GENETIC POLYMORPHISM OF GSTM1, GSTP1 IN LUNG CANCER PATIENTS OF BANGLADESH

A thesis submitted by

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ABSTRACT

Background

Lung cancer has been the most common cancer in the world for several decades, and by 2008, there were an estimated 1.61 million new cases, representing 12.7% of all new cancers. It was also the most common cause of death from cancer; with 1.38 million deaths (18.2% of the total). The majority of the cases now occur in the developing countries (55%). The impact of genetic polymorphisms in GSTM1, GSTT1, and GSTP1 on susceptibility to lung cancer has received particular interest since these enzymes play a central role in detoxification of major classes of tobacco carcinogens. In the current study we investigated the role of GSTM1, GSTT1 and GSTP1 polymorphisms as a genetic modifier of risk for individuals with lung cancer as susceptible genotypes.

Objective

Our main goal was to verify possible associations between polymorphisms of these genes and susceptibility to lung cancer. GSTM1 and GSTP1 codify conjugation enzymes associated with detoxification processes of free radicals, xenobiotics and cytotoxic drugs. GSTT1 is involved in the detoxification of some environmental carcinogens. Homozygous deletions or null genotypes of GSTT1 and GSTM1 genes and GSTP1 -313-A/G have been reported as strong predisposing risk factors for lung cancer in different populations.

Methods

100 lung cancer patients and 100 controls were enrolled in a case-control study to investigate allelic variants of GSTM1, GSTT1 and GSTP1 genes. The GSTM1

& GSTT1 were analyzed using polymerase chain reaction (PCR) while polymorphism of GSTP1 was analyzed using polymerase chain reaction (PCR) - Restriction fragment length polymorphism (RFLP).Risk of lung cancer was estimated as odds ratio (OR) at 95% confidence interval (CI) using unconditional logistic regression models adjusting for age, sex, and tobacco use.

Result

No significant difference was found (p = 0.7752) in GSTM1 homozygous deletion/null type genotype distribution between control and lung cancer cases as it was absent in 56% in control and 58% in patients (Adjusted Odds Ratio = 0.81, 95% CI = 0.43 to 1.52). The frequency of homozygous deletion/null type GSTT1 genotype among the lung cancer patients (72%) was also not significant (Adjusted Odds Ratio = 0.82, 95% CI = 0.43 to 1.56, p = 0.648), For GSTP1, wild (Ile/Ile), heterozygous (Ile/Val) and mutant (Val/Val) genotypes were observed for 63%, 29% and 8% individuals in patients and 87%, 11% & 2% individuals in control respectively. GSTP1 single nucleotide polymorphism (SNP) encoding A313G base change increases the susceptibility for lung cancer. A significantly elevated lung cancer risk was associated with GSTP1 heterozygous, mutant and combined heterozygous plus mutant variants of rs1695 (Adjusted OR = 3.81, 95% CI = 1.73-8.39, p = 0.001; OR = 5.98. 95% CI = 1.15-31.09, p = 0.33 and OR = 4.14. 95% CI = 1.99-8.59, p = 0.000 respectively).

On the other hand, no association with risk of lung cancer was found in case of tobacco users & nonusers carrying null & present genotype of GSTM1 & GSTT1. Lung cancer cases having distribution of variant genotypes of GSTP1 i.e. Heterozygous (HE),

Mutant Homozygote (MH) or Heterozygous + Mutant Homozygous genotype (OR=3.3793, 95% CI = 1.55 to 7.34, p=0.002; OR=7.96, 95% CI = 0.93 to 67.99, p=0.050; and OR=3.37, 95%; CI = 1.79 to 7.89, p= 0.0005 respectively) were found to be higher in the tobacco user cases leading to the increased risk of lung cancer, which is statistically significant . There is a 3 fold (approx.) increase in the risk of lung cancer in case of tobacco user of HE & HE+MH and an 8 fold increase in risk of lung cancer in cases of tobacco user of MH with respect to NH, which is statistically significant. No association with risk of lung cancer was found among variant genotypes of GSTP1 in case of tobacco nonusers.

Conclusion

Our observations showed that carrying the GSTM1 & GSTT1 null genotype is not a risk factor alone for lung cancer. Our findings also suggest that GSTP1 exon 5 polymorphism (Ile105Val) is associated with high risk of lung cancer especially adenocarcinoma.

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DECLARATION

Not any portion of this work referred to in this thesis paper has been submitted for another degree or qualification of the University of Dhaka or any other University or any other institute of learning.

DEDICATION

Dedicated to my parents, sister, brother and wife who always inspire me in every steps of my life

LIST OF ABBREVIATIONS

Name	Details
3HC	Trans-3'-Hydroxy cotinine
A	Adenine (where referring to a nucleotide)
AAs	Arylamine
ADRs	Adverse drug reactions
AHR	Aryl Hydrocarbon Receptor
ARNT	Aryl Hydrocarbon nuclear translator
Asn	Asparagine
Asp	Aspartic acid
ASW	African ancestry in Southwest
BP	Benzo[a]pyrene
bp	Base pair
BPDE	Benzo(a)pyrene-7,8 diol-9,10-epoxide
C	Cytosine (where referring to a nucleotide)
cDNA	Complementary DNA
CHB	Han Chinese in Beijing
CHD	Chinese in Metropolitan Denver
CI	confidence interval
Cl^-	Chloride ion
CMV	Cytomegalovirus

COPD	Chronic Obstructive Pulmonary Disease
COT	Cotinine
CYP	Cytochrome P450
Cys	Cysteine
D'	normalized disequilibrium constant
DF	Dilution factor
DMBA	Dimethl Benzanthracene
DMEs	Drug metabolizing enzymes
DNA	Deoxyribonucleic acid
dNTP	De-oxy nucleotide triphosphate
EGFR	Epidermal growth factor receptor
EM	Extensive metabolizer
EPA	Environmental Protection Agency
EtBr	Ethidium Bromide
FMO3	flavin-containing monooxygenase 3
FP	Forward Primer
G	Guanine (where referring to a nucleotide)
GATS	Global Adult Tobacco Survey
GC-MS	Gas chromatography–mass spectrometry
GIH	Gujarati Indians in Huston
GSTs	Glutathione S-transferases
GWAS	Genome-wide association studies
HE	heterozygote
HPLC	High-performance liquid chromatography

IARC	International Agency for Research on Cancer
Ile	Isoleucine
IM	Intermediate metabolizer
INCC	International Lung Cancer Consortium
IREB2	iron-responsive element-binding protein
JPT	Japanese in Tokyo
K^+	Potassium ion
L	Liter
LBAC	Localized Bronchioalveolar carcinoma
LC-MS	Liquid chromatography–mass spectrometry
LD	Linkage disequilibrium
LLL	Left Lower Lobe
LUL	Left Upper Lobe
LWK	Luhya in webuye, Kenya
Met	Methionine
MEX	Mexican ancestry in Los Angeles
mg	Miligram
$MgCl_2$	Magnesium Chloride
MH	mutant homozygote
ml	Milliliter
mM	Millimolar
MPI	Mannose phosphate isomerase
mRNA	Messenger Ribonucleic acid
Na^+	Sodium ion

nAChRs	Nicotinic acetylcholine receptors
NAS	National Academy of Sciences
NC	Not Calculated
NCBI	National Centre for Biotechnological Information
NCI	National Cancer Institute
NDEA	N-nitrosodiethylamine
NE buffer	New England Biolab buffer
NEB	New England Biolab
ng	Nanogram
NH	normal homozygote
NNK	4- (methylnitrosamine)-1-(3-pyridyl)-butanone
NNN	N-nitrosonornicotine
NSAID	Non-steroidal anti-inflammatory drug
NSCLC	Non-small Cell Lung Cancer
OD	Optical Density
OR	Odds Ratio
PAHs	Polycyclic aromatic hydrocarbons
PCR	Polymerase Chain Reaction
PM	Poor metabolizer
Pre-mRNA	preliminary-mRNA
Pro	Proline
REase	Restriction endonuclease
RefSeq	NCBI Reference Sequences

RFLP	Restriction Fragment Length Polymorphism
RLL	Right Lower Lobe
RML	Right Middle Lobe
RNA	Ribonucleic acid
RP	Reverse Primer
rs	DbSNP record ID number
RUL	Right Upper Lobe
SCLC	Small Cell Lung Cancer
Ser	Serine
SHS	second-hand smoke
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for the Social Sciences
SRS	Substrate recognition sites
T	Thymine (where referring to a nucleotide)
TAE	Tris-acetate-EDT
TE	Tris-EDTA
Thr	Threonine
TNM	tumor, node and metastases
Tris-HCL	Tris-Hydrochloride
TSI	Tuscan in Italy
TSNAs	Tobacco specific nitrosamines
U	unit
UGT	Uridine 5'-diphospho-glucuronosyltransferase

UM	Ultrarapid metabolizer
USA	United States of America
USDHHS	U.S. Department of Health and Human Services
USEPA	United States Environmental Protection Agency
UTR	3´-untranslated region
UV	Ultraviolet
Val	Valine
WCRF	World Cancer Research Fund
WHO	World Health Organization
WMADH	World Medical Association Declaration of Helsinki
YRI	Yoruban in Ibadan
α	Alpha
β	Beta
γ	Gamma
δ	Delta
µg	Microgram
µl	Micro liter

INTRODUCTION

1. Lung cancer continues to be the leading cause of cancer related deaths worldwide (Parkin et al., 2005). Lung cancer is considered to be the leading cancer site in males; it accounts 17% of the total new cancer cases and 23% of the total cancer deaths (Jemal et al., 2011). Among females, it was the fourth most commonly diagnosed cancer and the second leading cause of cancer death (Parkin et al., 2005). According to the latest WHO data published in April 2011 lung cancers deaths in Bangladesh reached 18,124 or 1.89% of total deaths (World Health Rankings, Lung Cancers in Bangladesh Cited on March 2012 Available on http://www.worldlifeexpectancy.com/bangladesh-lung-cancers). Total no of lung cancer patients aged ≥30 years was estimated to be 196,000 in Bangladesh (Sinha *et al.*, 2012). It was also the most common cause of death from cancer; with 1.38 million deaths (18.2% of the total). The majority of the cases now occur in the developing countries (55%). Lung cancer is still the most common cancer in men worldwide (1.1 million cases, 16.5% of the total), with high rates in central-Eastern and Southern Europe, Northern America and Eastern Asia. Very low rates are still estimated in Middle and Western Africa (ASRs 2.8 and 3.1 per 100,000 respectively). In females, incidence rates are generally lower but worldwide lung cancer is now the fourth most frequent cancer of women (516 000 cases, 8.5% of all cancers) and the second most common cause of death from cancer (427 000 deaths, 12.8% of the total). The highest incidence rate is observed in Northern America (where lung cancer is now the second most frequent cancer in women), and the lowest in Middle Africa (Cancer fact sheets 2008, GLOB0CAN).

Because of its high fatality (the ratio of mortality to incidence is 0.86) and the lack of variability in survival in developed and developing countries, the highest and lowest

mortality rates are estimated in the same regions, both in men and women (WHO, 2008). According to the latest WHO data published in April 2011 the age adjusted death rate which is 20.29 per 100,000 of population ranks Bangladesh at position 59 in the world (worldlifeexpectancy, 2008). A growing body of experimental and epidemiological evidence suggests that cancer risk as well as lung cancer susceptibility results from the combined effect of genes and environment. Knowledge of genetic risk factors may increase the statistical influence of future epidemiological studies, particularly those aimed at investigating common risk factors with only a moderate relation to lung cancer e.g. passive tobacco, air pollution (parera,1998). Predominant cause of lung cancer is exposure to tobacco smoke, with active smoking causing most cases but passive smoking also contributing to the lung cancer burden (Alberg *et al.*, 2007a).Tobacco smoking is the major risk factor for the development of lung cancer and about 80–90% of lung cancers are attributable to cigarette smoking (Tang *et al.*, 2010). Bangladesh is in a high risk for the prevalence of lung cancer due to high production and consumption of tobacco and high amount of environmental pollutants like polycyclic aromatic hydrocarbons (PAHs) (Rahman et al., 2003). Cigarette smoke contains about 4,000 chemicals at least 250 of them are known to be harmful and more than 60 are known to be carcinogenic (Zaga *et al.*, 2011). These have been detected in mainstream cigarette smoke and most of the same carcinogens are also present in passive smoking. Only one of ten lifetime smokers develops lung cancers, implying that the differential risk for lung cancer may be explained by genetic susceptibility factors (Perera, 1998). Lung cancer is a complex multifactorial disease (Holland et al., 2011), where many genetic and environmental factors might contribute to disease risk.

Metabolizing enzymes- mainly phase I enzymes metabolically activate tobacco carcinogens like PAHs, N-nitrosamines such as N-nitrosonornicotine (NNN), 4-(methylnitrosamine)-1-(3-pyridyl)-butanone (NNK), and aromatic amines to reactive intermediates which can react with DNA, resulting in the formation of covalently bound products known as DNA adducts and thereby potentially initiating the carcinogenic process (Hecht, 2002).

Phase II biotransformation enzymes generally act as inactivating enzymes to catalyze the binding of intermediary metabolites to cofactors, transform them into more hydrophilic products and thus facilitate their elimination. Both Glutathione S-transferases (GSTs) and N-terminal acetyltransferases (NATs) are phase II transformation enzymes involved in the detoxification of hazardous agents (Hirvonen *et al.*, 1996). The Glutathione S-transferase (GST) gene family encodes genes that are critical for certain life processes, as well as for detoxication and toxification mechanisms, via conjugation of reduced glutathione (GSH) with numerous substrates such as pharmaceuticals and environmental pollutants (Nebert et al., 2004). Human Genome Organization-sponsored Human Gene Nomenclature Committee database showed 21 acknowledged functional genes for GSTs (Nebert et al., 2004). Among these few are considered to have GST -like activity such as GST -kappa 1(GSTK1), prostaglandin E synthase (PTGES) and three microsomal GSTs (MGST1, MGST2, MGST3), but these genes are not evolutionarily related to the GST gene family . It is now established that GST gene family comprises 16 genes in six subfamilies —alpha (GSTA), mu (GSTM), omega (GSTO), pi (GSTP), theta (GSTT) and zeta (GSTZ). A family of enzymes, the Glutathione-S-transferases (GSTs) are divided into at least five

major classes (α, μ, π, θ, ζ) among which polymorphism have been detected in the genes encoding for GSTA1 (α class) GSTM1 (μ class), GSTP1 (π class), GSTT1 (θ class) and GSTZ1 (ζ class) (Stucker et al., 2002). Among them the GSTM1, GSTP1 and GSTT1 genotypes have been comprehensively studied recently for their polymorphisms as a genetic modifier of risk for individuals with lung cancer as susceptible genotypes (Stucker et al., 2002). The main role of GSTs is to detoxify xenobiotics by catalyzing the nucleophilic attack by GSH on electrophilic carbon, sulfur, or nitrogen atoms and convert to nonpolar xenobiotic substrates, thereby prevent their interaction with crucial cellular proteins and nucleic acids (Hayes *et al.*, 1986; Josephy, 2010a) *GSTM1, GSTT1* and *GSTP1* are dimeric enzyme members constituting *GST* super-family that catalyze the conjugation of Glutathione to bio transform toxic chemicals into non-toxic substances (Lewis *et al.*, 2002). GSTM1, GSTT1 and GSTP1 whether hetero or homodimeric in nature, contain a single non substrate binding site, as well as a GSH-binding site. Cytosolic mu and alpha classes form heterodimeric GST complexes, however, the cleft between the two subunits is home to an additional high-affinity non substrate xenobiotic binding site, which may account for the enzymes' ability to form heterodimers (Hayes et al., 1986). It provides a twofold action; firstly binds both the substrate at the enzyme's hydrophobic H-site and secondly GSH at the adjacent, hydrophilic G-site, which together form the active site of the enzyme; and subsequently activate the thiol group of GSH, allowing the nucleophilic attack upon the substrate.

The *GSTM1* gene is polymorphic and is represented by two active alleles and a non-functional null allele which results from the entire *GSTM1* gene deletion mutation. *GSTM1* may act as a determinant factor in susceptibility to the related disease and may

be a risk factor for cancer (Strange *et al.*, 1991). *GSTM1* are present in human lung tissue (Anttila *et al.*, 1995; Mainwaring *et al.*, 1996; Moscow *et al.*, 1989) and this suggests that these enzymes may provide protection against some, but not necessarily all, of the carcinogens present in cigarette smoke. Inter-individual variation in their expression or functional activity may be important in determining susceptibility to lung cancer (Houlston, 1999; Strange *et al.*, 1999). Between 38% and 67% of Caucasians carry a deletion in both alleles of the *GSTM1* gene resulting in a total absence of *GSTM1* enzyme activity (Rebbeck, 1997). Individuals lacking *GSTM1* have significantly lower levels of *GSTM3* in the lung than *GSTM1* gene carriers (Anttila *et al.*, 1995). Some studies suggest that the *GSTM1* null genotype confers an increased risk of lung cancer but this result has not been replicated by others (Houlston, 1999) and such differences may result from differences in study design and analysis (Houlston, 1999). A meta-analysis of more than 20 genotyping studies found only a slight increase in lung cancer risk associated with the homozygous null genotype with an odds ratio (OR) of 1.13 (95% confidence interval ,CI = 1.04–1.25); in Caucasians the OR was 1.08 (95% CI= 0.97–1.22) (Houlston, 1999).

GSTT1 on the other hand, is expressed at high levels in extra hepatic tissues, including the kidney, liver and the lung, suggesting an important role in the protection against carcinogens and other xenobiotics in these tissues (de Bruin *et al.*, 2000; J D Rowe, 1997; Pemble *et al.*, 1994). There are little evidences that *GSTT1* genotype is associated with lung cancer risk. Between 11% to 18% of Caucasians have the homozygous null deletion of the *GSTT1* gene. Deletion variants that are associated with a lack of enzyme function exist at this locus. Individuals who are carriers of homozygous

deletions in the *GSTM1* or *GSTT1 g*enes may have an impaired ability to metabolically eliminate carcinogenic compounds and may therefore be at increased cancer risk (Rebbeck, 1997). Molecular epidemiological studies have provided three pieces of information about the relationship of *GSTM1* and *GSTT1* with cancer susceptibility. First, the frequencies of homozygous *GSTM1* and *GSTT1* deletion carriers is very high (i.e., 20-50%) in most populations studied to date. Second, *GSTM1* and possibly, *GSTT1* may be involved in the etiology of cancer at more than one site. Third, the risk conferred to individuals who carry homozygous deletions in *GSTM1* or *GSTT1* appears to be small in magnitude (e.g., odds ratio of <2). However, the magnitude of risk is larger (e.g., odds ratio of 3-5) when interactions of *GSTM1* or *GSTT1* with other factors (e.g., cigarette smoking) are considered (Rebbeck, 1997). The combined *GSTM1* and *GSTT1* null genotype may be associated with increased lung cancer risk than for either null genotype alone (Jourenkova *et al.*, 1997).

GSTP1 is the major *GST* expressed in extra hepatic tissues such as the lungs and the esophagus with very little expression in the liver (Rowe *et al.*, 1997). *GSTP1* alleles have been described in four types, the wild-type allele and three variant alleles. Two single nucleotide polymorphisms (SNPs) in *GSTP1* resulting in amino acid substitutions that affect enzyme activity function are rs1695 (formerly rs947894), which is due to an A313G substitution resulting in an Ile105Val amino acid change and rs1138272 (formerly rs1799811), which is due to a C341T substitution resulting in an Ala114Val amino acid change (Ali-Osman *et al.*, 1997). In vitro cDNA expression, study suggests that substitution of these amino acid reduces enzyme activity (Zimniak *et al.*, 1994). An amino acid substitution from isoleucine to valine at residue 105 in the GSTP1 gene

(Ile105Val), reduces catalytic activity of the enzyme. The *GSTP1* polymorphism in exon 6 is less common than that in exon 5 (Yamamura *et al.*, 2000). Ala114Val polymorphism in exon 6 was less common than the Ile105Val in population and there was a non-significant trend toward lower mean GST enzyme activity among individuals with the 114 valine allele but *GST* activities in lung tissue were significantly lower among individuals with the 105Val allele. Thus, the polymorphism at codon 105 of human *GSTP1* gene results in active proteins with different enzyme activity (Ali-Osman *et al.*, 1997). The polymorphisms of human *GSTP1* gene for codon 105 present three genotypes: wild genotype (AA, Ile/Ile), heterozygous mutant genotype (AG, Ile/Val) and homozygous mutant genotype (GG, Val/Val). GSTP1 enzymes with 105Val allele showed different catalytic efficiency for various chemicals or drugs (Ryberg *et al.*, 1997). Individuals with the 105Val allele have a higher risk of developing lung cancer than individuals with the 105Ile allele (Ryberg *et al.*, 1997).

We conducted a case-control study to investigate the association between the risk of lung cancer and *GSTM1*, *GSTT1 and GSTP1* polymorphisms for first the time in Bangladesh. Although a series of studies have been conducted in Caucasians, Asians-mostly in Chinese, Japanese and Korean population, no study of *GSTM1, GSTP1* and *GSTT1* as risk factors for lung cancer has been conducted on a Bangladeshi population. A few case-control studies of *GSTM1* and *GSTT1* have been conducted in Indian sub-continent population, which is around one-sixth of the world's population, yet the South Asian countries has been sorely under-represented in genome-wide studies of human genetic variation. The International HapMap Project, for example, includes populations with African, East Asian and European ancestry — but no Indians.

Table 1.1: Description of our study genes

Gene	Known Alleles	Nucleotide change	Amino-acid change	Chromosome location
GSTM1	GSTM1*0	gene deletion in intron 6		1p13.3
	GSTM1*A	C (exon 7,534), wild type	Lys (codon 172)	
	GSTM1*B	G (exon 7,534)	Asn (codon 172)	
GSTP1	GSTP1*A	A (exon 5, 313), wild type	Ile(codon105)	11q13.3
		C (exon 6,341)	Ala(codon 114)	
	GSTP1*B	G(exon 5,313)	Val(codon 105)	
		C(exon 6,341)	Ala(codon 114)	
	GSTP1*C	G (exon 5,313)	Val(codon 105)	
		T(exon 6,341)	Val(codon 114)	
	GSTPI*D	A (exon 5,313)	Ile (codon 105)	
		T(exon 6,341)	Val(codon 114)	
GSTZ1	GSTZ1*A	A (exon 3,94)	Lys (codon 32)	14q24.3
		A (exon 3,124)	Arg (codon 42)	
	GSTZ1*B	A (exon 3,94)	Lys (codon 32)	
		G (exon 3,124)	Gly (codon 42)	
	GSTZ1*C	G (exon 3,94)	Glu (codon 32)	
		G (exon 3,124)	Gly (codon 42)	
GSTM3	GSTM3*A	Wild type		1p13.3
	GSTM3*B	3 bp deletion in intron 6		
GSTT1	GSTT1*0	gene deletion		22q11.2
	GSTT1*(positive)			

The closest the Human Genome Diversity Cell Line Panel of 51 global populations comes is Pakistan, India's western neighbor. The Indian Genome Variation database was launched in 2003 to fill the gap, but so far the project has studied only 420 DNA-letter differences, called single nucleotide polymorphisms (SNPs), in 75 genes (CONSORTIUM, 2008). The ancestry in Indian subcontinent is unique (Reich *et al.*, 2009). Researchers showed that most Indian populations are genetic admixtures of two ancient, genetically divergent groups, which each contributed around 40-60% of the DNA to most present day populations. One ancestral lineage — which is genetically similar to Middle Eastern, Central Asian and European populations — was higher in upper-caste individuals and speakers of Indo-European languages such as Hindi, other lineage was not close to any group outside the subcontinent, and was most common in people indigenous to the Andaman Islands, a remote archipelago in the Bay of Bengal (CONSORTIUM, 2008). The Indian population is believed to be most diverse because of different socio-cultural traditions. The variation in our population from the rest of the world population signifies the impact of ethnicity. Thus, this kind of study may form the basis for future establishment of epidemiological and clinical databases. This approach also has the potential for identifying susceptible individuals. The present work thus provides probably the first study of this nature from Bangladesh. We believe that further investigation of GSTM1, GSTT1 and GSTP1 allelic variants in Bangladesh should provide useful information for identification of founder mutations and ethnic predisposition alleles that result various cancerous disease phenotypes.

1.1 Lung Cancer

Cancer is a term used for diseases in which abnormal cells divide without control and are able to invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymph systems. Exposure to cigarette smoke, excess radiation and other environmental carcinogens along with genetic factors can cause malignant transformation (carcinogenesis) of normal cells. Malignant or cancerous cells grow and divide independent of the needs and limitations of the body, avoiding the resting state typical of normal cell. The transformation from a normal cell into a tumour cell is a multistage process, typically a progression from a pre-cancerous lesion to malignant tumours. These changes are the result of the interaction between a person's genetic factors and three categories of external agents, including:

- Physical carcinogens, such as ultraviolet and ionizing radiation.
- Chemical carcinogens, such as asbestos, components of tobacco smoke, aflatoxin (a food contaminant) and arsenic (a drinking water contaminant).
- Biological carcinogens, such as infections from certain viruses, bacteria (WHO, January 2013).

It is possible that genetic susceptibility to lung cancer may in part be determined by inter-individual variations in the genetic factors associated with cigarette smoking. Advances in molecular biology have led growing interest in investigation of biological markers, which may increase/decrease predisposition to smoking-related carcinogenesis.

Mortality, Incidence and Prevalence associated with lung cancer.

Globally in Males

I. 0.85 Million deaths in 2002.
II. 29.4 ages standardized death rates (world) per 100,000 in 2008.
III. 34.0 age standardized incidence rates (world) per 100,000 in 2008.
IV. One year prevalence of 0.38 million and five year prevalence of 0.93 million in 2002.

Globally in Females

I. 0.33 Million deaths in 2002.
II. 11.0 age standardized death rates (world) per 100,000 in 2008.
III. 13.5 age standardized incidence rates (world) per 100,000 in 2008.
IV. One year prevalence of 0.16 million and five year prevalence of 0.42 million in 2002.

1.2.1. Classification of lung cancer

Most lung cancer starts in the lining of the bronchi, but it can also start in other parts of the lung including in the trachea and bronchi (singular, bronchus) (ACS, February 16, 2010). The American Cancer Society (ACS) classifies lung cancer into two main subtypes:

I. Small Cell Lung Cancer (SCLC)
II. Non-Small Cell Lung Cancer (NSCLC)

Small Cell Lung Cancers are also known by Oat Cell Carcinoma and Small Cell Undifferentiated Carcinoma. This cancer often starts in the bronchi near the center of the chest. Although the cancer cells are small, they can divide quickly, form large tumors, and spread to lymph nodes and other organs throughout the body. Small Cell

type accounts for 10% to 15% of all lung cancers.

Non-Small Cell Lung Cancer has three subtypes. About 8 to 9 out of 10 cases of all lung cancers are the non-small cell type. The cells in these sub-types differ in size, shape, and chemical make-up. These three subtypes include:

1. Squamous Cell Carcinoma: About 25% to 30% of all lung cancers are of this kind. They are linked to smoking and tend to be found in the middle of the lungs, near a bronchus (February 02, 2010).

2. Adenocarcinoma: This type accounts for about 40% of lung cancers. It is usually found in the outer part of the lung (ACS, February 16, 2010.). Adenocarcinomas arise from the glandular cells located in the epithelium lining of the bronchi and are typically peripherally located, often near the pleural surface. Adenocarcinomas were sub typed by Noguchi into pathological sub-types A-F. These sub-types, A to F, are associated with progressively poorer prognoses in terms of long term survival:

A. Localized bronchioalveolar carcinoma (LBAC)
B. LBAC with foci of collapsed alveolar structures
C. LBAC with foci of active fibroblastic proliferation
D. Poorly differentiated adenocarcinoma
E. Tubular adenocarcinoma
F. Papillary adenocarcinoma with compressive and destructive growth

3. Large-Cell (undifferentiated) Carcinoma: About 10% to 15% of lung cancers are of this type. It can start in any part of the lung. It tends to grow and spread quickly, which makes it harder to treat (American Cancer Society. Overview: Lung cancer).

This distinction is important, because the treatment varies; non-small cell lung carcinoma (NSCLC) is sometimes treated with surgery, while small cell lung carcinoma (SCLC) usually responds better to chemotherapy and radiation. The most common symptoms are shortness of breath, coughing (including coughing up blood) and weight loss.

1.2.2 Clinical presentation

Most patients with lung cancer are symptomatic at the time of diagnosis. Unfortunately, their symptoms are often associated with locally advanced or distant disease, which may render them inoperable (Eric B. Haura).

Table 1.2 Lung cancer symptoms and signs (Radzikowska et al., 2002).

Systemic	Asymptomatic	Metastatic
Anorexia	Primary	Regional
Weight loss	Cough	Hoarseness
Fatigue	Wheeze and stridor	Superior vena cava syndrome
Weakness	Chest pain	Dysphagia
Finger clubbing	Shortness of breath	Horner's syndrome
Paraneoplastic endocrine syndromes	Hemoptysis	Brachial plexus pain
	Fever, chills, or sweats (associated pneumonia)	Chest wall pain

Specific signs and symptoms depend on the location of the tumor, its loco regional spread, and the presence of metastatic disease. In addition, Para neoplastic syndromes occur more frequently in lung cancer than in any other tumor. Also, some patients are totally asymptomatic and for unrelated reasons undergo incidental chest x-ray and are found to have an asymptomatic lesion. Unfortunately, many of the symptoms of lung cancer are nonspecific and in the elderly may be attributed to comorbid illness. This may result in a delay in diagnosis, which may have profound effects on the treatment options available for the patient.

Table 1.3. Lung cancer symptoms that may be confused with non-cancer symptoms in the elderly.

Lung Cancer Symposium	Cormorbid disease or "aging explanation"
Cough	Chronic bronchitis
Dyspnea	Emphysema, old age
Fever (post obstructive pneumonia)	Cold, flu
Weight loss	Depression, inactivity
Bone pain (bone metastases)	Arthritis
Altered mental status (brain metastases, hypercalcemia)	old age

Source: From Crawford et al. With permission.

Although mass screening for lung cancer has not been recommended, high-risk patients

over age 65 might benefit from screening to detect earlier-stage squamous cell carcinomas with favorable prognosis. It would seem logical that patients presenting with early-stage lung cancer are far more likely to be cured than those patients with advanced disease. However, over the past 30 years, the percentage of localized disease and respectability rates has remained unchanged at approximately 20%, indicating that screening and early detection programs have been unsuccessful. Several prospective randomized studies using serial chest x-rays and sputum cytologies to complement each other in early lung cancer diagnosis did not result in detection of lung cancers at a curable stage or demonstrate that intensive screening led to a lower death rate from lung cancer. However, a study conducted by O'Rourke et al suggested that lung cancer may present at a less advanced stage with increasing age. Information from the centralized cancer patient data system with a total of 22,874 cases showed that the percentage of lung cancer patients with local stage disease increased from 15.3% for patients aged 54 years or younger to 25.4% of those 75 years or older. An additional 6,332 patients who underwent surgical staging were analyzed and showed a greater likelihood of presenting with local disease with an increase in age. Therefore, in addition to having a higher age-specific incidence, older cancer patients may have a higher likelihood of local stage lung cancer. Thus, the older high-risk patient, smoker or former smoker, should be followed carefully for the development of lung cancer. In the absence of an official recommendation for routine screening, the physician should have a low threshold for obtaining a chest x-ray in these patients as symptoms develop (Eric B. Haura). The case for screening for lung cancer has recently resurfaced with the results of the Early Lung Cancer Action Project (ELCAP) group, who examined the usefulness of annual helical low-dose computed tomography (CT) scanning compared to chest x-ray in

heavy smokers over the age of 60. Cancers were detected in 2.7% of patients by CT scan compared to 0.7% by chest x-ray; 85% of the CT-detected tumors were stage I and all but one were respectable. The overall rate of detection by CT scanning was six times higher than by chest x-ray. To prevent a large number of questionable biopsies, recommendations were made by the ELCAP investigators to initially biopsy only nodules with no smooth edges or no calcified nodules 10 mm or larger. For smaller nodules, documented growth by high-resolution CT was recommended before biopsy. Biopsies were done on 28 of 233 patients with no calcified lesions, with 27 having malignant disease and 1 having a benign nodule. Despite these encouraging results, a number of problems still remain. First, the 27 cancers detected by CT scanning represented only one-quarter of all nodules found on CT scans, potentially necessitating a large number of follow-up scans. The need for biopsies, however, was maintained at a reasonable level, possibly because of recommendations offered by the investigators. Issues regarding cost also remain unclear although the investigators claim that the CT scan costs are only slightly higher than that of a chest x-ray and that only 20 s of CT time are required to obtain the images. Finally, the ability of annual screening CT scans to improve overall survival in smokers remains to be determined (Eric B. Haura, 2004).

1.2.3 Lung cancer staging

Lung cancer staging is a method by which the extent of disease is classified. This process is important in identifying appropriate treatment approaches and determining prognoses. All available factors, including clinical factors (physical exam, imaging and laboratory findings) and pathological finding (from tissue specimens obtained via bronchoscopy, mediastinoscopy or surgery are used to determine stage. The methods for staging differ

based on cellular classification. For non-small cell lung cancers (NSCLC) the American Joint Committee on Cancer has designated staging by tumor, node and metastases (TNM) classification. This staging system takes into account the extent of the tumor (T), the level of regional lymph node involvement (N) and the presence of metastases (M) (Tanoue, 2008)

1.2.3.1 Primary tumor (T)

i. TX: Positive malignant cytology finding with no observable lesion
ii. Tis: Carcinoma in situ
iii. T1: Diameter of 3 cm or smaller, is surrounded by lung or visceral pleura, and is without invasion more proximal than the lobar bronchus
iv. T2: Diameter greater than 3 cm and/or has extension to the visceral pleura, atelectasis, obstructive pneumonitis that extends to the hilar region but does not involve the whole lung or tumor of a main bronchus more than 2 cm distal from the carina
v. T3: A tumor of any size that directly invades any of the following: chest wall (including superior sulcus tumors), diaphragm, mediastinal pleura, parietal pericardium; or, associated atelectasis or obstructive pneumonitis of the entire lung or, tumor in the main bronchus less than 2 cm distal to the carina but without involvement of the carina
vi. T4: A tumor of any size that invades any of the following: mediastinum, heart, great vessels, trachea, esophagus, vertebral body, carina; or, separate tumor nodules in the same lobe; or, tumor with a malignant pleural effusion.

1.2.3.2 Regional lymph nodes (N)

vii. NX: Regional lymph nodes cannot be assessed
viii. N0: No regional lymph node metastasis
ix. N1: Metastasis to ipsilateral peribronchial and/or ipsilateral hilar lymph nodes, and intrapulmonary nodes including involvement by direct extension of the primary tumor
x. N2: Metastasis to ipsilateral mediastinal and/or subcarinal lymph node(s)
xi. N3: Metastasis to contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s)

1.2.3.3 Metastasis (M)

i. MX: Distant metastasis cannot be assessed
ii. M0: No distant metastasis
iii. M1: Distant metastasis present

Using the TNM classification to describe the level of tumor invasion, a staging system and these stages can be linked to estimated survival rates;

i. Stage 0: TisN0M0
ii. Stage IA: T1N0M0, 5 year survival rate of 60-80%
iii. Stage IB: T2N0M0, 5 year survival rate of 50-60%
iv. Stage IIA: T1N1M0, 5 year survival rate of 40-50%
v. Stage IIB: T2N1M0 or T3N0M0, 5 year survival rate of 25-40%
vi. Stage IIIA: T3N1M0 or T(1-3)N2M0, 5 year survival rate of 10-35%
vii. Stage IIIB: T4N(0-3)M0 or T(1-4)N3M0, 5 year survival rate of 5%

viii. Stage IV: T(1-4)N(0-3)M1, 5 year survival rate less than 5%

Due to the aggressive nature of small cell lung cancers (SCLC), the majority of diagnosed patients also have metastases and hence a simple two stage classification (limited versus extensive) is typically favored over the detailed TNM staging used for NSCLC. Limited stage SCLC is assigned for small tumors which are confined to the chest (including mediastinum and supraclavicular node) with no pleural effusion. Limited SCLC stage is associated with a 2 year survival rate of 20%. Extensive stage SCLC is assigned for an occurrence of distant metastases and/or for any tumor too extensive to be incorporated into the limited stage. The prognosis for extensive stage SCLC is a 2 year survival rate of 5% (2009); (Dollinger et al., 2002).

1.2.4 Causes of lung cancer

Cigarette smoking is by far the most important cause of lung cancer, and the risk from smoking increases with the number of cigarettes smoked and the length of time spent smoking (Services, 2004). Other recognized causes include radon (Biological Effects of Ionizing Radiation (BEIR) VI Report, 1999). secondhand smoke(USDHS, 2006) and some occupational chemicals and air pollutants like benzene, formaldehyde pollution (USEPA, November 30, 2009). Asbestos, a product used in insulation and manufacturing for years, is also an important cause of lung cancer (Centers for Disease Control and Prevention . malignant mesothelioma mortality — United States, April 2009)

It has been estimated that active smoking is responsible for close to 90 percent of lung cancer cases; radon causes 10 percent, occupational exposures to carcinogens account for approximately 9 to 15 percent and outdoor air pollution 1 to 2 percent. Because of the interactions between exposures, the combined attributable risk for lung cancer exceeds 100

percent (Alberg AJ, 2003).

1.2.4.1 Smoking and secondhand smoke

Cigarette smoking is the number one risk factor for lung cancer. In the United States, cigarette smoking causes about 90% of lung cancers. Tobacco smoke is a toxic mix of more than 7,000 chemicals. Many are poisons. At least 70 are known to cause cancer in people or animals. People who smoke are 15 to 30 times more likely to get lung cancer or die from lung cancer than people who do not smoke. Even smoking a few cigarettes a day or smoking occasionally increases the risk of lung cancer. The more years a person smokes and the more cigarettes smoked each day, the more risk goes up (Alberg *et al.*, 2007b). People who quit smoking have a lower risk of lung cancer than if they had continued to smoke, but their risk is higher than the risk for people who never smoked (WHO, 2003). Smoking can cause cancer almost anywhere in the body. Smoking causes cancer of the mouth, nose, throat, larynx, esophagus, bladder, kidney, pancreas, cervix, stomach, blood, and bone marrow (acute myeloid leukemia) (Eric B. Haura).

1.2.4.2. Environmental carcinogens

Environmental carcinogens can cause genetic damage contributing to the development of cancer. Some of the environmental carcinogens are- **Radon, Asbestos, Arsenic, Chromium, Nickel, Polycyclic Aromatic Hydrocarbons (PAHs).**

1.2.4.3. Other environmental lung carcinogens

Bis(chloromethyl)ether, chloromethyl methyl ether, ionizing radiation (x-rays), gamma radiation, mustard gas, vinyl chloride. There are also some suspected lung carcinogens- acrylonitrile, cadmium, beryllium, lead, ferric oxide dust.

1.2.4.4. Diet

Scientists are studying many different foods and dietary supplements to see whether they increase the risk of getting lung cancer. There is much we still need to know. We do know that smokers who take beta-carotene supplements have increased risk of lung cancer (WCRF, 2010).

1.2.4.5. Radiation therapy

Radiation therapy was recognized as a potential cause of cancer many years ago. In fact, much of what we know about the possible health effects of radiation therapy has come from studying survivors of atomic bomb blasts in Japan. We also have learned from workers in certain jobs that included radiation exposure, and patients treated with radiation therapy for cancer and other diseases (WCRF, 2010).

1.2.4.6. Lung diseases

Recent studies have shown an increased risk of lung cancer in patients with bronchial obstructive changes, including patients with COPD. It seems that there are common factors of pathogenesis of both diseases associated with oxidative stress. The genes linked to the repair of oxidative damage of DNA, associated with cancer, of iron metabolism and coding proteolytic enzymes were assessed in different studies (Grudny *et al.*, 2013).

1.2.4.7. Viruses

Many viruses infect humans but only a few viruses are known to promote human cancer. These include DNA viruses and retroviruses, a type of RNA virus. Viruses associated with cancer include human papillomavirus (genital carcinomas), hepatitis B (liver carcinoma), Epstein-Barr virus (Burkitt’s lymphoma and nasopharyngeal carcinoma), human T-cell leukemia virus (T-cell lymphoma); and, probably, a herpes virus called KSHV (Kaposi’s sarcoma and some B cell lymphomas). The ability of retroviruses to promote cancer is

associated with the presence of oncogenes in these viruses. These oncogenes are very similar to proto-oncogenes in animals. Retroviruses have acquired the proto-oncogene from infected animal cells. An example of this is the normal cellular c-SIS proto-oncogene, which makes a cell growth factor. The viral form of this gene is an oncogene called v-SIS. Cells infected with the virus that has v-SIS overproduce the growth factor, leading to high levels of Cell growth and possible tumor cells. Viruses can also contribute to cancer by inserting their DNA into a chromosome in a host cell. Insertion of the virus DNA directly into a proto-oncogene may mutate the gene into an oncogene, resulting in a tumor cell. Insertion of the virus DNA near a gene in the chromosome that regulates cell growth and division can increase transcription of that gene, also resulting in a tumor cell. Using a different mechanism, human papillomavirus makes proteins that bind to two tumor suppressors, p53 protein and RB protein, transforming these cells into tumor cells. Remember that these viruses contribute to cancer; they do not by themselves cause it. Cancer, as we have seen, requires several events (Gibbs, 2003).

1.3. Molecular Genetics of Lung Cancer

Lung cancer results from multiple changes in the genome of susceptible pulmonary cells caused by exposure to carcinogens found in tobacco smoke, the environment, or the workplace. Recent studies suggest that histologically apparent lung cancer is due to the sequential accumulation of specific genetic and morphologic changes to the normal epithelial cells of the lung. Positive signalers, such as those mediated by the oncogene RAS, and negative signalers, such as those mediated by the tumor suppressor retinoblastoma protein (RB), contribute to unchecked cell growth and proliferation. Other

key molecular derangements can also be considered hallmarks of cancer, including evasion of apoptosis and senescence, angiogenesis, tissue invasion, and metastases. Epigenetic inactivation of genes via DNA methylation provides another novel way of evading normal cellular control mechanisms. The new knowledge of the human genome coupled with global methods of detecting genetic abnormalities and profiling gene expression in tumor cells may enable us to understand the signaling pathways of lung cancer cells. These are molecular targets for new cancer therapeutics such as receptor tyrosine kinase inhibitors. This information could advance risk assessment, early detection, prognosis, and therapy for lung cancer (Sekido *et al.*, 2003). It is becoming clear that the genetic changes acquired by lung cancers are not only multiple but also complex and heterogeneous both in chronology and mechanistic pathways. Cancer cells may harbor a homogeneously amplified region of chromosome 8q, consistent with activation of the proto-oncogene MYC through copy number amplification. In addition, there may be frequent loss of heterozygosis at chromosomal loci. Such loss of heterozygosis indicates one of the two "hits" that are generally required to inactivate a tumor suppressor gene (TSG), e.g., 17p forp53, 9p21 for p14 ARF andp16INK4a, 13q14 for RB, and multiple loci of 3p for FHIT, RASSF1A, and/or other unidentified genes (Hanahan D, 2000). There are also emerging data on the molecular lesions that are specific to one of the two major lung cancer subtypes and those that are common to both. Some genes are targeted for both subtypes (and other solid human cancers), such as mutations ofp53. Others can be relatively specific to a subtype and may play a role in its differentiation. For example, SCLC features more frequent alteration in myc activation and RB inactivation than NSCLC, whereas NSCLC has more in ras activation and $p^{16\ INK4a}$ inactivation (Hanahan D, 2000).

Table 1.4 Frequent molecular genetic changes in lung cancer

	SCLC	NSCLC
Frequent allelic loss	3p,4p,4q,5q,8p,10q, 13q,17p,22q	3p,6q,8p,9p,13q, 17p,19q
RAS mutations	< 1%	15-20%
BCL2 expression	75-95%	10-35%
MYC family overexpression	15-30%	5-10%
RB1 inactivation	~90%	15-30%
p^{53} inactivation	80-90%	~50%
$p16^{INK4a}$ inactivation	0-10%	30-70%
RARβ	70%	40%
FHIT inactivation	~75%	50-75%

*Adapted from Sekido et al. [2001].

1.4 Genetic Polymorphisms and Lung Cancer Susceptibility

1.4.1 Metabolic polymorphisms

Polymorphism in drug metabolizing enzymes is caused by mutations in genes that code for specific biotransformation enzyme (Dykes CW 1966). Generally they follow the autosomal recessive trait that means that the mutations are not sex linked (autosomal) and that one mutated allele does not express the phenotype when combined with a normal, not mutated (dominant) allele (Weaver RF & Hedrick PW, 1977).

A large number of enzymes most of which are polymorphic participate in metabolism of xenobiotics such as drugs and carcinogens. In phase I, drug metabolizing enzymes DMEs,

mostly cytochromes P450 (CYPs), metabolically activate xenobiotics to reactive electrophilic forms which is then conjugates to some endogenous compound by Phase II DMEs; such as UDP-glucuronosyltransferases, (UGTs), N-acetyl transferases (NATs), glutathione S-transferases (GSTs), or others. Genetic polymorphism of these enzymes involved in this process leads to inter-individual variations in metabolism and pharmacokinetics of drugs and could therefore influence drug response. About 40 % of phase I metabolism of clinically used drugs is affected by polymorphic enzymes. (Nebert DW 1999). Among the conjugating enzymes in drug metabolism, NAT2 was one of the first to be found to have a genetic basis some 50 years ago. About 15 allelic variants have been identified, and some of which are without functional effect, but others are associated with either reduced or absent catalytic activity. Similarly, genetic variability in the catalytic activity of Glutathione S-transferases (GSTs) may be linked to individual susceptibility to drug toxicity (Tetsuo Satoh, 2007).

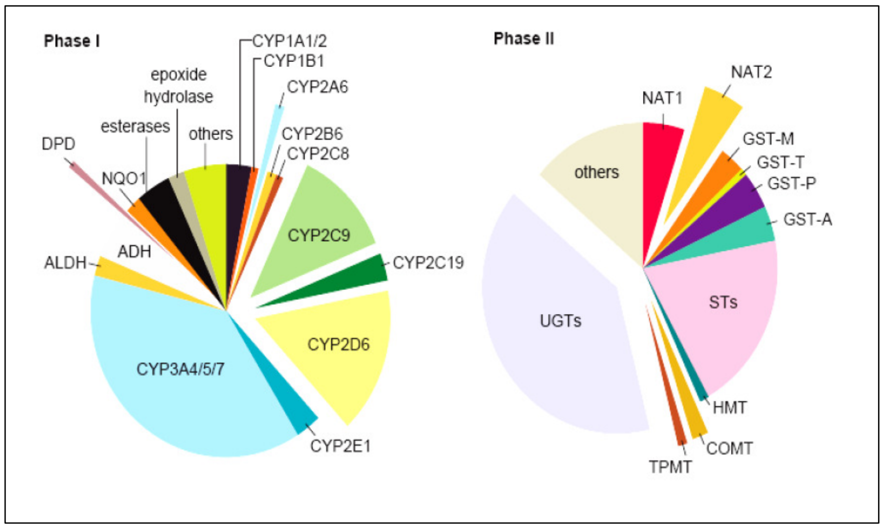


Figure 1.1: Genetic polymorphisms in drug metabolizing enzymes. Participation of specific human liver cytochrome P450 enzymes (left side) and phase-II-enzymes (right side) in drug

metabolism. The sizes of the segments refer to the relative number of drugs metabolized by the respective enzyme, e.g. about 40% of all currently used drugs are metabolized by cytochrome P4503A enzymes (bright blue segment) (Evans and Relling 1999).

1.4.1.1 Cytochrome P450s

Cigarette smoke contains several thousand chemicals, of which about 50 compounds are known carcinogens, including polycyclic aromatic hydrocarbons (PAHs), aromatic amines and N-nitroso compounds. Some of these compounds are reactive carcinogens, but most are procarcinogens, which need to be activated by Phase-I enzymes such as those encoded by the CYP supergene family, and converted into reactive carcinogens. All these reactive carcinogens can bind to DNA and form DNA adducts capable of inducing mutations and initiating carcinogenesis. CYPs are a multi-gene super-family of mixed function monooxygenases. Based on sequence homology, the CYP superfamily is divided into 10 subfamilies, CYP1/CYP10. Subfamilies CYP1CYP2, CYP3 and CYP4 are primarily involved in drug metabolism (Kiyohara *et al.*, 2006; Reszka *et al.*, 2006) (Gresner *et al.*, 2007). A positive association between development of lung cancer and the mutant homozygous genotype of CYP1A1 MspIorCYP1A1Ile-Val polymorphism has been reported in several Japanese populations but such an association has been observed in neither Caucasians nor American/Africans. The CYP1A1 MspI polymorphism has a higher variant allele frequency than the CYP1A1Ile-Val polymorphism. The relationship between CYP2D6 gene and lung cancer remains conflicting and inconclusive. Several polymorphisms have been identified at the CYP2E1 locus. No definitive link between the polymorphisms of CYP2E1 and the risk of lung cancer has, however, been identified. Some studies on

CYP2A6, CYP2C9 and CYP2C19 have indicated a relationship between lung cancer and the occurrence of a rare allele, although future research is needed in order to establish a significant relationship (Reszka *et al.*, 2006).

1.4.1.2 Glutathione S-transferases

Following Phase-I reaction, Phase- II enzymes such as GSTs are responsible for detoxification of activated forms PAH epoxides. GSTs are constitutively found in a wide variety of tissues, with different characteristic patterns of GST isozymes. GST genes form a super-family of at least 13 genes consisting of five distinct families, named alpha (GSTA), sigma (GSTS), mu (GSTM), pi (GSTP) and theta (GSTT). Certain genes within the GSTM, GSTT and GSTP subfamilies (GSTM1, GSTT1 and GSTP1) are polymorphic in humans and the levels of individual enzymes expressed can be influenced by induction and genetic polymorphism. The role of GSTM1, GSTT1 or GSTP1 polymorphism in modifying the lung cancer risk may be more limited than has been so far anticipated (Reszka *et al.*, 2006).

1.4.1.3 Combined phase I and II polymorphisms

Since genetic polymorphisms have been found for both phase I and II enzymes, risk assessment could be increased in sensitivity if polymorphisms in both phases of enzymes are taken into consideration as biomarkers for susceptibility to cancer. It is likely that an individual with the high risk genotype (either a genotype coding for a more active phase I enzyme or a less efficient phase II enzyme, or both of those) might be at higher risk of cancer than that with the opposite genotype (combination). The data in Asian population studies to date indicate the combined genotype between CYP1A1 MspI and GSTM1 polymorphisms reveals higher OR for lung cancer than a single locus (Reszka *et al.*, 2006). The association between the CYP1A1Ile-Val polymorphism and lung cancer varied by

GSTM1 status in a Chinese population with the mutant allele frequency of CYP1A1Ile-Val polymorphism intermediate between Japanese and Caucasian population (London *et al.*, 2000b). For non-Asian populations, the relevance of CYP1A1 MspI and GSTM1 polymorphisms to lung cancer is questionable (Alexandrie *et al.*, 1994).

1.4.1.4 Microsomal epoxide hydrolase

Microsomal epoxide hydrolase (mEH) is an important phase II biotransformation enzyme

Table 1.5: Selected phase I enzymes, tissue distribution of these enzymes and carcinogens activated by them (Hecht, 1999)

Genes	Chromosomal location	Tissue expression	Activation of constituents in tobacco smoke
CYP1A1	15q22-q24	Lung, skin, placenta, lymphocytes	PAHs
CYP2D6	22q13.1	Lung, liver, intestine, prostate	TSNAs
CYP2E1	10q24.3-qter	Lung, liver, brain, kidney	TSNAs
CYP2A6	19q13.2	Lung, nasal, pharyngeal	TSNAs
CYP2C9	10q24.1-q24.3	Liver, Intestine	PAHs
CYP2C19	10q24.1-q24.3	Liver, prostate	PAHs
EPHX1	1q42.1	Lung, liver, kidney, testis, lymphocytes	PAHs
NOQ1	16q22	Lung, liver, brain, kidney	PAHs, HCAs

MPO	17q21-23	Neutrophils, monocytes, macrophages	PAHs, HCAs, AAs

PAH: Polycyclic Aromatic Hydrocarbon, **TSNA-** Tobacco Specific Nitrosamines, **HCA:** Heterocyclic Amine, **AA**: Arylamine

and it is highly expressed in several human tissues including the lung, where it catalyzes the hydrolysis of various epoxides and reactive epoxide intermediates into less reactive and more water soluble dihydrodiols, which are then excreted from the body (Hassett *et al.*, 1994b; Seidegard *et al.*, 1997). Hence, mEH is a protective enzyme involved in general oxidative defenses against a number of environmental substances (Harrison *et al.*, 1999; Oesch, 1973). However, mEH is also involved in the xenobiotic activation of tobacco carcinogens. Combined with CYP, mEH can metabolize PAHs into highly mutagenic and carcinogenic diol epoxides (Gelboin, 1980; Miyata *et al.*, 1999; Pastorelli *et al.*, 1998). Thus, the activation or inactivation effects of mEH depend on the specific compounds being metabolized. The mEH gene is located on chromosome 1q42.1. There are two polymorphisms that affect enzyme activity in the human mEH gene. One variant is characterized by substitution of histidine for tyrosine (Tyr113His) in exon 3 (EH3) and the other is substitution of arginine for histidine (His139Arg) in exon 4 (EH4), conferring low and high enzymatic activity, respectively (Hassett *et al.*, 1994a). Lower activity mEH genotypes were associated with decreased lung cancer risk in several studies. French study found that lower activity of mEH3 genotype (His/His) was a protective factor for lung cancer (Wang *et al.*, 2013). Three Caucasian studies (London *et al.*, 2000a; Smith CA, 1997; To-Figueras *et al.*, 2001) two Chinese studies (Persson *et al.*, 1999; Yin *et al.*, 2001) and a

study of Wu et al. among African Americans and Mexican Americans (Wu *et al.*, 2001) found, however, no significant relationship between mEH3 genotype and lung cancer risk. A significant protective effect of low activity mEH3 genotype was observed among African/Americans (London *et al.*, 2000a) and Spaniards (To-Figueras *et al.*, 2001). The seven case-control studies of lung cancer and mEH3 genotype included 2626 subjects (1010 lung cancer cases and 1616 controls). The overall OR was 0.96 (95% CI=0.66-1.39). For EH4, six of seven studies could not found a significant association and the direction of the association was different among them (London *et al.*, 2000a; Persson *et al.*, 1999; Wu *et al.*, 2001; Yin *et al.*, 2001; Makowska *et al.*, 2010; Wang *et al.*, 2013). However, the exon 4 polymorphism associated with a significantly increased risk of lung cancer among Chinese and Mexican/Americans (Persson *et al.*, 1999; Wu *et al.*, 2001). The overall OR among the seven case-control studies with nine different ethnic populations (1010 lung cancer cases and 1616 controls) was 1.44 (95% CI=1.03-2.00), which was significantly different than 1.0. A test for heterogeneity suggested no significant heterogeneity when polymorphisms of EH3 and EH4 were combined, low activity genotype was significantly associated with a decreased risk of lung cancer among French Caucasian (Benhamou *et al.*, 1998). However, other studies among Caucasian populations (Harrison *et al.*, 1999; London *et al.*, 2000a; To-Figueras *et al.*, 2001) and among African/Americans (London *et al.*, 2000a) did not confirm the association. Although a Taiwanese study also found that low mEH activity genotype was not associated with decreased risk of lung cancer (OR =1.03, 95% CI=0.66-1.61), low activity genotype was significantly associated with a decreased risk of squamous cell carcinoma (OR=0.51, 95% CI =0.27-0.96) (Zhou W, 2001). The relationship between mEH genotypes and lung cancer risk has not been studied in a large

number of subjects. Recently, no relationship between the low activity genotype and lung cancer risk was found by a large American study. It also indicated that cumulative cigarette smoking exposures play pivotal roles in the association between both mEH polymorphisms and lung cancer risk, altering the direction of risk (in the case of combined low activity genotype in both EH3 and EH4 from a risk factor (OR=1.59, 95% CI=0.80-3.14) in non-smokers to a protective factor (OR=0.45, 95% CI=0.22-0.93) in heavy smokers (Zhou *et al.*, 2001). The six case-control studies in seven different ethnic populations of lung cancer and the combined genotype included 4381 subjects (1818 lung cancer cases and 1563 controls). The overall OR was 0.96 (95% CI=0.68-1.34). Differences in associations between ethnic subgroups or between study populations can result from linkage disequilibrium with additional allelic variants that modulate overall enzyme activity and may be present in different frequencies in the different groups or linkage disequilibrium with another gene that is casually related to lung cancer. Joint effects among mEH genotype, other genetic polymorphisms and cigarette smoking were investigated in additional studies.

1.4.1.5 NAD(P)H quinone oxidoreductase (DT-diaphorase)

NAD(P)H quinone oxidoreductase 1 (NQO1), formerly referred to as DT-diaphorase, is a cytosolic enzyme catalyzing the two-electron reduction of quinone substrates. NQO1 either metabolically activates or detoxifies carcinogens present in cigarette smoke. BP is one of the most important carcinogens and the formation of BP quinone/DNA adduct is prevented by NQO1(Joseph P, 1994). In contrast, carcinogenic heterocyclic amines present in smoke are metabolically activated by NQO1 (De Flora S, 1994). Therefore, this enzyme is thought to be involved in both metabolic activation and detoxification of carcinogenic agents that could be involved in lung carcinogenesis. The NQO1 gene is located on chromosome

16q22. Recently, a polymorphism of the gene encoding NQO1 has been described. The polymorphic variant of the gene (a C to T transition at base pair 609, codon 187) is associated with reduced NQO1 activity (D. Ross, 1996; Jaiswal *et al.*, 1988; Traver RD, 1992). The three genotypes of this gene are the homozygous wild-type C/C (normal activity), the heterozygous C/T genotype (mild activity) and the homozygous rare allele T/T genotype (2-4% of normal activity). There have been several studies examining the relationship between the NOQ1 polymorphism and lung cancer risk, but the conclusions have been contradictory (Rosvold et al., 2011). It is still unclear whether variant NQO1 genotype is associated with a decreased risk of lung cancer or not although a non-significant protective overall. NQO2 is a polymorphic gene that encodes an enzyme with similar activity to NQO1. NQO2 might be more important than NQO1 in determining lung cancer risk. As the role of NQO1 may be different among different histology and different ethnic groups, a larger study group is warranted to evaluate the effect of smoking amount on those parameters.

1.4.1.6 Myeloperoxidase (MPO)

Neutrophil recruitment into lung tissue occurs after exposure to variety of insults known to increase lung cancer risk, including tobacco smoke particles, infection, asbestos and ozone (G. W. Hunninghake, 1879; Schmekel *et al.*, 1990a; Schmekel *et al.*, 1990b). Following immunological or chemical insults, neutrophils release MPO and undergo a respiratory burst, which is characterized by a massive increase in oxygen consumption and a consequent NADPH-dependent production of superoxide and other free radicals (G. W. Hunninghake, 1879). MPO is present in the primary granules of neutrophils and catalyzes the production of the potent bacteriotoxic oxidizing agent hypochlorous acid (a one and

two-electron oxidant that can attack endogenous molecules including DNA) from hydroxyl radicals and chloride ions. A significant proportion (25-40%) of the hydrogen peroxide formed by activated neutrophils may be converted to hypochlorous acids (Foote *et al.*, 1983; Prutz, 1998; Tsuruta *et al.*, 1985). MPO metabolically activates a wide range of tobacco smoke mutagens and environmental pollutants to DNA-damaging metabolites, including aromatic amines (Tsuruta *et al.*, 1985) the promutagenic derivatives of PAHs and heterocyclic amines (Trush MA, 1985). The MPO gene is located on chromosome 17. A G to A transition at position 463 is the promoter region of the MPO gene.

1.4.2 DNA repair genes

Physiologically, the DNA repair capacity should be correlated with the level of proteins involved in DNA repair activity, which is controlled at the transcriptional level (Cleaver, 1968). Therefore, it is conceivable that the baseline transcriptional level of DNA repair genes reflects a cellular ability to meet repair demand once the cells are stimulated by carcinogen exposure. One major DNA repair pathway capable of removing a variety of structurally unrelated DNA lesions, including that induced by tobacco carcinogens, is nucleotide excision repair (NER). This complex DNA repair process consists of approximately 30 proteins involved in sequential damage recognition, chromatin remodeling, incision of the damaged DNA strand on both sides of the lesion, excision of the oligonuleotide containing the damage and gap-filling DNA synthesis followed by strand ligation (Asami *et al.*, 1997). For example, smoking-related bulky adducts induced by benzo(a)pyrene or other PAHs and arylamines are removed effectively by the NER pathway (Abrahams *et al.*, 1998). In xeroderma pigmentosum (XP), patients having extraordinarily higher rate of skin cancer because of a genetically determined defect in NER

(Cleaver, 1968). Other cancer prone patients who have deficient DNA repair also have a higher rate of internal cancer (Cleaver, 1968). Three rare, autosomal recessive inherited human disorders are associated with impaired NER activity: XP, Cockayne Syndrome (CS) and trichothiodystrophy (TTD). XP has been studied most extensively. Seven different human NER genes, which correct seven distinct genetic XP complementation groups (XPA, XPB, XPC, XPD, XPE, XPF and XPG) have been identified (de Boer *et al.*, 2000; Kenneth H. Kraemer, 2007). A number of genes that correct defective human NER have been designated as excision repair cross-complementing (ERCC) genes. The human gene responsible for XP groups B, D, F and G are identified as ERCC3, ERCC2, ERCC4 and ERCC5, respectively. ERCC1 has not been found to be involved in any XP, CS or TTD (Van Duin M, 1989), because defects in ERCC1 resulting from mutations or deletions of this cause early death before the disease develop (McWhir J, 1993). ERCC6 is identical to CSB and mutations in this gene are involved in CS (Troelstra *et al.*, 1992). Concerning DNA repair genes, 11 genes have been reported to date nine NER genes (ERCC1-6, XPA, XPE and XPF), a gene involved in double-strand break repair/recombination genes (X-ray cross-complementing group 3, XRCC3) and a gene functioning in base excision repair and the repair of radiation-induced damage (XRCC1). Polymorphisms in DNA repair genes may be associated with differences in the repair efficiency of DNA damage and may influence an individual's risk of lung cancer because the variant genotype in those polymorphisms might destroy or alter repair function.

1.4.2.1 Glutathione peroxidase 1 (GPX1) and human 8-oxoguanine/DNA glycosylase 1 (hOGG1)

In view of its abundance and mutagenicity, a number of defense mechanisms operate to

minimize 8-hydro-xyguanine (oh8Gua) accumulation within the genome. oh8Gua is a major DNA lesion produced by oxygen-radicals. Primary defense mechanisms include anti-oxidants and enzymes such as glutathione peroxidase (Halliwell, 1994; Kasai *et al.*,1993; Wang *et al.*,1998). Glutathione peroxidases reduce organic peroxides and hydrogen peroxides through the coupled oxidation of reduced glutathione. Glutathione peroxidase 1 (GPX1) is the major cytosolic form of this enzyme, but other isozymes are found in the plasma and phospholipid membranes (Moscow JA, 1994). The cytosolic form of human GPX1 belongs to a family of selenium dependent peroxidases that include another cytosolic forms, hGXP2 (Chu FF, 1993), the plasma-based hGXP3 (Takahashi K, 1990) and the phospholipids hydroperoxidase hGPX4 (Maiorino M, 1991) Polymorphisms in GPX1 are characterized by a variable polyalanine repeat and the six-alanine repeat form ALA6, instead of ALA5 or ALA7 with the wild-type proline also contains a proline to leucine substitution at codon 198 towards the C-terminus (Moscow *et al.*, 1994). Recently, this polymorphism showed no functional effect on erythrocyte GPX1 activity (Forsberg *et al.*, 2000). In addition, the presence of the ALA6 allele the Leu allele was non-significantly associated with reduced levels of oh8Gua levels (Hardie *et al.*, 2000). Although biochemical characterization of the human GPX1 enzymes encoded by the distinct genotype of the GPX1 polymorphism is still unclear, constitutive genotype may play a significant role in determining oh8Gua levels within tissue DNA. The formation of oh8Gua in DNA causes G:C to T:A transversion, since oh8Gua pairs with adenine as well as cytosine (Cheng *et al.*, 1992; Shibutani *et al.*, 1991). The human 8-oxoguanine DNA glycosylase 1 (hOGG1) gene encodes base excision repair proteins for oh8Gua in double-stranded DNA (Aburatani *et al.*, 1997). The OGG1 protein possesses the ability to excise oh8 Gua paired with cytosine

(Aburatani *et al.*, 1997). The hOGG1 Ser326Cys polymorphism was initially identified by Kohno et al. (Kohno *et al.*, 1998). Preliminary evidence from Escherichia coli complementation assay suggested that the hOGG1Cys isoform exhibited reduced oh8Gua repair activity (Kohno *et al.*, 1998). However, Hardie et al (Kohno *et al.*, 1998) suggested that differences in oh8Gua glycosylase activity within hOGG1 polymorphic variants were insufficient to impact on tissue oh8 Gua levels because levels of oh8Gua did not vary with hOGG1 genotype. Both GPX1 and hOGG1 locate to regions of chromosome 3p (3p21 and 3p25/26, respectively) which are subjected to frequent and early loss of heterozygosity during lung cancer development (Hibi *et al.*, 1992; Lu *et al.*, 1997; Moscow *et al.*, 1994).

1.4.3 Germline polymorphism of tumor suppressor gene

The p53 tumor suppressor gene is one of the most commonly mutated genes in all types of human cancer. Recent studies of the function of the wild-type p53 demonstrated that its anti-proliferative effect is mediated by stimulation of a 21-kDa protein (p21cip/ waf) that inhibits cyclin-dependent kinase activity and, thereby, cell division. This negative cell cycle controller effect may explain why the wild-type p53 gene can suppress the transformation of malignant cells in vitro (Dulic *et al.*, 1994; Frebourg *et al.*, 1993; Marx, 1993). Analysis of somatic tissue from many human cancers has shown that the wild-type p53 allele is lost frequently and a mutant allele retained, providing a growth advantage for malignant cells in vitro (Hussain *et al.*, 2000; Jensen *et al.*, 1993; Vogelstein *et al.*, 1992). The mutation of the p53 gene can damage its DNA-binding properties and cell cycle control and in cell proliferation (Roy *et al.*, 1994). Somatic p53 mutations are found more frequently in squamous cell carcinoma than in adenocarcinoma, although this may be a function of higher exposures to tobacco in patients with squamous cell carcinoma (Fujita *et al.*, 1999).

The p53 gene is located on chromosome 17p13. This gene is a key and potent mediator of cellular response against genotoxic insults (Oren *et al.*, 1999).

Table 1.6: Summery of candidate genes involved in lung cancer risk

Category	Candidate genes
Phase I metabolic genes	CYP1A1, CYP1A2, CYP2A6, CYP2D6, CYP2E1, CYP2C9 ,CYP2C19 , ADH2,EPHX1, NOQ1, NOQ2
Phase II metabolic genes	ADH3, MPO ,EPHX1, GSTM1, GSTT1, GSTP1, NAT2, ADH2, SOD2, NOQ1, NOQ2, SULT1A1, SULT1A2, SULT1A3
DNA repair genes	XRCC1, XRCC3, XPD, XPF, ERCC1
Cell cycle control genes	TP53, HRAS
Addiction genes	CYP2A6,CYP2D6, DRD2, DRD4, DRD5, nAChR, SLC6A3, 5HTT

1.5 Analysis of the Glutathione S-transferase (GST) Gene Family

The glutathione S-transferase (GST) gene family encodes genes that are critical for certain life processes, as well as for detoxication and toxification mechanisms, via conjugation of reduced glutathione (GSH) with numerous substrates such as pharmaceuticals and environmental pollutants. The GST genes are up regulated in response to oxidative stress

and are in explicably overexpressed in many tumors, leading to problems during cancer chemotherapy. An analysis of the GST gene family in the Human Genome Organization-sponsored Human Gene Nomenclature Committee database showed 21 putatively functional genes. Upon closer examination, however, GST -kappa 1(GSTK1), prostaglandin E synthase (PTGES) and three microsomal GSTs (MGST1, MGST2, MGST3)were determined as encoding membrane-bound enzymes having GST -like activity, but these genes are not evolutionarily related to the GST gene family. It is concluded that GST gene family comprises 16 genes in six subfamilies —alpha (GSTA), mu (GSTM), omega (GSTO), pi (GSTP), theta (GSTT) and zeta (GSTZ) (Nebert *et al.*, 2004).

1.5.1 The glutathione-S-transferases (GSTs) enzymes

A family of enzymes, the glutathione-S-transferases (GSTs), has the general function of conjugating glutathione with electrophilic substances that are capable of generating free radicals, thus leading to detoxification of their effects. Genetic polymorphisms associated with reduced activity of GSTs are therefore of interest in the study of disease susceptibility (Minelli *et al.*, 2010). The cytosolic isoenzymes of GST are divided into at least five major classes (α, µ, π, θ, ζ) among which polymorphism have been detected in the genes encoding for GSTA1 (α class) GSTM1 (µ class), GSTP1 (π class), GSTT1 (θ class) and GSTZ1 (ζ class) (Stucker *et al.*, 2002). Among them the GSTM1, GSTP1 and GSTT1 genotypes have been extensively studied during recent years for their potential modulating role in individual susceptibility to environmentally-induced diseases, including cancer (Stucker *et al.*, 2002).

Table 1.7 : Glutathione-S-transferases (GSTs) nomenclature and functional significance

Protein name	**Protein structure and isoenzymes**	**Gene symbol /location**	**Known functions**	**Gene variations and significance**
GST	Eight GST isoenzyme classes, usually homodimer, but also heterodimer: alpha (liver), kappa, mu, pi, sigma, theta, zeta, and omega	*GSTM1,2,3,4, and 5*—1p13.3; *GSTT1 and 2*—22q11.2; *GSTP1*—11q13; *GSTA1,2,3,4,* and *5*—6p12; *GSTK1*—ND; *GSTZ1*—14q24.3; *GSTS1*—4q21-22; *GSTO1, 2*—10q24.3	Conjugate glutathione with toxic compounds	*GSTM1—GSTM1-0, GSTM1a, GSTM1b; GSTM1-0* no function allele; No functional difference between *GSTM1a* and *GSTM1b; GSTT1—GSTT1-0, GSTT1-1; GSTT1-0* homozygous deletion, no function allele *GSTP1*: *GSTP1a, GSTP1b, GSTP1c*; *GSTP1a* has 3-4 fold higher activity than *GSTP1b* or *GSTP1c*; *GSTA1—GSTA1_A* and *GSTA1_B* based on promoter variations, _A type has higher gene activity than _B type; *GSTO1*—two functional SNPs, one causing the deletion of Glu155 and another the substitution of Ala140Asp; *GSTZ1*—three nonsynonymous SNPs

1.5.2 Tissue distribution of the glutathione S-transferase (GSTs)

Tissue distribution data obtained primarily from Comstock *et al.,* 1993; Hayes and Pulford, 1995; Rowe *et al.,* 1997. Useful marker substrates for specific isoforms.

Table 1.8 : Tissue distribution of GSTs

Sub unit	MW kDa	Chromosome	Primary tissues	Preferred substrates
A1	25.6	6p12	Liver, testis>>>kidney, adrenal>pancreas>>lung, brain>heart	CDNB (moderate) DCNB (low) CHP (mod-high) ECA (low-mod)
A2	26	6p12	Liver, pancreas, testis>kidney>adrenal>brain, lung, heart	CHP (high) CDNB (moderate) DCNB (moderate) ECA (low-mod)
A3	?	?	Placenta	Unknown
A4	25.7	?	Small intestine>spleen	4-hydroxynonenal CDNB (low) CHP (low) ECA (moderate)
(M1a, M1b)	26.7	1p13.3	(M1+) liver>>testis>brain, adrenal, kidney, pancreas>lung, heart	Trans-stilbene oxide , CDNB (high), DCNB (moderate), ECA (moderate), CHP (low)
M2	26.70	1p13.3	Brain>testis>heart> pancreas kidney>adrenal>lung, liver	Catecholamine quinones ,CDNB (high) DCNB (high),
M3	26.7	1p13.3	Testis>>>brain, spleen>>others	CDNB (low) DCNB (low) ECA (low) CHP (low)

M4	26.7	1p13.3	Liver, skeletal muscle>heart, brain>>pancreas>>lung, kidney, placenta	CDNB (low) DCNB (low) ECA (low) CHP (low
M5	26	1p13.3	Brain, testis, lung	CDNB (moderate)
Pi P1 (P1a, P1b, P1c, P1d)	23	11q13	Brain>lung, heart, testis>adrenal, kidney, pancreas>liver	BPDE CDNB (moderate); DCNB (low) ECA (high) CHP (low)
Theta T1	27	22q11.2	Kidney, liver>small intestine>brain, spleen, prostate, pancreas, testis>heart, lung	CDNB (0) ECA (low) CHP (mod-high) Dichloromethane
T2	27	22q11.2	liver	1-menaphthyl sulfate CDNB (0) CHP (high)
Zeta Z1	24.2	14q24.3	?	Dichloroacetic acid,

CDNB (1-chloro-2,4-dinitrobenzene) activities: high, above 150, moderate, 50–150, low, less than 50 {mol/min/mg**;** **DCNB** (1,2-dichloro-4-nitrobenzene) activities: high, above 2, moderate, 0.5–2, low, less than 0.5 {mol/min/mg; ECA activities: high, above 1; moderate, 0.1–1; low, less than 0.1 {mol/min/mg; **CHP** (cumene hydroperoxide) activity: high, >2, moderate, 0.5–2; low <0.5 {mol/min/mg; EPNP (1,2-epoxy-3-(*p*-nitrophenoxy)propane.

1.5.3 Structure and function of glutathione and its associate enzymes

Glutathion is a tripeptide composed of g-glutamate, Cysteine and Glycine the sulfhydryl side chains of the Cysteine residues of two glutathione molecules form a disulfide bond (GSSG) during the course of being oxidized in reactions with various oxides and peroxides in cells. Its synthesis starts with the conjugation of cysteine and glutamate by glutamyl

cysteine synthetase (GCS). Glutathione synthetase catalyzes the addition of glycine toglutamylcysteine to form glutathione. When glutathione becomes oxidized by glutathione peroxidase (GPX), two molecules are linked together to form glutathione disulfide. This process can be reversed by glutathione reductase (GSR). Reduction of GSSG to two moles of GSH is the function of Glutathione reductase , an enzyme that requires coupled oxidation of NADPH. The activity of GSTs is dependent upon a steady supply of GSH from the synthetic enzymes gamma-glutamylcysteine synthetase and glutathione synthetase, as well as the action of specific transporters to remove conjugates of GSH from the cell. The primary role of GSTs is to detoxify xenobiotics by catalyzing the nucleophilic attack by GSH on electrophilic carbon, sulfur, or nitrogen atoms of said nonpolar xenobiotic substrates, thereby preventing their interaction with crucial cellular proteins and nucleic acids (Hayes *et al.*, 2005; Josephy, 2010b). Specifically, the function of GSTs in this role is twofold: to bind both the substrate at the enzyme's hydrophobic H-site and GSH at the adjacent, hydrophilic G-site, which together form the active site of the enzyme; and subsequently to activate the thiol group of GSH, enabling the nucleophilic attack upon the substrate (Eaton *et al.*, 1999b). Both subunits of the GST dimer, whether hetero or homodimeric in nature, contain a single non substrate binding site, as well as a GSH-binding site. In heterodimeric GST complexes such as those formed by the cytosolic mu and alpha classes, however, the cleft between the two subunits is home to an additional high-affinity non substrate xenobiotic binding site, which may account for the enzymes ability to form heterodimers (Hayes *et al.*, 2005). Glutathione forms conjugates with xenobiotics, toxic superoxides, or antineoplastic agents in reactions catalyzed by glutathione-S-transferases (GSTs). This conjugation reaction renders the compound more anionic, a form that can be exported from cells by the

adenosine triphosphate-dependent GS-X pump. Glutathione also detoxifies peroxides generated from oxygen radicals and reduces oxidized centers on DNA, proteins, and other biomolecules through transhydrogenases. Each member in the glutathione system has its unique role, but their significance varies in drug metabolism and, as a result, in antineoplastic drug resistance and toxicity. GCS, the rate-limiting enzyme in the synthesis of glutathione, and the GSTs, the critical enzymes for conjugation, appear to be the key functional enzymes in the system.

Table1.9: Structure, location, and functions of metabolic enzymes in the glutathione pathway

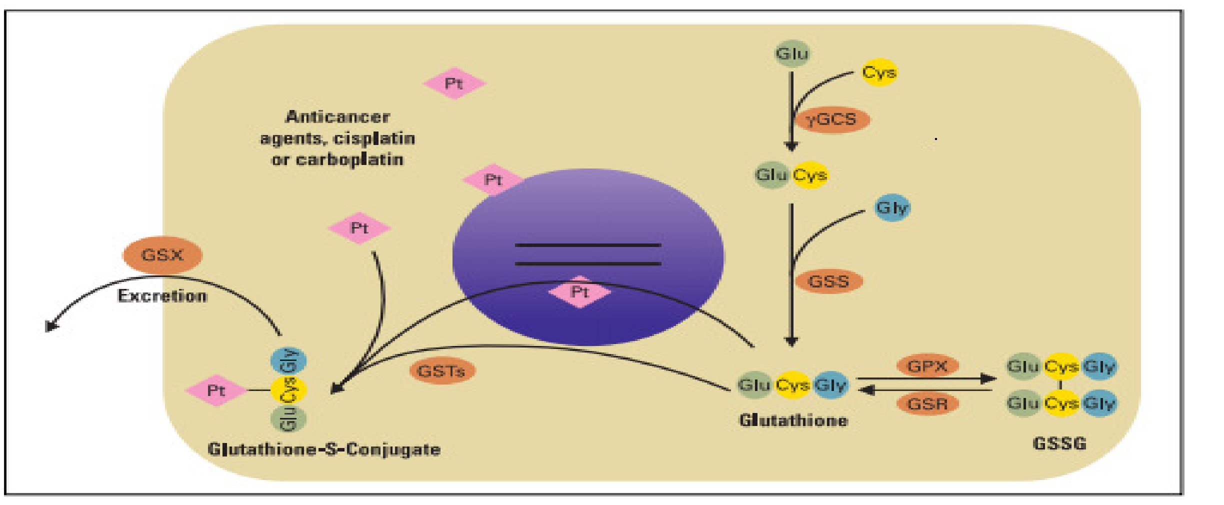
Protein name	**Protein structure and isoenzymes**	**Gene symbol/location**	**Known functions**	**Gene variations and significance**
gGCS	A heterodimer of two subunits,a heavy catalytic subunit anda light regulatory subunit	GCLC—6P12 heavy unit; GCLM—1P22.1 light unit	A rate limiting enzyme of glutathione synthesis; reduced apoptosis	A followed by T mutation causes the enzyme deficiency; trinucleotide (GAG) repeat in GCLC, associated with glutathione level and antineoplastic drug resistance; and 588T of GCLMis associated with

				lower plasma glutathione
GSS		GSS—20q11.2	Addition of glycine to GCS to produce GSH	Multiple mutations cause abnormal GSS, which leads to 5-oxoprolinuria
GPX	GPX 1-7, distributed in different organs, coded by different genes located at different chromosomes	GPX1—3p21.3;GPX2—14q24.1; GPX3—5q23; GPX4—19p13.3; GPX5—6p21.32; GPX6—1p32; GPX7—unknown	Against cytotoxic lipid peroxidation, inhibit apoptosis induced by CD95 ligation, prevent absorption of hydro-peroxides; differential distribution in normal and cancer	Three alleles with base substitution in lung tumors; frequent LOH in lung adenocarcinoma; Pro198Leu variant associated with increased lung cancer risk; in-frame GCG repeats
GSR		GSR—8p21.1	Deficiency may cause hemolytic anemia	10 SNPs: Eight nonsynonymous and two synonymous
GS-X pump	Two major types, cMOAT in liver and MRP1 in other cells	ABCC1(MRP1)—16p13.12-13; ABCC2(cMOAT MRP2)—	Transport glutathione-conjugated complex	Two nonsynonymous SNPs, but no functional

		10q24; ABCC3(MRP3)—17q22; ABCC4(MRP4)—13q31	out of cells	significance

Abbreviations: GCS, gamma-glutamylcysteine synthetase; GSS, glutathione synthetase; GSH, glutathione; GPX, glutathione peroxidase; LOH, loss of heterozygosity; GSR, glutathione reductase; SNP, single nucleotide polymorphisms; GST; glutathione-S-transferase

Figure 1.2 Glutathione and its associated enzymes (Ping Yang, Jon O. Ebbert, Zhifu Sun, and Richard M. Weinshilboum, 2004)



The glutathione pathway and its role in detoxification. Free platinum compound in the cytoplasm and probably in the nucleus can be conjugated with glutathione and excretedfrom cells. Glu, glutamate; Cys, cysteine; Pt, platinum-based anticancer drugs; GCS, gamma-glutamylcysteine synthetase; GSS, glutathione synthetase; Gly, glycine;

GPX, glutathione peroxidase; GSR, glutathione reductase; GSTs, glutathione-S-transferases; GSX, glutathione-conjugation complex export pump; GSSG, glutathione disulfide.

Table 1.10: Summary of chemotherapy drug resistance and the glutathione system in cell line studies (Ping Yang, Jon O. Ebbert, Zhifu Sun, and Richard M. Weinshilboum, 2004)

Glutathione molecules or glutathione system enzymes	Level and/or activity in tumor cell lines	Response to chemotherapy drugs	Type or site of cancer
GSH (intracellular molecule)	Down or GSH depletion UP	Resistance	Lung, NSCL; SCLC; leukemia; melanoma; breast; colon; Lung, NSCLC; SCLC; bladder; brain; head and neck; colon; ovarian
GSTs (protein)	UP or present	Resistance	Lung, NSCLC; SCLC; bladder; ovarian
GSR (protein)	Down, absent, or GST inhibition	Resistance	NSCLC; bladder
GPX (protein)	UP or Down	Resistance Sensitive	NSCLC; bladder
GCS (protein, expressed mRNA)	UP or Down GCS inhibition	Resistance Sensitive	SCLC; colon; ovarian
MRP (1,2,3; expressed mRNA	Up Down (caused by GCS expression inhibitor)	Resistance Sensitive	NSCLC; colon Colon

Abbreviations: GSH, glutathione; NSCLC; non–small-cell lung cancer; SCLC; small-cell lung cancer; GST; glutathione-S-transferase; GSR, glutathione reductase; NR, not reported; GPX, glutathione peroxidase; GCS, gamma-glutamylcysteine synthetase; MRP, multidrug resistance protein

1.6 The Biology of Genetic Polymorphism

The term ‘polymorphism’ is often used in rather vague and facile ways by geneticists. Technically, a polymorphic locus is one whose alleles or variants are such that the most common variant among them occurs with less than 99% frequency in the population at large (e.g. if the locus is biallelic, the rarer allele must occur with a frequency greater than 1% in the population). However, use of polymorphism in modern genetic initiatives ultimately emanated from the study of physiological and bio-chemical variation, such as that exhibited by protein isoforms and blood group antigens (Botstein *et al.*, 1980; Cargill *et al.*, 1999; Cooper DN, 1999; Schull, 1973). The Human Genome Project showed that we humans are 99.9% similar at the level of our DNA. However, that remaining 0.1% makes us unique - not only in our appearance and behavior, but also in our risk to develop certain diseases and our response to different types of medication. Genetic Polymorphism is a difference in DNA sequence among individuals, groups, or populations. Sources include SNPs, sequence repeats, insertions, deletions and recombination (e.g., a genetic polymorphism might give rise to blue eyes versus brown eyes, or straight hair versus curly hair). Genetic polymorphisms may be the result of chance processes, or may have been induced by external agents (such as viruses or radiation). If a difference in DNA sequence among individuals has been shown to be associated with disease, it will usually be called a genetic mutation. Changes in DNA sequence which have been confirmed to be caused by external agents are also generally called "mutations" rather than "polymorphisms" (Smith, 2002). Genetic polymorphisms of drug-metabolizing enzymes give rise to distinct subgroups in the population that differs in their ability to perform certain drug biotransformation reactions. Polymorphisms are generated by mutations in the genes for

these enzymes, which causes decreased, increased or absent enzyme expression or activity by multiple molecular mechanisms. Moreover, the variant alleles exist in the population at relatively high frequency. Genetic polymorphisms have been described for most drug metabolizing enzymes (Meyer *et al.*, 1997).

1.7.1 Polymorphic expression of GST

A significant number of genetic polymorphisms among the soluble GSTs have been described (Hayes and Strange ,2000). Importantly, variation in GST alleles is very common in the population and will presumably make as significant contribution to interindividual differences in drug metabolism. Gene deletions have been reported for GSTM1 and GSTT1, and alterations in amino acid coding sequences have been demonstrated for GSTA2, GSTM1, GSTP1, GSTT2 and GSTZ1 (Hayes et al., 1986). One of the allelic forms of GSTT2 encodes a truncated protein (Coggan et al., 1998). An allelic variation occurs in intron 6 of GSTM3 with one form of the gene lacking a YYI transcription factor binding site (Inskip et al., 1995). Allelic variations have also been found among MAPEG members, though these occur in then on-coding regions of MGST-I, LTC4S and FLAP. For the most part, polymorphisms in individual GST genes do not obviously confer a markedly increased risk of cancer. Typically, odds ratios associated with any single variant GST allele and the development of particular neoplastic diseases are found to be less than 3.0 (Hayes et al., 1986). However, combinations of variant GST alleles, either with other polymorphic GST or with alleles of other detoxication or antioxidant genes, are likely to have an additive effect in conferring predisposition to the nucleotide number quoted is that found in the cDNA.

Table 1.11: Polymorphic expression of GST (daniel w. nebert, 2004)

Class or super family	Gene	Allele	Alteration in gene or nucleotides	Protein or amino acid affected
Alpha	GSTA2	GSTA2*A	C335, A629	Thr112, Glu210
		GSTA2*B	G335, C629	Ser112, Ala210
Mu	GSTM1	GSTM1*A	G519	Lys173
		GSTM1*B	C519	Asn173
		GSTM1*0	Gene deletion	No protein
	GSTM3	GSTM3*A	Wildtype	Wildtype protein
		GSTM3*B	3 bp deletion in intron 6	Primary structure
				Unaltered
	GSTM4	GSTM4*A	Wildtype	Wildtype
		GSTM4*B	Changes in introns	Unchanged
Pi	GSTP1	GSTP1*A	A313, C341, C555	Ile105, Ala114, Ser185
		GSTP1**B	G313, C341, T555	Val105,Ala114, Ser185
		GSTP1*C	G313, T341, T555	Val105, Val114, Ser185
		GSTP1*D	A313, T341	Ile105, Val114
Theta	GSTT1	GSTT1*A	Unique gene	Unique protein
		GSTT1*0	Gene deleion	No protein
	GSTT2	GSTT2*A	A415	Met139
		GSTT2*B	G415	Ile139
Zeta	GSTZ1	GSTZ1*A	A94; A124; C245	Lys32; Arg42; Thr82
		GSTZ1*B	A94; G124; C245	Lys32; Gly42; Thr82
		GSTZ1*C	G94; G124; C245	Glu32; Gly42; Thr82
			G94; G124; T245	Glu32; Gly42; Met82

1.8 Pharmacogenomics , Personalized Medicine & Drug Safety

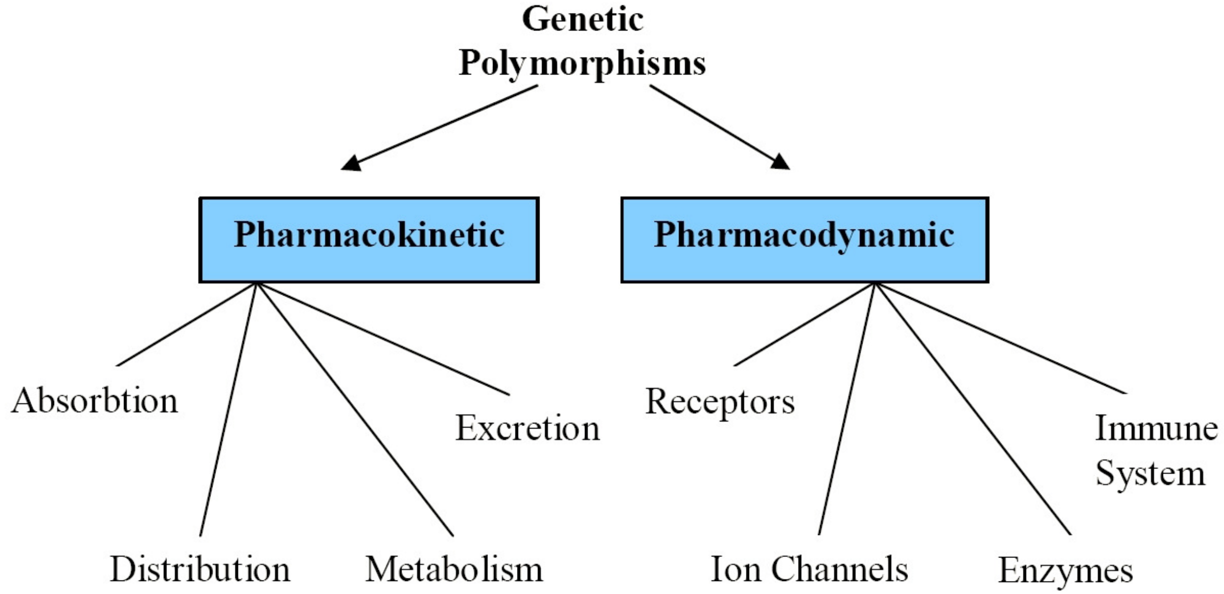
Personalized medicine is based on using an individual's genetic profile to make the best therapeutic choice by facilitating predictions about whether that person will benefit from a particular medicine or suffer serious side effects. Drugs are generally tested on a large population of people and the average response is reported. This sort of evidence based medicine (that is, medical decision making based on empirical data) relies on the law of averages; personalized medicine, on the other hand, recognizes that no two patients are alike (Adams, 2008).

1.8.1 Basics of pharmacogenomics

In pharmacogenomics, genomic information is used to study individual responses to drugs. When a gene variant is associated with a particular drug response in a patient, there is the potential for making clinical decisions based on genetics by adjusting the dosage or choosing a different drug, for example. Scientists assess gene variants affecting an individual's drug response the same way they assess gene variants associated with diseases: by identifying genetic loci associated with known drug responses, and then testing individuals whose response is unknown. Modern approaches include multi gene analysis or whole-genome single nucleotide polymorphism (SNP) profiles, and these approaches are just coming into clinical use for drug discovery and development.

When studying drug action in individuals, researchers focus on two major determinants: (1) how much of a drug is needed to reach its target in the body, and (2) how well the target cells, such as heart tissue or neurons, respond to the drug. The scientific terms for these two determinants are pharmacokinetics and pharmacodynamics, and both are critical considerations in the field of pharmacogenomics (Goldstein *et al.*, 2003a).

Figure 1.3: How pharmacology and other branches are related to pharmacogenetics and pharmacogenomics (Srivastava et al., 2003).



1.8.2 Pharmacokinetics

Pharmacokinetics encompasses four processes: absorption, distribution, metabolism, and excretion, which are often abbreviated as ADME.

1.8.3 Pharmacodynamics

As previously mentioned, pharmacodynamics is the molecular action of a drug on its target, whether this is a cell surface target (e.g., a receptor), an ion channel, or an intracellular target (e.g., an enzyme or regulatory protein). For example, the beta-2 adrenergic receptor is the target of both beta-agonists in the treatment of asthma and beta-blockers in the treatment of hypertension, and this receptor has polymorphisms that have been associated with response to these drugs (Goldstein *et al.*, 2003).

1.8.4 Drug safety

Within the United States, serious side effects from pharmaceutical drugs occur in 2 million people each year and may cause as many as 100,000 deaths, according to the Food and Drug Administration. Costs associated with adverse drug reactions (ADRs) are estimated at $136 billion annually. ADRs come in two forms. One form results from misuse, such as taking too much of a medication or taking the medication too often or for too long. The second form involves the mysterious, idiosyncratic effects of various drugs. The term "idiosyncratic" is used because these (often serious) side effects are not related to drug dose and are thought to be unpredictable. Scientists believe many idiosyncratic effects result from individual variation that is encoded in the genome. Thus, genetic variations in genes for drug-metabolizing enzymes, drug receptors, and drug transporters have been associated with individual variability in the efficacy and toxicity of drugs. Genetics also underlies hypersensitivity reactions in patients who are allergic to certain drugs, such as penicillin, wherein the body mounts a rapid, aggressive immune response that can cause not only a rash, but can also hinder breathing and cause edema to the point of cardiovascular collapse. Predicting serious ADRs is a priority for pharmacogenomics research. For example, the enzyme CYP2D6, one of a class of drug-metabolizing enzymes found in the liver, breaks down and terminates the action of certain antidepressant, antiarrhythmic, and antipsychotic drugs. Molecular cloning and characterization studies of the gene that codes for this enzyme have described more than 70 variant alleles (Meyer, 2000). These alleles contain one or more point mutations, only some of which affect enzyme activity; however, some of these alleles involve gene deletions and duplications that can lead to increased enzyme activity. Individuals who are homozygous or

heterozygous for the wild-type or normal activity enzymes (75%–85% of the population) are called extensive metabolizers; intermediate (10%–15%) or poor (5%–10%) metabolizers are carriers of two alleles that decrease enzyme activity (Ingelman-Sundberg, 1999); and ultrarapid metabolizers (1%–10%) are carriers of duplicated genes. The most common alleles can be detected by DNA chip microarrays, allowing most patients to be assigned to a particular phenotype group (Goldstein *et al.*, 2003b; Meyer, 2000; Paik *et al.*, 2004; Pirmohamed *et al.*, 2001) .

1.9 Personalized Medicine and Lung Cancer

Following advancements in diagnostic science and early detection markers, a number of cancer types can be detected before pathological symptoms develop. These markers are biochemical, epigenetic, genetic, imaging, metabolomics, and proteomic. Technologies can be used to detect these markers in clinical samples with an option of multiplexing. Use of more than one marker in the same sample generally increases the sensitivity and specificity of cancer detection and helps a physician to diagnose early and accurately. This information is of great significance because individual specific treatment regimens can be designed based on the presence and stage of cancer as concluded from profiles of markers. Pathological diagnosis is still gold standard in clinical practice; however, molecular diagnosis with additional information may be different from pathological diagnosis. Genetic aberrations, either somatic or hereditary, may lead to cancer. Hereditary cancers, which are a major part of medical genetics, can be understood by following cancer genetics. Familial cancers cover only 10–15% of total cancers, and the remaining cancers are influenced by environmental factors, infections, and lifestyle. This information helps scientists to determine the risk of cancer development in an individual's lifetime (Stricker *et al.*, 2011).

However, there are only a few cancer-disposing syndromes in which an allele is segregated in an autosomal-dominant fashion, thereby contributing to a high risk of cancer development. Furthermore, non-genetic factors contribute to mutations or other genetic changes. Cancer also has been observed to develop in individuals who have no family history of cancer. Along with genetic variations in tumors per se, inherited genetic variants in genes that metabolize and process drugs also influence response to treatment. These variants may increase the toxicity of specific drugs. This knowledge has enabled the development of the science of "pharmacogenomics," which identifies individuals who, based on their genotype information, will respond to a specific therapy (Schroth *et al.*, 2009). The goal of personalized medicine is to use the right drug at the right dose, with minimal or no toxicity, for the right patient at the right time (Hohl, 2013).

Figure 1.4: The clinical effects of genotypic influences on phenotype

Phenotype	Genotype	Enzyme activity	Substrate	Therapeutic Response
Poor metabolizer (PM)	or	None	Active parent drug	Failure
Intermediate metabolizer (IM)	or	Reduced	Active parent drug	Reduced
Extensive metabolizer (EM)	or or	Normal	Active parent drug	Expected
Ultrarapid metabolizer (UM)	or	Excessive	Active parent drug	Failure

Inactive allele
Active allele
Increased activity of allele
Partially active allele
Whole gene deletion

1.9.1 Examples of personalized medicine in lung cancers

The design of personalized health care is based on prevention or therapeutic approaches in conjunction with current knowledge of the cancer type (Baehner *et al.*, 2011). Because of the heterogeneity of cells, it is extremely difficult to treat lung cancer. Regular treatment techniques, mainly surgical and chemotherapy have been used to treat lung cancer. Based on recent data and understanding of the genetic basis of lung cancer, EGFR, K-ras, ALK, MET, CBL, and COX2 are being used as therapeutic targets (Salgia *et al.*, 2011) . Curran (Maemondo *et al.*, 2010) recently demonstrated utilization of crizotinib in the treatment of NSCLC. Crizotinib is an inhibitor of anaplastic lymphomakinase (ALK) and has showed promising results. Other investigators have also observed benefits of using crizotinib for lung cancer treatment (Maemondo *et al.*, 2010). Erlotinib and EGFR mutated lung cancer has also provided significant clinical results (Chmielecki *et al.*, 2012). FLEX trial has also demonstrated promising results. Data from histopathological examination and the patient's history also is considered in evaluating the state of the disease and its aggressiveness. Nybergetal (Nyberg *et al.*, 2011) studied association between SNPs and acute interstitial lung disease in Japanese population undergoing treatment with gefitinib. This research provided basis for further research. In Chinese population, ABCC1 polymorphism was found to be associated with lung cancer susceptibility in patients undergoing chemotherapy (Yin *et al.*, 2011)

ABCC1 polymorphism Arg723Gln (2168G – A) is associated with lung cancer susceptibility in a Chinese population (Yin *et al.*, 2011). Genomic variations in EGFR and ERCC1 have also been correlated with drug response in small cell lung cancer patients (Osawa; Wu *et al.*, 2011).

1.10 SNPs

A Single Nucleotide Polymorphism is a source variance in a genome. A SNP ("snip") is a single base mutation in DNA. SNPs are the most simple form and most common source of genetic polymorphism in the human genome (90% of all human DNA polymorphisms). There are two types of nucleotide base substitutions resulting in SNPs:

A transition in human, the transitional SNP (C↔T/G↔A) is the most common, representing over two-thirds of the total (Holliday R & Grigg GW, 1993). Transition substitution occurs between purines (A, G) or between Pyrimidines (C, T). This type of substitution constitutes two third of all SNPs (Richard M Twyman & Sandy B Primrose, 2003; Schwartz *et al.,* 2003).

A transversion substitution occurs between a purine and a pyrimidine. (C↔A/G↔T, C↔G/G↔C and T↔A/A↔T) & together account for the remaining third (Richard M Twyman & Sandy B Primrose, 2003).

1.10.1 Sequence variation

Sequence variation caused by SNPs can be measured in terms of nucleotide diversity, the ratio of the number of base differences between two genomes over the number of bases compared. This is approximately 1/1000 (1/1350) base pairs between two equivalent chromosomes.

1.10.2 Distribution of SNPs

SNPs are not uniformly distributed over the entire human genome, neither over all chromosomes and neither within a single chromosome. There are one third as many SNPs within coding regions as non-coding region SNPs. It has also been shown that sequence

variation is much lower for the sex chromosomes. Within a single chromosome, SNPs can be concentrated about a specific region, usually implying a region of medical or research interest. For instance, the sequence that encodes proteins that present antigens to the immune system in chromosome 6 displays very high nucleotide diversity compared to the other areas of that chromosome.

1.10.3 SNPs position & classification

SNPs are found throughout the genome, e.g. in exons, introns, intergenic regions, in promoters or enhancers, etc. Hence, they are more likely to yield, upon collection, a functional or physiologically relevant allele than other sorts of polymorphism. What is of extreme interest in this regard is the nature of the effect that a simple base pair substitution can have on a trait or disease. Thus, a SNP in coding region may directly impact a relevant protein, an intronic SNP can influence splicing (Krawezak M et al., 1992), a SNP in a promoter can influence gene expression (Drazen JM et al., 1999) etc. Of the SNPs that are near or in a gene, their effect on function is difficult to determine. SNPs are generally classed by genomic location (Sean Mooney, 2005).

Table 1.12: Functional classification of SNPs

Coding SNPs	cSNP	Positions that fall within the coding regions of genes
Regulatory SNPs	rSNP	Positions that fall in regulatory regions of genes
Synonymous SNPs	sSNP	Positions in exons that do not change the codon to substitute an amino acid

Non-synonymous SNPs	nsSNP	Positions that incur an amino acid substitution
Intronic SNPs	iSNP	Positions that fall within introns

However, the degree to which each kind of SNP influences phenotypic expression is likely to receive a great deal of attention as more and more SNPs are identified and studied (Schork NJ et al., 2000).

1.10.4 Coding region SNPs

A SNP in a coding region may have two different effects on the resulting protein:

Synonymous this substitution caused no amino acid change to the protein it produces. This is also called a silent mutation. There are several ways an nsSNP can affect gene product function. The most probable effect is a partial or complete loss of function of the mutated gene product. A less likely possibility is a gain of function mutation, such as those that have been observed in somatic mutations of the androgen receptor ligand binding domain (Zhao *et al.,* 2000) or the activation (by loss of GTPase activity) of the RAS oncogene (Quilliam *et al.,* 1995)

Non-Synonymous this substitution results in an alteration of the encoded amino acid. A missense mutation changes the protein by causing a change of codon. A nonsense mutation results in a misplaced termination codon. One half of all coding sequence SNPs results in non-synonymous codon changes.

SNPs may occur in regulatory regions of genes. These SNPs are capable of changing the amount of timing of a proteins production. Such SNPs are much more difficult of find and

understand and gene regulation itself is not yet clearly understood (Jin *et al.,* 1999).

1.10.5 STRs, VNTRs

Other types of genetic polymorphism result from the insertion or deletion of a section of DNA. The most common type of such 'insertion/deletion' polymorphism is the existence of variable numbers of repeated base or nucleotide patterns in a genetic region (Cooper DN & Krawczak M 1999). Repeated base patterns range in size from several hundreds of base pairs, known as 'variable number of tandem repeats' (VNTRs or 'mini satellites'), to the more common 'microsatellites' consisting of two, three or four nucleotides repeated some variable number of times. Microsatellites are often referred to as 'simple tandem repeats' (STRs). Repeat polymorphisms often result in many alleles or variants (e.g. several different repeat sizes) within the population and are thus considered 'highly polymorphic (Schork NJ *et al.,* 2000).

1.10.6 Phenotype, genotype and haplotype

Phenotype, genotype and haplotype are the most important and a basic concept related to SNPs. It is important to have a clear understanding of each term and the processes of genotyping and haplotyping.

Phenotype The observable properties of an individual as they have developed under the combined influences of the individual's genotype and the effects of environmental factors (Purves et al., 2004)

Genotype An exact description of the genetic constitution of an individual, with respect to a single trait or a larger set of traits. (Purves et al.,2004). The genetic constitution of an organism as revealed by genetic or molecular analysis, i.e. the complete set of genes, both dominant and recessive, possessed by a particular cell or organism.

Genotyping is normally defined as detecting the genotypes of individual SNPs. In diploid organisms (have alternative alleles of SNPs), such as humans, the linkage of particular SNP genotypes on each chromosome in a homologous pair (the haplotype) may provide additional information not available from SNP genotyping alone.

Haplotype (haploid genotype) A particular pattern of sequential SNPs (or alleles) found on a single chromosome. These SNPs tend to be inherited together over time and can serve as disease gene markers. The examination of single chromosome sets (haploid sets), as opposed to the usual chromosome pairings (diploid sets), is important because mutations in one copy of a chromosome pair can be masked by normal sequences present on the other copy. A combination of alleles of closely linked loci that are found in a single chromosome and tend to be inherited together. Haplotype analysis is useful in identifying recombination events (Purves et al., 2004).

Haplotyping involves grouping subjects by haplotypes, or particular patterns of sequential SNPs, found on a single chromosome. Genomic variation, and thus SNPs, is responsible for diversity in the human species. It follows that since SNPs account for diversity in human genotypes, they can be mapped to account for diversity in phenotypes. An "individual SNPs may serve as signposts for disease genes, haplotypes are believed to be superior for this purpose. The study of haplotypes within genes, which is also of great current interest, provides the opportunity to discover reliable markers of various phenotypes. This relation forms the basis and motivation for the identification & genotyping of SNPs.

1.11 Restriction Fragment Length Polymorphisms (RFLPS)

One of the earliest and most widely used genotyping methods, restriction fragment length polymorphism (RFLP) analysis, works on the principle of allele-specific enzymatic cleavage. An RFLP is generated when an SNP occurs at a restriction endonuclease recognition sequence, and one allele preserves the sequence while the other destroys it. If we consider any DNA fragment with three adjacent restriction sites, with the middle one containing an SNP, then digestion of amplified genomic DNA with the appropriate restriction endonuclease will produce either a single large fragment (if the central restriction site is absent) or two smaller fragments (if the central restriction site is present and cleavage occurs (Richard M. Twyman, 2005). The fragments are then separated by gel electrophoresis and can be compared to others to detect the differences. These differences are called restriction fragment length polymorphisms (RFLPs) (Saiki *et al.,* 1985, Osborn *et al.,* 2000)

1.12 Lung Cancer Prevalence

Table 1.13 : Worldwide incidence, mortality, 5 years prevalence of different types of cancers in males

Cancer	Incidence		Mortality		5-year prevalence	
	Number	ASR (W)	Number	ASR (W)	Number	Prop
Lip, oral cavity	170496	5.2	83109	2.6	401075	11.7
Nasopharynx	57852	1.7	35984	1.1	153736	4.5
Other pharynx	108588	3.4	76458	2.4	229030	6.7
Oesophagus	326245	10.1	276007	8.5	335707	9.8
Stomach	640031	19.7	463930	14.2	1050306	30.8
Colorectum	663904	20.3	320397	9.6	1765422	51.7
Liver	523432	16	478134	14.5	433207	12.7
Gallbladder	58375	1.8	42949	1.3	69252	2
Pancreas	144859	4.4	138377	4.2	91997	2.7
Larynx	129651	4.1	70336	2.2	366566	10.7
Lung	1092056	33.8	948993	29.2	1121619	32.8
Melanoma of skin	101807	3.1	25860	0.8	385064	11.3
Prostate	899102	27.9	258133	7.4	3200372	93.7
Testis	52322	1.5	9874	0.3	201562	5.9
Kidney	169155	5.2	72019	2.2	466631	13.7
Bladder	294345	8.9	112308	3.3	904169	26.5
Brain, nervous system	126815	3.8	97251	2.9	171827	5
Thyroid	49211	1.5	11206	0.3	195097	5.7
Hodgkin lymphoma	40265	1.2	18256	0.5	114537	3.4
Non-Hodgkin lymphoma	199736	6	109484	3.3	427038	12.5
Multiple myeloma	54923	1.7	37795	1.1	112421	3.3
Leukaemia	195456	5.8	143555	4.3	278754	8.2
All cancers excl. non-melanoma skin cancer	6617844	202.8	4219626	127.9	13514868	395.8

Figure 1.5 [Incidence and mortality data for males, 5-year prevalence for adult population only, ASR (W) and proportions per 100,000] 2010; http://globocan.iarc.fr/factsheet.asp, accessed date: 9 July, 2012)

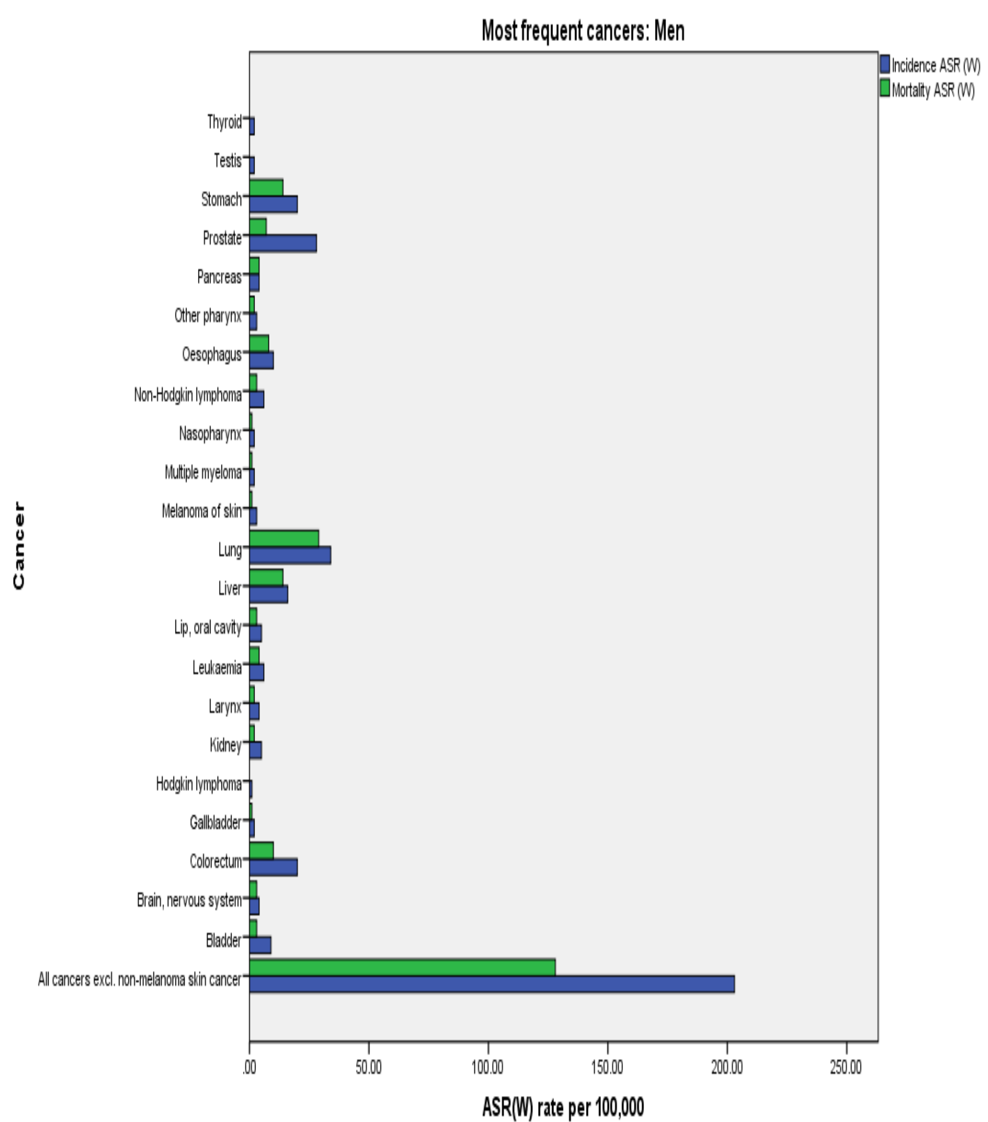


Figure 1.6 [Incidence and mortality data for women, 5-year prevalence for adult population only, ASR (W) and proportions per 100,000] (Fearly *et al.*, 2010; http://globocan.iarc.fr/factsheet.asp, accessed date: 9 July, 2012)

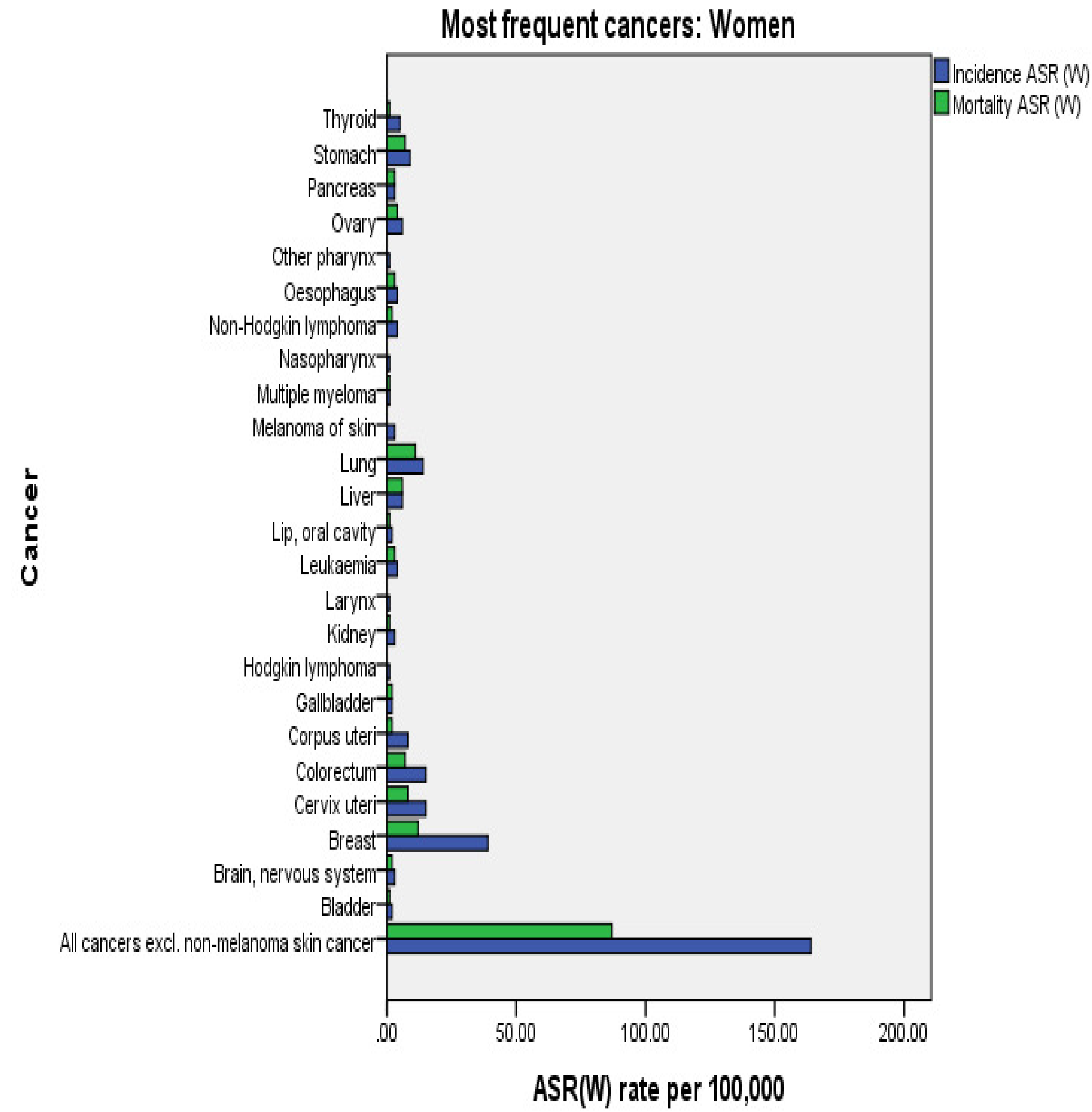


Table1.14: Worldwide incidence, mortality, 5 years prevalence of different types of cancers in females, most frequent cancers: Women

Cancer	Incidence		Mortality		5-year prevalence	
	Number	ASR (W)	Number	ASR(W)	Number	Prop
Lip, oral cavity	92524	2.5	44545	1.2	209581	6.2
Nasopharynx	26589	0.8	15625	0.4	68975	2.1
Other pharynx	28034	0.8	19092	0.5	59008	1.8
Oesophagus	155400	4.2	130526	3.4	146438	4.4
Stomach	348571	9.1	273489	6.9	548134	16.3
Colorectum	571204	14.6	288654	7	1495468	44.5
Liver	226312	6	217592	5.7	180006	5.4
Gallbladder	86828	2.2	66638	1.7	99206	3
Pancreas	133825	3.3	128292	3.1	80495	2.4
Larynx	21026	0.6	11556	0.3	59131	1.8
Lung	515999	13.5	427586	10.9	555797	16.5
Melanoma of skin	97820	2.7	20512	0.5	370814	11
Breast	1384155	38.9	458503	12.4	5189028	154.5
Cervix uteri	530232	15.2	275008	7.8	1555341	46.3
Corpus uteri	288387	8.2	73854	1.9	1097620	32.7
Ovary	224747	6.3	140163	3.8	549850	16.4
Kidney	104363	2.8	44349	1.1	277559	8.3
Bladder	88315	2.2	37974	0.9	268292	8
Brain	111098	3.1	77629	2.2	145212	4.3
Thyroid	163968	4.7	24177	0.6	667377	19.9
Hodgkin lymphoma	27654	0.8	11646	0.3	81808	2.4
Non-Hodgkin lymphoma	156695	4.2	82115	2.1	344983	10.3
Multiple myeloma	47903	1.2	34658	0.9	98276	2.9
Leukaemia	154978	4.3	113606	3.1	221120	6.6
All cancers excl. non-melanoma skin cancer	6044710	164.4	3345176	87.2	15288300	455.2

Table1.15: Worldwide Incidence, Mortality, 5 years prevalence of top 5 Cancers in both sexes

Cancer	Incidence		Mortality		5-year prevalence	
	Number	ASR (W)	Number	ASR (W)	Number	Prop
Lip, oral cavity	263020	3.8	127654	1.9	610656	9
Nasopharynx	84441	1.2	51609	0.8	222711	3.3
Other pharynx	136622	2	95550	1.4	288038	4.3
Oesophagus	481645	7	406533	5.8	482145	7.1
Stomach	988602	14	737419	10.3	1598440	23.6
Colorectum	1235108	17.2	609051	8.2	3260890	48.1
Liver	749744	10.8	695726	9.9	613213	9.1
Gallbladder	145203	2	109587	1.5	168458	2.5
Pancreas	278684	3.9	266669	3.7	172492	2.5
Larynx	150677	2.2	81892	1.2	425697	6.3
Lung	1608055	22.9	1376579	19.3	1677416	24.8
Melanoma of skin	199627	2.8	46372	0.6	755878	11.2
Breast	1384155	38.9	458503	12.4	5189028	76.6
Cervix uteri	530232	15.2	275008	7.8	1555341	23
Corpus uteri	288387	8.2	73854	1.9	1097620	16.2
Ovary	224747	6.3	140163	3.8	549850	8.1
Prostate	899102	27.9	258133	7.4	3200372	47.2
Testis	52322	1.5	9874	0.3	201562	3
Kidney	273518	4	116368	1.6	744190	11
Bladder	382660	5.3	150282	2	1172461	17.3
Brain	237913	3.5	174880	2.5	317039	4.7
Thyroid	213179	3.1	35383	0.5	862474	12.7
Hodgkin lymphoma	67919	1	29902	0.4	196345	2.9
Non-Hodgkin lymphoma	356431	5.1	191599	2.7	772021	11.4
Multiple myeloma	102826	1.4	72453	1	210697	3.1
Leukaemia	350434	5	257161	3.6	499874	7.4
All cancers excl. non-melanoma skin cancer	12662554	180.8	7564802	105.6	28803166	425.2

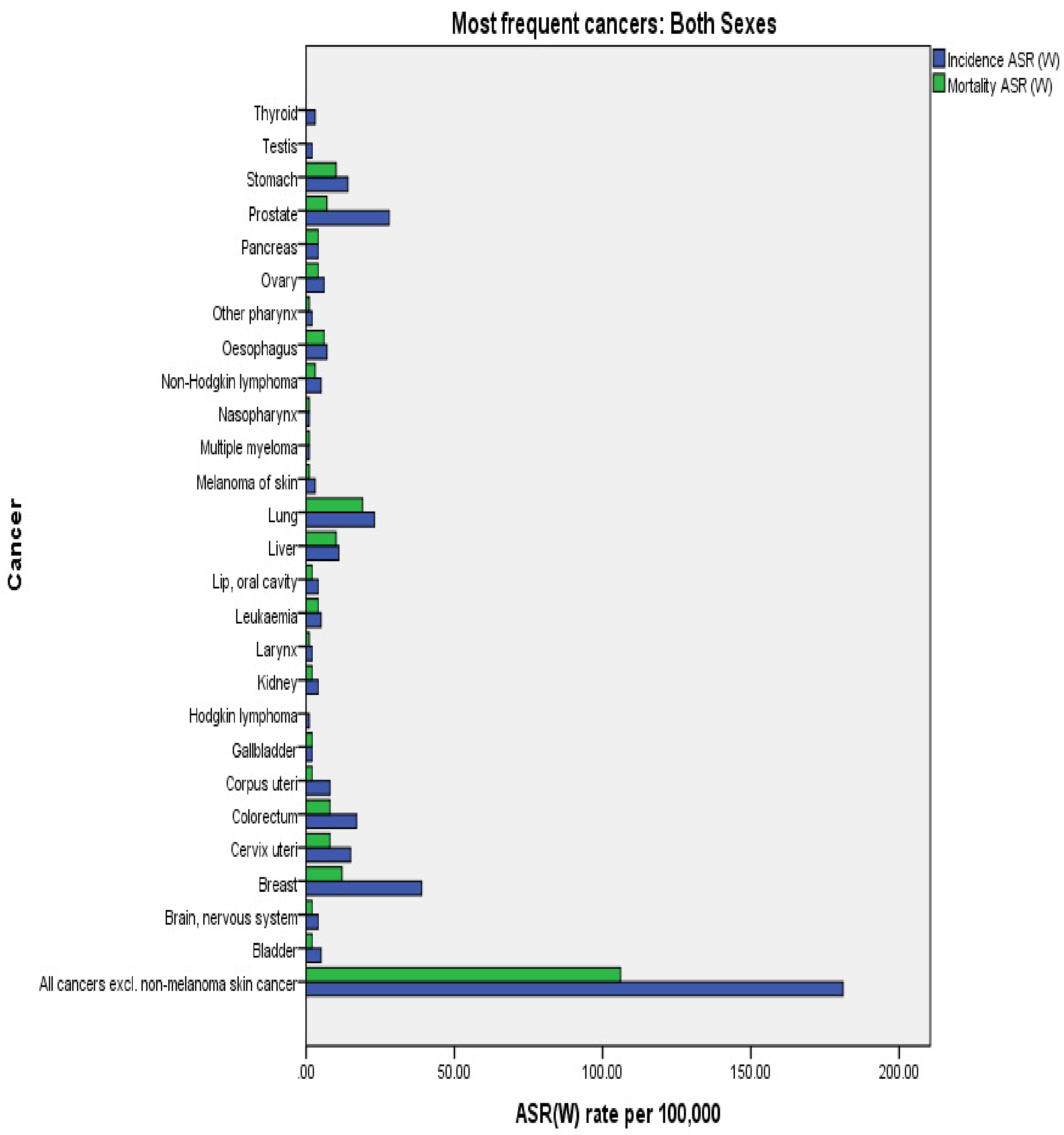


Figure 1.7 Incidence and mortality data for all ages, 5-year prevalence for adult population only, population only, ASR (W) and proportions per 100,000] (Fearly et al., 2010; http://globocan.iarc.fr/factsheet.asp,accessed date:9 July, 2012 1.12

1.13 Prevalence in Bangladesh

There is no population-based tumour registry in the country. The numbers and percentages of the top five malignancies in men, women, and both sexes are given in table 1.15, as per the annual report 2007 of National Institute of Cancer Research Hospital (NICRH), the only tertiary-level cancer care center of the country (A. F. M. Kamal Uddin, 2013). According to GLOBOCAN 2008, the 5-year cancer survival prevalence is 291.2 thousand (Ferlay *et al.*, 2010). The number of new cases per year is 141.1 thousand and the number of cancer deaths is 103.3 thousand. New and old cases constitute 124.8 thousand. The top five malignancies are lung, breast, cervix uteri, lip and oral cavity, and esophagus, considering both sexes. Lung cancer tops the list in the case of men and breast cancer tops the list in the case of women (Mahmud *et al.*, 2013). The estimated total lung cancer patients in Bangladesh were 196,000 among those aged 30 years and above (Haque, 2011; WHO, 2007).

Table 1.16 The top five malignancies in men, women and both sexes in Bangladesh.

Men n (%)	Women n (%)	Both sexes
Lung (25.5)	Breast(25.6)	Lung (17.3)
Lymphoma(7.4)	Cervix uterine(21.5)	Breast (12.3)
Esophagus(5.9)	Esophagus(3.4)	Cervix (9.1)
Larynx(5.4)	Lung (5.6)	Lymphoma(6.0)
Stomach(5.1)	Lymphoma(4.1)	Esophagus(4.6)
NICRH= National Institute of Cancer Research Hospital		

Figure 1.8 Top-10 Leading cancer prevalence during 2008 to 2010 in NICRH

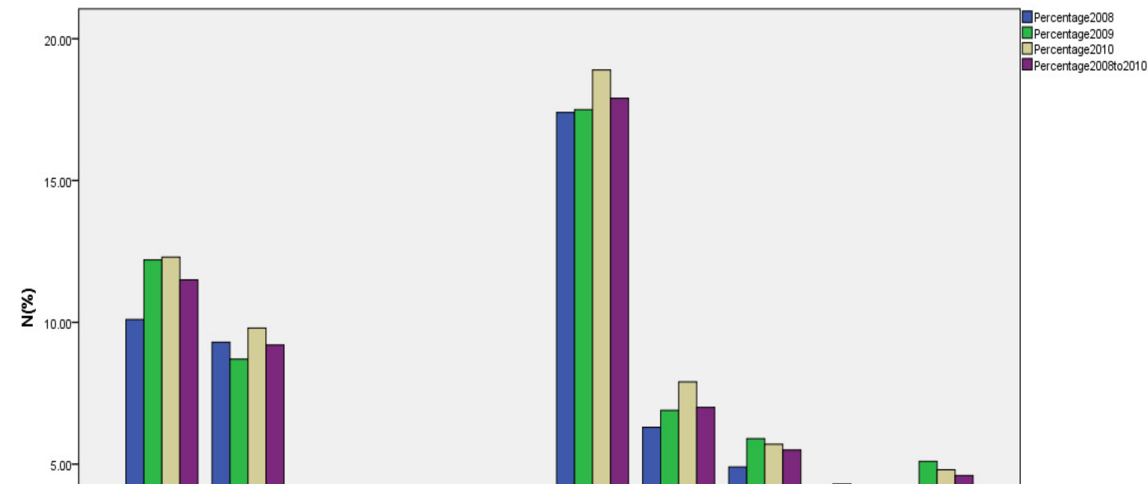


Figure showed different types of cancer based on ICD-O-3rd. It is evident that majority, 6304 (23.1%), of the cancer involving respiratory system and intra thoracic organs is followed by digestive organs, (18.5%) and female genital organs, (11.9%). Breast cancer was steadily increasing during the past three years. The figures on 2008, 2009 and 2010 were 759 (10.2%), 1196 (12.3%) and 1242 (12.3%), respectively. We found a significant upward trend of lung cancer rate during 2008-2010. Lung cancer 4915 (17.9%), breast cancer 3185 (11.5%), cervix cancer 2532 (9.2%), lymphnode and lymphatics cancer 1948 (7.0%), oesophagus cancer 1437 (5.5%), and stomach cancer 1193 (4.6%) were the top six cancer throughout the three years. Top 10 cancers in Bangladesh were shown in the figure (Mohammad Abul Bashar Sarker, 2010).

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CHAPTER 2

LUNG CANCER RISK IN RELATION TO GSTM1 & GSTT1 GENOTYPE IN LUNG CANCER PATIENTS OF BANGLADESH

2. GSTM1 & Allele of GSTM1

Phase II biotransformation enzymes generally act as inactivating enzymes to catalyze the binding of intermediary metabolites to cofactors, transform them into more hydrophilic products and thus facilitate their elimination. GSTs are phase II transformation enzymes involved in the detoxification of hazardous agents (Hirvonen *et al.*, 1996). GSTM1 catalyzes the detoxification of genotoxins including aromatic hydrocarbon epoxides and products of oxidative stress such as DNA hydroperoxides(Heagerty *et al.*, 1994) ,The GSTM1 gene is polymorphic and is represented by two active alleles and a non-functional null allele which results from the entire GSTM1 gene deletion mutation. GSTM1 may act as a predisposing factor in different diseases and may be a risk factor for cancer (Strange *et al.*, 1991). Five mu class genes are situated in tandem (5/-GSTM4-GSTM2-GSTM1-GSTM5-GSTM3-3/) kb cluster on chromosome 1p13.3. The GSTM1 gene on chromosome 1p13, according to the three alleles, can be grouped into two classes: GSTM1-null homozygote for the null allele (GSTM1-0), nonfunctional class and GSTM1-1 with at least one of the GSTM1a or GSTM1b allele i.e. functional class (Seidegard *et al.*, 1985).GSTM1*0 is deleted and homozygotes (GSTM1null genotype) express no protein. GSTM1*A and GSTM1*B differ by one base in exon 7 and encode monomers that form active homo and hetero-dimeric enzymes. The catalytic effectiveness of the enzymes encoded by these alleles is similar (McLellan *et al.*, 1997) . GSTM1 and the clinical con-sequences of genotypes resulting from combinations of the GSTM1*0, GSTM1* A and GSTM1*B alleles have been intensively investigated.

Table 2.1 Alleles nomenclature and functional consequences of GSTM1 (Sherratt et al., 2002)

Class or Super family	Gene	Allele	Alteration in gene Or nucleotides	Protein or amino acid affected
Mu	GSTM1	GSTM1*A	G519	Lys173
		GSTM1*B	C519	Asn173
		GSTM1*0	Gene deletion	No protein
	GSTM3	GSTM3*A	Wild type	Wild type protein
		GSTM3*B	3 bp deletion in intron 6	Primary structure
				Unaltered
	GSTM4	GSTM4*A	Wild type	Wild type
		GSTM4*B	Changes in Introns	Unchanged

2.1.1 Inter-ethnic variability of GSTM1

The variations in frequency distributions of the alleles are ethnic dependent and are even responsible for the efficacy and toxicity with various drugs (Kurose et al., 2012). Inter-individual variation in their expression or functional activity may be important in determining susceptibility to lung cancer (1996; Houlston, 1999; Strange et al., 1999). Between 38% and 67% of Caucasians carry a deletion in both alleles of the GSTM1 gene resulting in a total absence of GSTM1 enzyme activity (Rebbeck, 1997). Intra Ethnic Differences at GSTM1 null type frequency among Indian population is given in Table 2.2

AGAAAACACAGACCACATTTCCTTTACTCTGGCCCTTTTCCTGGGGGTCCTTCCTATACC
ACTGACACTGTTCCTGTGTAGGCGGGGCTAGAGGGGAGACTAAGCCCTGGGAGTAGCTTT
CGGATCAGAGGAAGTCCTGCTCTTACAGTGACAGGGGCTGAATTAAATTCCCAGGTTGGG
GCCACCACTTTTTAGTCTGACCCCTGCAGCCGGAGTCTCCCAGAGCCCTTGGGAACTCGG
CAGCGGAGAGAAGGCTGAGGGACACCGCGGGCAGGGAGGAGAAGGGAGAAGAGCTTTGCT
CCGTTAGGATCTGGCTGGTGTCTCAAGCGCACAGCCAAGTCGCTGTGGACCTAGCAAGGG
CTGGATGGACTCGTGGAGCCTCAGGGCTGGGTAGGGAAGCTGGCGAGGCCGAGCCCCGCC
TTGGGCTTCTGGGCGCCCTGACTTCGCTCCCGGAACCCTCGGGCCTGGGAGGCGGGAGGA
AGTCTTACTGAGTGCAGCCCCAGGCGCCCTCTCCCGGGCCTCCAGAATGGCGCCTTTCGG
GTTGTGGCGGGCCGAGGGGCGGGGTCGCAGCAAGGCCCCGCCTGTCCCCTCTCCGGAGCT
CTTATACTCTGAGCCCTGCTCGGTTTAGGCCTGTCTGCGGAATCCGCACCAACCAGCACC
ATGCCCATGATACTGGGGTACTGGGACATCCGCGGGGTGAGCGAGGGTCCGCTGGACGGT
GGGACGAGGGCGCAGGGGAGGGAAGTGCGAAGCAGCTGCGGGACGGACTCTAGGGACCGT
TCCTCTTCAGGGCTGCCCGCCTCAGAAGGGCCTGTGCATGCCGCTGTGTGTGTGTTGGGG
GTGTGGGCGGGTAGAGGAGGCAACGGGTACGTGCAGTGTAAACTGGGGGCTTCCCTGGTG
CAG**ACAAAGTCAGGGACCCTCCATCTCTGACGCGACCTGCGGGCCATCTCTCCCAGCTGG**
CCCACGCCATCCGCCTGCTCCTGGAATACACAGACTCAAGCTATGAGGAAAAGAAGTACA
CGATGGGGGACGGTAATGGCACCCTCGTGTTCGGGCTCTGCCCACTCACGCTAAGTTGGC
ACCAAGCAACCCATGGTGGCCACCTGTGGCTGCCTCTGCAGGCCTCCCCTGCTGGAGCTG
CAGGCTGTCTCTTCCCTGAGCCCCGGTGAGGGAGCCCTCTGGCCTTGCAAGGCAGAATGC
TGGGGTGGGATGCTGGGCCCCCTGTCTAATTGGGACGGGTGTCCCTCAGGGCTTGCCTAA
ACCCTGGAAGCCTTAGCTGTGTGGGGTCCAGAGCCCTCAGCGGGATTCTTTGTCCCTGAA
CCCTGGGATGTGGGACTGAGTGGTCAGATTCTAGATCCACCTGTCTCAGGGATCTTGCCA
CTGGCTCCGTGGGAGGGTCCCCGGGAAGGAGGGCTGGGCTCTGGGGAGGTTTGTTTTCAC
TTCTTCTTCCCCACCACAGCTCCTGATTATGACAGAAGCCAGTGGCTGAATGAAAAATTC
AAGCTGGGCCTGGACTTTCCCAATGTAGGTGCAGGGGAAGGGGCGGTTTTGGGGGAAAGT
GCAACGTGTCTCTGACTGCATCTCCTCTCCCCAGCTTAGAGGTGTTAAGATCAGGAGTCT
TCTGCCCAATTCCTCTCACTCCTGGCTGTCTAAACAGTCCTTCCATGATGTTCTGTGTCC
ACCTGCATTCGTTCATGTGACAGTATTCTTATTTCAGTCCTGCCATGAGCAGGCACAGTG
AGTGCCCGGTCTCCTCTCTGCTCTTGCTTATGGGAAG**GGGATGCTGGGGAGCCTGGTGGC**
CCAACTGAGCTTCGCCGGTTTCCCATCCATCCAGCTGCCCTACTTGATTGATGGGGCTCA
CAAGATCACCCAGAGCAACGCCATCTTGTGCTACATTGCCCGCAAGCACAACCTGTGTGA
GTGTGGGTGGCTGCAATGTGTGGGGGGAAGGTGGCCTCCTCCTTGGCTGGGCTGTGATGC
TGAGATTGAGTCTGTGTTTTGTGGGTGGCAG**GTGGGGAGACAGAAGAGGAGAAGATTCGT**
GTGGACATTTTGGAGAACCAGACCATGGACAACCATATGCAGCTGGGCATGATCTGCTAC
AATCCAGAATTTGTGAGTGTCCCCAGTGAGCTGCATCTGACAGAGTTTGGATTTGGGGCC

GSTM1*A
and
GSTM1*B
(C/G)

Figure 2.1: GSTM1*A and GSTM1*B polymorphisms obtained from ensembl (http://www.ensembl.org, accessed date: May 8, 2010)

Given the functional importance of GSTM1 in cellular protection from environmental and oxidative stress, genetic variations that alter this gene expression or enzyme activity may play an important role in both risk of disease development and cellular sensitivity to drugs. Two polymorphisms of the GSTM1 gene, namely GSTM1*0 and GSTM1*A/GSTM1*B, have been identified. GSTM1*0 is a deleted allele, and the homozygous allele (GSTM1-null genotype) expresses no GSTM1 protein. Most studies of GSTM1 polymorphism have compared the homozygous deletion

genotype with genotypes containing at least one functional allele. Alleles GSTM1*A and GSTM1*B differ by a C-G substitution at base position 534. This C-G substitution results in the substitution of Asn/Lys at amino acid 172; however, this substitution results in no functional difference between the two alleles. As a result, GSTM1*A and GSTM1*B are categorized together as non-null conjugated phenotypes (R. Stephanie Huang et al.).

Table 2.2: GSTM1 genotype frequencies among Indian populations

Location	Sample No.	GSTM1 Null (%)	p value	Literature
Gujarat	504	20	0.679	KP Senthilkumar et al.,2009
Karnataka	110	36.4	0.01183*	Naveen et al., 2004
Lucknow	200	36.5	0.01773*	Konwar et al., 2010
Pradesh	115	33	0.054	Naveen et al., 2004
Kerala	122	31.9	0.0756	Naveen et al., 2004
North India	370	33	0.1125	Mishra et al., 2004
South India	772	27.72	0.2463	Naveen et al., 2004, Vetriselvi et al., 2006
Central India	883	26.6	0.317	Buch et al., 2001
Tamilnadu-Pondicherry	170	23.5	0.6089	Naveen et al., 2004
Orissa	72	23.8	0.6089	Roy et al., 1998

*Significance at $p<0.05$

Table 2.3: GSTM1 genotype frequencies among different world populations

Country/ Population	No (N)	GSTM1 (%)		p	Reference
		Null	Present		
North India	370	33	67	0.1125	Dhruba Mishra et al., 1999
Newcastle, England	178	50.8	49.2	0.01	Welfare et al., 1999
Central Europe	127	45	55	0.11	Steinhoff C et al., 2000
Turkish	133	51.9	48.1	0.01	Ada et al., 2004
Italians	273	46.9	53.1	0.06	D’Alo et al., 2004
South India	517	30.4	69.6	0.76	Naveen et al, 2004
Chinese	477	51	49	0.01	Sctiawan et al., 2000
Caucasian	166	48.8	51.2	0.03	Gsur et al., 2001
Japanese	88	55.7	44.3	0.01	Kiyohara et al., 2000
Finnish Caucasians	478	41.8	58.2	0.24	Mitrunen et al., 2001
African Americans	271	28	72	0.54	Millikan et al., 2000
White (USA)	392	52	48	0.01	Millikan et al., 2000
Brazilian Non Whites	272	34.2	65.8	1.00	Rossini et al., 2002
Brazilian Whites	319	48.9	51.1	0.02	Rossini et al., 2002

2.1.2 GSTM1 substrates

GSTs metabolize many drugs, exogenous substances and play an important role in the bio activation of procarcinogens.

Table 2.4 List of GSTM1 substrates

Superfamily	Class	Chromosomes	Enzyme	Substrates
GST	Mu	1p13.3	GSTM1-1	CDNB; trans-4-phenyl-3buten-2-one; aflatoxin B_1-epoxide; trans-stilbene oxide
			GSTM2-2	High with CDNB; 1'2-dichloro-4-nitrobenzene; aminochrome
			GSTM3-3	Low towards CDNB; H_2O_2
			GSTM4-4	Not determined
			GSTM5-5	Low towards CDNB

Table 2.5 Substrates for Glutathione- S-transferases (Eaton *et al.*, 1999).

Environmental carcinogens/toxicants	Pesticides	Drugs	Endogenous molecules
Benzopyrene 7,8-dihydrodiol-9,10-epoxide (BPDE)	Lindane Alachlor Atrazine	Cis-platin Chlorambucil	4-Hydroxy-2-nonenal
AFB-8,9-epoxide Styrene oxide 5-Hydroxymethyl-chrysene sulfate	DDT	Cyclophosphamide BCNU (Bis-chloro-methyl nitrosourea)	Cholesterol-5,6-oxide Adenine
7Hydroxymethylbenz(a)-anthracene sulfate4-Nitroquinoline oxide	Methyl parathion EPN (*O*-ethyl-*O*-4 nitrophenyl phenyl-	Thiotepa	propenal 9-Hydroperoxy-linoleic acid
Acrolein		Fosfomycin	Dopaminochrome

	phosphonothioate)	Ethacrynic acid	
Hexachlorobutadiene		Nitroglycerine Menadione	aminochrome Catechol estrogens (quinones derived from 2 and/or 4 hydroxyestradiol)
Butadiene		Acetaminophen (NAPQI)	
Trichloroethylene		Mitozantrone	Malelylacetoacetate
Methylene chloride			
Ethylene oxide		Adriamycin	
PhiP (2-amino-1-methyl-6-phenylimidazo[4,5*b*]-pyridine)			

2.1.3 GSTT1 & allele of GSTT1

GSTT1 are enzyme members constituting GST super-family that catalyze the conjugation of glutathione to bio transform toxic chemicals into non-toxic substances (Strange *et al.*, 1991). GSTT1 enzymes show important differences in their catalytic activity compared with other GSTs: they have lower glutathione binding activity (Meyer, 1993), with increased catalytic efficiency GSTT1 plays a major role in phase II biotransformation of a number of drugs and industrial chemicals, e.g., cytostatic drugs, hydrocarbons, and halogenated hydrocarbons (Bolt HM, 2006). GSTT1 is involved in the metabolism of smaller compounds, such as mono halomethane and ethylene (Landi, 2000). GSTT1 also metabolize compounds formed during oxidative stress, such as hydroperoxides and oxidized lipids, and they are transcriptionally activated during oxidative stress oxide (Hayes *et al.*, 2005). Two theta-class GSTs, GSTT1 and GSTT2, have been identified in the human liver, and the corresponding genes are localized in the same region on

human chromosome 22, specifically in the sub band 22q11 (Tan *et al.*, 1995). Theta is considered as the most ancient of the GSTs, and theta-like GSTs are found in almost all organisms investigated (Meyer, 1993). The encoded GSTT1 human subunit is about 25,300 Da ; the gene is 8.1 kb long (Jemth *et al.*, 1997). GSTT1 null phenotype appears to increase the susceptibility to some types of cancer (Brockmoller et al., 1996). GSTT1 is polymorphic and represented by a functional (wild) allele and a non-functional null allele. This null allele results from total or partial deletion of the gene and presents two possible phenotypes: GSTT1 null, which is the homozygote of the deleted allele and GSTT1-positive, which is the phenotype that at least one copy of the gene is intact (Pemble *et al.*, 1994). The null genotype of GSTT1 ranges from 9 to 64% in different populations (Kiyohara *et al.*, 2002). Certain genetic variants in the glutathione -S-transferase genes, such as the GSTM1 and GSTT1null polymorphisms, are prevalent among 50% and 20% of Caucasians, respectively (Garte *et al.*, 2001). Meta-analyses have indicated that the carriers of GSTM1null or GSTT1null genotypes have a slightly higher risk of developing lung cancer compared to carriers of at least one functional allele (Garte *et al.*, 2001; Raimondi *et al.*, 2006; Ye *et al.*, 2006).

Table 2.6: Alleles nomenclature and functional consequence of GSTT1 ***(Sherratt et al., 2002)***

Class or Super family	Gene	Allele	Alteration in gene Or nucleotides	Protein or amino acid affected
Theta	GSTT1	GSTT1*A	Unique gene	Unique protein
		GSTT1*0	Gene deletion	No protein
	GSTT2	GSTT2*A	A415	Met139
		GSTT2*B	G415	Ile139

2.1.4 GSTT1 substrates

Table 2.7 GSTT1 substrates

Superfamily	Class	Chromo Somes	Enzyme	Substrates
Soluble	Theta	22q11	GSTT1-1	1,2-epoxy-3-(p-nitrophenoxy)propane; dichloromethane; dibromoethane
			GSTT2-2	1-menaphthyl sulphate; cumene hydroperoxide

2.1.5 Inter-ethnic variability of GSTT1

GSTT1 null genotype appears to increase the susceptibility to some types of cancer (J. Brockmoller, I. Cascorbi, R. Kerb,.et al). GSTT1 variants include homozygous deletion of the gene or a null genotype (Pemble et al., 1994) and the prevalence has been found to vary among ethnic groups (Nelson et al., 1995; Kimyohara, 2000). About 35-60% of individuals (Katoh et al., 1996; Chenevix-Trench et al., 1995; Bell et al. 1993) and 10-65% (Nelson et al., 1995, Chenevix-Trench et al., 1995) have been reported to possess null genotypes for GSTM1 and GSTT1 respectively. Nelson et al. reported that the null genotype of GSTT1 was present in 64% of Chinese, 60% of Koreans, 28% of Caucasians and in 22% of African-Americans. This null genotype is more common in the Asians than Caucasians, strengthening the idea that polymorphisms in enzymes that metabolize tobacco carcinogens have a strong ethnical link. Table 2.9 shows that GSTT1 null frequency (72%) was different from 13-19.1% of various Indian ethnic's (Roy et al., 1998; Buch et al., 2001; Mishra et al., 2004; Naveen et al., 2004; Vetriselvi et al., 2006; Konwar et al., 2010)

Table 2.8: GSTT1 genotype frequencies among different world populations

Country/ Population	No (N)	GSTT1 (%)		p	Reference
		Null	Presence		
North India	370	18.4	81.6	0.0064	Dhruba Mishra et al., 1999
Newcastle, England	178	16.9	83.1	1.00	Welfare et al., 1999
Central Europe	127	13	87	0.43	Steinhoff C et al., 2000
Turkish	133	17.3	83.7	1.00	Ada et al., 2004
Italians	273	19	81	1.00	D'Alo et al., 2004
South India	517	16.8	83.2	1.00	Naveen et al., 2004
Chinese	477	46	54	<0.01	Sctiawan et al., 2000
Caucasian	166	19.9	80.1	0.86	Gsur et al., 2001
Japanese	88	44.3	55.7	<0.01	Kiyohara et al., 2000
Finnish Caucasians	478	13.2	86.8	0.43	Mitrunen et al., 2001
African Americans	271	17	83	1.00	Millikan et al., 2000
White (USA)	392	16	84	0.85	Millikan et al., 2000
Brazilian Non Whites	272	25.7	74.3	0.23	Rossini et al., 2002
Brazilian Whites	319	25.1	74.9	0.30	Rossini et al., 2002

Table 2.9. Intra ethnic differences at GSTT1 null type frequency among Indians

Location	Sample No	GSTT1 Null%	p Value	Literature
Gujarat	504	35.5		KPSenthilkumar et al 2012
Tamilnadu-Pondicher	170	13.0	0.0002481***	Naveen et al., 2004
Western Central India	883	13.0	0.0002481***	Buch et al., 2001
Lucknow	200	14.0	0.0005253***	Konwar et al., 2010
Kerala	122	15.6	0.002018**	Naveen et al., 2004
South India	772	17.09	0.003691**	Naveen et al., 2004, Vetriselvi et al., 2006
North India	370	18.4	0.006471**	Mishra et al., 2004
Karnataka	110	19.1	0.01091*	Naveen et al., 2004
Andhra Pradesh	115	18.8	0.01091*	Naveen et al., 2004

*Significance at $p<0.05$, **Significance at $p<0.01$, ***Significance at $p<0.001$

2.1.6 Glutathione-S-transferase T1 (GSTT1) and lung cancer risk:

It is hypothesized that reduced GSTs activity is associated with higher incidence of cancer that seems a results of decreased elimination of electrophilic carcinogens (Strange *et al.*, 2000). The gene and associated proteins were found in a definite percentage of the human population with different ethnicities. The GSTT1*0 genotype has been found to elevate baseline level of sister chromatid exchange (SCE) frequently after exposure to 1, 3-butadiene and haloalkanes. It has been found SCE, in turn, has strong positive correlation with cancer risk (Norppa, 2004) . Among known substrates metabolized by GST theta, dichloromethane (DCM) is

one of the most thoroughly studied (Olvera-Bello *et al.*, 2010). Erythrocytes from some subjects ("conjugators") catalyze the conjugation of DCM with GSH, whereas the remaining individuals ("non-conjugators") do not. Formaldehyde obtained from metabolism of DCM covalently binds to cellular macromolecules like single-stranded DNA, serum albumin, or the N-terminal valine of hemoglobin and form molecular adducts (Casanova *et al.*, 1997). Formaldehyde-adducted RNAs were found in DCM- exposed isolated hepatocytes GSTT1*1 (positive) Human but not in hepatocytes from GSTT1*0(negative) donors (Casanova *et al.*, 1997). 1,3-Butadiene, ethylene, drinking water after chlorination, and metabolites of aflatoxin are other substrates for GSTT1 and genetic polymorphism in metabolism of these compounds modifies the risk of encephalopathy in Human(Landi, 2000). Styrene as carcinogen that is used worldwide in the production of different polymers is also metabolized by GSTT1 enzymatic activity causing DNA and hemoglobin adducts (2008). In vivo studies are required to differentiate the genotype of GSTT1 positive and null forms to determine the enzymatic activity of GSTT1 whether it acts as conjugator (GSTT1*1) or non conjugator enzyme (GSTT1*0)

(GSTT1*A)
C/G

```
AGGCTCCATGGCAGCAAGGG                  CTTACGGCTCAGCAGATGAATCCAGGGGG
GTGGACTTGCAAGGACTCTG                  CTTCCCTGTCATTTTAAAGATGGAGAAAC
ACAGGCCTGGAGAAAGGAAG                  GGGAATGCGATAGATGGCACTGTGCTGGC
AGGGGCAGGAAGACCAAGGCTGTGACATTCCAGGGAGTGCGACTGCAGTGAAGCGAGTGA
CCACAAAACACTGAACGTAAGCCCTGAGGCTTTGCCACTTGCTGGCACCAAGGACTCTGC
AGTGTTGAGCCAAGAGGATGGACTTTGGAGCCAGACTTTCTGGGTCTATAATCATTCAGT
GTCTGTCTCCTCCTCTAAAATGCGGACGGTGGGAAATTCTGACACACGCTTCAACACAGA
TGCACCTTGAAGACATTATGCTAAGTGAAATGAGCGAGCCAGGCACGAAAGGGATGGGAC
TGGGGCCTCTCTGTGCGTCCCCCTGAGAGACAACGGTCTCTTGGCCACCCACCCTGATCC
CACAGGAGCCAGGCGGGCCCAGCCCTGAGACAGGCCGCCCGCCGCCCGCAATTGGACTAA
AGAGTGTCCCAGGCGTCCGTGCCGCCCAATGGGGCACAGCGGTCGGGGTGCGTAGCCGCA
GGGGCGTGGTCTGAGGTCCGAGACCCGAGTCCTGGCACTGGAGTTTGCTGACTCCCTCTG
GTTTCCGGTCAGGTCGGTCGGTCCCCACTATGGGCCTGGAGCTGTACCTGGACCTGCTGT
CCCAGCCCTGCCGCGCTGTTTACATCTTTGCCAAGAAGAACGACATTCCCTTCGAGCTGC
GCATCGTGGATCTGATTAAAGGTAGGTCCAGCCTCGGGTTTGGGGAACCGAAAAGTCAGG
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Figure 2.2: GSTT1*0 and GSTT1*A polymorphisms obtained from ensembl (http://www.ensembl.org, accessed date: june10, 2006)

2.2. Materials and Methods

2.2.1. Materials

Instruments	Sources
UNIVERSAL 240V 50i60Hz Refrigerated Bench-Top Centrifuge	Hettich GmbH & Co., Germany
MJ Mini Gradient Thermal Cycler	Bio-Rad Laboratories, USA
Alpha Imager® HP (Gel Doc. System)	Alpha Innotech Corporation, USA
Gel Electrophoresis Machine (Elite)	Wealtech, Germany
UV Probe V.2.1 Spectrophotometer	Shimadzu, USA
pH Meter (Cyber Scan 500)	Eutech ,Singapore
Water Bath	Siemens, USA
Micropipette	Bio-Rad Laboratories, USA
Distillation Plant (Distinction D4000)	Bibby Sterlin Ltd., UK
Ultrapure Water System (Arium® 611)	Sartorius, Germany
Microcentrifuge Machine (Mikro 20)	Hettich GmbH & Co., Germany
Freeze (– 40^0 C)	Siemens, USA
Freeze (– 80^0 C)	DAIREI, Sweden
Autoclave Machine	Yongfeng Enterprise Co., UK
Heidolph Unimax-2010 Incubator	Wolf Laboratories Ltd., UK
Reagent Bottle (250, 500, 1000 ml)	Schott GL-45, Germany
Conical Flasks	Schott GL-45, Germany
Pipettes (Precicolor)	HBG, Germany
Eppendorf Tube (1.5 ml)	Hamburg, Germany
Pipette Tips	ALA, USA
PCR Tubes (0.2/0.5 ml)	Bio-Rad Laboratories, USA
Falcon Tubes (50 ml)	Hamburg, Germany
Polypropylene Tubes (15 ml)	Hamburg, Germany

2.2.2. Chemicals and reagents

2.2.2.1. Agarose

Type	DNA Size	Gel Strength
HS	0.5-30	>2000 (1.5%)
H	1-200	>2800 (1.5%)
X	0.01-1	>1000 (3%)
1600	0.01-1	>1400 (1.5%)

2.2.2.2. Other reagents

Reagents	Sources
Triton-X 100	Sigma Chemical Company, USA
Sodium Lauryl Sulphate	Sigma Chemical Company, USA
Ethanol	Sigma Chemical Company, USA
Chloroform	Sigma Chemical Company, USA
Sodium Perchlorate	Sigma Chemical Company, USA
Glacial Acetic Acid	Sigma Chemical Company, USA
Sodium Chloride	Sigma Chemical Company, USA
Sucrose	Sigma Chemical Company, USA
Magnesium Chloride	Sigma Chemical Company, USA
Tris-HCl	Sigma Chemical Company, USA
EDTA-Na2	Sigma Chemical Company, USA
Nuclease Free Water	Promega Corporation, USA
Ethidium Bromide	BDH, UK
Sample Loading Dye,6x	Promega Corporation, USA
Taq DNA Polymerase	NEB, USA
Standard reaction buffer	NEB, USA
$MgCl_2$ Solution	NEB, USA
Deoxynucleotide Solution Mix (dNTP)	NEB, USA
Quick-Load® 50 bp DNA Ladder	NEB, USA
Quick-Load® 2-Log DNA Ladder(0.1-10.0 kb)	NEB, USA
100 bp DNA Ladder	NEB, USA

2.2.2.3. Restriction Enzymes (Res)

Genes	RE	Recognition sites	Source
GSTP1	BsmAI	5'...GTCTC(N)₁▼...3' 3'...CAGAG(N)₅▲...5'	NEB

2.2.2.4. Buffers: (supplied with REs)

Buffer name	Composition	Applicable for enzymes
1X NE Buffer 3	50 mM Tris-HCl	BsmA1
	100 mM NaCl	
	10 mM $MgCl_2$	
	1 mM Dithiothreitol (pH 7.9)	
1X NE Buffer 4	20 mM Tris-acetate	BsmAI
	50 mM Potassium acetate	
	10 mM Magnesium	
	1 mM Dithiothreitol (pH 7.9)	

2.3. Selection of Lung Cancer Patients and Control Subjects

The study was a case-control study conducted on 100 lung cancer patients and 100 healthy volunteers matched by age, sex and smoking status. Lung cancer patients were recruited from Ahsania Mission Cancer and General Hospital, Dhaka Medical College Hospital and Bangabandhu Sheikh Mujib Medical University (PG Hospital), Dhaka, Bangladesh. Patients were histologically diagnosed with lung cancer according to the International Association of Lung Cancer (Travis, 2011) between the periods of January 2009 to December, 2011. After physical examination controls were selected by matching age, sex and

smoking status to lung cancer patients. Smoking information, demographic characteristics, lifestyle factors were collected through interviews by trained nurses in presence of expert physicians. Current smokers had been smoking regularly and nonsmokers had never smoked or not smoked a cigarette a day or 1 cigarette a week for 6 months at any period during his/her lifetime. Those smokers who quit for more than 1 year before the recruitment were considered as former smokers. The study protocol was approved by the ethical committees of the respective hospitals and the study was conducted in accordance with the declaration Helsinkis and its subsequent revisions (WMADH, 2008). Each patient and control subject signed an informed consent document after briefing the purpose of the study.

2.3.1. Blood collection

Venous blood (3 ml) was collected from all cases and controls in sterile tubes containing EDTA-Na_2 and stored at -80^0C until DNA extraction.

2.3.2. Preparation of DNA isolation reagents

2.3.2.1. Cell Lysis Buffer

To prepare 1 L buffer, 10 mM Tris-(hydroxymethyl)-amino methane, 320 mM Sucrose, 5 mM $MgCl_2$ were taken in a 1L buffer container and it was diluted to 850 ml with Milli-Q water. P^H was adjusted to 8.0 by adding Glacial acetic acid. After autoclaving 1% Triton X-100 was added to it and the total solution was made up to 1L by Milli-Q water and it was stored at 4^0C.

2.3.2.2. Nuclear Lysis Buffer

400 mM Tris-(hydroxymethyl)-amino methane, 60 mM EDTA-Na_2, 150 mM Sodium chloride were taken in a 1L buffer container and it was added to 850 ml with Milli-Q water. P^H was adjusted to 8.0 by adding Glacial acetic acid. After

autoclaving, 1% Sodium lauryl sulphate was added to it and the total solution was made up to 1L by adding Milli-Q water and stored at room temperature.

2.3.2.3. Sodium perchlorate (5m)

61.22 gm of Sodium perchlorate was dissolved in 100 ml Milli-Q water and stored at 4^0C

2.3.2.4. Tris-EDTA (TE) Buffer (1x)

10 ml of 1M Tris - (Hydroxymethyl) - amino methane and 2 ml of 500 mM EDTA-Na_2 were mixed in buffer container and then diluted to 1L by adjusting pH to 8.0 and stored at 4^0C. The final concentration of Tris - (Hydroxymethyl) - amino methane and EDTA- Na_2 were 10Mm and 1mM respectively.

2.3.2.5. TAE buffer (10x)

0.4 M Tris -(Hydroxymethyl)-amino methane, 11.4 %(v/v)/0.2 M Glacial acetic acid and 0.01 M EDTA-Na_2 were taken in a buffer container and diluted to 1L after adjusting pH to 7.6 and stored at room temperature.

2.4. Genomic DNA Isolation

DNA was isolated from blood by previously published method (Daly et al., 1998). Briefly, 3 ml blood was taken in a 50 ml Falcon centrifuge tube. 20 ml Lysis Buffer was added to it. Then it was mixed gently for 2 minutes by inversion and It was then centrifuged for 10 minutes at 3000 rpm at 4°C by using UNIVERSAL 240V 50-60Hz Refrigerated Bench- Top Centrifuge Machine (Hettich GmbH & Co., Germany). The supernatant was discarded into a bottle containing enough savlon. The pellet was collected. 2 ml Nuclear Lysis Buffer and 0.5 ml of 5 M Sodium perchlorate were added to it. Then the tube was mixed in a rotary mixture at room temperature for about 15 min so that pellet was dissolved completely. The sample tube was then

incubated at 65℃ for 30 min. (Heidolph Unimax-2010 Incubator, Wolf Laboratories Limited, UK). Then 2.5 ml of chilled Chloroform was added to it. It was then mixed in a rotary mixture for 10 min at room temperature. Then the tube was centrifuged at 1500 rpm for 5 min (37℃). The DNA containing phase (uppermost phase) was transferred to a fresh autoclaved 15 ml polypropylene tube using a disposable Pasteur pipette. Two volumes of Ethanol (double that of DNA phase) was added to it. It was then mixed immediately by slow gentle inversion until all cloudiness was disappeared. DNA was seen to come out of the solution as a white 'cotton-wool' pellet. The white 'cotton-wool pellet' was collected with a disposable microbiology loop. The loop was air dried. The DNA was dissolved in 200µl TE Buffer contained in a 1.5 ml screw cap tube. Then the tube was kept at 65℃ overnight. Then it was taken back and was stored in freezer (-40℃).

2.5. Quantification of Genomic DNA

A vortex shaker for approximately 30 minutes before measurements was taken to confirm the complete sample homogeneity that is critical when measuring genomic DNA concentration and purity with this instrument. Working solutions of genomic DNA were made up to a standard concentration of 50 ng/µl with nuclease free water, except in cases where the sample had an initial concentration of less than 50 ng/µl, in which case an undiluted aliquot was taken as a working solution. A sample volume of 1.5 to 2 µl was pipetted onto the fibre optic measurement surface.

For calculation of the concentration of RNA free DNA, the following conversion factor is used: 1 OD260 = 50 mg of DNA/ml. DNA concentration in µg/µl was calculated as follows:

$$\text{DNA Concentration } (\mu g/\mu l) = \frac{\text{OD } 260 \times 50 \text{ (dilution factor)} \times 50 \ \mu g/ml}{1000}$$

A value out of this range is not acceptable due to the contamination (i.e., protein) in the DNA sample that may inhibit subsequent reactions. The purity and integrity of isolated genomic DNA were also assessed by means of Agarose Gel Electrophoresis. A sample volume of 5 µl (50-70 ng/µl) was resolved on a 1% (w/v) agarose gel.

2.6. Genotyping Overview

In case of GSTM1 & GSTT1 the genotyping of DNA samples collected from cases and controls were analyzed using polymerase chain reaction (PCR) while polymorphism of GSTP1 was analyzed using polymerase chain reaction (PCR) - Restriction fragment length polymorphism (RFLP) due to simplicity, affordability, ease of use and reliability and cost effectiveness of this method in comparison with other methods. In this method PCR amplified product is digested or fragmented with restriction endonuclease (REase). The resulting DNA fragments are then separated by length through agarose gel electrophoresis. The digestion or lack of digestion, of PCR amplification product due to the presence or absence of an SNP within the REase recognition site allows for accurate and reliable genotyping and the consequent determination of SNP frequencies within a sample cohort. The classification of an SNP genotype as 'wild-type' or 'variant' was done according to accepted nomenclature and the relevant reference sequences available from the National Centre for Biotechnological Information(NCBI) Entrez Nucleotides Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide).Primers were designed from the literature study and sequence from the ensembl (http://www.ensembl.org/index.html) and NCBI Reference Sequences (RefSeq).

2.7. Agarose Gel Electrophoresis Procedure

Agarose gel electrophoresis is one of several physical methods for determining

the size of DNA and proteins. This simple but precise, analytical procedure is routinely used for the preparation and analysis of DNA in most every molecular biology research laboratory. It is a powerful separation method frequently used to analyze DNA fragments generated by restriction enzymes, and it is a convenient analytical method for determining the size of DNA molecules in the range of 500 to 30,000 base pairs. It can also be used to separate other charged biomolecules such as dyes, RNA and proteins. Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field. Agarose is a polysaccharide purified from seaweed. An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool. The result is a flexible gelatin-like slab. During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode. The DNA to be analyzed is forced through the pores of the gel by the electrical current. Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black). Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules. Smaller DNA molecules move through the agarose faster than larger molecules. DNA itself is not visible within an agarose gel. DNA is visualized by the use of a dye that binds to DNA. On completion of the PCR reaction, it is often useful to determine whether the amplification has been successful using gel electrophoresis before additional analysis is performed. The precise type of gel and electrophoresis conditions depend on several factors including the size of the fragments to be analyzed and whether separation of a number of fragments of similar molecular weight is required. It is essential that molecular weight standards be run on all gels. For most purposes, we use 100-bp DNA ladder markers.

2.7.1. Agarose gel electrophoresis protocol

2.7.1.1. Preparation of agarose Gel

1. An appropriate amount of agarose powder (1gm for 2% agarose gel) was added in a conical flask containing appropriate volume of 1x TAE buffer (50 ml for 2% agarose gel). The amount of agarose powder and gel volume will vary depending on the size of the casting tray.

2. The solution is then heated in a microwave until the solution becomes clear.

3. Then the solution was allowed to cool to about 50°-55°C and swirling the flask occasionally to ensure cool evenly.

4. The casting tray was prepared by sealing the two ends with two layers of tape.

5. Prior to pouring the gel, Ethidium bromide was added to the dissolved agarose and swirled to mix.

6. The gel was poured into the casting tray and the comb was adjusted to keep the wells perpendicular. The gel was allowed to cool and was hardened (20-30minutes) prior to use.

7. The gel was placed in the electrophoresis chamber by pulling out the comb and removing the tape and enough TAE (1x) buffer was added so that there is about 2-3 mm of buffer over the gel.

2.7.2. Loading and running the gel

1. An appropriate volume of loading dye was added to PCR product or digested product (1µl of dye with each 6 µl sample).

2. 6-20µl of sample loading buffer mixture was placed into separate wells in the gel.

3.10 µl of the DNA ladder standard was loaded into at least one well of each row on the gel.

4. After the gel had been loaded, the cover was gently placed on the apparatus and

the power leads were hooked up. The power was adjusted to 80 volts (constant voltage). The gel was run until the first dye front (bromophenol blue) had migrated about two-thirds the length of the gel and the second dye front (xylene cyanol) had migrated approximately one-third of the length of the gel.

5. The power was turned off before removing the gel for photographing. The gel was placed on the UV transilluminator to visualize the DNA.

2.8. Genotyping of GSTM1 & GSTT1

After collecting 3 ml of venous blood in a sterile eppendorf tube containing EDTA-Na_2 from each of cases and controls, it was stored at -80^0C until DNA extraction. Genomic DNA was extracted from blood samples of all subjects (Daly et al., 1998). Quantification and purity determination were performed by UV Spectrophotometric method at 260 and 280 nm. PCR method was employed for genotyping due to its simplicity, affordability and reliability and cost effectiveness. Primers were designed from the published papers. All DNA samples (100 lung cancer cases and 100 controls) were analyzed for the genotyping of GSTM1 by the previously reported method with slight modification (Abdel-Rahman SZ et al, 1996).

In case of GSTM1, the primers used were- FP: 5-CTGCCCTACTTGATTGATGGG-3(sense) and 5-CTGGATTGTAGCAGATCATGC-3 (antisense) whereas in case of GSTT1 the primers used were GSTT1 (F)5-TTCCTTACTGGTCCTCACATCTC-3 and GSTT1 (R)5-TCACCGGATCATGGCCAGCA-3 The basic steps for all PCR reactions were: 1) initial denaturation; 2) denaturation; 3) annealing; 4) extension; 5) an allele-specific number of cycled repeats of steps 2-4 (denaturation, annealing, extension); and 6) final extension (first amplification only). The PCR conditions were mentioned in Table 2.10 & 2.11. PCR was carried out in total volume of 25 µl containing 1 µL genomic DNA samples (50-70 ng/µl), 2.5 µl of 10x standard Taq

reaction buffer (with MgCl2), 0.5 µl dNTPs (10 mM), 0.5 µl of each primer (10 mM), 0.13 µl Taq DNA polymerase (5U/ µl) (NEB, USA) and 20 µl nuclease free water.

Markers used for the study

PCR amplified product BRCA2 has been used as a marker for GSTM1 and PCR amplified product of CYP3A5*3 has been used as a marker for GSTT1

1. An appropriate volume of loading dye was added to PCR product or digested product (1µl of dye with each 6 µl sample).

2. 6-20µl of sample loading buffer mixture and 5 µl of marker were placed into the same wells in the gel.

3.10 µl of the DNA ladder standard was loaded into at least one well of each row on the gel.

4. After the gel had been loaded, the cover was gently placed on the apparatus and the power leads were hooked up. The power was adjusted to 80 volts (constant voltage). The gel was run until the first dye front (bromophenol blue) had migrated about two-thirds the length of the gel and the second dye front (xylene cyanol) had migrated approximately one-third of the length of the gel.

Table 2.10: PCR conditions for amplification of alleles of *BRCA2* & CYP3A5*3

ALLELE	**PCR CONDITIONS**	**SIZEOF PCR PRODUCTS**
BRCA2	94^0 C for 1 min 59^0 C for 30sec 72^0 C for 1 min	346
CYP3A5*3	94^0C for 1 min 57^0C for 1 min 72^0C for 1 min	196

Table 2.11: PCR conditions for amplification of alleles of *GSTM1 & GSTT1*

Allele	PCR conditions	Size of PCR products
GSTM1	94^0 C for 1 min 59^0 C for 1 min 72^0 C for 1 min	273
GSTT1	94^0 C for 1 min 59^0 C for 1 min 72^0 C for 1 min	459

2.9. Gel Electrophoresis and Visualization of PCR Product

After PCR amplification, 5µl of PCR product was mixed with 1µl of 6x blue orange loading dye. 2% agarose gel was prepared from the agarose powder (Bioline, UK) with 1x TAE buffer. The gel was stained with ethidium bromide (EtBr) (BDH, UK) (1 µg of EtBr per ml agarose solution from the stock solution of 10 mg/ml. After gel loading samples were electrophoresed at 80 V for 60 minutes and visualized on the UV transilluminator (Alpha Innotech Corporation, San Leandro, California).

2.10. Restriction Enzyme Digestion and Visualization After PCR Amplification

PCR product of GSTM1 and GSTT1 were not digested with restriction endonucleases.

2.11. Direct sequencing

In case of GSTP1 All mutant homozygous and 20% of heterozygotes analyzed twice to confirm genotype and were also subject to direct sequencing by standard Kit of ABI PRISM BigDye Terminator (Applied Biosystems, USA) with 100% concordance.

2.12. Statistical Analysis

Distributions of demographic variables were compared between cases and controls using $\chi 2$- tests and two-sided unpaired t-tests. For the assessment of the deviation from Hardy–Weinberg equilibrium in the reported genotype frequencies among controls, the appropriate goodness-of-fit $\chi 2$-test was carried out. Genotype and allelic frequencies were reported as percentage. The distribution of geneotype and haplotype frequency was also compared by $\chi 2$- test. Unconditional logistic regression was used to estimate crude odds ratio (OR), adjusted OR and their 95% confidence intervals (CIs), with adjustment for age, sex and smoking status using the statistical software package SPSS version 20.0 (SPSS, Inc., Chicago, IL).

2.13. Result

2.13.1. Cases and controls characteristics

The distributions of demographic characteristics and clinical data among study subjects are summarized in Table 2.12. This case-control study consisted of 100 lung cancer cases and 100 controls. The related factors such as gender, age and smoking history of cases and controls were compared to confirm the observed effects were solely due to the genotype frequency. There were no significant differences in gender (p =0. 828), mean age (p = 0.579) and smoking status (p = 0.228) between the two groups.

2.13.2. Histological subtype of lung cancer

Among 100 cases, the histological subtypes of lung cancer were squamous cell Carcinoma (44%), adenocarcinoma (36%), small cell carcinoma (17%), large cell carcinoma (2%) and adenosquamous cell carcinoma (1%) (Table 2.12.).

2.13.3. Smoking status

The observed ever smoking rate was 80% in the cases and 79% in controls. Among the ever smokers 53% and 27% were current smokers & Ex-smokers in

cases and 63% and 13% were current smokers & ex-smokers in controls, respectively. There is no significant difference between current smoker, Ex-smoker never smokers & chewing tobacco groups between cases and controls (p= 0.228) (Table-2.12).

Table 2.12: Distribution of demographic variables of the lung cancer patients and controls

Variables	Cases (n=100) (%)	Control (n=100) (%)	p-value
Gender, n (%)			
Male	87	89	0.828[a]
Female	13	11	
Age(years)			
Mean age ,n (±SD)	57.81(±10.315)	59.10(±9.739)	0.579[b]
Range	18-80	20-87	
Smoking status, n (%)			
Current smoker	53	63	0.228[a]
Ex-smoker	27	16	
Never smoker	8	11	
Chewing tobacco	12	10	
Ever smoker	80	79	
Histological Type, n (%)			
Adenocarcinoma	36		
Squamous cell carcinoma	44		
Small cell carcinoma	17		
Large cell carcinoma	2		
Adenosquamous cell carcinoma	1		

[a] Chi-square test, [b]Unpaired t test

2.13.4. Genomic DNA extraction

Genomic DNA was isolated from 100 lung cancer patients and 100 controls successfully (Daly *et al*., 1998). The purity (OD 260/OD 280) of all the genomic DNA samples was found to be in the range between 1.7 to1.9 and the average concentration was 50 to 70 ng/ml.

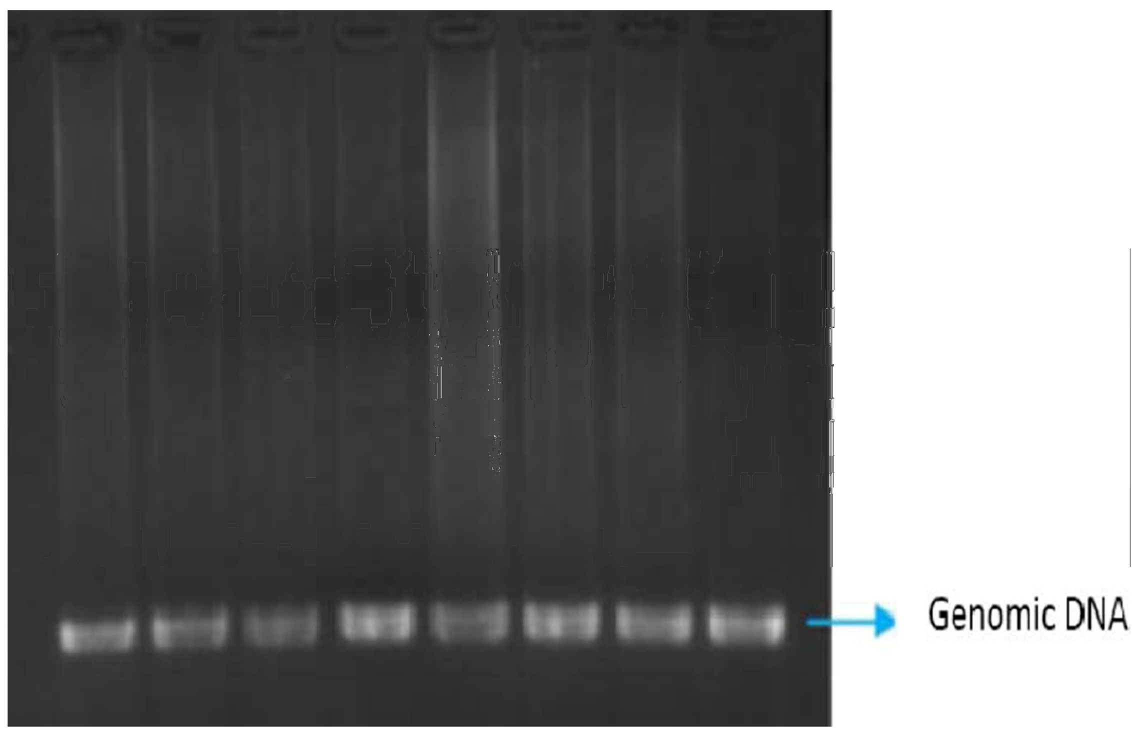
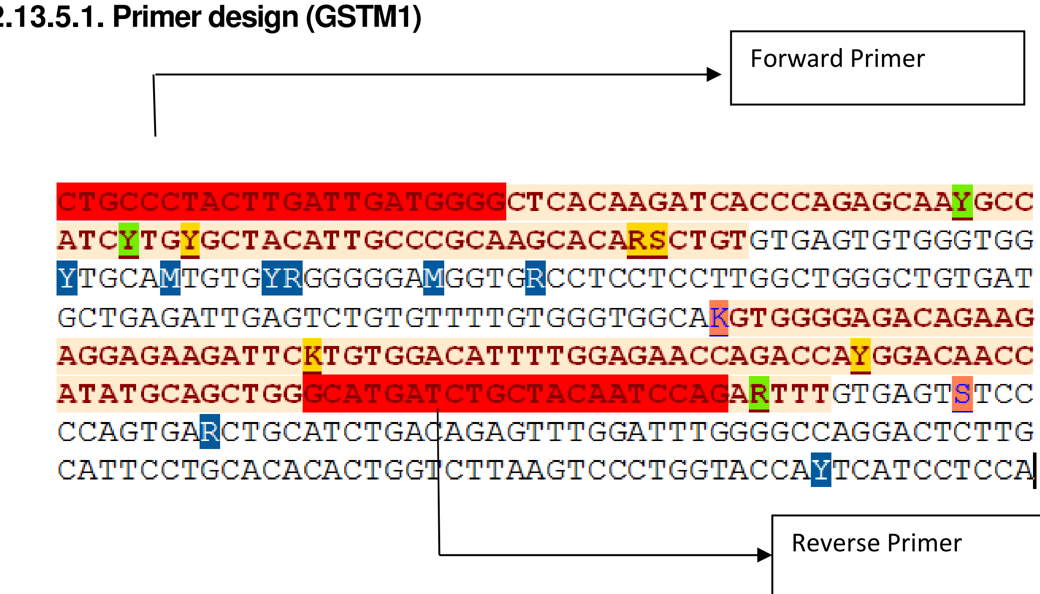


Figure 2.3: Agarose gel electrophoresis (1% w/v agarose) of genomic DNA

2.13.5. Genotyping of GSTM1 gene

PCR method was carried out to detect the GSTM1 gene in the lung cancer cases and controls after PCR amplification amplified PCR products were not digested by Restriction Endonucleases.

2.13.5.1. Primer design (GSTM1)



There are some guidelines for primer design:

• PCR primers should be generally 15-30 nucleotides long.

• Optimal GC content of the primer is 40-60%. Ideally, C and G nucleotides should be distributed uniformly along the primer.

• Should avoid placing more than three G or C nucleotides at the 3'-end to lower the risk of non-specific priming.

• Should avoid primer self-complementarity or complementarity between the primers to prevent hairpin formation and primer dimerization.

• Should check for possible sites of non-desirable complementarity between primers and the template DNA.

• Differences in melting temperatures (Tm) of the two primers should not exceed 5°C.

By considering all the factors, the primers for the study were designed. The sequences of the primers used and their sizes are presented in

After completing PCR amplification with appropriate reagents a PCR product of *GSTM1* was obtained.

```
CTGCCCTACT TGATTGATGG GGCTCACAAG ATCACCCAGA
GCAAYGCCAT CYTGYGCTAC ATTGCCCGCA AGCACARSCT
GTGTGAGTGT GGGTGGYTGC AMTGTGYRGG GGGAMGGTGR
CCTCCTCCTT GGCTGGGCTG TGATGCTGAG ATTGAGTCTG
TGTTTTGTGG GTGGCAKGTG GGGAGACAGA AGAGGAGAAG
ATTCKTGTGG ACATTTTGGA GAACCAGACC AYGGACAACC
ATATGCAGCT GGGCATGATC TGCTACAATC CAG
```

The PCR product size was 273 bp along with marker was visualized in 2% (w/v) agarose gel.

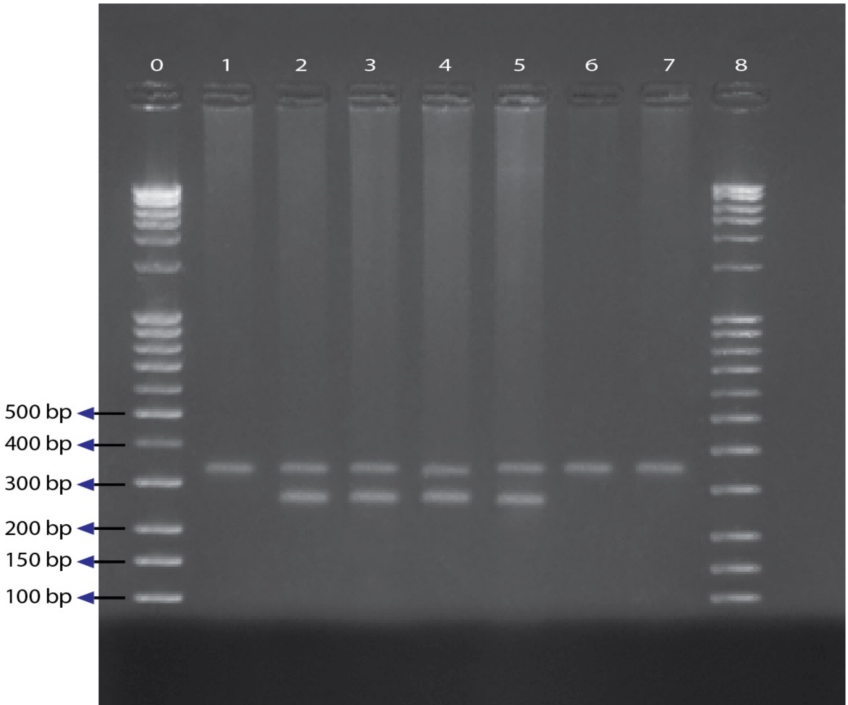


Figure 2.4: Polymerase chain reaction assay for GSTM1 gene polymorphism. Lanes 2, 3, 4.5; GSTM1 positive genotype (273 bp), lanes 1, 6, 7; GSTM1 null genotype, M: marker. BRCA2 gene was used as an internal positive control (346bp).

2.13.5.2 Primer design for BRCA2 *gene (Marker)*

PCR method was carried out to detect the GSTM1 genotype in the lung cancer cases and controls PCR amplified product of BRCA2 was used as a marker in gel documentation

Table 2.13 Primer sequence of BRCA2

NO	Allele	Primer sequence	MT (℃)	Size (bp)
1.	BRCA2	FP 5' TGG AAT ACA GTG ATA CTG AC 3'	63.08	20
		RP 5' TTG GAT TAC TCT TAG ATT TG 3'	65.58	20

2.13.5.3 PCR product of BRCA2

TGGAATACAGTGATACTGACTTTCAATCCCAGAAAAGTCTTTTATATGATCATG
AAAATGCCAGCACTCTTATTTTAACTCCTACTTCCAAGGATGTTCTGTCAAACC
TAGTCATGATTTCTAGAGGCAAAGAATCATACAAAATGTCAGACAAGCTCAAAGG
TAACAATTATGAATCTGATGTTGAATTAACCAAAAATATTCCCATGGAAAAGAATCAAGATGTAT
GTGCTTTAAATGAAAATTATAAAAACGTTGAGCTGTTGCCACCTGAAAAATACATGAGAGTAGC
ATCACCTTCAAGAAAGGTACAATTCAACCAAAACATTGGATTACTCTTAGATTTG

By using the appropriate pair of primer and other PCR reaction program parameters the PCR product of **BRCA2** was obtained. The PCR product size was 346 bp. The PCR product was visualized in 2% (w/v) agarose gel along with PCR product of GSTM1.

2.13.5.4 Genotyping of GSTT1 gene

PCR method was carried out to detect the GSTT1 gene in the lung cancer cases and controls

2.13.5.5 Primer design (GSTT1)

```
TTCYTTACTGGTCCTCWCATCTCCTTAGCTGACCTCRTAGCCATCACGRAGCTGATGCATG
TGARTGCTGTGGGCAGG GAACCCACTAGGCAGGGGGCCCTGGCTAGTTGCTGAAGTCCTG
CTTATGCTGCCACACCGGGCTATGGCACTGTGCTTAAGTGTGTGTGCAAACACCTCCTGGA
GATCTGTGGTCCCCAAATCAGATGCTGCCCATCCCTGCCCTCACAACCATCCATCCCCAGT
CTGTACCCTTTTCCCCACAGCCCGTGGGTGCTGGCTGCCAAGTCTTCRAAGGC GACCCAA
GCTGGCCACATGGCGGCAGCRCGTGGAGGCAGCAGTGGGGRAKGACCTCTTCCAGGAGGCC
CATGAGGTCATTCTGAAGGCCAAGGACTTCCCACCTGCAGACYCCACCATAAARCAGAAGC
TGATGCCCTGGGTGCTGGCCATGATCYGGTG GCTGGGAAACCTCACCCTTGCACCGTCCT
CAGCAGTCCACAAAGCATTTTCATTTCTAATGGCCCATGGAGCCAGGCCCAGAAAGCAGG
AATGGCTTGCYTAAGACTTGCCCAAGTCCCAGAGCACCTCACCTCCCGAAGCCACCATCCC
CACCCTGTCTTCCACAGCCGCCTGAAAGCCACAATGAGAATGATGCACACTGAGGCCTTGT
GTMCTTTAATCACTGCATTTCATTTTGATTTTGGATAATAAACCTGGGCTCAGCCTGAGCC
TCTGCTTCTAACTCTAATGTGTGATTTATTTGACTTTCCTCTGTCCCAGACCTGGTCATGG
TCTCTAT
```

Forward Primer

Reverse Primer

Primer sequence | Possible SNPs

Exon sequence | SNP of interest

Table 2.14 Primer sequence of GSTT1

No	Allele	Primer sequence	MT (℃)	Size (bp)
1.	GSTT1	FP 5' TTCCTTACTGGTCCTCACATCTC 3'	73.08	20
		RP 5' TCACCGGATCATGGCCAGCA 3'	75.58	20

2.13.5.6 PCR product (GSTT1)

By using the appropriate pair of primer and other PCR reaction program parameters the PCR product of **GSTT1** was obtained. The PCR product size was 459 bp.

```
TTCCTTACTGGTCCTCACATCTCCTTAGCTGACCTCGTAGCCATCACGGAGCTGATGCATC
CCGTGGGTGCTGGCTGCCAAGTCTTCGAAGGCCGACCCAAGCTGGCCACATGGCGGCAGCG
CGTGGAGGCAGCAGTGGGGGAGGACCTCTTCCAGGAGGCCCATGAGGTCATTCTGAAGGCC
AAGGACTTCCCACCTGCAGACCCCACCATAAAGCAGAAGCTGATGCCCTGGGTGCTGGCCA
TGATCCGGTGA
```

2.13.5.7 Primer design for CYP3A5*3 *gene (Marker)*

PCR method was carried out to detect the GSTT1 genotyping in the lung cancer cases

and controls. PCR amplified product of **CYP3A5*3** was used as a marker in gel documentation

Table 2.15 primer sequence of CYP3A5*3

Allele	Primer sequence	MT (℃)	Size (bp)
CYP3A5*3	**FP** 5'-CCTGCCTTCAATTTTTCACT-3'	58.0	20
CYP3A5*3	**RP** 5'- GGTCCAAACAGGGAAGAGGT-3'	65.0	20

2.13.5.8. PCR product of CYP3A5*3 gene (Marker)

By using the appropriate pair of primer and other PCR reaction program parameters the PCR product of CYP3A5*3 was obtained. The PCR product size was 196 bp. The PCR product was used as a marker

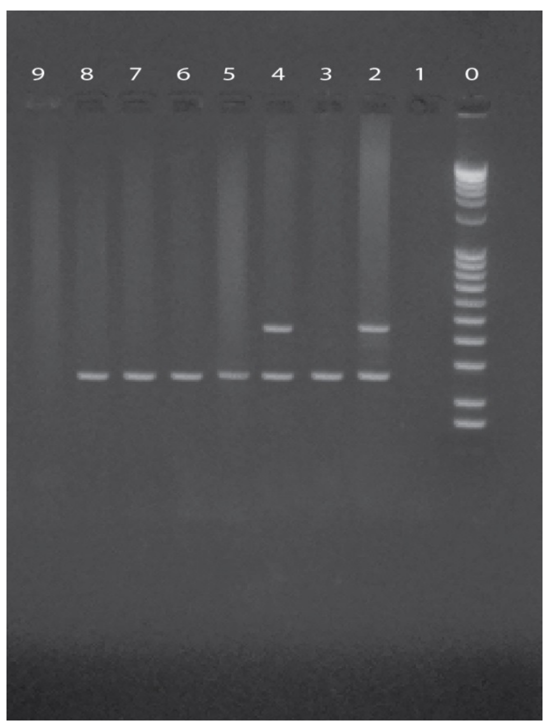


Figure 2.5: Polymerase chain reaction assay for GSTT1 gene polymorphism. Lane 2,4 ; GSTT1 positive genotype, lanes 3,5,6,7,8 ; GSTT1 null genotype, 0: marker. **CYP3A5*3** gene was used as an internal positive control.

PCR of CYP3A5*3 (rs776746)

CCTGCCTTCAATTTTTCACTGACCTAATATTCTTTTTGATAATGAAGTATTTTAA
ACATAKAAAACATTATGGAGAGTGGCA9TAGGAGAKACCCACGTATGTACCAC
CCAGCTTAACGAATGCTCTACTGTCATTTCTAACCATAATCTCTTTAAAGAGCT
CTTTTGTCTTTCARTACCTCTTCCCTGTTTGGACC

Primer sequence	Possible SNPs
Exon sequence	SNP of interest

2.14. Genotype and Allele Frequency of GSTM1

Table 2.16 The genotype frequency of GSTM1

Genotype	Patient	Control	Odd Ratio	95%CI	p
Null genotype	58	56	1.08	(0.61 to 1.89)	0.775
GSTM1-positive	42	44			

Among the 100 cases, 58% were carrying null genotype, and 42% were GSTM1 positive. Among the 100 controls 56% were carrying null genotype, and 44% were GSTM1 positive. No significant difference was found between the genotype frequency distribution of the two groups (p = 0.775) carrying null genotype. Risk of lung cancer by GSTM1 null genotype is not statistically significant (OR = 1.08, 95% CI = (0.61 to 1.89), p = .775)

Using unconditional logistic regression models adjusting for age, sex, and tobacco use following is obtained.

Genotype	Patient	Control	Odd ratio	95%CI	p
Null genotype	72	76	.81	(0.43 to 1.52)	0.519
GSTT1-positive	28	24			

2.15 .Genotype and Allele Frequency of GSTT1

Table 2.17: The genotype frequency of GSTT1

Genotype	Patient	Control	Odd Ratio	95%CI	p
Null genotype	72	76	.821	(0.43 to 1.56)	0.550
GSTT1-positive	28	24			

Among the 100 cases 72% were carrying null genotype, and 28% were GSTT1 positive. Among the 100 controls 76% were carrying null genotype, and 24% were GSTT1 positive. No significant difference was found between the genotype frequency distribution of the two groups (P = 0.519) carrying null genotype. Risk of lung cancer by GSTT1 null genotype is not statistically significant (OR = 0.81, 95% CI =0.43 to 1.52, p = 0.519)

Using unconditional logistic regression models adjusting for age, sex, and tobacco use following is obtained

Genotype	Patient	Control	Adjusted Odd Ratio	95%CI	p
Null genotype	72	76	0.81	(0.43 to 1.52)	0.519
GSTT1-positive	28	24			

2.16 Association Between Lung Cancer Risk and Tobacco Consumption

Table 2.18 GSTM1 genotype among lung cancer patients and controls

Tobacco use status		GSTM1 Patient		
		Null	Present	Total
	Chewing tobacco	8	4	12
	Current smoker	33	20	53
	Ex-smoker	13	14	27
	Never smoker	4	4	8
Total		58	42	100

Tobacco use status		GSTM1 Control		
		Null	Present	Total
	Chewing tobacco	4	6	10
	Current smoker	31	32	63
	Ex-smoker	13	3	16
	Never smoker	8	3	11
	Total	56	44	100

Table 2.19 GSTT1 genotype among lung cancer patients and controls

Tobacco use status		GSTT1 Control		Total
		Null	Present	
	Chewing tobacco	7	5	12
	Current smoker	41	12	53
	Ex-smoker	17	10	27
	Never smoker	7	1	8
Total		72	28	100

Tobacco use status		GSTT 1 Patient		Total
		Null	Present	
	Chewing tobacco	9	1	10
	Current smoker	47	16	63
	Ex-smoker	13	3	16
	Never smoker	7	4	11
	Total	76	24	100

Table 2.20 Effect of GSTM1 & GSTT1 genotypes on the association of smoking with lung cancer.

Genotype	Tobacco user		OR (95% CI)	p	Non user		OR (95% CI)	p
	Patients (%) (n=100)	Controls (%) (n=100)			Patients (%) (n=100)	Controls (%) (n=100)		
GSTM1								
Present	38	41	1		4	3	1	
Null	54	48	1.21 (0.67 to 2.18)	0.518	4	8	0.37 (0.05 to 2.55)	0.316
GSTT1								
Present	27	20	1		1	4	1	
Null	65	69	0.69 (0.35 to 1.36)	0.292	7	7	4 (0.35 to 45.38)	0.263

According to table 2.20, lung cancer cases having distribution of variant genotypes of GSTM1 i.e. homozygous deletion of the gene or a null genotype & present (OR=1.21, 95% CI = 0.67 to 2.18, p=0.518; OR=0.37, 95% CI = 0.05 to 2.55 ,p=0.316,respectively) and in case of GSTT1 homozygous deletion of the gene or a null genotype & Present (OR = 0.69 95% CI = 0.35 to 1.36, p=0.292; OR= 4 , 95% CI = 0.35 to 45.38, p=0.263, respectively) were not found to be higher in the

Tobacco user cases leading to the increased risk of lung Cancer, which is statistically not significant. There is no increase in the risk of lung cancer in case of non-smoker of null genotype & present .No association with risk of lung cancer was found with the distribution of variant genotypes of GSTT1 in case of non-user.

Table 2.21 Distribution of GSTM1 & GSTT1 combined genotype among lung cancer patients and their association with lung cancer histology

Type of Lung Cancer		GSTM1 &GSTT1				Total
		(++)	(+-)	(-+)	(--)	
	Adenocarcinoma	4	11	4	17	36
	Adenosqumous	0	0	1	0	1
	Large cell	1	1	0	0	2
	Small cell	1	7	3	6	17
	Squmous cell	6	11	8	19	44
Total		12	30	16	42	100

(++)= GSTM1 positive & GSTT1 Positive, (+-) = GSTM1 positive & GSTT1 null, (-+) = GSTM1 null & GSTT1 Positive, (--) GSTM1 null & GSTT1 null.

As tobacco use is the potential risk factors to lung cancer, we further calculated the modifying effect of GSTM1 & GSTT1 genotypes on the association of tobacco use with lung cancer in table 2.22

Table 2.22 : Modifying effect of GSTM1 & GSTT1 genotypes on the association of smoking with lung cancer

Genotype	Chewing tobacco		OR (95% CI)	p	Current smoker		OR (95% CI)	p	Ex-smoker		OR (95% CI)	p	Never smoker		OR (95% CI)	p
	Patients (%) (n=100)	Controls (%) (n=100)			Patients (%) (n=100)	Controls (%) (n=100)			Patients (%) (n=100)	Controls (%) (n=100)			Patients (%) (n=100)	Controls (%) (n=100)		
GSTM1																
Present	4	6	1		20	32	1		14	3	1		4	3	1	
Null	8	4	3 (0.52 to 17.15)	0.216	33	31	1.70 (0.81 to 3.58)	0.160	13	13	0.21 (0.04 to 0.92)	0.039	4	8	0.37 (0.05 to 2.55)	0.316
GSTT1																
Present	5	1	1		12	16	1		10	3	1		1	4	1	
Null	7	9	0.15 (0.01 to 1.65)	0.122	41	47	1.16 (0.49 to 2.74)	0.729	17	13	0.39 (0.08 to 1.72)	0.214	7	7	4 (0.35 to 45.38)	0.263

2.17 Discussion

A number of studies have tried to establish links between polymorphic expression of different GSTs and lung cancer risk in different ethnic populations and the results have been conflicting. One reason for the differences could be the fact that most studies were conducted in different populations. However, none of the main characteristics of the subjects explain satisfactorily the apparent differences (i.e. race, histological type and level of smoking). Different histological subtypes of lung cancer, in particular may also be related to respective exposures or factors, and thus need to be analyzed separately. The M1 and T1 variant of GSTs detoxifie of major classes of tobacco procarcinogens such as aromatic amines and PAHs. Carriers of homozygous deletion in GSTM1 and GSTT1 genes have an absence of GST-m and GST-q enzyme activity, respectively. These deletion variants are very useful in epidemiological studies of cancer because they divide individuals in two well-defined susceptibility classes: those who are and those who are not able to detoxify potential carcinogens by the metabolic pathways regulated by GSTM1 and GSTT1 genes. The relationship between GSTM1 polymorphism and lung cancer has already been observed in two large studies from Japan (Hayashi S et al., 1992; Kihara et al.1995) and two from China. Furthermore, in a study in Caucasians, a significant association of lung adenocarcinoma with the GSTM1 null genotype was reported (Lan et al., 2000; Sun et al., 1997; Woodson et al., 1999). In a meta-analysis study by Williams et al, it was shown that GSTM null allele was a risk factor for the development of lung cancer (Williams et al., 1997.).

A meta-analysis of 11 studies found an OR of 1.6 (95% CI= 1.26–2.04) for an association between the GSTM1 null genotype and lung cancer risk (Errico et al., 1997.). In another meta-analysis study, it was reported that there was no statistically significant relationship between the individuals carrying GSTM1 null genotype and susceptibility to lung cancer but

the number of patients carrying this genotype was greater in the lung cancer group (Benhamou et.al.,2009).

GSTM1 null allele in the present study is 58%, which is not similar to the frequencies reported in Indian subcontinent**.** The Comparative analysis of the GSTM1 null allele frequency of Bangladeshi patient, according to the two sided Fisher's test as shown in Table was unexpectedly not significantly different to the previous reports from India for various Indian ethnic's frequency of 23-33% (Roy et al., 1998; Buch et al., 2001; Mishra et al., 2004; Naveen et al., 2004; Vetriselvi et al., 2006; Konwar et al., 2010).

Table 2.23: GSTM1 genotype frequencies with compare to Indian ethnic group

Location	Sample No.	GSTM1 null(%)	p value	Literature
Bangladesh	100	58	0.519	Present study
Gujarat	504	20	0.679	KP Senthilkumar et al
Karnataka	110	36.4	0.01183*	Naveen et al., 2004
Lucknow	200	36.5	0.01773*	Konwar et al., 2010
Pradesh	115	33	0.054	Naveen et al., 2004
Kerala	122	31.9	0.0756	Naveen et al., 2004
North India	370	33	0.1125	Mishra et al., 2004
South India	772	27.72	0.2463	Naveen et al., 2004, Vetriselvi et al., 2006
Central India	883	26.6	0.317	Buch et al., 2001
Tamilnadu-Pondicherry	170	23.5	0.6089	Naveen et al., 2004
Orissa	72	23.8	0.6089	Roy et al., 1998

*Significance at $p<0.05$

In our study, we found no significant relation between GSTM1 null genotype and susceptibility to lung cancer. Additionally, the rate of GSTM1 null genotype was higher in the control group with compare to other control groups in different Indian ethnic population. GSTM1 locus is entirely absent in approximately 50% of Caucasians. GSTM1 null genotype

has been shown in 31% to 66% of Asians, Indians and Caucasians (Ozturk et al., 2003; Hong YS et al., 1998; Persson et al., 1999.).

On the other hand, the frequency of GSTM1 deletion polymorphism for African-Americans was found to be 23% to 35 % (Kelsey et al., 2000) and this figure was 21% for Chileans (Quinones et al., 1999). In the study by Öztürk and coworkers, GSTM1 null genotype incidence was found to be very similar in the control subjects and in lung cancer patients (respectively 51.7% and 51.5%) in the Turkish population (Ozturk et al., 2003). In our study, the rate of GSTM1 null genotype was detected as 58% in cases. The incidence of the GSTT1 null allele differs among global populations. Significant differences in GSTT1 null allele frequencies were observed between Caucasian, Asian, African and African American populations (Lee et al., 2008) and the prevalence of GSTT1 null allele in the present study is 72%, which is not similar to the frequencies reported in Indian subcontinent (Roy et al., 1998; Buch et al., 2001; Mishra et al., 2004; Naveen et al., 2004; Vetriselvi et al., 2006; Konwar et al., 2010 ; Roy et al., 1998; Buch et al., 2001; Mishra et al., 2004; Naveen et al., 2004; Vetriselvi et al., 2006; Konwar et al., 2010).

Table 2.24. GSTT1 null genotype frequencies with compare to Indian ethnic group

Location	Sample No	GSTT1 Null%	p Value	Literature
Bangladesh	100	72	0.519	Present study
Gujarat	504	35.5		KPSenthilkumar et al., 2012
Tamilnadu-Pondicher	170	13.0	0.0002481***	Naveen et al., 2004
Western Central India	883	13.0	0.0002481***	Buch et al., 2001
Lucknow	200	14.0	0.0005253***	Konwar et al., 2010
Kerala	122	15.6	0.002018**	Naveen et al., 2004
South India	772	17.09	0.003691**	Naveen et al., 2004, Vetriselvi et al., 2006
North India	370	18.4	0.006471**	Mishra et al., 2004
Karnataka	110	19.1	0.01091*	Naveen et al., 2004
Andhra Pradesh	115	18.8	0.01091*	Naveen et al., 2004

*Significance at p<0.05, **Significance at p<0.01, ***Significance at p<0.001

Among world population Korean population showed higher frequency of (45.3%) of GSTT1 null allele compared with the white Americans (20.4%), African Americans (21.8%), and Mexican-Americans (9.7%) (Hoglund et al., 2009; Marinkovic et al., 2008) and Turkish populations (10.8–28.3%) (Oke et al.,1998 ; Shchipanov et al., 2008; Sura et al., 2008.). The GSTT1 null allele frequency in Native Russians is very close to allelic frequencies observed in some European populations (Baysal et al., 2008). GSTT1 null allele in Ouangolodougou, a north Ivory Coast population, is significantly higher (33.1%) than in Chinese, Japanese and Pakistani populations (Santovito et al., 2010; Shaikh et al., 2010.). In the HapMap CEU population, it was demonstrated that the SNP rs2266633 (Asp141Asn) is the “tagging SNP” of the GSTT1 homozygous deletion (Zhao et al., 2009). In our study, we found no statistically significant relation between GSTT1 null genotype and susceptibility to lung cancer. Additionally, the frequency of GSTM1 null genotype was higher with compare to different Indian ethnic population as well as world population.

2.18 Conclusion

Our observations showed that carrying the GSTM1 & GSTT1 null genotype is not a risk factor alone for lung cancer. Large scale multicenter studies are necessary to obtain more reliable and correct results.

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CHAPTER THREE

LUNG CANCER RISK IN RELATION TO GSTP1 GENOTYPE IN BANGLADESHI PATIENTS

3. GSTP1 and Allele of GSTP1

Glutathione-S-transferases (GSTs) are major phase II detoxifying enzymes and are able to perform a wide variety of functions. Phase II enzymes catalyze conjugation of activated xenobiotics with an endogenous water soluble substrate, such as glutathione (GSH), UDP-glucuronic acid or glycine (Miller *et al*., 1988). GSTP1 codes for enzyme glutathione S-transferase pi and is located on chromosome 11q13. It is also subjected to polymorphic variation(Hayes *et al*., 1995). Four GSTP1 alleles have been identified (Clapper, 2000; Harris *et al*., 1998; Lo *et al*., 1997). The wild type allele (GSTP1*A) differs by an A:G transition at nucleotide 313 (Val105-Ala114) from GSTP1*B and from GSTP1*C by this transition and a C:T transition at 341 (Val 105 –Val 114). A GSTP1*D allele (Ile 105 –Val 114) has also been identified.

Table 3.1: Alleles nomenclature and functional consequence of GSTP1*(Sherratt et al., 2002)*

Class or Super family	Gene	Allele	Alteration in gene Or nucleotides	Protein or amino acid affected
Pi	GSTP1	GSTP1*A	A313, C341, C555	Ile105, Ala114, Ser185
		GSTP1**B	G313, C341, T555	Val105,Ala114, Ser185
		GSTP1*C	G313, T341, T555	Val105, Val114, Ser185
		GSTP1*D	A313, T341	Ile105, Val114

3.1.1 Glutathione-S-transferase P1 (GSTP1) and lung cancer risk:

Glutathione-S-transferase P1 (GSTP1) may be important in determining risk for lung cancer because it is the most prevalent GST in lung tissue (Anttila *et al*., 1993; Hayes *et al*., 1995). It has the highest specific activity towards the active benzo(a)pyrene diol

epoxide metabolite of cigarette, benzo(a)pyrene-7b,a-dihydrodiol-9a,10a-epoxide (BPDE) and is almost exclusively active towards the (+)-enantiomer of anti-BPDE, thought to be the ultimate mutagenic form of benzo(a)pyrene(Robertson *et al.*, 1986) and shown to be a direct cause of lung cancer (Denissenko *et al.*, 1996; Tang *et al.*, 1995). Two commonly expressed GSTP1 variants have differing specific activities and affinities for electrophilic substrates resulting from a single base pair difference (nucleotide 313) and an amino acid substitution (Ile0/Val) at codon 105, site close to the hydrophobic binding site for electrophilic substrates(Zimniak *et al.*, 1994). Gene frequency for the val (or GSTP1b) variant has been reported at 0.11-0.34 among healthy control populations (British).(Harries *et al.*, 1997; Harris *et al.*, 1998) The val variant has generally lower activity towards polycyclic aromatic hydrocarbon diol epoxides, especially BPDE and, thus, has been predicted to have lower detoxification potential and greater risk for cancer (Coles *et al.*, 2000). DNA-adducts occur more frequently in GSTP1val (GSTP1b) individuals than in those with GSTP1iso (GSTP1a) (Ryberg *et al.*, 1997). However, the val variant has higher activity than the isoleucine variant towards some other diol epoxides in tobacco smoke, including 5-methylchrysene and benzo(c)phenanthrene (Coles *et al.*, 2000; Hu *et al.*, 1997; Hu *et al.*, 1998a; Hu *et al.*, 1998b; Kihara *et al.*, 1999; Sundberg *et al.*, 1998). Enzymes with Val 105 have a 7-fold higher efficiency for PAH diol epoxides than the enzymes with Ile105 In contrast, the Val105 enzyme is 3-fold less effective using 1-chloro-2,4-dinitrobenzene (Harris *et al.*, 1998). The Val105 substitution results in steric restriction of the H-site due to shifts in the side chains of several amino acids. Thus, the Val105 variant allozyme may be able to accommodate less bulky substrates than the Ile105 allozyme and, as a result,

may display substrate specificities that differ from those of the wild-type (WT) allozyme (Ali-Osman *et al.*, 1997). In addition, the thermal stability of the codon 105 variant allozyme differs from that of the WT (Johansson *et al.*, 1998). These characteristics may be responsible, in part, for the reported association between this allozyme and carcinogenesis or variation in response to antineoplastic drugs (Stoehlmacher *et al.*, 2002). There have been some reports of an excess of val variant homozygotes among lung cancer cases relative to controls (Harries *et al.*, 1997) and among squamous cell cancer cases only (Kihara *et al.*, 1999). There have also been several reports of no association in varied ethnic study populations (Harris *et al.*, 1998; Jourenkova-Mironova *et al.*, 1998; Saarikoski *et al.*, 1998).

Table 3.2 GSTP1 genotype frequencies among different world populations

Country/ Population	No (N)	Genotype (%)			Deviation (p)	Allele (%)		Deviation (p)	References
		Ile/Ile	Ile/ Val	Val/ Val		Ile	Val		
North India	370	44.3	50.3	5.4	Ref.	69.5	30.5	Ref.	Mishra et al
Newcastle England	178	44.9	43.4	11.7	0.18	66.6	33.4	0.76	Welfare et al, 1999
Caucasian	166	39.2	47.3	13.3	0.13	63	37	0.37	Gsur et al, 2001
Japanese	88	70.5	29.5	0	<0.00	85.3	14.7	0.02	Kiyohara et al, 2000
Finnish Caucasians	481	55.3	37.6	7.1	0.23	74.1	25.9	0.64	Mitrunen et al, 2000
African Americans	271	22	55	23	<0.00	49.5	50.5	0.006	Millikan et al, 2000

White (USA)	392	40	49	11	0.28	64.5	35.5	0.55	Rossini et al, 2002
Brazilian Non White	272	47.8	42.6	9.6	0.36	69.1	30.9	1	Rossini et al, 2002
Brazilian White	319	51.4	34.2	14.4	0.2	68.5	31.5	1	Rossini et al, 2002

3.2.1 PCR amplification

All DNA samples (100 lung cancer cases and 100 controls) were analyzed for the genotyping of GSTP1 by the previously reported method (Harries et al., 1997) with slight modification. The primers used for genotyping were FP: Sense (5-ACCCCAGGGCTCTATGGGAA-3) RP: antisense (5-TGAGGG CAC AAG AAG CCC CT-3) A 25 µl PCR mixture consisted of 1 µL genomic DNA samples (50-70 ng/µl), 2.5 µl of 10x standard Taq reaction buffer (with MgCl2), 0.5 µl dNTPs (10 mM), 0.5 µl of each primer (10 mM), 0.13 µl Taq DNA polymerase (5U/ µl) (NEB, USA) and 20 µl nuclease free water. The initial denaturation was performed at 95^0C for 5 min, followed by 30 cycles each consisting of denaturation at 95^0 C for 30 sec, annealing at 56^0C for 30 sec, and extension at 72^0 C for 1 min and the final extension was performed at 72^0 C for 10 min. PCR condition and size of PCR product are given in Table 3.3

Table 3.3: PCR conditions for amplification of alleles of GSTP1

Gene	PCR conditions	Amplified fragment
GSTP1	95^0 C for 30 sec	176
	56^0 C for 30 sec	
	72^0 C for 1 min	

3.2.2 Gel electrophoresis and visualization of PCR product

After PCR amplification, 5µl of PCR product was mixed with 1µl of 6 blue orange loading dyes. 2% agarose gel was prepared from the agarose powder (Bioline, UK) with 1x TAE buffer. The gel was stained with ethidium bromide (EtBr) (BDH, UK) (1 µg of EtBr per ml agarose solution from the stock solution of 10 mg/ml). After gel loading samples were electrophoresed at 80 V for 60 minutes and visualized on the UV transilluminator (Alpha Innotech Corporation, San Leandro, California).

3.2.3 Restriction enzyme digestion and visualization

After PCR amplification 15 µl of PCR product was digested with 2 µl each of restriction endonucleases (NEB, USA). The digestion condition and possible fragments are mentioned in Table 3.5. After completing digestion the sample was electrophoresed and visualized like the aforementioned visualization of PCR products.

Table 3.4: Restriction enzymes (RE), digestion condition and length of the expected fragments on digestion of GSTP1

RE	Digestion condition	DNA fragments
BsmAI	incubated over night with BsmAI at 55^{0}C	Homozygous for of 176 bp.
		Heterozygous (ile/val) 176, 93 & 83
		Homozygotes Mutant(val/val) showed two bands of 93and 83

3.2.4 Statistical analysis

Distributions of demographic variables were compared between cases and controls using χ2- tests and two-sided unpaired t-tests. Genotype and allelic frequencies were reported as percentage. Differences in the frequencies of genotypes and alleles

between cases and controls were compared by χ^2- test. Unconditional logistic regression was used to estimate crude odds ratio (OR), adjusted OR and their 95% confidence intervals (CIs), with adjustment for age, sex and smoking status. OR with 95% confidence intervals (CIs), of tobacco users and nonusers were calculated by χ^2-test. All calculations were performed by using the statistical software package SPSS version 20.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ (two-tailed) was considered statistically significant.

3.2.5 Genomic DNA extraction

Genomic DNA was isolated from 100 lung cancer patients and 100 controls successfully (Daly et al., 1998). The purity (OD 260/OD 280) of all the genomic DNA samples were found to be in the range between 1.7 to 1.9 and the average concentration was 50 to 70 µg/ml.

3.2.6 Genotyping of GSTP1 gene

PCR-RFLP method was carried out to detect the allelic variation of GSTP1 gene in the lung cancer cases and controls.

3.2.7 PCR-RFLP of GSTP1 gene

After completing PCR amplification with appropriate reagents, a PCR product of GSTP1 was obtained. The PCR product size was 176 bp. The PCR product was visualized in 2% (w/v) agarose gel.

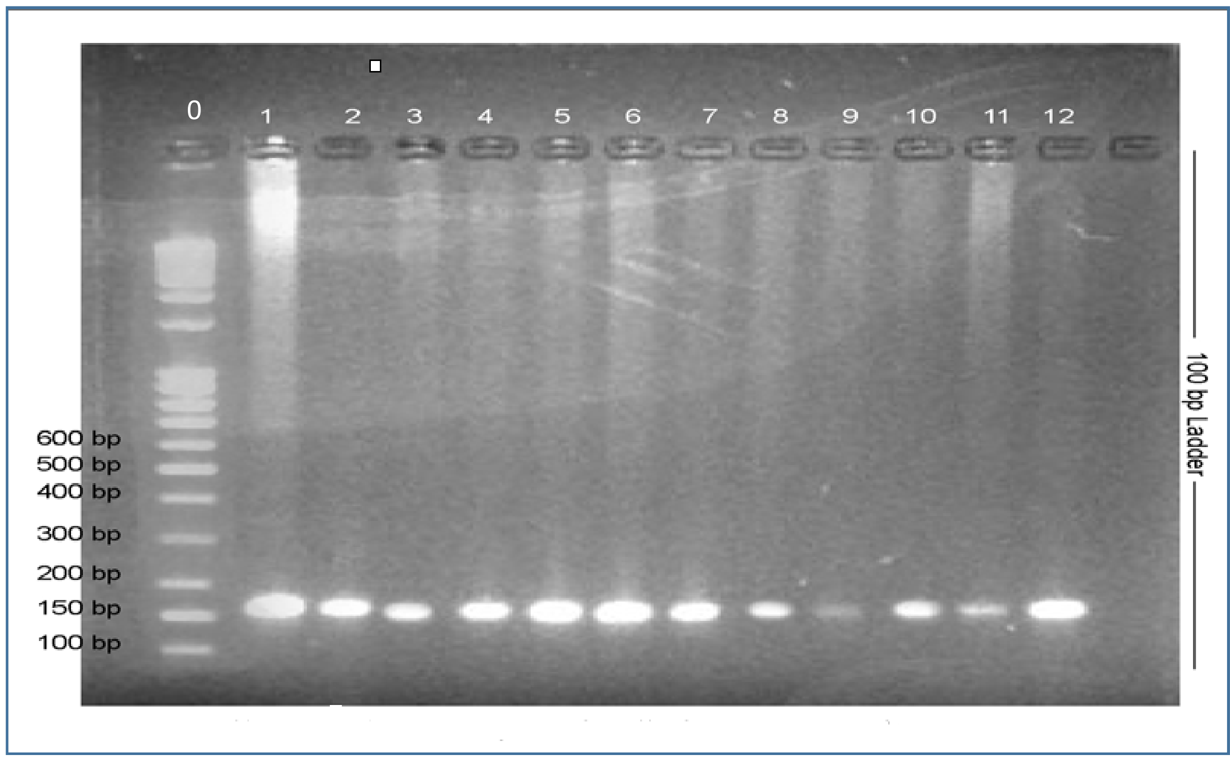
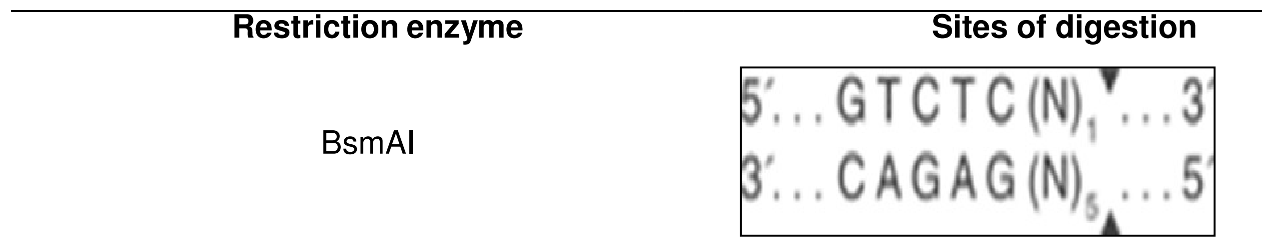


Figure 3.1: PCR product of GSTP1 (Lane 1 to 12) (2% agarose gel) (Lane-0 contains molecular ruler).

3.2.8 Fragmentation pattern

The fragments were visualized in agarose gel (2%) after digestion of the PCR product with BsmAI .

Table 3.5: Name of the restriction enzyme with its sites of digestion



Restriction enzyme	Sites of digestion
BsmAI	5′...GTCTC(N)$_1$▼...3′ 3′...CAGAG(N)$_5$▲...5′

Figure 3.2 In case of GSTP1 (cutting by BsmAI)

```
TGGGAAAGAGGGAAAGGCTTCCCCGGCCAGCTGCGCGGCGACTCCGGGGACTCCAGGGCGCCCCTCTGCG
GCCGACGCCCGGGGTGCAGCGGCCGCCGGGGCTGGGGCCGGCGGGAGTCCGCGGGACCCTCCAGAAGAGC
GGCCGGCGCCGTGACTCAGCACTGGGGCGGAGCGGGGCGGGACCACCCTTATAAGGCTCGGAGGCCGCGA
GGCCTTCGCTGGAGTTTCGCCGCCGCAGTCTTCGCCACCAGTGAGTACGCGCGGCCCGCGTCCCCGGGGA
TGGGGCTCAGAGCTCCCAGCATGGGGCCAACCCGCAGCATCAGGCCCGGGCTCCCGGCAGGGCTCCTCGC
CCACCTCGAGACCCGGGACGGGGGCCTAGGGGACCCAGGACGTCCCCAGTGCCGTTAGCGGCTTTCAGGG
GGCCCGGAGCGCCTCGGGGAGGGATGGGACCCCGGGGGCGGGGAGGGGGGGCAGACTGCGCTCACCGCGC
CTTGGCATCCTCCCCCGGGCTCCAGCAAACTTTTCTTTGTTCGCTGCAGTGCCGCCCTACACCGTGGTCT
ATTTCCCAGTTCGAGGTAGGAGCATGTGTCTGGCAGGGAAGGGAGGCAGGGGCTGGGGCTGCAGCCCACA
GCCCCTCGCCCACCCGGAGAGATCCGAACCCCCTTATCCCTCCGTCGTGTGGCTTTTACCCCGGGCCTCC
TTCCTGTTCCCCGCCTCTCCCGCCATGCCTGCTCCCCGCCCCAGTGTTGTGTGAAATCTTCGGAGGAACC
TGTTTCCCTGTTCCCTCCCTGCACTCCTGACCCCTCCCCGGGTTGCTGCGAGGCGGAGTCGGCCCGGTCC
CCACATCTCGTACTTCTCCCTCCCCGCAGGCCGCTGCGCGGCCCTGCGCATGCTGCTGGCAGATCAGGGC
CAGAGCTGGAAGGAGGAGGTGGTGACCGTGGAGACGTGGCAGGAGGGCTCACTCAAAGCCTCCTGCGTAA
GTGACCATGCCCGGGCAAGGGGAGGGGGTGCTGGGCCTTAGGGGGCTGTGACTAGGATCGGGGGACGCCC
AAGCTCAGTGCCCCTCCCTGAGCCATGCCTCCCCCAACAGCTATACGGGCAGCTCCCCAAGTTCCAGGAC
GGAGACCTCACCCTGTACCAGTCCAATACCATCCTGCGTCACCTGGGCCGCACCCTTGGTGAGTCTTGAA
CCTCCAAGTCCAGGGCAGGCATGGGCAAGCCTCTGCCCCCGGAGCCCTTTTGTTTAAATCAGCTGCCCCG
CAGCCCTCTGGAGTGGAGGAAACTGAGACCCACTGAGGTTACGTAGTTTGCCCAAGGTCAAGCCTGGGTG
CCTGCAATCCTTGCCCTGTGCCAGGCTGCCTCCCAGGTGTCAGGTGAGCTCTGAGCACCTGCTGTGTGGC
AGTCTCTCATCCTTCCACGCACATCCTCTTCCCCTCCTCCCAGGCTGGGGCTCACAGACAGCCCCCTGGT
TGGCCCATCCCCAGTGACTGTGTGTTGATCAGGCGCCCAGTCACGCGGCCTGCTCCCCTCCACCCAACCC
CAGGGCTCTATGGGAAGGACCAGCAGGAGGCAGCCCTGGTGGACATGGTGAATGACGGCGTGGAGGACCT
CCGCTGCAAATACATCTCCCTCATCTACACCAACTATGTGAGCATCTGCACCAGGGTTGGGCACTGGGGG
CTGAACAAAGAAAGGGGCTTCTTGTGCCCTCACCCCCCTTACCCCTCAGGTGGCTTGGGCTGACCCCTTC
TTGGGTCAGGGTGCAGGGGCTGGGTCAGCTCTGGGCCAGGGGCCCAGGGGCCTGGGACAAGACACAACCT
GCACCCTTATTGCCTGGGACATCAACCAGCCAAGTAACGGGTCATGGGGGCGAGTGCAAGGACAGAGACC
TCCAGCAACTGGTGGTTTCTGATCTCCTGGGGTGGCGAGGGCTTCCTGGAGTAGCCAGAGGTGGAGGAG
ATTTGTCGCCAGTTTCTGGATGGAGGTGCTGGCACTTTTAGCTGAGGAAAATATGCAGACACAGAGCACA
TTTGGGGACCTGGGACCAGTTCAGCAGAGGCAGCGTGTGTGCGCGTGCGTGTGCATGTGTGTGCGTGTGT
GTGTGTACGCTTGCATTTGTGTCGGGTGGGTAAGGAGATAGAGATGGGCGGGCAGTAGGCCCAGGTCCCG
AAGGCCTTGAACCCACTGGTTTGGAGTCTCCTAAGGGCAATGGGGGCCATTGAGAAGTCTGAACAGGGCT
GTGTCTGAATGTGAGGTCTAGAAGGATCCTCCAGAGAAGCCAGCTCTAAAGCTTTTGCAATCATCTGGTG
AGAGAACCCAGCAAGGATGGACAGGCAGAATGGAATAGAGATGAGTTGGCAGCTGAAGTGGACAGGATTT
GGTACTAGCCTGGTTGTGGGGAGCAAGCAGAGGAGAATCTGGGACTCTGGTGTCTGGCCTGGGGCAGACG
GGGGTGTCTCAGGGGCTGGGAGGGATGAGAGTAGGATGATACATGGTGGTGTCTGGCAGGAGGCGGGCAA
GGATGACTATGTGAAGGCACTGCCCGGGCAACTGAAGCCTTTTGAGACCCTGCTGTCCCAGAACCAGGGA
GGCAAGACCTTCATTGTGGGAGACCAGGTGAGCATCTGGCCCCATGCTGTTCCTTCCTCGCCACCCTCTG
CTTCCAGATGGACACAGGTGTGAGCCATTTGTTTAGCAAAGCAGAGCAGACCTAGGGGATGGGCTTAGGC
CCTCTGCCCCCAATTCCTCCAGCCTGCTCCCGCTGGCTGAGTCCCTGGCCCCCCTGCCCTGCAGATCTCC
TTCGCTGACTACAACCTGCTGGACTTGCTGCTGATCCATGAGGTCCTAGCCCCTGGCTGCCTGGATGCGT
CGGGAAACAGTGAGGGTTGGGGGGACTCTGAGCGGGAGGCAGAGTTTGCCTTCCTTTCTCCAGGACCAAT
AAAATTTCTAAGAGAGCTACTATGAGCACTGTGTTTCCTGGGACGGGGCTTAGGGGTTCTCAGCCTCGAG
GTCGGTGGGAGGGCAGAGCAGAGGACTAGAAAACAGCTCCTCCAGCACAGTCAGTGGCTTCCTGGAGCCC
TCAGCCTGGCTGTGTTTACTGAACCTCACAAACTAGAAGAGGAAGAAAAAAAAAGAGAGAGAGAAACAAA
AGCATCTGGCCCCATGCTGTTCCTTCCTCGCCACCCTCTGCTTCCAGATGGACACAGGTGTGAGCCATTT
GTTTAGCAAAGCAGAGCAGACCTAGGGGATGGGCTTAGGCCCTCTGCCCCCAATTCCTCCAGCCTGCTCC
CGCTGGCTGAGTCCCTGGCCCCCCTGCCCTGCAGATCTCCTTCGCTGACTACAACCTGCTGGACTTGCTG
CTGATCCATGAGGTCCTAGCCCCTGGCTGCCTGGATGCGTTCCCCCTGCTCTCAGCATATGTGGGGCGCC
TCAGTGCCCGGCCCAAGCTCAAGGCCTTCCTGGCCTCCCCTGAGTACGTGAACCTCCCCATCAATGGCAA
CGGGAAACAGTGAGGGTTGGGGGGACTCTGAGCGGGAGGCAGAGTTTGCCTTCCTTTCTCCAGGACCAAT
AAAATTTCTAAGAGAGCTACTATGAGCACTGTGTTTCCTGGGACGGGGCTTAGGGGTTCTCAGCCTCGAG
GTCGGTGGGAGGGCAGAGCAGAGGACTAGAAAACAGCTCCTCCAGCACAGTCAGTGGCTTCCTGGAGCCC
TCAGCCTGGCTGTGTTTACTGAACCTCACAAACTAGAAGAGGAAGAAAAAAAAAGAGAGAGAGAAACAAA
```

Figure 3.3: Fragments obtained after PCR RFLP by BsmAI in case of GSTP1

PCR Product

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGA
ATGACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCCCTCWTCTAYASCAACTA
TGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAACAAAGAAAGGGGCTTC
TTGTGCCCTCA

Fragment: 1

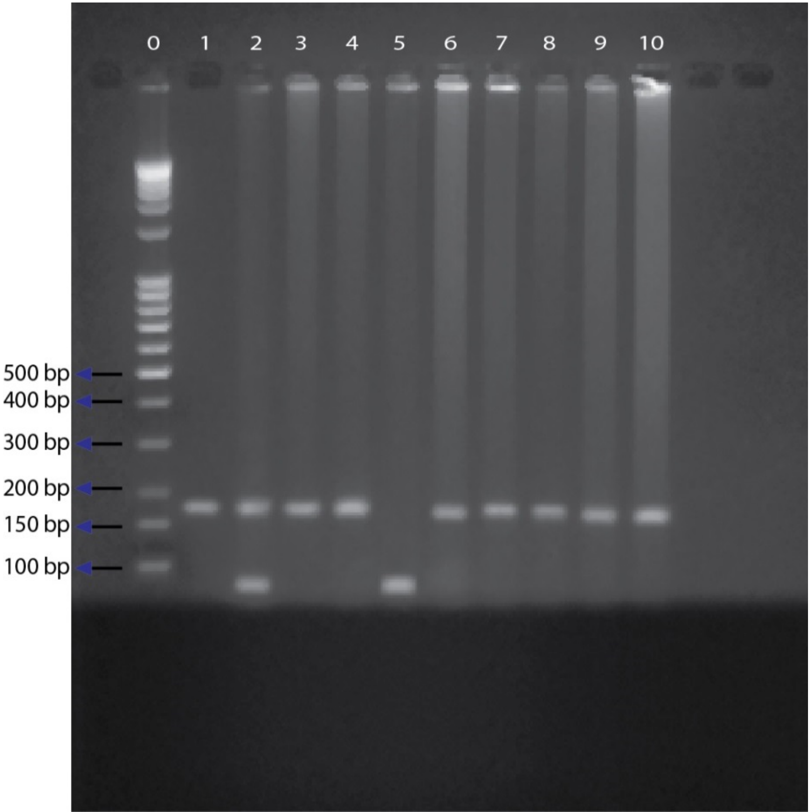
ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGAAT
GACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCC = 93 bp

Fragment: 2

CTCWTCTAYASCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAAC
AAAGAAAGGGGCTTCTTGTGCCCTCA **= 83bp**

Figure 3.4: Restriction endonuclease digestion fragment of GSTP1 in 3% agarose gel

Electrophoresis of the digested PCR products showed individuals homozygous (ile/ile) for the GSTP1 BsmAI polymorphism as one band of 176 bp. Heterozygous (ile/val, val) for the polymorphism resulted in three bands of 176, 91 and 85. Homozygotes mutant (val/val) showed two bands of 91 and 85 bp (which appear as one band due to close molecular size).



Gel electrophoresis of the digested PCR products showing individuals DNA for the GSTP1 polymorphism. Heterozygous polymorphism (lane 2; ile/val Heterozygous), Homozygous polymorphism (lane1, 3, 4, 6, 7, 8, 9, 10; val/val), Mutant Homozygous (lane5)

Table 3.6: Restriction enzyme and site of digestion for restriction enzymes

Restriction enzyme	**Sites of digestion**
BsmAI	5'….GTCTC $(N)_1$…3' 3'… .CAGAG $(N)_5$ …..5'

3.3 Expected result after digestion for GSTP1

Table 3.7 Type of nucleotide changes, cutting sites and fragments of the allele in case of GSTP1

SNP	Cutting site	Fragments	Type
When R=A (AA)	No	176	**Normal Homozygote**
When R=G (GA)	313,None	176,83,93	**Heterozygote**
When R=G (GG)	313,313	83,93	**Mutant Homozygote**

3.4 When X= A in both of the sister chromosomes: (NORMAL HOMOZYGOTE) (A/A)

There is no cutting site. So there will be one fragment for each chromosome.

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGA
ATGACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCCCTCWTCTAYASCAACTA
TGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAACAAAGAAAGGGGCTTC
TTGTGCCCTCA

3.5 When X=G in one of the sister chromosome: (HETEROZYGOTE) (A/G)

There is no cutting site for one chromosome (X=A), but for the polymorphic chromosome X=G) there will be one cutting site (93). So, there will be 3 fragments for two sister chromosomes.

Fragment: 1

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGA

ATGACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCCCTCWTCTAYASCAACTA

TGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAACAAAGAAAGGGGCTTC

TTGTGCCCTCA (176bp)

Cutting site (Yellow---BSMAI Recognition site)

5'….GTCTC $(N)_1$…3

3'… .CAGAG $(N)_5$ …..5'

Fragment: 2

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGAAT GACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCC = (93 bp)

Fragment : 3

CTCWTCTAYASCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAAC AAAGAAAGGGGCTTCTTGTGCCCTCA **= (83bp)**

3.6 When X=G in both of the sister chromosomes: (Mutant Homozygote) (G/G)

There will be cutting sites (93) for both the chromosomes. So there will be two

fragments for each chromosome.

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGA

ATGACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCCCTCWTCTAYASCAACTA

TGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAACAAAGAAAGGGGCTTC

TTGTGCCCTCA

Cutting site (Yellow---BSMAI Recognition site)

5'….GTCTC $(N)_1$…3

3'… .CAGAG $(N)_5$ …..5'

Fragment: 1

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGAAT GACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCC = (93 bp)

Fragment : 2

CTCWTCTAYASCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAAC AAAGAAAGGGGCTTCTTGTGCCCTCA **= (83bp)**

Fragment: 3

ACCCCAGRGCTCTRTGGGAAGGACCAKCAGGAGGCAGCCCYGGTGGACAYGGTGAAT GACGGCRTGGAGGACCTCCGCTGCAAATACRTCTCC = (93 bp)

Fragment : 4

CTCWTCTAYASCAACTATGWRAGCATCTGCACCAGGGTTGGGCACKGGGRGCTGAAC AAAGAAAGGGGCTTCTTGTGCCCTCA **= (83bp)**

Observed results

Restriction enzyme digestion products were visualized in agarose (2%) gel.

Table 3.8: Name of the allele, PCR product size, and restriction enzyme, length of the expected and observed fragments on digestion

Allele Name	PCR Product	RE	Expected Fragments(bp)	Observed Fragments(bp)
GSTP1	176	**BSMAI**	**NH(176)**	NH,HE,MH
			HE(176,83,93)	
			MH(83,93)	

NH = Normal Homozygote, HE=(Ile/Val)Heterozygote, MH = (Val/Val) Mutant Homozygote

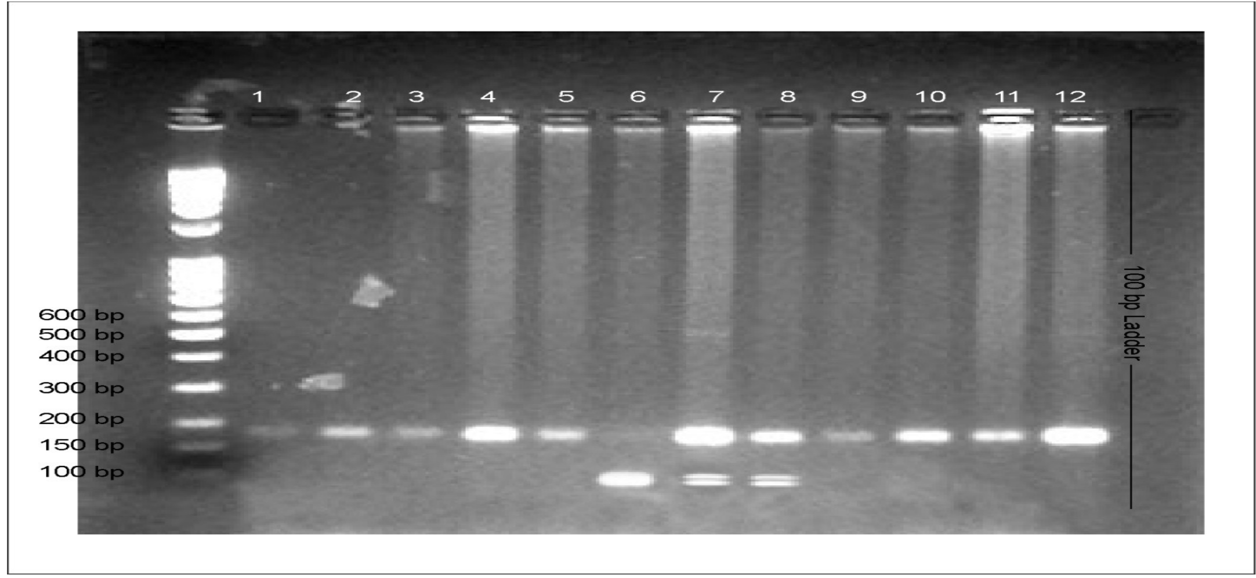


Figure 3.5: Restriction Enzyme (BSMA1) digestion fragment of GSTP1 (lane 1 to 12) (2% agarose gel); lane (7, 8): Heterozygous, lane-6: Mutant Homozygous; Uncut PCR product at lane-(1-5) and (9-12): Normal Homozygote (176 bp); lane-0: Molecular ruler

3.7 Observed result for GSTP1 patients

Table 3.9 GSTP1 genotype among lung cancer patients and controls and their association with lung cancer

Genotype	Patients	Control	OR	95%CI	p
NH	63	87	1	-	-
HE	29	11	3.64	1.69-7.83	0.001
MH	8	2	5.52	1.13-26.9	0.019
HE+MH	37	13	3.93	1.93-8.00	0.000

NH = Normal Homozygote, HE=(Ile/Val) Heterozygote, MH = (Val/Val) Mutant Homozygote

Compared to the AA (NH) genotype of GSTP1 rs1695, Heterozygous(AG), Mutant(GG) and combined Heterozygous plus Mutant variants (AG+GG) increased the risk of lung cancer (OR = 3.64. 95% CI = 1.69-7.63, p = 0.001,OR = 5.52. 95% CI = 1.13-26.90, p = 0.019, OR = 3.93. 95% CI = 1.93-8.00, p = 0.000 respectively). The distribution of the GSTP1 genotypes were significantly different between the cases and controls [AA (NH), AG(HE) and GG(MH)] genotypes; 63%, 29% and 8 % vs 87%, 11 % and 2 %). Compared to the AA (NH) genotype AG (HE) and GG (MH) genotypes increased the risk of lung cancer approximately 4 and 6 times respectively (adjusted OR= 3.81, 95% CI = 1.73-8.39, p = 0.001; adjusted OR= 5.98, 95% CI = 1.15-31.09, p = 0.033, for AG

and GG respectively). Whereas AG+GG (HE+MH) combined genotype has 4 times more risk of lung cancer compared to AA (NH) genotype (adjusted OR= 1.96, 95% CI = 1.14 - 3.36, p = 0.015).

Table 3.10 GSTP1 genotype among lung cancer patients and controls and their association with lung cancer with adjusted odd ratios

Genotype	Patients	Control	Crude OR	95%CI	p	Adjusted OR	95%CI	p
NH	63	87	1	-	-	-	-	-
HE	29	11	3.64	1.69-7.83	0.001	3.81	1.73—8.39	.001
MH	8	2	5.52	1.13-26.9	0.019	5.98	1.15-31.09	.033
HE+MH	37	13	3.93	1.93-8.00	0.000	4.14	1.99-8.59	.000

The frequency distribution of LC patients obeys the Hardy-Weinberg equilibrium (χ^2 = 2.83, p= 0.092), whereas that of controls deviate from the equilibrium (χ^2 = 4.29, p= 0.038)

Table 3.11 Association between GSTP1 and tobacco consumption

		GSTP1 Patient			Total
		NH	HE	MH	
	Chewing tobacco	4	7	1	12
	Current smoker	37	14	2	53
	Ex-smoker	17	7	3	27
	Never smoker	5	1	2	8
Total		63	29	8	100

Tobacco using status		GSTP1 control			Total
		NH	HE	MH	
	Chewing tobacco	9	1	0	10
	Current smoker	53	9	1	63
	Ex-smoker	15	1	0	16
	Never smoker	10	0	1	11
Total		87	11	2	100

NH = Normal Homozygote, HE= (Ile/Val) Heterozygote, MH = (Val/Val) Mutant Homozygote

Table 3.12 Distribution of GSTP1 genotype among lung cancer patients and their association with lung cancer histology

GSTP1					
		Genotyping GSTP1			Total
		HE	MH	NH	
Type of LC	Adenocarcinoma	10	2	24	36
	Adenosqumous	0	1	0	1
	Large cell	1	0	1	2
	Small cell	4	0	13	17
	Squmous cell	14	5	25	44
Total		29	8	63	100

NH = Normal Homozygote, HE=(Ile/Val) Heterozygote, MH = (Val/Val) Mutant Homozygote

As smoking is the potential risk factor for lung cancer, we further calculated the modifying effect of GSTP1 genotypes associating tobacco use with lung cancer.

Table 3.13: Modifying effect of GSTP1 genotypes on the associating tobacco use with lung cancer.

Geno type	Chewing tobacco		OR (95% CI)	p	Current smoker		OR (95% CI)	p	Ex-smoker		OR (95% CI)	p	Never smoker		OR (95% CI)	p
	Patients (%) (n=100)	Controls (%) (n=100)			Patients (%) (n=100)	Controls (%) (n=100)			Patients (%) (n=100)	Controls (%) (n=100)			Patients (%) (n=100)	Controls (%) (n=100)		
GSTP1																
NH	4	9	1		37	53	1		17	15	1		5	10	1	
HE	7	1	15.75 (1.42 to 174.25)	0.025	14	9	2.22 (0.87 to 5.68)	0.094	7	1	6.17 (0.67 to 56.15)	0.106	1	0	-	0.309
MH	1	0	-	0.286	2	1	2.86 (0.25 to 32.76)	0.397	3	0	6.20 (0.29 to 129.75)	0.239	2	1	4 (0.28 to 55.47)	0.302

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Table 3.14 Interaction of genotype of GSTM1 and smoking with lung cancer risk

Geno type	Tobacco user		OR (95% CI)	p	Non user		OR (95% CI)	p
	Patients (%) (n=100)	Controls (%) (n=100)			Patients (%) (n=100)	Controls (%) (n=100)		
GSTP1								
NH	58	77	1		5	10	1	
HE	28	11	3.37 (1.55 to 7.34)	0.0021	1	0	5.72 (0.19 to 165.35)	0.309
MH	6	1	7.96 (0.93 to 67.99)	0.050	2	1	4 (0.28 to 55.47)	0.301
HE + MH	34	12	3.76 (1.79 to 7.89)	0.0005	3	1	6 (0.49 to 73.45)	0.160

NH = Normal Homozygote, HE=(Ile/Val) Heterozygote, MH = (Val/Val) Mutant Homozygote

According to table, lung cancer cases having distribution of variant genotypes of GSTP1 i.e. Heterozygous, Mutant Homozygote or Heterozygous + homozygous mutant genotype (OR=3.37, 95% CI = 1.55 to 7.34, p=0.002; OR=7.96 95% CI = 0.93 to 67.99, p=0.050; OR=3.37, 95%; CI = 1.79 to 7.89, p= 0.0005 respectively) were found to be higher in the tobacco user cases leading to the increased risk of lung cancer, which is statistically significant . There is a 3 fold (approx.) increase in the risk of lung cancer in case of tobacco user of HE & HE+MH and an 8 fold increase in risk of lung cancer in cases of tobacco user of MH with respect to NH, that are statistically significant. No association of risk of lung cancer was found with the distribution of variant genotypes of GSTP1 in case of tobacco nonusers.

3.8 Discussion

In this study, we investigated the association of the GSTP1 Ile105Val polymorphism with predisposition to lung cancer in the Bangladeshi population. Common polymorphisms exist in genes coding for various GSTs including Glutathione S transferase M1 (GSTM1) and P1 (GSTP1). GSTP1 is the most abundant isoform in the lung and is also involved in response to oxidative stress(Zimniak *et al.*, 1994). GSTP1 Ile105Val polymorphism is an adenine (A) to guanine (G) transition within the substrate binding domain of GSTP1 at position +313 within exon 5 (+313 A —> G) that results in an amino acid change from isoleucine to valine at codon 105, which forms part of the active site for binding of hydrophobic electrophiles, and affects substrate-specific catalytic activity thus results in a significantly lower conjugating activity among individuals who carry one or more copies of the G allele (Ile/Val or Val/Val) compared with those who have the A/A (Ile/Ile) genotype (Sergentanis *et al.*, 2010). Having at least one copy of the G allele at this locus is also associated with increased levels of hydrophobic adducts in the lung and higher levels of PAH-DNA adducts in human lymphocytes (Butkiewicz *et al.*, 2000). The study included 100 patients with bronchogenic carcinoma and 100 healthy control subjects of matched age and sex. lung cancer cases having distribution of variant genotypes of GSTP1 i.e. Heterozygous, Mutant Homozygote or Heterozygous + Homozygous Mutant genotype (OR=3.37, 95% CI = 1.55 to 7.34, p=0.002; OR=7.96, 95% CI = 0.93 to 67.99, p=0.050; OR=3.37, 95%; CI = 1.79 to 7.89, p= 0.0005 respectively) were found to be higher in the tobacco user cases leading to the increased risk of lung cancer. Which indicated that cigarette smoking is the greatest risk factor associated with lung cancer development (Ramalhinho *et al.*, 2011).

.The distribution of the GSTP1 genotypes was significantly different between the cases and controls [AA(NH), AG(HE) and GG(MH)] genotypes; 63%, 29% and 8 % vs 87%, 11 % and 2 %) with respect to AA (NH) genotype. Our results revealed that the frequency of GSTP1 105Val was significantly increased in lung cancer patients compared to healthy controls, and in NSCLC and squamous cell carcinoma cases compared to SCLC cases. Adenocarcinoma accounts for 40% of non-small-cell lung cancers(Travis, 2002). Most cases of adenocarcinoma are associated with smoking; however, among people who have never smoked, adenocarcinoma is the most common form of lung cancer (Subramanian *et al.*, 2007). In our current study we observed that adenocarcinoma was accounted for 36 % of lung cancer burden. In Meta and pooled analysis of GSTP1 polymorphism and lung cancer, we found an overall association between lung cancer and carriage of the GSTP1 Val/Val or Ile/Val genotype compared to those carrying the Ile/Ile genotype. Increased risk also varied by the histologic type. Among whites, there were no statistically significant differences in risk associated with the GSTP1 gene polymorphism and adenocarcinoma, squamous cell carcinoma, or small cell carcinoma. In Asian populations, individuals carrying at least 1 valine allele were at increased risk of adenocarcinoma (Cote *et al.*, 2009).

In conclusion, our findings suggest that GSTP1 exon 5 polymorphism (Ile105Val) is associated with high risk of NSCLC especially adenocarcinoma not significantly related to tobacco use. As, other GST polymorphisms play important overlapping roles in detoxifying tobacco carcinogens and because risk might be associated with these polymorphisms, further larger populations studies of risk associated with multiple polymorphisms are needed to fully understand the genetic interactions under-lying risk susceptibility.

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CHAPTER FOUR

SUMMARY

SUMMARY

Phase II biotransformation enzymes generally act as inactivating enzymes to catalyze the binding of intermediary metabolites to cofactors, transform them into more hydrophilic products and thus facilitate their elimination. GSTs are phase II transformation enzymes involved in the detoxification of hazardous agents (Hirvonen et al., 1996). The glutathione-S-transferase (GST) gene family encodes genes that are critical for certain life processes, as well as for detoxication and toxification mechanisms, via conjugation of reduced glutathione (GSH) with numerous substrates such as pharmaceuticals and environmental pollutants(Nebert et al., 2004). The susceptibility to cancers especially in those which are environmentally determined like lung cancers can be influenced by Inter–individual variability in glutathione-S-transferase (GST) enzyme (Rebbeck, 1997). The M1 and T1 variant of GSTs detoxifies of major classes of tobacco procarcinogens such as aromatic amines and PAHs. Carriers of homozygous deletion in GSTM1 and GSTT1 genes have an absence of GST-m and GST-q enzyme activity, respectively. These deletion variants are very useful in epidemiological studies of cancer because they divide individuals in two well-defined susceptibility classes: those who are and those who are not able to detoxify potential carcinogens by the metabolic pathways regulated by GSTM1and GSTT1genes.

GSTP1 is the most abundant isoform in the lung and is also involved in response to oxidative stress(Zimniak *et al.*, 1994). It has the highest specific activity towards the active benzo(a)pyrene diol epoxide metabolite of cigarette, benzo(a)pyrene-7b,a-

dihydrodiol-9a,10a-epoxide (BPDE) and is almost exclusively active towards the (+)-enantiomer of anti-BPDE, thought to be the ultimate mutagenic form of benzo(a)pyrene(Robertson *et al.*, 1986). GSTP1 Ile105Val polymorphism is an adenine (A) to guanine (G) transition within the substrate binding domain of GSTP1 at position +313 within exon 5 (+313 A —> G) that results in an amino acid change from isoleucine to valine at codon 105, which forms part of the active site for binding of hydrophobic electrophiles, and affects substrate-specific catalytic activity , results in a significantly lower conjugating activity among individuals who carry one or more copies of the G allele (Ile/Val or Val/Val) compared with those who have the A/A (Ile/Ile) genotype. Having at least one copy of the G allele at this locus is also associated with increased levels of hydrophobic adducts in the lung and higher levels of PAH-DNA adducts in human lymphocytes (Butkiewicz *et al.*, 2000).

A number of studies have tried to establish links between polymorphic expression of different GSTM1 and GSTT1 with lung cancer risk in different ethnic populations and the results have been conflicting. One reason for the differences could be the fact that most studies were conducted in different populations. However, none of the main characteristics of the subjects explain satisfactorily the apparent differences (i.e. race, histological type and level of smoking). A significant association of lung adenocarcinoma with the GSTM1 null genotype was reported in two large studies from Japan (Hayashi *et al.*, 1992; Kihara *et al.*, 1999) and two from China. Furthermore, in a study in Caucasians, a significant association of lung adenocarcinoma with the GSTM1 null genotype was reported (Lan *et al.*, 2000; Sun *et al.*, 1997; Woodson *et al.*, 1999).

Two meta-analysis one comprises of 11 studies found an OR of 1.6 (95% CI= 1.26–2.04) for an association between the GSTM1 null genotype and lung cancer risk (D'Errico *et al.*, 1996)) another study by Williams at al, it was shown that GSTM null allele was a risk factor for the development of lung cancer (McWilliams *et al.*, 1995). In another meta-analysis study, it was reported that there was no statistically significant relationship between the individuals carrying GSTM null genotype and susceptibility to lung cancer but the number of patients carrying this genotype was greater in the lung cancer group (Benhamou *et al.*, 2002). In our study GSTM1null allele in the present study is 58%, which is not similar to the frequencies reported in Indian Subcontinent (Buch *et al.*, 2001; Konwar *et al.*, 2010; Mishra *et al.*, 2004; Naveen *et al.*, 2004; Roy *et al.*, 1994; V *et al.*, 2006). In our study, we found no significant relation between GSTM1 null genotype and susceptibility to lung cancer. Additionally, the rate of GSTM1 null genotype was higher in the control group with compare to other control group in different Indian ethnic population.

The incidence of the GSTT1 null allele differs among global populations. Significant differences in GSTT1null allele frequencies were observed between Caucasian, Asian, African and African American populations (Lee et al., 2008) and the prevalence of GSTT1null allele in the present study is 72%, which is not similar to the frequencies reported in Indian Subcontinent (Roy et al., 1998; Buch et al., 2001; Mishra et al., 2004; Naveen et al., 2004; Vetriselvi et al., 2006; Konwar et al., 2010 Roy et al., 1998; Buch et al., 2001; Mishra et al., 2004; Naveen et al., 2004; Vetriselvi et al., 2006.). In our study, we found no statistically significant relation between GSTT1 null genotype and

susceptibility to lung cancer. Additionally, the frequency of GSTM1 null genotype was higher with compare to different Indian ethnic population as well as world population.

In case of GSTP1 our results revealed that the frequency of GSTP1 105Val significantly increased in lung cancer patients compared to healthy controls, and in NSCLC and squamous cell carcinoma cases compared to SCLC cases.

We further compared individual, multiple and combination of three high risk genotypes with compare to no risk genotypes and the results are given in table 5.1

Table 5.1The distribution, as n (%), of GST genotypes in the patients with Lung cancer and controls

Table 3.15 shows the frequencies of GSTM1, GSTT1 and GSTP1 alleles and genotypes by case-control status and the association of GST genotypes with Lung cancer risk.

Genotype	Controls	Patients	P	OR (95% CI)
N Patients	100	100		
GSTM1	44	42	1	
Null	56	58	0.775	1.08 (0.61 to 1.89)
GSTT1	24	28	1	
Null	76	72	0.519	0.81 (0.43 to 1.52)
GSTP1				
I/I	87	63	1	
I/V	11	29	0.0009	3.64 (1.69 to 7.83)
V/V	2	8	0.034	5.52 (1.13 to 26.90)
MT Both	11	12	1	
Either Null	46	46	0.852	0.91 (0.36 to 2.28)
Both Null	43	42	0.814	0.89

				(0.35 to 2.25)
MP				
M1(+/+) & P(I/I)	37	28	1	
M1(+/+) & P(I/V or V/V)	6	12	0.082	2.64 (0.88 to 7.90)
M1(-/-) & P(I/I)	50	35	0.815	0.92 (0.48 to 1.77)
M1(-/-) & P(I/V or V/V)	7	25	0.001	4.71 (1.78to 12.46)
TP				
T1(+/+) & P(I/I)	20	17	1	
T1(+/+) & P(I/V or V/V)	5	17	0.022	4 (1.21 to 13.12)
T1(-/-) & P(I/I)	67	46	0.575	0.80 (0.38 to 1.70)
T1(-/-) & P(I/V or V/V)	8	20	0.042	2.94 (1.03 to 8.35)
Triple				
M1 & T1(+/+) & P(I/I)	9	8	1	
M1 & T1(+/+) & P(I/V or V/V)	2	4	0.414	2.25 (0.32 to 15.75)
M1 (-/-) & T1(+/+) & P(I/I)	11	9	0.900	0.92 (0.25 to 3.36)
M1(-/-) & T1(+/+) & P(I/V or V/V)	2	7	0.143	3.93 (0.62 to 24.73)
M1 (+/+) & T1(-/-) & P(I/I)	28	20	0.699	0.80 (0.26 to 2.44)
M1(+/+) & T1(-/-) & P(I/V or V/V)	5	10	0.268	2.25 (0.53 to 9.45)
M1 (-/-) & T1(-/-) & P(I/I)	39	26	0.599	0.75 (0.25 to 2.19)
M1(-/-) & T1(-/-) & P(I/V or V/V)	4	16	0.042	4.5 (1.05 to 19.21)

GSTM1null (-/-), GSTM1present (+/+), GSTT1null (-/-), GSTT1 Present (+/+), GSTP1 Normal Homozygote (I/I), Heterozygote (I/V), Mutant Homozygote (V/V).

For GSTM1 among the 100 cases, 58% were carrying null genotype, and 42% were GSTM1 positive. Among the 100 controls 56% were carrying null genotype, and 44%

were GSTM1 positive. In case of GSTT1 among the 100cases 72% were carrying null genotype, and 28% were GSTT1 Positive. Among the 100 controls 76% were carrying GSTT1 null genotype, and 24% were GSTT1 positive. With GSTP1, wild (Ile/Ile), heterozygous (Ile/Val) and mutant (Val/Val) genotypes were observed in 63, 29 and in lung cancer patients and in 87, 11& 2 individuals in controls respectively.

There was no significant association of null genotypes of the GSTM1 and GSTT1 and with lung cancer risk. A significantly elevated lung cancer risk was associated with GSTP1 heterozygous, mutant and combined heterozygous plus mutant variants of rs1695.

The combination of the two high risk genotype GSTM1 null and GSTP1 (I/V or V/V) contributed to lung cancer risk (OR= 4.71, 95%CI =1.78 to 12.46, P=0.001) with compare to no-risk genotypes (M1(+/+) & P(I/I) .Combination of GSTT1 null and GSTP1 (I/V or V/V) also contributed to lung cancer risk (OR= 2.94, 95%CI =1.03 to 8.35, P=0.04) with compare to no-risk genotypes T1(+/+) & P(I/I).

We further investigated the risk associated with all the three high risk GST genotypes compared to no-risk genotypes (positive genotypes of GSTM1 and GSTT1 and 313 A/A genotype of GSTP1 were designated as the reference group). The combination of three high risk GST genotypes GSTM1null (-/-) & GSTT1null (-/-) & GSTP1 (I/V or V/V) is significantly associated with lung cancer risk (OR = 4.5. 95% CI = 1.0537 to 19.2174, p = 0.0423).

To our knowledge this is the first genetic study of Lung cancer in the Bangladeshi patients, and showed that the GSTM1 & GSTT1 null genotype is not a risk factor alone for lung cancer, GSTP1-313 G allele (Val), Is a strong predisposing risk factors for Lung

cancer ,The combination of the two high risk genotype GSTM1 null and GSTP1 (I/V or V/V) contributed to lung cancer risk with compare to no-risk genotypes (M1(+/+) & P(I/I) and the combination of three GST genotypes (GSTM1null (-/-) & GSTT1null (-/-) & GSTP1 (I/V or V/V)) further increase the risk of Lung cancer in Bangladeshi patients.

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CHAPTER
FIVE

APPENDIX

PATIENT/ VOLUNTEER CONSENT FORM

I, the undersigned, authorize the research student to consider me as a lung cancer patient/volunteer for his research work. I understand that I can change my mind at any time to withdraw myself as volunteer during this research work.

Patient/ Volunteer consent to study treatment

Please tick as appropriate

1. Do you have complete idea about the type, ultimate goal and methodology of the research?	Yes	No
2. Are you aware that you don't have to face any physical, mental and social risk for this?	Yes	No
3. There will be no chance of injury in any of your organs; are you aware of this?	Yes	No
4. Have you got any idea about the outcome of this experiment?	Yes	No
5. Have you decided intentionally to participate in this experiment?	Yes	No
6. Do you think this experiment violate your human rights?	Yes	No
7. Are you sure that all the information regarding you will be kept Confidentially?	Yes	No
8. No remuneration will be provided for this experiment, are you aware of this?	Yes	No

After reading the above mentioned points, I am expressing my consent to participate in this experiment as a lung cancer patient / volunteer.

Volunteer signature and Date: ________________________________

Volunteer's Name: ____

Address: ____

Witness: ____

[Please return the signed copy to the research student and keep an extra copy for yourself]

BLOOD SAMPLE COLLECTION FORM

Questionnaires:

1. Identification

1.1 I.D. Code:

1.2 Name:

1.3 Father's/ Husband's Name:

1.4 Sex: **Male** **Female**

1.5 Marital Status:

Married **Unmarried**

1.6 Date of Birth (dd/mm/yy): **1.7 Age (yr):**

1.8 Mailing address

1.9 Permanent address

1.10 Telephone No.

1.11 Religion

1.12 Nationality

2. Personal History

2.1 Area of residence:	**Rural**	**Urban**	**S-Urban**	**Others**
Where have you spent your boyhood (1-15 y)?				
Where have you spent at least ¾th or more of your life time?				

2.2 Education level

- Illiterate
- Can read only
- Can write a letter
- SSC or equivalent
- HSC or equivalent
- Graduate or higher
- Other

2.3 Occupation

- Student
- Professional
- Business
- Technical
- Unemployed
- Housewife
- Skilled worker
- Other

2.4 Family expense/month

2.5 Impression about social class

- Rich
- Upper middle
- Lower middle
- Poor

2.6 Smoking habit

- Never
- Ex-smoker Sticks/day
- Current smoker Sticks/day
- Duration of smoking (when necessary)

3. Biophysical Characteristics

3.1 Height (cm)

3.2 Weight (kg)

3.4 Temperature (^{0}C)

3.5 BP (Sys/Dias)

3.3 Pulses/min

4. **Family history of lung cancer**

5. **Date of Confirmation of Lung cancer**

6. Histopathology of Lung cancer

6.1 NSCLC Adenocarcinoma

Squamous cell Carcinoma

Bronchoalveolar Carcinoma

Large Cell Carcinoma

Adenosquamous

6.2 SCLC

6.3 Treatment period

Investigated by

Name **Signature**

Date