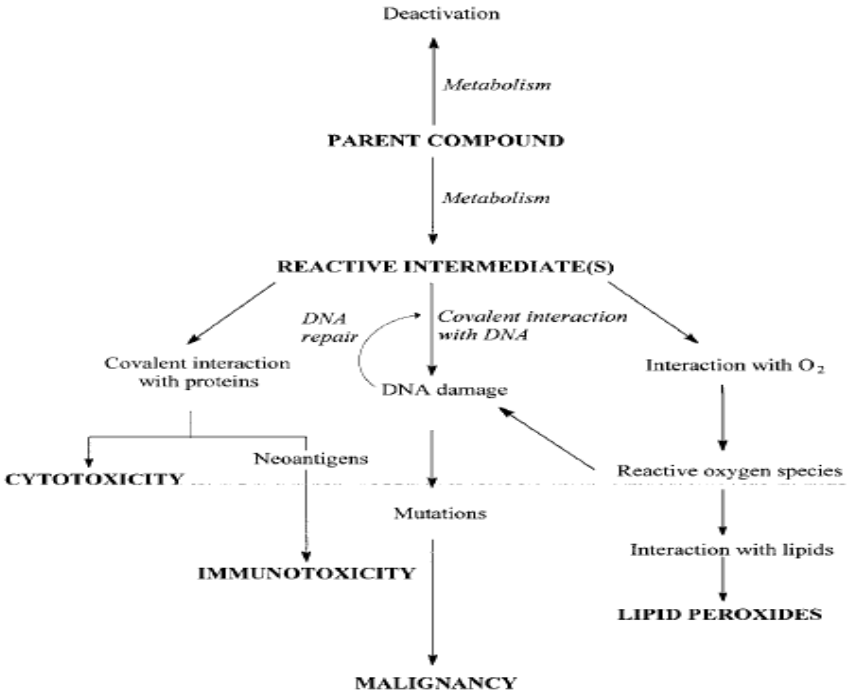

Figure: 2.1 Bioactivation of Xenobiotics biotransformation (Ioannides et al., 2004)

2.2 Biotransformation reactions

The biotransformation reactions of xenobiotic compounds divided into two phases-

- Phase I reactions
- Phase II reactions

These reactions catalyzed by various xenobiotic biotransforming enzymesas shown in Table 2.1 (Williams, 1971). Phase I reactions involvehydrolysis, reduction, and oxidation. These

reactions expose or introducea functional group (–OH, –NH2, – SH or –COOH), andusually result in only a small increase in hydrophilicity. Phase IIbiotransformation reactions include glucuronidation, sulfonation(more commonly called sulfation), acetylation, methylation, conjugationwith glutathione (mercapturic acid synthesis), and conjugationwith amino acids (such as glycine, taurine, and glutamicacid).The cofactors for these reactions react withfunctional groups that are either presents on the xenobiotic or areintroduced/exposed during phase I biotransformation. Most phaseII biotransformation reactions result in a large increase in xenobiotichydrophilicity, hence they greatly promote the excretion of foreignchemicals.

Phase I biotransformation reactions

Phase I reaction involves series of three chemical reaction, hydrolysis, reduction and oxidation. The enzymes of these chemical reactions expose or introduce some polar functional group on the xenobiotic compounds which slightly increase the hydrophilicity of the chemical compounds.

In*Hydrolysis*reactions, a variety of hydrolytic enzymes such as *carboxylesterases, cholinesterases and organophosphatases*hydrolyse the xenobiotics containing such functional groups as a carboxylicacid ester (procaine), amide (procainamide), thioester(spironolactone), phosphoric acid ester (paraoxon), and acid anhydride[diisopropylfluorophosphate (DFP)] (Satoh and Hosokawa, 1998). Certain xenobiotic compounds or environmental metals like pentavalent arsenic contain functional groups like aldehyde, ketone, disulfide, sulfoxide, quinone, N-oxide,alkene, azo, or nitro group areoften reduce in vivo by enzymatically or non-enzymatically through interaction with reducing agents like FMN, FAD and NAD(P). These functional groups can either reduce by *reduction reactions* like azo reduction, nitro reduction, disulfide reduction, sulfoxide reduction etc. or *oxidised* by catalysing various oxidising enzymes like Cytochrome P450, aldehyde oxidase, alcohol dehydrogenase, aldehyde dehydrogenase etc.

Phase II biotransformation reactions

Phase II biotransformation reactions include glucuronidation, sulfonation(more commonly called sulfation), acetylation, methylation,conjugation with glutathione (mercapturic acid synthesis), andconjugation with amino acids (such as glycine, taurine, and glutamicacid) (Paulson *et al*., 1986).The cofactors for these reactionsreact with functional groupsthat are

either present on the xenobiotic or are introduced/exposedduring phase I biotransformation. With the exception of methylationand acetylation, phase II biotransformation reactions result ina large increase in xenobiotic hydrophilicity, so they greatly promotethe excretion of foreign chemicals.Mostphase II biotransforming enzymes are mainly located in the cytosol;a notable exception is the UDP-glucuronosyltransferases,which are microsomal enzymes (Table 2.1). Phase II reactions generallyprocseed much faster than phase I reactions, such as thosecatalyzed by cytochrome P450. Therefore, the rate of eliminationof xenobiotics whose excretion depends on biotransformation bycytochrome P450 followed by phase II conjugation is generally determinedby the first reaction (Androutsopoulos *et al*., 2009).

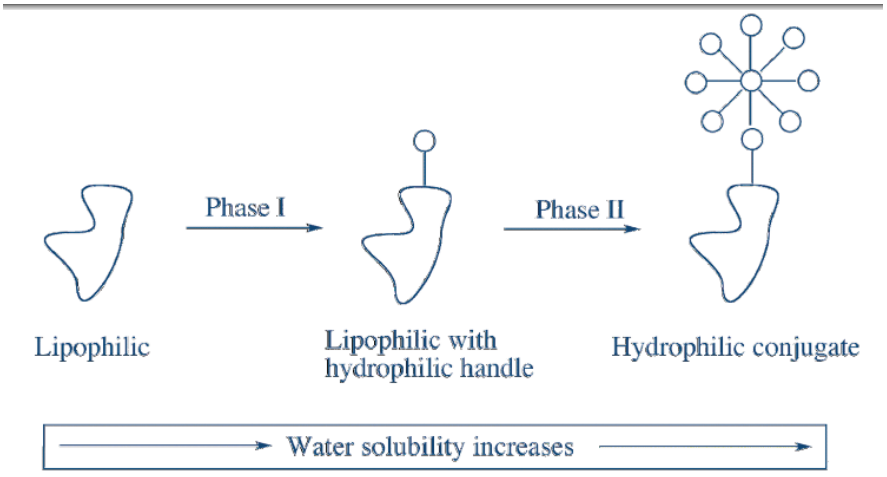

Figure 2.2 An overview of Biotransformation reactions (Centre for analysis and synthesis)

Table: 2.1 Enzymes in Biotransformation reactions (Williams., 1971)

Reaction	Enzyme	Localization
Phase I enzymes		
Hydrolysis	Esterase	Microsomes, cytosol, lysosome, blood
	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro-reduction	Microflora, microsomes, cytosol
	Carbonyl reduction	Cytosol, blood, microsomes
	Disulfide reduction	Cytosol
	Sulfoxide reduction	Cytosol
	Quinone reduction	Cytosol, microsomes
	Reductive dehalogenation	Microsomes
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Prostaglandin H synthase	Microsomes
	Flavin-monooxygenases	Microsomes
	Cytochrome P450	Microsomes
Phase II reaction		
	Glucuronide conjugation	Microsomes
	Sulfate conjugation	Cytosol
	Glutathione conjugation	Cytosol, microsomes
	Amino acid conjugation	Mitochondria, microsomes
	Acylation Mitochondria	cytosol
	Methylation	Cytosol, microsomes, blood

2.3 Cytochrome P450 Enzyme

Among the enzymes of Phase I reactions Cytochrome p450 enzymes play a key role in the biotransformation. It detoxifies or activates the reactive intermediates of chemical reactions (Guengerich, 1987; Waterman andJohnson, 1991). The highest concentration of P450 enzymes involvedin xenobiotic biotransformation is found in liver endoplasmicreticulum (microsomes), but P450 enzymes are present in virtuallyall tissues. Cytochrome P450 enzyme is a large family of heme- containing proteins. The hemeiron in cytochrome P450 is usually in the ferric ($Fe3^{+}$) state. When reduced to the ferrous ($Fe2^{+}$) state, cytochrome P450 can bind ligandssuch as O2 and carbon monoxide (CO). The complex betweenferrous cytochrome P450 and CO absorbs light maximally at 450nm, from which cytochrome P450 derives its name (Negishi *et al*., 1996).By competing with oxygen, CO inhibits cytochrome P450.The inhibitory effect of carbon monoxide can be reversed by irradiationwith light at 450 nm, which photodissociates the cytochromeP450–CO complex. This inhibition established cytochrome p450 as the microsomal and mitochondrial enzymeinvolved in drug biotransformation and steroid biosynthesis(Omura, 1999). Cytochrome p450 enzyme often exhibit broad substrate specificity, thus it may act on many xenobiotic compounds; consequently, different P450smay catalyze formation of the same product. Due to the extreme versatile nature of p450 enzyme, it can catalyse approximately 60 different types of reactions in biotransformation. However, basically they catalyze monooxygenation reactions involving introduction of one atom of oxygen into thesubstrate, designated as RHand the other is reduced to water with reducingequivalents derived from NADPH, as follows:

$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^{+} \longrightarrow \text{Product (ROH)} + H_2O + NADP^{+}$$

The Liver microsomes from all mammalian species contain numerousP450 enzymes, each with the potential to catalyze the various types of oxidation reactions, including:

- Hydroxylation of an aliphatic or aromatic carbon
- Epoxidation of a double bond
- Heteroatom (S-, N-, and I-) oxygenation and N-hydroxylation
- Heteroatom (O-, S-, N- and Si-) dealkylation
- Oxidative group transfer

- Cleavage of esters
- Dehydrogenation

The liver microsomal P450 enzymes involvedin xenobiotic biotransformation belong to three main P450 genefamilies, namely CYP1, CYP2, and CYP3. Liver microsomes alsocontain P450 enzymes encoded by the CYP4 gene family, substratesfor which include several fatty acids and eicosanoids butrelatively few xenobiotics. In humans approximately 60 genes have been found to encode more than 15 different Cytochrome p450 enzymes that biotransform xenobiotic compounds (*CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, 4A9, and 4A11*) (Guengerich, 1994; Wrighton and Stevens, 1992).

In mammalian species *CYP1A1* is most predominately expressed in lung epithelium, skin, intestine, lymphocytes and placenta particularly in cigarette smokers and is widely studied gene in smokers.

In humans *CYP1A1* gene consists of seven exons and six introns and is located on chromosome 15q24.1 (Murray *et al*., 2001; Nelson *et al*., 2004). The enzyme encoded by *CYP1A1* gene known as Aromatic Hydrocarbon Hydroxylase (AHH) constitutes of 512 amino acids having size of 58kD. This enzyme mainly catalyzes the O-dealkylation of7-methoxyresorufin and 7-ethoxyresorufin. Reactionspreferentially catalyzed by *CYP1A1* include the hydroxylationand epoxidation of benzo[a]pyrene and theepoxidation of the leukotriene D4 receptor antagonist, verlukast.

CYP1A1 gene contains AhR binding sites, which are involved in transcriptional activation of these enzymes (Schults *et al*., 2010). Induction of *CYP1A1* involvesboth derepression and activation of transcription by the Ah receptor.Although this cytosolic receptor binds several aromatic hydrocarbons,such as 3-methylcholanthrene and benzo[a]pyrene, theligand with the highest binding affinity is TCDD, which is why theAh receptor is also known as the dioxin receptor (Whitlock, 1993).

The Ah receptor is normally complexes in a 1:2 ratio with heatshock protein (hsp90), which dissociate upon binding of ligand tothe Ah receptor, enabling the receptor to be phosphorylated by tyrosinekinase. The activated Ah receptor then enters the nucleusand forms a heterodimer complex with the Ah-receptor-nucleartranslocator Arnt. Inside the nucleus, the Ah receptor-Arnt complexbinds to regulatory sequences [known as dioxin-responsive elements(DRE) or xenobiotic responsive elements (XRE)] and enhancesthe transcription of the *CYP1A1* gene and other genes withan XRE or XRE-like sequence in their upstream enhancer region[namely CYP1A2, DT-diaphorase, glutathione S-transferase,

UDPglucuronosyltransferase(UGT1A6 and UGT1A7), and aldehydedehydrogenase]. The XRE is only a small segment of DNA (theconsensus sequence is 5-TXGCGTG-3, where X is normally Tor A), which can be located more than a thousand bases from theinitiation site for transcription. The enhancer region of the *CYP1A1*gene contains multiple XREs, which accounts for the marked (<100-fold) increase in *CYP1A1* mRNA and protein levels followingexposure to ligands for the Ah receptor.Arnt was initially thought to be a cytosolic protein that simplyfacilitates the translocation of the ligand-bound Ah receptorinto the nucleus. It is now recognized as an important componentof the receptor complex that binds to DNA and activates transcriptionof genes under the control of the Ah receptor. For heterodimerformation, the Ah receptor must be bound to ligand andpossibly phosphorylated, and Arnt must be phosphorylated, apparently by protein kinase C. The Ah receptor is often comparedwith the steroid/thyroid/retinoid family of receptors, which alsobind ligands in the cytoplasm and are translocated to the nucleuswhere they bind to DNA and enhance gene transcription. However,the Ah receptor is a novel ligand-activated transcription factor, verydistinct from these other receptors. Whereas the steroid/thyroid/retinoid receptors have "zinc-finger" DNA-binding domains andform homodimers, the Ah receptor forms a heterodimer with Arnt;both of these contain a basic helix-loop-helix (bHLH) domainnear their N-terminus. The basic region binds DNA and the helixloop-helix is involved in protein–protein interactions. The XRErecognized by the Ah receptor-Arnt complex contains a sequenceof four base pairs (5-GCGT-3) that is part of the recognitionmotif for other bHLH proteins (Whitlock, 1993). Further interactions of AhR/arnt heterodimer with transcription factors such as Sp1 and NF-1 are essential to enhance the expression of *CYP1A1* gene. Other proteins which possess HAT (Histone Acetyl transferase) activity and act as co-activators include SRC-1 (Steroid receptor co-activator), NcoA2 (Nuclear co activator 2), p/CIP and p300. SRC-1, NcoA2 and p/CIP have been shown to associate with the mouse *CYP1A1* enhancer region and to enhance XRE-driven reporter gene transcription (Mimura *et al*., 2003).

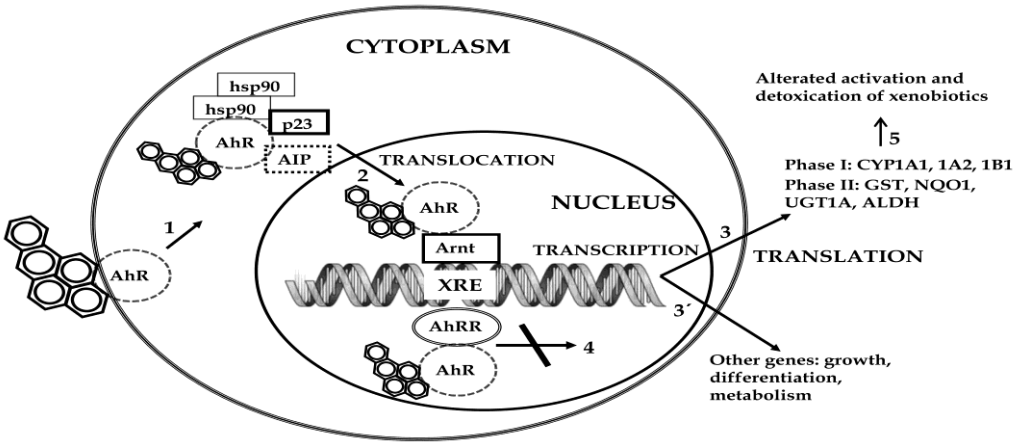

Figure 2.3 Activation of the AhR and the AHR pathway. PAH interact with the receptor AhR (1) an it is assisted with other proteins as hsp90, p23 and AIP. AhR-PAH complex is translocated to the nucleus (2) and it makes complex with Arnt in order to activate transcription of several genes as Phase I and Phase II (3), and other genes participating in other cellular responses (3´). If AhRR interacts with the AhR-PAH complex, the transcription is inhibited (4). The transcription is performed in order to response the detoxification of xenobiotics and other molecules (5) (Arenas-Huerteroet al.).

Mechanism of activation of procarcinogenes by CYP1A1

CYP1A1 is one of the significant cytochrome p450 that is involved in metabolic activation of environmental carcinogens to highly reactive metabolites that are capable of causing oncogenic mutations. *CYP1A1* metabolizes carcinogens to epoxide intermediates, which are further activated to diol epoxides by the enzyme epoxide hydrolase. Following is a demonstration of metabolic activation by Benzopyrene to the ultimate carcinogen, the diol-epoxide (Androutsopoulos *et al*., 2009)

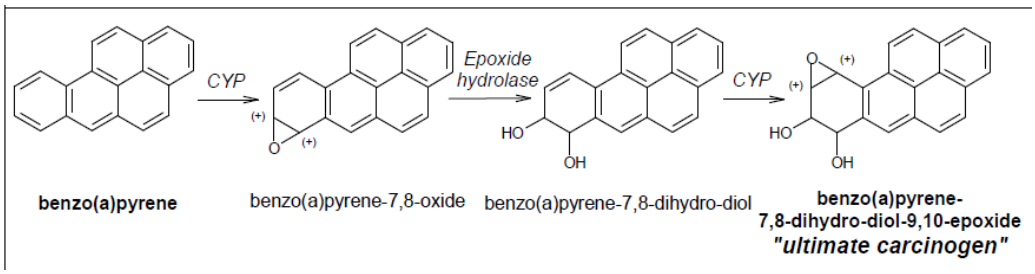

Figure: 2.4 Metabolic activation of Benzopyrene (Shimada et al., 1989)

The major steps involved in mechanism of Benzopyrene metabolic activation are:

1. Oxidation of B[a]P to B[a]P-7,8 oxide by *CYP1A1* enzyme.
2. Hydrolysis of B[a]P-7,8 oxide to B[a]P-7,8-diol and the two enatiomers (+)-B[a]P-7,8-diol and (-)-B[a]P-7,8-diol by epoxide hydrolase (Shimada and Fujii., 2004).

3. Last step involves oxidation into four diol-epoxide by *CYP1A1* enzyme. These diol-epoxides are considered as the ultimate carcinogens, capable of causing oncogenic mutations in specific parts of DNA

2.4 Nicotine and its metabolism

Nicotine is natural occurring clear to pale yellow liquid alkaloid found in plants of Solanaceae family like tobacco plants, tomato plant, and bellanoid plant (Nightshade plant). It constitutes approximately 5% of the dry weight of tobacco and present in various other plants of Solanaceae family in the range of 2–7 µg/kg. According to the IUPAC nomenclature nicotine in named as (S)-3-[1-Methylpyrrolidin-2-yl] pyridine with chemical formula C10H14N2. In its chemical structure nicotine is a bicyclic compound with a pyridine cycle and a pyrrolidine cycle. The nicotine molecule possesses an asymmetric carbon atom and therefore it exists in two enantiomeric forms. In nature, nicotine only exists in the S shape (Berrendero *et al*., 2010).

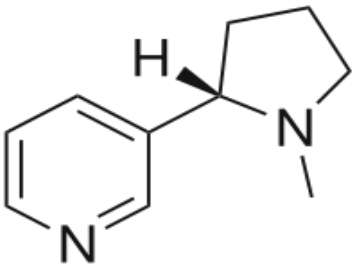

Figure: 2.5 Chemical structure of Nicotine molecule

Nicotine is absorbed through the skin and mucosal lining of the nose and mouth or in the lungs. Nicotine is a weak base having pKa value 8.0 which means 50% is ionized at pH 8.0. Therefore, absorption of nicotine through cellular membranes depends on change in pH value. If the pH is acidic, nicotine is ionized and does not easily pass through membranes. At physiologic pH (pH = 7.4), 31% of nicotine is not ionized and easily passes through membranes. In lungs, nicotine is quickly absorbed by the systemic circulation. Absorbed nicotine is rapidly distributed among all the organs, and it reaches the brain within only ten seconds.Active form of nicotine has cationic charge on nitrogen of pyrrole ring due to which its structure is similar to acetylcholine, a neurotransmitter in autonomic nervous system (ANS). Acetylcholine can bind to two different kinds of receptors: nicotinic receptors, which are activated by nicotine, and muscarinic receptors, which are activated by muscarine. Nicotine and muscarine are thus specific agonists of

one kind of cholinergic receptors (an agonist is a molecule that activates a receptor by reproducing the effect of the neurotransmitter. (Colquhoun *et al*., 2003)

Nicotine competitively binds to nicotinic cholinergic receptors. The binding of the agonist to the nicotinic receptor triggers off a conformation change of the architecture of the receptor, which opens the ionic channel during a few milliseconds. This channel is selective for cations (especially sodium). Its opening thus leads to a brief depolarization. Then, the channel closes and the receptor transitionally becomes refractory to agonists. This is the state of desensitization. Then, the receptor usually goes back to a state of rest, which means that it is closed and sensitive to the agonists. In case of continuous exposure to agonists (even in small doses), this state of desensitization will last long (long-term inactivation).

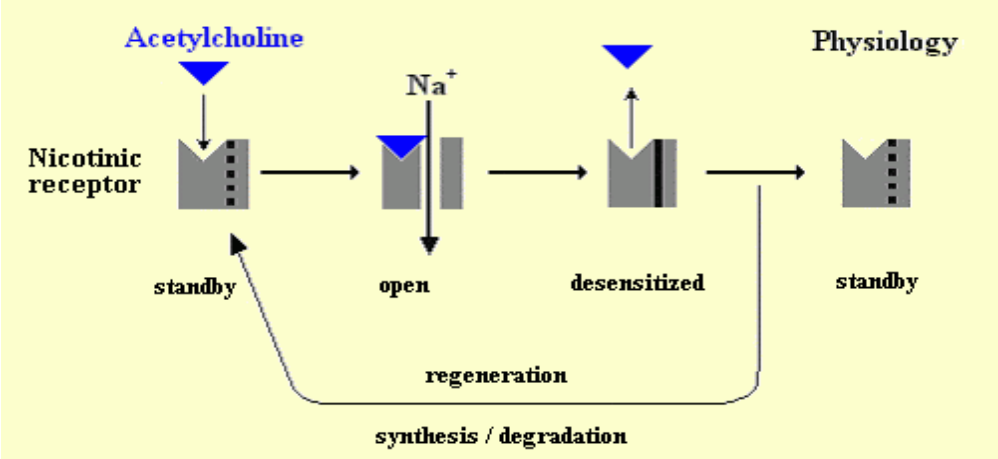

Figure 2.6 Signal transduction through nAChRs in Physiological normal conditions: After the opening of the canal by binding to acethylcholine, the receptor becomes desensitized before it goes back to the state of rest or it is regenerated.

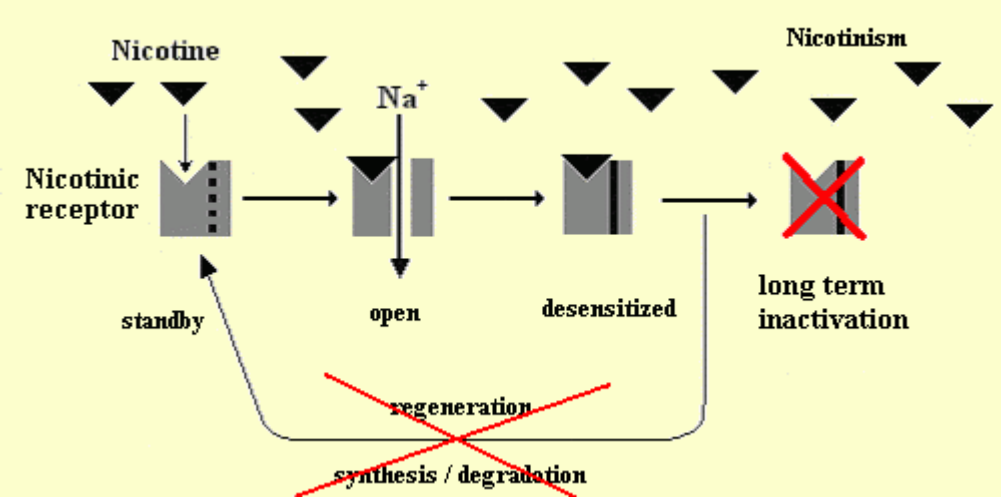

Figure 2.7 Effect of Continuous exposure to tobacco on nAChRs: Nicotine substitutes for acetylcholine and over stimulates the nicotinic receptor. Then, the receptor is long-term inactivated and its regeneration is prevented by nicotine.

Nicotine is mainly transformed in liver but lungs and kidneys can also participate in the transformation of nicotine. Nicotine gets oxidize by cytochrome p450 in hypatocytes. Oxidation of nicotine yields two primary metabolites i.e. cotinine and nicotine N-oxide.In humans, about 70-80% of nicotine is converted to cotinine. This transformation involves two steps. The first is mediated by a cytochrome P450 system to produce nicotine-1' (5') - iminium ion, which is in equilibrium with 5'-hydroxynicotine. The second step is catalyzed by a cytoplasmic aldehyde oxidase. (Janne Hukkanen, Peyton Jacob III, and Neal L. Benowitz 2005). Nicotine N'- oxide is another primary metabolite of nicotine metabolism, although only 4-7% of nicotine is get metabolized via this route. This conversion involves flavin – containing monooxygenase 3 (FMO3), a flavoprotein.

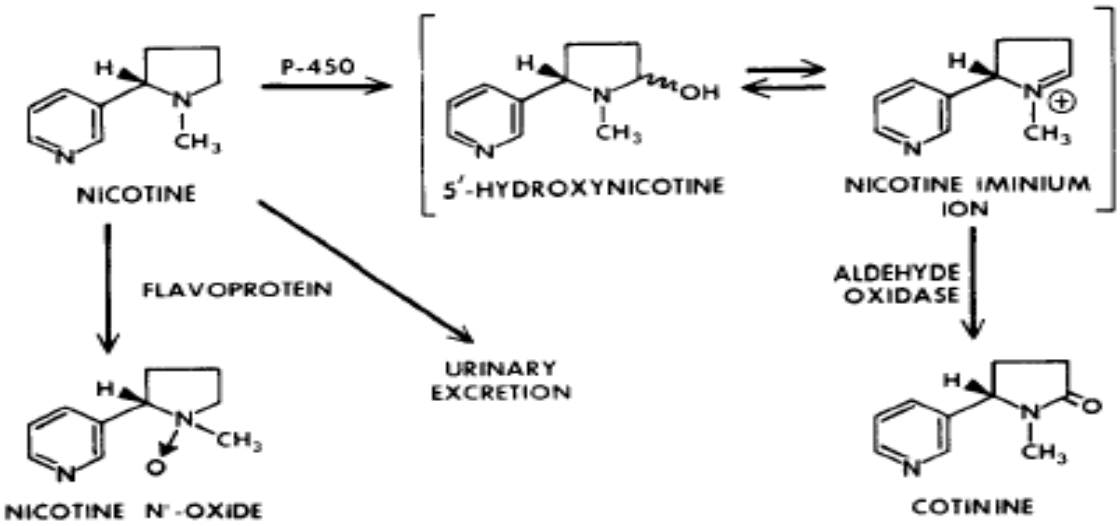

Figure 2.8 Metabolic reactions of Nicotine transformation

Nicotine and its nitroso derivatives are carcinogenic in nature. N- Nitroso derivatives of nicotine arise by the action of nitrous acid on nicotine. Nicotine can undergo several kinds of transformation like a pyrrole cycle opening. The methyl group on this cycle can become a very powerful alkylating agent when removed from the cycle.

The amine function of nicotine may react with nitrogen monoxide or with nitrous acid in order to form a "nitrosonium" type molecule. This compound may then be transformed by the body, which means oxidized and opened. This opening leads to two isomers, two "nitrosamino" type molecules (R2N-N=O) where one of the two R group is a methyl.

In acidic medium, the oxygen of the "nitrosamino" group is protonated and the double bond moves to the central nitrogen, which becomes positively charged. This new molecule is a methyl source. The "nitrosamino" group can then react with another amine, which removes the positive charge from the nitrogen. If the amine that reacts is a part of the structure of the DNA, an irreversible alkylation of the DNA occurs. This alkylation is really noxious and may help in the development of cancer as it prevents the normal development of the cell.

Nicotinic acetylcholine Receptors (nAChRs)

Nicotine and its nitroso derivatives is agonist to nicotinic acetylcholine receptors. These chemical compounds after metabolism binds to these receptors and distorted the signalling pathways. nAChRs are a heterogeneous family of ligand- gated cation channels activated by the endogenous neurotransmitter acetylcholine (Ach) and exogenous chemical such as nicotine and its metabolites (Ma. Reina D. Improgo, 2011). Nicotinic acetylcholine (ACh) receptors are responsible for transmission of nerve impulses from motor nerves to muscle fibres (muscle types) and for synaptic transmission in autonomic ganglia (neuronal types). These receptors are expressed in various cell types and tissues including endothelial cells, gastrointestinal tissue, glia, immune cells, keratinocytes and lung tissues.

nAChRs are classified into two main categories-

1. Neuronal acetylcholine receptors (present in brain)
2. Non- Neuronal or muscle acetylcholine receptors (present in muscles).

Structure nicotine acetylcholine receptors:

The nAChR is a glycoprotein complex (~290kDa) which consists of five subunits (2α, β, δ, ε) arranged around a central membrane-spanning pore. Each subunit comprises a large extracellular amino-terminal domain, four predicted membrane-spanning segments (*M1*-M4) and a long cytoplasmic loop between M3 and M4.Each subunit is coded by different subfamily of genes present on different chromosome number. Depending upon the type of cell there are different subunits are expressed in the nAChRs. Transfection studies have shown that the ratio of α/β subunits in nAChRs subunits depends on the ratio of expression of the encoding nAChR subunit genes. It has been shown that the nAChR α7

in normal human bronchial epithelial cells is up regulated by exposure to nicotine. In mammalian system, there are basically 9α subunits (α1-α7, α9, α10) and 4β subunits (β1-β4) coded by *CHRNA1-CHRNA7, CHRNA9, CHRNA10* and *CHRNB1*-CHNB4 genes located on different chromosomes.

Subunit*	Gene	Chromosome location**		
		Mouse	Rat	Human
α2	*CHRNA2*	14	15p12	8p21
α3	*CHRNA3*	9	8q24	15q24
α4	*CHRNA4*	2	3q43	20q13.2-q13.3
α5	*CHRNA5*	9	8q24	15q24
α6	*CHRNA6*	8	16q12.3	8p11.21
α7	*CHRNA7*	7	1q22	15q14
α9	*CHRNA9*	5	14p11	4p14
α10	*CHRNA10*	7	1q32	11p15.5
β2	*CHRNB2*	3	2q34	1q21.3
β3	*CHRNB3*	8	16q12.3	8p11.2
β4	*CHRNB4*	9	8q24	15q24

Figure 2.9 Chromosome location of genes encodes the subunits of nAChRs (**Manglott et al; 2011)

nAChRs function can be modulated by phosphorylation by the activation of second messenger- dependent protein kinases. It involves in the regulation of signaling mechanism in sensory epithelia and other non- neuronal cell types.Non neural nAChRs in the lung act most frequently as calcium channels and have been linked to regulatory proteins such as src and phosphotidylinositol – 3 –kinase, which can control proliferations.nAChRs are also involves in the regulation of apoptotic and agiogenic signaling pathways. It involves in the channel for Ca2+- dependent mechanisms, including activation of second messengers such as PKA, PKC, PI3K/Akt, and MAPK. These biological roles of receptors dependent on the expression of receptor subunits and their binding affinity towards nicotine and its derivatives. *CHRNA5* subunit predominantly expressed in lung cancer tissues and plays a remarkable role in modifying the calcium permeability of receptors and its affinity towards agonist.

2.5 Polymorphism in CYP1A1 and CHRNA5 gene and lung cancer

Lung cancer increases the mortality rate worldwide and its major cause of occurrence is smoking which produce highly carcinogenic compounds in our body. The mechanism of metabolism of carcinogens is not completely understood. Not all the smokers could develop

the lung cancer; fewer than 20% of smoker could develop the disease (Carlsten *et al*., 2008), in spite to believe that cigarette smoking is major cause of lung cancer which suggests that there could be the other factors that involve in cause of lung cancer. The susceptibility to develop the disease is largely effected by the genetically determined variants that involve in the metabolism of carcinogens produce by tobacco smoking. The identification and understanding of different allelic variants that involve in the genetic susceptibility factors for lung cancer is important to prevent the occurrence of the disease.It is typically elucidated as a sample of interest comprising of a polymorphism seen in atleast 1% of the population. In this approach we assume that the low or medium penetrant genes account for the majority of the lung cancer susceptibility rather than those which are highly penetrating. A single nucleotide polymorphism (SNP) is an alteration in a single nucleotide present in the DNA sequence.

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

- Either we will see a nucleotide variation in the coding region of a gene therefore altering the enzyme activity due to the amino acid substitution.
- There might be a deletion in the coding region of the gene leading to an inactive enzyme or even lack of protein synthesis.
- Variation in the polyadenylation site causing post transcriptional repression hence affecting the quantity of the transcript formed. (Boffeta 2000)

The *CYP1A1* gene is polymorphic and has been shown to have 15 different allelic variants (Androutsopoulos *et al*., 2009). Among the different polymorphic variants of the *CYP1A1* gene four of these variants designated as *m1*, *m2*, m3 and m4 have been widely studied in Asian and Caucasian populations as a risk factor for lung carcinogenesis. The *m1* and *m2* polymorphism are found to have involvement in lung carcinogenesis (Shah *et al*., 2008; Xu etal., 1996). The *CYP1A1m1*(rs4646903)polymorphism has been associated with elevated enzyme activity due to a single base point mutation at nucleotide position 3801 which results in transition of thymine to cytosine in the 3' untranslated region. The *CYP1A1m2*polymorphism (rs1048943) involves a base substitution from adenine (A) to guanine (G) at codon 462 in exon 7, resulting in substitution of isoleucine with valine (Ile462Val) which results in the increase in enzyme activity (12).Conventional theory regarding the effect of polymorphisms on *CYP1A1* suggests that the variants affect the function of the enzyme by altering the level of gene expression or the mRNA stability,

although the results in the literature appear to be contradictory (Ji *et al*., 2012). For example, the Ile462Val polymorphism was found to confer increased levels of induced or basal *CYP1A1* mRNA as the number of Val variants increased in one study, whereas in purified Escherichia Coli, no difference in the metabolismof benzo[a]pyrene between the Ile and Val variants was noted. Similarly, a high activity or lack of correlation has been suggested with the 3801TC and Ile462Val polymorphisms regarding the activity of mutant enzymes in lymphocytes (Zhan *et al*., 2007).

On the other hand, Prolonged or repeated exposure to a stimules often results in decreased responsiveness of that receptor toward a stimulus, termed desensitization. PKA and PKC have been shown to phosphorylate the receptors resulting in desensitization. It has been reported that, after prolonged receptor exposure to agonist, the agonist itself causes an agonist- induced conformational change in the receptor, resulting in desensitization. The long term desensitization of the receptors results in polymorphism of receptor subunits. This polymorphism increases the risk of lung cancer due to distortions in signalling pathways (Cattaneo *et al*., 1997).

Polymorphism in genes of encoding α and β- subunits types of receptors effect on gene expression or protein functions. In lung cancer expression of α3, α5 and α7 is predominant. The variations in these receptors are strong candidate of risk factors for nicotine addiction and lung cancer (Amos *et al*., 2010).

Multiple genome-wide association studies (GWAS) have implicated the *CHRNB4*/A3/A5 locus in nicotine dependence and lung cancer (Amos *et al*., 2008; Hung *et al*., 2008; Thorgeirsson *et al*., 2008). The gene that codes for α5 subunit (*CHRNA5*) contains the SNP 1192G>A, characterized by asubstitution of the aminoacid aspartic acid/D (coded by the G allele) to asparagine/N (coded by A, the risk allele) in position 398 (D398N) of the fifth exon of *CHRNA5* protein. This gene variant is localized in the central portion of the second intracellular loop and although the function of this loop and the biological consequences of this alteration have not been completely understood yet, this aminoacid is highly conserved among species, suggesting it might have functional importance 10-12. The asparagine risk allele is associated with decreased maximal response to agonists, indicating altered receptor function (Bierut *et al*., 2008; George *et al*., 2012). Additionally, the genotype in this locus appears to corre- late with mRNA levels suggesting that rs16969968 may influence *CHRNA5* expression as well (Falvella *et al*., 2009; Wang *et al*., 2009).

2.6 Population Studies in CYP1A1 and CHRNA5 gene polymorphism in lung cancer

Various studies have been done on *CYP1A1* gene having *m1* and *m2* polymorphism as well as G-A polymorphism in *CHRNA5* gene.

Table: 2.2 Distribution of CYP1A1 MspI and CYP1A1 Ile^{462}Val genotypes among lung cancer cases and control (Ji et al., 2012)

S.No.	First author- year	Ethnicity (Country of origin)	Total sample size (Case/control)
1	Kawajiri K-1990	Asian (Japan)	68/104
2	Tefre T-1991	Caucasian (Norway)	221/212
3	Hirvonen A - 1992	Caucasian (Finnish)	87/121
4	Shields PG -1993	Mixed population	56/48
5	Nakachi K- 1993	Asian (Japan)	31/127
6	Alexandrie AK- 1994	Caucasian (Sweden)	296/329
7	Kelsey KT - 1994	Mixed (African Americans)	72/97
8	Kihara M-1995	Asian (Japan)	97/258
9	Xu XP -1996	Caucasians (USA)	207/238
10	Garcia- ClosaM- 1997	Mixed population	416/446
11	Hong YS - 1998	Mixed (Mexican and African)	171/295
12	Taioli E-1998 Asia	Mixed population	105/307
13	Le Marchand L- 1998	Mixed population	341/456
14	Hu YL -1999	Asian (China)	59/132
15	Dresler CM-2000	Caucasian (USA)	158/149
16	Song N- 2001	Asian (China)	217/40
17	Quinones L- 2001	Caucasians (Chile)	60/174
18	Yin LH- 2002	Asian (China)	84/84
19	Zhou XW- 2002	Asian (China)	92/98
20	Cai XL-2003	Asian (China)	91/138
21	Kiyohara C- 2003	Asian (Japan)	158/259
22	Taioli E-2003	Mixed population	109/424, 110/707 exon7
23	Wang J- 2003	Asian (China)	162/181
24	Dialyna IA-2003	Caucasians (Greek)	122/178

25	Gu YF- 2004	Asian (China)	180/224
26	Liang GY- 2004	Asian (China)	152/152
27	Chen SD-2004	Asian (China)	58/62
28	Sobti RC-2004	Asian (India)	100/76
29	Wenzlaff AS- 2005	Caucasian (USA)	128/181
30	Wrensh MR- 2005	Mixed population	371/944 MspI 363/930 Exon 7
31	Ng DP-2005	Asian (Singapore)	126/162
32	Raimondi S-2005	Caucasians	165/519 MspI 175/723 Exon7
33	Raimondi S- 2005 - 2	Asians	46/138
34	Sreeja L-2005	Asian (Indian)	146/146
35	Adonis M-2005	Mixed population	57/103
36	Belogubova-2006	Caucasians (Russian)	141/450
37	Pisani P-2006	Asian (Thailand)	211/408
38	Tao WH-2007	Asian (China)	47/94
39	Cote ML-2007	Mixed population	354/440
40	Xia Y-2008	Asian (China)	58/116
41	Qi XS-2008	Asian (China)	53/72
42	Shah PP-2008	Asian (China)	200/200
43	Cote ML-2009	Mixed population	502/523
44	Honma HN- 2009	Mixed population	200/264
45	Klinchid J-2009	Asian (Thailand)	85/82
46	Shaffi SM-2009	Asian (India)	109/163
47	Jin Y-2010	Asian (China)	124/154
48	Wright CM- 2010	Caucasians (Australians)	1040/784
49	Mota P-2010	Caucasians (Portugal)	175/217
50	Zhu XX-2011	Asian (China)	160/160

Table: 2.3 Different SNP studies in 15q25 chromosomal region to lung cancer (Trovo de Marqui et al., 2011)

Gene	SNP identifier (dbSNP)/ Nucleotide change	Sample size	Study type/ Main findings	References
CHRNA5 CHRNA3	16969968/1192G>A 1051730/645C>T	1,154 smokers of European origin 1,137 population based control 711 patients with lung cancer 362 population based controls	Case-control/Increased risk for lung cancer	*Amos et al, 2008*
CHRNA3	1051730/645C>T	13,945 smokers 4,302 non-smokers 655 patients with lung cancer 28,752 population based controls	Case- control/ Association with the quantity of cigarettes	*Thorgeirsson et al.,2008*
CHRNA5	16969968/1192G>A	2,284 individuals dependent on alcohol and their families	Cohort/ functional analysis of polymorphism-variants do not differ in expression (p=0.007)	*Bierut et al.,2008*
CHRNA5	16969968/1192G>A	Pulmonary tissues of 68 patients who underwent lobotomy	Case-control/mRNA levels 2.5 folds lower in individuals homozygous for the non- risk allele.	*Falvella et al.,2009*

CHRNA5	16969968/1192G>A	302 patients with lung adenocarcinoma	Cohort/SNP is rare in Japanese population and correlates with reduced survival (log rank test p= 0.0146)	*Sasaki et al., 2010*
CHRNA3	1051730/645C>T	467 patients with lung cancer 388 African-American controls	Case- control/ association of the variant to lung cancer in individuals that never smoked	*Amos et al., 2010*
CHRNA5 CHRNA3	16969968/1192G>A 1051730/645C>T	819 smokers from Hawaii 99 patients from the Tobacco Reduction Intervention study Program 137 smoker patients	Cohort/ elevated risk for lung cancer (P= 0.003)	*Le Marchand et al., 2008*

CHAPTER 3

OBJECTIVES

3. Objectives of the Study

The present piece of work is an attempt to study the following aspects that might be associated towards the associative risk for lung cancer.

1. The epidemiological factors associated with lung cancer in North Indian population.
2. To study the genotypic frequencies of *CYP1A1m1* and *m2* gene in lung cancer cases and controls.
3. To find a correlation between genetic polymorphism of *CYP1A1* gene towards the risk for lung cancer and also the clinico-pathological features associated with it.
4. To find the association between *CYP1A1m1* and *m2* gene polymorphism as individual as well as combine and risk for lung cancer in smokers and non-smokers.
5. To study the effect on polymorphism in *CHRNA5* gene on risk of lung cancer associated with different histological subtypes and smoking status.
6. The combine effects of polymorphism in *CYP1A1m1*, *m2* and *CHRNA5* on risk of lung cancer in smokers, non-smokers and different histological subtypes of lung cancer.

CHAPTER 4

STUDY DESIGN

4. Material and Methods

4.1 Sample Collection

The current study enrolled 353 lung cancer patients who were recruited from the Department of Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, India. This study has been reviewed and approved by the Institute ethics committee of PGIMER. Informed written consent was obtained from all participants or their representatives. All the recruited patients were histopathologically diagnosed as having NSCLC and SCLC. Patients under study having a prior history of cancer were excluded from the study. There was no age, gender, smoking, histological or TNM stage restrictions. The control group of the study consisted of 351 unrelated volunteers having no lung cancer history at the time of blood collection; they entered the hospital for health check-ups. The controls were pair-matched for sex, age (±10 years) and smoking parameters in order to avoid any sampling bias. Each participant filed up the detailed questionnaire with the help of trained interviewer. The questionnaire included information on demographic and smoking characteristics like tobacco habits such as smoking of beedi/cigarette etc. Individuals having regular smoking habits were classified as smokers. They were further stratified as light and heavy smokers on the basis of pack years (PY) that were calculated by the formula: [(cigarettes or beedis per day/20)*years smoked], PY≤25 were light smokers and PY>25 were heavy smokers. The medical information of cases such as histology, TNM classification, clinical staging, primary tumor size, involvement of lymph node and metastasis were obtained from medical records of the hospital.

4.2 DNA extraction

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 (Field *et al* 1999).

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 Preparation of washing buffer

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

Table no. 4.2 Preparation of lysis buffer

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

Procedure of DNA Isolation

- Took 5ml of blood and 5ml of Washing Buffer was added and mixed thoroughly. Centrifuged it at 3500rpm for 5 minutes.
- Discarded the supernatant and added 5ml of Washing buffer (1.6ml 1M Sucrose,0.5 ml Triton X-100, 0.25ml $MgCl_2$, 0.5 ml 100mM Tris HCl and 0.26ml of water) to the pellet and re suspended the pellet in the Buffer and centrifuged again (repeat this step thrice).

- Dissolved the pellet in 5ml of Lysis buffer (1 M Tris HCl 2ml,10% SDS 0.5ml ,0.5 M EDTA 0.6ml, 5M NaCl 0.15ml,10mg/ml Proteinase-K 0.05ml and water 1.7ml) and incubated at 44 °C overnight.
- Added an equal volume of Phenol: Chloroform: Isoamyl alcohol (PCI) 25:24:1(25ml Phenol, 2.4 ml Chloroform and 0.1ml isoamyl alcohol) and mixed the contents slowly.
- Centrifuged at 8000rpm for 10minutes at 4°C. Took the upper aqueous layer and again add PCI mix and centrifuged.
- Took the aqueous layer and added equal volume of Chloroform: Isoamyl alcohol (24:1).
- Centrifuged it at 6500 rpm for 5 minutes and took the upper layer.
- To the aqueous layer added equal volume of chilled Isopropanol or 2.5 times volume of absolute Ethanol and mixed it gently.
- Freeze it at -20°C for 1-2 hours.
- Centrifuged it at 12,000 rpm for 10 min at 4°C.The supernatant was discarded and the pellet of DNA was washed with chilled 70% Ethanol twice at 10,000 rpm for 5 minutes.
- Decant ethanol and air dry the pellet.
- Dissolved the pellet in 50µl-150µl Tris-EDTA buffer depending on the size of DNA pellet (Bartlett & White, 2003).

4.3 Quantitative and qualitative estimation of DNA template

The quantitative estimation of DNA was done by using UV spectrophotometer and the absorbance was recorded at two wavelengths A_{260} nm and A_{280} nm. The ration of absorbance at 260nm and 280 nm is used to assess the purity of DNA. If the ration is ~1.8 the DNA sample is considered to be pure and free from contaminations of RNA and proteins. A ratio of ~2.0 or >1.8 indicates RNA contamination in the sample, whereas a ratio <1.8 indicates protein contamination in the sample. The concentration of pure double standard DNA with an A260 of 1.0 is 50µg/ml. DNA concentration of the solution was determined by using formula:

Concentration (µg/ml) = A_{260} X 50 µg/ml X Dilution factor

Procedure

- Pipetted 1µl of Deionised water onto the lower optical surface of Nanodrop (Thermo Scientific) to clean it
- Opened the Nanodrop software and select Nucleic acid Module
- Performed a blank measurement by loading 1µl of TE and selecting "blank" from the screen
- Measured the Nucleic acid sample by loading 1µl of DNA sample and selecting "measure"

Concentration and purity of DNA samples were calculated automatically.

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

Requirements

- Electrophoresis buffer (TAE or TBE)
- Ethidium bromide solution
- Electrophoresis-grade Agarose
- 6X loading dye
- DNA molecular weight markers
- Horizontal gel electrophoresis apparatus
- Gel casting platform
- Gel combs (slot formers)
- DC power supply

Preparing 5X TBE (1000ml)

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

Preparing 6X Loading Dye (20ml)

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

Preparation of the Agarose Gel for Electrophoresis

- Prepared an adequate volume of electrophoresis buffer.
- Added the desired amount of Electrophoresis-grade Agarose to a volume of Electrophoresis buffer sufficient for preparing the gel. For example, for genomic DNA 0.7% gel (0.7g agarose in 100ml 0.5X TBE) was prepared while for the PCR products 1.7% gel (1.7g agarose in 100ml 0.5X TBE buffer) was prepared.
- Melted agarose was cooled to 55°C in a water bath before pouring onto the gel platform to prevent warping of the gel apparatus.
- Before pouring Ethidium bromide solution was added to the melted well mixed agarose gel to a final concentration of 0.3µg/ml to facilitate visualization of DNA when seen under UV Transilluminator.
- Poured the melted Agarose onto the gel casting apparatus between 0.5 and 1 cm thick and with the gel comb inserted prior to pouring, making sure that no bubbles are trapped underneath the combs and all bubbles on the surface of the surface of the agrose were removed before the setting of the gel.

Loading and running the gel

- After the gel got solidified, the gel comb was withdrawn with proper care without disrupting the sample wells.
- Placed the gel casting platform containing the set gel in the Electrophoresis tank. Added sufficient Electrophoresis buffer to cover the gel until the tops of the wells are submerged. Made sure no air pockets were trapped within the wells.

- DNA samples were prepared by mixing 5µl DNA with 2µl of 6X loading dye and 2ul water in case of genomic DNA or by mixing 5µl DNA with 2µl of 6X loading dye in case of PCR product.
- Samples were typically loaded into the wells with micropipette. Care was taken to prevent mixing of the samples between wells.
- Appropriate DNA molecular weight marker were also loaded in case of PCR and digestion products
- Connected the electrodes to a power pack and allowed the Electrophoresis apparatus to run at 60 V until the marker dyes migrated the desired distance.
- Turned off the electric power, disconnected the leads, and discarded the electrophoresis buffer from the reservoirs
- DNA was visualized by placing the gel on a UV transilluminator and then photographed using Gel Documentation.

4.5 ***Genotyping of CYP1A1m1 polymorphism by PCR-RFLP***

To analyze the T to C polymorphism in *CYP1A1* gene at *m1* site a PCR-RFLP technique was used followed by digestion with *Msp1* restriction enzyme. PCR amplification of 899bp long DNA fragment was done in 25µl reaction mixture containing 300ng of DNA template, 0.5µmol each of forward 5-GGCTGAGCAATCTGACCCTA-3 and reverse primer 5-TAGGAGTCTTGTCTCATGCCT-3 (Cascorbi *et al*., 1996) with 1.5mM $MgCl_2$, 0.2mM dNTPs, 1x PCR buffer and 1U Taq DNA polymerase. PCR was performed with initial denaturation step of 5minutes at 95ºC then followed by 30 cycles of 1 minute at 94ºC, 30 seconds at 60ºC as annealing step and 45 seconds at 72ºC followed by final extension step of 5mins at 72ºC. The amplification was confirmed by using agarose gel electrophoresis.

Table no 4.3 - Reaction mixture of PCR- RFLP

Reagent	**Stock concentration**	**Final Reaction concentration**	**Quantity Used**
Additive 1 BSA	1000 µg/ml	100µg/ml	44µl
PCR Buffer (Mg Conc.)	10X, 25mM	1X 1.5mM total	44µl
Primer (forward)	10µM	0.5µl	22 µl
Primer (reverse)	10µM	0.5µl	22 µl
Taq Polymerase	2.0U	1.5µl	22 µl
dNTPs	10mM each	0.2	8.8 µl
PCR Grade Water			189.2 µl
DNA Template	100ng/µl	300ng	3 µl

The PCR product was then subjected to restriction digestion with 5U of *Msp1* enzyme (New England Biolabs, Ipswich, MA, USA). After digestion the samples were then run on a 2.5% agarose gel electrophoresis and visualized in UV light. PCR fragments of 899bp was digested into two fragments of 693bp and 206bp in case of mutant genotype (CC) and undigested fragment of 899bp in size was indicative of wild type genotype (TT). The heterozygous group was represented by fragment size of 899, 693 and 206bp respectively. To ensure quality control, genotyping was performed without knowledge of the subjects' case/control status and a 15% random sample of cases and controls was genotyped twice by different persons; reproducibility was 100%.

4.6 ***CYP1A1m2 genotyping by PCR-RFLP***

The genotyping of *CYP1A1m2*gene was also evaluated by using PCR-RFLP method according to the protocol of Oyama *et al*., 1994. The product size of the ampliconthat was generated by PCR is 187bp in size which was amplified by using the following primer sequences 5-GAACTGCCACTTCAGCTGTCT-3 and 5-GAAAGACCTCCCAGCGGTCA-3. The PCR conditions were: denaturation at 95ºC for 1 minute, annealing at 55ºC for 30 seconds and extension at 72ºC for 45 seconds for 30 cycles with final extension at 72ºC for 5 minutes. The restriction digestion of PCR product was done using 5U of *HincII* enzyme incubated at 37ºC (New England Biolabs, Ipswich, MA, USA). The digested PCR products were then separated on a 10% PAGE gel and silver staining was done to observe the digested bands. The restriction digestion pattern observed in 10% acrylamide gel was as follows a wild genotype (AA) gave two bands of 139 and 48bp, heterozygous genotype (AG) gave four bands of size 139, 120, 48 and 19 bps and mutant genotype (CC) gave three bands of 120, 48 and 19 bps as shown in Figure 3.

Requirements of PAGE

- 30% Acrylamide solution (29:1 w/v; Acrylamide: Bisacrylamide)
- TEMED
- Ammonium persulphate 10%
- 5X TBE solution

Table: 4.4 Gel concentration and components for PAGE

Gel%	30% acrylamide (ml)	H2O (ml)	5X TBE (ml)	10% APS (µl)	TEMED (µl)
6%	2.4	7.2	2.4	200	10
8%	3.2	6.4	2.4	200	10
10%	4.0	5.6	2.4	200	10
12%	4.8	4.8	2.4	200	10

Reagents of Silver Staining

- Fixative: Glacial acetic acid, water, methanol in the ration of 10:50:40.
- Staining solution: 0.1% AgNO3, 150µl HCHO in 100 ml of distilled water.
- Developing solution: 3g Na2CO3, 150µl HCHO, 20µl of 10mg/ml sodium thiosulphate in 100ml of distilled water.

Procedure of Silver Staining

- Insert the gel in fixative for at least 15 minutes after run on electrophoresis.
- Rinse it with sterile deionised water at twice.
- Put the gel staining solution in dark for 30 minutes
- Rinse with sterile deionised water twice.
- Transfer the gel in developing solution and shake it for few minutes for gel to be developed.

4.7 ***Genotyping of CHRNA5 gene***

To analyze the G to A polymorphism in *CHRNA5* gene a PCR-RFLP technique was used followed by digestion with Taq1 restriction enzyme (New England Biolabs, Ipswich, MA, USA) for digestion. PCR amplification of 435bp long DNA fragment of *CHRNA5* gene was done in 25µl reaction mixture containing 400ng of DNA template, 0.5µmol of both forward primers 5--CGCCTTTGGTCCGCAAGATA-3 and reverse 5--TGCTGATGGGGGAAGTGGAG-3 (Sa M *et al*., 2012) with 1.5mM MgCl2, 0.2mM dNTPs, 1x PCR buffer and 1U Taq DNA polymerase. PCR was performed with initial denaturation step of 5minutes at 95ºC then followed by 30 cycles of 1 minute at 94ºC, 30 seconds at 60ºC as annealing step and 45 seconds at 72ºC followed by final extension step of 5mins at 72ºC. The amplification was confirmed by using agarose gel electrophoresis. The PCR product of

gene was then subjected to restriction digestion with 5U of Taq1 enzyme incubated overnight at 65°C (New England Biolabs, Ipswich, MA, USA). After digestion the samples were then run on a 2.5% agarose gel electrophoresis and visualized in UV light. PCR fragments of 435bp was digested into two fragments of 290bp and 145bp in case of wild genotype (GG) and undigested fragment of 435bp in size was indicative of mutant type genotype (AA). The heterozygous group was represented by fragment size of 435bp, 290bp, and 145bp respectively (GA).

4.8 ***Statistical analysis***

Differences in the distribution of demographic and genotypic characteristics of cases and controls were evaluated using statistical software Medcalc 15.5.5 (Medcalc software, Ostend, Belgium) and SPSS version 20.0. (Chicago, IL, USA) statistical software. For the categorical data the Chi-square test ($\chi 2$ test) and for continuous variables *t*-test was done. To calculate the genotyping frequency of both the polymorphism the Hardy-Weinberg equilibrium theory ($p2+2pq+q2=1$; where p is the frequency of the wild-type allele and q is the frequency of the variant allele) was used both in cases and controls using $\chi 2$ test. Haplotype analysis for the two SNPs studied and the D' value and r2 were calculated with the SHEsis software (Li *et al*., 2009) (http://202.120.31.177/myAnalysis.php). To identify the risk of lung cancer due to the allelic variants the Odds ratios along with 95% Confidence Interval (CI) and P-value at the level of significance <0.05 were calculated by using logistic regression analysis with adjustment for possible confounders such as age and pack years of smoking as continuous variables and gender as a nominal variables. In addition to overall association analysis, stratifiedanalysis was performed to estimate risk for subgroups made on the basis of smoking status, tumor histology and gender.

CHAPTER 5

RESULTS

5. Results

5.1 Genomic DNA Isolation

The genomic DNA isolated from blood samples of lung cancer patients and controls were qualitatively analyzed on 1.7% agarose gel prepared in 0.5X TBE buffer.

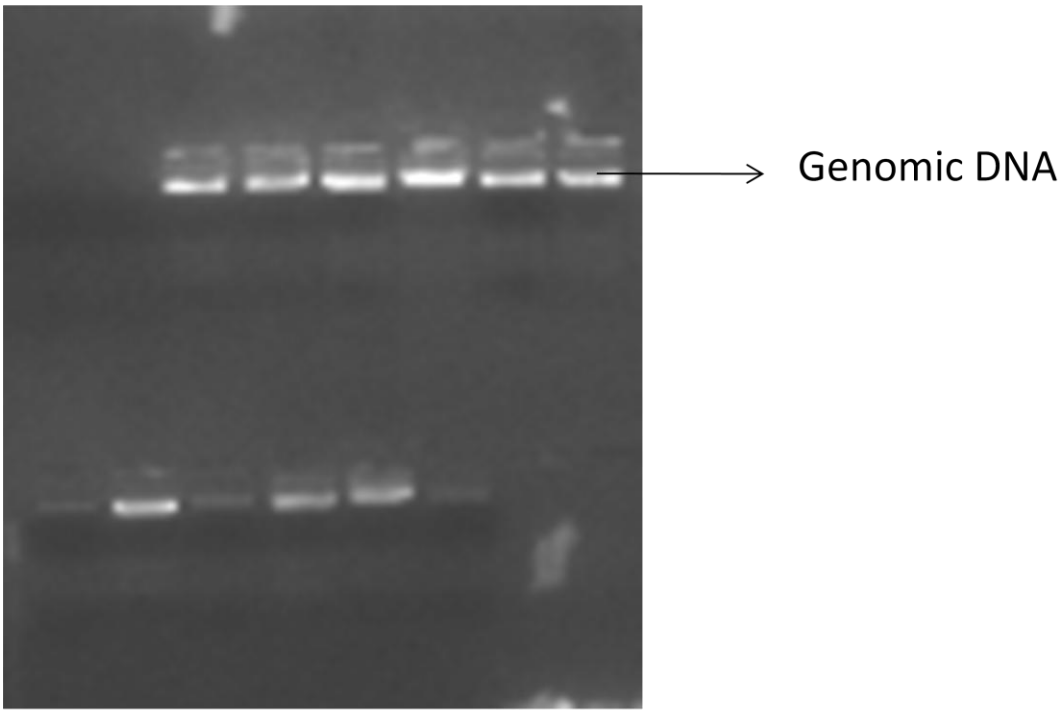

Figure 5.1: Confirmation of genomicDNAisolation using Gel Electrophoresis

5.2 Restriction Fragment length polymorphism of CYP1A1m1, m2 and CHRNA5 gene

The digestion of PCR product of *CYP1A1m1* gene polymorphism with Msp1 (10U/µl) NEB enzyme resulted in two smaller fragments of 693 bp and 206 bp in case of mutant genotype, three fragments of 899bp, 693bp and 206bp in case of heterozygous genotype and one fragment of 899bp in size in case of wild wild genotype when subjected to 2.5 % agarose gel containing ethidium bromide in 0.5X TBE buffer at 60 volts as shown in figure 5.2.

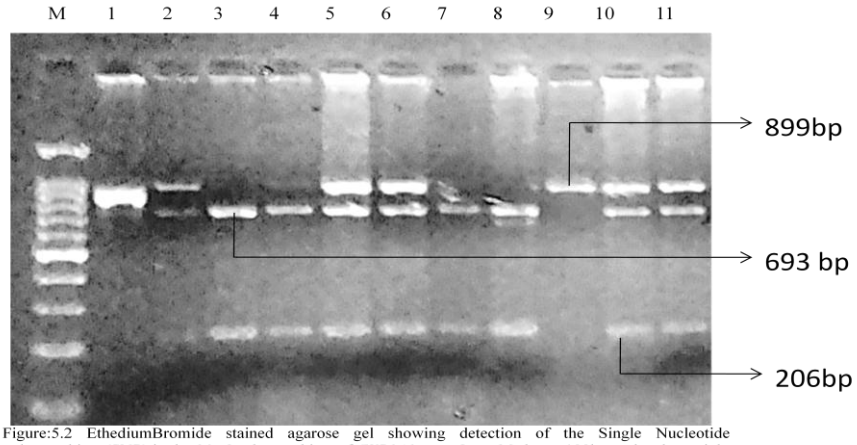

Figure:5.2 EthediumBromide stained agarose gel showing detection of the Single Nucleotide polymorphism (SNP) in the *MspI* polymorphism of *CYP1A1* gene, Lane M shows 100bp molecular weight (MW) marker, Lane 1 shows 899 bp uncut product, Lane 9 represent 899bp homozygous wild genotype (TT), Lane 2,4-6,10,11 show heterozygous genotype (TC) and Lane 3,7 and 8 represent mutant genotype (TT) of MspI polymorphism.

In case of *m2* polymorphism of *CYP1A1* gene the size of PCR product was 187 bp. The digestion of PCR product was done using HincII (10U/µl) NEB enzyme incubated at 37°C temperature. The digested bands were too small to observe in 2.5% agarose gel, therefore PAGE and silver staining was done to observe the digested bands. The restriction digestion pattern observed in 10% acrylamide gel, a wild genotype gave two bands of 139 and 48bp , heterozygous genotype gave four bands of size 139,120,48 and 19 bps and mutant genotype gave three bands of 120,48 and 19 bp. The band pattern has shown in figure 5.3.

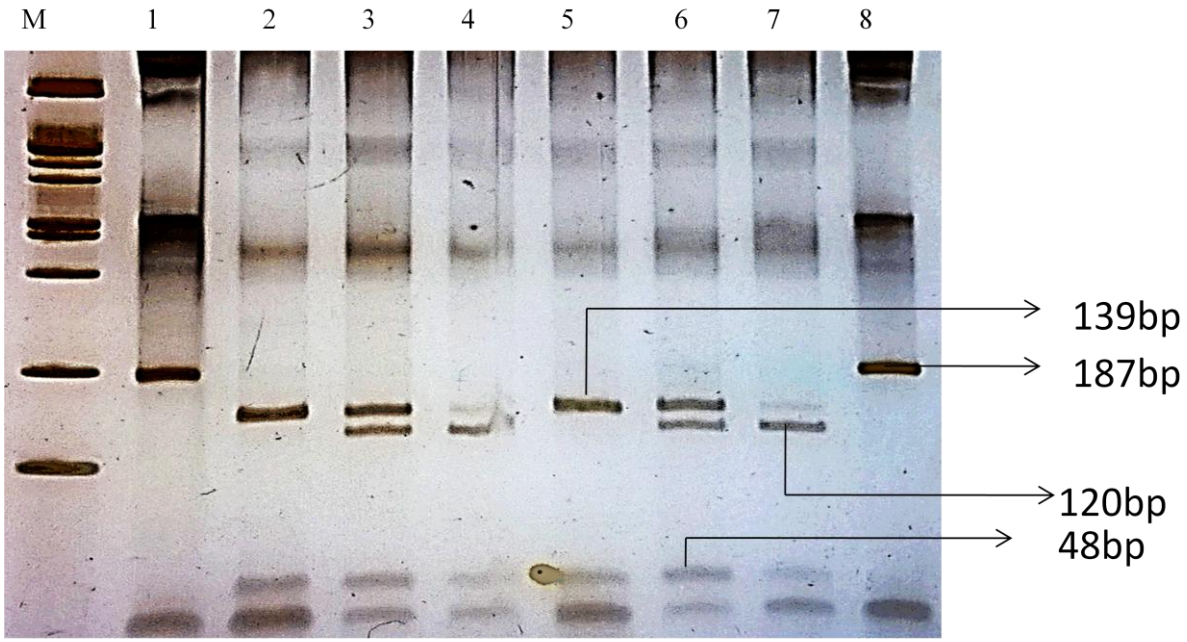

Figure: 5.3Polyacrylamide gel electrophoresis (PAGE) showing CYP1A1 $Ile^{462}Val$ genotype analysis by PCR-RFLP, Lane M shows 100bp molecular weight (MW) marker, Lane 1 and 8 represent the uncut 187 bp product, Lane 2 and 5 represent the 139, 48 bp Homozygous wild genotype (AA), lane 3 and 6 represent the 139,120,48 and 19 bp heterozygous genotype (AG), lane 4 and 7 shows 120, 48 and 19 bp homozygous mutant genotype (GG) of m2 polymorphism.

To observe the digestion pattern of *CHRNA5* the PCR product was incubated with TaqI enzyme at 65°C temperature. After digestion the samples were then run on a 2.5% agarose gel electrophoresis and visualized in UV light. PCR fragments of 435bp was digested into two fragments of 290bp and 145bp in case of wild genotype (GG) and undigested fragment of 435bp in size was indicative of mutant type genotype (AA). The heterozygous group (GA) was represented by fragment size of 435bp, 290bp, and 145bp respectively. As shown in figure 5.4.

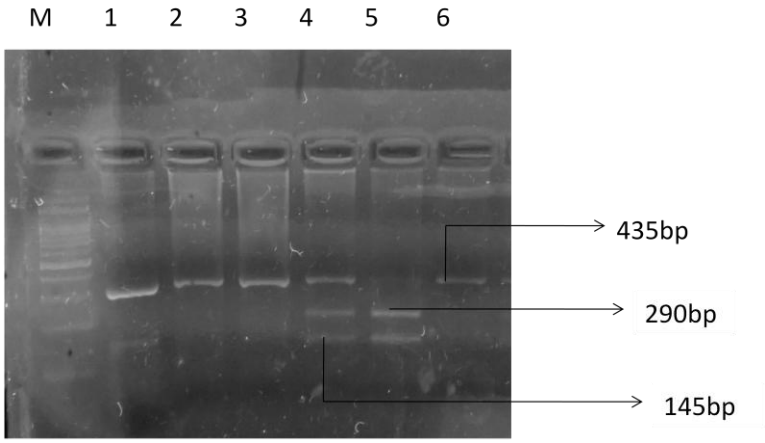

Figure:5.4 Ethedium Bromide stained agarose gel showing detection of the Single Nucleotide polymorphism (SNP) in the *Taq*Ipolymorphism of *CHRNA5* gene, Lane M shows 100bp molecular weight (MW) marker, Lane 1 shows 435 bp uncut product, Lane 5 represent 290bp and 145bp homozygous wild genotype (GG), Lane 4show heterozygous genotype (GA) and Lane 2,3 and 6 represent mutant genotype (AA) of *CHRNA5* polymorphism.

5.3 ***Demographic characteristics and clinical data of studied subjects (Table 5.1)***

The demographic characteristics and clinical data of the studied subjects are shown in table 1. A total of 353 cases and 351 controls were recruited and the mean age of the cases was 57.55± 10.69 (range 28–86) and that of the controls was52.84 ±10.80 (range 19–83). The study included 86.4 males and 13.6% females in cases where as 85.47% males and 14.53% females were present in the control group. There was no significant difference in distribution of males and females between controls and patients suggesting adequate matching (p=0.804).In the present study the number of smokers was represented more in the cases as compared to the controls (78.75 vs 71.23%), whereas the frequency of non-smokers was more in the control group as compared to cases (28.77 vs 21.25%, p=0.026).When stratified on the basis of pack years; there was a significant difference in distribution between the pack years of cases and controls (PY 27.59 vs 17.52, p<0.0001). When the lung cancer cases were stratified on basis of histology41.33% were of SQCC, 33.14% of Adenocarcinoma (ADCC) and 22.23% Small Cell Lung Carcinoma (SCLC) patients. These were further classified

according to their Tumour Node Metastatic (TNM) staging. Among the cases, 0.85% patients were of stage I, 3.6% of stage II of patients, 46% and 42% were of stage III and stage IV respectively, whereas the rest of 7% patients were unclassified. Tumor size, lymph node

involvement and metastasis data was also included in the study. Tumor size of patients varied from T1- T4, 4% patients had T1 type of tumor size, 12% had T2, 24% had T3 and maximum patients had T4 (45%) type of tumor size. Similarly 14% patients had no (N0) lymph node involvement, 11% had N1, 38% had N2, and 25% had N3 lymph node involvement. Among the all the lung cancer patients that had TNM data 50% of them had no metastasis whereas 40% of the patients showed distant metastasis (*M1*).

Table: 5.1 – Demographic characteristics and clinical data of study subjects

Variables	**Cases, (n %) N=353**	**Controls, (n %) N=351**	***p*- Value**
Age			
Mean ±SD	57.55±10.69	52.84±10.80	<0.0001
Range	28 - 86	19 - 83	
Gender			
Male	305 (86.4)	300 (85.47)	
Female	48 (13.6)	51 (14.53)	0.8047
Smoking Status			
Smokers	278 (78.75)	250 (71.23)	
Non-smokers	75 (21.25)	101 (28.77)	0.0264
Pack Years			
Mean ± SD	27.59 ± 34.28	17.52 ± 19.74	<0.0001
Histology Types			
SQCC	148 (41.93)		
ADCC	117 (33.14)		
SCLC	82 (22.23)		
TNM Staging			
I	3 (0.85)		
II	13 (3.68)		
III	163 (46.17)		
IV	149 (42.2)		
Unclassified	25 (7.08)		
Tumor Size			
T1	16 (4.53)		
T2	44 (12.46)		
T3	88 (24.93)		
T4	162 (45.89)		
Tx	10 (2.8)		
Unknown	33 (9.3)		
Lymph node			
N0	52 (14.73)		
N1	41 (11.61)		
N2	137 (38.81)		
N3	90 (25.5)		
Unknown	33 (9.3)		
Metastasis			
M0	178 (50.42)		
M1	142 (40.22)		
Unknown	33 (9.3)		

Abbreviations: SD = Standard Deviation, n = total number of cases patients and control subjects
***p*-values were derived from Pearson Chi- square test except age and PY; student T- test used for age and pack years. All *p*-values are two- sided test,**
***p*<0.05 was considered statistically significant.**

5.4 Distribution of allelic and genotypic frequencies of CYP1A1m1and m2 and their association with risk for lung cancer and histological sub-types (Table 5.2 and 5.3)

The allelic and genotypic frequencies were calculated according to the Hardy–Weinberg equilibrium (HWE) analysis. The data showed that the allelic frequency of *CYP1A1m1*in cases (X^2= 0.76; df=1; p=0.38) and control group (X^2 = 3.05; df =1; p=0.08) were in HWE;

suggesting that there was no population stratification and no sample bias. Similarly in *CYP1A1m2* the allelic frequencies of control group followed the HWE(X^2=1.81; df=1; *p*=0.17). The minor allelic frequency (MAF) of the C allele of *m1* polymorphism was 0.35 in cases and 0.26 in control group, whereas MAF of G allele of *CYP1A1m2* polymorphism was 0.18 in cases and 0.11 in controls. As shown in table 5.2 the genotypic frequencies and the ORs of *CYP1A1m1* and *m2* genes were calculated after adjusting for age, sex and smoking status using logistic regression analysis so as to evaluate the risk of occurrence of lung cancer. In case of *CYP1A1m1* polymorphism the frequency of the heterozygous variant allele was same in both patients and controls; however the frequency of mutant genotype (CC) were over represented in the patients as compared to controls (13 vs 5%). Taking the homozygous wild genotype (TT) as reference, it was observed that there was a threefold increased risk for lung cancer in individuals carryingthe homozygous mutant genotype (OR=3.15; 95%CI=1.75-5.71; *p*=0.0001) in the case of *m1* polymorphic site of *CYP1A1* gene. Such an association was not observed in case of subjects with heterozygous (TC) genotype. When both the mutant and heterozygous alleles were combined as a single genotype a marginal risk was observed (OR=1.4; 95%CI=1.03-1.91; *p*=0.02).Similarly in case of *CYP1A1m2* polymorphism it was observed that the frequency of heterozygous allele was highly represented in cases as compare to controls (32% vs 13%). Taking the wild genotype as a reference (AA) it was observed that subjects with the heterozygous genotype (AG) showed two fold increased risk of occurrence of lung cancer (OR=2.3; 95%CI = 1.6-3.3; *p*=<0.0001) as compared to individuals with the wild genotype (AA). In our study the mutant genotype was not associated with any risk towards lung cancer development.

Table 5.2: Distribution of allelic and genotypic frequencies of CYP1A1m1 and m2

	Controls, N=351(%)	**Cases, N=353(%)**	**OR[a] (95% CI)**	***p*- value**
CYP1A1m1				
TT	181 (51.5)	155 (43.9)	1.00(Ref.)	Ref.
TC	151 (43.0)	152 (43.05)	1.19(0.86 – 1.6)	0.27
CC	19 (5.4)	46 (13.03)	3.15(1.74 – 5.7)	0.0001
TC+CC	170 (48.4)	198 (56.09)	1.40(1.03– 1.9)	0.02
T	514(73.2)	460(65.3)		
C	188 (26.8)	244(34.7)		
MAF	0.26	0.34		
CYP1A1m2				
AA	282 (80.3)	233 (66)	1.00(Ref.)	Ref.
AG	62 (17.6)	116 (32.8)	2.32(1.62 – 3.3)	<0.0001
GG	7 (1.9)	4 (1.13)	0.78(0.22 – 2.7)	0.701
AG+GG	69 (19.6)	120 (33.9)	2.18(1.53– 3.1)	<0.0001
A allele	626(89.2)	580(82.4)		
G allele	76(10.8)	124(17.6)		
MAF	0.11	0.18		

[a]**Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.**

Furthermore as shown in table 5.3, when stratified according to the different histological types of lung cancer; the less number of SQCC, ADCC and SCLC patients have been observed having mutant genotype as compare to wild and heterozygous genotype in *CYP1A1m1* polymorphism. Data has shown 18.2% patients in SQCC, 11.1% in ADCC and 7.3% in SCLC patients having mutant genotype. In case of, *CYP1A1m2* polymorphism the number has decreased even more as there was no SCLC patients have observed in this polymorphism. It was observed that SQCC subjects with the mutant genotype (CC) of *CYP1A1m1* gene had a four-fold elevated risk (OR=4.41; 95% CI=2.19-8.86; p=0.0001); whereas a two-fold risk was observed in ADCC patients which was found to be marginally significant (OR=2.2; 95%CI=0.9-5.0; p=<0.05). No such association was observed in case of SCLC. In case of *CYP1A1m2* polymorphism, an elevated risk was seen in SQCC with the heterozygous genotype (AG) which was found to be significant (OR=2.65; 95%CI 1.68-4.18, p=0.04) as well as ADCC patients (OR=2.5; 95%CI=1.54-4.06; p=0.0001). No significant association was observed in SCLC patients in both the polymorphic variants.

Table 5.3: The association of genotypic frequencies of CYP1A1m1 and m2 with risk for lung cancer according to histological sub-types

	Controls, N=351(%)	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell lung carcinoma**		
		N:148 (%)	**OR[a] (95% CI)**	***p*-value**	**N:117 (%)**	**OR[a] (95% CI)**	***p***	**N:82 (%)**	**OR[a] (95% CI)**	***p***
CYP1A1m1										
TT	181 (51.5)	61 (41.2)	1.00 (Ref.)	Ref.	57 (48.7)	1.00 (Ref.)	Ref.	34 (41.4)	1.00 (Ref.)	Ref.
TC	151 (43.0)	60 (40.5)	1.17 (0.76-1.8)	0.46	47 (40.1)	0.99 (0.6-1.5)	0.97	42 (51.2)	1.54 (0.9-3)	0.10
CC	19 (5.4)	27 (18.2)	4.40 (2.19-8.86)	<0.0001	13 (11.1)	2.21 (0.9 – 5.0)	0.05	6 (7.3)	2.11 (0.7-6.1)	0.16
TC+CC	170 (48.4)	87 (58.7)	1.53 (1.02-2.29)	0.03	60 (51.2)	1.12 (0.7 – 1.7)	0.58	48 (58.5)	1.58 (0.9-2.6)	0.07
CYP1A1m2										
AA	282 (80.3)	93 (62.8)	1.00 (Ref.)	Ref.	74 (63.2)	1.00 (Ref.)	Ref.	61 (74.3)	1.00 (Ref.)	Ref.
AG	62 (17.6)	52 (35.1)	2.65 (1.68-4.18)	<0.0001	42 (35.8)	2.50 (1.54-4.0)	0.0002	21 (25.6)	1.75 (0.9-3.1)	0.06
GG	7 (1.9)	3 (2.0)	1.74 (0.41-7.46)	0.442	1 (0.85)	0.53 (0.06-4.6)	0.573	0 (0)	0.00 (0.0-0.0)	0.99
AG+GG	69 (19.6)	55 (37.1)	2.58(1.6-4.03)	<0.0001	43 (36.7)	2.31 (1.4-3.7)	0.0006	21 (25.6)	1.75 (0.9-3.1)	0.06

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

5.5 Haplotype and Linkage Disequilibrium Analysis in CYP1A1m1 and CYP1A1m2 Gene Polymorphisms (Table 5.4)

We obtained the inferred haplotypes of *CYP1A1* by using SHEsis program platform (SHEsis). Haplotype approach revealed that four possible haplotypes were observed both in the patients and the controls as shown in table 5.4. Haplotypes with frequencies <0.03 among both cases and controls were omitted from the analysis. The haplotype, T-A was considered to be the reference carrying wild type alleles. The frequency of the other three haplotypes (C-A, C-G, T-G)and their distribution among cases and controls is shown in Table 3.Comparisons of overall haplotype distribution profiles revealed a statistically significant difference between cases and controls (global test:X^2= 17.91, df=3, p= 0.01). The haplotype T-G was associated with an increased risk of lung cancer (OR=2.0, 95%CI=1.13—3.53, p=0.0004). No associations were observed for any of the other haplotypes. For haplotype analysis for the two loci of *CYP1A1* gene polymorphism, linkage disequilibrium between *m1* and *m2* is shown in figure 3 (D:0.591, r^2=0.131)

Table: 5.4 Haplotype Analysis

***CYP1A1* Haplotypes**	**Control Frequency n (%)**	**Case Frequency n (%)**	**OR (95% CI)**	***p* value**
C-A	0.19 (134)	0.22 (156)	1.20 (0.927-1.556)	0.165
C-G	0.078 (55)	0.125 (88)	1.67 (1.178-2.394)	0.003
T-A	0.701 (492)	0.604 (426)	0.65 (0.521-0.811)	0.0001
T-G	0.030 (21)	0.051 (36)	1.73 (1.001-2.997)	0.0469

5.6 ***The association between combined CYP1A1m1 and m2 genotypes and risk for lung cancer and different histological sub-types (Table 5.5 and 5.6)***

In the present study we also evaluated the combined effect of *CYP1A1m1* and *m2* polymorphic sites towards lung cancer risk as shown in table 5. There were total 192 cases and 214 controls having common genotype for both *m1* and *m2* sites of *CYP1A1* gene. In lung cancer cases there were 66.67% subjects who were carrying homozygous wild allelesfor both *m1* and *m2*, 31.77% of subjects had heterozygous alleles for both polymorphic sites and 1.56% was having the mutant alleles. When compared to the lung cancer cases the control group had higher representation (78.5%) for bothhomozygous wild alleles, whereas the frequency of heterozygous alleles for both the *CYP1A1* polymorphic sites was less as compared to cases (20 vs 31.77%). It was observed that individuals having heterozygous

alleles for both polymorphic sites had a 2-fold risk for lung cancer which was found to be significant (OR=1.99; 95%CI=1.2-3.1; *p*=0.004]. But no significant association was observed in subjects having homozygous mutant alleles for both polymorphisms.

***Table: 5.5- The association between combined CYP1A1m1 and m2 genotypes and risk for lung cancer*.**

	Controls, N=214(%)	**Cases, N=192 (%)**	**OR[a] (95% CI)**	***p*-value**
CYP1A1m1+m2				
0*	168 (78.5)	128 (66.6)	1.00(Ref.)	Ref.
1*	43 (20)	61 (31.7)	1.99 (1.24 – 3.1)	0.0040
2*	3 (1.4)	3 (1.5)	1.49 (0.28 – 7.9)	0.6350
3*	46 (21.4)	64 (33.3)	1.96 (1.24 – 3.1)	0.0040

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

*** 0 –TT+AA (Wild genotype*m1&m2*), 1–TC+AG (Heterozygous genotype, *m1&m2*), 2– CC+GG (Mutant genotype, *m1&m2*), 3 TC+CC+AG+GG (Heterozygous genotype combine with mutant genotype, *m1&m2*).**

Furthermore, when stratified according to histological sub-types, very few approximately 4.1% SQCC patients have been found to have mutant genotype whereas no ADCC as well as SCLC patients have been found to have mutant genotype on other hand maximum number of patients found to have wild type genotype. The given data showed that there was two-fold increased risk for SQCC (OR=2.0; 95%CI=1.97-3.81; *p*=0.028] as well as in SCLC patients (OR=2.2; 95%CI=1.04-4.7; *p*=0.037]. However, there was no significant association observed in ADCC patients.

Table: 5.6- The association between combined CYP1A1m1 and m2 genotypes and different histological sub-types.

	Controls, N=214(%)	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell lung carcinoma**		
		N:73 (n%)	**OR[a] (95% CI)**	***p***	**N:68 (%)**	**OR[a] (95% CI)**	***p***	**N: 47(%)**	**OR[a] (95% CI)**	***P***
CYP1A1 m1+m2										
0*	168 (78.5)	47(64.4)	1.00(Ref.)	Ref.	46(67.6)	1.00(Ref.)	Ref.	32(68)	1.00(Ref.)	Ref.
1*	43 (20)	23 (31.5)	2.02 (1.07-3.8)	0.02	22(32.3)	1.85 (0.96-3.5)	0.06	15(31.9)	2.22 (1.0-4.7)	0.03
2*	3 (1.4)	3(4.1)	5.55 (0.8-36.4)	0.07	0(0)	0.00 (0.0-0.0)	0.99	0(0)	0.00 (0.0-0.0)	0.99
3*	46 (21.4)	26 (35.6)	2.19 (1.1-4.0)	0.01	22(32.3)	1.74 (0.91-3.3)	0.09	15 (31.9)	2.11 (1.0-4.4)	0.04

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

*** 0 –TT+AA (Wild genotype*m1&m2*), 1–TC+AG (Heterozygous genotype, *m1&m2*), 2– CC+GG (Mutant genotype, *m1&m2*), 3 TC+CC+AG+GG (Heterozygous genotype combine with mutant genotype, *m1&m2*).**

5.7 Interaction of CYP1A1 genotypes and smoking status with risk towards lung cancer and according pack years (Table 5.7& 5.8)

The risk of lung cancer in relation to both the *m1* and *m2* polymorphism of the *CYP1A1* gene was further examined after stratification based upon smoking status and cumulative smoking dose as shown in table 5.7 & 5.8. It was observed that smokers with the mutant genotype (CC) for *m1* of the *CYP1A1* gene had a threefold increased risk for lung cancer (OR=3.44;95%CI= 1.66-7.13, *p*=0.0009)which was found to be highly significant as compared to smokers with the wild type genotype (TT), however there was no significant association found in non-smokers. Similarly in *CYP1A1m2* polymorphic site, smokers with the heterozygote (*Ile/Val*) genotype have a two-fold increased risk for lung cancer (OR=2.44; 95%CI=1.60-3.73, *p*=<0.0001). On the contrary no such association has been seen in case of non-smokers.

Further stratifying the smokers into light and heavy according to the pack years as shown in Table 8, it was revealed that light smokers who smoked≤25 pack years and carrying the mutant genotype (CC) for *CYP1A1m1* the OR for lung cancer was 2.6(95%CI=1.3-5.2; *p*=0.004) and was statistically significant. However, the OR increased to 5.6(95%CI=1.5-20.9; *p*=0.009) in those individuals who were heavy smokers and carried the homozygous mutant genotype (CC). Similarly when analysed in *CYP1A1m2*polymorphism; light smokers who carried the heterozygous genotype (*Ile/Val*) showed approximately two-fold increased risk for lung cancer (OR=1.9; 95%CI=1.28-3.1; *p*=0.002), while for heavy smokers a threefold increased risk towards lung cancer was observed with individuals carrying the heterozygous genotype (OR=3.0; 95%CI=1.6-5.9; *p*=0.0008).

5.8 Association between combine genotype of CYP1A1 gene and risk of lung cancer in tobacco smokers

In the present study we also have evaluated the role of combined polymorphic sites of *CYP1A1* gene along with smoking status (Table 5.9). It was observed that individuals who were smokers and carried the variant allele of each polymorphic site had elevated risk for lung cancer (OR=2.3; 95%CI= 1.31-4.01; *p*=0.002) as compared to those subjects with the same genotype but who were non-smokers (OR=1.3; 95%CI= 0.51-3.4; *p*=0.554).

5.9 Interaction of CYP1A1 genotypes and tobacco smoke according to histological sub-types of lung cancer

The interaction of the *CYP1A1* polymorphisms and tobacco smoke was also assessed separately for SQCC, ADCC and SCLC histological sub-types as shown in table 5.10. Compared with individuals with the *CYP1A1m1* wild type genotype (TT and who were smokers), it was observed that SQCC subjects carrying the mutant genotype (CC) for *m1*site had an elevated risk towards lung cancer (OR=5.4; 95%CI=2.4-11.9; *p*=<0.0001) which was found to be significant, whereas no such association was observed in both ADCC and SCLC. In case of *CYP1A1m2* polymorphism, SQCC patients who were smokers showed approximately threefold increased risk for lung cancer with the heterozygous genotype (AG) (OR=2.8; 95%CI=1.72-4.6; *p*=<0.0001]. Similar results were observed in case of ADCC (OR= 2.2; 95%CI=1.2- 4.2; *p*=0.009] and SCLC (OR=2.1; 95%CI= 1.15-4.0; *p*=0.015] but the effect was less pronounced as compared to SQCC patients.

In case of non–smokers the sample size has been reduced as in SCLC patients there was no*CYP1A1m2* polymorphism observed and no significant association and risk was observed with any histological sub-types and the variant *CYP1A1m1* genotype. However for the *CYP1A1m2* polymorphism ADCC patients having the heterozygous allele for *CYP1A1m2* gene (*Ile/Val*) showed approximately 3-fold increased risk (OR=2.9; 95%CI=1.3-6.5; *p*=0.008) [Table 5.11].

Furthermore, the smokers were stratified into light and heavy smokers according to pack-years. Heavy smokers had more risk for lung cancer when both the variant alleles of the two polymorphic sites of *CYP1A1* gene were combined as a single genotype and compared with the combined wild type genotype (OR=2.8; 95%CI=1.24-6.5, *p*=0.013).

Furthermore, we also stratified light smokers and heavy smokers on the basis of histological subtypes (Table 5.12). It was observed that as compared to the *CYP1A1m1* wild type genotype subjects who were light smokers, the odds of SQCC for the mutant *CYP1A1* genotype was 3.9 (95%CI=1.7-8.8, *p*=0.001)whereas no significant association was seen in other histological subtypes of light smokers in *CYP1A1m1* polymorphism. Similarly in the case of *m2* polymorphism, ADCC subjects who were classified as light smokers based on pack years had a high risk for lung cancer (OR=2.5; 95%CI=1.49-4.4;*p*= 0.0007) as

compared to subjects who had same exposure of smoking but with the wild type (*Ile/Ile*) genotype.

In case of heavy smokers (Table 5.13), a positive and strong association was observed for SQCC and SCLC subjects where smoking dose was above PY>25 and who were carrying the mutant genotype for *CYP1A1* gene (CC) (OR=7.5; 95%CI=1.8-30.9; *p*=0.004) as compared to SQCC subjects with the wild-type genotype (TT) of the *CYP1A1* gene. In case of *CYP1A1m2* polymorphism, among heavy smokers, SQCC patients showed a threefold increase risk in lung cancer with heterozygous allele (*Ile/Val*) (OR=3.5; 95%CI=1.69-7.5; *p*=0.0008), whereas no association was observed in ADCC and SCLC, respective.

Table: 5.7– Interaction of CYP1A1 genotypes and smoking status with risk towards lung cancer

Smoking status-Smokers & Non-smokers								
	Cases (Smokers) = 278 (%)	**Controls(Smokers) N=250(%)**	**OR[a] (95% CI)**	***p***	**Cases (Non-Smokers) N=75(%)**	**Controls, (Non-Smokers) N=101 (%)**	**OR[a] (95% CI)**	***p***
CYP1A1m1								
TT	117(42)	131 (52.4)	1.00(Ref.)	Ref.	38(50.6)	50(49.5)	1.00(Ref.)	Ref.
TC	128(46)	107 (42.8)	1.39 (0.9-2.0)	0.073	24(32)	44(43.5)	0.70(0.35-1.38)	0.308
CC	33(11.8)	12 (4.8)	3.44 (1.6-7.1)	0.0009	13(17.3)	7(6.9)	2.55(0.89-7.3)	0.079
TC+CC	161 (57.9)	119 (47.6)	1.59(1.1-2.2)	0.009	37(49.3)	51(50.4)	0.96(0.51-1.79)	0.903
CYP1A1m2								
AA	184 (66.1)	203 (81.2)	1.00(Ref.)	Ref.	49 (65.3)	79(78.21)	1.00(Ref.)	Ref.
AG	91 (32.7)	43 (17.2)	2.44 (1.6-3.7)	<0.0001	25(33.3)	19(18.81)	2.04(0.99-4.2)	0.051
GG	3 (1.07)	4 (1.6)	1.05(0.2-4.9)	0.94	1(1.3)	3(2.9)	0.52(0.05-5.5)	0.592
AG+GG	94 (33.8)	47(18.8)	2.34(1.5-3.5)	0.0001	26(34.67)	22(21.78)	1.83(0.91-3.6)	0.088

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

Table: 5. 8– Interaction of CYP1A1 genotypes and smoking status after stratification to pack years.

Smoking status-Smokers & Non-smokers								
	Cases (Light Smokers; PY≤25) N= 220(%)	**Controls, (Light Smokers; PY≤25) N=254(%)**	**OR[a] (95% CI)**	***p***	**Cases (Heavy-Smokers; PY>25) N= 133(%)**	**Controls (Heavy-Smokers; PY>25) N=97 (%)**	**OR[a] (95% CI)**	***p*-**
CYP1A1m1								
TT	102(46.3)	128(50.4)	1.00(Ref.)	Ref.	53(39.8)	53(54.6)	1.00(Ref.)	Ref.
TC	88(40)	110(43.3)	1.01 (0.68-1.5)	0.94	64(48.1)	41(42.3)	1.73 (0.97-3)	0.059
CC	30(13.6)	16(6.3)	2.64 (1.3-5.2)	0.004	16(12)	3(3)	5.62 (1.5-20.9)	0.009
TC+CC	118(53.6)	126 (49.6)	1.20 (0.8-1.74)	0.31	80(60.1)	44(45.3)	2.00 (1.15-3.4)	0.013
CYP1A1m2								
AA	151(68.6)	203(79.9)	1.00(Ref.)	Ref.	82(61.6)	79(81.4)	1.00(Ref.)	Ref.
AG	66(30)	46(18.1)	1.99 (1.28-3.1)	0.002	50(37.6)	16(16.5)	3.09 (1.6-5.9)	0.0008
GG	3(1.3)	5(1.9)	0.92(0.21-4)	0.91	1(0.7)	2(2)	0.51(0.04-6.2)	0.59
AG+GG	69(31.3)	51(20)	1.90 (1.2-2.9)	0.003	51(38.3)	18(18.5)	2.82 (1.49-5.3)	0.0014

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtype.

Table: 5.9– Interaction of combined CYP1A1 genotypes and its association with lung cancer risk stratified according to smoking status and pack years

	Cases (Smokers) N= 149(%)	**Controls (Smokers) N=155 (%)**	**OR[a] (95% CI)**	***p***	**Cases (Non-Smokers) N= 43 (%)**	**Controls (Non-Smokers) N=59 (%)**	**OR[a] (95% CI)**	***P***
CYP1A1m1+m2								
0*	97(65.1)	123(79.3)	1.00(Ref.)	Ref.	31(72)	45(76.2)	1.00(Ref.)	Ref.
1*	49(32.8)	31(20)	2.31 (1.3-4.01)	0.002	12(27.9)	12(20.3)	1.33 (0.51-3.4)	0.55
2*	3(2.01)	1(0.64)	4.20 (0.4-42.9)	0.22	0(0.0)	2(3.38)	0.00 (0.0-0.0)	0.99
3*	52(34.8)	32(20.6)	2.38(1.3-4.1)	0.001	12(27.9)	14(23.7)	1.18 (0.4-3.03)	0.72
	Cases N= 125 (%) (light Smokers; PY≤25)	**Controls N=149 (%) (light Smokers; PY≤25)**	**OR[a] (95% CI)**	***p***	**Cases N= 67 (%) (Heavy-Smokers; PY>25)**	**Controls N=65 (%) (Heavy-Smokers; PY>25)**	**OR[a] (95% CI)**	***P***
CYP1A1m1+m2								
0*	87(69.6)	117(78.5)	1.00(Ref.)	Ref.	41(61.2)	51(78.4)	1.00(Ref.)	Ref.
1*	36(28.8)	30(20.1)	1.70 (0.95-3.03)	0.06	25(37.3)	13(20)	2.86 (1.24-6.5)	0.01
2*	2(1.6)	2(1.3)	1.64 (0.21-12.5)	0.63	1(1.5)	1(1.5)	1.24 (0.05-27.5)	0.88
3*	38(30.4)	32(21.4)	1.70 (0.97-2.9)	0.06	26(38.8)	14(21.5)	2.78 (1.2-6.3)	0.01

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes. *0 –TT+AA (Wild genotype, *m1&m2*), 1–TC+AG (Heterozygous genotype, *m1&m2*), 2– CC+GG (Mutant genotype, *m1&m2*), 3-TC+CC+AG+GG (Heterozygous genotype combine with mutant genotype, *m1&m2*).

Table: 5.10– Interaction of CYP1A1 genotypes and tobacco smoke according to histological sub-types of lung cancer.

	Controls, (Smokers) N=250 (%)	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell Lung Carcinoma**		
		N:134 (%)	**OR[a] (95% CI)**	***p*-value**	**N:65 (%)**	**OR[a] (95% CI)**	***p***	**N:74 (%)**	**OR[a] (95% CI)**	***P***
CYP 1A1 m1										
TT	131 (52.4)	53(39.5)	1.00(Ref.)	Ref.	33(50.7)	1.00(Ref.)	Ref.	29(39.1)	1.00(Ref.)	Ref.
TC	107 (42.8)	57(42.5)	1.31 (0.82-2)	0.24	28(43)	1.04 (0.58-1.8)	0.88	40(54)	1.75 (0.9-3.1)	0.05
CC	12 (4.8)	24(17.9)	5.40 (2.4-11.9)	<0.0001	4(6.1)	1.55(0.45-5.2)	0.47	5(6.7)	1.96 (0.5-6.8)	0.28
TC+ CC	119 (47.6)	81(60.4)	1.69(1.0-2.6)	0.017	32(49.2)	1.08 (0.62-1.8)	0.77	45(60.8)	1.77(1.0-3.0)	0.04
CYP 1A1 m2										
AA	203 (81.2)	83(61.9)	1.00(Ref.)	Ref.	44(67.6)	1.00(Ref.)	Ref.	53(71.6)	1.00(Ref.)	Ref.
AG	43 (17.2)	48(35.8)	2.83 (1.7-4.6)	<0.0001	21(32.3)	2.28 (1.2-4.2)	0.0098	21(28.3)	2.16 (1.1-4.0)	0.01
GG	4 (1.6)	3(2.23)	2.34 (0.4-11.2)	0.287	0(0)	0.00 (0.0-0.0)	0.99	0(0)	0.00 (0.0-0.0)	0.99
AG+ GG	47(18.8)	51(38)	2.81(1.73-4.5)	<0.0001	21(32.3)	2.13 (1.1-3.9)	0.01	21 (28.3)	2.02(1.0-3.7)	0.02

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

Table: 5.11-Interaction ofCYP1A1 genotypes and non-smokers risk towards lung cancer risk on the basis of histology

	Controls (Non-Smokers) N=101 (%)	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell Lung Carcinoma**		
		N=14 (%)	**OR[a] (95% CI)**	***p***	**N=52 (%)**	**OR[a] (95% CI)**	***p***	**N=8 (%)**	**OR[a] (95% CI)**	***P***
CYP1A1 m1										
TT	50(49.5)	8(57.1)	1.00(Ref.)	Ref.	24(46.1)	1.00(Ref.)	Ref.	5(62.5)	1.00(Ref.)	Ref.
TC	44(43.5)	3(21.4)	0.34(0.08-1.45)	0.14	19(36.5)	0.88 (0.4-1.9)	0.74	2(25)	0.53(0.09-3.1)	0.48
CC	7(6.9)	3(21.4)	1.68(0.28-10)	0.56	9(17.3)	3.23 (0.98-10.6)	0.05	1(12.5)	6.38 (0.36-111.7)	0.20
TC+CC	51(50.49)	6(42.8)	0.67(0.21-2.1)	0.5	28(53.8)	1.18(0.57-2.4)	0.64	3(37.5)	0.73(0.15-3.4)	0.69
CYP1A1 m2										
AA	79(78.21)	10(71.4)	1.00(Ref.)	Ref.	30(57.6)	1.00(Ref.)	Ref.	8(100)	--	--
AG	19(18.81)	4(28.5)	1.59(0.44-5.7)	0.47	21(40.3)	2.94 (1.3-6.5)	0.008	0(0)	--	--
GG	3(2.9)	0(0)	0.00 (0.0-0.0)	0.99	1(1.9)	0.85 (0.07-9.5)	0.90	0(0)	--	--
AG+GG	22(21.78)	4(28.5)	1.42(0.4-5.0)	0.58	22(42.3)	2.66 (1.2-5.8)	0.014	8(100)	--	--

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

Table: 5.12 –Interaction of CYP1A1m1 and m2 genotypes for Light smokers after stratifying on basis of lung cancer histology

	Controls, (light Smokers) N=254 (%)	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell Lung Carcinoma**		
		N=78 (%) (light Smokers)	**OR[a] (95% CI)**	***p***	**N=90 (% (light Smokers)**	**OR[a] (95% CI)**	***p***	**N=49 (%) (light Smokers)**	**OR[a] (95% CI)**	***P***
CYP1A 1m1										
TT	128(50.4)	35(44.8)	1.00(Ref.)	Ref.	41(45.6)	1.00(Ref.)	Ref.	24(48.9)	1.00(Ref.)	Ref.
TC	110(43.3)	27(34.6)	0.88 (0.49-1.5)	0.67	38(42.2)	1.08 (0.64-1.83)	0.75	22(44.9)	1.09(0.5-2.1)	0.78
CC	16(6.3)	16(20.5)	3.91 (1.7-8.8)	0.001	11(12.2)	2.23 (0.9-5.5)	0.08	3(6.1)	1.34 (0.32-5.5)	0.68
TC+CC	126(49.6)	43(55.1)	1.24(0.73-2.0)	0.41	49(54.4)	1.23 (0.74-2.0)	0.41	25(51)	1.10 (0.58-2.0)	0.76
CYP1A 1m2										
AA	203(79.9)	52(66.7)	1.00(Ref.)	Ref.	56(62.2)	1.00 (Ref.)	Ref.	40(81.6)	1.00(Ref.)	Ref.
AG	46(18.1)	24(30.7)	2.14 (1.1-3.8)	0.011	33(36.7)	2.59 (1.49-4.4)	0.0007	9(18.4)	1.12 (0.5-2.5)	0.77
GG	5(1.9)	2(2.5)	2.41 (0.41-13.8)	0.32	1(1.1)	0.71(0.07-6.4)	0.76	0(0)	0.00 (0.0-0.0)	0.99
AG+GG	51(20)	26(33.3)	2.16 (1.2-3.8)	0.008	34(37.8)	2.41 (1.4-4.1)	0.001	9(18.4)	1.03 (0.46-2.3)	0.93

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

Table: 5.13 – Interaction of CYP1A1m1 and m2 genotypes for Heavy smokers after stratifying on basis of lung cancer histology

	Controls, N= (%) (Heavy-Smokers)	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell Lung Carcinoma**		
		N:70 (%)	**OR[a] (95% CI)**	***p***	**N:27 (%)**	**OR[a] (95% CI)**	***p***	**N:33 (%)**	**OR[a] (95% CI)**	***P***
CYP1 A1m1										
TT	53(54.6)	26(37.1)	1.00(Ref.)	Ref.	16(59.2)	1.00(Ref.)	Ref.	10(30.3)	1.00(Ref.)	Ref.
TC	41(42.3)	33(47.1)	1.75(0.8-3.4)	0.10	9(33.3)	0.82(0.31-2.1)	0.68	20(66.7)	2.79 (1.1-6.8)	0.02
CC	3(3)	11(15.7)	7.57 (1.8-30.9)	0.004	2(7.4)	2.13 (0.3-14.7)	0.44	3(9.0)	5.60 (0.95-32.8)	0.05
TC+ CC	44(45.3)	44(62.8)	2.14 (1.1-4.1)	0.02	11(40.7)	0.92(0.37-2.2)	0.85	23(76.7)	2.98 (1.2-7.2)	0.01
CYP1 A1m2										
AA	79(81.4)	41(58.6)	1.00(Ref.)	Ref.	18(66.7)	1.00(Ref.)	Ref.	21(63.6)	1.00(Ref.)	Ref.
AG	16(16.5)	28(40)	3.58 (1.69-7.5)	0.0008	9(33.3)	2.25 (0.8-6.2)	0.11	12(36.3)	3.29 (1.3-8.2)	0.01
GG	2(2)	1(1.4)	1.00 (0.07-12.8)	0.99	0(0)	0.00 (0.0-0.0)	0.99	0(0)	0.00 (0.0-0.0)	0.99
AG+ GG	18(18.5)	29(41.4)	3.33 (1.6-6.9)	0.0012	9(33.3)	2.07 (0.75-5.7)	0.15	12(36.3)	2.99 (1.2-7.3)	0.01

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

5.10 Demographic studies of case- control study of CHRNA5 gene (Table 5.14)

The demographic characteristics and clinical data of the studied subjects are shown in table 5.14. A total of 206 cases and 307 controls were recruited and the mean age of the cases was 57.55± 10.69 (range 19–86) and that of the controls was 53.19 ±11.10 (range 19–83). The study included 84.95% males and 15.05% females in cases where as 84.04% males and 15.96% females were present in the control group. There was no significant difference in distribution of males and females between controls and patients suggesting adequate matching (p=0.8767).In the present study the number of smokers was represented more in the cases as compared to the controls (79.13 vs 71.66%), whereas the frequency of non-smokers was more in the control group as compared to cases (20.87 vs 28.34%, p=0.0715).When stratified on the basis of pack years; there was not significant difference in distribution between the pack years of cases and controls (PY 30.20 vs 17.52, p<0.0001). When the lung cancer cases were stratified on basis of histology 37.38% were of SQCC, 35.92% of Adenocarcinoma (ADCC) and 23.79% Small Cell Lung Carcinoma (SCLC) patients. These were further classified according to their Tumour Node Metastatic (TNM) staging. Among the cases, 0.48% patients were of stage I, 1.46% of stage II of patients, 48.06% and 43.69% were of stage III and stage IV respectively, whereas the rest of 6.31% patients were unclassified. Tumor size, lymphnode involvement and metastasis data was also included in the study. Tumor size of patients varied from T1- T4, 5.83% patients had T1 type of tumor size, 11.65% had T2, 21.84% had T3 and maximum patients had T4 (48.54%) type of tumor size. Similarly 11.16% patients had no (N0) lymph node involvement, 9.22% had N1, 42.72% had N2, and 28.16% had N3 lymph node involvement. Among the all the lung cancer patients that had TNM data 51.46% of them had no metastasis whereas 39.80% of the patients showed distant metastasis (*M1*).

Table: 5.14 – Demographic characteristics and clinical data of study subjects

Variables	**Cases, (n %) N=206**	**Controls, (n %) N=307**	***p*- Value**
Age Mean ±SD Range	 58.22 ±11.01 19-86	 53.19±11.10 19-83	 <0.0001
Gender Male Female	 175 (84.95) 31 (15.05)	 258 (84.04) 49 (15.96)	 0.8767
Smoking Status Smokers Non-smokers	 163 (79.13) 43 (20.87)	 220 (71.66) 87 (28.34)	 0.0715
Pack Years Mean ± SD	 30.20±40.11	 17.52±19.69	 <0.0001
Histology Types SQCC ADCC SCLC Unclassified	 77 (37.38) 74 (35.92) 49 (23.79) 6 (2.91)		
TNM Staging I II III IV Unclassified	 1 (0.48) 3 (1.46) 99 (48.06) 90 (43.69) 13 (6.31)		
Tumor Size T1 T2 T3 T4 Tx Unknown	 12 (5.83) 24 (11.65) 45 (21.84) 100 (48.54) 8 (3.88) 17 (8.25)		
Lymph node Involvement No N1 N2 N3 NX Unknown	 23 (11.16) 19 (9.22) 88 (42.72) 58 (28.16) 1 (0.49) 17 (8.25)		
Metastasis M0 M1 MX Unknown	 106 (46) 82 (39.80) 1(0.49) 17 (8.25)		

Abbreviations: SD = Standard Deviation, n = total number of cases patients and control subjects
******p*-values were derived from Pearson Chi- square test except age and PY; student T- test used for age and pack years. All *p*-values are two- sided test,**
***p*<0.05 was considered statistically significant.**

5.11 Distribution of allelic and genotypic frequencies of CHRNA5 and their association with risk for lung cancer and histological sub-types (Table 5.14)

The association study of CHRNA 5 gene polymorphism with increase risk of lung cancer included 307 control subjects and 206 cases subjects. The data has observed that there was less number of patients having wild genotype in controls (48.5%) as compare to cases (52.9%) where as patients with heterozygous genotype was more in controls (46.3%) than in cases (40.3%). Similarly the patients with mutant genotype in less in controls (5.2%) as compare to cases (6.8%). The given data has shown that the polymorphism in *CHRNA5* gene has no significant effect on North Indian population. As there were no significant Odds ratios was obtained in any genotype of *CHRNA5* polymorphism as shown in table 5.14. This shows that this polymorphism has not any significant role in increase risk of lung cancer.

Table 5.15: Association of CHRNA5 gene polymorphism with risk of lung cancer.

NA5	**Controls, (%) N=307**	**Cases, (n= %) N= 206**	**OR[a] (95% CI)**	***p*-value**
GG	149 (48.5)	109 (52.9)	1.00 (Ref.)	Ref.
GA	142 (46.3)	83 (40.3)	0.83 (0.57-1.2)	0.35
AA	16 (5.2)	14 (6.8)	1.22 (0.56-2.6)	0.60
GA+AA	158 (51.5)	97 (47.1)	0.87 (0.6-1.2)	0.47

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

To further study the association of *CHRNA5* gene polymorphism with increase risk of lung cancer, the study has also stratified the data according to the histological cancer subtypes. The histological data revealed that the SQCC patients having mutant genotype as more in cases (6.5%) as compare to controls (5.2%). But there was no significant association seen in SQCC patients (OR=1.23; 95%CI= 0.4-3.6; p=0.70). On the other hand, there was more number of ADCC patients (8.1%) in cases compare to controls. Similar in this the association between genotype of *CHRNA5* and increase risk of lung cancer has shown no significant relationship (OR= 1.97; 95% CI= 0.69-5.6; p=0.20). Similar scenario was observed in SCLC patients (OR= 0.99; 95%CI= 0.2-3.7; p= 0.99). Data has shown in table 5.15.

Table 5.16: The association of genotypic frequencies of CHRNA5 with risk for lung cancer according to histological subtypes

NA5	Controls, (%) N=307	Squamous cell Carcinoma			Adenocarcinoma			Small cell lung carcinoma		
		N:77 (n%)	OR[a] (95% CI)	*p*-value	N:74 (n%)	OR[a] (95% CI)	*p*-value	N:49 (n%)	OR[a] (95% CI)	*p*-value
GG	149 (48.5)	37 (48.1)	1.00 (Ref.)	Ref.	41 (55.4)	1.00 (Ref.)	Ref.	29 (59.2)	1.00 (Ref.)	Ref.
GA	142 (46.3)	35 (45.4)	1.02 (0.5-1.7)	0.93	27 (36.5)	0.75 (0.4 -1.3)	0.31	17 (34.7)	0.61 (0.3-1.2)	0.15
AA	16 (5.2)	5 (6.5)	1.23 (0.4-3.6)	0.70	6 (8.1)	1.97 (0.69-5.6)	0.20	3 (6.1)	0.99 (0.2-3.7)	0.99
GA+AA	158 (51.5)	40 (51.9)	1.04 (0.6-1.7)	0.86	33 (44.6)	0.85 (0.5-1.4)	0.56	20 (40.8)	0.66 (0.3-1.2)	0.21

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

5.12 Interaction of CHRNA5 genotype and smoking status with risk towards lung cancer and their histological subtypes. (Table: 5.17 and 5.18)

The study has also evaluated the effect of smoking on lung cancer risk having *CHRNA5* polymorphic geneas shown in table 15. It has been observed that the smokers having mutant genotype were more in cases (8.6%) than in controls (5.4%) but they have not shown any significant relationship between the genotype of *CHRNA5* polymorphism and increase risk of lung cancer (OR= 1.46; 95%CI= 0.6-3.3; p= 0.37). Similarly in case of non – smokers no smokers have been found having mutant genotype and the association was not significant between the genotype of non- smokers and increase risk of lung cancer.

The histological data of smokers has observed that the number of SQCC smokers with heterozygous allelic variants has increase in cases (47.3%) compare to controls (45.4%). But on the other hand the number of ADCC (35%) and SCLC (34.1%) smokers were observed less in number than controls (45.4%) whereas smokers with mutant genotype allelic variant were more in SQCC (6.8%), ADCC (15%) and SCLC (6.8%) cases compare to controls (5.4%). These allelic variants of *CHRNA5* polymorphic gene in SQCC smokers patients has shown no significance in relation to lung cancer risk with OR=1.13, 95%CI value= 0.65-1.97 and p value= 0.64 in heterozygous variant and OR= 1.31, 95% CI= 0.43-3.99 and p value= 0.63 in case of mutant variant. Similarly, in case of ADCC smokers patients there was no association seen with heterozygous variant (OR=0.78; 95%CI=0.37-1.64; p value= 0.51) of *CHRNA5* gene towards increase risk of lung cancer whereas ADCC smokers having mutant genotype observed to have twofold increase in OR but no significant have been shown with this risk of lung cancer. On the other hand, in case of SCLC smokers, similar scenario was observed which has shown no significant relationship between genotype of *CHRNA5* gene polymorphism and increase risk of lung cancer in smokers.

Consistently, in case of non-smokers stratified according to histological subtype of lung cancer, it was seen that the there has been no effect of polymorphism on lung cancer risk in either of SQCC, ADCC or SCLC patients of non-smokers. Moreover, the number of patients has been observed too low to observe the significant data in non-smokers.

5.13 Association between CHRNA5 polymorphism and smokers stratified according to pack years (Table: 5.20)

The smokers were also measured according to pack years to observe the effect of quantity of exposure of nicotine in individuals having *CHRNA5* polymorphic gene. The data of present study has shown that the individuals who have PY<25 and considered as light smokers were observed to have less number of heterozygous variants in cases (37.5%) than in controls (43.5) but more number of mutant variants in cases (7%). On the other hand the heavy smokers having PY>25 that excessively exposed to nicotine quantity were obtained less number of heterozygous variants (44.9%) and more number of mutant variants (6.4%)) in cases compare to controls (53.6%; 4.7%).

The risk involved in case of light smokers were not significant in heterozygous variant of *CHRNA5* gene (OR=0.84; 95%CI= 0.52-1.33; p value=0.46) as well as in mutant variant of *CHRNA5* gene (OR=1.32; 95%CI= 0.51-3.38; p value= 0.55) compare to wild genotype. In case of heavy smokers, the scenario was observed to be consistent with it but different OR values, OR= 0.68; 95%CI= 0.34-1.37; p value= 0.29 in case of heterozygous and OR= 1.20; 95%CI= 0.26-5.59 and p value = 0.80 in case of mutant genotype of *CHRNA5* polymorphism. The association has no significant value with *CHRNA5* polymorphic allelic variants and lung cancer risk in heavy smokers.

Table 5.17: Interaction of CHRNA5 genotypes and smoking status

SMOKING STATUS								
NA5	**Controls, (n %) N=220 (Smokers)**	**Cases. (n %) N= 163 (Smokers)**	**OR[a] (95% CI)**	***p*-Value**	**Controls, (n %) N=87 (Non-Smokers)**	**Cases. (n %) N= 43 (Non-Smokers)**	**OR[a] (95% CI)**	***p*-Value**
GG	108 (49.1)	82 (50.3)	1.00 (Ref.)	Ref.	41 (47.1)	27 (62.8)	1.00 (Ref.)	Ref.
GA	100 (45.4)	67 (41.1)	0.88 (0.5-1.3)	0.57	42 (48.3)	16 (37.2)	0.62 (0.28-1.3)	0.23
AA	12 (5.4)	14 (8.6)	1.46 (0.6-3.3)	0.37	4 (4.6)	0 (0)	0.0 (0.0-0.0)	0.99
GA+AA	112 (50.9)	81 (49.7)	0.94 (0.6-1.4)	0.80	46 (52.9)	16 (37.2)	0.58 (0.26-1.27)	0.17

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

Table 5.18: Interaction of CHRNA5 genotypes and tobacco smoke according to histological sub-types of lung cancer.

	Controls, (n %) N=220 (Smokers)	Squamous cell Carcinoma			Adenocarcinoma			Small cell Lung Carcinoma		
		N:74 (n%)	OR[a] (95% CI)	*p*-value	N:40 (n%)	OR[a] (95% CI)	*p*-value	N:44 (n%)	OR[a] (95% CI)	*p*-value
CHRNA5										
GG	108 (49.1)	34 (45.9)	1.0 (Ref.)	Ref.	20 (50)	1.0 (Ref.)	Ref.	26 (59.1)	1.0 (Ref.)	Ref.
GA	100 (45.4)	35 (47.3)	1.13 (0.65-1.97)	0.64	14 (35)	0.78 (0.37-1.64)	0.51	15 (34.1)	0.52 (0.25-1.09)	0.08
AA	12 (5.4)	5 (6.8)	1.31 (0.43-3.99)	0.63	6 (15)	2.30 (0.72-7.34)	0.15	3 (6.8)	1.05 (0.27-4.08)	0.93
GA+AA	112 (50.9)	40 (54.1)	1.16 (0.68-1.97)	0.58	20 (50)	0.94 (0.47-1.86)	0.86	18 (40.9)	0.59 (0.29-1.18)	0.13

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

Table: 5.19 Interaction of CHRNA5 genotypes and tobacco smoke according to histological sub-types of lung non- cancer.

	Controls, (n %) N=87 (Non-Smokers)	Squamous cell Carcinoma			Adenocarcinoma			Small cell Lung Carcinoma		
		N:3 (n%)	OR (95% CI)	*p*-value	N:34 (n%)	OR[a] (95% CI)	*p*-value	N:5 (n%)	OR[a] (95% CI)	*p*-value
CHRNA5										
GG	41 (47.1)	3 (100)	1.0 (Ref.)	Ref.	21 (61.8)	1.00 (Ref.)	Ref.	3 (60)	1.0 (Ref.)	Ref.
GA	42 (48.3)	0	0.0 (0.0-0.0)	0.99	13 (38.2)	0.67 (0.28-1.59)	0.36	2 (40)	0.80 (0.11-5.47)	0.83
AA	4 (4.6)	0	NS	NS	0 (0)	0.00 (0.0-0.0)	0.99	0 (0)	--	--
GA+AA	46 (52.9)	0	NS	NS	13 (38.2)	0.67 (0.28-1.59)	0.36	2 (40)	0.80 (0.11-5.47)	0.83

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes.

Table: 5.20– Interaction of CHRNA5 genotypes and smoking status after stratification to pack years.

	SMOKING STATUS							
	Controls, (n %) N=223 (light Smokers; PY≤25)	Cases. (n %) N= 128 (light Smokers; PY≤25)	OR[a] (95% CI)	*p*-Value	Controls, (n %) N=84 (Heavy-Smokers; PY>25)	Cases. (n %) N= 78 (Heavy-Smokers; PY>25)	OR[a] (95% CI)	*p*-Value
CHRNA5								
GG	114 (51.1)	71 (55.5)	1.0 (Ref)	Ref.	35 (41.7)	38 (48.7)	1.0 (Ref.)	Ref.
GA	97 (43.5)	48 (37.5)	0.84 (0.52-1.33)	0.46	45 (53.6)	35 (44.9)	0.68 (0.34-1.37)	0.29
AA	12 (5.4)	9 (7.0)	1.32 (0.51-3.38)	0.55	4 (4.7)	5 (6.4)	1.20 (0.26-5.59)	0.80
GA+AA	109 (48.9)	57 (44.5)	0.89 (0.57-1.39)	0.61	49 (58.3)	40 (51.3)	0.73 (0.37-1.44)	0.37

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtype

5.14 The association between combined CYP1A1m1, m2 and CHRNA5 genotypes and risk for lung cancer and different histological sub-types (Table 5.21)

CYP1A1 enzyme also involved in the metabolism of nicotine and its derivatives, the polymorphism in *CYP1A1* gene as well as *CHRNA5* gene may have greater effect on the risk of lung cancer, therefore to find out the this effect on North Indian population, study has also evaluated the combine effects of *CYP1A1m1* polymorphism with *CHRNA5* as well as *CYP1A1m2* polymorphism with *CHRNA5*. Further, study has also given the data of triple combination of both polymorphism of *CYP1A1* gene and *CHRNA5* gene.

According to the data, number of wild type individual observed in *CYP1A1m1* and *CHRNA5* polymorphic combination was more in cases (58.1%) as compare to controls (53.9%) whereas heterozygous genotype individuals more in controls (45.4%) than in cases (40.7%). The study has observed that the association between genotype of *CHRNA5* polymorphism and risk of lung cancer has no any significance (OR=2.00; 95%CI= 0.1 -34.5; p=0.63). On the other hand, when study has combine *CYP1A1m2* polymorphism with *CHRNA5* polymorphism, it was observed that there was not a single individual found having mutant genotype of both polymorphism in cases as well as in controls whereas heterozygous genotype individuals were more in cases (25.5%) compare to controls (18.1%). Similarly in this combination there were no significant association found between the genotype of both polymorphic gene and risk of lung cancer (OR= 1.80; 95%CI=0.9-3.4; p=0.07). Further, the study has given the data to triple combination of *CYP1A1m1*, *m2* and *CHRNA5* polymorphism which has shown that there were more number of heterozygous individuals in cases (24.6%) compare to controls (22%). This combination also has no significance relationship between the genotype of combine three polymorphism and risk of lung cancer (OR= 1.44; 95%CI= 0.6 -3.2; p=0.38).

Table 5.21 Association between genotypic combination of CYP1A1m1, m2 and CHRNA5 polymorphism and risk of lung cancer and its histological subtypes.

CYP1A1 m1 vs NA5	**Controls, (%) N=141**	**Cases, (%) N=86**	**OR[a] (95% CI)**	***p*-value**
GG+TT	76 (53.9)	50 (58.1)	1.00 (Ref.)	Ref.
GA+TC	64 (45.4)	35 (40.7)	0.87 (0.4-1.5)	0.65
AA+CC	1 (0.7)	1 (1.2)	2.00 (0.1 -34.5)	0.63
GA/AA+ TT/TC	65 (46.1)	36 (41.9)	0.89 (0.5-1.5)	0.68
CYP1A1 m2 vs NA5	**Controls, (%) N=138**	**Cases, (%) N=94**	**OR[a] (95% CI)**	***p*-value**
GG+AA	113 (81.9)	70 (74.5)	1.00 (Ref.)	Ref.
GA+AG	25 (18.1)	24 (25.5)	1.80 (0.9-3.4)	0.07
AA+GG	0 (0)	0 (0)	0.0 (0.0-.0.0)	NS
GA/AA+ AG/GG	25 (18.1)	24 (25.5)	1.8 (0.9-3.4)	0.07
CYP1A1 vs NA5	**Controls, (%) N=91**	**Cases, (%) N= 57**	**OR[a] (95% CI)**	***p*-value**
0	71 (78.0)	43 (75.4)	1.00 (Ref.)	Ref.
1	20 (22.0)	14 (24.6)	1.44 (0.6 -3.2)	0.38
2	0 (0)	0 (0)	NS	NS
3	20 (22.0)	14 (24.6)	1.44 (0.6 -3.2)	0.38

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes. NS= Not Significant.

In different histological subtypes of lung cancer the genetic combination of *CYP1A1m1*, *m2* and *CHRNA5* polymorphism may show its effect on increase risk of lung cancer. In the data of present study revealed that in double combination of *CYP1A1m1* polymorphism and *CHRNA5* polymorphism the number of SQCC patients having heterozygous genotype have reduced in cases (44.1%) compare to controls (45.4%). Similarly, ADCC as well as SCLC patients having heterozygous genotype were less in cases (31.8%, 41.4% respectively) than in controls (45.4%). But no ADCC and SCLC patients have found to have mutant genotype. The risk involve with SQCC patients with both genetic polymorphism was observed to have no significance (OR= 1.05; 95%CI= 0.4- 2.3; p= 0.89). Likewise, ADCC patients (OR= 0.60; 95%CI= 0.2- 1.6; p= 0.31) and SCLC patients (OR= 0.91; 95%CI= 0.3-2.1; p= 0.83) having combine genetic variants of *CYP1A1* and *CHRNA5* polymorphism were observed to have no risk of lung cancer.

In another case, the double combination of *CYP1A1m2* and *CHRNA5* polymorphism, the study has observed that there were no any patients seen having mutant genotype. There was

no risk involve in ADCC (OR= 1.45; 95%CI= 0.5 -4.1; p=0.48) and SCLC (OR= 1.22; 95%CI= 0.4 -3.7; p=0.71) patients having both genotypic combination of *CYP1A1m2* and *CHRNA5* polymorphism. But in case of SQCC patients having heterozygous genotypic variant of both polymorphism were observed to have approximately three fold increase risk of lung cancer (OR= 2.87; 95%CI= 1.1 -7.1; p=0.02). According to the study, triple combination of *CYP1A1m1*, *m2* and *CHRNA5* polymorphism has observed to have more number of SQCC (26.3%), ADCC (25%) as well as SCLC (22.7%) patients having heterozygous genotype as compare to controls (22.0%). But no significance has been seen in all the histological subtypes of lung cancer having triple combination of *CYP1A1m1*, *m2* and *CHRNA5* polymorphic variations.

5.15 Association between combine genotype of CYP1A1 gene and CHRNA5 gene polymorphism and risk of lung cancer in tobacco smokers (Table: 15.23)

The study has further stratified the data of combine *CYP1A1* and *CHRNA5* gene polymorphism on the basis of smoking status of cases and controls. Study has included both smokers as well as non smokers to find out the association between genetic polymorphism of different combination of *CYP1A1* gene and *CHRNA5* gene and risk of lung cancer. The results has shown that in combination of *CHRNA5* with *CYP1A1* gene having *m1* polymorphic site has more number of smokers in cases (45.8%) having heterozygous genotype where as number has been decreased in non smokers (cases = 14.3%) compare to controls (44.3% smokers, 47.7% non smokers). It was observed that the association between genetic variants of combine polymorphism of *CYP1A1m1* and *CHRNA5* gene and increase risk of lung cancer was not at the level of significance both in smokers (OR= 1.15; 95%CI= 0.6 -2.1; p= 0.64) as well as in non-smokers (OR= 0.19; 0.03 - 1.0; p=0.05). Unlikely, in case of combination of *CYP1A1m2* and *CHRNA5* genetic polymorphism there were significant association was seen in smokers having heterozygous genotype of both polymorphism (OR= 2.2; 95%CI= 1.0 - 4.8; p=0.04) where as no association was seen in non smokers having *CYP1A1m2* and *CHRNA5* combination. On the other hand, the triple combination of *CYP1A1m1*, *m2* and *CHRNA5* genetic polymorphism has been observed to have no significant association between genetic polymorphism and increase risk of lung cancer in smokers (OR= 1.76; 95%CI= 0.6 - 4.6; p= 0.25) and non-smokers (OR= 0.65; 95%CI= 0.1 - 3.9; p=0.64).

Table: 15.22 Risk involve in different genetic combination of CYP1A1m1, m2 and CHRNA5 polymorphism with different histological subtypes of lung cancer.

CYP1A1m1 vs NA5	**Controls, (%) N=141**	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell lung carcinoma**		
		N=34 (%)	**OR[a] (95% CI)**	***p***	**N=22 (%)**	**OR[a] (95% CI)**	***p***	**N=29 (%)**	**OR[a] (95% CI)**	***p***
GG+TT	76 (53.9)	18 (52.9)	1.00 (Ref.)	Ref.	15 (68.2)	1.00 (Ref.)	Ref.	17 (58.6)	1.00 (Ref.)	Ref.
GA+TC	64 (45.4)	15 (44.1)	1.05 (0.4- 2.3)	0.89	7 (31.8)	0.60 (0.2- 1.6)	0.31	12 (41,4)	0.91 (0.3-2.1)	0.83
AA+CC	1 (0.7)	1 (2.9)	NS	NS	0 (0)	0.0 (0.0-0.0)	0.99	0 (0)	0.0 (0.0-0.0)	0.99
GA/AA+TT/TC	65 (46.1)	16 (47)	1.12 (0.5-2.4)	0.77	7 (31.8)	0.58 (0.2-1.5)	0.28	12 (41.4)	0.90 (0.3 -2.1)	0.81
CYP1A1m2 vs NA5	**Controls, (%) N=138**	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell lung carcinoma**		
		N=31 (%)	**OR[a] (95% CI)**	***p***	**N=30 (%)**	**OR[a] (95% CI)**	***p***	**N=32 (%)**	**OR[a] (95% CI)**	***p***
GG+AA	113 (81.9)	20 (64.5)	1.00 (Ref.)	Ref.	23 (76.7)	1.00 (Ref.)	Ref.	26 (81.3)	1.00 (Ref.)	Ref.
GA+AG	25 (18.1)	11 (35.5)	2.87 (1.1 -7.1)	0.02	7 (23.3)	1.45 (0.5 -4.1)	0.48	6 (18.7)	1.22 (0.4 -3.7)	0.71
AA+GG	0 (0)	0 (0)	NS	NS	0 (0)	NS	NS	0 (0)	NS	NS
GA/AA+AG/GG	25 (18.1)	11 (35.5)	2.8 (1.1 -7.1)	0.02	7 (23.3)	1.45 (0.5 -4.1)	0.48	6 (18.7)	1.22 (0.4 -3.7)	0.71
CYP1A1 vs NA5	**Controls, (%) N=91**	**Squamous cell Carcinoma**			**Adenocarcinoma**			**Small cell lung carcinoma**		
		N=19 (%)	**OR[a] (95% CI)**	***p***	**N=16 (%)**	**OR[a] (95% CI)**	***p***	**N=22 (%)**	**OR[a] (95% CI)**	***P***
0*	71 (78.0)	14 (73.7)	1.00 (Ref.)	Ref.	12 (75)	1.00 (Ref.)	Ref.	17 (77.3)	1.00 (Ref.)	Ref.
1*	20 (22.0)	5 (26.3)	1.37 (0.4 -4.5)	0.60	4 (25)	1.69 (0.4 -6.6)	0.44	5 (22.7)	1.38 (0.3 -5.1)	0.62
2*	0 (0)	0 (0)	NS	NS	0 (0)	NS	NS	0 (0)	NS	NS
3*	20 (22.0)	5 (26.3)	1.37 (0.4 -4.5)	0.60	4 (25)	1.69 (0.4 -6.6)	0.44	5 (22.7)	1.38 (0.3 -5.1)	0.62

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding p-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes. NS= Not Significant.

*** 0 –TT+AA+GG (Wild genotype*m1*, *m2* and *CHRNA5*), 1–TC+AG+GA (Heterozygous genotype, *m1*, *m2* and *CHRNA5*), 2– CC+GG++AA (Mutant genotype, *m1*, *m2*, *CHRNA5*), 3 TC+CC+AG+GG+GA+AA (Heterozygous genotype combine with mutant genotype, *m1*, *m2* and *CHRNA5*).**

Table 15.23 Interaction between risk of lung cancer and combine genetic polymorphism of CYP1A1 and CHRNA5 gene in smokers and non-smokers

SMOKING STATUS								
NA5+CYP 1A1m1	**Controls, (n%) N=97 (Smokers)**	**Cases. (n %) N= 72 (Smokers)**	**OR[a] (95% CI)**	***p*-Value**	**Controls, (n %) N=44 (Non-Smokers)**	**Cases. (n %) N= 14 (Non-Smokers)**	**OR[a] (95% CI)**	***p*-Value**
GG+TT GA+TC AA+CC GA/AA+TC/CC	54 (55.7) 43 (44.3) 0 (0) 43 (44.3)	38 (52.8) 33 (45.8) 1 (1.4) 34 (47.2)	1.00 (Ref.) 1.15 (0.6 -2.1) NS 1.19 (0.6 -2.2)	Ref. 0.64 NS 0.57	22 (50) 21 (47.7) 1 (2.3) 22 (50)	12 (85.7) 2 (14.3) 0 (0) 2 (14.3)	1.00 (Ref.) 0.19 (0.03 - 1.0) 0.0 (0.0-0.0) 0.18 (0.03 - 0.9)	Ref. 0.05 0.99 0.04
NA5+CYP 1A1m2	**Controls, (n%) N=103 (Smokers)**	**Cases. (n %) N= 70 (Smokers)**	**OR[a] (95% CI)**	***p*-Value**	**Controls, (n %) N=35 (Non-Smokers)**	**Cases. (n %) N= 24 (Non-Smokers)**	**OR[a] (95% CI)**	***p*-Value**
GG+AA GA+AG AA+GG GA/AA+AG/GG	86 (83.5) 17 (16.5) 0 (0) 17 (16.5)	51 (72.9) 19 (27.1) 0 (0) 19 (27.1)	1.00 (Ref.) **2.2 (1.0 - 4.8)** NS 2.2 (1.0 - 4.8)	Ref. **0.04** NS 0.04	27 (77.1) 8 (22.9) 0 (0) 8 (22.9)	19 (79.2) 5 (20.8) 0 (0) 5 (20.8)	1.00 (Ref.) 0.93 (0.2- 3.4) NS 0.93 (0.2- 3.4)	Ref. 0.92 NS 0.92
NA5+CYP 1A1m1+m2	**Controls, (n%) N=65 (Smokers)**	**Cases. (n %) N= 44 (Smokers)**	**OR[a] (95% CI)**	***p*-Value**	**Controls, (n %) N=26 (Non-Smokers)**	**Cases. (n %) N= 13 (Non-Smokers)**	**OR[a] (95% CI)**	***p*-Value**
0 1 2 3	51 (78.5) 14 (21.5) 0 (0) 14 (21.5)	32 (72.7) 12 (27.3) 0 (0) 12 (27.3)	1.00 (Ref.) 1.76 (0.6 - 4.6) NS 1.76(0.6 - 4.6)	Ref. 0.25 NS 0.25	20 (76.9) 6 (23.1) 0 (0) 6 (23.1)	11 (84.6) 2 (15.4) 0 (0) 2 (15.4)	1.00 (Ref.) 0.65 (0.1 - 3.9) NS 0.65 (0.1 - 3.9)	Ref. 0.64 NS 0.64

[a]Adjusted odds ratio, 95% confidence intervals and their corresponding *p*-values were calculated by unconditional logistic analysis after adjusting for age, gender, smoking status and histological subtypes. NS= Not Significant.

*** 0 –TT+AA+GG (Wild genotype*m1,m2 and CHRNA5*), 1–TC+AG+GA (Heterozygous genotype, *m1,m2 and CHRNA5*), 2– CC+GG++AA (Mutant genotype, *m1,m2, CHRNA5*), 3 TC+CC+AG+GG+GA+AA (Heterozygous genotype combine with mutant genotype, *m1,m2 and CHRNA5*).**

CHAPTER 6

DISCUSSION

6. Discussion

Lung cancer initiation can be mediated by exposure to carcinogens such as PAH, and aromatic amines, etc. present in cigarette or beedi smoke. However, these pro-carcinogens have to be activated by metabolic enzymes coded by family of *CYP1A1*gene (Nebert and Gonzalez, 1987). *CYP1A1* gene is polymorphic in nature and might play an important role in lung carcinogenesis and hence lung cancer. Several studies have been conducted both in Asian and Caucasian populations to access whether genetic variability in *CYP1A1* gene plays an important role towards susceptibility for lung cancer risk (Houlston *et al*., 2000; Shia *et al*., 2007). These studies have shown the different associations between the genotypes of *CYP1A1* polymorphism and risk for lung cancer. However, the results so far have been inconsistent between different populations and also within the same populations. This discrepancy might be due to varying factors like the studies were carried out in different ethnic populations with different heredity backgrounds, different life style or habits like smoking etc. The different histological subtypes of lung cancer might also be a reason for inconsistency in the results.

In our study we have observed a significant difference in distribution of the frequency of the mutant genotype for the *CYP1A1m1*genotype between the cases and controls. Furthermore, our data clearly demonstrates a significant association between the *CYP1A1m1*mutantgenotype and lung cancer risk and thisbecame more pronounced in SQCC. The present findings are consistentwith previous studies conducted in various ethnic groups. Studies conducted in North Indian populations have shown an elevated risk of lung cancer between *CYP1A1m1*(TC) variant allele (Kumar *et al*., 2009; Shaffi *et al*., 2009; Sobti *et al*., 2004). Similarly Sreeja*et al*., 2005 reported a high frequency of variant *CYP1A1m1* genotype with risk for lung cancer in South Indian population. Thus, the data from this study revealed an association between genetic polymorphisms in the *CYP1A1* locus and lung cancer risk among Indians. Also previous studies conducted in different Asian populations such as Chinese (Song *et al*., 2001) Japanese (Hayashi *et al*., 1992; Nakachi *et al*., 1991; 1993), Mongolians (Jiang*et al*., 2014) and Koreans (Hong *et al*. 1998) exhibited a positive association between *m1* polymorphic site and lung cancer risk. However on the contrary, some studies in Asian populations have reported a lack of association between *CYP1A1m1* genotype and lung cancer risk (Kihara *et al*., 1995; Kiyohara *et al*., 2003; Qi *et al*., 2007; Tao *et al*., 2007). The results presented in the current study are inconsistent with those reported by

the Caucasians. This might be attributed of very low frequency of the variant allele *CYP1A1* gene (Bouchardy*et al*., 2001;Cote etal., 2007; Hirvonen *et al*., 1992; Tefre *et al*., 1991; Vineis*et al*., 2002;).

In contrast to the significant association of *CYP1A1*mutant *m2*genotype with lung cancer in Asians (Dong *et al*., 2004;Raimondi *et al*., 2005; Xue *et al*., 2001), no significant association of was observed with mutant genotype of *CYP1A1m2* and lung cancer risk in the present study. It has been reported that the frequency of the mutant (*Val/Val*) genotype is highly represented in Japanese and Chinese as compared to Indian population (Zhan *et al*., 2011). In our study we report that the heterozygous genotype for *CYP1A1m2* (*Ile/Val*) to be positively associated with lung cancer risk. The data is consistent with other Indian studies who also have reported an association for the heterozygous genotype of *CYP1A1m2* gene towards risk for lung and also studied in Egyptian population (Hussein *et al*., 2014; Shaffi*et al*., 2009; Shah *et al*., 2008; Sreeja *et al*., 2005).However, Sobti *et al*., (2004) reported a high frequency of mutant genotype. It has to be noted that in Caucasians the frequency of the mutant genotype for *CYP1A1m2* is less represented as compared to East Asians (Wright *et al*., 2009).

Plethora of data points out to the fact that since lung cancer histologically is heterogeneous in nature it is imperative that the different pathological sub-types of lung cancer should be studied separately in relation to the polymorphisms present within in the *CYP1A1* gene. When lung cancer was sub-grouped according to histology, it was observed that *CYP1A1m1*variant polymorphism was found to be significantly associated with SQCC and not ADCC or SCLC.Similarly our data also suggests a significant role of *CYP1A1m2* heterozygous genotype towards risk for SQCC. The results are consistent with the results obtained by previous studies (Le Marchand *et al*., 2003; Lin *et al*., 2014; Motovali-Bashi *et al*., 2012; Shaffi*et al*., 2009; Song *et al*., 1999) who found significant association between *CYP1A1* polymorphisms and elevated risk for SQCC type of lung cancer but not with ADCC type.No significant association was found between the *CYP1A1* polymorphisms andSCLC.Some previous studies expressed that there was no significant risk for SQCC and*CYP1A1m1* polymorphism in Caucasian populations. In contrast,another study exhibited the *m1* polymorphism was associatedwith ADCC (Sugimura *et al*., 1998). A study conducted in Spanish populationrevealed that *m2* polymorphism contributes to increase risk for SQCCand SCLC susceptibility but not for ADCC(Jose *et al*., 2010; Lopez-Cima *et al*., 2012).

Our study has also evaluated the combined effect of both the polymorphic variants of the *CYP1A1m1* and *m2* genotypes towards susceptibility for lung cancer. From our current study we infer that individuals who carried the variant alleles of both the *m1* and *m2* genotype were found to be more susceptible towards lung cancer and this risk was found to be 2-fold in cases of SQCC. Our data is consistent with other studies done (Hung *et al*., 2003;Mota *et al*., 2010; Quionenes *et al*., 2001). Since *CYP1A1* is a key enzyme involved in xenobiotic metabolism, evidence from expression studies have conferred that the variant allele of the *CYP1A1m1* site might affect the inducibility of the enzyme by affecting the transcriptional control elements. Furthermore the *m2* polymorphism might result in increased activity of the enzyme because of substitution of the isoleucine to Valine in heme binding region, which then enhances the enzyme activity (Zhang *et al*., 1996). Thus individuals having more risk alleles, therefore have more ability to convert these pro-carcinogen into carcinogens(He *et al*., 2013).

Cigarette smoke contains highly carcinogenic compounds like PAH such as benzo(a)pyrene etc., and is regarded as a major cause for lung cancer as these PAHs once acted by AHH forms PAH metabolites which act as strong carcinogens and are responsible for countless mutations in the lung (Bartsch *et al*., 2000). The study performed analyses on the basis of smoking habits to determine its impact on lung cancer risk due to the presence of polymorphism. We found that*CYP1A1m1* polymorphism was significantlyrelated towards risk for lung cancer especially with individuals havingthe mutant genotype (p=0.0009) and who were smokers, whereas it was observed that thepatients who were also life time smokers and carrying the heterozygous variant allele for *CYP1A1m2*gene wasalso strongly associated towards risk for lung cancer (p<0.0001). The findings are consistent with previous conducted studies where the risk for lung cancer with variant *CYP1A1* genotypes was closely associated with smokers only (Adonis *et al*., 2005; Honma*et al*., 2009; Jin *et al* 2010; Shah *et al*., 2008; Taioli *et al*., 1998; Zhu *et al*., 2010) and not with non-smokers. Biological and epidemiological data suggest that among the smokers the different lung cancer pathologiesbe analyzed separately (Sato *et al*., 1994).When stratified on histological sub-types of lung cancer, we observed that patients who were smokers and having variant genotypes for both the *CYP1A1* polymorphic sites were strongly associated with increased susceptibility for SQCC. No association was observed for non-smokers and SQCC. On the contrary we observed that patients with the either the mutant *CYP1A1m1* or heterozygous *m2* genotype who were life-time non-smokers were at the highest risk for developing ADCC. It may be

plausible that there might be other carcinogenic factors that are involved in development of ADCC among non-smokers which probably may also be substrates for *CYP1A1*. The findings were in line as compare with the Taiwanese population (Lin *et al*., 2000) where it was observed that elevated risk for SQCC and not ADCC was significantly associated with polymorphisms in the *CYP1A1* and epoxide hydroxylase genes. Our current findings corroborate previous studies (Munish *et al*., 2009; Shaffi *et al*., 2009; Sobti *et al*., 2004; Song *et al*., 2001). Their data supports the evidence that *CYP1A1* polymorphisms are important determinants in susceptibility to tobacco induced lung cancer. We also stratified the smoking status on the basis of pack years which included both light smokers (PY≤25) and heavy smokers (PY>25). We observed that there was five-foldriskforlung cancer for individuals who were heavy smokers and carrying the homozygous mutant genotype in case of*CYP1A1m1* polymorphism, whereas it was seen that heavy smokers who were carrying the heterozygous variant allele for*CYP1A1m2*sitehad a three-fold increase risk for lung cancer which was significant (p=0.0008). We also observed that heavy smokers with either the mutant *m1* or the *Ile/Valm2* genotype were strongly associated towards risk for SQCC as compared to ADCC or SCLC. These findings are in agreement with the studies done by Song *et al*.,1999 and Dong *et al*., 2004 in Chinese population. However Sugimura *et al*. (1995) reported that light smokers were atan increase for lung cancer risk with the mutant *m2* genotype of the *CYP1A1* gene. SimilarlyIchibe *et al*.,1997conferred thatat low smoking levels, the *CYP1A1m1* and *m2*genetic polymorphisms confer susceptibility to lung cancer.Garcia-Closas *et al*. (1997) could not find substantial evidence of the effect of tobacco pack-years towards lung cancer risk by the *CYP1A1m1* genotypes.

In combined site of *m1* and *m2* polymorphism increase risk of lung cancer was present in heterozygous allelic variants in case of heavy smokers only whereas no association was observed in light smokers. This result was seen consistent with Kashmiri population (Shaffi *et al*., 2009). It was also observed that patients who were heavy smokers and carrying the variant alleles either for *m1* or *m2*wereat higher risk to develop SQCC than light smokers. The limitation of our study was sample size, no matter it was larger than the previous studies that have been done on Indian population but the number of subjects in some of the subcategories was rather small which can be considered as a drawback of study. Secondly,findings from the present study were only from a North Indian population, so it is uncertain whether these results are generalizableto the general population of other regional areas of India.Thirdly the controls in our study were recruited from health centre that might

inherit limitations that introduced selection bias as compare to population based studies.However, potential confounding bias might have been minimized by matching the controls to the cases on age, sex, area of residence and byfurther adjustment for the confounding factors in data analysis.

Different studies have suggested that the α5 subunit of nAChRs coded by *CHRNA5* gene is predominantly expressed in both NSCLC and SCLC type of lung cancer. This has been postulated that, *CHRNA5* may have direct role in lung cancer. Moreover, smokers and non smokers also expressed different levels of *CHRNA5* subunits (Kummer *et al*., 2008).

Studies have been shown that mis-sense G>A mutations in *CHRNA5* (rs16969968) appeared to have association with carcinogenic pathways in lungs and air ways tissues. This mutation modulated the calcium permeability of nAChRs and also affects the nicotine dependence in brain. Due to this the influx of excessive Ca2+ ions supposed to transmit the signal to activate the cell proliferation cascade and suppression of apoptotic pathway. It may also facilitate the neoplastic transformation (Amos *et al*., 2008). Therefore if we assume that *CHRNA5* protein subunit generally transmit a signal of nicotine binding on nAChRs in tobacco/ cigarette smokers and this SNP in *CHRNA5* supposed to associate with lung cancer risk especially in smokers. Various studies have been done to find out the relationship between genetic variants of *CHRNA5* polymorphism and risk of lung cancer in different ethnic population (Cattaneo *et al* 1997). Although, CHRNA 5 expressed in lung cancer tissue of all type of population but its association towards increase risk of lung cancer may vary population to population. The various factors have been involved in this variation like age, sex, smoking, hereditary back ground etc.

The present study has been done to find out interaction between the SNP of *CHRNA5* (rs16969968) gene and lung cancer risk in North Indian population. The results observed are found to be inconsistent with various studies done on Asian and Caucasian population (Sa, M., *et al*. 2012; Li *et al*., 2010; Spitz *et al*., 2008). The studied data has revealed that the frequency of mutant allele in North Indian population was low as compare to wild genotype. Whereas, heterozygous genotype is predominantly expressed in this population. This may be because of the unique ancestry and high genetic differentiation has been seen in Indian sub continental due to racial admixture (Reich *et al*. 2009). The study has shown no significant relationship between genotype of *CHRNA5* polymorphism and risk of lung cancer. This result has shown consistency with study done on Bangladeshi population which has also observed

to have similar type of racial admixture and genetic diversity (Sa, M., *et al* 2012). It has also been found that SNP rs16969968 of *CHRNA5* was express rare in Asian population (Truong *et al*., 2010). On the contrary, in Caucasian population *CHRNA5* polymorphism has been observed to have strong association towards the risk of lung cancer. Bierut *et al*., 2008 has shown that the frequency of *CHRNA5* polymorphism was 0% in Africans and 37% in European population which has showed inconsistency with the result of present study (Bierut et at., 2008). A study on African-American population have also seen the strong association of *CHRNA5* polymorphism toward lung cancer which is contradict the result observed in present study on North Indian population (Walsh *et al*., 2012).

Some findings have also observed that nAChRs may implicated in the growth regulation of lung cancer because it has been given that nicotine might stimulate the DNA synthesis of different histological cell lines of human lung cancer (Manechjee *et al*., 1990). Different findings have been studied to find out the involvement of *CHRNA5* SNP in occurrence of SCLC and NSCLC type of lung cancer (Singh *et al*., 2011). It was observed that the association of genetic variants of *CHRNA5* polymorphism towards the risk of different histological subtypes of lung cancer has shown no significant relationship in North Indian population. Very few studies have given the data of *CHRNA5* polymorphism risk related with different histological subtypes. One of the studies on Japanese population has given the consistent results with no significant relationship of polymorphism towards the lung cancer risk in different histological subtypes (Ito *et al*., 2012). These findings have also suggested that there has been no study reported on the Indian sub continental population to investigate the risk associated in increase of lung cancer due to *CHRNA5* polymorphism except one that has been done on Gujarati Indian in USA which observed to have consistent data with the present study. Unlikely, the Caucasians have shown significant effect of SNP in increase of different histological subtype of lung cancer (Bierut *et al*., 2010; Thorgeirrson *et al*. 2008)

Nicotine and its derivatives are components of tobacco plant and excessively present in cigarette smoking. Therefore, it has been postulated that nAChRs may highly expressed in the lungs of smokers (Lindstrom *et al*., 2003). This principle may increase the doubt on the expression of nAChRs in smokers and non smokers that whether the expression pattern of nAChRs is different in smokers compare to non-smokers or not (Steven *et al*., 2008; Sun *et al*., 2007). This contradiction has supported the findings that nAChRs play an important role in lung cancer risk especially in smokers. Most of the studies have given the contradictive results in smokers having *CHRNA5* polymorphism. The study has given that In case of North

Indians, it has been seen that there was no significant level of genetic distribution of *CHRNA5* polymorphic gene in acquiring the disease in smokers. Similarly, the studies on Asian population like in Chinese and Japanese population have also observed no significance of the polymorphism in risk of lung cancer in smokers (Ito *et al*., 2012; Munfo *et al*., 2012 Sherva *et al*., 2008; Wu *et al*., 2009). But in case of Caucasians particularly in Europeans, UK and African Americans have observed to have strong association between genetic polymorphism of *CHRNA5* and lung cancer risk in case of smokers (Li *et al*., 2010; Lips *et al*., 2010; Macqueen *et al*., 2014; Ware *et al*.,2011) . The present study has also showed that the relationship between *CHRNA5* polymorphism and lung cancer risk was not significant even in case of non-smokers. Further, data has also shown the stratification of smokers and non smokers on the basis of histological subtypes of lung cancer to reveal the effect of smoking on SQCC, ADCC and SCLC lung cancer patients. The study has observed no significance in the genetic variant of *CHRNA5* polymorphism on increase in lung cancer risk.

Some findings have also given that the exposure period of nicotine may also get effected by the polymorphism in *CHRNA5* gene (Lee *et al*., 2011). To find out this effect in North Indian population, the present study has stratified data on the basis of pack years and classified the cases and controls on the basis of light and heavy smokers. The data has revealed that there has been no significant association between genetic variant of *CHRNA5* polymorphism and lung cancer risk in light smokers as well as in heavy smokers. It has been shown that the pack years are imperfect measurement of smoking status, therefore, it has no significant relationship between the polymorphism and lung cancer risk. Chanock *et al*., 2008 has given that in Asian population, smokers were not significantly affected by lung cancer having polymorphic gene of *CHRNA5* (Marchand *et al*., 2008; Wessennar *et al*., 2011). But the results remain contradictive in relation to other population like Europeans and Koreans (Woja- Krawczyk *et al*., 2012; Li *et al*., 2010).

CHAPTER 7

CONCLUSION

7. Conclusion

In conclusion, our study demonstrated that *CYP1A1* (rs4646903) and (rs1048943) polymorphism play an important factor contributing to increased susceptibility and pathological development of lung cancer in North Indian population.

On the other hand *CHRNA5* (rs16969968) polymorphism does not give their significant contribution to increase susceptibility and development of lung cancer. Even the combination of *CHRNA5* (rs16969968) polymorphism with *CYP1A1* (rs4646903) and (rs1048943) polymorphism does not have much important role in risk of lung cancer except with *CYP1A1*(rs1048943) in SQCC as well as in smokers.

Validation of these findings with functional evaluation and larger studies with more rigorous study designs are needed. Studies are also required to assess the carcinogenesis role of passive smoking, diet and cooking, pollution, occupational exposure, other environmental factors, in addition to genetic factors, so to establish a risk profile for each individual or sub-group in affirmation of the prevention scope.

CHAPTER 8

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8. References

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APPENDIX-1

1. 0.5M EDTA: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionised water, and then adjusted the pH to 8.0 by 1 M sodium hydroxide. Sterilized the solution by autoclaving.
2. 10% SDS: Dissolved 1g of SDS in 10ml of deionised water.
3. 100mM Tris-Cl (pH 8.0): Dissolved 0.32g of Tris-Cl in 10 ml of deionised water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilized the solution by autoclaving.
4. 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilized the solution by autoclaving.
5. 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionised sterile water and kept at 4 C overnight.
6. 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionised water. Sterilized the solution by autoclaving.
7. 5X TBE buffer: Dissolved 54g of Tris base and 27.5g of boric acid in 980ml of double distilled water and then added 20ml of 0.5 EDTA. Sterilized the solution by autoclaving.
8. Ethidium Bromide (10mg/ml): Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.
9. Magnesium chloride (MgCl2) (100mM): Dissolved 0.41gms of MgCl2 in 20ml of deionised water and sterilized by autoclaving.
10. Sucrose (1M): Dissolved 3.41 g of sucrose in 10 ml of deionised water and sterilized by autoclaving.
11. TE buffer (pH 8.0): Added 1ml of 100mM Tris-Cl (pH 8.0) and 200 µl of 0.5M EDTA solution to 8.8 ml of deioinsed water. Sterilized the solution.
12. Triton X- 100 (10%): Took 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.