GENETIC VARIATION IN *CYP1A1* GENE TOWARDS RISK OF OCCURRENCE OF LUNG CARCINOMA IN NORTH INDIAN POPULATION

A

Dissertation submitted

in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

IN

MICROBIOLOGY

BY

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CERTIFICATE

This is to certify that the dissertation entitled **"Genetic variation in *CYP1A1* gene towards risk of occurrence of lung carcinoma in North Indian population"** being submitted by Ms Nerrittee (Roll no. 301105009) in partial fulfillment of the requirements for the award of degree of Master of Science in Microbiology, Thapar University, Patiala is a bonafide work carried out under the esteemed supervision and conception of Dr. Siddharth Sharma and that no part of this dissertation has been submitted for the award of any other degree.

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CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the dissertation entitled **"Genetic variation in *CYP1A1* gene towards risk of occurrence of lung carcinoma in North Indian population"** in partial fulfillment of the requirements for the award of degree of Masters in Microbiology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala is my own laboratory work during the period of January 2013 to June 2013, under the conception and supervision of Dr. Siddharth Sharma, Assistant University Professor, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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ABSTRACT

Lung cancer is the most common cause of death throughout the world with cigarette smoking being established as the major etiological factor in lung cancer. The *CYP1A1* category of enzymes plays a central role in the metabolic activation of major tobacco carcinogens. Several polymorphisms within the *CYP1A1* locus have been identified and have been shown to be associated with lung cancer risk, particularly in Asian populations. Here we focused on the influence of one polymorphism on lung cancer in ethnic North Indian Population, genotyping 84 lung cancer cases and 86 healthy controls by PCR-RFLP methods. Allele frequency of *CYP1A1*2A/*2A* were 16.66% in cases and 5.81% in controls. The *CYP1A1*2A/*2A* variant was significantly associated with lung cancer susceptibility (OR=3.818, 95% CI= 1.215-11.650, p-value= **0.014).** This risk was prominent in case of SQCC compared with AC or other types of lung cancer (OR=5.25, 95% CI=1.413-19.505, *p*-value= **0.008**). Stratified analysis showed a significant association between tobacco smoking and variant *CYP1A1M1* genotype on the risk of SQCC. Among smokers those carrying *CYP1A1*2A/*2A* genotype had almost five fold increase in lung cancer. Smokers having with *CYP1A1*2A/*2A* mutant genotype had 7 fold elevated risk of SQCC (OR=7, 95% CI=0.743-65.943, *p-value* = 0.0614). Our findings thus support the conclusion that *CYP1A1*2A/*2A* polymorphism is associated with the smoking related lung cancer risk in North Indian population. Also concluding that the *CYP1A1*2A* mutant allele is an important risk factor of lung cancer especially for SQCC.

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LIST OF ABBREVATIONS

Abbreviations	*Description*
AC	Adenocarcinoma
AHH	Aryl hydrocarbon hydroxylase
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon nuclear translocator
CI	Confidence interval
CYP1A1	Cytochrome P450 1A1
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra cyclic acetic acid
IARC	International Agency of Research on Cancer
NNK	4- methyl nitroso-amino-1,3-pyridyl-1-butanone
NNN	Nitrosonornicotine
NSCLC	Non small cell lung carcinoma
PAHs	Polycyclic aromatic hydrocarbons
OR	Odds ratio
PCR	Polymerase chain reaction
RFPL	Restriction fragment length polymorphism
SQCC	Squamous cell carcinoma
SCLC	Small cell lung carcinoma
TBE	Tris Borate EDTA
TE	Tris-Cl EDTA
XRE	Xenobiotic response element

CHAPTER-1

INTRODUCTION

Cancer is the most lethal disease of all known diseases. It is mainly characterized by uncontrolled cell division and growth, resulting in the formation of a mass of tissue known as a "tumor". Cancer is majorly attributed to mutations in DNA or damage to the genes involved in cell division, carcinogen metabolism. Lung cancer is the most common cancer after prostate gland cancer and breast cancer worldwide. Cigarette smoking is the strongest established risk factor for lung cancer, but genetically determined variations in metabolism of tobacco derived carcinogens may affect individual susceptibility to lung cancer. However, although risk of lung cancer has been exclusively associated with tobacco smoking, but fewer than 20% of smokers develop the disease (Carlsten *et al.*, 2008), indicating that there may be important genetic components involved in the etiology; which makes identification of genetic susceptibility factors for lung cancer important in order to understand and prevent occurrence of the disease.

Tobacco, asbestos and radon are the major causes of lung cancer. Human body has an inbuilt mechanism of detoxification for such carcinogens to prevent cancer. Xenobiotics metabolizing enzymes are responsible for metabolism of many exogenous chemicals that are toxic, mutagenic or carcinogenic. Carcinogen detoxifying enzymes include the phase I enzymes involved in the detoxification of carcinogens and either neutralize them or change them into electrophilic compounds that are detoxified by the phase II enzymes (Rajani *et al.*, 2003).

The principal enzymes responsible for phase I reaction belong to cytochrome P-450 multigene family. The cytochrome P450 1A1 enzyme functions by the addition of oxygen atom into the toxic chemical and initiate detoxification and elimination by increasing hydrophilicity (Guengerich and Shimada 1991). Cytochrome P450 1A1 gene (*CYP1A1*) is located on chromosome 15q22-24 and encodes aromatic hydrocarbon hydroxylase that converts polycyclic aromatic hydrocarbons (PAHs) (Shimada *et al.*, 1989) to carcinogen and is predominantly expressed in extra hepatic tissues including lungs (Anttila *et al.*, 1992).

Several studies have suggested that genetic polymorphism in genes controlling carcinogen metabolism underlie individual variation in cancer susceptibility (Nebert *et al*., 1996; Gonzalez *et al*., 1997). Several metabolic enzymes have been investigated for their possible role in lung cancer susceptibility, including members of CYP450 super family. As phase 1 enzymes, these catalyze one of the first steps in the metabolism of carcinogens. This oxidizing step often creates more reactive intermediates that are capable of binding with DNA and causing genetic mutations (Gonzalez *et al*., 1994; Bartsch *et al*., 2000). The Cyp super family of enzymes are the primary agents involved in oxidizing carcinogens found in tobacco smoke, like polycyclic aromatic hydrocarbons (PAHs), nitrosamines and aryl amines (Spivack *et al*, 2001; Hukkanen *et al*., 2002).

CYP1A1 polymorphisms were the first in the CYP genes to be associated with lung cancer. Several important single nucleotide polymorphisms have been identified in the *CYP1A1* locus. *CYP1A1* genes contain 7 exons and have been localized to human chromosome no.15 (15q22). The *CYP1A1*2A* allele has a T-C mutation in 3'noncoding region, which has been associated with elevated enzyme activity (Petersen *et al*., 1991; Crofts *et al*., 1994; Kiyohara *et al*., 1994; Landi *et al*., 1994). An A-G transition in exon 7 creates the second allelic variant (M2), which leads to an amino acid substitution of Val for Ile in the heme binding region and results in an increase in microsomal enzyme activity (Cosma *et al*., 1993; Crofts *et al*., 1994; Kiyohara *et al*, 1998).The variant M3 has a mutation in intron 7 (Crofts *et al*., 1993). Another polymorphism (M4), located two bases upstream of the M2 site, also causes an amino acid substitution of the Asn in heme binding region of the enzyme (Cascorbi *et al*., 1996).

The transcriptional activation of the *CYP1A1*gene is mediated by the binding of environmental pollutants and inhalation chemicals, notably substrates of the CYP1A1enzyme, to the cytosolic receptor *AhR* and is also mediated by its translocation to the nucleus and subsequent formation of a dimer, which interacts with the corresponding xenobiotic response elements to activate transcription. Increased transcription of *CYP1A1* by *AhR* leads to activation of procarcinogens into carcinogenic form, which in turn results in the formation of DNA adducts. Therefore the major objective of our study focuses on *CYP1A1*2A* polymorphism, the genetic variation of *CYP1A1* gene towards the risk of lung cancer and effect of smoking on *CYP1A1* towards the risk of lung cancer and also the clinico-pathological features associated with it.

CHAPTER- 2

LITERATURE REVIEW

LUNG CANCER

Lung cancer is the most common cause of cancer deaths worldwide. It is the 3rd most common cancer after prostate gland cancer and breast cancer. The major risk factors related with lung cancer are smoking, genetic factors, some occupational hazards like asbestos and radon.

1.1 SYMPTOMS:

Characteristic symptoms of lung cancer are:

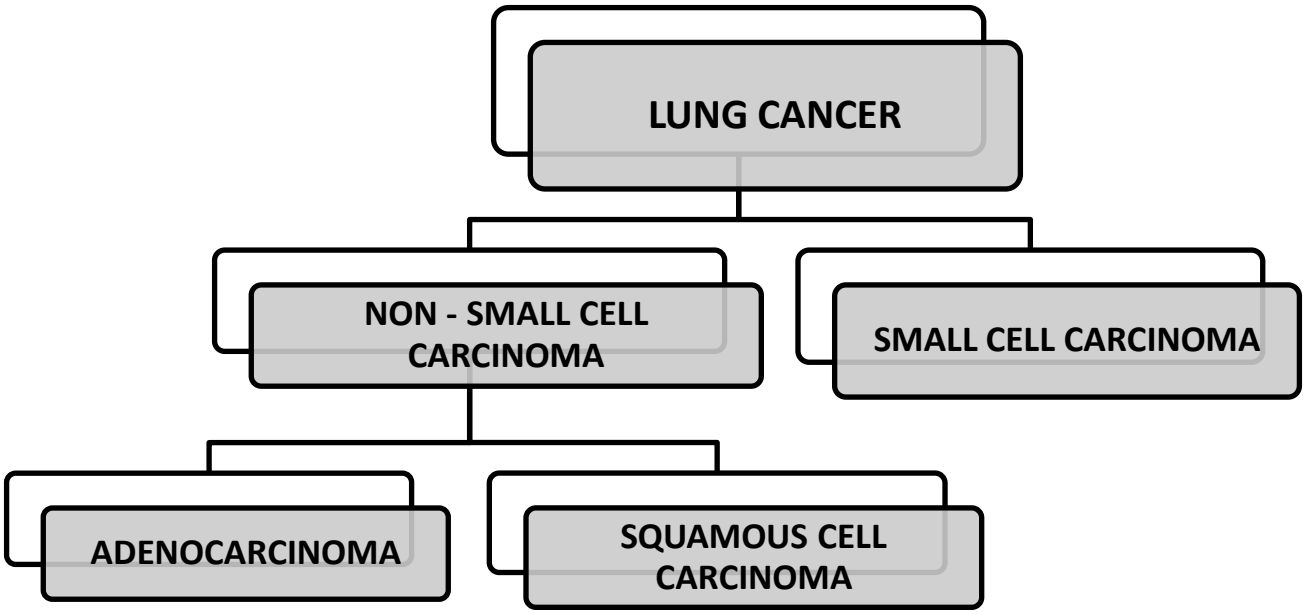
- A cough that doesn't go away and gets worse over time
- Constant chest pain
- Coughing up blood
- Shortness of breath, wheezing, or hoarseness
- Repeated problems with pneumonia or bronchitis
- Swelling of the neck and face
- Loss of appetite or weight loss
- Fatigue

1.2 DIAGNOSIS:

Lung cancer is diagnosed by microscopic examination of a piece of the abnormal mass of tissue in lungs (biopsy). Lung cancer can also be diagnosed by endoscopy. After diagnosis type and stage of cancer is determined using TNM staging. TNM staging method helps to know how extensive a cancer is best treatment for the type of cancer and predicts the average prognosis of lung cancer.

1. 3 HISTOLOGY:

Lung cancer is majorly classified into two main types:



- NSCLC accounts for about 80% of all lung cancers and spreads very slowly.
- SCLC, also known as " **oat cell carcinoma**"; the name is attributed to oat shaped cells which are smaller than normal cells due to failure of size control mechanism of the cells.
- SCLC accounts for 20% of all lung cancers and spreads quickly.

1.4 INCIDENCE:

1.4.1 WORDWIDE:

Tobacco use is responsible for five million deaths (one in ten adults) in the world every year with 2.41 million being attributed to developing countries and 2.43 million in developed countries (Thankappan & Thresia., 2007), (Ezzati & Lopez., 2003). The death toll due to lung cancer is projected to rise to ten million 2030 with 7 out of 10 deaths in the developing world (Thankappan & Thresia., 2007) (Osler M., 2001).

Estimated New Cases

Males				Females		
Prostate	35,430	37%		Breast	27,060	33%
Lung & bronchus	13,110	14%		Lung & bronchus	10,980	13%
Colon & rectum	9,280	10%		Colon & rectum	8,830	11%
Kidney	4,400	5%		Uterine corpus	5,690	7%
Liver & intrahepatic bile duct	3,270	3%		Thyroid	3,850	5%
Non-Hodgkin lymphoma	2,960	3%		Kidney	3,030	4%
Pancreas	2,670	3%		Pancreas	2,930	4%
Oral cavity & pharynx	2,460	3%		Non-Hodgkin lymphoma	2,590	3%
Urinary bladder	2,380	2%		Myeloma	2,170	3%
Leukemia	2,220	2%		Uterine cervix	2,060	3%
All Sites	**94,540**			**All Sites**	**82,080**	

Estimated Deaths

Males				Females		
Lung & bronchus	9,430	29%		Lung & bronchus	6,830	21%
Prostate	4,980	15%		Breast	6,080	19%
Colon & rectum	3,600	11%		Colon & rectum	3,250	10%
Liver & intrahepatic bile duct	2,240	7%		Pancreas	2,390	7%
Pancreas	2,110	6%		Uterine corpus	1,500	5%
Stomach	1,100	3%		Ovary	1,330	4%
Leukemia	1,040	3%		Myeloma	940	3%
Myeloma	950	3%		Leukemia	890	3%
Esophagus	900	3%		Liver & intrahepatic bile duct	830	3%
Kidney	820	2%		Stomach	760	2%
All Sites	**32,970**			**All Sites**	**31,910**	

Fig. 1: Leading Sites of New Cancer Cases and Deaths among African Americans, 2013 Estimates (Santis *et al.*, 2013)

1.4.2 INDIA:

- Relative risk of developing lung cancer in India are- (Khurana S),(Notani & Sanghavi., 1974)
  - Beedi smokers: 2.64
  - Cigarette smokers: 2.23
  - Overall: 2.45
- Incidence rates (age-standardized) per 100,000 population (2008) (SIRO, 2011).
  - Males: 10.9
  - Females: 2.5
- Increase in new cancer cases per 100,000 population each year over 24 years (till 2005) (Kirmani, Jamil, Naidu, 2010).
  - Chennai: 160%
  - Bangalore: 100%
  - Delhi: 40%
  - Mumbai: (-) 60%
- Most prevalent is NSCLC amongst lung cancers (Kenfield *et al.*, 2008)
  - Squamous cell carcinoma: 44.73%
  - Adenocarcinoma: 30.26%

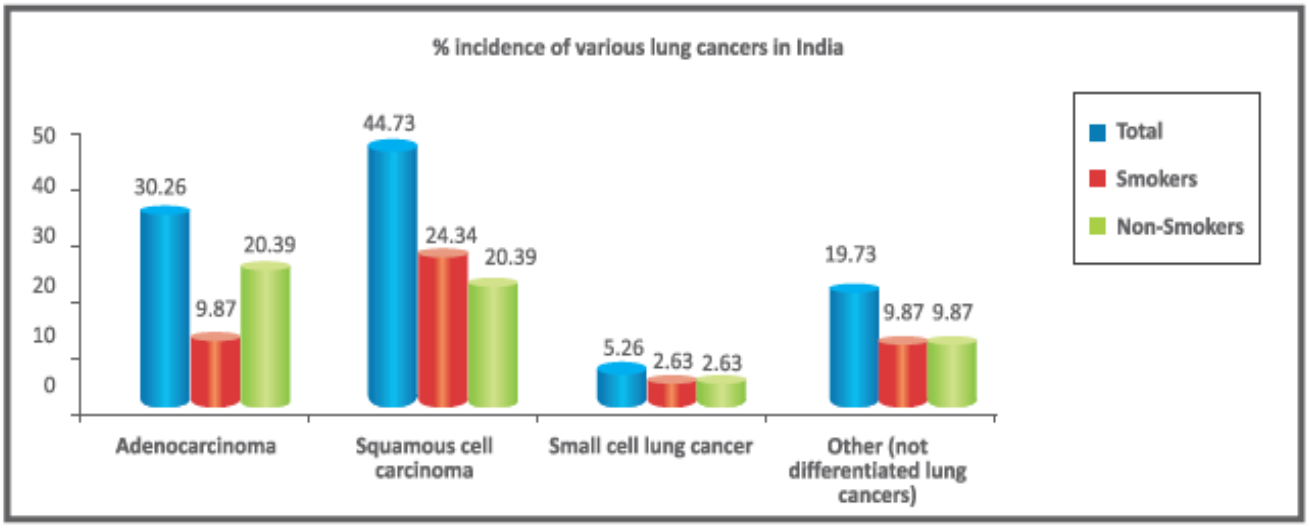
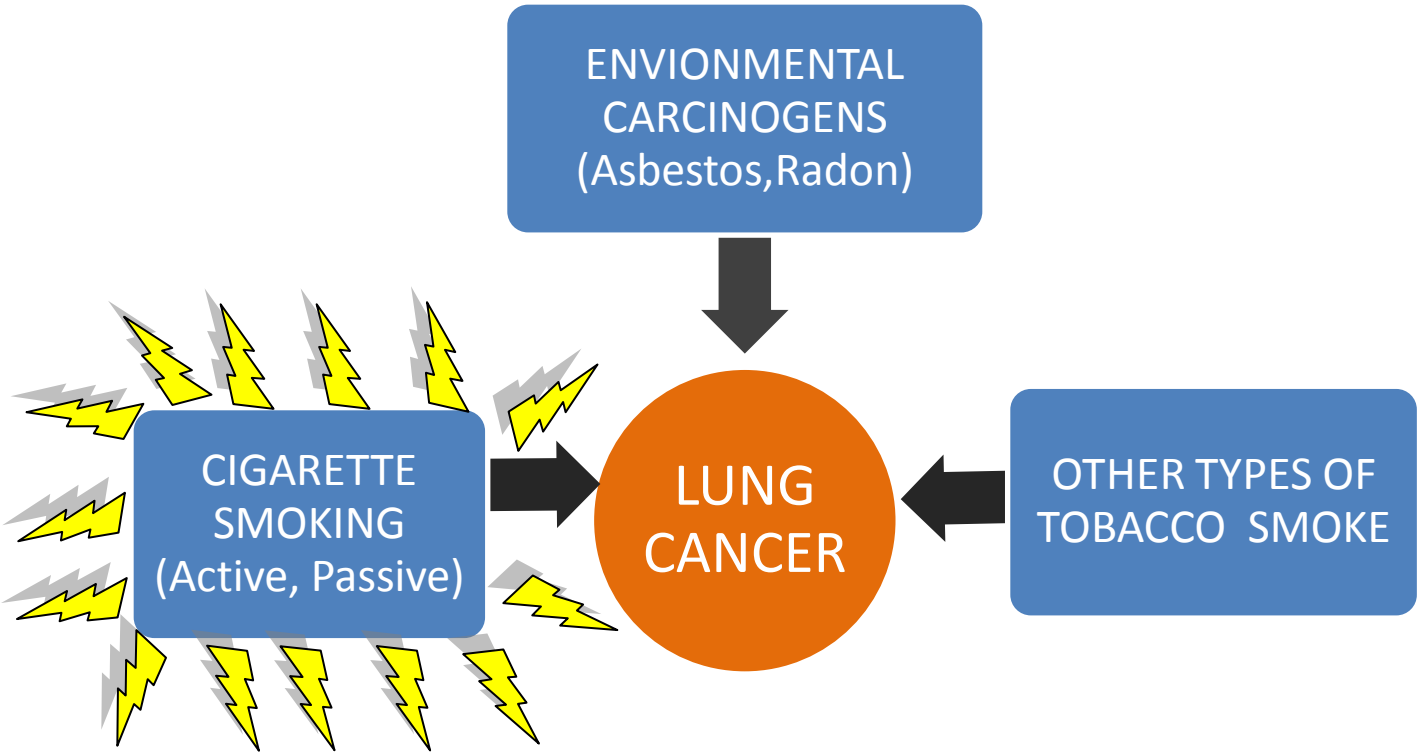


Fig. 2: Percentage incidence of various lung cancers in India

1.7 EPIDEMIOLOGY:

The major contributing factors towards the risk of lung cancer are:



Of the above mentioned risk factors, cigarette smoke is most potent in causing lung cancer. Out of the 36 carcinogenic chemicals listed by International Agency of Research on Cancer (IARC), cigarette smoke contains 10 of these compounds (IARC, 1999). Major carcinogens in cigarette smoke are:

a. Polycyclic Aromatic Hydrocarbons (PAHs) :

Polycyclic aromatic hydrocarbons are generated as a result of incomplete combustion of coal, oil, garbage etc. One of the most toxologically potent PAH is Benzopyrene. PAHs also form an important constituent of cigarette smoke.

b. Nitrosamines:

Some of the nitrosamines exist in tobacco and tobacco smoke. Nitrosamines have the ability to form DNA adducts and cause mutations. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and nitrosonornicotine (NNN) have the greatest mutagenic potential and are classified as probable human carcinogens (Hecht, 1999; IARC 1999).

c. Chlorinated Dioxins and Furans :

Dioxins are produced during incomplete combustion including cigarette smoking, vehicle emissions, industrial processes such as smelting, refining. TCDD (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin) is the most toxic and potent dioxin.

1.6 GENETIC FACTORS :

Some of the risk factors for lung cancer can cause certain changes in the DNA of lung cells. These changes can lead to abnormal cell growth and, sometimes, cancer. Some genes are responsible for giving signals that are important for controlling cell growth, division into new cells, and death. Genes that help cells grow, divide, or stay alive are called *oncogene*. Genes that slow down cell division or cause cells to die at the right time are called *tumor suppressor genes*.

Cancers can be caused by DNA changes that turn on oncogene or turn off tumor suppressor genes. Some people inherit DNA mutations (changes) from their parents that greatly increase their risk for developing certain cancers. Some people seem to inherit a reduced ability to break down or get rid of certain types of cancer-causing chemicals in the body, such as those found in tobacco smoke. This could put them at higher risk for lung cancer. Some inherit faulty DNA repair mechanisms that make it more likely they will end up with DNA changes. Acquired mutations in lung cells often result from exposure to factors in the environmental carcinogens such as tobacco smoke. Acquired changes in certain genes, such as the *TP53* or *p16* tumor suppressor genes and the *K-RAS* oncogene, are thought to be important in the development of non-small cell lung cancer (WIKIPEDIA).

1.7 THE DETOXIFICATION SYSTEM :

The human body is exposed to a wide array of xenobiotics in one's lifetime, from food components to environmental toxins to pharmaceuticals, and has developed complex enzymatic mechanisms to detoxify these substances.

1.7.1 The Phase I System:

The Phase I detoxification system, composed mainly of the cytochrome P450 supergene family of enzymes, is generally the first enzymatic defense against foreign compounds. Most pharmaceuticals are metabolized through Phase I biotransformation (Liska, 1998). Cytochrome P450 uses oxygen and

NADPH as a cofactor to add a reactive group in a typical Phase 1 reaction. This step results in the formation reactive molecules which are more toxic than the parent molecule. If these reactive molecules are not further metabolized by Phase II conjugation, they may cause damage to proteins, RNA, and DNA within the cell (Vermeulen, 1996).

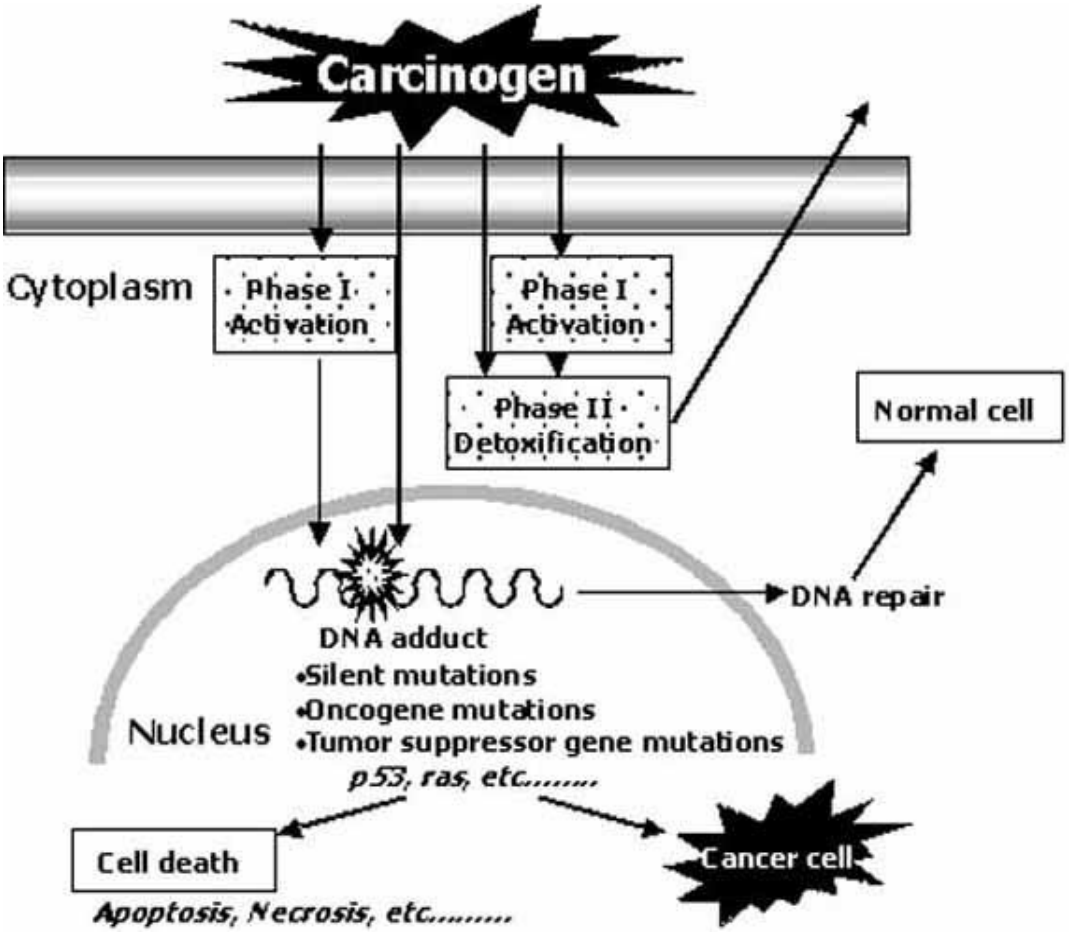


Fig. 3: Metabolic pathways of carcinogen (Kawamoto *et al.*, 2004)

1.7.2 PHASE I ENZYME (CYP1A1):

INTRODUCTION:

Cytochrome P450 constitutes the major enzyme family capable of catalyzing oxidative biotransformation of most drugs and lipophilic xenobiotics (Nelson, 2006; Guengerich, 2008; Zanger *et al.*, 2008). In humans they are classified into 18 families and 44 subfamilies. Cytochrome P450s catalyze reactions involved in biosynthesis of steroid hormones, metabolism of xenobiotics, oxidation of unsaturated fatty acids to intracellular messengers (Hasler *et al.*, 1999), out of which CYP1A1, 2 &3

families are responsible for biotransformation of majority of drugs and other xenobiotics (Zanger and Schwab., 2012) . *CYP1A1*gene contains AhR binding sites; which are involved in transcriptional activation of *CYP1A1*enzymes (Schults *et al*., 2010).

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). *CYP1A1*enzyme is primarily expressed in extra hepatic tissues. The protein encoded by *CYP1A1*gene constitutes of 512 amino acids having size of 58 kD.*CYP1A1*gene encodes an enzyme known as **A**romatic **H**ydrocarbon **H**ydroxylase (AHH). CYP1A1 enzyme is an important phase-I metabolism enzyme involved in xenobiotic metabolism. CYP1A1enzymes are also associated with induction of procarcinogens into carcinogenic compounds.

The induction of CYP1A1 expression is mediated through a specific cytosolic receptor, the Aryl hydrocarbon receptor or AhR. AhR exists as part of a cytosolic protein complex, which consists of two Hsp-90 heat-shock proteins, a Hsp-90-interacting co-chaperone p23 and an immunophillin-like protein XAP2 or AIP (Puga *et al*., 2009). In the presence of an exogenous ligand such as B[*a*]P or the industrial byproduct 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) the receptor complex translocates to the nucleus, where it heterodimerizes with another protein, the aryl hydrocarbon nuclear translocator or ARNT (Figure 4). This heterodimer binds to consensus regulatory sequences termed AhREs (Aryl hydrocarbon response elements) XREs (Xenobiotic response elements) or DREs (Dioxin response elements), located in the promoter region of AhR target genes such as CYP1A1 and CYP1A2 and initiates their transcription by recruiting RNA polymerase II (Puga *et al*., 2009). The transcription of CYP1A1 is inhibited by the AhR-related factor Aryl hydrocarbon receptor repressor or *AhRR*, which localizes in the nucleus in the form of a dimeric protein along with ARNT. The *AhRR/ARNT* heterodimer acts as a repressor both by stopping transcription initiated at the XREs and by competing with AhR for heterodimer formation with *ARNT*. All *AhR, ARNT* and *AhRR* are members of the bHLH (basic helix loop- helix) PAS (Per-ARNT-Sim) family of proteins. Heterodimerisation of *AhR/ARNT* is facilitated by interactions between bHLH and PAS domains. Further interactions of the *AhR/ ARNT* heterodimer with trascription factors such as Sp1 and NF-1 are essential to enhance the expression of the *CYP1A1* gene. Other proteins which possess HAT (Histone Acetyl trasnferase) 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).

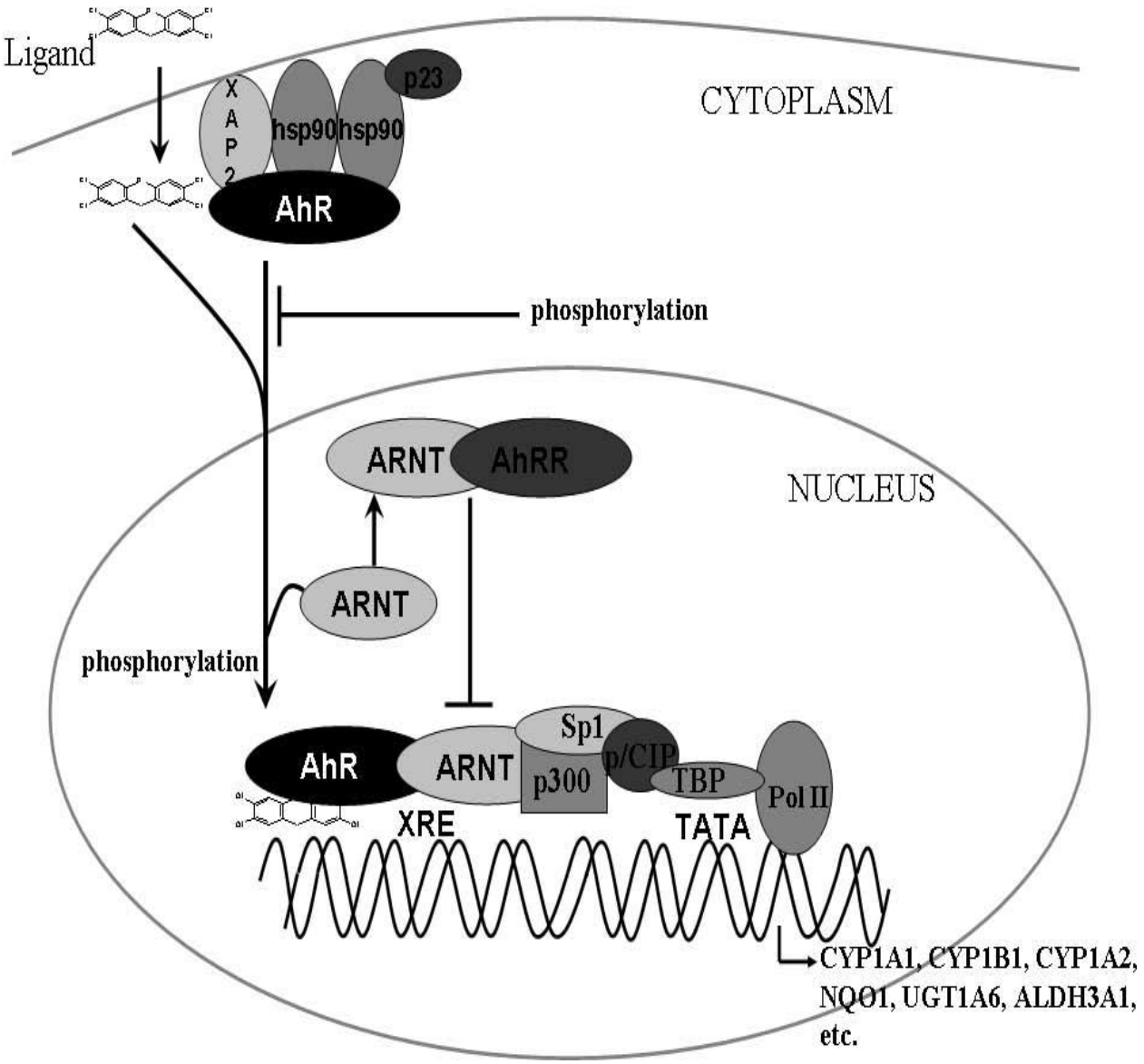


Fig. 4: AhR ligand-mediated activation of phase I and II metabolizing enzyme genes (Androutsopoulos *et al*., 2009)

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 of Benzopyrene to the ultimate carcinogen, the diol- epoxide (Androutsopoulos *et al*., 2009).

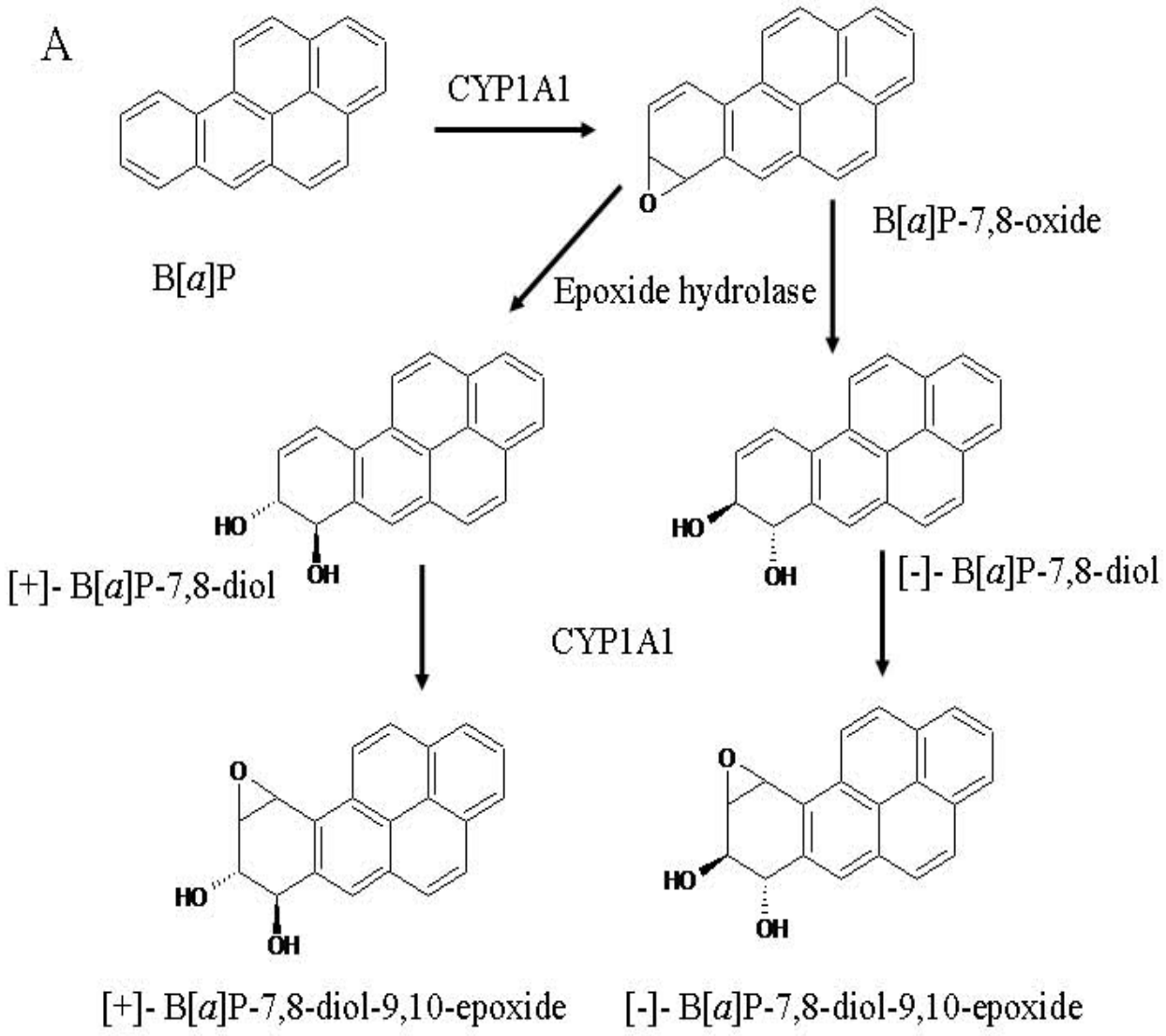


Fig. 5: Metabolic activation of Benzopyrene (Shimada *et al*., 1989)

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 CYP1A1enzyme.

2. Hydrolysis of B[a]P-7,8 oxide to B[*a*]P-7,8-diol and the two enantiomers (+)-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-epoxides by CYP1A1enzyme. These diol- epoxides are considered as the ultimate carcinogens, capable of causing oncogenic mutations in specific parts of DNA.

- **GENETIC POLYMORPHISM:**

*CYP1A1*exhibits several genetic polymorphisms out of which most studied polymorphisms are:

Table 2.1: Polymorphisms exhibited by *CYP1A1* gene. (Bartsch *et al*., 2000)

SNo.	POLYMORPHISM	SUBSTITUTION	FEATURES
1.	M1 /*CYP1A1*2*	T→C substitution at 3801 in the 3'-non-coding region downstream of the polyadenylation site. (Bale *et al* ., 1987)	*CYP1A1*2A* allele is related with increased catalytic activity and a higher inducibility by TCDD (Landi *et al*., 1994). *CYP1A1*2A* variant alleles are also known to be associated with higher levels of benzo[a] pyrene related DNA adducts in human white blood cells (Persson I, Johansson I, Ingelman-Sundberg M., 1997)
2.	M2/*CYP1A1*3*	A→G substitution at nucleotide 2455 in the hemebinding region of exon 7 induces an amino acid change of isoleucine to valine at codon 462 (Kawajiri *et al*., 1999)	Substitution leads to an amino acid change of isoleucine to valine at codon 462 and results in increase in microsomal enzyme activity (Crofts *et al*., 1994). It was also hypothesized that the substitution has an adverse effect on *CYP1A1* m-RNA stability (Alexandrov *et al*., 2002)
3.	M3/*CYP1A1*4*	T→C substitution at nucleotide 3205 in the 3'-non-coding region.	It is specific to African American population (Crofts *et al*., 1994).
4.	M4/*CYP1A1*5*	C→A substitution at nucleotide 2453.	The substitution leads to an amino acid change of threonine to asparagines at codon 461.

1.7.5 *CYP1A1* AND CANCER

1.7.5.1 *CYP1A1* AND LUNG CANCER:

Lung cancer remains the most lethal cancer worldwide, despite improvements in diagnostic and therapeutic techniques. (Alberg AJ, Samet JM., 2003). Cigarette smoking is the major cause of lung cancer, but not all smokers develop lung cancer (Alberg AJ, Brock MV, Samet JM., 2005). Many environmental carcinogens require metabolic activation by drug-metabolizing enzymes. Members of CYP450 super family have been investigated for their possible role in lung cancer susceptibility. *CYP1A1*being a phase 1 enzyme catalyze one of the first steps in the metabolism of carcinogens. This oxidizing step often creates more reactive intermediates that are capable of binding with DNA and causing genetic mutations (Gonzalez *et al*., 1994; Bartsch *et al*., 2000).

The Cyp super family of enzymes are the primary agents involved in oxidizing carcinogens found in tobacco smoke, like polycyclic aromatic hydrocarbons (PAHs), nitrosamines and aryl amines (Spivack *et al,* 2001;Hukkanen *et al*., 2002). Another study (Parag *et al*, 2008) showed that *CYP1A1*polymorphism is an important modifying factor in determining susceptibility to lung cancer significant increase in the risk in the individuals carrying variant genotypes of *CYP1A1*and GSTM1 have further provided evidence that gene–gene interaction may play an important role in the development of lung cancer. Likewise significant interactions of *CYP1A1*genotypes with tobacco, both in the form of tobacco smoking or tobacco chewing and alcohol have demonstrated the importance of gene and environment interactions in modifying the susceptibility to lung cancer.

1.5.2 *CYP1A1*POLYMORPHISM IN VARIOUS POPULATIONS:

The relationship between *CYP1A1*polymorphism and lung cancer risk in various ethnic populations have been investigated in several studies (Bartsh *et al*., 2000). In Asian populations *CYP1A1*M1 and M2 polymorphisms have generally been associated with moderate to increased risk of lung cancer compared to Caucasians and African-American, where these polymorphisms are much less common (Cosma *et al*.,1993; Garte *et al*.,1998). In Japanese and Chinese, *CYP1A1*polymorphisms have been associated with increased lung cancer risk, especially in relation to tobacco smoking (Hong *et al*., 1998; Sugimura *et al*., 1998; Bartsch *et al*., 2000; Song *et al*., 2001). The studies on North Indian populations (Sobti *et al*., 2003; 2004) have reported that CYP1A1*2A and CYP1A1*2C polymorphism is not significantly associated with lung cancer risk, though the risk was found higher in heavy smokers.

Similarly a study on South Indian population (Sreeja *et al*., 2005) reported significant association of CYP1A1*2A polymorphism with lung cancer risk.

Table 2.2: Distribution of *CYP1A1MspI* genotypes among lung cancer cases and controls (Ji *et al*., 2012)

SNO.	First author-year	Ethnicity (country of origin)	Total sample size (case/control)
1.	Kawajiri K-1990	Asia (Japan)	68/104
2.	Tefre T-1991	Caucasian (Norway)	221/212
3.	Hirvonen A-1992	Caucasian (Finnish)	87/121
4.	Shields PG-1993	Mixed populations	56/48
5.	Nakachi K-1993	Asia (Japan)	31/127
6.	Alexandrie AK-1994	Caucasian (Sweden)	296/329
7.	Kelsey K.T -1994	Mixed (African Americans)	72/97
8.	Kihara M-1995	Asia (Japan)	97/258
9.	Xu XP-1996	Caucasian (USA)	207/238
10.	Garcia-ClosaM-1997	Mixed populations	416/446
11.	Hong YS-1998	Mixed (Mexican and African)	171/295
12.	Taioli E-1998 Asia	Mixed populations	105/307
13.	Le Marchand L-1998	Mixed populations	341/456
14.	Hu YL-1999	Asia (China)	59/132
15.	Dresler CM-2000	Caucasian (USA)	158/149
16.	Song N-2001	Asia (China)	217/404
17.	Quinones L-2001	Caucasians (Chile)	60/174
18.	Yin LH-2002	Asia (China)	84/84

19.	Zhou XW-2002	Asia (China)	92/98
20.	Cai XL-2003	Asia (China)	91/138
21.	Kiyohara C-2003	Asia (Japan)	158/259
22.	Taioli E-2003	Mixed populations	109/424 MspI 110/707exon7
23.	Wang J-2003	Asia (China)	162/181
24.	Dialyna IA-2003	Caucasians (Greek)	122/178
25.	Gu YF-2004	Asia (China)	180/224
26.	Liang GY-2004	Asia (China)	152/152
27.	Chen SD-2004	Asia (China)	58/62
28.	Sobti RC-2004	Asia (India)	100/76
29.	Wenzlaff AS-2005	Caucasian (USA)	128/181
30.	Wrensch MR-2005	Mixed populations	371/944 MspI 363/930exon7
31.	Ng DP-2005	Asia (Singapore)	126/162
32.	Raimondi S-2005	Caucasians	165/519 MspI 175/723exon7
33.	Raimondi S-2005–2	Asians	46/138 MspI
34.	Sreeja L-2005	Asia (Indian)	146/146
35.	Adonis M-2005	Mixed populations	57/103
36.	Belogubova-2006	Caucasians (Russian)	141/450
37.	Pisani P-2006	Asia (Thailand)	211/408
38.	Tao WH-2007	Asia (China)	47/94
39.	Cote ML-2007	Mixed populations	354/440
40.	Xia Y-2008	Asia (China)	58/116
41.	Qi XS-2008	Asia (China)	53/72

42.	Shah PP-2008	Asia (India)	200/200
43.	Cote ML-2009	Mixed populations	502/523
44.	Honma HN-2009	Mixed populations	200/264
45.	Klinchid J-2009	Asia (Thailand)	85/82
46.	Shaffi SM-2009	Asia (India)	109/163
47.	Jin Y-2010	Asia (China)	124/154
48.	Wright CM-2010	Caucasians (Australian)	1040/784
49.	Mota P-2010	Caucasian (Portugal)	175/217
50.	Zhu XX-2011	Asian (China)	160/160

1.5.3 *CYP1A1* AND OTHER CANCERS:

*CYP1A1*M1 polymorphism is not associated with lung cancer only, rather its association with other types of cancers have been reported. *CYP1A1*M1 polymorphism is also associated with cervical cancer (Teresa *et al*., 2007), head and neck cancer (Masood *et al*., 2011), breast cancer (Li *et al*., 2004), oral and pharyngeal cancers (Varela-Lema *et al.,* 2008*)* and even with coronary heart diseases (Achour *et al*., 2011*).*

CHAPTER-3

AIM OF STUDY

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

1. The epidemiological factors associated with lung cancers in North Indian population
2. To study the genotypic frequencies of the *CYP1A1*2A* gene in lung cancer cases and controls.
3. To find a correlation between genetic polymorphisms of *CYP1A1*2A* gene towards the risk for lung cancer and also the clinico-pathological features associated with it.

CHAPTER-4

METHODOLOGY

4.1. STUDY SUBJECTS AND SAMPLE COLLECTION:

The study consists of 84 lung cancer cases and 86 cancer free controls. The cases with histologically confirmed primary lung cancer were recruited from September 2012 to May 2013 from Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, Punjab. The current study was ethically approved by the Institute ethics committee of PGIMER, Chandigarh. Written informed consent was obtained from all participants or from patients representatives if direct consent could not be obtained. Each participant was personally interviewed to obtain detailed information on demographic characteristics and lifetime history of tobacco use. 5ml of blood sample was withdrawn from each individual.

4.2. *CYP1A1*2A* GENOTYPING:

DNA ISOLATION: Genomic DNA was isolated using standard Proteinase K digestion, phenol / chloroform extraction and ethanol precipitation method from whole blood samples of both cases and controls. The constituents of washing and lysis buffer are as follows:

Table 4.1: Constituents of washing buffer

SNO.	STOCK CONCENTRATION	WORKING CONCENTRATION
1.	1M Sucrose	320mM Sucrose
2.	100% Triton X-100	1% Triton X-100
3.	100mM $MgCl_2$	5mM $MgCl_2$
4.	100mM Tris Cl (pH=8)	10mM Tris Cl (pH=8)

Table 4.2: Constituents of Lysis buffer

SNO.	STOCK CONCENTRATION	WORKING CONCENTRATION
1.	1M Tris Cl (pH=8)	400mM Tris Cl (pH=8)
2.	10% SDS	1% SDS
3.	0.5M EDTA	60mM EDTA
4.	5M NaCl	150mM NaCl
5.	10mg/ml Proteinase K	100µg/ml Proteinase K

The first step of isolation involved washing of blood with washing buffer (APPENDIX) and centrifuged it at 3500 rpm for 5 minutes. Discard the supernatant and added equal volume of washing buffer as earlier to the pellet and resuspend the pellet in the buffer and centrifuged again (repeated this step thrice). Dissolved the pellet in Lysis buffer and incubated at 44 °C overnight. Added an equal volume of Phenol: chloroform: Isoamyl alcohol (25:24:1) and mixed the contents slowly. Centrifuged at 8000 rpm for 10 minutes at 4°C. Took the upper aqueous layer and again added PCI mix and centrifuge. 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 and mixed it gently. Freeze it at -20°C for 1-2 hours. Discarded the supernatant and dissolved the pellet in TE buffer.

QUANTITATIVE AND QUALITATIVE ESTIMATION: 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 ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA. If the ratio is ~1.8 the DNA sample is considered to be pure and free from contaminations of RNA and protein. 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-stranded DNA with an A_{260} of 1.0 is 50µg/ml. DNA concentration of the solution was determined by using the formula:

$$\text{Concentration } (\mu g/ml) = A_{260} \times 50\ \mu g/ml \times \text{Dilution factor}$$

AGAROSE GEL ELECTROPHORESIS: 0.8% agarose gel containing ethidium bromide (EtBr) of concentration 0.5µg/ml was made in 0.5X TBE buffer. The gel was allowed to solidify and the comb was removed carefully. 5 X TBE buffer was poured into the electrophoretic tank so as to fully immerse the gel. The DNA samples were mixed with 6X loading dye and then loaded to the wells and were allowed to run at 50 volts. The gel was observed under UV transilluminator and the gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software.

PCR-RFLP: *CYP1A1* genotypes at M1 sites were analyzed by PCR-RFLP methods (Cascorbi *et al*, 1996; Nakachi *et al*, 1991). A DNA fragment of 899 bp was amplified in 20µL containing 400ng of genomic DNA, 0.5µmol of primers M3F (5'GGCTGAGCAATCTGACCCTA3') and P80 (5' TAGGAGTCTTGTCTCATGCCT3') (Cascorbi *et al*., 1996) (Euro fins) 0.2mM dNTPs, 1.5 mM $MgCl_2$ and 0.8 U Taq DNA polymerase. PCR was performed for 30 cycles of 5 minutes at 94°C, 30 second at 60°C, and 45 second at 72°C. The PCR product (10µL) was digested with *Msp*1 (NEB) (3U, 37°C). The restriction enzyme *Msp*1 is obtained from *Moraxella* sp. and its sequence length is 262 amino acids. *Msp*1 recognizes the double-stranded sequence 5' C|CGG 3' and cleaves after C-1 (UNIPROT). resulting in two smaller fragments (693 and 206 bp) in case of the mutant genotype, three fragments (899,693 and 206 bp) in case of heterozygous genotype, one fragment (899bp) in case of wild genotype when subjected to electrophoresis on a 2% agarose gel in 0.5X TBE buffer at 60 volts. The gel was observed under UV transilluminator and the gel imaging was performed under UV light in Bio- Rad Gel documentation System using Quantity-1-D analysis software.

STATISTICAL ANALYSIS: Pearson's ($\chi 2$) test was used to examine differences in distribution of genotypes studied between cases and controls. Tests for Hardy-Weinberg equilibrium among cases and controls were conducted using observed genotype frequencies and a $\chi 2$ test with two degree of freedom. The crude odd ratios (ORs) were calculated by Wolf's method, The Odds ratios (ORs) with 95% confidence interval (CI) calculated were computed to estimate the association between certain genotypes or tobacco smoking and disease. Smokers were considered current smokers if they smoked up to one year before the date of diagnosis for cancer or up to the date of interview for controls. Information was collected on the number of cigarettes smoked per-day, the age at which the subject

started smoking and the age at which the subject stopped smoking if the person was an ex-smoker. All the statistical analysis was performed with SNP Analyzer 2 and Med Calc (Sobti *et al*., 2003).

CHAPTER-5

RESULTS AND DISCUSSIONS

5.1 GENOMIC DNA ISOLATION:

The genomic DNA isolated from blood samples of lung cancer patients were qualitatively analyzed on 0.7% agarose gel prepared in 0.5X TBE buffer.

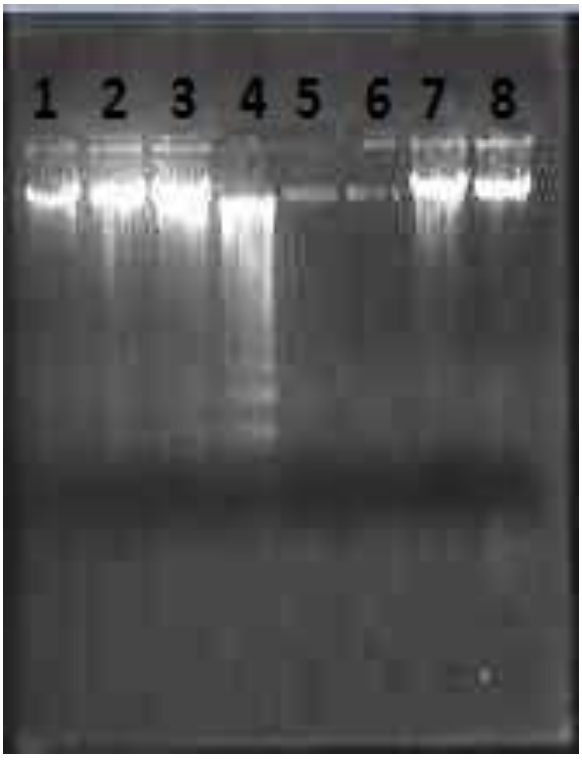


Fig. 6: Lanes 1-8 shows isolated genomic DNA on 0.7% agarose gel. 2µl of DNA was loaded in each well.

5.2 PCR AMPLIFICATION:

PCR amplified products were resolved on 1.7% agarose gel with a 100bp ladder. The size of product was 899bp.

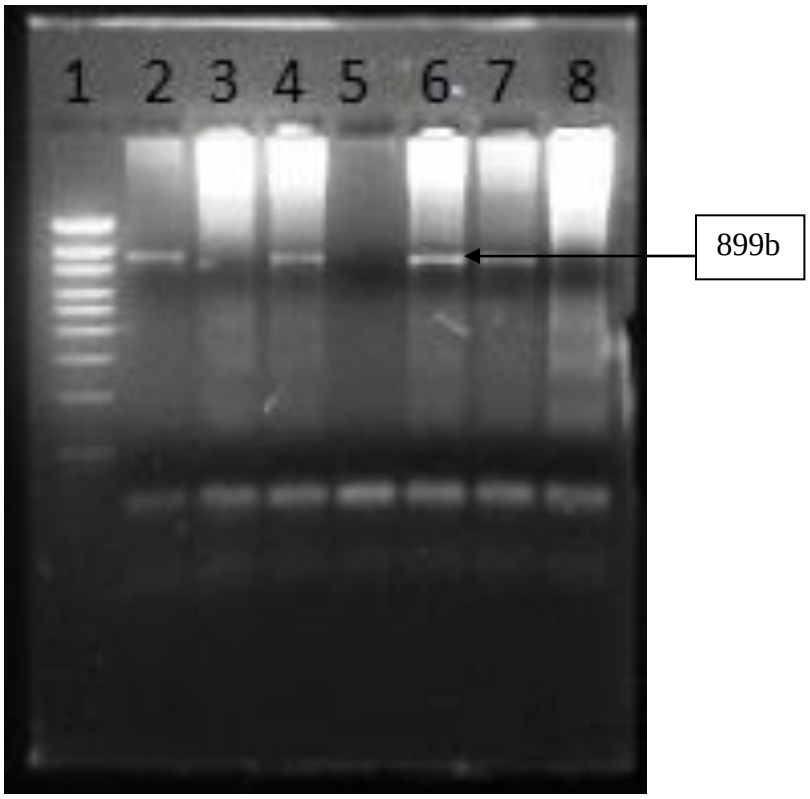


Fig. 7: Shows PCR-RFLP analysis of *CYP1A1* (*Msp1*): 100bp ladder in lane 1 and 899 bp PCR product in lanes 2-8.

5.3 Restriction Fragment Length Polymorphism:

The restriction enzyme Msp1 was used to distinguish the M1 polymorphism; gain of a Msp1restriction site occurs in the polymorphic allele, the wild type allele shows a single band representing the entire 899bp fragment, variant allele results in two fragments of 693bp and 206bp and heterozygous allele results in three fragments of 899bp, 693bp, 206bp.

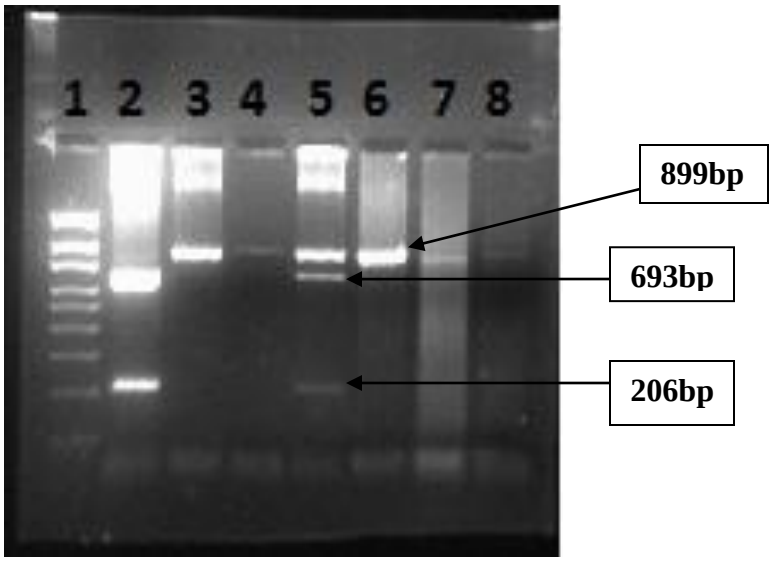


Fig. 8: *CYP1A1* (*Msp1)* genotype analysis of lung cancer controls

Lane 1= 100bp ladder (G-Biosciences)

Lane2 =Mutant genotype (693, 206bp)

Lane 3, 4, 6, 7, 8= Homozygous wild type genotype (899 bp)

Lane 5= Heterozygous genotype (899, 693, 206 bp)

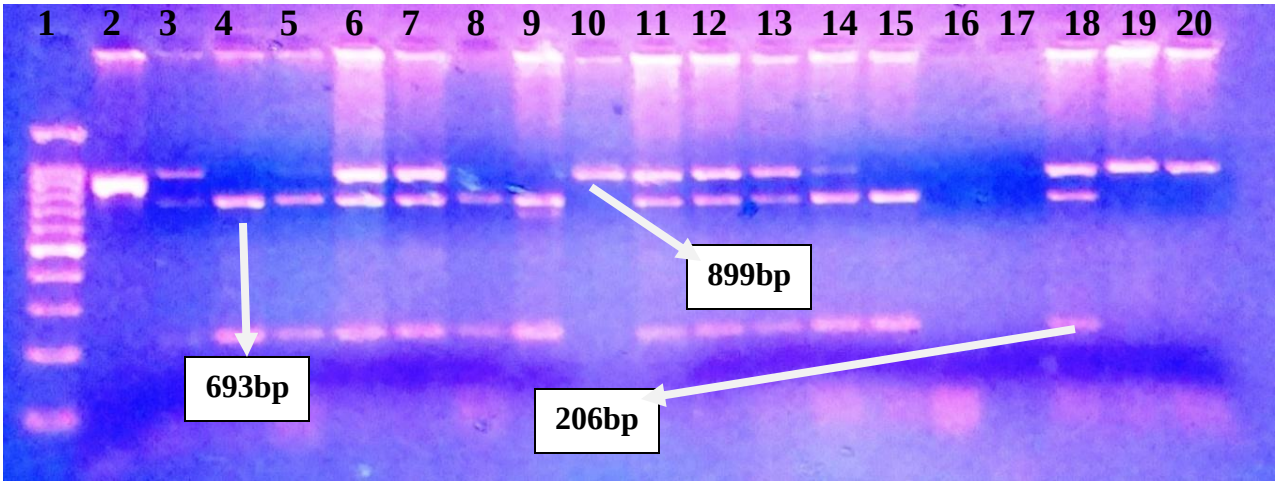


Fig. 9: *CYP1A1* (*Msp1*) genotype analysis of lung cancer cases

Lane 1= 100bp ladder (G-Biosciences)

Lane 2, 10, 16, 17, 19, 20 = Homozygous wild type genotype (899 bp)

Lane 3, 5, 6, 7, 11, 12, 13, 18 = Heterozygous genotype (899, 693, 206 bp)

Lane 4, 8, 9, 15 =Mutant genotype (693, 206bp)

5.4 Epidemiology:

The study consists of 86 cancer free controls and 84 lung cancer cases. The demographic characteristics of study subjects are shown in Table 5.1. The mean average age was 59.88 ± 9.95 years and 41.88 ± 6.29 years among cases and controls respectively. There was no significant difference in gender distribution with 11.90% females and 88% males in case group and 8.13% females, 76% males among control group. The gender specific OR is 1.726, *p-value*= 0.027, for men and OR= 1.167, *p-value*= 0.869 for women. Although an effort was made to obtain a frequency match on smoking status between cases and controls, more smokers were present in case group compared to controls (OR=2.043 , $\chi 2$=3.226, *p-value*=**0.072**) with a percentage of 83.33 % in case group and 25.58 % in control group.

Table 5.1: **Demographic Characteristics of the Study Subjects**

CHARACTERSTIC	**CASES (n%)**	**CONTROLS (n%)**	**OR(95%CI)**	***p-value***	**Chi-square**
GENDER					
MALE	73 (88.0)	76(91.86)	1.726	**0.027**	4.886
FEMALE	11 (11.90)	7 (8.13)	1.167	0.869	0.027
AGE*					
<50	15 (17.85)	57 (66.27)	1.362 (0.586-3.168)	0.472	0.518
≥50	68 (80.95)	15 (17.44)	3.193 (1.151-8.856)	**0.02**	5.379
RANGE	35-80	30-54			
Mean Age ± S.D	59.88 ± 9.95	41.88 ±6.29			
SMOKING*					
NO	14 (16.66)	64 (74.42)	1.211 (0.501-2.924)	0.671	0.181
YES	68 (80.95)	21 (25.58)	2.043 (0.928-4.499)	**0.072**	3.226
HISTOLOGICAL TYPE*					

SQCC	33 (40.47)				
AC	14 (16.66)				
SCLC	10 (11.90)				
OTHERS	4 (4.76)				
TNM STAGING					
I	1 (1.19)				
II	7 (8.33)				
III	22 (26.19)				
IV	29 (34.52)				
DATA NA	25 (29.76)				

* missing data is not available
n=total number of case patients or control subjects
Others include bronchogenic lung carcinoma
p- value <0.05
NA= not available

Table 5.1 shows that 16.66 % of the cases and 74.42 % of the controls were non smokers (OR=1.211, 95% CI, $\chi 2$=0.181, *p-value*=0.671). When stratified according to histology, 40.47 % of cases had squamous cell carcinoma, 16.66% had adenocarcinoma, 11.90% had small cell carcinoma and 4.76 % had other type of lung cancer, whereas the prevalence of these histological types is much higher (SQCC=59.9%, AC=29.5%) in Chinese population (Song *et al*., 2001). A study on African Americans consisted of cases with SQCC= 35.2%, AC= 39.1%, SCLC =6.7% (Taioli *et al*., 1998), whereas in Kashmiri population prevalence of SQCC was more than Adenocarcinoma; SQCC=63.3%, AC= 16.5% (Shaffi *et al*., 2009). On stratifying on the basis of TNM staging, stage IV was highly prevalent among lung cancer patients (34.52%) followed by stage III (26.19%) and stage II (8.33%).

5.5 Relationship between *CYP1A1* (Msp1) genotype and lung cancer:

Table 5.2: *CYP1A1* Genotype Frequencies in different histological types

	CYP1A1 *1/*1	***CYP1A1 *1/*2A***	***CYP1A1 *2A/*2A***	**OR (95%)**	***p-value***
CONTROL (86)	45 (52.32)	36 (41.86)	5 (5.81)	1.00	
CASES (84)	33 (39.28)	37 (44.04)	14 (16.66)	3.818 (1.215-11.650)	**0.01413**
SQCC (33)	12 (36.3)	14 (42.4)	7 (21.2)	5.250 (1.413-19.505)	**0.0086**
AC (14)	5 (35.71)	8 (57.14)	1 (7.14)	1.800 (0.174-18.638)	0.61786
SCLC (10)	2 (20)	6 (60)	2 (20)	9.00 (1.031-78.574)	**0.02192**
OTHERS (4)	2 (50)	2 (50)	-		

Others include bronchogenic lung carcinoma

p- value <0.05

The genotyping results from Table 5.2 shows that the frequency of *CYP1A1 *2A/*2A* mutant allele of *CYP1A1* gene in lung cancer cases was far more than in controls (16.66% and 5.81% respectively). Despite having small sample size, those who carried the mutant *CYP1A1*2A/*2A* allele were at 4 fold elevated risk for lung cancer (OR=3.818, 95% CI= 1.215-11.650, p-value= **0.014**) which was statistically significant. A study carried out in Chinese population, however shows the frequency of the mutant genotype *i.e. CYP1A1*2A/*2A* more represented in the controls as compared to the lung cancer cases (12.9% and 13.9%) (Song *et al*., 2001).The frequency of *CYP1A1*1/*1* was more represented among controls than cases 52.32% and 39.28% respectively. The frequency of *CYP1A1*1/*2A* among cases was slightly more represented than in controls (44.04% and 41.86% respectively). Studies carried out on north Indian population demonstrated the same trend as *CYP1A1*2A/*2A* mutant allele had a frequency of 6% and 6.6% among cases and controls while the variant allele *CYP1A1*1/*2A* was more represented in cases (45% in cases and 38.2% in controls) (Sobti *et al*., 2003). The frequency of

*CYP1A1*2A/*2A* mutant allele in another study on north Indian population was 69% in controls and 53% in controls (Shah *et al*., 2007). Studies carried out in Caucasians reported lesser representation of the mutant genotype. The frequency of *CYP1A1 *2A/*2A* mutant allele in Germans 0.7% (Drakoulis *et al*., 1994), 1.3% in Swedes (Alexandrie *et al*., 1994), 2% in French (Bouchardy *et al*., 1997), 1.7% in Finnish (Hirvonen *et al*., 1992), 2.3% in Belgians (Jacquet *et al*., 1996), 1% in Americans (Tefre *et al*., 1991), 76.63% in cases and 82.39% in controls in Australians (Wright *et al*., 2010), 81.14% in Portuguese (Mota *et al*., 2010*)*. Studies carried out in Chinese population (Jin *et al*., 2010) represented the *CYP1A1*2A/*2A* mutant allele among cases (63.70%) and controls (51.94%), whereas Zhu *et al*., 2011 showed a higher frequency of *CYP1A1*2A/*2A* mutant allele among controls (42.5%) than in cases (34.37%) in Chinese population. Similarly, the frequency of *CYP1A1*2A/*2A* mutant allele in a study carried out on Russian population (Belogubova *et al*., 2006) was 73.75% among cases and 79.33% among controls.

From the results obtained it is considered that the *CYP1A1*2A/*2A* is a susceptible risk factor for lung cancer in the present population attributed to a high odds ratio (OR=3.818, 95% CI= 1.215-11.650, *p-value* =**0.014**). When both the mutant *CYP1A1*2A/*2A* and *CYP1A1*1/*2A* heterozygous genotypes were combined as a single genotype (*CYP1A1*1/*2A/*2A/*2A*), they had a slight association with lung cancer in the present study (OR= 1.4, 95% CI). However the results were not statistically significant due to small sample size of the study subjects. A study on north Indian population revealed significant association towards the lung cancer risk (OR: 1.93, 95%CI: 1.28–2.89, *p-value* = 0.002) with *CYP1A1*1A/*2A/*2A* variant genotypes (combined heterozygous and mutant genotypes) (Shah *et al*., 2007). However, a study on Chinese population (Jin *et al*., 2010) and (Zhu *et al*., 2011) with *CYP1A1*2A/*2A* mutant genotype showed higher association towards the risk of lung cancer (OR=1.03, 95%CI=0.65-1.62), (OR=1.41, 95%CI=0.90-2.22) respectively. Some of the studies on Caucasian population (Belogubova *et al*., 2006), (Mota *et al*., 2010), (Wright *et al*., 2010) also exhibited a significant association of *CYP1A1*2A/*2A* mutant genotype with lung cancer.

The allele frequencies for *CYP1A1*2A/*2A* were 73.25 % and 38.69 % in cases and controls respectively. In another case control study on Chinese population the allele frequencies were much less than the present study (35.6% in control, 42.6% cases). The distribution of *CYP1A1* genotypes at M1 site when compared among cases and controls, was found that 60.17% of cases carried *CYP1A1*1/*2A*

or *CYP1A1*2A/*2A* genotype, which was significantly higher than that of controls (47.67%) ($\chi 2$, *p-value* =**0.01413**).

When stratified according to histology, the frequency of *CYP1A1*2A/*2A* mutant allele was slightly higher in SQCC (21.2%) than SCLC (20%) as compared to Adenocarcinoma (7.14%). On the contrary, a study conducted in a Chinese population showed that the frequency of *CYP1A1*2A/*2A* mutant allele was found to be much lower among SQCC cases (11.5%) (Song *et al*., 2001). Association of the *CYP1A1*2A/*2A* genotype with risk towards SQCC has been observed in Hawaiian and some Caucasian populations (Marchand *et al*., 1998). Individuals who were carrying the mutant genotype *CYP1A1*2A/*2A* had a 5-fold increased risk for developing SQCC (OR=5.25, 95% CI=1.413-19.505, *p*-value= **0.008**) which was statistically significant. The difference in the genotypic trends may be attributed the small sample size of our study in comparison to the other studies. The individuals having SCLC with *CYP1A1*2A/*2A* mutant genotype have 9 fold elevated risk towards lung cancer (OR=9.00, 95% CI= 1.031-78.574, *p*-value= **0.02**). In spite of having a small sample size the data is statistically significant. An association of *CYP1A1*1/*2A* and increased risk of adenocarcinoma has been studied in African-Americans but due to small sample size the results were not statistically significant (Taioli *et al.*, 1998).

In Japanese population an association between *CYP1A1* polymorphisms and lung cancer was stronger for SQCC compared to AC (Nakachi *et al.*, 1991; 1993; Hayashi *et al.*, 1992). Belogubova *et al*., 2006 have reported a higher risk for lung cancer especially for SQCC (OR= 2.13, 95%CI), but not for AC. On the contrary, (Mota *et al*., 2010) have pointed an elevated risk for AC (OR= 1.03, 95% CI) than SQCC (OR=0.52) and SCLC (OR= 0.51). Thus, it is assumed that since SQCC is epithelial in origin is associated with the cigarette/ beedi smoke, as a result of which increased exposure to tobacco smoke leads to development of lung cancer. Therefore the individuals lacking the *CYP1A1* gene have reduced capacity to detoxify carcinogens present in cigarette smoke are thus more susceptible to develop SQCC but not AC and SCLC.

Table 5.3: Genotype Frequencies of *CYP1A1* Polymorphisms for Different Smoking Categories

			CASES			CONTROL				
	n	***CYP1A1* *1/*1**	***CYP1A1* *1/*2A**	***CYP1A1* *2A/*2A**	**n**	***CYP1A1* *1/*1**	***CYP1A1* *1/*2A**	***CYP1A1* *2A/*2A**	**OR (95%)**	***p-value***
TOTAL	84	33	37	14	86	45	36	5	3.818	**0.01413**
NON-SMOKERS	14	8	3	3	64	32	28	4	3.00	0.187
SMOKERS	68	25	33	10	21	12	8	1	4.800	0.126

n=total number of case patients or control subjects

When lung cancer risk was examined with stratification of smoking status, among smokers it was found those carrying *CYP1A1*2A/*2A* genotype (OR= 4.800, 95% CI= 0.549-41.948, *p-value*= 0.126) had almost five fold increase in lung cancer but this increase in risk associated with tobacco smoking was found to be statistically non significant for *CYP1A1* msp1 due to small sample size (Table 5.3).

A study on north Indian population showed that cigarette smoking in patients with either heterozygous or homozygous mutant genotype *CYP1A1*1/*2A* /*CYP1A1*2A/*2A* resulted in almost six fold increase in the lung cancer risk (OR= 6.13, 95%CI: 3.04–12.39) which was also found to be statistically significant (Shah *et al*., 2007). When lung cancer risk was examined in Kashmiri population with stratification of smoking status ,among smokers it was found those carrying *CYP1A1*1/*2A* or *CYP1A1*2A/*2A* genotype had an elevated risk compared to those carrying wild type *CYP1A1*1/*1* genotype (OR=3.12 , 95% CI=1.656-5.876) (Shaffi *et al*., 2009). A significant increase in lung cancer risk was found in subjects having smoking habit (OR=3.14, 95%CI=1.939–5.087, *p-value*= 0.001) in studies on South Indian population (Sreeja *et al*., 2005) and a study on Russian population (Belogubova *et al*., 2006) (OR=1.52, 95%CI= 0.91-2.53). Whereas a study on Chinese population (Zhu *et al*., 2011) have reported a higher risk towards lung cancer among non smokers (OR= 1.41, 95%CI= 0.90-2.22).

Table 5.4: Interaction of *CYP1A1* Genotypes and Tobacco Smoking on the Risk of Squamous Cell Carcinoma

		CASES			CONTROLS		
	n	***CYP1A1* *1/*1**	***CYP1A1* *2A/*2A**	**n**	***CYP1A1* *1/*1**	***CYP1A1* *2A/*2A**	**OR(95%CI)**
Total	33	12	7	86	45	5	3.818
Non smokers	2	2	-	64	32 (50)	4 (6.25)	-
Smokers	31	12 (38.7)	7 (22.58)	21	12 (57.14)	1 (4.76)	7.00

n=total number of case patients or control subjects

Table 5.4 shows the interaction of *CYP1A1* polymorphism and tobacco smoking in relation to SQCC. The frequency of *CYP1A1*2A/*2A* mutant allele in SQCC cases was 22.58%. It was found that OR for combined smoking and variant allele was 7.00 (0.743-65.94), indicating that the individuals who are smokers having SQCC with *CYP1A1*2A/*2A* mutant genotype have 7 fold elevated risk of lung cancer (OR=7, 95% CI=0.743-65.943, *p-value* = 0.0614). This increase in risk associated with tobacco smoking was found to be statistically significant for *CYP1A1*2A/*2A*. Stratified analysis on Kashmiri population showed a multiplicative interaction between tobacco smoking and *CYP1A1*1/*2A* genotype on the risk of SQCC. The ORs of SQCC for non-smokers were 2.08 and 3.15 for smokers (Shaffi *et al.*, 2009).

Table 5.5: Interaction of *CYP1A1* Genotypes and Tobacco Smoking on the Risk of Small cell lung carcinoma

	CASES				**CONTROLS**		
	n	***CYP1A1* *1/*1**	***CYP1A1* *2A/*2A**	**n**	***CYP1A1* *1/*1**	***CYP1A1* *2A/*2A**	**OR(95%CI)**
Total	14	4	2	86	45	5	3.800
Non smokers	1	-	1	64	32	4	3.00
Smokers	9	2	1	21	12	1	6.00

n=total number of case patients or control subjects

When the interaction of *CYP1A1* polymorphisms and tobacco smoking was assessed separately for SCLC (Table 5.5), the frequency of mutant allele in SCLC cases was 20 %. It was found that OR for combined smoking and variant allele was 6.00, indicating that the individuals who are smokers having SCLC with *CYP1A1*2A/*2A* mutant genotype have six times increased risk of lung cancer (OR=6, 95% CI= 0.257-140.045, *p-value* =0.22). The increase in risk towards SCLC associated with tobacco smoking was not statistically significant for *CYP1A1 *2A/*2A*.

Table 5.6: Genotype Frequencies of *CYP1A1* Polymorphisms for different stages

	n	***CYP1A1* *1/*1**	***CYP1A1* *1/*2A**	***CYP1A1* *2A/*2A**
TOTAL	84	33	37	14
IA	1	-	1	-
II	7	2 (28.57)	3 (42.85)	2 (28.57)
III	22	7 (31.81)	11 (50)	4 (18.18)
IV	29	13 (44.82)	14 (48.27)	2 (6.89)
Data NA	25	11 (44)	8 (32)	6 (24)

When case group was stratified according to the TNM staging (Table 7), the frequency of mutant allele (*CYP1A1**2A/*2A) was highest in stage II (28.57%) when compared with stage III (18.18%) and stage IV (6.89%). The variant allele (*CYP1A1*1/*2A*) is highly represented in stage III (50%) in comparison to stage IV (48.277%) and stage II (42.85).

CHAPTER- 6

CONCLUSION

- From the demographic characteristics it is evident that majority of the lung cancer patients were above 50 years of age, indicating that lung cancer is a disease of middle and old age.

- The mutant *CYP1A1*2A/*2A* genotype is associated with an increased risk for lung cancer.

- Smoker individuals with *CYP1A1*2A/*2A* genotype had a higher risk towards lung cancer.

- The presence of *CYP1A1*2A/*2A* mutant allele in smokers have more risk for SQCC than SCLC.

CHAPTER-7

REFERENCES

1. Alexandrie, A. K., Sundberg, M. I., Seidegård, J., Tornling, G., and Rannu, A., (1994). Genetic susceptibility to lung cancer with special emphasis on *CYP1A1*and *GSTM1*: a study on host factors in relation to age at onset, gender and histological cancer types. *Carcinogenesis,* **15** (9): 1785-1790.

2. Alexandrov, K., Cascorbi, I., Rojas, M., Bouvier, G., Kriek, E., and Bartsch, H., (2002). *CYP1A1* and *GSTM1* genotypes affect benzo[*a*]pyrene DNA adducts in smokers' lung: comparison with aromatic/hydrophobic adduct formation. *Carcinogenesis,* **23**:1969–1977.

3. Alberg, A. J., Samet J. M., (2003). Epidemiology of lung cancer. *Chest,* **123**:21-49.

4. Alberg, A. J., Brock, M. V., Samet, J. M., (2005). Epidemiology of lung cancer: looking to the future. *Journal of Clinical Oncology*, **23**:3175-85.

5. Achour, A., *et al*., (2011). Role of *CYP1A1* (T6235C) polymorphism and cigarette smoking in the development of coronary heart disease in Tunisian population. *Journal of Medical Genetics*, **90**: 303–307.

6. Anttila, S., Vainio, H., Hietanen, E., Camus, A. M., Malaveille, C., Brun, G., (1992). Immunohistochemical detection of pulmonary cytochrome P-4501A and metabolic activities associated with P4501A1 and P4501A2 isozymes in lung cancer patients. *Enviromental Health Perspect,* **98**: 179-182.

7. Androutsopoulos, V. P., Tsatsakis, A. M., Spandidos, D. A., (2009). Cytochrome P450 CYP1A1: wider roles in cancer progression and Prevention. *Bio Med Central Cancer*, **9**:187

8. Bale, A. E., Nebert, D. W., McBride, O. W., (1987). Subchromosomal localization of the dioxin-inducible P1450 locus (CYP1) and description of two RFLPs detected within a 3' P1450 cDNA probe. *Cytogenetics and Cell Genetics*, **46**:574-575.

9. Bartsch, H., Nair, U., Risch, A., *et al* (2000). Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. *Cancer Epidemiol Biomarkers Prev*, **9**: 3-28.

10. Belogubova, E. V, Ulibina, Y. M., Suvorova, I. K., (2006). Combined CYP1A1/GSTM1 at risk genotypes are overrepresented in squamous cell lung carcinoma patients but underrepresented in elderly tumor-free subjects .*Journal of Cancer Research and Clinical Oncology*, **132**: 327–31.

11. Bouchardy, C., Benhamou, S., Dayer, P., Hirvonen, A., (2001). Metabolic genetic polymorphism and susceptibility to lung cancer. *Lung cancer*, **32**: 109-1112.

12. Carlsten, C., Sagoo, G. S., Frodsham, A. J., (2008). Glutathione S-transferase M1 (GSTM1) polymorphisms and lung cancer: a literature-based systematic huge review and meta analysis. *American Journal of Epidemiology*, **167**: 759-774.

13. Crofts, F., Taioli, E., Trachman, J., Cosma, 0. N., Currie, D., Toniolo, P., and Garte, S. J (1994). Functional significance of different humanCYPJA1 genotypes. *Carcinogenesis (Land.)*, **15**: 2961-2963.

14. Cascorbi, I., Brockmöller, J., and Roots, I., (1996). A C4887A Polymorphism in Exon 7 of Human CYP1A1: Population Frequency, Mutation Linkages, and Impact on Lung Cancer Susceptibility. *Cancer Research*, **56**: 4965-4969.

15. Crofts, F., Cosma, G. N., Currie, D., *et al* (1993). A novel *CYP1A1*gene polymorphism in African-Americans. *Carcinogenesis*, **14**: 1729-1731.

16. Drakoulis, N., Cascorbi, I., Brockmoller, J., Gross, C. R., Roots, I., (1994).Polymorphism in the human *CYP1A1*gene as susceptibility factors for lung cancer: exon 7 mutation (4889 A to G) and a T to C mutation in the 3' flanking region. *Carcinogenesis*, **72**:240-248.

17. Ezzati, M., & Lopez, A. D., (2003). Estimates of global mortality attributable to smoking in 2000. *Lancet*, **362**: 847-852.

18. Gonzalez, F. J., Gelboin, H. V., (1994). Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metabolism Reviews*, **26**:165-183.

19. Guengerich, F. P., Shimada, T., (1998). Activation of procarcinogens by human cytochrome P450 enzymes. *Mutation Research*, **400**: 201-213.

20. Gonzalez, F. J., Gelboin, H. V., (1994). Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metabolism Reviews*, **26**: 165-83.

21. Gonzalez, F. P., (1997). The role of carcinogen-metabolizing enzyme polymorphisms in cancer susceptibility. *Reproductive Toxicology*, **11**: 397-412.

22. Guengerich, F. P., Shimada, T., (1991). Oxidation of toxic and carcinogenic chemicals by human cytochrome P-450 enzymes. *Chemical Research in Toxicology*, **4**: 391-407.

23. Guengerich, F.P. (2008). Cytochrome P450 and chemical toxicology. *Chemical Research in Toxicology*, **21**:70–83.

24. Hayashi, S., Watanabe, J., Nakachi, K., Kawajiri, K., (1991). Genetic linkage of lung cancer-associated MspI polymorphisms with amino acid replacement in the heme binding region of the human cytochrome P4501A1 gene. *Journal of Biochemistry*, **110**:407-411.

25. Hecht, S., 1999. DNA adduct formation from tobacco-specific N-nitrosamines. *Mutation Research*, **424**(1-2):127-142.

26. Hukkanen, J., Pelkonen, O., Hakkola, J., Raunio, H., (2002). Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung. *CRC Critical Reviews in Toxicology*, **32**: 391-411.

27. http://www.cancer.org/cancer/lungcancer-non-smallcell/detailedguide/non-small-cell-lung-cancer-what-causes.

28. https://docs.google.com/viewer?a=&q=cache:kcjOH8ZHPzMJ:sancd.org/uploads/pdf/lungcancer-factsheet.

29. Hirvonen, A., Husgafvel, P. K., Antilla, S., Vanio, H., (1993). The *GSTM1* null genotype as a potential risk modifier for squamous cell carcinoma of the lung. *Carcinogenesis*, **14** (7): 1479-1481.

30. Hong, Y. S., Chang, J. H., Kwon OJ, Ham YA, Choi JH (1998).Polymorphism of the *CYP1A1*and glutathione-S transferase. *Molecular Medicine*, **30**: 192-198.

31. Cascorbi, I., Ãller, J. B., Roots, I., (1996). A C4887A Polymorphism in Exon 7 of Human CYP1A1: Population Frequency, Mutation Linkages, and Impact on Lung Cancer Susceptibility. *Cancer Research*, **5**:4965-4969.

32. Hasler, J. A., Estabrook, R., Murray, M., Pikuleva, I., Waterman M., Capdevila, J.,(1999). Human cytochromes P450. *Molecular Aspects of Medicine*, **20**: 137.

33. Jacquet, M., Lambert, V., Baudoux, E., Muller, M., Kremers, P., and Gielen, J., (1996). Correlation Between P450 CYPlAl Inducibility, MspI Genotype and Lung Cancer Incidence. *Europeon Jounal of Cancer*, **32A**: 1701-1706.

34. Ji, Y., Wang, Q., Lin, X., Suo, L., (2012). *CYP1A1*MspI polymorphisms and lung cancer risk: An updated meta-analysis involving 20,209 subjects. *Cytokine*, **59**: 324–334.

35. Jin, Y., Xu, H., Zhang, C., Kong, Y., Hou, Y., Xu, Y., (2010). Combined effects of cigarette smoking, gene polymorphisms and methylations of tumor suppressor genes on non small cell lung cancer: a hospital-based case–control study in China. *Bio Med Central Cancer*, **10**: 422.

36. Khurana, S., Lung cancer. (https:// docs .google.com /viewer? a=v &q=cache: sancd.org /uploads /pdf/ lung cancer-factsheet).

37. Kawajiri, K., (1999). CYP1A1. In Vineis P, Malats N, Lang M., d'Errico A, Caporaso N, Cuzick J. and Boffetta P. Metabolic Polymorphisms and Susceptibility to Cancer. *IARC Scientific Publications*, **148** :159±172.

38. Kagawa, N., Kunugita, N., Kitagawa, K., Ogawa, M., Yamaguchi, T., Suzuki, R., Kinaga, T., Yashima, Y., Ozaki, S., Isse, T., Kim, Y., Kim, H., Kawamoto, T., (2004). Expression of cytochrome p450 in tumor tissues and its association with cancer development. *Frontiers in Bioscience*, **9**:1967-1976.

39. Kenfield, S. A., Wei, E. K., Stampfer, M. J., Rosner, B. A., Colditz, G. A., (2008). Comparison of Aspects of Smoking Among Four Histologic Types of Lung Cancer. *Tobacco Control*, **17**(3): 198–204.

40. Kirmani, N., Jamil, K., Naidu, M. U. R., (2010). Occupational and environmental carcinogens in epidemiology of lung cancer in South Indian population. *Biology and Medicine* **2**(4):1-11.

41. Kiyohara, C., Hirohata, T., Inutsuka, S., (1996). The relationship between aryl hydrocarbon hydroxylase and polymorphisms of the CYP1A1 gene. *Japanese Journal of Cancer Research*, **87**: 18-24.

42. Kiyohara, C., Nakanishi, Y., Inutsuka, S., et al., (1998). The relationship between CYP1A1 and aryl hydrocarbon hydroxylase activity and lung cancer in a Japanese population. *Pharmacogenetics*, **8**: 315-23.

43. Landi MT, Bertazzi PA, Shields PG, et al (1994). Association between CYP1A1 genotype, mRNA expression and enzymatic activity in humans. *Pharmacogenetics,* **4**: 242-6.

44. Liska., (1998). The Detoxification Enzyme Systems. *Alternative Medical Reviews,* **3**(3):187-198).

45. Li, Y., Millikan, R. C., Bell, D. A., Cui, L., Tse, C. H., Newman, B., Conway, K., (2004). Polychlorinated biphenyls, cytochrome P450 1A1 (*CYP1A1*) polymorphisms, and breast cancer risk among African American women and white women in North Carolina: a population-based case-control study. *Breast Cancer Research*, **7** No 1.

46. Marchand, Le., Sivaraman, L., Pierce, L., *et al.*, (1998). Association of CYP1A1, GSTM1, and CYP2E1 polymorphisms with lung cancer suggests cell type specificities to tobacco carcinogens. *Cancer Research,* **58**: 4858-4863.

47. Masood, *et al.,* (2011). A novel *CYP1A1*gene polymorphism and the risk of head and neck cancer in Pakistani population. *African Journal of Biotechnology*, **10(27)**: 5273-5280.

48. Mimura J, Fujii-Kuriyama Y (2003). Functional role of AhR in the expression of toxic effects by TCDD. *Biochemical Pharmacology*, **1619**:263-268.

49. Mota, P., Moura, D. S., Vale, M. G, Coimbra, H., Carvalho, L., Regateiro, F., (2010). CYP1A1 M1 and M2 polymorphisms: genetic susceptibility to lung cancer. *Review of Portuguese Pneumology*, **16**(1):89–98.

50. Murray, G. I., Melvin, W. T., Greenlee, W. F., & Burke, M. D., (2001). Regulation, function and tissue - specific expression of cytochrome P450 CYP1B1. *Annual Review of Pharmacology and Toxicology,* **41**: 297– 316.

51. Nebert, D. W., Mckinnon, R. A., Puga, A., (1996). Human drug metabolizing enzyme polymorphisms: effects on risk of toxicity and cancer. *DNA and Cell Biology*, **15**: 273-80.

52. Nelson, D. R., (2006). Cytochrome P450 nomenclature (2004). *Methods in Molecular Biology,* **320**: 1–10.

53. Notani, P., & Sanghavi, L. D., (1974). A retrospective study of lung cancer in Bombay. *British Journal of Cancer,* **29**: 477-482.

54. Osler, M., (2001). Tobacco control in developing countries. *British Journal of Cancer,* **322**: 869.

55. Persson, I., Johansson, I., Ingelman, S. M., (1997). In vitro kinetics of two human CYP1A1 variant enzymes suggested to be associated with inter individual differences in cancer susceptibility. *Biochemical and Biophysical Research Communications,* **231**: 227–230.

56. Petersen, D. D., McKinney, C. E, Iyeka, K., et al., (1991). Human CYP1A1 gene: Co-segregation of the enzyme inducibility phenotype and an RFLP. *American Journal of Human Genetics,* **48**, 720-5.

57. Puga, A., Ma, C., Marlowe, J. L., (2009). The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. *Biochemical Pharmacology,* **77**:713-722.

58. Rajani, B., Aparna, K., Shama, B., (2003). Polymorphism at CYP and GST gene loci and susceptibility to tobacco related cancers. *Proceedings of the Indian National Science Academy,* **69(1)**: 35-48.

59. Santis, *et al.,* (2013). Cancer Statistics for African Americans. *Cancer Journal for Clinicians,* 000:000-000.

60. Schults, M. A., Timmermans, L., Godschalk, R. W., Theys, J., Wouters, B. G., Van Schooten, F. J., (2010). Diminished carcinogen detoxification is a novel mechanism for hypoxia inducible factor 1-mediated genetic instability. *Journal of Biomolecular Chemistry,* **285**: 14558–14564.

61. Sobti, R. C., Sharma, S., Joshi, A., Jindal, S. K., Janmeja, A., (2003). *CYP1A1*and CYP2D6 polymorphism and risk of lung cancer in a North Indian population. *Biomarkers,* **8**, 415-28.

62. Sobti, R. C., Sharma, S., Joshi, A., Jindal, S. K., Janmeja, A., (2004). Genetic polymorphism of the CYP1A1, CYP2E1, GSTM1 and GSTT1 genes and lung cancer susceptibility in a North Indian population. *Molecular Cell Biochemistry*, **266**, 1-9.

63. Shimada, T., Martin, M. V., Pruess, S. D, Marnett, L. J., Guengerich, F. P., (1989). Roles of individual cytochrome P-450 enzymes in the bioactivation of benzo(a)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(a) pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Research,* **49**: 6304-6312.

64. Shimada, T., Fujii, K. Y., (2004). Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Science*, 95:1-6.

65. Shah, P. P., Singh, A. P., Singh, M., *et al*., (2008). Interaction of cytochrome P4501A1 genotypes with other risk factors and susceptibility to lung cancer. *Mutation Research*, **639**: 1-10.

66. Shaffi, S. M., Shah, M. A., Bhat, I. A., Koul, P., Ahmad, S. N., Siddiqi, M. A., (2009). CYP1A1 Polymorphism and Risk of Lung Cancer in the Ethnic Kashmiri Population. *Asian Pacific Journal for Cancer Prevention*, **10**: 651-656

67. Song, N., Tan, W., Xing, D., Lin, D., (2001). *CYP1A1*polymorphism and risk of lung cancer in relation to tobacco smoking: a case control study in China. *Carcinogenesis*, **22**:11-16.

68. Spivack, S. D., Hurteau, G. J., Reilly, A. A., *et al.,* (2001). CYP1B1 expression in human lung. *Drug Metabolism and Disposition*, **29**:916-22.

69. Sreeja, L., Syamala, V., Hariharan, S., *et al.,* (2005). Possible risk modification by CYP1A1, GSTM1 and GSTT1 gene polymorphisms in lung cancer susceptibility in a South Indian population. *Journal of Human Genetics*, **50**:618-27.

70. Tefre, Toril., Ryberg, D., Haugen, A., Nebert, D. W., Skaug, V. B., Anton, B., Anne-Lise Human *CYP1A1*(cytochrome P1450) gene: lack of association between the Msp I restriction fragment length polymorphism and incidence of lung cancer in a Norwegian population. *Pharmacogenetics*, **1**: 20-25.

71. Teresa, *et al.,* (2007). The risk of developing cervical cancer in Mexican women is associated to *CYP1A1* MspI polymorphism. *European journal of cancer* **43**: 1590 –1595.

72. Thankappan, K. R., & Thresia, C. U., (2007). Tobacco use & social status in Kerala. *Indian Journal of Medical Research,* **126**: 300-308.

73. Varela, L., (2008). Meta-analysis and pooled analysis of GSTM1 and *CYP1A1*polymorphisms and oral and pharyngeal cancers: a Huge-GSEC review. *Genetics in Medicine*, **10**(6):369–384.

74. Vermeulen, N. P. E., (1996). Role of metabolism in chemical toxicity. In: Ioannides C, ed. *Cytochromes P450: Metabolic and Toxicological Aspects. Boca Raton, FL: CRC Press, Inc*: 29-53.

75. Wright, C. M., Larsen, J. E., Colosimo, M. L., Barr, J. J., Chen, L., McLachlan, R. E., *et al*., (2010). Genetic association study of CYP1A1 polymorphisms identifies risk haplotypes in nonsmall cell lung cancer. *European Journal of Respiratory Diseases,* **35**:152–159.

76. Zanger, U., (2008). The *CYP2D* Subfamily. In: C. Ioannides (Ed.), Cytochromes P450: Role in the metabolism and toxicity of drugs and other xenobiotics. *Royal Society of Chemistry*, 241-275.

77. Zhu, X. X., Hu, C. P., Gu, Q. H., (2010). CYP1A1 polymorphisms, lack of glutathione S transferase M1 (GSTM1), cooking oil fumes and lung cancer risk in nonsmoking women. *Zhonghua Jie He He Hu Xi Za Zhi*, **33**(11):817–22.

APPENDIX

1. Sucrose (1M): Dissolved 3.41 g of sucrose in 10 ml of deionised water and sterilized by autoclaved.
2. Magnesium chloride ($MgCl_2$) (100mM): Dissolved 0.41gms of $MgCl_2$ in 20ml of deionised water and sterilized by autoclaved.
3. Triton X- 100 (10%): Taken 100 µl of TritonX-100 and mixed with 900 µl of deionised water and mixed properly.
4. 100mM Tris-Cl (pH 8.0): Dissolved 0.32g of Tris-Cl in 10 ml of deionsed water, then adjusted the pH to 8.0 by 1M sodium hydroxide. Sterilize the solution by autoclaving.
5. 10% SDS: Dissolved 1g of SDS in 10ml of deionsed water.
6. 10mg/ml Proteinase K: Dissolved 10mg Proteinase K in 1ml of double distilled water. Sterilize the solution by autoclaving.
7. 0.5M EDTA: Dissolved 9.306g of disodium salt of EDTA in 20ml of deionsed water, and then adjusted the pH to 8.0 by 1 M sodium hydroxide. Sterilize the solution by autoclaving.
8. 5M Sodium chloride (NaCl): Dissolved 5.85g of sodium chloride in 20ml of deionsed water. Sterilize the solution by autoclaving.
9. 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 deionsed water. Sterilize the solution by autoclaving.
10. 1mg/ml BSA: Dissolved 100mg of BSA in 100ml of deionsed sterile water and kept at 4 ℃ overnight.
11. Ethidium Bromide (10mg/ml): Dissolved 1g of ethidium bromide in 100ml of water. Mixed the solution properly.

12. 5% DMSO: Mixed 50ml of 100% DMSO in 50ml of deionsed sterile water. Sterilize the solution by autoclaving and stored at -20˚C.
13. 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. Sterilize the solution by autoclaving.