MOLECULAR STUDIES ON ORAL SQUAMOUS CELL CARCINOMA

Thesis submitted for the award of the degree of

Doctor of Philosophy

in

Cell Biology and Molecular Genetics

under the Faculty of Allied Health and Basic Sciences

by

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2019

Declaration by the candidate

I, **Mrs. Deepa Mugadur**, hereby declare that this thesis entitled **"Molecular studies on oral squamous cell carcinoma"** is a bonafide and genuine research work carried out by me in the Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, under the supervision of **Dr. A. V. Moideen Kutty,** Professor of Cell Biology and Molecular Genetics, **Dr. Sharath B.,** Associate Professor of Cell Biology and Molecular Genetics, and **Dr. Azeem Mohiyuddin S. M.,** Professor of Otorhinolaryngology and Head and Neck Surgery. No part of this thesis has formed the basis for the award of any degree or fellowship previously. Plagiarism check was carried out on the thesis and the report was found to be satisfactory.

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ABBREVIATIONS

OSCC	Oral squamous cell carcinoma
WHO	World health organization
HPV	Human papillomavirus
ROS	Reactive oxygen species
ELB	Erythrocyte cell lysis buffer
SDS	Sodium dodecyl sulfate
NaCl	Sodium chloride
DNA	Deoxyribonucleic acid
TE	Tris-EDTA
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase chain reaction
dNTP	Deoxyribonucleotide triphosphate
$MgCl_2$	Magnesium chloride
TAE	Tris-Acetate-EDTA
RFLP	Restriction fragment length polymorphism
RMT	Retromolartrigone
GST-pi	Glutathione S-transferase-pi
SNP	Single nucleotide polymorphism
IHC	Immunohistochemistry
FFPE	Formalin-fixed paraffin embedded
TBS	Tris-buffered saline
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
h	Hour
s	Second
min	Minute
µl	Micro litre
ng	Nano gram
DAB	3,3'- diaminobenzidinetetrahydrochloride
LD	Linkage disequilibrium

MAF	Minor allele frequency
nAChR	Nicotinic acetyl choline receptor
CHRN	Cholinergic receptor nicotinic gene
NNN	N-Nitrosonornicotine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PI3K	Phosphoinositide 3-kinase
Akt	Protein Kinase B
NF- κB	Nuclear factor kappa B
Bp	Base pair
p53	Phosphoprotein53
MDM2	Mouse double minute 2 homolog
NLS	Nuclear localization signal
NES	Nuclear export signal
DBD	DNA binding domain
Ile	Isoleucine
Val	Valine
Ala	Alanine
Arg	Arginine
Trp	Tryptophan

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Abstract

Tobacco is a major risk factor for carcinogenesis. The mechanisms behind the association of tobacco use and oral carcinogenesis remain largely unknown. The aim of this thesis was to determine the association of gene polymorphisms in the xenobiotic metabolism and nicotine signaling with oral carcinogenesis.

A case-control study was carried out by recruiting 100 cancer patients with oral squamous cell carcinoma (OSCC) subtype and 200 healthy controls. The data analysis indicated that the tobacco use and not human papillomavirus infection were found to be the major risk factor in the patient group.

The association of functional single nucleotide polymorphisms (SNPs) in the *GSTP1* gene with the predisposition to OSCC was evaluated. *GSTP1* gene codes for glutathione S-transferase -pi which is the major xenobiotic detoxification enzyme in the oral mucosa. It was hypothesized that functional SNPs in the *GSTP1* gene that reduces its enzymatic activity and therefore, the detoxification capacity of the oral mucosa should be enriched in OSCC patients than in the healthy individuals. Three functional SNPs in the *GSTP1* gene *viz*., Ile105Val, Ala114Val, and Arg187Trp were analyzed by using the PCR-RFLP method. Ala114Val and Arg187Trp but not Ile105Val were associated with the predisposition to OSCC. In addition, it was deciphered that Arg187Trp was linked to the duration of tobacco use indicating the role of gene-environment interaction as a predisposing factor in the development of OSCC.

This was followed by an association study of Asp398Asn SNP in the *CHRNA5* gene with the predisposition to OSCC. *CHRNA5* gene codes for the

cholinergic receptor nicotinic alpha 5 subunit. Nicotine, the major psychoactive agent present in tobacco serves as an agonist for the nicotinic receptor. Activation of the nicotine receptor by nicotine and its metabolites induces cell proliferation, inhibition of apoptosis and transformation. 398Asn SNP reduces the sensitivity of the nicotinic receptor by nearly half. It was hypothesized that the functional SNP in the *CHRNA5* gene that reduces the sensitivity of the nicotinic receptor should be less common among oral cancer patients than among healthy individuals. Asp398Asn was analyzed by using the PCR-RFLP method. The data obtained indicated that there was no association between Asp398Asn and predisposition to oral cancer.

Further, an evaluation of the mutation profile of the *TP53* gene in OSCC was carried out. *TP53* gene codes for the p53 protein which is a transcription factor that plays a key role in maintaining the genomic integrity of the cell. The hypothesis was that the mutation of NLS and NES motifs of *TP53* gene would be a common feature in OSCC specimen. Nuclear localization is an important prerequisite for p53 function and this is determined by the NLS and NES motifs. Mutations of these motifs can abrogate p53 function. It was observed that the mutations were localized to the transcriptionaly active DNA binding domain and were absent in the NLS and NES motifs.

This study shows that tobacco use and deficiency of xenobiotic detoxification are important factors in the predisposition to oral cancer.

Chapter I

Introduction

Oral cancer is a common head and neck cancer that arises from the tissues of the oral cavity. Oral cancer arises mainly as a primary lesion. It may also arise as an extension from the nasal cavity or as a secondary lesion due to metastasis from a different site of origin [Heroiu et al. 2013; Montero et al. 2015]. There are several types of oral cancers such as squamous cell carcinoma, basal cell carcinoma, verrucous carcinoma, salivary gland carcinoma, lymphoma, and sarcoma. Among these types, squamous cell carcinoma is the most common malignancy that accounts for 90 - 95 % of oral cancers [Choi et al. 2008; Parkin et al. 2005]. Oral squamous cell carcinomas (OSCC) originates from the squamous epithelium of the oral cavity [Tumuluri et al. 2010].

1.1. Epidemiology

Oral cancer is the sixth most common cancer in the world accounting for 2 – 4 % of all the cancers and constitutes a major public health burden [Petersen. 2005]. The incidence of oral cancer is significantly high globally with an estimated 650, 000 new cases and 350, 000 cancer deaths each year [Parkin et al. 2005]. Oral cancer accounts for 50 - 70 % of total cancer mortality and has the highest incidence in Asia [Khandekar et al. 2006]. It is the most common cancer in South East Asia (India, Bangladesh, Pakistan, and Sri Lanka) where it accounts for one- third of all cancers [Singh et al. 2014; Khan et al. 2017]. According to WHO, the age-standardized incidence rate of oral cancer in India is 12.6 per 1,00, 000 population [Petersen. 2005].

OSCC occurs in almost all the regions of the oral cavity (Figure 1.1). In India, the commonly affected site is the buccal mucosa followed by the alveolar

surface, hard palate, anterior 2/3rd of tongue and floor of mouth [Montero et al. 2015]. Majority of the OSCC patients present with locally advanced disease. Despite improvements in surgical and therapeutic approaches, less than 60 % of the patients survive for more than 5 years [Rosebush et al. 2011].

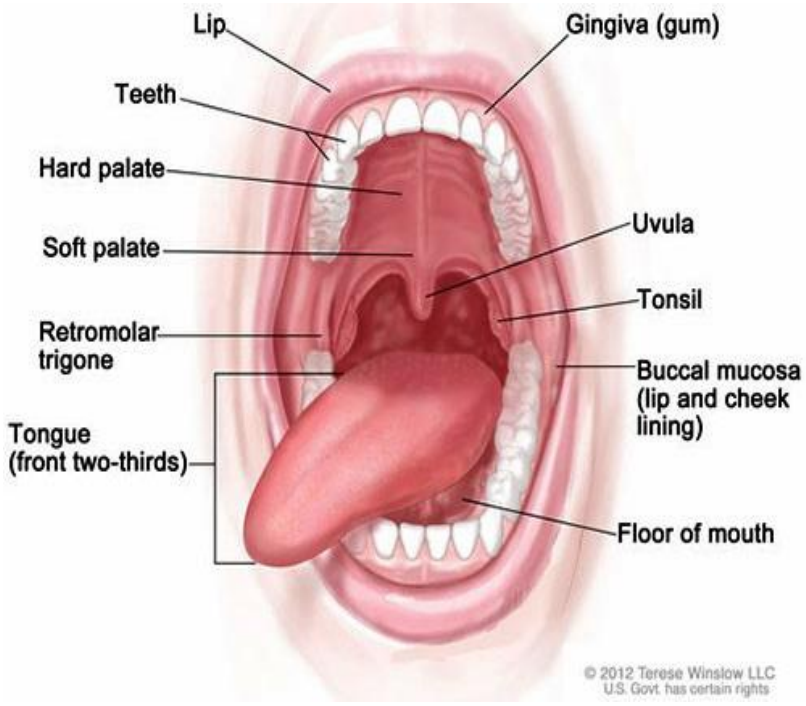


Figure 1.1: Oral cancer regions

1.2. Risk factors

OSCC is a multi-factorial disease with environmental, lifestyle, and genetic factors which interact to cause the malignant stage [Ram et al. 2011]. Environmental factors mainly include infectious agents like human papillomavirus (HPV). HPV is classified as an oncovirus. HPV proteins E6 and E7 inactivate tumor suppressor proteins p53 and pRb respectively [Chocolatewala et al. 2009; Yim et al. 2005]. The inactivation of tumor suppressor proteins reduces the capacity of the infected cell to defend against DNA damage. Unrepaired DNA damages result in nucleotide

misincorporation during replication. HPV infection thus ultimately leads to carcinogenesis [Pinidis et al. 2016]. Infection with high - risk strains of HPV like 16 and 18 are observed in about 20 - 50 % of OSCC patients across the world.

The major lifestyle factors in the development of OSCC are tobacco and alcohol use [Zygogianni et al. 2011]. Tobacco is used either in smoke or smokeless forms. Chewing tobacco along with *betel* quid and snuff are the main smokeless form. The risk of OSCC due to tobacco use is well established with both smoke and smokeless forms. The relationship between smoking and oral cancer has been established strongly by epidemiological studies [Khandekar et al. 2006; Gupta et al. 1995; Jussawalla et al. 1971; Mehta et al. 1972]. The risk of developing OSCC in tobacco chewers is 4.8 times higher compared to non-tobacco chewers [Khan et al. 2014].

The genetic factors in the development of OSCC involve polymorphisms in the genes that regulate various aspects of carcinogenesis. Unlike mutations, polymorphisms do not cause cancer but constitute a predisposition to develop cancer [Malhotra et al. 2014; Kang et al. 2001]. Gene polymorphism is defined as the variation in the sequence of DNA that occurs with a frequency greater than 0.5 % [1000 Genomes Project Consortium et al. 2015; Wang et al. 2010; Panagiotou et al. 2010]. Gene polymorphisms in DNA repair and apoptosis pathways have been associated with predisposition to OSCC [Kiyohara et al. 2012]. Polymorphisms in the genes that code for the enzymes involved in xenobiotic metabolism are also suspected to play a key role in the genetic predisposition to tobacco-induced head and neck cancers [Lacko et al. 2009]. Xenobiotics are foreign substances that are non-physiological in origin and are often hydrophobic in nature. As a consequence,

they cannot be easily removed from the human system [Patterson et al. 2010]. Tobacco is a rich source of xenobiotic carcinogens like nicotine, polycyclic aromatic hydrocarbons, nitrosamines, metals, and aldehydes. In addition, stable and unstable free radicals and reactive oxygen species (ROS) with the potential for biological oxidative damage are known to occur in the tobacco (Figure 1.2) [Xue et al. 2014]. Therefore, gene polymorphisms linked to xenobiotic metabolism may interact with tobacco use to increase the predisposition to develop cancer. In this light, the overall aim of this study was to understand the role of xenobiotic metabolism linked gene polymorphisms in the development of OSCC with emphasis on glutathione S-transferase.

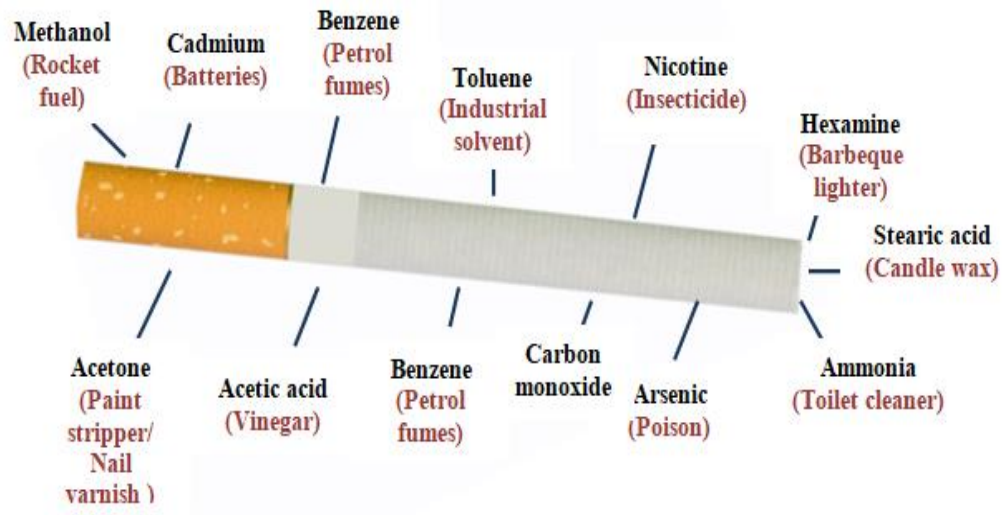


Figure 1.2: Tobacco carcinogens

Chapter II

Review of literature

Oral cancers are caused due to predisposing genetic factors and exposure to the carcinogens. Tobacco smoking is responsible for 20 – 30 % of overall cases [Hashibe et al. 2007; Rahman et al. 2005]. In geographical areas where tobacco chewing is a standard cultural practice, 90 % of oral cancer among women and 50 % of oral cancer among men are attributed to frequent tobacco chewing [Balaram et al. 2002]. Heavy alcohol drinking is linked to 7–19 % of the cases [Hashibe et al. 2007; Room et al. 2005]. Micronutrient deficiency accounts for 10–15 % of the cases [Wiseman et al. 2008]. Human papillomavirus (HPV) infection accounts for about 3 % of the cases [Parkin and Bray. 2006]. Additionally, exposure to two or more of these risk factors has a collective effect in increasing predisposition to oral cancer risk [Applebaum et al. 2007; Boccia et al. 2008].

OSCC accounts for more than 90 % of the malignant tumors of the oral cavity and constitutes the most malignant tumors of the head and neck [Diez-Perez et al. 2011]. OSCC originates from the oral keratinocyte. In the Indian subcontinent, oral cancer is among the most common malignancies, mainly due to tobacco and alcohol consumption [Leon-Barnes et al. 2005]. However, not all tobacco and alcohol users develop OSCC. Absorption, metabolism, and elimination of carcinogenic constituents of tobacco depend on the activity of xenobiotic metabolism pathways. Interindividual differences in the xenobiotic metabolism therefore constitutes an important factor in the development of OSCC.

2.0. Biological significance of xenobiotic metabolism

The human body is constantly exposed to xenobiotics that include both synthetic and natural chemical substances like drugs, industrial chemicals, pesticides,

pollutants, pyrolysis products in cooked food, secondary metabolites in food, and microbial toxins. Skin, respiratory and digestive tract are the major sites for the absorption of xenobiotics [Park et al. 2014; Koppel et al. 2017]. These substances are generally poorly soluble in water and therefore resist elimination. The nonpolar nature imparts xenobiotics with lipophilic property. As a consequence xenobiotics tend to accumulate in cell membrane and fat tissue. The progressive accumulation can lead to toxic build-up and this phenomenon is known as biomagnification [Patterson et al. 2010; Croom. 2012]. This is prevented by a physiological defense mechanism called as xenobiotic metabolism. This process converts lipophilic xenobiotics into water soluble compounds so that they can be easily eliminated by the excretory system [Omiecinski et al. 2010; Patterson et al. 2010].

2.1. Phases of xenobiotic metabolism: The xenobiotic metabolism is classified into three phases based on the nature of the conversion reaction. Phase I metabolism involves functionalization of the xenobiotics with reactive and polar groups. The main reactions of the phase I metabolism are oxidation, reduction, and hydrolysis. Phase II metabolism involves conjugation of the phase I activated xenobiotics with charged moieties like glutathione, sulfate, glycine or glucuronic acid. Phase III metabolism involves removal of the conjugated xenobiotics from the cell by active transport. The anionic groups on the conjugated xenobiotics increases their affinity to membrane transport proteins like ATP-binding cassette transporter [Xu et al. 2005; Omiecinski et al. 2010]. The reactions of xenobiotic metabolism are shown in Table 2.1.

Table 2.1: Reactions of xenobiotic metabolism and their major subcellular location

Reaction	Enzyme	Localization
Phase I		
Hydrolysis	Esterase	Microsomes, cytosol, blood
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro-reductase	Microflora, microsomes, cytosol
	Aldo - ketoreductase	Cytosol, blood, microsomes
Oxidation	Cytochrome P450	Microsomes, mitochondria
	Alcohol dehydrogenase	Cytosol
	Flavin- monooxygenases	Microsomes
	Monoamine oxidase	Mitochondria
	Aldehyde dehydrogenase	Mitochondria, cytosol
Phase II		
Glucuronidation	UDP-glucuronic acid	Microsomes
Sulfonation	Sulfotransferases	Cytosol
Glutathionylation	Glutathione S-transferases	Cytosol, microsomes
Amino acid conjugation	Amino acid transferases	Mitochondria, microsomes
Acylation	N-acetyl transferases	Mitochondria, cytosol
Methylation	Methyl transferases	Cytosol, microsomes, blood
Phase III		
ATP-binding cassette transporters		Cell membrane

2. 2. Reactions of the Phase I metabolism

2.2.1. Oxidation

This is the most common phase I reaction. Oxidation results in increasing the water solubility of the xenobiotics by introducing polar functional group such as the hydroxyl group. Oxidation reactions are catalyzed by various oxidoreductase enzymes [Grant. 1991].

2.2.1.1. Enzymes catalyzing oxidative phase I reactions

2.2.1.2. Cytochrome P450: This is a superfamily of heme containing enzyme that is responsible for majority of the phase I reactions. Cytochrome P450 is found in most of the cells with the highest concentration in the hepatocytes. They are mainly localized in the microsomes and mitochondria. The most common reaction catalyzed by cytochrome P450 is a monooxygenase reaction [Iyanagi et al. 2007; Hart et al. 2008; Zanger et al. 2013]. This involves addition of an atom of oxygen into the aliphatic position of an organic substrate (RH) while the other oxygen atom is reduced to water:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

2.2.1.3. Alcohol dehydrogenase: This is a zinc-containing enzyme present in several tissues with the highest levels in the liver and the gastric mucosa. This enzyme catalyses the oxidation of ethanol into acetaldehyde:

$$CH_3CH_2OH + NAD^+ \rightarrow CH_3CHO + NADH + H^+$$

The physiological function of alcohol dehydrogenase (ADH) is to metabolize both consumed alcohol and that present naturally in food and also produced by bacteria in the digestive tract [Cederbaum. 2013].

2.2.1.4. Flavin monooxygenases: These enzymes catalyze oxidation of xenobiotics by using FAD as the prosthetic group. They are mainly involved in the oxidation of nucleophiles like amines, sulfides and phosphites. Flavin monooxygenases are found in fungi, yeast, plants, mammals, and bacteria [Krueger et al. 2005; Mitchell. 2008].

2.2.1.5. Monoamine oxidase: This enzyme is involved in the oxidative deamination of primary, secondary, and tertiary amines (Benedetti and Dostert. 1994). They are found in the brain and also in the liver, kidney, intestine, and blood platelets mainly in the outer mitochondrial membrane. Oxidative deamination of amine produces ammonia and aldehyde.

2.2.1.6. Aldehyde dehydrogenase: This enzyme catalyzes the oxidation of aldehydes using NAD+ as the cofactor:

$$RCHO + NAD^+ + H_2O \rightarrow RCOOH + NADH + H^+$$

These enzymes are found in many tissues of the body but are at the highest concentration in the liver. They participate in a wide variety of biological processes including the detoxification of exogenously and endogenously generated aldehydes [Chen et al. 2014].

2.2.2. Reduction

The reduction reactions plays an important role in the metabolism of xenobiotics containing carbonyl, nitro and azo groups.

2.2.2.1. Enzymes catalyzing reduction phase I reactions

2.2.2.2. Azo- and Nitro - reductase: These enzymes are involved in the detoxification of xenobiotics such as azo dyes, nitro-aromatic, and azoic drugs which are derived from paint, textile, cosmetics, pharmaceutical industries and untreated wastewater from industries. Nitro and amine moieties of azo dyes and synthetic nitro aromatic compounds are potential carcinogenic and mutagenic. The enzymes are normally present in gastrointestinal microflora [Chengalroyen et al. 2013].

2.2.2.3. Aldo - ketoreductase: These enzymes catalyze the reduction of certain aldehydes to primary alcohols and of ketones to secondary alcohols [Weiner and Flynn. 1989]. These are NADPH-dependent enzymes present in blood and the cytosolic fraction of the liver, kidney, brain, and other tissues [Penning et al. 2015].

2.2.3. Hydrolysis

These reactions commonly involve a large chemical change in the substrate in which the addition of water splits the xenobiotic into two fragments. The hydroxyl group is incorporated into one fragment and the hydrogen atom is incorporated into the other. Larger chemical substances such as esters, amines, hydrazines, and carbamates are generally metabolized by hydrolysis.

2.2.3.1. Enzymes catalyzing hydrolytic phase I reactions

2.2.3.2. Esterase: This includes a variety of hydrolytic enzymes like carboxylesterases, cholinesterases and organophosphatases. These enzymes

hydrolyzexenobiotics containing functional groups such as carboxylic acid ester, amide, thioester, phosphoric acid esterase, and acid anhydride:

RC (O) OR + H2O RCOOH + HOR Carboxylester hydrolysis

RC (O) NRR + H2O RCOOH + HNRR Carboxyamide hydrolysis

RC (O) SR + H2O RCOOH + HSR Carboxythioester hydrolysis

These enzymes are located in various tissues, serum and blood.

2.2.3.3. Epoxide hydrolase: Electrophilic epoxides and arene oxides are constantly produced during the cytochrome P450-dependent oxidation of unsaturated aliphatic and aromatic xenobiotics and are highly reactive with cellular macromolecules such as DNA and protein. Epoxide hydrolases can rapidly convert these potentially toxic metabolites to the corresponding dihydrodiols, which are less reactive and easier to excrete. These enzymes are found in almost all tissues, including the liver, testis, ovary, lung, kidney, skin, intestine, colon, spleen, thymus, brain, and heart [Beetham et al. 1995; Fretland and Omiecinski. 2000].

Example for phase I reaction: Phase I xenobiotic detoxification by CYP450 involve oxidation, hydroxylation, and reduction (Figure 2.1).

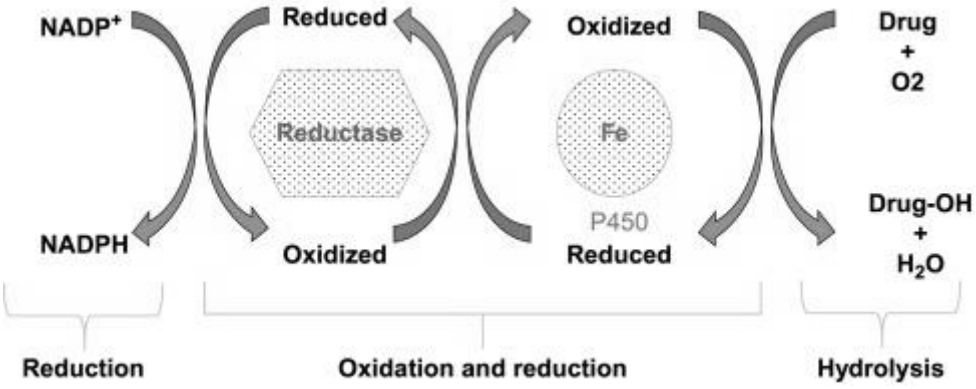


Figure 2.1: Phase I metabolism catalysed by cytochrome P450

2. 3. Reactions of the phase II metabolism

2.3.1. Glucuronidation: The reaction involves membrane-bound conjugating enzymes that catalyze the transfer of the glucuronic acid component of UDP-glucuronic acid to hydrophobic molecule:

ROH +UDP-glucuronic acid → RO-glucuronide +UTP

The reaction results in the formation of less biologically and chemically reactive products. This, increases their polarity and susceptibility to excretion contributing to the detoxification of xenobiotics. Glucuronidation is a major Phase II reaction with approximately 40–70% of all clinical drugs being affected by it. The enzyme is mainly found in the liver, intestine, kidney and other tissues [Miners and Mackenzie. 1992; Rowland et al. 2013; Tukey et al. 2002].

2.3.2. Sulfation: The reaction is catalyzed by various types of sulfotransferases. Reactions of the sulfotransferase enzyme involves various xenobiotics including alcohols, arylamines, and, phenols resulting in the production of water soluble compounds that are readily eliminated from human system. These are cytosolic enzymes found primarily in the brain, liver, intestine, lung, and kidney [Nagata and Yamazoe. 2000; Chen et al. 2015].

2.3.3. Acetylation: N-Acetylation reaction involves metabolism of xenobiotics containing an aromatic amine or a hydrazine group, which are converted to aromatic amides and hydrazides, respectively [Spielberg. 1996]. The reaction is catalyzed by N-acetyltransferases and requires acetyl-coenzyme A (acetyl-CoA) as the cofactor. These are cytosolic enzymes found in liver and many other tissues.

2.3.4. Methylation: This is a common minor pathway of xenobiotic detoxification. The reaction involves transfer of a methyl group from S -adenosylmethionine to xenobiotics that contain, - C, - O, - N, or - S functional groups. Unlike other conjugation reactions, methylation of xenobiotics results in more hydrophobic metabolites, except for cases of N - methylation of pyridine - containing xenobiotics such as nicotine and S- methylation of thioethers. The enzymes are found in liver and kidney [Weinshilboum. 1989; Wang and Weinshilboum. 2006].

2.3.5. Amino Acid Conjugation: Amino acid conjugation occurs with substrates containing carboxylic acid and aromatic hydroxylamines. Xenobiotics containing carboxylic acid group are conjugated with the amino group of amino acids such as glycine and glutamine [Parkinson and Ogilvie. 2008]. Examples of compounds that undergo amino acid conjugation are benzoic acid (glycine), valporic acid (glutamine, glutamate, and glycine), and ibuprofen (taurine). The enzymes are localized in mitochondria and microsomes.

2.3.6. Glutathionylation: This is the key pathway for detoxification of chemically reactive electrophilic xenobiotics. The reaction involves conjugation of xenobiotics (X) with the tripeptide glutathione (GS-), catalyzed by a family of glutathione S-transferases (Z).

$$X\text{-}Z + GS\text{-} \rightarrow GS\text{-}X + Z\text{-}$$

Glutathione conjugates produced in the liver are excreted in bile, or they are converted to mercapturic acids in the kidney and excreted in urine. These enzymes are located mainly in the tissues such as liver, intestine, kidney, adrenal, and lung, where they are localized in the cytoplasm and endoplasmic reticulum. GSTs play a

major role in the detoxification of epoxides derived from polycyclic aromatic hydrocarbons and alpha-beta unsaturated ketones [Townsend et al. 2009; Parkinson and Ogilvie. 2008].

2.4. Classification of GSTs

Human GSTs are classified into three main families *viz.*, cytosolic GSTs, mitochondrial and microsomal GSTs (Table 2.2). The cytosolic and mitochondrial GSTs are soluble enzymes and share a similar three-dimensional structural similarity. On the basis of the primary structure, the human cytosolic GST superfamily consists of 16 genes which are further subdivided into 8 separate classes designated as α (alpha), μ (mu), π (pi), ω (omega), σ (sigma), θ (theta), ζ (zeta), and κ (kappa). Microsomal GSTs are also called as MAPEGs (membrane associated proteins in eicosanoid and glutathione metabolism) which are structurally different from cytosolic GSTs [Hayes et al. 2005]. Each of these GST classes are coded by different genes which are located on different chromosomes [Nissar et al. 2017].

Table 2.2: The glutathione S-transferase supergene family

Sl. No.	Class	Enzyme
1	Cytosolic	*Alpha* *Mu* *Pi* *Sigma* *Theta* *Zeta* *Omega*
2	Mitochondrial	*Kappa*
3	MAPEG	*FLAP* *MGST1* *MGST2* *MGST3* Prostaglandin E synthase *1*

* *MGST:* Microsomal glutathione S-transferase

* *FLAP:* 5-lipoxygenase activating protein

2.5. Reactions of the phase III metabolism

Phase III metabolism is involved in actively removing xenobiotics from the cells [Xu et al. 2005]. Water-soluble compounds are moved out of the cells with the help of transport protein. Phase III transporters belong to a family of proteins called as the ATP-Binding Cassette transporters (ABC transporters), because they require ATP to actively pump xenobiotics out of the cell membrane [Liang et al. 2015; Mizuno et al. 2003]. They are also referred as Multidrug Resistance Proteins (MRPs) since, drug - resistant cancer cells use them as protection against chemotherapy drugs [Keppler. 2011]. In the liver, phase III transporters move glucuronide, glutathione,

and sulfate conjugates out of the cells into the bile for elimination. In the kidney and intestine, phase III transporters remove xenobiotics from the blood for elimination from the body [Klaassen and Lu. 2008]. Phase III transporters such as P-glycoprotein, MRPs, and organic anion transporting polypeptide 2 are expressed in many tissues such as the liver, intestine, kidney, and brain [Miller et al. 2008].

2. 6. Variability in xenobiotic metabolism

The magnitude of xenobiotic detoxification is not a uniform property but shows significant inter-individual variation [Zanger et al. 2013]. The phenotypes of the xenobiotic metabolism may be classified into 4 groups on the basis of detoxification capacity as follows:

i) Slow metabolizer: detoxification is reduced or no enzyme activity

ii) Intermediate metabolizer: reduced detoxification capacity

iii) Extensive metabolizer: normal detoxification capacity

iv) Rapid metabolizer: elevated detoxification capacity

2.7. Gene polymorphism as the basis of variability in xenobiotic metabolism

Gene polymorphism is defined as the variation in the sequence of DNA that occurs with a frequency greater than 0.5 % [1000 Genomes Project Consortium et al. 2015]. Functional polymorphisms occur in the genes that code for xenobiotic metabolizing enzymes (XME). These polymorphisms reduce the enzymatic activity by reducing the stability or by altering the active site of XME [Moyer et al. 2008]. A large number of polymorphisms in XME genes occur commonly in the general

population and have been linked to variability in the xenobiotic metabolism. Some of the common polymorphisms in the major XME genes are listed in Table 2.3.

Table 2.3: Common polymorphisms in the XME genes

Family	Gene	SNP	Reference
CYP450	*CYP1A1* *CYP1B1*	Ile462Val Ala119Ser	Hung et al. 2003 Sasaki et al. 2003
NAT	*NAT2*	Arg268Lys	Khlifi et al. 2014
ALDH	*ADH1B1*	Arg47His Arg369Cys	Edenberg, 2007 Edenberg et al. 2006
UDP-glucuronosyltransferase	*UGT1A7*	Gly115Ser Asn129Lys	Carlini et al. 2005
GST	*GSTA*	Thr112Ser Pro110Ser	Ning et al. 2004
	GSTM	Lys173Asn Null	Moyer et al. 2007 Sharma et al. 2015
	GSTP	Ile105 Ala114 Ser185	Hayes et al. 2005
	GSTT	Null	Sharma et al. 2015
	GSTO	Ala140Asp Asn142Asp	Xu et al. 2014

2.8. Relationship between xenobiotic metabolism and pharmacogenomics

Pharmacogenomics is the understanding of how individuals differ in their response to drug therapy and the mechanisms underlying variable drug response by utilizing genomics, proteomics, transcriptomics, and metabolomics-based knowledge. Individuals vary significantly in their clinical responses to administered

drugs and the outcomes. The difference may be inherited or acquired. Pharmacogenomics deals specifically with the inherited differences in the response to a drug [Ahmed et al. 2016].

Gene polymorphisms can influence the effect of a drug by altering its pharmacokinetics, pharmacodynamics, or both (Figure 2.2). Pharmacokinetics and pharmacodynamics are the two major determinants of the interindividual differences in drug response. Pharmacokinetics deals with how much of a drug is required to reach its target site in the body, while pharmacodynamics deals with how well the target such as receptor, ion channel, and enzyme responds to drugs [Pirmohamed. 2014; Pirmohamed and Park. 2001]. Drugs are xenobiotic in nature and therefore gene polymorphisms in XMEs can alter this pharmacokinetic and pharmacodynamic processing [Ahmed et al. 2016].

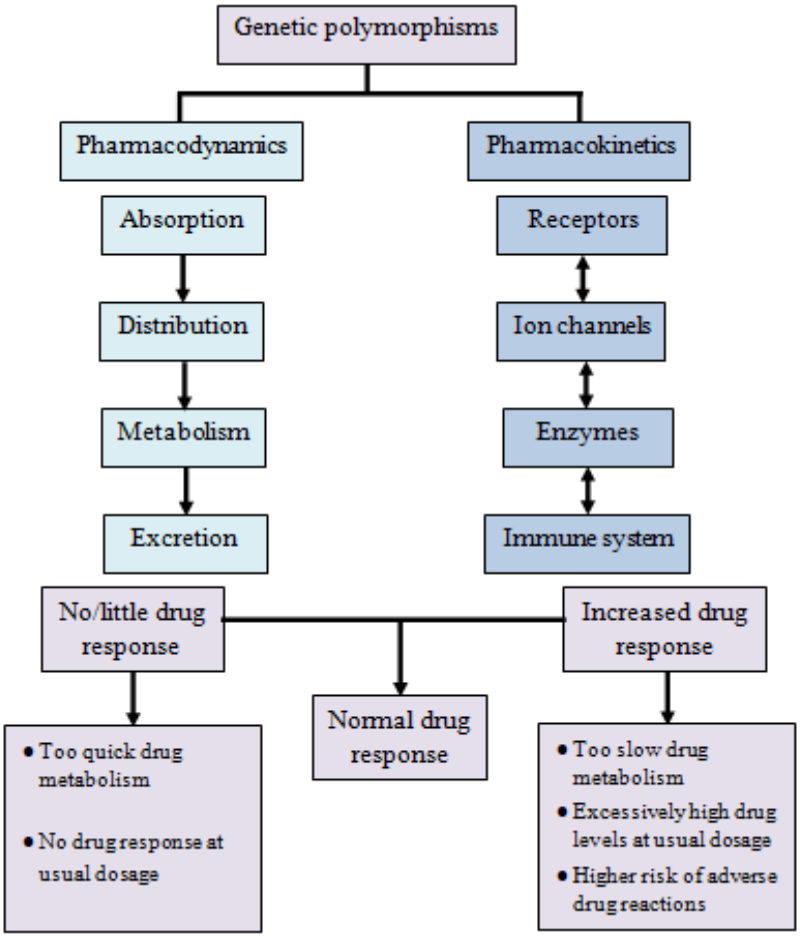


Figure 2.2: Effect of gene polymorphism on drug metabolism

2.9. Role of XMEs in chemotherapy failure

The relationship between XME and pharmacogenomics plays an important role in chemotherapy. XME recognizes chemotherapeutic agents as xenobiotics and catalyze their metabolism. As a result, XMEs play an important role in decreasing the intracellular concentration of chemotherapeutic drugs. XMEs that catalyze oxidation and conjugation reactions are particularly involved in chemotherapy failure [Housman et al. 2014].

Among various XMEs, GSTs are mainly implicated in the resistance to chemotherapeutic drugs. GSTs are involved in the metabolism of various

chemotherapeutic agents like cisplatin, adriamycin, busulfan, chlorambucil, carmustine, cyclophosphamide, ethacrynic acid, and melphalan [Xu et al. 2005; Paumi et al. 2001]. Cell culture studies have shown that overexpression of GST-Pi increases the resistance to anticancer drugs [Ban et al. 1996; Wang et al. 2007]. The overexpression of *GSTA* has also been associated with the drug resistance in MCF-7 breast cancer and in H69 small cell lung cancer cell lines [Sharma et al. 2006]. Increased GST-Pi expression has also linked to drug resistance in the cancers of the ovary, head and neck and lung [Perquin et al. 2001; Mayr et al. 2000; Shiga et al. 1999; Inoue et al. 1995].

2.10. Association of XME gene polymorphism the predisposition to cancer

Several xenobiotics are carcinogenic in nature. Therefore, fully functional xenobiotic metabolism is vital for the detoxification of carcinogenic xenobiotic and maintaining the genomic integrity. Factors that compromise the detoxification capacity thus contribute towards carcinogenesis. Polymorphisms in the XME genes reduce their functional capacity. As a result, polymorphisms in several XME genes have been associated with the predisposition to cancer. Some of the major polymorphisms in the common XME genes and their association with various cancers is shown in Table 2.4.

Table 2.4: Gene polymorphism in XMEs and the associated cancer risk

Cancer type	Ref	Popln.	Case/ Control	Genes	SNP site	Risk
CYP450						
Lung						
	Kumar et al. 2009	North Indian	93/253	*CYP1A1*	A2454G	Increased
	Sreeja et al. 2005	South Indian	146/146	*CYP1A1*	T3698C	Increased
	Shah et al. 2008	South Indian	200/200	*CYP1A1*	T3698C	Increased
	Sobti et al. 2008	North Indian	ND	*CYP1A1*	A2454G	Increased
	Pappireddy et al. 2003	South Indian	246/250	*CYP1A1*	T3801C	Increased
				CYP2D6		Increased
	Shah et al. 2008	North Indian	200/200	*CYP1B1*	R48G A119S L432V A453S	Increased Increased Decreased Decreased
Head and Neck						
	Sharma et al. 2010	North Indian	203/201	*CYP1A1*	T3801C	Increased
	Sharma et al.2010	North Indian	203/201	*CYP2A13*	T478C, T494C	Decreased
	Ruwali et al.2009	ND	350/350	*CYP2A6*	*1B and * 4C	Decreased
	Ruwali et al. 2009	ND	350/350	*CYP2E1*	*5B	Increased
	Yadav et al. 2008	ND	ND	*CYP2C19*	2	Increased
UADT	Sam et al. 2008	South India	408/220	*CYP1A1*	T3698C	Increased
Esophageal	Jain et al. 2007	North India	161/201	*CYP1A1*	T3698C	Decreased
	Bhat et al. 2010	North India	492/492	*CYP2C19* *CYP2D6*		Increased
	Bhat et al. 2015	Kashmir	7.3/1664	*CYP2C19* *CYP2D6* *CYP2E1*	*1/*1, G/G, c1/c1	Increased
	Shah et al. 2015	Kashmir	404/404	*CYP1B1*	L432V	Decreased
EADC	Malik et al.2010	Kashmir	135/195	*CYP2E1*	RsaI/PstI	Increased
Gastric	Malik et al.2009	Kashmir	108/195	*CYP2E1*	RsaI/PstI	Increased

Gall Bladder	Pandey et al. 2008	North India	142/171	*CYP1A1*	T3698C	Increased
Breast						
	Surekha et al. 2009	ND	250/250	*CYP1A1*	A2454G	Increased
	Singh et al. 2007	North India	ND	*CYP1A1*	T3698C A2454G T3204C C2452A	Decreased Increased Decreased Decreased
Prostate						
	Vijayalakshmi et al. 2005	ND	ND	*CYP1A1*	T3698C	Increased
					A2454G	Decreased
	Sobti et al. 2006	North India	100/100	*CYP17*		Increased
				CYP1B1	L432V	Increased
	Sobti et al. 2009	North India	157/170	*CYP17*		Increased
Leukemia						
ALL	Joseph et al. 2004	ND	118/118	*CYP1A1*	W3698C A2454G	Increased
	Nida et al. 2017	Kashmir valley	200/200	*CYP1A1*	*2A	Increased
				CYP2D6	*4	Increased
CML	Bajpai et al. 2009	North India	183/208	*CYP3A5*	*6	Decreased
GSTs						
Lung						
	Kumar et al. 2009	North India	93/53	*GSTT1*	Null	Increased
	Sreeja et al. 2008	ND	ND	*GSTT1*	Null	Increased
				GSTP1	A105G	Increased
	Sreeja et al. 2005	South India	146/146	*GSTM1*	Null	Decreased
	Peddireddy et al. 2016	South Indian	246/250	*GSTT1*	Null	Increased
Head and Neck						
	Sabitha et al. 2008	ND	120/130	*GSTM1*	Null	Increased
				GSTT1	Null	Increased
	Singh et al. 2008	ND	175/200	*GSTM1*	Null	Increased
				GSTT1	Null	Increased
	Sharma et al. 2010	North India	203/201	*GSTM1*	Null	Increased
						Increased
GIT						

UADT	Soya et al. 2007	South India	408/220	*GSTT1*	Null	Increased
Esophageal	Jain et al. 2007	North India	149/200	*GSTM3*	A/B	Increased
ESCC	Malik et al. 2010	Kashmir	135/195	*GSTP1*	I105V	Increased
				GSTM3	A/B	Decreased
Gastric	Malik et al. 2009	Kashmir valley	108/195	*GSTM1*	Null	Increased
	Tripathi et al. 2008	ND	76/100	*GSTM1*	Null	Decreased
				GSTT1	Null	Increased
				GSTP1	I105V	Decreased
Gall Bladder	Pandey et al. 2006	North India	106/201	*GSTM1*	Null	Decreased
				GSTT1	Null	Decreased
				GSTP1	I105V	Increased
				GSTM3		Decreased
HCC	Asim et al. 2010	ND	254/524	*GSTM1*	Null	Increased
				GSTT1	Null	Increased
	Kiran et al. 2008	ND	63/169	*GSTT1*		Increased
Liver	Mansoori et al. 2017	MP	121/145	*GSTT1*	Null	Increased
				GSTM1	Null	Increased
Breast						
	Samson et al. 2007	South India	250/500	*GSTM1*	Null	Decreased
				GSTP1	I105V	Decreased
	Saxena et al. 2009	North India	413/410	*GSTM1*	Null	Increased
				GSTT1	Null	Increased
				GSTP1	I105V	Increased
	Syamala et al. 2008	ND	22sp*,125fl/25o	*GSTM1*	Null	Increased
				GSTP1	Null	Increased
				GSTP1	I105V	Decreased
Cervical	Singh et al. 2008	North India	150/168	*GSTM1*	Null	Increased
				GSTT1	Null	Increased
				GSTM3	A/B	Increased
Prostate	Mittal et al. 2004	North India	103/117	*GSTM1*	Null	Increased
				GSTT1	Null	Increased
	Srivastava et al. 2005	North India	127/144	*GSTM1*	Null	Increased
				GSTT1	Null	Increased
				GSTP1	I105V	Increased

	Kesarvani et al. 2009	North India	135/169	*GSTM3*	A/B	Increased
ALL	Suneetha et al. 2008	South India	92/150	*GSTM1*	Null	Increased
				GSTP1	I105V	Decreased
	Joseph et al. 2004	ND	118/118	*GSTM1*	Null	Increased
CML	Bajpai et al. 2006	ND	75/78	*GSTM1*	Null	Increased
				GSTT1	Null	Decreased
AML	Majumder et al. 2008	Eastern India	110/144	*GSTM1*	Null	Increased
Bladder	Srivastava et al. 2004	North India	106/182	*GSTM1*	Null	Increased
				GSTT1	Null	Increased
	Srivastava et al. 2005	North India	106/370	*GSTM1*	Null	Decreased
				GSTT1	Null	Decreased
				GSTP1	I105V	Increased
	Mittal et al. 2005	North India	106/162	*GSTP1*	I105V	Increased
						Increased
NAT2						
Esophageal	Jain et al. 2007	North India	126/164	*NAT2*	Slow	Decreased
	Malik et al. 2009	Kashmir	123/182	*NAT2*	Slow	Decreased
Prostate	Srivastava and Mittal 2005	North India	130/140	*NAT2*	Rapid Acetylator genotype	Decreased
AML	Majumdar et al. 2008	Eastern India	110/144	*NAT2*	2*6B	Increased
D.*SULT*						
MPN	Kotnis et al. 2008	Mixed	132/198	*SULT1A1*	R213H	Increased
Lung	Pachouri et al. 2006	North India	103/122	*SULT1A1*	G638A	Increased
Breast	Chacko et al. 2004	ND	140/140	*SULT1A1*		Increased
E. *UGT*						
Head and Neck	Sharma et al. 2010	North India	203/201	*UGT1A7*	Low activity genotype	Increased
F.*EPHX1*						
Esophageal	Ihsan et al. 2010	ND	142/185	*EPHX1*	W113H	Decreased
					H139R	Increased
ESCC	Jain et al.	ND	107/320	*EPHX1*	Y113H	Increased

	2008					
					H139R	Decreased
Hepatitis related HCC	Kiran et al. 2008	ND	63/169	*EPHX*	H139R	Increased

ND : Not determined

2.11. Tobacco as a source of xenobiotics

Tobacco is rich source of xenobiotics. About 4000 different chemical substances are present in tobacco. The chemical composition depends on the growing conditions of the tobacco plant, curing, processing, fermentation, and storage [Peele et al. 2001; Bush et al. 2001]. Combustion that occurs during the smoking of tobacco results in the formation of more than 2000 compounds. About 28 compound in raw tobacco have been identified as carcinogenic in nature. There are N-nitrosamines, N-nitrosoamino acids, aldehydes, aromatic hydrocarbons including benzo[a]pyrene, metals (Cd, Cr, Pb, and Ni), lactones, urethane, mercury, chromium, uranium -235, and polonium- 210 [Hoffmann and Djordjevic. 1997].

2.12. Nicotine addiction

Nicotine is the major psychoactive chemical present in tobacco [Benowitz, 2010]. It is absorbed into the blood through oral tissues, and then it reaches the brain. Chewable tobacco releases more nicotine than cigarette. Therefore people habituated to tobacco chewing may find difficulty in quitting tobacco chewing than smoking. Quitting tobacco chewing causes similar symptoms as quitting cigarette such as increased appetite, intense cravings, irritability, anxiety, and depression [Sangar. 2016].

2.13. Tobacco induced carcinogenesis

Nicotine addiction causes continual tobacco use and chronic exposure to carcinogens. The metabolism of many tobacco carcinogens takes place in through xenobiotic detoxification pathway. In this pathway the phase I reactions, mediated by cytochromome P450 family and microsomal epoxide hydrolase, result in the formation of phenols, epoxides and other reactive intermediates that can be transformed into highly carcinogenic electrophilic compounds such as BaP-diolepoxides [Hoffmann and Djordjevic. 1997]. These compounds, in the absence of a rapid further intervention by phase II conjugating enzymes such as GSTs and UGTs, may form DNA adducts. The DNA adducts can be repaired by enzymes of the DNA repair pathway. However, failure to repair DNA adducts results in misincorporation of nucleotides during DNA replication. This results in permanent mutation in the DNA sequence. Tobacco induced mutations can alter the genetic landscape by activating the oncogenes and inactivating the tumor-suppressor genes. This results in the loss of normal regulation of cell proliferation. Nicotine can also bind directly to cellular receptors and lead to activation of the PI3K/AKT and PKC/ERK1/2 pathways. This, in turn, results in increased cell proliferation and decreased apoptosis, which are hallmarks of a cancer cell [Wen et al. 2011; Schaal and Chellappan. 2014].

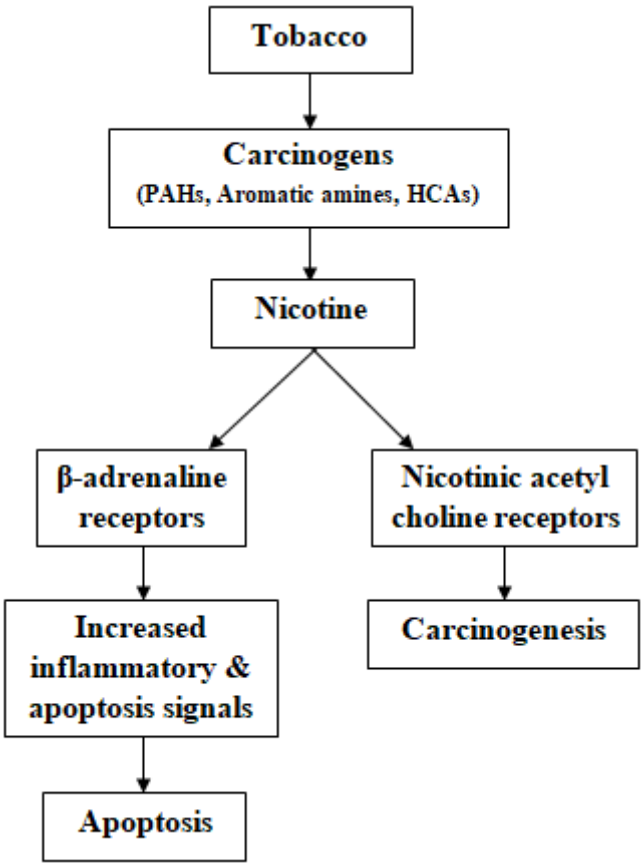


Figure 2.4: Cellular pathway of tobacco metabolism by xenobiotic enzymes

2.14. Association of polymorphisms in XME genes with the predisposition to oral cancer

The oral mucosa is in direct contact with potentially toxic or carcinogenic agents ingested through tobacco chewing. The upper aerodigestive mucosa acts as a first-line barrier. Substances that enter the mucosa and systemic circulation are then processed by XMEs. It is well established that gene polymorphisms occur in XMEs that affects their functional capacity. These functional polymorphisms appear to be responsible for the differences in the inter-individual susceptibility for the

development of oral cancer. Functional polymorphisms in XME genes linked with the susceptibility to develop cancer are summarized in Table 2.5.

Table 2.5: XME gene polymorphism associated with oral cancer

Genes	SNP site	Case/ Control	Risk	Ref
CYP1A1	A2454G	98/60	Increased	Sreelekha et al. 2001
CYP1A1	T3698C	446/727	Decreased	Devasena et al. 2007
	C/C		Increased	
CYP1A1	T3698C	ND	Decreased	Chatterjee et al. 2009
GSTM1	Null	297/450	Increased	Buch et al. 2002
GSTT1	Null	40/87	Increased	Sharma et al. 2006
GSTM1	Null		Decreased	
GSTM1	Null	451/727	Increased	Devasena et al. 2007
GTT1	Null	456/726	Increased	
GSTM3	A/B	63/132	Increased	Park et al. 2000
GSTP1	I105V	35/60	Decreased	Yaghmaei et al. 2015
GSTT1	Null	35/60	Increased	
GSTM1	Null	35/60	Increased	
GSTP1	I105V	310/389	Increased	Datta et al. 2007

ND: Not determined

2.14. Role of p53 in xenobiotic metabolism

p53 is a tumor suppressor protein coded by the *TP53* gene. It plays a key role in maintaining the genomic integrity of the cell and hence it is referred to as the molecular policeman of the cell. Functionally, it is a transcription factor that regulates DNA repair, apoptosis and cell cycle. *TP53* gene is commonly mutated in

almost 50 % of cancers. p53 protein plays an important role in the regulation of cellular response to benzo[a]pyrene which is a major carcinogen in tobacco [Zheying. 2015; Yeo. et al. 2016; Xiao and Singh. 2007]. Studies have also shown a strong correlation between mutational hotspots and DNA adduct formation due to polycyclic aromatic hydrocarbons present in tobacco. Earlier it was assumed that mutations caused by chemical carcinogens in the *TP53* gene occurred randomly. However, recent studies have shown that benzo[a]pyrene, binds specifically to the sites that are hotspots for OSCC. While, OSCC related *TP53* mutations occur at several positions, majority of the hotspot mutations are found in codons R175, G245, R248, R282, H179, and R273 [Rivlin et al. 2011; Cho et al. 1994].

The review of the literature shows that there are several aspects that contribute to carcinogenesis which include environmental, genetic and lifestyle factors.

Chapter III

Aims and Objectives

3.1. Aims

To elucidate the genetic predisposition to oral squamous cell carcinoma with emphasis on tobacco mediated carcinogenesis

3.2. Objectives

1. To evaluate the association of Ile105Val, Ala114Val, and Arg187Trp polymorphisms in the *GSTP1* gene with predisposition to OSCC.

2. To evaluate the association of Asp398Asn polymorphism in the *CHRNA5* gene with predisposition to OSCC.

3. To evaluate the profile of *TP53* gene mutations in OSCC.

Chapter IV

Materials and Methods

4.1. Study design

The study was conducted by adopting a case-control study design. A total of 100 oral cancer patients and 200 healthy controls were enrolled in the study. The study design is given in Figure 4.1. Patients meeting the inclusion and exclusion criteria were recruited after obtaining informed consent. A structured questionnaire was used to collect information regarding demographic parameters (age, gender), habits (tobacco use, smoking, and alcohol) and family history from both patients and healthy controls. Clinical and histopathological details were collected from patients medical records.

4.2. Study population

Oral cancer patients were recruited from the Department of Otorhinolaryngology and Head and Neck Surgery, R.L. Jalappa Hospital and Research Centre, the teaching hospital of the Sri Devaraj Urs Medical College, Kolar, Karnataka, India. Age and gender-matched controls were recruited from the local population. The period of study was from 2014 to 2017.

4.3. Criteria for patient selection

4.3.1. Inclusion criteria - Patients diagnosed with squamous cell carcinoma of the oral cavity; patients of both gender and age > 18 years.

4.3.2. Exclusion criteria - Head and neck squamous cell carcinoma other than OSCC and T_1 OSCC.

4.3.3. Diagnostic criteria: Patients were diagnosed with OSCC on the basis of clinical and histopathological evaluation. The staging was done according to the 7th edition of American Joint Committee on Cancer TNM (T – Primary tumor staging, N – Nodal status, M – Metastasis) staging system for OSCC.

4.4. Ethical issues

The study was initiated after obtaining permission from the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, India. Informed consent was obtained from the study participants in writing before recruitment.

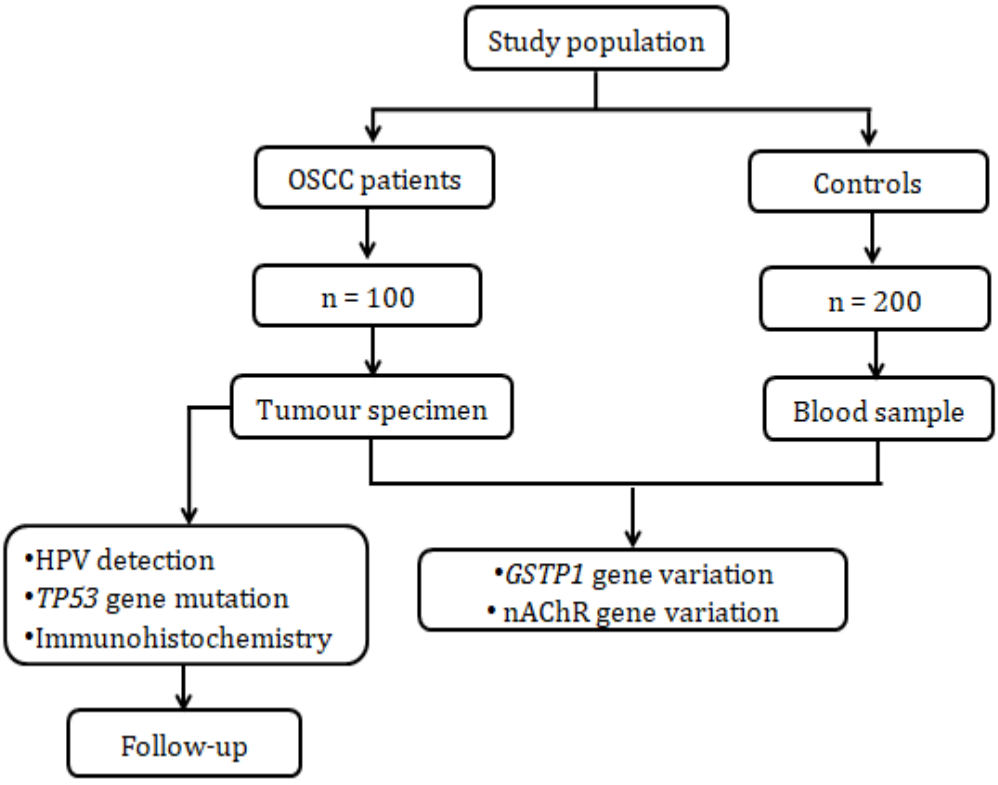


Figure 4.1: Schematic representation of the study design

4.5. Clinical specimen

A surgically resected tumor sample was collected from the oral cancer patient. The sample was divided into two parts. One portion was stored in -80 ºC while the other portion was immediately stored in formalin. Fresh frozen sample was used for the preparation of genomic DNA. The peripheral blood sample was collected from the control individuals.

4.6. Preparation of genomic DNA

4.6.1. Preparation of genomic DNA from tissue specimen: About 50 mg of frozen cancer tissue was homogenized with 1 ml ELB. The homogenate was transferred into a sterile 15 ml Falcon tube containing 3 ml of ELB. The mixture was vortexed for 10 s and to this 270 µl of SDS and 40 µl of proteinase K were added. The tubes were mixed gently and incubated overnight at 37 °C in a water bath. If the digestion was incomplete, an additional 40 µl of proteinase K was added and the incubation was continued overnight. This process was repeated for about 4 - 5 days to ensure complete digestion of the tissue. After the complete digestion, 500 µl of NaCl and an equal volume of isopropyl alcohol were added to precipitate the DNA. The DNA precipitate was transferred into a 1.5 ml microcentrifuge tube containing 500 µl of freshly prepared 80 % ethanol and repeated 3 times by centrifugation. DNA was then air dried and suspended in 500 µL of TE buffer. The suspension was incubated in a water bath at 65 °C for 30 min and then placed in a rotator until the DNA was completely dissolved. The final preparation was stored at -20 °C.

4.6.2. Preparation of genomic DNA from blood sample: Genomic DNA was isolated from the peripheral blood by using the salting-out method (Miller et al. 1988). 3 ml of peripheral blood was collected in sterile EDTA vacutainer from the controls and stored at 4 °C until processing. The vacutainer was vortexed and the blood was transferred into a sterile 15 ml Falcon tube. ELB was added to the falcon tube in 1:4 ratio and mixed by vortexing. The tube was maintained on ice for 30 - 45 min to facilitate hemolysis. The tube was then centrifuged at 3000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in 2.5 ml of ELB, vortexed and the final volume was made to 10 ml with a further addition of ELB. The sample was again centrifuged at 3000 rpm for 10 min and the supernatant was discarded. ELB washing was repeated 2 more times. The supernatant was discarded and the pellet was suspended in 1.8 ml of ELB. The suspension was vortexed and the volume was made to 5 mL with ELB. To this, 270 µL of SDS and 30 µL of proteinase K were added. The tube was mixed gently and incubated overnight at 37 °C in a water bath. Next day, 500 µl of NaCl and an equal volume of isopropyl alcohol were added to the lysate. The tubes were mixed gently with a slow and swirling motion. At this stage, genomic DNA begins to appear in the forms of mucoid threads. The DNA was transferred into a 1.5 ml microcentrifuge tube containing 500 µl of freshly prepared 80 % ethanol and incubated for 15 min at room temperature. The tube was centrifuged for 5 min at 12,000 rpm. The supernatant was discarded and the alcohol wash was repeated 3 more times. The DNA precipitate was then air dried and suspended in 500 µl of TE buffer. The suspension was incubated at 65 °C in a water bath for 30 min. The tube was then kept in a rotator until the DNA was completely dissolved (~ 2 days). The final preparation was stored at -20°C.

4.6.3. DNA estimation and purity analysis: Concentration and purity of the DNA preparation were estimated by UV spectrophotometry (Perkin Elmer model Lambda 35, Waltham, MA, USA). The amount of DNA was quantified using the formula: dsDNA concentration = 50 µg/mL × OD_{260} × dilution factor. The ratio of absorbance (at 260 nm and 280 nm) between 1.7 – 2.0 was considered pure.

4.7. Polymerase Chain Reaction (PCR)

All PCR reactions were carried out on a gradient thermal cycler (Bio-Rad, California, USA). The PCR reaction was performed in a final volume of 25 µl comprising of 1 pM of each primer, 1mM dNTPs, 1.5 mM $MgCl_2$, 100-300 ng of genomic DNA and 1 unit of *Taq* DNA polymerase (Bangalore Genei, India). Negative controls were prepared by excluding the template DNA. The PCR products were analyzed on 1 % agarose gel electrophoresis and visualized under UV illumination.

4.8. Agarose gel electrophoresis

Agarose gel of appropriate concentration (as required by individual experiment) was prepared in TAE buffer. Ethidium bromide was used to visualize the DNA under UV illumination.

4.9. RFLP analysis

10 µl of the PCR amplicon was incubated with 10 U of the respective restriction enzyme (New England Biolabs, USA) for 16 h at the optimal temperature specified by the manufacturer (Table 4.1). The reaction mix was then analyzed on 2 % agarose gel. DNA sample showing complete digestion (homozygous genotype for

the allele cut by the restriction enzyme) was used as the positive control. 10 % of the samples were randomly chosen and the RFLP analysis was repeated to confirm the accuracy of genotyping.

Table 4.1: Standard condition for the RFLP analysis

Component	Volume
PCR product	10 µl
10 X Reaction Buffer	2 µl
Restriction enzyme (10 U/ µl)	1 µl
MilliQ water	7 µl
Total	20 µl

4.10. HPV PCR

Genomic DNA prepared from the tumor specimen was amplified with GP5+/GP6+ primers [Pezeshkpoor et al. 2012]. The primer set is specific to the L1 consensus region of the viral DNA. The use of primers for the consensus region ensured that all the strains of HPV were tested irrespective of whether they were high risk or low risk. The primer sequences are given in Table 4.2. The PCR reaction was carried out under standard conditions as described in section 4.3. Reaction mix without template DNA was included as the negative control. A reaction containing 1:1 mix of genomic DNA of HPV 18 positive cervical carcinoma and SiHa cell line was used as positive control. SiHa cell line was obtained from National Centre for Cell Science, Pune, India. PCR products were analyzed on 2 % agarose gel (Figure 4.2).

Table 4.2: Primer sequences used for HPV PCR

Gene	Primer sequence (5' - 3')	PCR (bp)
GP5+/6+	**F:** TTT GTT ACT GTG GTA GAT ACT AC **R:** GAA AAA TAA ACT GTA AAT CAT ATT C	149
HCP5	**F:** TCA TTG TGT GAC AGC AGC C **R:** TCC CAT TCC TTC AAC TCA CC	268

Table 4.3: PCR program used for HPV PCR

Step	Temperature	Duration
Initial denaturation	95 ºC	5 min
Cycle denaturation	94 ºC	1 min
Annealing	40 ºC	2 min
Extension	72 ºC	1.5 min
Cycle repetitions	-	35 times
Final extension	72 ºC	5 min

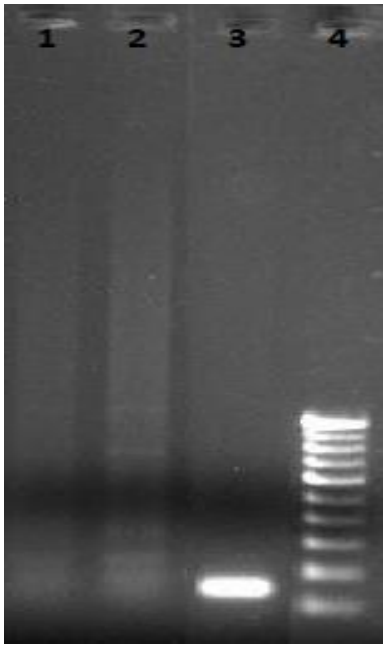


Figure 4.2: Representative agarose gel image showing band pattern in HPV PCR. Lane 1: negative control, Lane 2: OSCC sample, Lane 3: HPV positive control Lane 4: 100 bp ladder.

4.11. Statistical analysis

Statistical analysis was carried using the web based programme available at www.OpenEpi.com (updated 2013/04/06) and SPSS software version 20. P-value ≤0.05 was considered as statistically significant.

Chapter V

Characteristics of the study groups

5.1. Demographic parameters of the study participants

The case group comprised of 100 OSCC patients and the control group comprised of 200 age and gender-matched healthy individuals. The baseline demographic parameters of the study participants are summarised in Table 5.1. Age of the patients ranged from 21 to 80 years with a mean of 53.8 ± 10.7 years. Majority of the patients were females (79 %).

Table 5.1: Demographic and risk profile of the study participants

Parameters	Patient (n = 100)	Control (n = 200)
Male	21	44
Female	79	156
Age (mean ± SD; y)	54 ± 12	55 ± 13

5.2. Clinicopathological parameters of the OSCC patients

The clinicopathological parameters are summarised in (Table 5.2). Buccal mucosa was the most common site of tumor (65 %) (Figure 5.1); the other sites of the lesion were lower alveolus, anterior 2/3rd of the tongue, retromolar trigone (RMT) and floor of the mouth. 11 patients in the study had locally advanced disease involving buccal mucosa and lower alveolus and it was difficult to identify the epicenter of the disease. These tumors are described as lower gingivobuccal sulcus (GBS) cancers which are unique to the Indian subcontinent. Staging of the tumor indicated that the majority of the patients had stage IVa tumor (59 %) followed by stage III (21 %) and stage II (20 %). Squamous cell carcinoma was found to be well

differentiated in 71 %, moderately differentiated in 27 % and poorly differentiated in 2 % of the patients.

Table 5.2: Clinicopathological parameters of the OSCC patients

Parameters	Patient (n = 100)
Site of primary tumour	
Buccal mucosa	65
Floor of mouth	1
Anterior 2/3rd of tongue	7
Lower alveolus	14
Lower GBS	11
RMT	2
Tumour Grade	
Grade 1	71
Grade 2	27
Grade 3	2
TNM Stage	
T_{1-2}	8
T_{3-4}	92
Stage	
II	20
III	21
IV	59

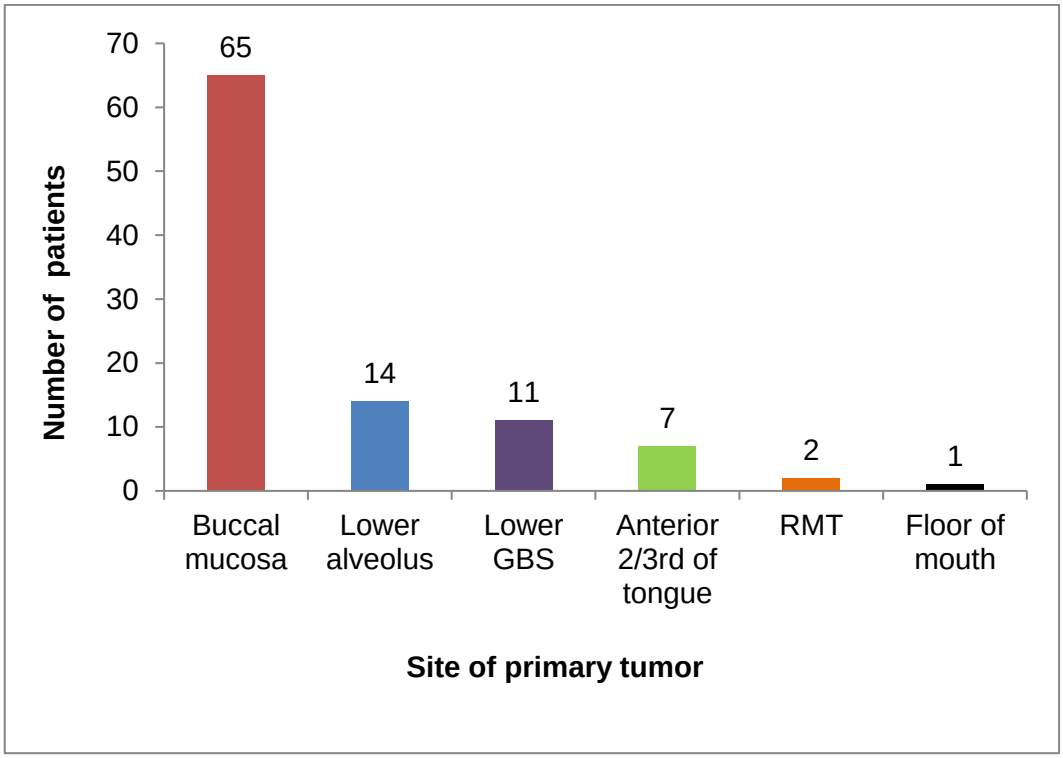


Figure 5.1: Site of primary tumour in the OSCC patients

5.3. Cancer-related risk habits in the patient group

All the patients included in the study were found to be habituated to one or more carcinogenic substances like betel-nut, chewable tobacco, *gutkha*, smoking or alcohol (Figure 5.2). Chewing betel nut and tobacco quid was the most common habit seen in all the patients. The median duration of tobacco use was 31 years. Only 7 % of the patients used tobacco in both smoke and chewable form. 6 % of the patients were habituated to tobacco chewing along with *gutkha* and alcohol consumption.

5.4. HPV screening of the OSCC specimens

All the OSCC DNA samples were tested for HPV infection and none of the samples showed HPV positive reaction. 20 % of the samples were randomly chosen

and re-tested. Results of the second test were 100 % concordant with the first set. Therefore, tobacco use in chewable form was the major risk factor in the patient group (Figure 5.2).

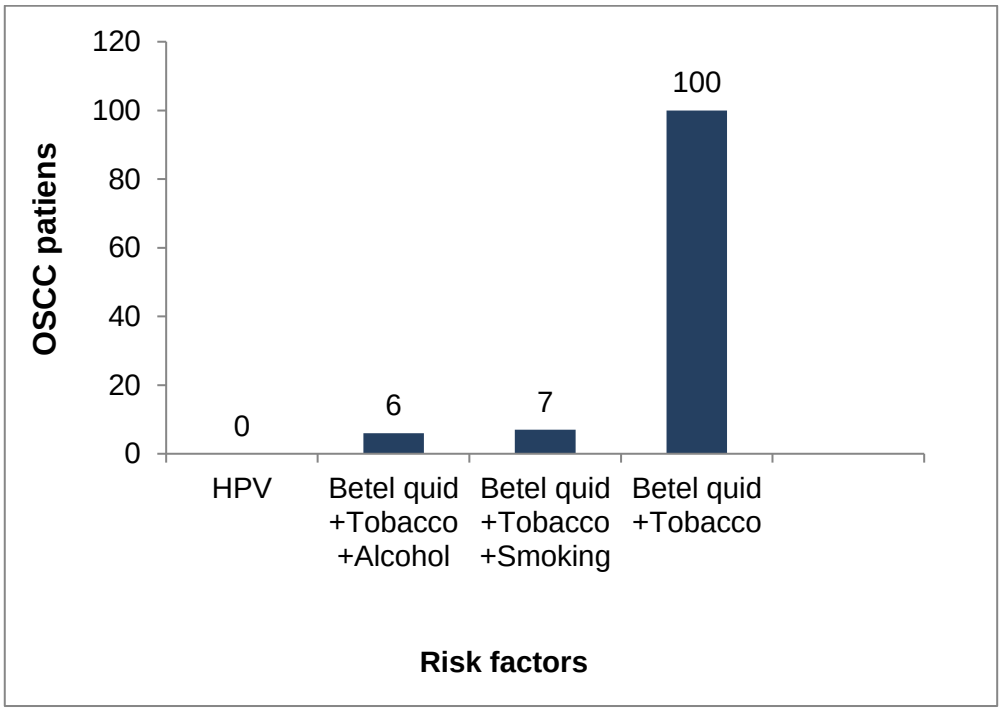


Figure 5.2: Risk factors in the study group

5.5. Recurrence-free survival of the OSCC patients

The patients were followed for a period of 3 years (Figure 5.3). 10 patients were lost to follow-up. Of the remaining 90 patients, 10 patients died (cause not known), 18 patients presented with recurrence and 62 patients showed recurrence-free survival. Thus, the frequency of recurrence was 18 % and recurrence-free survival was 60 %.

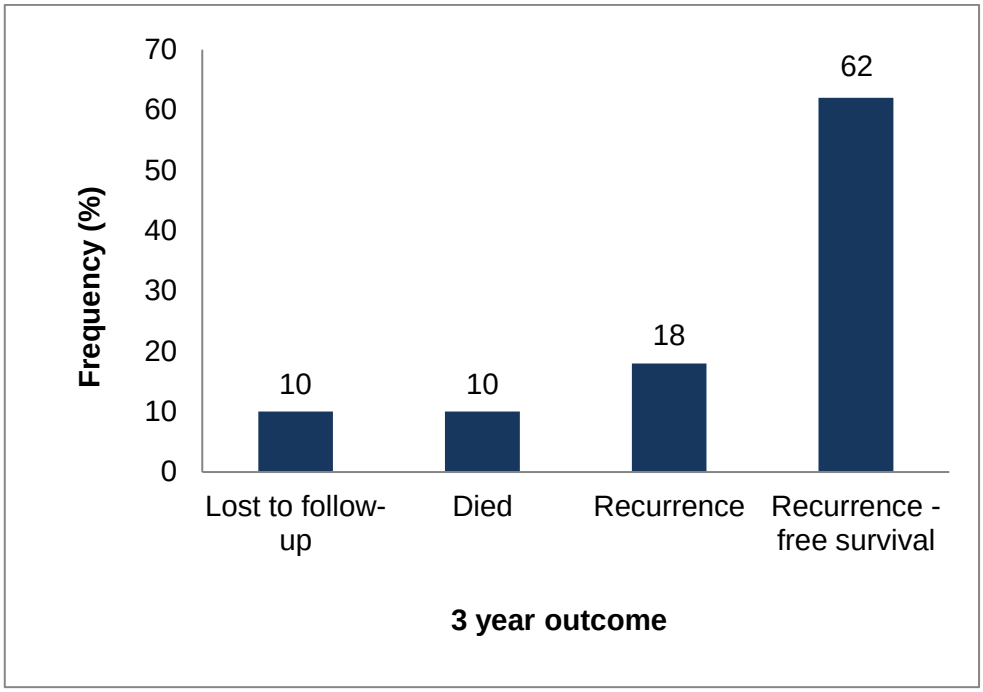


Figure 5.3: Recurrence-free survival of the OSCC patients

5.6. Discussion

The salient characteristics of the OSCC patients included in the study are: (i) majority of the patients were women, (ii) buccal mucosa was the commonly affected site, (iii) majority of the patients were of advanced stage, (iv) tobacco use in the chewable form was an invariant risk habit, and (v) frequency of HPV infection was 0 %.

Data available indicated that the frequency of women suffering from OSCC ranges from 20 – 40 % suggesting OSCC more common among men than in women [Singh et al. 2015; Nagpal et al. 2002; Elango et al. 2011; Tandon et al. 2017]. In contrast, a higher frequency of women were found affected with OSCC in this study. This observation corroborates with the previous observation from this laboratory [Kalyani et al. 2010]. OSCC can arise from buccal mucosa, anterior 2/3rd of a tongue,

lower alveolus, a floor of mouth, gingiva, and RMT. Of these, buccal mucosa is the commonly affected site followed by and lower alveolus [Montero et al. 2015]. Even in this study, a majority of the tumors were located in the buccal mucosa (Table 5.1). This may be due to the tendency of the patients to keep the tobacco in the form of quid in the buccal sulcus with close proximity to the alveolus. This leads to constant irritation of the buccal mucosa along with chemical and physical injury to the gingiva. The preponderance of OSCC of the advanced stage is the hallmark of this patient group. This may be due to the ignorance of patients about the symptoms and lack of concern for the disease.

Tobacco use in the chewable form was an invariant risk habit in the patient group. This is in agreement with the overall trend observed in several Indian studies [Kannan et al. 1999; Munirajan et al. 1996; Heinzel et al. 1996; Ralhan et al. 2001; Saranath et al. 1999]. Tobacco use was mainly in a chewable form in most of the previous Indian studies.

The HPV status of OSCC has been evaluated in several Indian studies (Table 5.3). The frequency ranges from 0 – 74 %. More than one-third of the tumors were infected in most of the studies. This is exceptionally high compared to the global prevalence of 3 % [Parkin et al. 2005; Parkin et al. 2006]. The frequency of 0 % observed in this study disagrees with the general trend in India but agrees with the study from Gujarat and worldwide profile [Patel et al. 2014; Chen et al. 2016; Akhter et al. 2013]. To the best of our knowledge, this is the first study to report a total absence of HPV in OSCC in the patient group from South India.

Table 5.3: List of Indian studies on the prevalence of HPV in oral cancer

Study center	Sample size (n)	HPV prevalence (%)	Reference
Trivandrum	91	74	Elango et al. 2011
Bangalore	60	48.3	Balaram et al. 1995
Bhubaneshwar	37	33.6	Nagpal et al. 2002
Chandigarh	111	32.4	Barwad et al. 2012
Navi Mumbai	102	31	Koppikar et al. 2005
Gujarat	97	0	Patel et al. 2014

5.7. Conclusion: Tobacco use in chewable form and not human papillomavirus infection was the major risk factor in the patient group.

Chapter VI

GSTP1 gene polymorphisms in OSCC

6.1. Introduction

Glutathione S-transferase pi (GST-pi) is the main enzyme involved in the detoxification of xenobiotics in the oral mucosa [Wang et al. 2010]. It mediates detoxification by conjugating xenobiotic substances with glutathione. Glutathione is a cysteinyl tripeptide that serves as major anti-oxidant in the mammalian cell (Figure 6.1). GST-pi is a phase II detoxification enzyme. It is responsible for the conjugation of glutathione to the hydrophobic and electrophilic compounds. Thus, GST-pi protects the cellular macromolecules from oxidative damage from electrophilic hetero atoms like -O, -N, and -S [Kanwal et al. 2014]. It also provides protection against tobacco constituents [Hayes et al. 2005]. Tobacco contains several xenobiotics and carcinogenic substances like nicotine, polycyclic aromatic hydrocarbons, nitrosamines, metals, aldehydes, several stable and unstable free radicals [Xue et al. 2014]. Several studies have shown that the down-regulation of GST-pi has been shown to increase DNA damage on exposure to carcinogens [Kanwal et al. 2014]. Therefore, factors that affect the expression and functional integrity of GST-pi can reduce the detoxification capacity of the oral mucosa and increase the risk of carcinogenesis [Wang et al. 2010].

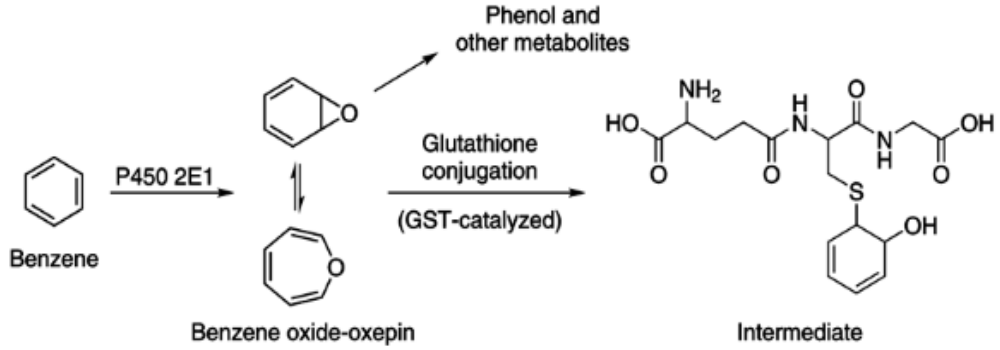


Figure 6.1: Illustration of GST catalyzed conjugation of benzene (a tobacco carcinogen)

GST-pi enzyme is coded by the *GSTP1* gene. The gene is located on chromosome 11q13, spans 2.8 kb, contains 7 exons, and codes for a protein with 209 amino acids (Figure 6.2) [Song et al. 2002]. Several SNPs have been described in the *GSTP1* gene [Moyer et al. 2008]. Some of the functional SNPs are known to be associated with the predisposition to cancers like carcinomas of head and neck, thyroid, breast, lung, stomach, liver, and prostate [Ruwali et al. 2011; Li et al. 2010; Marciniak et al. 2006; Lee et al. 2008; Yan et al. 2016; Miller et al. 2003; Zhou et al. 2009; Chen et al. 2010; Rybicki et al. 2006]. Furthermore, SNPs in the *GSTP1* gene have also been associated with an increased risk of cancer in tobacco users [Uddin et al. 2014; Fontana et al. 2009; Rybicki et al. 2006]. Cell culture studies have shown that SNPs in the *GSTP1* gene affect the efficiency of detoxification of electrophilic compounds [Kanwal et al. 2012].

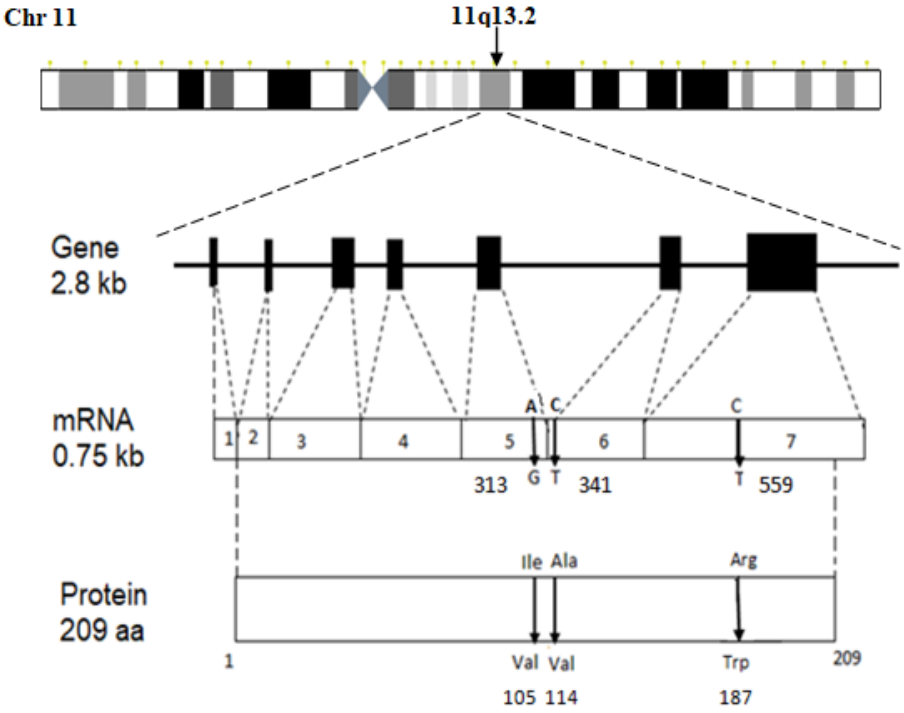


Figure 6.2: Organisation of the *GSTP1* gene. Loci of the 3 SNPs selected for this study are marked.

Biochemical studies have been carried out to determine the functional impact of SNPs in the *GSTP1* gene [Moyer et al. 2008]. This has resulted in the identification of 3 SNPs in the *GSTP1* gene that contributes to the significant loss of enzymatic activity (Figure 6.3). The three debilitating SNPs in the order of occurrence in the GST-pi polypeptide chain are Ile105Val (c.313A>G; rs1695), Ala114Val (c.341 C>T; rs1138272), and Arg187Trp (c.559 C>T; rs45549733). The residual enzyme activity of these SNPs is 21.8 % (Ile105Val), 79.9 % (Ala114Val), and 55.2 % (Arg187Trp).

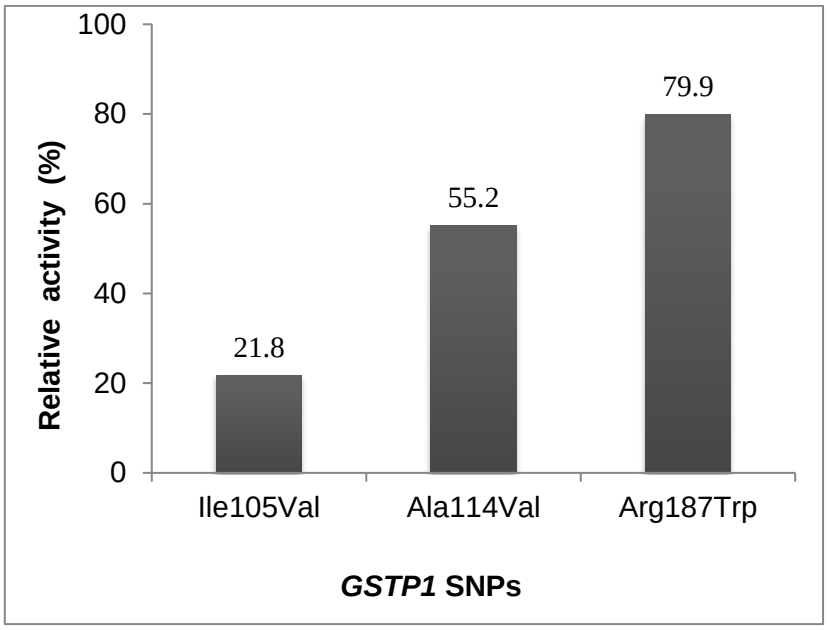


Figure 6.3: Comparison of the functional impact of the *GSTP1* SNPs on the enzyme activity in relation to the major allele

6.2. Rationale: Ile105Val, Ala114Val and Arg187Trp SNPs in the *GSTP1* gene reduce the specific activity of GST-pi enzyme. GST-pi is the major xenobiotic detoxifying enzyme in the oral epithelium. Therefore, these functional SNPs in the *GSTP1* gene reduces the detoxification capacity of the oral epithelium. Reduced detoxification results in the elevated levels of the carcinogenic xenobiotics in the oral

epithelium. Elevated levels of carcinogens contribute to an increased risk of carcinogenesis. The rationale of this chapter is schematically illustrated in Figure 6.4.

6.3. Hypothesis: The hypothesis of this study was that the minor allele frequencies of Ile105Val, Ala114Val, and Arg187Trp would be higher in the OSCC patients than in the control group.

6.4. Objective: The objective of this study was t*o* evaluate the association of Ile105Val, Ala114Val, and Arg187Trp SNPs in the *GSTP1* gene with the predisposition to OSCC.

6.5. Novelty: Several studies have shown that the functional SNPs in the *GSTP1* gene are linked with the predisposition to OSCC [Li et al. 2010; Karen-Ng. 2011; Cho et al. 2006]. These studies were focussed only on Ile105Val. Also, the putative association has failed to be reproduced when the combined data were subjected to meta-analysis [Lang et al. 2012]. Prior to this study, there was no information on the association of Ala114Val and Arg187Trp SNPs with OSCC. Therefore, this study was carried out as a comparative evaluation of all the 3 known functional SNPs in the *GSTP1* gene for association with the predisposition to OSCC.

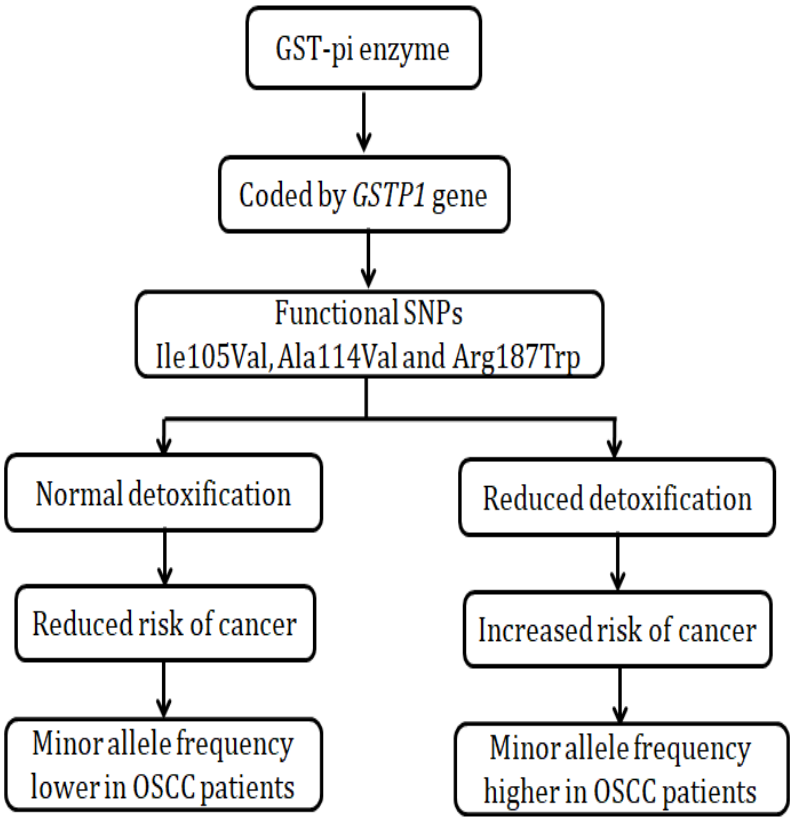


Figure 6.4: Schematic representation of the rationale for the evaluation of the association between *GSTP1* SNPs and OSCC

Table 6.1: Details of the *GSTP1* SNPs selected for the association study

Gene	Locus	SNP	Polymorphism	Amino acid substitution	Residual activity*
GSTP1	11q13	rs1695	c.313A>G	Ile105Val	21.8 %
		rs1138272	c.341 C>T	Ala114Val	55.2 %
		rs45549733	c.559 C>T	Arg187Trp	79.9 %

* With reference to the major allele

6.6. Materials and methods

6.6.1. Genotyping of *GSTP1* SNPs: *GSTP1* SNPs (Ile105Val, Ala114Val, and Arg187Trp) gene were genotyped by using PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) method.

6.6.2. PCR conditions: The PCR reaction was performed in a final volume of 25 µl containing 1 pM of each primer, 1 mM dNTPs, 1.5 mM $MgCl_2$, 100 - 300 ng of genomic DNA, and 1 unit of *Taq* DNA polymerase (Bangalore Genei, India). Negative controls were included in all reactions to prevent misjudging following contamination of the samples. PCR amplification program comprised of initial denaturation at 95 ºC for 3 min followed by 35 cycles at 95 ºC for 30 min, annealing temperature (Table 6.2) for 30 s and 72 ºC for 1 min; final extension involved 5 min at 72 ºC. PCR products were analyzed on 1 % agarose gel electrophoresis and visualized under UV illumination. An aliquot of the amplicon in each case was incubated with 10 units of the respective restriction enzyme for 16 h and the digestion pattern was analyzed on 3 % agarose gel. Details of primer sequences and PCR conditions are summarized in Table 6.2.

Table 6.2: PCR parameters used for genotyping of *GSTP1* SNPs

SNP	Primer sequence (5' - 3')	Annealing temperature (ºC)	Amplicon size (bp)
Ile105Val	**F:** ACG CAC ATC CTC TTC CCC TC **R:** TAC TTG GCT GGT TGA TGT CC	64	440
Ala114Val	**F:** CAA GGA TGG ACA GGC AGA ATG G **R:** ATG GCT CAC ACC TGT GTC CAT C	63	367
Arg187Trp	**F:** GTG TGA GCC ATT TGT TTA GC **R:** TGG AGA AAG GAA GGC AAA CTC	64	356

6.6.3. RFLP conditions: Restriction digestion of the amplified fragments was carried out for the above polymorphism in Touch Thermal Cycler (Chapter 4, section 4.9). Restriction enzymes sensitive to the sequence modification due to the target SNP were selected by using the web-tool *NEBcutter*, version 1.0 (Available at http://www.labtools.us/nebcutter-v2-0/). RFLP conditions used in the study are summarised in Table 6.3. An aliquot of the amplicon in each case was incubated at with 10 units of respective restriction enzyme (New England Biolabs, USA) for 16 h and the digestion pattern was analyzed on 2 % agarose gel (Figure 6.5). The genotyping results were confirmed by repeated analysis with 10 % of randomly chosen samples.

Table 6.3: RFLP parameters used for the genotyping of *GSTP1* SNPs

SNP	Restriction enzyme	Incubation temperature (ºC)	RFLP pattern (bp)		
			Maj/Maj	Maj/Min	Min/Min
Ile105Val	*BsmAI*	55	440	440 + 227 + 213	227 + 213
Ala114Val	*AciI*	37	172 + 195	367 + 172 + 195	367
Arg187Trp	*MspI*	37	232 + 124	356 + 232 + 124	356

bp: base pairs

Maj: Major allele

Min: Minor allele

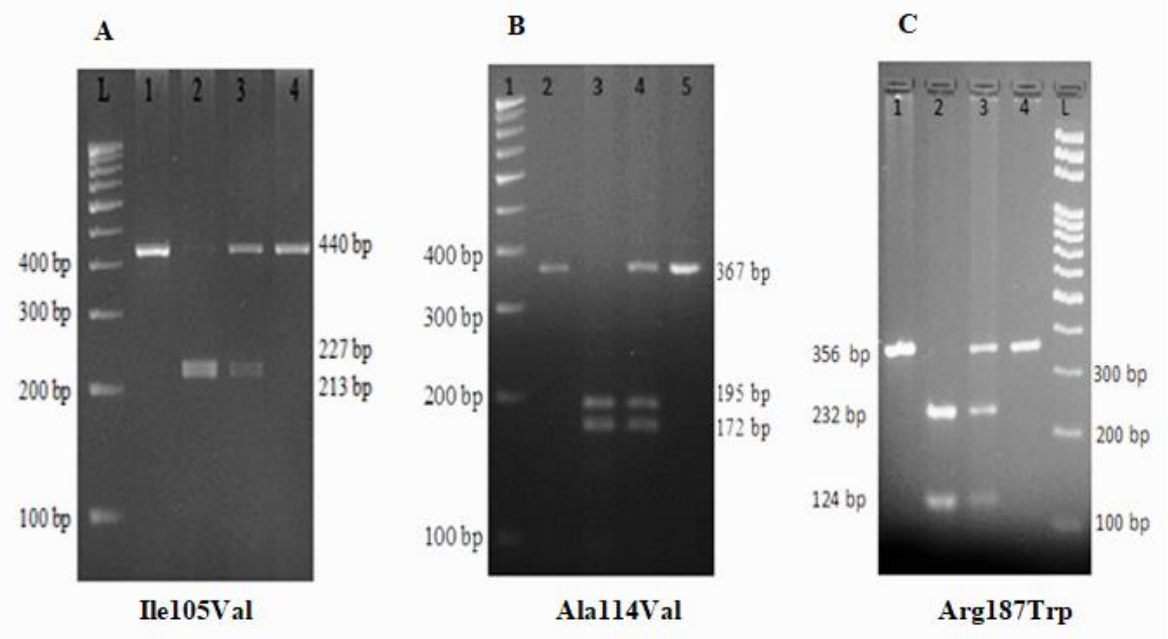


Figure 6.5: Representative image of agarose gel electrophoresis showing PCR-RFLP pattern for *GSTP1* SNPs.

(A) Lane L: 100 bp DNA ladder; Lane 1: PCR amplicon (440 bp); Lane 2:Ile/Ile genotype (227+213 bp); Lane 3:Ile/Val genotype (440+227+213 bp); Lane 4: Val/Val genotype (440 bp).

(B) Lane 1: 100 bp DNA ladder; Lane 2: PCR amplicon (367 bp); Lane 3: Ala/Ala genotype (195+172 bp); Lane 4: Ala/Val genotype (367+195+172 bp); Lane 5: Val/Val genotype (367 bp).

(C) Lane 1: PCR amplicon (356 bp); Lane 2: Arg/Arg genotype (232 +124 bp); Lane 3: Arg/Trp genotype (356+232+124 bp); Lane 4: Trp/Trp genotype (356 bp); Lane L: 100 bp DNA ladder.

6.6.4. Linkage Disequilibrium: The analysis was carried out by using the web-tool SHEsis (Available at https:/analysis.bio-x.cn/).

6.6.5. Immunohistochemistry (IHC): Resected tumor specimens were collected in formalin for IHC analysis. Formalin-fixed paraffin-embedded blocks (FFPE) were prepared from formalin-fixed tissues. 3 μm thick sections were mounted on poly-L-lysine coated slides and incubated at 60 °C overnight. Sections were deparaffinized in xylene and rehydrated in decreasing gradients of alcohol. For antigen retrieval, slides were boiled in a microwave oven with Tris-EDTA buffer (pH 9.0) for 20 min and further the sections were treated with 3 % H_2O_2 for 30 min to inhibit endogenous peroxidase activity. The tissue sections were washed with Tris-Buffered Saline for 15 min. The sections were further incubated with the primary anti-GSTP1 antibody (1:50 dilution; #SC-66000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h, washed with TBS buffer, followed by incubation with mouse/rabbit HRP secondary antibody (Abcam, Cambridge, UK) for 30 min. The immunostain was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin, dehydrated and mounted for analysis. Negative controls were included with the omission of the primary antibody with buffer. The slides were assessed using light microscopy. IHC slides were scored independently by two researchers who were blinded w.r.t., the *GSTP1* genotype of the tumor specimen.

6.6.6. Statistical analysis: The methods employed for statistical analysis is described in Chapter 4 section 4.11.

6.7. Results

A total of 100 OSCC tumor biopsies and peripheral blood of 200 age and gender-matched healthy controls were available for the analysis. The clinical,

pathological and demographic details of the study groups are summarized in Chapter 5, section 5.2.

6.7.1. The allele frequency of *GSTP1* gene SNPs in OSCC patients and healthy controls

The distribution of the alleles of the *GSTP1* SNPs in the patient and control groups are given in Table 6.4. The minor allele frequency (MAF) of Ile105Val, Ala114Val, and Arg187Trp among OSCC patients were 34 %, 15.5 %, and 11 %. These frequencies were 1.2, 4.1, and 6.3 times higher than that in the control group (Figure 6.6). The difference in the distribution of allelic frequencies was analyzed for statistical significance by means of a 2X2 contingency table. Significant differences were observed with Ala114Val and Arg187Trp but not with Ile105Val. *Post hoc* analysis was carried out to determine the power of data. The power, based on normal approximation with continuity correction, was 86.8 % and 98.8 % with respect to Ala114Val and Arg187Trp.

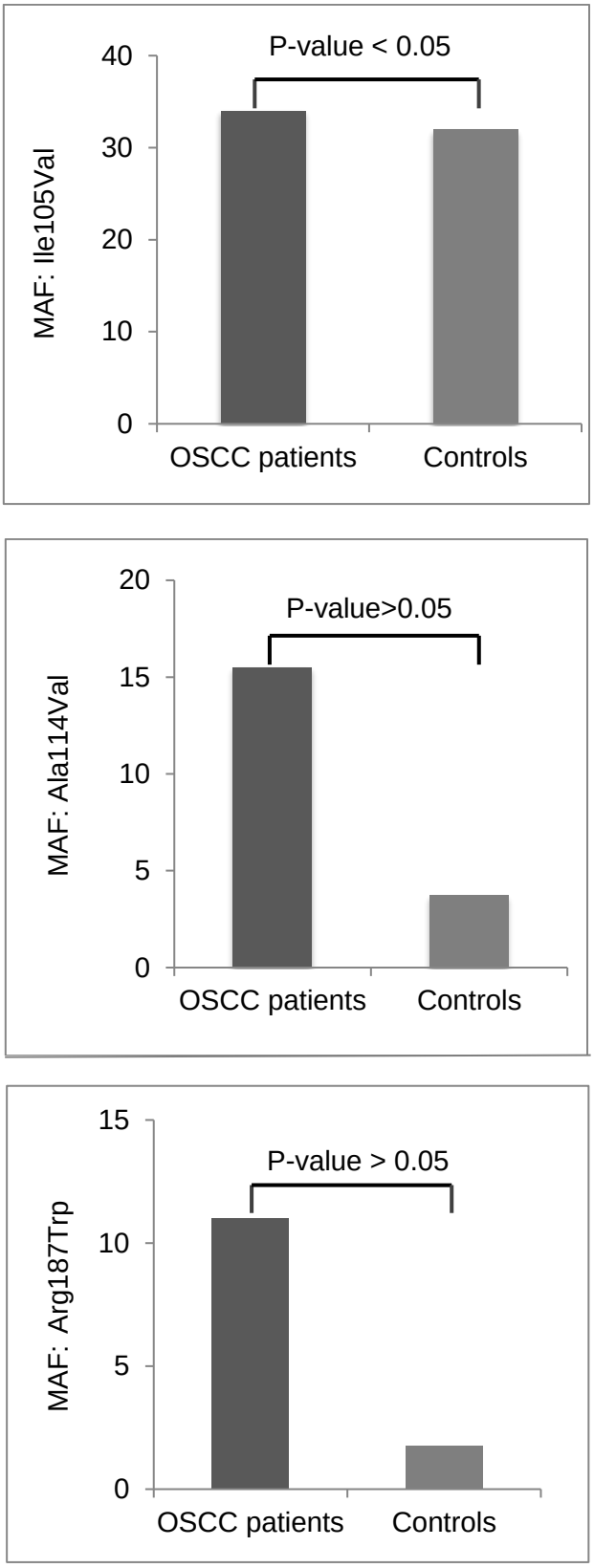


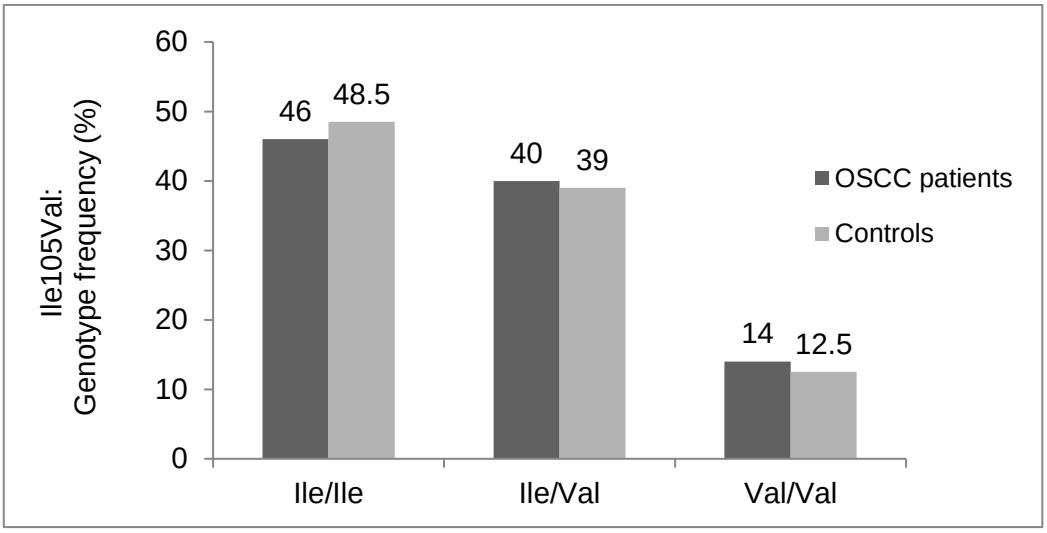
Figure 6.6: Distribution of the minor alleles of the *GSTP1* SNPs in study groups

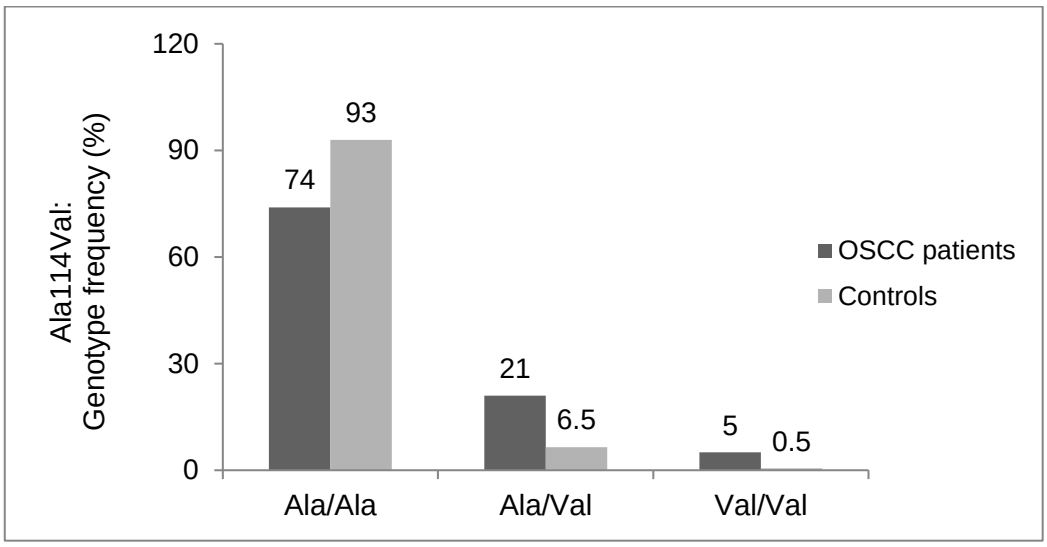
6.7.2. Genotype frequency of *GSTP1* SNPs in OSCC patients and healthy controls

Patient and control groups were also compared at the level of genotype distribution. The distribution of genotypes of the three SNPs is shown in Table 6.4. The distribution of the genotypes of all the three SNPs in the control group was in agreement with Hardy-Weinberg equilibrium ($\chi^2 \leq 3.84$).

The frequencies of all the 3 genotypes of Ile105Val in the patient and control groups were similar. Hence, a statistically significant difference was not observed between the two groups (P-value = 0.71). In the case of Ala114Val, the genotype frequencies of heterozygous genotype (Ala/Val) and homozygous minor allele genotype (Val/Val) in the patient group was 3.2 and 10 times higher than in the control group respectively. In the case of Arg187Trp, the frequency of heterozygous genotype (Arg/Trp) was 5.7 times higher in the patient group than in the control group. Homozygous minor allele genotype (Trp/Trp) was observed in the patient group (1 %) but not in the control group (Figure 6.7).

The difference in the distribution of the genotypes between the patient and control groups was compared by means of a 2X3 contingency table. Again, a statistically significant difference was observed only with Ala114Val and Arg187Trp and not with Ile105Val.





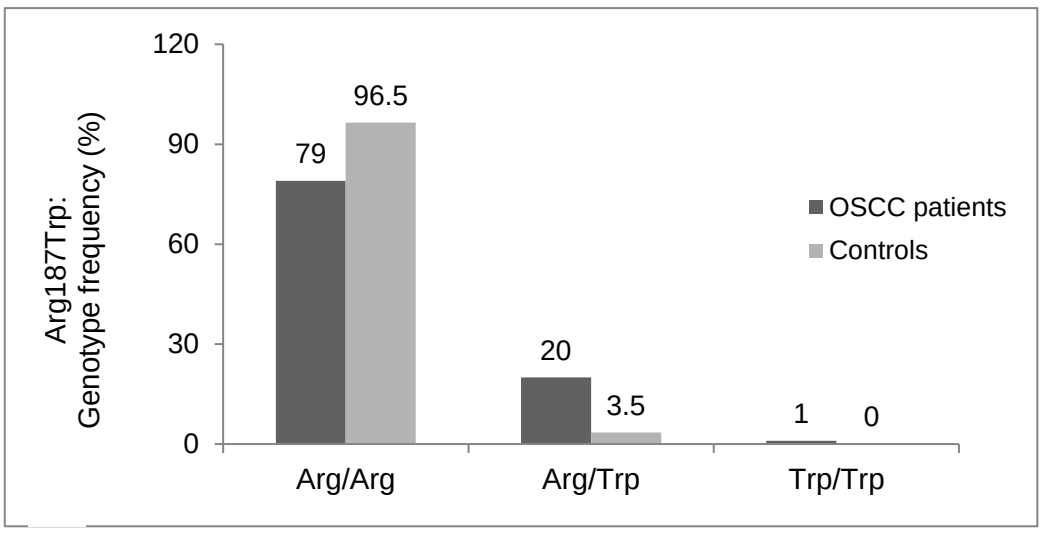


Figure 6.7: Distribution of the genotypes of the *GSTP1* SNP in the study groups

Table 6.4: Profile of allele and genotype frequencies of *GSTP1* SNPs in the study groups

***GSTP1* SNP**	**Allele/ Genotype**	**Patient (n = 100)**	**Control (n =200)**	**P-value†**	**HWE (χ^2)**
Ile105Val	Ile	132	272	0.64 OR: 1.09 (0.95 CI: 0.76 - 1.56)	2.16
	Val	68	128		
	Ile/Ile	46	97	0.71	
	Ile/Val	40	78		
	Val/Val	14	25		
Ala114Val	Ala	169	385	9.16×10^{-7} OR: 4.7 (0.95 CI: 2.47 – 8.95)	1.98
	Val	31	15		
	Ala/Ala	74	186	1×10^{-5}*	
	Ala/Val	21	13		
	Val/Val	5	1		
Arg187Trp	Arg	178	393	2×10^{-6} * OR: 6.9 (0.95 CI: 2.9 – 16.5)	0.06
	Trp	22	7		
	Arg/Arg	79	193	2×10^{-6} *	
	Arg/Trp	20	7		
	Trp/Trp	1	0		

* Significant ($P \leq 0.05$)

†Chi-square test - Fisher's exact

HWE: Hardy-Weinberg Equilibrium

6.7.3. Association between *GSTP1* SNPs and OSCC in different genetic models

Alleles occur in pairs as genotypes. Comparison of allele frequencies may not represent true biological conditions. Therefore, genotype data was examined in various genetic models to confirm the presence and the magnitude of the association. The results are summarised in Table 6.5. The evaluation was carried out by using a 2X2 contingency table. It was observed that Ile105Val was not associated with OSCC under any of the genetic models tested ($P > 0.5$). Both Ala114Val and Arg187Trp showed a statistically significant difference between patient and control groups in all the four genetic models tested ($P \leq 0.5$). In both the cases, the highest degree of association was observed with dominant and additive genetic models.

Table 6.5: Evaluation of association between *GSTP1* SNPs and OSCC risk under different genetic models.

SNP	Genetic model	Genotype	P-value†
Ile105Val	Dominant$	(Ile/Val + Val/Val) *vs*. (Ile/Ile)	0.71
	Recessive$	(Val/Val) *vs*. (Ile/Val + Ile/Ile)	0.85
	Additive†	(Val/Val) > (Ile/Val) > (Ile/Ile)	0.89
	Over-dominant$	(Ile/Val) *vs.*(Ile/Ile + Val/Val)	1.0
Ala114Val	Dominant$	(Ala/Val + Val/Val) *vs*. (Ala/Ala)	6 X 10^{-5}*
	Recessive$	(Val/Val) *vs.*(Ala/Val +Ala/Ala)	0.01*
	Additive†	(Val/Val) > (Ala/Val) > (Ala/Ala)	3 X 10^{-5}*
	Over-dominant$	(Ala/Val) *vs*. (Ala/Ala + Val/Val)	1 X 10^{-3}*
Arg187Trp	Dominant$	(Arg/Trp + Trp/Trp) *vs*. (Arg/Arg)	2 X 10^{-6}*
	Recessive$	(Trp/Trp) *vs.*(Arg/Trp + Arg/Arg)	6 X 10^{-1}*
	Additive†	(Trp/Trp) > (Arg/Trp)> (Arg/Arg)	2 X 10^{-6}*
	Over-dominant$	(Arg/Trp) *vs.*(Arg/Arg + Trp/Trp)	7 X 10^{-6}*

* Significant ($P \leq 0.05$)

$Chi-squared test -Fisher's exact

† Chi-square test for linear trend

6.7.4. Linkage disequilibrium (LD) between *GSTP1* SNPs

We found that 2 SNPs in the *GSTP1* gene (Ala114Val and Arg187Trp) were associated with the predisposition to OSCC suggesting that these two alleles could have been associated with the same condition due to a linkage. This is a genetic phenomenon involving co-inheritance of alleles due to their non-segregation during meiosis. This arises due to the physical proximity of alleles on the same chromosome. This results in reduced recombination between the alleles and their non-segregation. A linkage between the alleles is evaluated by LD analysis. This results in two indices namely D' and r^2. D' represents a deviation of the observed frequency of a haplotype from the expected frequency, while r^2 represents a correlation between a pair of loci. The results of the LD analysis of the genotype data are summarised in Table 6.6. Both D' and r^2 for all the 3 combinations were <50 %. This indicates that there was no strong linkage between the 3 alleles of the *GSTP1* gene. (Figure 6.8).

Table 6.6: Linkage disequilibrium analysis of *GSTP1* SNPs

SNP Pair	D'	r^2
Ile105Val + Ala114Val	0.523	0.04
Ala114Val + Arg187Trp	0.41	0.105
Ile105Va + Arg187Trp	0.280	0.007

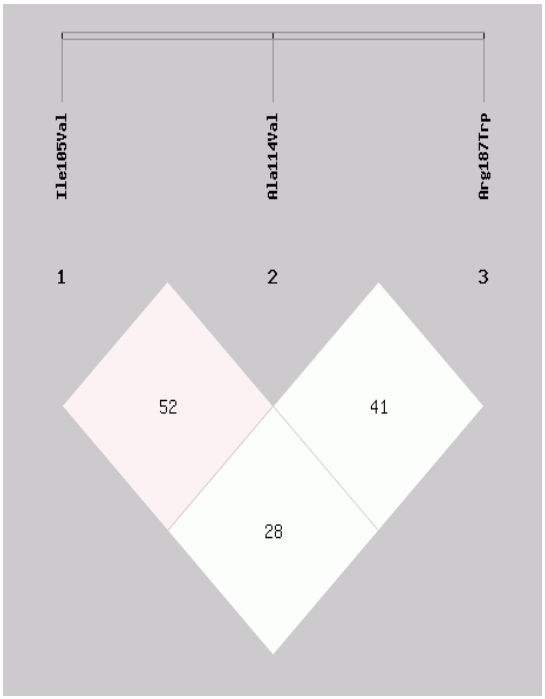


Figure 6.8: Linkage disequilibrium analysis of *GSTP1* SNPs. The color scale ranges from red to white. [color intensity decreases with decreasing D' value: white (D=0), pink (0<D<1) and red (D=1)]

6.7.5. Univariate-multivariate analysis of the association between *GSTP1* SNPs and OSCC

The association of *GSTP1* SNPs with OSCC in conjunction with the confounding factors like demography, clinicopathological parameters, and habit. Demographic factors included age and gender. Clinicopathological factors included TNM stage; and tumor grade. Habit included duration of tobacco usage. Univariate and multivariate analysis were carried out by means of logistic regression. The results are summarised in Table 6.7. Of the 3 SNPs evaluated, only Arg187Trp was significantly associated with the duration of tobacco usage in both univariate and multivariate analysis.

6.7.6. Tumor recurrence and its associated factors

The OSCC patients were followed for a period of 3 years. 10 patients were lost for follow-up study. Of the remaining 90 patients, 10 patients died (cause not known), 18 patients presented with recurrence and 62 patients showed disease-free survival. Thus the frequency of recurrence was 20 %.

Univariate and multivariate analysis were carried out to evaluate the association of tumor recurrence with *GSTP1* SNPs, demographic factors, clinicopathological factors, and habit. Demographic factors included age and gender. Clinicopathological factors included TNM stage; and tumor grade. Habit included a duration of tobacco use. Univariate and multivariate analysis were carried out by means of logistic regression. The results are summarised in Table 6.8.

The data suggested that the tumor recurrence was not associated with *GSTP1* SNPs, demographic factors, or clinicopathological factors. However, a statistically significant association was found with the duration of tobacco use.

Table 6.7: Univariate and multivariate analysis of the association between OSCC and *GSTP1* SNPs

Parameter	Univariate[†](P-value)	Multivariate[#](P-value)
Ile105Val		
Age[$] ≥50 years <50 years	0.95	0.96
Gender Male Female	0.75	0.76
Tumor grade G1 G2+G3	0.06	0.07
Duration of tobacco use[$] ≥31 years <31 years	0.89	0.91
Ala114Val		
Age[$] ≥50 years <50 years	0.33	0.29
Gender Male Female	0.72	0.68
Tumor grade G1 G2+G3	0.20	0.14
Duration of tobacco use[$] ≥31 years <31 years	0.82	0.74
Arg187Trp		
Age[$] ≥50 years <50 years	0.33	0.62
Gender Male Female	0.22	0.25
Tumor grade G1 G2+G3	0.58	0.59
Duration of tobacco use[$] ≥31 years <31 years	0.01* OR: 3.87 (0.95 CI: 1.35 - 11.05)	0.04* OR: 3.62 (0.95 CI 1.08- 12.16)

* Significant ($P \leq 0.05$)

[†]Chi-squared test – Fisher's exact

[#]Logistic regression analysis

[$] Dichotomised by median

Table 6.8: Univariate and multivariate analysis of tumor recurrence with different risk factors

Risk factor	Univariate P-value[†]	Multivariate P-value[#]
Gender Male Female	0.2	0.1
Age[@] ≥50 years <50 years	0.70	0.61
TNM Stage T_{1-2} T_{3-4}	0.93	0.69
Tumor grade G1 G2+G3	0.53	0.56
Ile105Val[$]	0.41	0.40
Ala114Val[$]	0.71	0.69
Arg187Trp[$]	0.26	0.26
Duration of tobacco use[@] ≥31 years <31 years	0.05* OR: 2.92 (0.95 CI:0.85 - 9.74)	0.03* OR: 5.06 (0.95 CI: 1.13 - 22.61)

*Significant ($P \leq 0.05$)

[†] Chi-square test (Fisher's exact)

[#] Logistic regression

[$] P value calculated using a dominant genetic model

[@]Dichotomised by median

6.7.7. Correlation between *GSTP1* SNPs and expression of GST-pi

Expression of GST-pi protein in the tumor specimen was evaluated by IHC. Representative photomicrographs of IHC analysis are shown in Figure 6.9. Immunoreactivity for GST-pi protein was noticed in the tumor cells and not in the surrounding normal squamous epithelium. Immunoreactivity of the tumor samples was assessed with respect to the intensity of staining and the area of tumor tissue being stained.

A random selection of 5 tumor specimens was done from each genotype of Ala114Val and Arg187Trp. Ile105Val was not considered since it was not associated with OSCC. The relationship between GST-pi protein expression score and the genotypes of Ala114Val and Arg187Trp was evaluated by using a 2X2 contingency table. The results are summarised in Table 6.9. No statistically significant correlation was observed between these two SNPs and GST-pi protein expression.

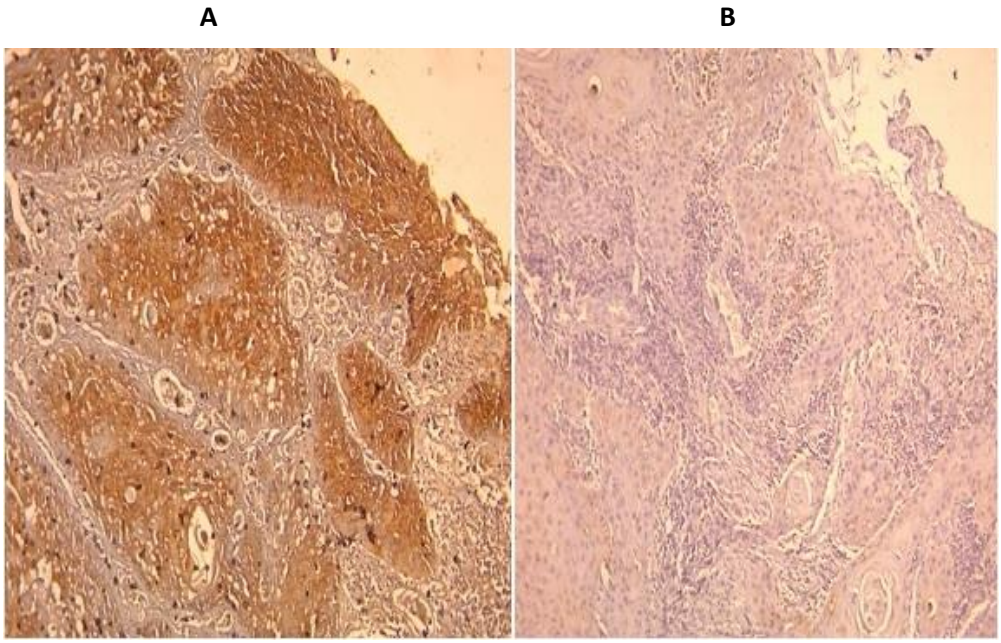


Figure 6.9: Photomicrograph of OSCC sections showing immunohistochemical staining of GST-pi. Panels 'A' shows high expression and 'B' shows low expression (10x magnification).

Table 6.9: Association between *GSTP1* SNPs and GST-pi expression in the tumor specimen

SNP	Genotype	GST-pi expression		P-value[#]
		Low	High	
Ala114Val	Ala/Ala	3	2	0.82*
	Ala/Val	2	3	
	Val/Val	2	2	
Arg187Trp	Arg/Arg	3	4	0.54*
	Arg/Trp	2	1	
	Trp/Trp	1	3	

[#]Chi-square test

* Not significant (P ≥ 0.05)

6.8. Discussion

The aim of this chapter was to evaluate the association of functional SNPs in the *GSTP1* gene with the predisposition to OSCC. So far, three functional SNPs have been documented in the *GSTP1* gene. These SNPs are Ile105Val, Ala114Val, and Arg187Trp. The association of Ile105Val, Ala114Val, and Arg187Trp with OSCC in a cohort of Indian patients was evaluated. The findings of the study are: (i) both Arg187Trp and Ala114Val were associated with the predisposition to OSCC but not Ile105Val, (ii) Arg187Trp, Ala114Val and Ile105Val were not in linkage disequilibrium (iii) duration of tobacco use and not *GSTP1* SNPs was associated with tumor recurrence, and (iv) GST-pi expression was not associated with *GSTP1* SNPs. Together, these results provide the evidence to implicate Ala114Val and Arg187Trp as independent risk loci in the predisposition to OSCC.

Prior to this, several studies have been reported on the association of SNP in the *GSTP1* gene and predisposition to OSCC. All these studies were carried out only with Ile105Val and not with Ala114Val and Arg184Trp. To the best of our knowledge, this is the first study to elucidate the association of Ala114Val and Arg184Trp with the predisposition to OSCC.

The various studies on the association of Ile105Val with the predisposition to OSCC have been subjected to meta-analysis [Li et al. 2010; Karen-Ng, 2011; Cho et al. 2006]. Though a positive association was observed in several reports, the same was not evident at the level of meta-analysis [Lang et al. 2012]. The lack of association at the level of meta-analysis carries two implications; variation in the

GSTP1 gene may not be associated with the predisposition to OSCC or variations other than Ile105Val might be involved.

Selection of a limited panel of SNPs is the major draw of the candidate gene approach. In a complex disease like OSCC, each SNP makes an incremental contribution to the predisposition. Therefore, we reasoned that a panel of functional SNPs would be more appropriate for evaluating the association. There was no association between Ile105Val and OSCC even in this study. In contrast, the positive association was found with Ala114Val and Arg184Trp. It is interesting to note that functionally, Ile105Val is the most devastating variant of the three SNPs. Also, the MAF of Ile105Val in the control group, which represents the general population was the highest (32 %). By convention, a gene polymorphism is defined as common if its MAF is >5 % and are chosen for genetic association study [Wang et al. 2010; Panagiotou et al. 2010]. The MAFs of Ala114Val and Arg187Trp in the control group were 3.75 % and 1.75 % which are less than 5 % conventional cut-off. Allele frequency with MAF between 0.5 – 5 % is defined as intermediate. We were perplexed as to why an SNP and intermediate frequency should show association rather than the commoner minor allele. This prompted us to consider other risk factors in the patient group to see if the observed association was linked to gene-environment interaction.

It was shown in chapter 5 that tobacco use and not HPV infection is the major exogenous risk factor in the patient group selected for this study. The high frequency of tobacco use in the patient group may have contributed to the observation of the positive association. Therefore, we carried out univariate-multivariate analysis to

examine if tobacco use and other factors like demography and clinicopathological parameters affected the association.

Univariate analysis is the simplest form of data analysis carried out with just one variable. In contrast, the multivariate analysis examines three or more variables to see if one or more of them are predictive of a certain outcome. The multivariate analysis gives a vital tool to examine relationships between variables and to quantify the relationship between those variables. Therefore, multivariate analysis was used to evaluate the relationship between *GSTP1* SNPs and other variables.

Univariate-multivariate analysis showed that Arg187Trp was significantly higher among patients with longer duration of tobacco use. Thus, the positive association observed with Arg187Trp may be due to habituation to tobacco use. However, a similar trend was not observed in the case of Ala114Val. This prompts us to reason that the association of *GSTP1* SNPs with the predisposition to OSCC may be modified or influenced by tobacco use. We reviewed the literature to see if the gene-environment interaction was observed with the *GSTP1* SNPs before. We found that the association of the *GSTP1* SNPs with the predisposition to cancer is often influenced by environmental factors. For instance, the association of Ile105Val and gastric cancer risk is higher in patients with risk factors like *Helicobacter pylori* infection, smoking, or alcohol use [Zhang et al. 2012]; the association of Ile105Val with the predisposition to lung and bladder cancers is higher in smokers compared to non-smokers [Uddin et al. 2014; Fontana et al. 2009]; the association of Ile105Val with predisposition to prostate cancer was higher in patients exposed to carcinogens like polycyclic aromatic hydrocarbons [Rybicki et al. 2006].

Our observation of the interaction between *GSTP1* SNP and tobacco use assumes significance in the light of the magnitude of the habit in the Indian population. Tobacco chewing has been reported to be responsible for more than 66 % of the total oral cancer cases in India. National Family Health Survey and Global Youth Tobacco Survey conducted in 18 Indian states show that tobacco chewing is highly prevalent in the younger demographic group (average prevalence of tobacco chewing was 29 % compared to 13 % for smoking) [Singh et al. 2014]. While the role of tobacco smoking is well established with lung cancer, its role in oral cancer is not clear. Tobacco chewing increases the duration of carcinogenic exposure of buccal mucosa. As a consequence tobacco chewing is the main risk factor for oral malignancy in India [Singh et al. 2014; Singh et al. 2015]. The inter-individual differences in the detoxification capacity of carcinogenic substances may determine whether a tobacco user develops cancer or not. The results of this study show that the *GSTP1* gene plays a crucial role in determining such an inter-individual difference in predisposition.

SNPs in the *GSTP1* gene have been evaluated for association with various biological phenomena like the predisposition to carcinogenesis, therapeutic response to pharmacological drugs, autoimmunity, infertility, and radiotoxicity [McIlwain et al. 2006; Yun et al. 2005; Yarosh et al. 2015; Ma et al. 2017]. Most of these association studies were carried out with Ile105Val and to a lesser extent with Ala114Val. In contrast, there is a paucity of literature on Arg187Trp. We found only one reference for Arg187Trp in a patent application wherein the SNP was part of a panel designed for predicting the predisposition to skin damage and using that information for personalized skin care (US Patent number US20160068904A1). To

the best our knowledge, this is the first study establishing the importance of Arg187Trp in predisposition to any cancer.

Two or more gene polymorphisms may be associated with a disease if they are located in the proximity of each. The closeness in their physical location ensures that the two variants fail to separate during meiotic recombination. This phenomenon of coinheritance of two or more alleles is called as linkage disequilibrium (LD) [Yang et al. 2014]. We were interested to see if the observed association of Ala114Val and Arg187Trp with OSCC was an independent phenomenon or a consequence of linkage. LD analysis was carried out and indicated that linkage between the Ala114Val and Arg187Trp was weak. This observation lead to the inference that the association of the two SNPs are independent phenomena.

GST-pi is linked with the carcinogen detoxification capacity and protection against carcinogenesis in the normal oral mucosa [Wang et al. 2010]. However, GST-pi has an opposite effect in the tumor tissue where it is often found to be overexpressed [Masood et al. 2011]. Increased expression of GST-pi contributes to tumor survival by neutralizing electrophilic chemotherapeutic agents like cisplatin and by minimizing the DNA damaging effects of radiation therapy [Allocati et al. 2018]. Examination of Ala114Val and Arg187Trp for association with the expression of GST-pi in the tumor specimen failed to find any evidence for the statistically significant association between immunohistochemical grade of GST-pi in tumor samples and Ala114Val and Arg187Trp. This indicates that the mechanism that results in GST-pi overexpression in the tumor tissue may be distinct and not linked to the genotype of *GSTP1* SNPs.

6.9. Conclusion

The results presented herein show that Ala114Val and Arg187Trp SNPs but not Ile105Val in the *GSTP1* gene influence the predisposition to OSCC. This association underlines the significance of GST-pi and detoxification pathway in the pathogenesis of OSCC.

Chapter VII

Nicotinic receptor gene variation in OSCC

7.1. Introduction

Nicotine is the main psychoactive substance present in tobacco [Crocq et al. 2003]. The chemical structure of nicotine closely resembles the structure of the acetylcholine neurotransmitter. As a result, nicotine is also capable of activating the nicotinic acetylcholine receptor (nAChR). These are ligand-gated ion channels found in the central and peripheral nervous system, muscle, and several other tissues. They are mainly responsible for the neurotransmission at the neuromuscular junction, autonomic ganglia and few at sites in the central nervous system [Albuquerque et al. 2009; Dani et al. 2015; Changeux et al. 2018]. The mode of action of nAChR is shown in Figure 7.1.

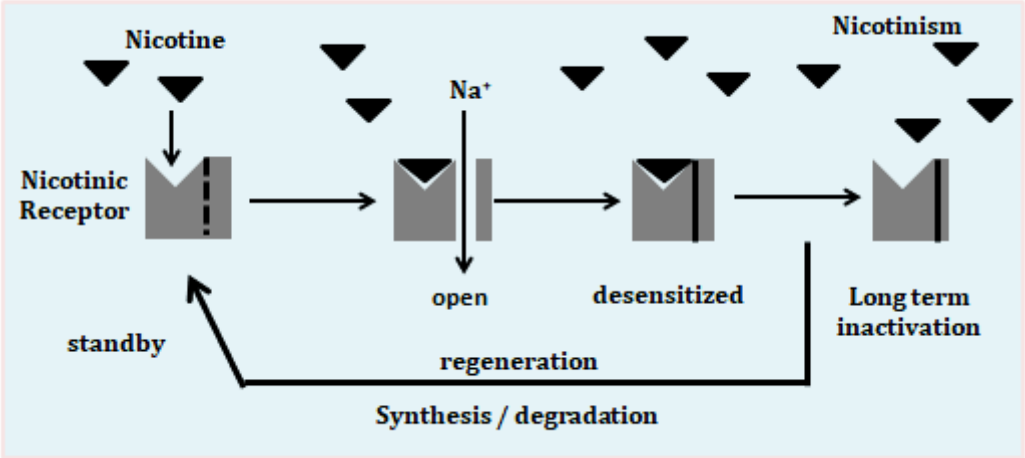


Figure 7.1: Schematic representation of the mechanism of action of nAChR

Human nAChR is composed of five protein subunits arranged around a central pore. There are 16 different types of proteins that can form the subunits and are coded by their respective Cholinergic Receptor Nicotinic (*CHRN*) gene (Table 7.1) [Albuquerque et al. 2009; Hendrickson et al. 2013; Dani et al. 2015].

Table 7.1: Classification of nAChR subunit proteins

Nicotinic receptor subunit protein	Gene
α 1 -10*	*CHRNA1–CHRNA10*
β 1 - 4	*CHRNB1–CHRNB4*
γ	*CHRNG*
δ	*CHRNAD*
ε	*CHRNE*

* α8 protein is not expressed in human. It is present only in an avian system

Nicotine contributes to carcinogenesis through direct and indirect means [Egleton et al. 2008]. By indirect means, nicotine, due to its psychoactive nature, induces addiction to tobacco use. The substance abuse then ensures chronic exposure to the tobacco carcinogens [Brunzell et al. 2015]. By direct means, nicotine contributes to carcinogenesis by activating tumorigenic pathways in the target cell. Activation of nAChR with nicotine has been shown to alter the intracellular calcium and the resultant activation of the downstream signaling cascades linked to cell proliferation, invasion, and angiogenesis (Figure 7.2) [Zhao et al. 2016; Hecht et al. 2003]. Nicotine could lead to mutagenesis indirectly due to the DNA damaging effects of tobacco-specific carcinogens like nitrosamines. In contrast, nicotine induces carcinogenesis in the direct mode without the involvement of mutagenesis. Therefore, direct means of nicotine-induced carcinogenesis may involve lower mutation load [Xue et al. 2014].

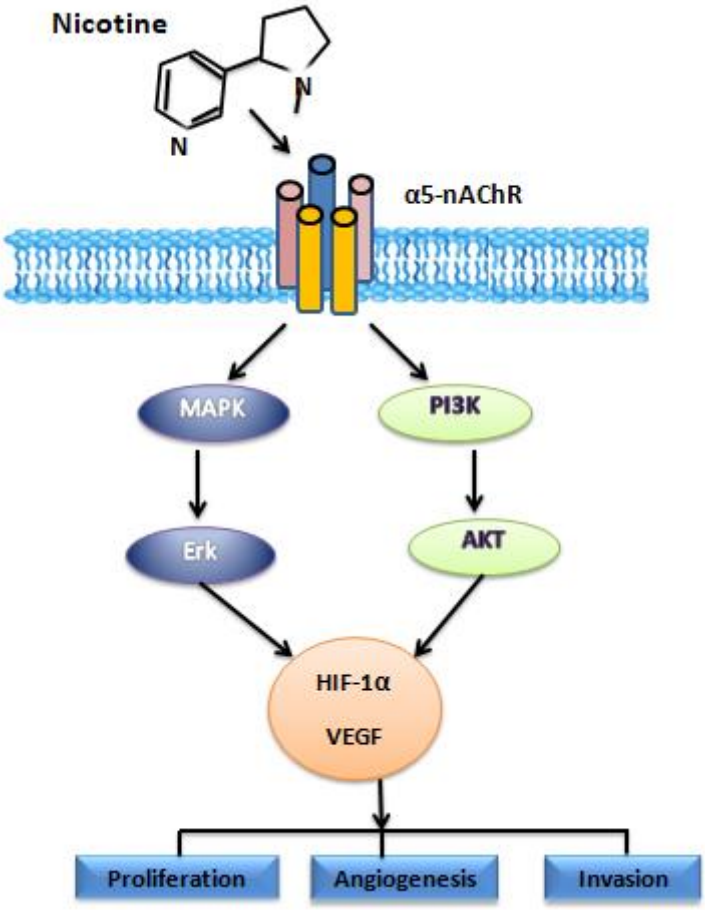


Figure 7.2: Schematic representation of the role of nAChR in the direct mode of tobacco induced carcinogenesis

There are several SNPs in the nAChR genes and these have been evaluated for association with nicotine dependence and cancer risk [Mobascher et al. 2016; MacQueen et al. 2014; Zhang et al. 2010]. Most notably, Asp398Asn SNP in the *CHRNA5* gene has been shown to be associated with nicotine dependence and lung cancer [Xu et al. 2015]. *CHRNA5* gene codes for the cholinergic receptor nicotinic alpha 5 subunit. Cell culture studies by Bierut et al. has shown that the sensitivity of the nAChR carrying the 398Asn polymorphism reduced by nearly half (Figure 7.3) [Bierut et al. 2008].

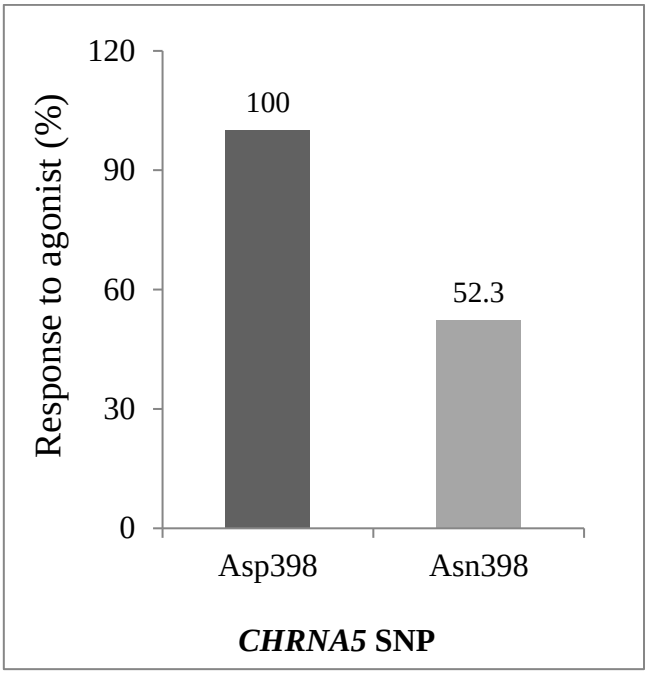


Figure 7.3: Graphical comparison of the impact of *CHRNA5* Asp398Asn polymorphism on the functional capacity of nAChR

7.2. Rationale: The risk of developing OSCC is 4.8 times higher in tobacco chewers than in non-chewers [Khan et al. 2014]. Chronic exposure of oral epithelial cells to nicotine upregulates the expression of the α5 subunit of nAChR [Arredondo et al. 2017]. Activation of nAChR by nicotine and its metabolites like NNN and NNK induces cell proliferation, inhibition of apoptosis and transformation [Zhao et al. 2016; Hecht et al. 2003]. Cell proliferation is mediated through PI3K/Akt pathway while apoptosis inhibition is mediated through the NF-kB pathway. Asp398Asn is a functional SNP in the *CHRNA5* gene that codes for the α5 subunit [Arredondo et al. 2017; Gabrielsen et al. 2013]. The minor allele 398Asn is nearly 50 % less competent in stimulating nAChR. Therefore, 398Asn should have a protective role in the carcinogenesis while the major allele Asp398 should be a risk factor. The rationale of this study is schematically illustrated in Figure 7.4.

7.3. Hypothesis: The hypothesis of this study was that the functional major allele Asp398 is higher in OSCC patients than in controls.

7.4. Objective: The objective of this study was to evaluate the association of Asp398Asn SNP in the *CHRNA5* gene with the predisposition to OSCC.

7.5. Novelty: Several studies have shown that the functional SNP in the *CHRNA5* gene is linked to tobacco dependence and predisposition to lung cancer [Xu et al. 2015; Gabrielsen et al. 2013; Schaal et al. 2014]. However, the association of Asp398Asn with OSCC is not known.

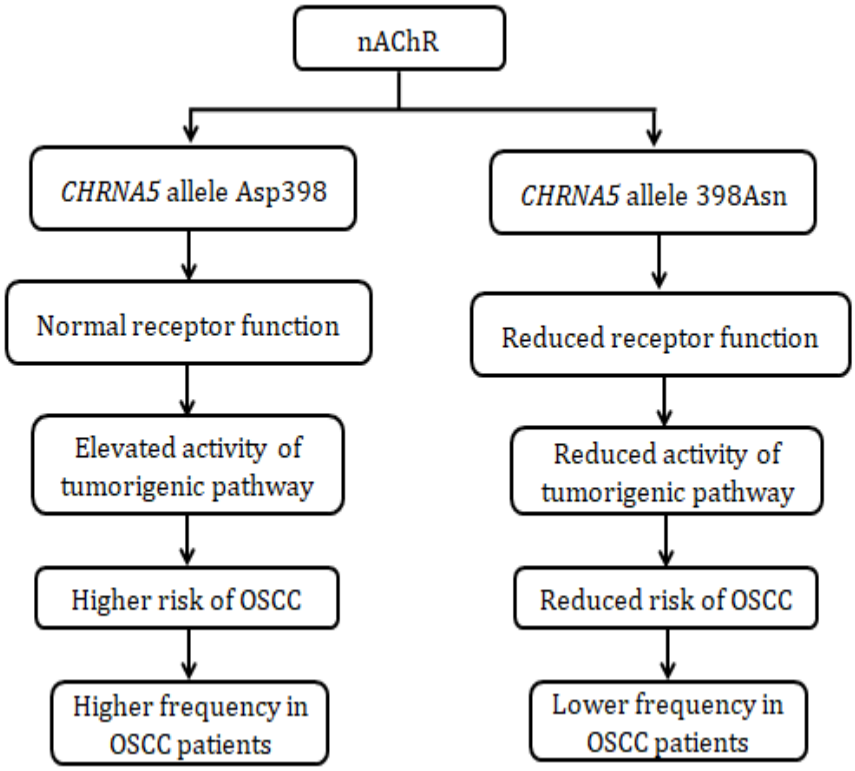


Figure 7.4: Schematic representation of the rationale for the evaluation of the association between *CHRNA5* Asp398Asn and OSCC

7.6. Materials and methods

7.6.1. Genotyping of *CHRNA5* Asp398Asn SNP

This was carried out by using a PCR-RFLP method as previously described by Bierut et al. The primer pairs used were 5'- CGC CTT TGG TCC GCA AGA TA -3' (forward) 5' - TGC TGA TGG GGG AAG TGG AG - 3' (reverse) [Bierut et al. 2008]. 25 µl of the reaction mixture contained 1 X assay buffer, 150 ng genomic DNA, 0.2 mM dNTP, 1 picomole of each primer, 1.5 mM $MgCl_2$ and 1 unit *Taq* DNA polymerase (Bangalore Genei, India). The program comprised an initial denaturation at 95 ºC for 5 min followed by 30 cycles at 94 ºC for 1 min, 60 ºC for 30 s and 72 ºC for 45 s; final extension involved 5 min at 72 ºC. The PCR product was analyzed on 1 % agarose gel an amplicon size of 435 bp.

The 435 bp amplicon was subjected to restriction digestion with 10 units of *Taq1* restriction enzyme (New England Biolabs, USA) for 16 h. The digestion product was then analyzed on 2 % agarose gel with ethidium bromide staining (Table 7.2). Representative agarose gel picture is shown in Figure 7.5. 10 % of the samples were randomly selected and subjected to PCR - RFLP for confirmation and the results were 100 % concordant. Sanger sequenced samples were used as positive controls.

7.6.2. Statistical analysis: The methods employed for statistical analysis is described in Chapter 4 section 4.11.

Table 7.2: RFLP parameters used in the genotyping *CHRNA5* Asp398Asn

SNP	Enzyme	Incubation temperature	RFLP pattern (bp)		
			Maj/Maj	Maj/Min	Min/Min
CHRNA5 Asp398Asn	*TaqI*	65 ºC	288 + 147	435 + 288 + 147	435

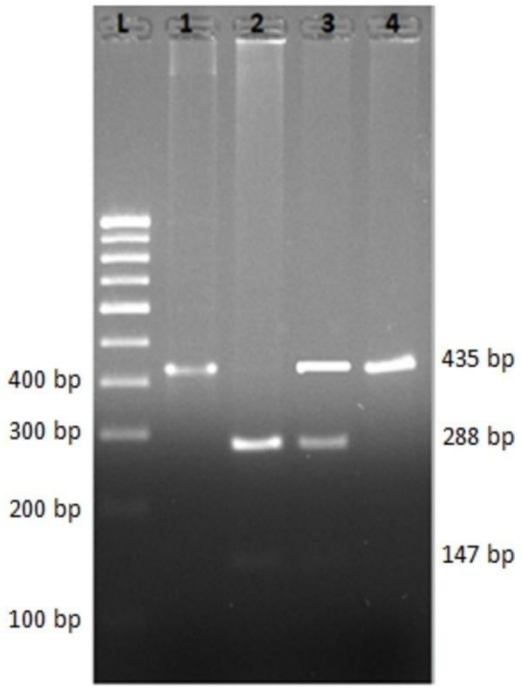


Figure 7.5: Representative image of agarose gel electrophoresis showing band pattern of PCR-RFLP analysis of *CHRNA5* Asp398Asn SNP. Lanes: L - 100 bp DNA ladder; 1 - PCR amplicon (435 bp); 2- Asp/Asp genotype (288+147 bp); 3- Asp/Asn genotype (435+288+147 bp); 4- Asn/Asn genotype (435bp)

7.7. Results

The distribution of the Asp398Asn genotypes in the control population was in agreement with Hardy-Weinberg equilibrium (χ^2 = 0.16).

7.7.1. Frequency of Asp398Asn alleles in the OSCC patients and the healthy controls

The frequency Asp398Asn alleles in the study groups are shown in Table 7.3. Minor allele frequency (398Asn) was 22 % in the patient group and 26 % in the control group (Figure 7.6). The difference in the distribution of Asp398Asn alleles in the patient and control groups was compared by means of a 2X2 contingency table. The P-value for the difference was 0.4 and therefore, the difference in the minor allele frequencies was not statistically significant.

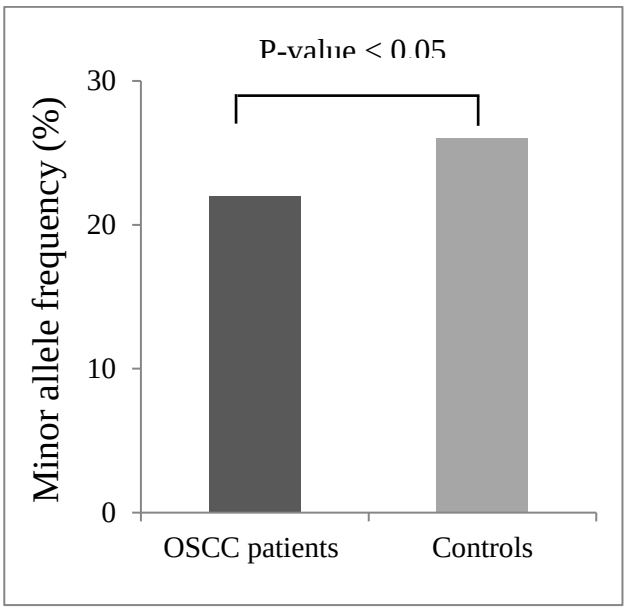


Figure 7.6: Distribution of the minor alleles of the Asp398Asn in study groups

7.7.2. Frequency of Asp398Asn genotypes in the OSCC patients and the healthy controls

The profile of the genotype frequency in the study groups is shown in Table 7.3. The major allele (Asp) was present mainly in the homozygous condition (57 % in the patient group and 54 % in the control group). Figure 7.7 depicts the frequency of the minor allele (Asn) in the homozygous condition and was found to be comparatively lower in the patient group (1 %) than in the control group (6 %) (Figure 7.7). The difference in the distribution of the genotypes in both the groups was compared by means of a 2X3 contingency table which did not show a statistically significant difference.

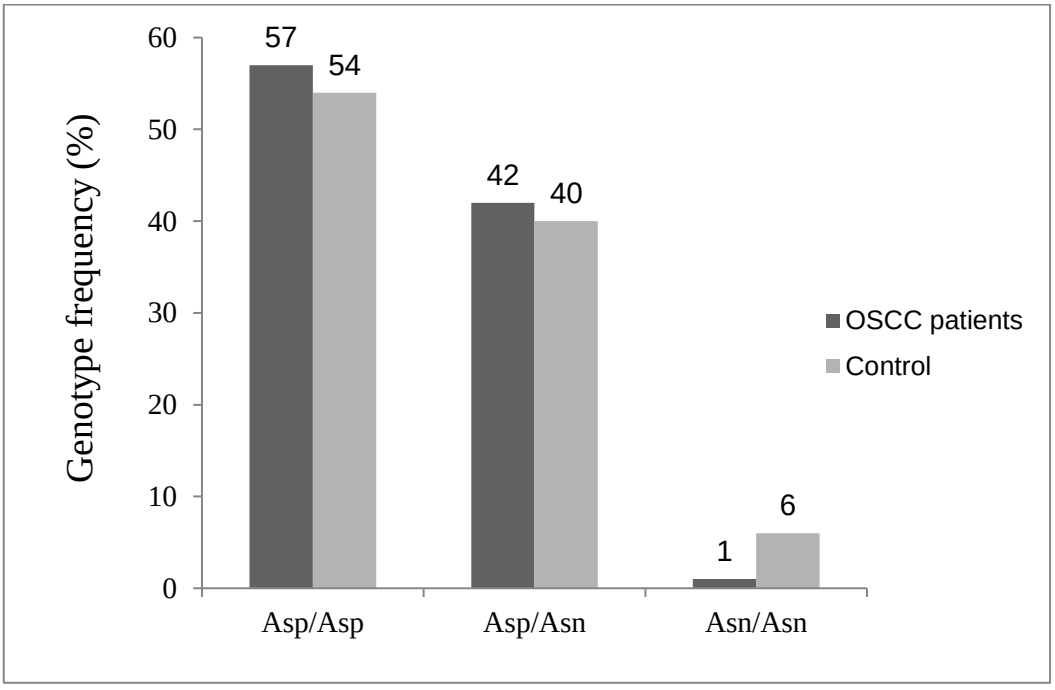


Figure 7.7: Comparison of the genotype frequencies of *CHRNA5* Asp398Asn polymorphism in the study groups

Table 7.3: Distribution of allele and genotype frequencies of *CHRNA5* Asp398Asn in the study groups

Allele/ Genotype	Patients (n = 100)	Controls (n =100)	P-value*
Asp	156	148	0.4
Asn	44	52	
Asp/Asp	57	54	0.19
Asp/Asn	42	40	
Asn/Asn	1	6	

*Chi-square test (Fisher's exact)

7.7.3. Association of Asp398Asn with OSCC under different genetic models

The genotype frequencies in the patient and control groups were compared by considering various genetic models. The outcome of this analysis is summarised in Table 7.4. There was no statistically significant difference between the study groups in any of the four models.

Table 7.4: Evaluation of the association between *CHRNA5* Asp398Asn and OSCC under different genetic models.

Model	Genotype	P-value
Dominant	Asp/Asp *vs.* Asp/Asn + Asn/Asn	0.77*
Recessive	Asn/Asn *vs.* Asp/Asn + Asp/Asp	0.1*
Additive	Asn/Asn> Asp/Asn> Asp/Asp	0.15†
Over-dominant	Asp/Asn *vs.* Asp/Asp +Asn/Asn	0.77*

* Chi-square test (Fisher's exact)

† Chi-square test for linear trend

7.7.4. Univariate and multivariate analysis of the association between *CHRNA5* Asp398Asn and OSCC

Univariate-multivariate analysis was carried out to evaluate the impact of the covariate factors on the genetic association. This approach permits elucidation of gene-environment interaction in the association. The association between the two variables was present only in the presence of another factor. The outcome of the analysis is summarised in Table 7.5. The results show that the association was not modified by any of the known risk factors and clinicopathological parameters.

Table 7.5: Univariate and multivariate analysis of the association between clinico-pathological parameters of *CHRNA5* Asp398Asn and OSCC.

Parameter	N	Univariate Analysis[#]	Multivariate Analysis*
		P-value	P-value
Age[†]			
≥54 years	54	0.84	0.66
<54 years	46		
Gender			
Male	21	0.63	0.53
Female	79		
TNM Stage			
T_{1-2}	8	0.28	0.28
T_{3-4}	92		
Grading			
G1	72	0.66	0.67
G2+G3	28		
Duration of tobacco[†]			
≥31 years	46	0.15	0.23
<31 years	54		

[†]In years

[#]Chi-square test (Fisher's exact)

*****Regression analysis

7.8. Discussion

The aim of this chapter was to evaluate the association of Asp398Asn SNP in the *CHRNA5* gene with the predisposition to OSCC. The main findings of this study were: (i) the frequency of the Asp398 allele was not higher in OSCC patients, (ii) there was no association even when the genotype frequencies were analyzed by assuming genetic models, and (iii) there was no association when the genotype frequencies were subjected to multivariate analysis. These results indicate that *CHRNA5* Asp398Asn is unlikely to be involved in the predisposition to OSCC.

Bierut and co-workers determined the global distribution of the minor allele frequency of Asp398Asn and found that it varies from population to population. The lowest frequency of 0 % was seen in the African population while the highest frequency of 37 % was seen in the European population. In this study, the minor allele frequency in the control group was 26 % [Bierut et al. 2008]. This indicates that the minor allele may be common in the Indian population.

Association of SNPs in the nAChR genes has been linked to tobacco dependence. Studies by Weiss and co-workers showed that specific haplotypes of *CHRNA5-A3-B4* genes are associated with nicotine addiction in an age-dependent manner. The *CHRNA5-A3-B4* haplotypes are consistently related to the severity of nicotine dependence among long-term smokers of European-American descent who began daily smoking at or before age 16 but not among those who began smoking daily after age 16 [Weiss et al. 2008]. Further studies by Schaepfer and co-workers showed that SNPs in the *CHRNA5-A3-B4* genes are linked to early alcohol and tobacco initiation [Schlaepfer et al. 2007]. A large European population-based study

on the genetic association of smoking has been carried out by the Consortium for the Genetic Analysis of Smoking Phenotypes. Genome-wide analysis showed that the SNPs in nAChR genes are linked to tobacco dependence and the strong linkage disequilibrium between SNPs in the *CHRNA5* and *CHRNA3* genes [Saccone et al. 2010]. Studies by Li-Shiun Chen has shown that *CHRNA5* Asp398Asn is linked to heavy smoking and delayed quitting [Chen et al. 2015].

CHRNA5 Asp398Asn has also been evaluated for association with the predisposition to lung cancer since smoking is one of its major risk factor [Xu et al. 2015]. Asp398Asn is linked to a higher likelihood and also the early onset of lung cancer. Asn/Asn genotype is associated with a four-year delay in the median age of quitting compared with the Asp/Asp genotype and also lung cancer diagnosis is four years earlier among smokers carrying Asn/Asn genotype compared with the low-risk genotype (Asp/Asp) [Chen et al. 2015].

Asp398Asn has also been evaluated for association with cancers of the upper aerodigestive tract [Lips et al. 2010]. Asp398Asn has been linked to oral and laryngeal cancers particularly in tobacco smokers [Anantharaman et al. 2014; Chen et al. 2011]. A major difference between this study and the studies by Lips et al. was in the mode of tobacco use by the cancer patients. Tobacco was used in the smokeless chewable form by the patients in this study while it was used in the smoke form in the previous study. The lack of association observed in this study may be due to the difference in the mode of tobacco use. Combustion of tobacco during smoking results in the conversion of several constitutes into highly carcinogenic products like tar, hydrogen cyanide, polonium-210, formaldehyde etc [Pappas et al. 2006; Khater

et al. 2004; Stabbert et al. 2003]. The positive association may be due to the action of nicotine in the indirect mode i.e., by increasing the dependence on tobacco use and the dependent exposure to tobacco carcinogens [Egleton et al. 2008]. Tobacco use in the chewable form may be less carcinogenic due to the absence of combustion products. Nicotine in chewable tobacco may be contributing to carcinogenesis through the direct mode since it is maintained for a longer duration in the oral cavity by the user. Thus smoke and smokeless tobacco appear to involve differently in the mode of carcinogenesis. These differences may be the basis for lack of positive association in this study.

7.9. Conclusion

The results of this study suggest that *CHRNA5* gene Asp398Asn SNP may not be an indicator for the predisposition to OSCC. Lung cancer and OSCC may share tobacco use as the common risk factor, but they appear to differ with respect to their predisposing genetic factors.

Chapter VIII

TP53 gene mutation in OSCC

8.1. Introduction

TP53 gene codes for the p53 protein which is a transcription factor that plays a key role in maintaining the genomic integrity of the cell [Levin et al. 2006]. It is located on the short arm of chromosome 17 (17p13.1) and spans approximately 20 kb. The gene contains 11 exons of which exons 2 – 11 code for the protein product while exon 1 is a non-coding exon [Belyi et al. 2010]. The p53 protein contains 393 amino acid and weighs 53 kDa (Figure 8.1) [Joerger et al. 2008]. The p53 protein is organized into functional domains as follows:

- Transactivation domain: Provides a binding site for the negative regulator *viz.*, MDM2 protein and plays a critical role in the regulation of the p53 protein [Raj et al. 2017].
- Proline-rich domain: It is comprised of the repeated PXXP motif (P, Proline and X, any amino acid). It is involved in the transactivation independent induction of cell cycle arrest.
- DNA binding domain: It is involved in the transcriptional regulation by binding to p53 responsive genes [Sullivan et al. 2018].
- Oligomerization domain: This domain facilitates the tetramerization of p53 protein.
- Nuclear Localization Signals (NLS): There are 3 NLS motifs that are involved in the translocation of the p53 protein from the cytosol to the nucleus.
- Nuclear Export Signal (NES): There are 2 NES motifs that serve to retain the p53 in the nucleus by suppressing its translocation to the cytosol [Marchenko et al. 2010].

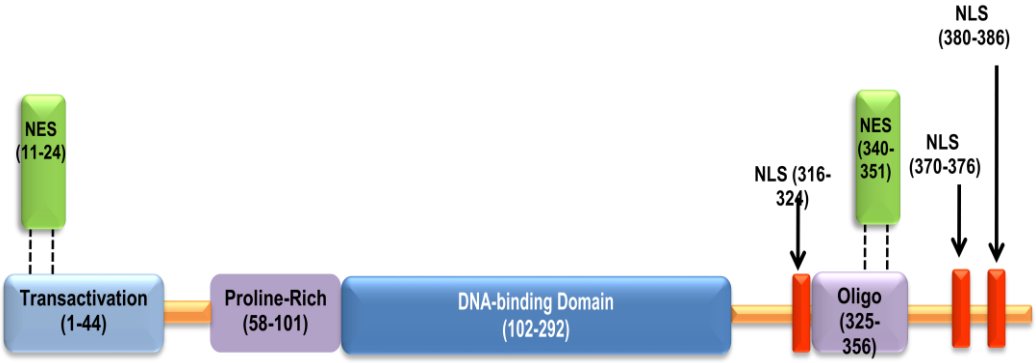


Figure 8.1: Structural organization of the p53 protein

The biological role of p53 protein is to the protect cells from DNA damage caused due to genotoxic stress factors like radiation and chemical carcinogens [Pflaum et al. 2014]. Activation of p53 results in the arrest of the cell cycle at the G1/S checkpoint. This ensures that cells carrying DNA damage do not enter S-phase and also provides time for the DNA repair pathways to remove the damages. If the DNA repair pathways fail to remove the damages, then p53 induces the arrested cells to undergo apoptosis. Thus, the main role of p53 is to prevent the formation of mutations. Lack of p53 function results in the accumulation of mutations which further leads to the transformation of the normal cell into a cancer cell [Giono et al. 2006; Zilfou et al. 2009; Rivlin et al. 2011].

TP53 gene is classified as a tumor suppressor gene since its protein product is involved in protecting the cell against genotoxic insult. *TP53* gene is commonly mutated in the cancer cell since it plays a crucial role in preventing carcinogenesis [Ozaki et al. 2011]. Somatic mutations in the *TP53* gene occur in almost all the types of cancers with frequency ranging from 38 – 50 %. Mutation in *TP53* gene has been observed in 30 - 70 % of OSCC [Amelia et al. 2009].

Germ-line mutations of the *TP53* gene lead to hereditary cancer like Li-Fraumeni syndrome [Malkin. 2011]. Several polymorphisms both in the coding and the noncoding regions functionally affect the gene and have been associated with predisposition to cancer [Bandele et al. 2010]. Inactivation of p53 protein is involved in human papillomavirus-mediated carcinogenesis. E6 protein from the human papillomavirus binds with high affinity to p53 protein and promotes its degradation [Buitrago-Pérez et al. 2009].

8.2. Rationale: *TP53* gene plays a key role in controlling the transformation of a normal cell into the cancer cell. Therefore, *TP53* is commonly mutated in about 50 % of all cancers. Mutations are mainly in the transcriptionally active DNA binding domain [Olivier et al. 2010]. The nascent p53 protein is produced in the cytosol but the mature protein is functionally active in the nucleus. Therefore, nuclear localization in an important prerequisite for p53 function and this is determined by the NLS and NES [Mandic et al. 2005]. Mutations of these motifs can thus abrogate p53 function. The rationale of this chapter is schematically illustrated in Figure 8.2.

8.3. Hypothesis: The hypothesis of this chapter was that the mutation of NLS and NES motifs of the *TP53* gene is common in OSCC specimen.

8.4. Objective: The objective of this chapter was to evaluate the mutation profile of the *TP53* gene in OSCC.

8.5. Novelty: Several attempts have been made to determine the mutational status of the *TP53* gene in Indian patients with OSCC. However, these studies were focussed only on the exons 5 – 8. As a result, there is a paucity of information on the

mutational status of exons 1 - 4 and 9 – 11 which contains functionally important NLS and NES motifs.

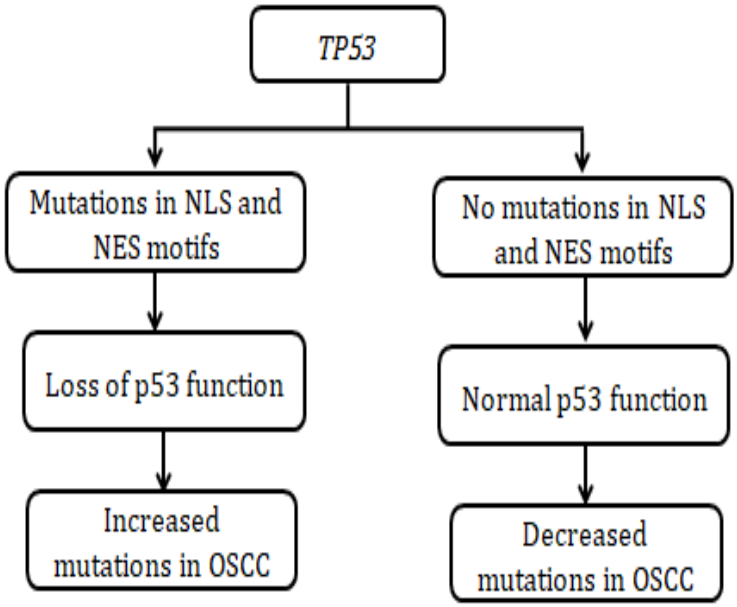


Figure 8.2: Schematic representation of the rationale for the evaluation of *TP*53 mutations in NLS and NES motifs in OSCC

8.6. Materials and Methods

8.6.1. PCR amplification of the *TP53* gene

Genomic DNA was isolated from the tumor specimen using the protocol detailed in Chapter 4. The PCR reaction was carried out in 25 µl final volume containing 1 pM of each primer, 1mM dNTPs, 1.5 mM $MgCl_2$, 100-300 ng of genomic DNA and 1 unit of *Taq* DNA polymerase. The PCR conditions were as follows. For exons 1 - 10: denaturation step at 95 °C for 3 min; followed by 34 cycles of denaturation at 95 °C for 30 s; annealing temperature depending on the primer sequence for 30 s; elongation at 72 °C for 1 min and a final extension at 72 °C for 7 min. Touchdown PCR was used for the amplification of exon 11. The PCR

conditions were as follows: 1 cycle of denaturation (12 min at 95 °C), 15 cycles of denaturation (95 °C for 30 s), annealing temperature (65.5 °C for 30 s, with - 0.5 °C per cycle), and extension (72 °C for 30 s), followed by 20 cycles of denaturation (95 °C for 30 s), annealing (58 °C, 30 s), and extension (72 °C for 30 s), and a final extension cycle of 72 °C for 10 min. The samples were run on 1 % agarose gel and the bands were visualized using ethidium bromide (Figure 8.3). A sequence of the primer and their annealing temperatures are given in Table 8.1.

Table 8.1: Details of the primers used for the PCR amplification of *TP53* gene

Exon	Primer (5' – 3')	Annealing temp (ºC)	Amplicon size (bp)
1	**FP:** CAC AGC TCT GGC TTG CAG A **RP:** AGC GAT TTT CCC GAG CTG A	59	442
2	**FP:** AGC TGT CTC AGA CAC TGG CA **RP:** GAG CAG AAA GTC AGT CCC ATG	62	319
3-4	**FP:** GAC CTA TGG AAA CTG TGA GTG GA **RP:** GAA GCC AAA GGG TGA AGA GGA	59	649
5-6	**FP:** CGC TAG TGG GTT GCA GGA **RP:** CAC TGA CAA CCA CCC TTA AC	66	550
7	**FP:** CTG CTT GCC ACA GGT CTC **RP:** TGG ATG GGT AGT AGT ATG GAA G	64	283
8-9	**FP:** GTT GGG AGT AGA TGG AGC CT **RP:** GGC ATT TTG AGT GTT AGA CTG	64	455
10	**FP:** CAG TTT CTA CTA AAT GCA TGT TGC T **RP:** ACA CTG AGG CAA GAA TGT GGT TA	57	421
11	**FP:** TCC CGT TGT CCC AGC CTT **RP:** TAA CCC TTA ACT GCA AGA ACA T	65.5	476

FP: Forward primer; RP: Reverse primer

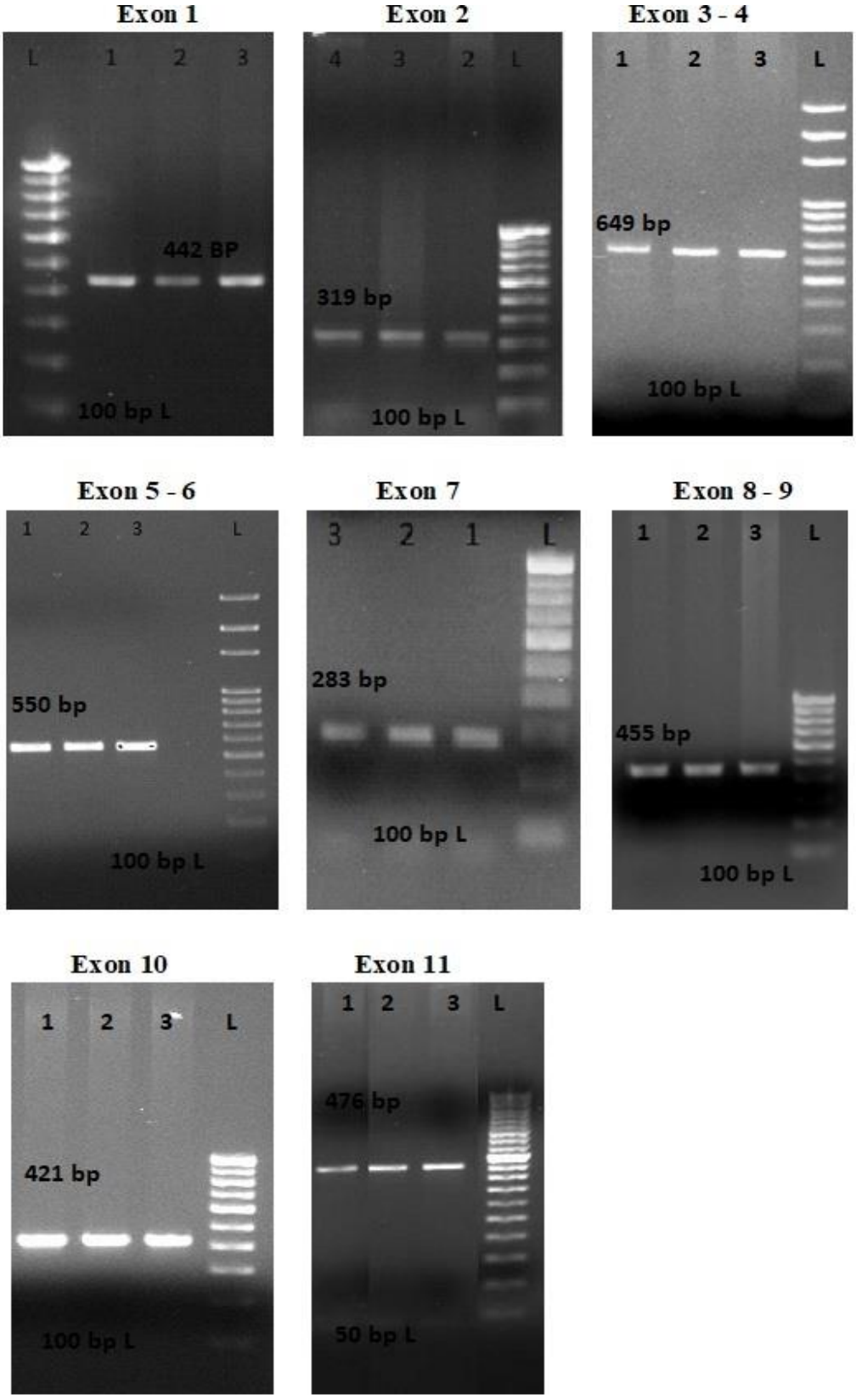


Figure 8.3: Representative agarose gel image showing PCR amplicons of *TP53* gene exons 1 – 11

8.6.2. DNA Sequencing: The PCR amplicons were purified using a commercial silica column (GeneJET PCR purification kit; Thermo Fisher Scientific, USA). Purified PCR amplicons were used for Sanger sequencing. Dideoxy chemistry was carried out using BigDye Terminator v3.1 Cycle Sequencing kit following manufacturer's instructions (Applied Biosystems, Foster City, California, USA) and analyzed using ABI-3500 Genetic Analyzer (ThermoFisher Scientific, CA, USA).

8.6.3. Sequence analysis: The sequence obtained by the genetic analyzer was compared with the reference sequence for any genetic variation. Sequence ID: NC_000017.11 from the National Center for Biotechnology Information was used as the reference sequence. The mutations were further confirmed by using Variant reporter v1.1 and SecScape v2.7 software. The *in-silico* analysis of the mutation impact was carried out by using web-based tools *viz.,* PolyPhen-2 (Available at http://genetics.bwh.harvard.edu/pph2/) and Human Splicing Finder (Available at http://www.umd.be/HSF/). Population frequency of SNPs was obtained Single Nucleotide Polymorphism Database maintained by the National Center for Biotechnology Information (Available athttps://www.ncbi.nlm.nih.gov/).

8.6.4. Results

A total of 60 randomly selected OSCC tumor DNA specimens were selected for evaluating the mutation profile of the *TP53* gene. All the coding exons of the *TP53* gene (exon 2 – 11) were sequenced by Sanger's method. Ten unique sequence variations were identified in fifteen tumor specimens. The genetic variations were classified as mutation or polymorphism on the basis of their population frequency. Out of ten sequence variations, seven were mutations and three were a

polymorphism. The details of these sequence variants are summarised in Tables 8.2 and 8.3.

All the 7 mutations were located in exons corresponding to the DNA binding domain (exons 5 – 8). Location of the observed mutations in the *TP53* gene is illustrated in Figure 8.4. No mutations were detected in the NES and the NLS motifs. The functional impact of the seven mutations was analyzed by *in-silico* method. Six of the mutations were functionally disruptive and one was benign. Images of Sanger sequencing electropherogram is given in Figure 8.5.

The polymorphisms were located in the exon 4 and the splice site region of intron 2 and 9. Functional impact of these variations was evaluated by *in-silico* methods. Exon 4 variant was predicted to be benign. The splice-site variants 2 and 9 were predicted to function as an enhancer and a silencer motif respectively.

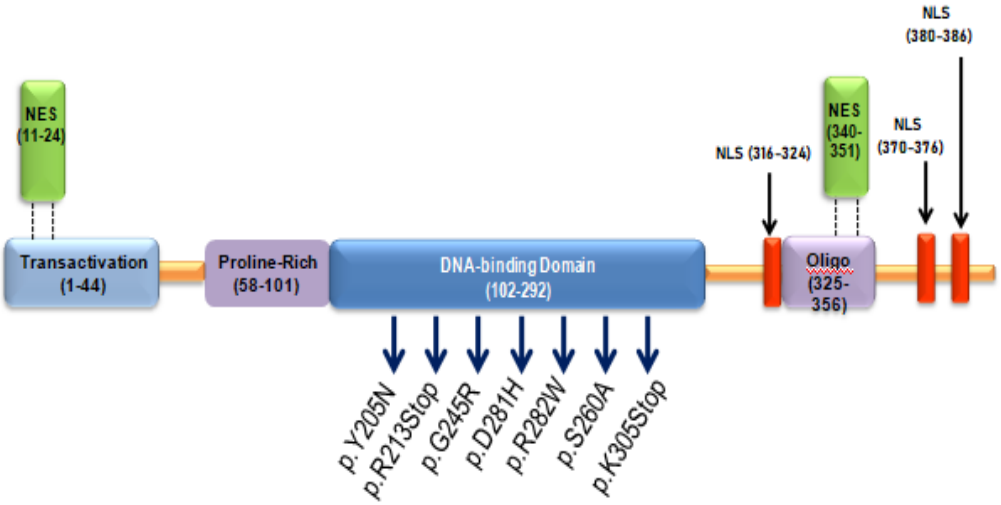


Figure 8.4: Location of the *TP53* gene mutations observed in the study

Table 8.2: Profile of the *TP53* gene mutations in the OSCC specimens

Locus	Frequency* (%)	Mutation		Functional impact
		Nucleotide sequence	Protein sequence	
Exon 6	3.3	c.613 T>A	Tyr205Asn	Probably damaging
		c.637 C>T	Arg213Stop	Probably damaging
Exon 7	3.3	c.778 T>G	Ser260Ala	Benign
		c.733 G>C	Gly245Arg	Probably damaging
Exon 8	5	c.844 C>T	Arg282Trp	Probably damaging
		c.841 G>C	Asp281His	Probably damaging
		c.913 A>T	Lys305Stop	Probably damaging

* Sample size = 60

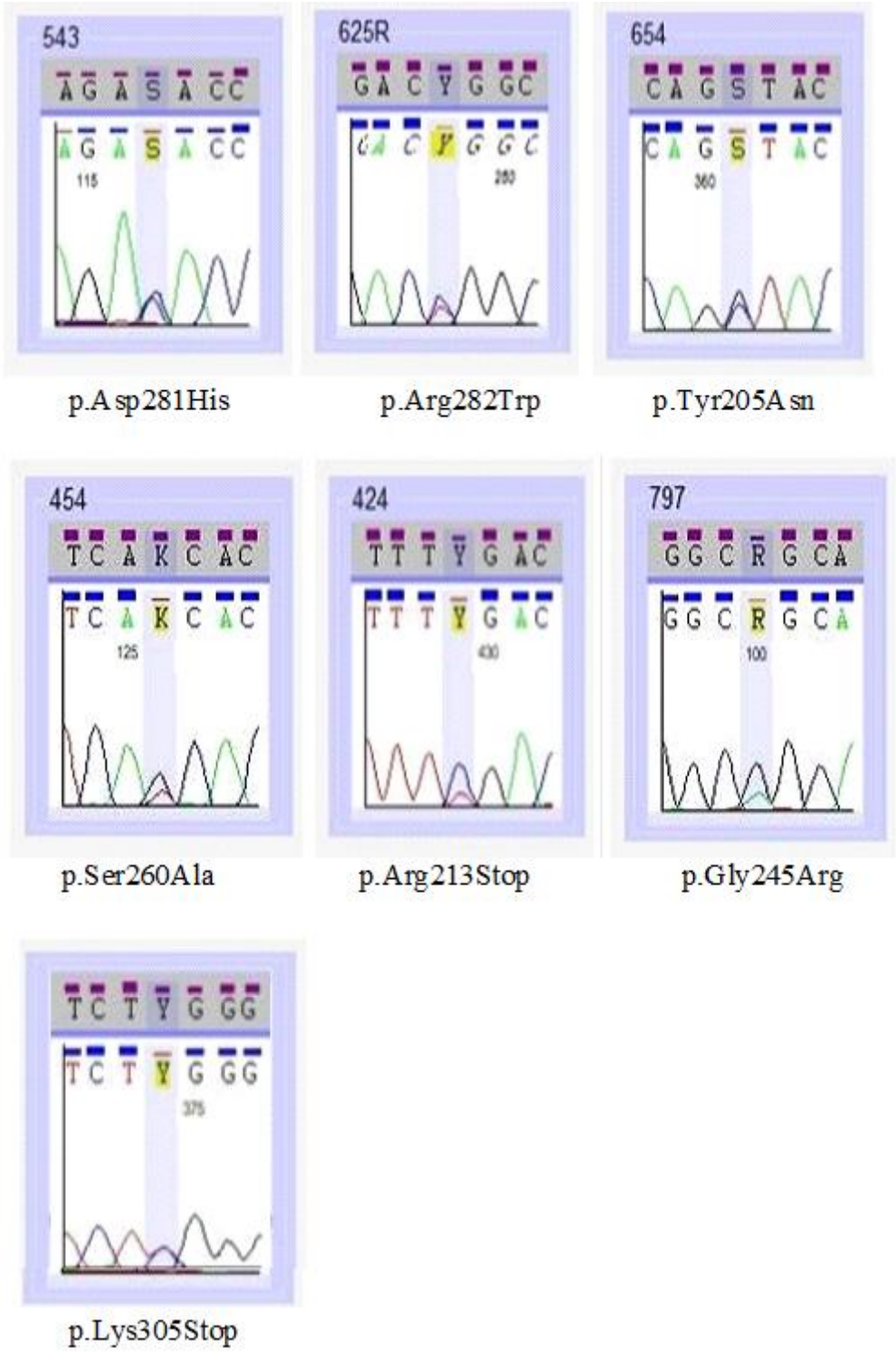


Figure 8.5: Electropherograms of *TP53* gene mutations observed in the study

Table 8.3: Profile of the *TP53* gene polymorphisms in the OSCC specimens

Locus	Frequency* (%)	Mutation		Functional impact
		Nucleotide Sequence	Protein sequence	
Intron 2	5	g.16068 C>G	-	Benign
Exon 4	6.7	c.216 C>G	Pro72Arg	Benign
Intron 9	1.6	g.19017 G>C	-	Pathogenic

* Sample size = 60

8.7. Discussion

The aim of this chapter was to evaluate the mutational profile of the *TP53* gene in OSCC with special emphasis on NLS and NES motifs. The main findings of the study are: (i) the frequency of *TP53* gene mutations was 11.7 %, (ii) the mutations were restricted to the central DNA binding domain, and (iii) mutations were absent in the NLS and NES motifs. These results prompt us to conclude that mutations of the nuclear localization signals are not frequent in OSCC.

The mutation profile of *TP53* gene in OSCC has been evaluated in several studies from India. The observations of these studies are summarised in Table 8.4. The mutation frequency showed vast variation from 17 – 110 %. The mutations were located in the DNA binding domain since only this region was screened. OSCC was linked to tobacco use in a smokeless chewable form in most of the studies. In contrast, the mutational frequency observed in this study is very low (11.7 %) despite sequencing the complete gene.

A mutational frequency of *TP53* gene is linked to tobacco use. The frequency is 3 - 4 fold higher in smokers than in non smokers in the case of lung cancer [Husgafvel-Pursiainen et al. 2000; Kreuzer et al. 2002]. The mutational frequency observed in this study is considerably lower than that of other studies despite tobacco use being a common risk habit in all the patients. Previous studies have also found that the association of *TP53* mutation frequency with tobacco use in the case of oral cancer may not be significant [Heinzel et al. 1996; Munirajan et al. 1996; Kannan et al. 1999].

Table 8.4: List of Indian studies on the mutational analysis of the *TP53* gene in OSCC

Center	N	Frequency (%)	Locus	Mode of tobacco use	Reference
Madurai	72	21	DBD	Smokeless	Kannan et al. 1999
Madurai	53	21	DBD	Smokeless	Munirajan et al. 1996
Gujarat	46	110.1*	NA	NA	Singh et al. 2016
Trivandrum	91	17	DBD	Smokeless	Heinzel et al. 1996
Delhi	30	23	DBD	Smokeless	Ralhan et al. 2001
Bombay	83	46	DBD	Smokeless	Saranath et al. 1999
Bhubaneshwar	22	20	DBD	NA	Patnaik et al. 1999

NA : not available

DBD: DNA binding domain

* 51 mutations from a total of 46 tumor specimens

A mutational frequency of *TP53* gene is also affected by HPV infection of the tumor [Mitra et al. 2006; Tommasino et al. 2003; Klug et al. 2001; Yim et al. 2005]. Multicentric studies by the International Agency for Research on Cancer found an inverse association between the presence of *TP53* gene mutations and HPV infections in the cancers of the oral cavity and oropharynx (Dai et al. 2004). The frequency of HPV infection was 0 % in this study and yet the *TP53* mutational frequency was low. This indicates that HPV status is not related to *TP53* mutational frequency in OSCC.

8.8. Conclusion

The results of this study show that the mutations of the *TP53* gene including the DNA binding domain and the nuclear localization signals are not common in OSCC. The tumorigenic pathway in OSCC linked to tobacco use in the chewable form may thus involve a *TP53* independent pathway.

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Appendix I

Composition of buffers and reagents

1	Erythrocyte Cell Lysis Buffer (ELB)	155 mM NH_4Cl 10 mM $KHCO_3$ 1 mM EDTA pH 7.4
2	NaCl	5 M in water
3	SDS	20 % Sodium Dodecyl Sulphate in water
4	Proteinase K	20 mg Proteinase K in water
5	TE Buffer	10 mM Tris base 1 mM EDTA pH8.0
6	TAE Buffer	40 mM Tris 20 mM Acetic acid 1 mM EDTA
7	TBS Solution	50 mM Tris-Cl 150 mM NaCl pH 7.6

Appendix II

Ethics clearance certificate and forms

	SRI DEVARAJ URS MEDICAL COLLEGE TAMAKA, KOLAR- 563 101	Format no	IEC 00
		Issue No	01
	Institutional Ethics Committee	Revision No	00
		Date	01/11/05

No. DMC/ KLR/ IEC/ 151 /2015-16 Date: 03-02-2016

From:
The Institutional Ethics Committee,
Sri Devaraj Urs Medical College,
Tamaka, Kolar- 563 101

To:
Mrs. Deepa Rajesh,
Ph.D. Scholar,
Dept. of Cell Biology & Molecular Genetics,
Sri Devaraj Urs Academy of Higher Education & Research,
Tamaka, Kolar - 563 101

Subject: Ethical Clearance to start the Ph.D work

This is to certify that the institutional ethics committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Ph.D. synopsis entitled **"Studies on mutational and biochemical markers in Oral Squamous Cell Carcinoma"** of Mrs. Deepa Rajesh, Ph.D. Scholar in the Department of Cell Biology & Molecular Genetics, Under the guidance of Dr. AV Moideen Kutty[1], Dr. Sharath B[1] and Dr. S M Azeem Mohiyuddin[2] in the Department of Allied Health Sciences[1] and ENT[2] at Sri Devaraj Urs Academy of Higher Education & Research, Kolar

Member Secretary
Member Secretary
Ethical Committee
SDUMC, Kolar.

Chairman
CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College,
Tamaka, Kolar

Sl. No-

INFORMED CONSENT FORM

Name of Organization: Sri Devaraj Urs Academy of Higher Education and Research
Title of the study: Molecular studies on oral squamous cell carcinoma
Name of the Investigator: Mrs. Deepa
Name of Participant: _____

The following has been explained to me:

1. Resected tumor specimen will be collected.

2. For optimal test interpretation, clinical information and family history are often necessary.

3. Participation is totally voluntary and there would be no payment for sample collection. All tests results are treated with medical confidentiality and will not be disclosed to any outsider except if it is required by the law.

I give my consent to use tumor samples for aforesaid and further research studies and allow my (or my child's) anonymous sample, photographs, pictures to be used for medical research, test validation, or education as long as my privacy is maintained.

I understand that I remain free to withdraw from this study at any time and this will not change my future care. I have read and received a copy of this consent form.

I understand the information provided in this document and I have had the opportunity to ask questions.

Patient's (or his / her parent) signature:
Date:

Witness name and signature: 1.
Date:

2.

Date

Person obtaining consent form & his/her signature:
Date:

For any clarification you are free to contact the Investigator:
Principal Investigator
Mrs. Deepa

Signature of the Investigator: Date:

PROFORMA

Name : **Date:**
Age : **IP/OP No. :**
Occupation : **Male / Female**
Phone No. :
Address :

Chief complaints:

Duration

Ulcer in cheek

Loose teeth

Inability to open mouth

Change in voice

Difficulty swallowing

Neck swelling

Weight loss

Associated complaints

Present history:

Past History:

Family History:

Clinical details:

Habits : ●Smoking ●Alcohol ●Gutka
● Pan masala ● Tobacco chewing

Tumor location : ●Buccal mucosa ●Lower alveolus
●Upper alveolus ●Anterior 2/3 of tongue
●Floor of mouth ●Hard palate
●Retromolar trigone

Tumor Size : ●<4 cm ●≥4cm

Lymph node metastasis: ●N_0 ●N_1 ●N_{2a} ●N_{2b} ●N_{2c} ●N_3

Distant metastasis : ●Present ●Absent

Histological grading : ●Well-G1 ●Moderate-G2 ●Poor-G3

Tumor depth : ●<3 mm ●3 – 5 mm ●>5 mm

Diagnosis :

Stage : ●T_1 ●T_2 ●T_3 ●T_{4a} ●T_{4b}

Resectability : ●Yes ●No

Residual tumor : ●R_0 ●R_1 ●R_2

Treatment : ●Surgery + RT
●Surgery + RT + Chemo
●Neoadjuvant chemo + Surgery + post-op RT +chemo
●Neoadjuvant chemo + Surgery + post-op RT –chemo
●Pallative RT + chemo

Surgery :

Histopathology report:

***TP53* mutation status:** ●Exon 1 ●Exon 5
●Exon 2 ●Exon 6
●Exon 3 ●Exon 7
●Exon 4 ●Exon 8

Chemo : ●Cisplatin ●Carboplatin ●5 FU ●Paclitaxel

Chemo response : ●Complete response ●Partial response
●Progressive disease ●No response

Post chemo symptoms: ●Giddiness ●Tinnitus
●Hearing loss ●Vomitting
●Parasthesia ●Oliguria, Anuria
●Mucositis

Recurrence : ● Present ●Absent

If yes,
● Local
●Regional
● Loco-Regional
●Distant metastasis

Last follow up : Date:

Status: ●Normal without disease
● Alive with disease
● Died due to disease
● Died of other causes
● Lost to follow up

Appendix III: Master chart

Sl. No	MGGL No.	Gender	Age*	Stage	H grading	Duration of tobacco use*	*GSTP1*			*CHRNA5*	*TP53* mutation	Recurrence
							Ile105Val	Ala114Val	Arg187Trp	Asp398Asn		
1	424	F	60	T4	G1	49	AG	CC	CC	GG	p.R213Stop	Died
2	438	F	50	T4	G1	35	AA	CC	CC	GA	p.Pro72Arg	No
3	440	F	48	T4	G1	20	AG	CC	CC	GG	NM	No
4	442	F	60	T4	G1	45	AA	CC	CC	GG	NM	No
5	443	F	75	T4	G1	30	AA	CC	CC	GA	NM	Yes
6	449	F	57	T4	G1	55	AA	CC	CC	GG	NM	Yes
7	454	F	49	T4	G1	40	AA	CC	CC	GA	p.G245R	Died
8	488	F	55	T4	G2	30	AG	CC	CC	GG	g.16068 C>G	Yes
9	491	M	55	T3	G2	24	AA	CC	CC	GA	p.Y205N	No
10	509	F	45	T4	G1	30	AA	CC	CC	GG	NM	No
11	515	F	45	T4	G1	25	AG	CC	CC	GA	NM	Yes
12	523	M	64	T4	G2	40	AA	CT	CC	GG	NM	No
13	525	F	62	T4	G1	40	AA	CC	TT	GA	NM	Died
14	526	F	65	T4	G2	40	AG	CC	CC	GG	NM	No
15	527	M	65	T3	G2	30	AG	CC	CC	GG	NM	No
16	529	M	55	T3	G1	10	AA	CC	CC	GG	NM	No
17	540	F	40	T4	G1	21	AA	CC	CC	GA	NM	No
18	543	F	70	T4	G1	50	AA	CC	CC	GG	p.D281H	No
19	545	F	62	T4	G2	40	GG	CC	CC	GA	NM	NA
20	547	M	68	T4	G1	50	AA	CC	CC	GA	NM	No
21	555	F	57	T4	G1	24	AG	CC	CC	GA	g.19017 G>C	No
22	557	F	47	T3	G2	26	AA	CC	CC	GG	NM	Yes
23	560	F	62	T4	G2	20	GG	CC	CC	GA	NM	No
24	575	F	69	T2	G1	15	AA	CT	CC	GA	NM	No
25	576	F	46	T4	G2	45	AA	CT	CT	GA	NM	No
26	581	F	42	T4	G2	30	GG	CT	CC	GG	NM	NA
27	584	M	58	T4	G1	38	AA	CT	CC	GG	NM	No
28	585	F	63	T3	G1	50	GG	CC	CC	GG	NM	No
29	587	F	70	T4	G1	43	AG	CC	CC	GG	NM	Died
30	588	F	49	T4	G1	10	AA	CC	CC	GG	NM	No
31	593	F	56	T4	G1	40	AG	CC	CT	GG	NM	Yes

32	597	M	45	T4	G1	25	AG	CC	CC	GG	NM	No
33	601	F	59	T4	G1	20	AG	CC	CC	GG	g.16068 C>G	No
34	603	F	47	T4	G1	10	AA	CC	CC	GG	NM	NA
35	622	M	37	T4	G1	10	GG	CC	CC	GG	g.16068 C>G	Yes
36	624	F	58	T2	G1	20	AG	CC	CC	GG	NM	No
37	625	F	55	T4	G2	50	AA	CC	CT	GG	p.K305Stop	No
38	627	F	47	T4	G2	20	AA	CC	CC	GA	NM	Yes
39	636	F	62	T4	G2	25	AG	CC	CC	AA	NM	Yes
40	644	F	66	T4	G1	37	AA	CC	CT	GG	NM	NA
41	645	M	80	T2	G1	48	AG	CC	CC	GA	NM	Died
42	647	F	55	T4	G2	35	AG	TT	CT	GA	NM	No
43	649	M	40	T4	G1	10	AA	CT	CC	GA	NM	No
44	651	F	60	T4	G1	20	AA	CC	CC	GA	NM	No
45	652	M	40	T4	G1	6	AG	CT	CC	GG	NM	Yes
46	654	F	60	T4	G1	40	AA	CT	CC	GG	p.R282W	NA
47	655	F	45	T3	G3	31	GG	CT	CC	GG	NM	No
48	656	F	55	T3	G1	35	AA	CC	CT	GA	NM	No
49	664	F	49	T2	G2	38	GG	CC	CC	GG	NM	No
50	667	F	70	T4	G1	50	GG	CT	CT	GA	NM	Died
51	668	F	70	T4	G1	57	AG	CC	CC	GA	NM	NA
52	674	F	62	T4	G1	37	AG	CT	CT	GG	NM	Yes
53	680	M	57	T3	G1	25	AG	CC	CT	GA	p.Pro72Arg	No
54	684	F	48	T2	G1	20	AG	CT	CC	GA	NM	Yes
55	685	F	60	T3	G1	45	GG	CC	CC	GA	NM	No
56	686	F	67	T4	G1	20	AA	TT	CT	GA	p.Pro72Arg	No
57	687	F	38	T3	G1	25	AA	CC	CT	GG	NM	Yes
58	691	F	44	T4	G1	36	AA	CC	CT	GG	p.Pro72Arg	No
59	698	F	50	T4	G1	15	AA	CT	CC	GA	NM	No
60	704	F	42	T4	G3	25	AG	TT	CT	GG	NM	Yes
61	705	F	43	T3	G1	10	AA	CC	CC	GA	NM	No
62	706	M	56	T4	G1	18	AA	CC	CC	GA	NM	No
63	712	M	56	T4	G2	36	GG	CC	CC	GG	NM	No
64	713	F	60	T3	G2	40	AG	TT	CC	GA	NM	NA
65	722	F	50	T3	G1	31	AA	CC	CT	GA	NM	No
66	727	F	70	T3	G1	30	AG	CT	CC	GG	NM	Died
67	729	F	62	T4	G2	20	AA	CC	CC	GG	NM	Died
68	730	F	61	T4	G1	28	AG	CC	CC	GG	NM	No

69	734	F	65	T4	G1	35	AG	CC	CC	GG	NM	NA
70	737	M	21	T3	G1	11	AA	CT	CC	GA	NM	No
71	738	M	65	T3	G2	58	GG	CC	CT	GG	NM	No
72	739	F	50	T3	G1	25	AG	CC	CC	GA	NM	NA
73	743	M	27	T4	G1	10	AG	CC	CC	GA	NM	No
74	747	M	38	T4	G1	22	AA	CC	CC	GG	NM	No
75	748	F	38	T4	G1	28	AG	CC	CC	GG	NM	Died
76	749	F	50	T4	G1	25	AG	CC	CC	GG	NM	No
77	750	F	50	T4	G2	31	AA	CC	CT	GA	NM	No
78	751	F	61	T3	G1	40	AG	CC	CC	GA	NM	NA
79	755	F	51	T4	G1	30	AA	CC	CC	GG	NM	No
80	773	F	39	T2	G1	40	AG	CC	CC	GA	NM	No
81	774	F	50	T4	G1	30	AG	CC	CC	GA	NM	Yes
82	781	M	39	T3	G2	29	AG	TT	CC	GG	NM	No
83	796	F	45	T2	G1	20	AA	CT	CC	GG	NM	Yes
84	797	F	60	T4	G1	40	AG	CC	CT	GG	p.S260A	No
85	798	F	64	T4	G1	44	AA	CC	CT	GG	NM	Yes
86	799	M	40	T4	G1	20	AG	CC	CC	GG	NM	No
87	800	F	40	T2	G1	23	AA	CC	CC	GA	NM	No
88	801	F	40	T4	G1	20	AA	CC	CC	GA	NM	No
89	809	F	48	T4	G2	32	AG	CC	CT	GA	NM	No
90	810	F	70	T4	G1	40	GG	CT	CC	GG	NM	No
91	811	F	65	T4	G2	45	GG	CT	CC	GG	NM	No
92	812	F	50	T4	G1	25	AA	CT	CT	GA	NM	No
93	825	M	50	T4	G1	10	AA	CC	CC	GG	NM	No
94	826	F	46	T4	G1	30	AG	CC	CC	GG	NM	No
95	827	F	59	T3	G2	10	AA	CC	CC	GA	NM	No
96	829	F	50	T4	G2	23	AG	CT	CC	GG	NM	Yes
97	830	F	55	T4	G1	35	GG	CC	CC	GG	NM	No
98	831	F	70	T4	G1	40	AG	CC	CC	GG	NM	No
99	832	F	45	T4	G2	32	AG	CC	CC	GG	NM	Died
100	846	F	48	T4	G1	34	AA	CT	CC	GG	NM	No

* Years; NM: No Mutation

Contributions to literature

Publications:

1. Rajesh D, Mohiyuddin SMA, Kutty AVM, Balakrishna S. Prevalence of Human Papilloma Virus in Oral Squamous Cell carcinoma: A rural teaching hospital based cross-sectional study. Indian J Cancer. 2017;54(3):498-501.

2. Rajesh D, Balakrishna S, Azeem Mohiyuddin SM, Suresh TN, Moideen Kutty AV. *GSTP1* c.341C>T gene polymorphism increases the risk of oral squamous cell carcinoma. Mutat Res. 2018;831:45-49.

3. Rajesh D, Balakrishna S, Mohiyuddin SMA, Suryanarayana R, Kutty AVM. Novel association of oral squamous cell carcinoma with *GSTP1* Arg187Trp gene polymorphism. Journal of Cellular Biochemistry. 2018 Oct 30. doi: 10.1002/jcb.27877.

4. Rajesh D, Mohiyuddin SMA, Balakrishna S, Kutty AVM. Nicotinic acetylcholine receptor gene polymorphism is not associated with tobacco related oral squamous cell carcinoma. Indian Journal of Cancer, 2018 (Accepted).

5. Mutational spectrum of *TP53* gene in HPV negative oral squamous cell carcinoma patients with tobacco chewing habit (Manuscript under preparation).

Patents:

1. Deepa Rajesh, Sharath Balakrishna, AVM Kutty, Azeem Mohiyuddin SM. Method of determining cancer risk using SNPrs45549733. App. No. 201841025451.

2. Deepa Rajesh, Sharath Balakrishna, AVM Kutty, Azeem Mohiyuddin SM. Single Nucleotide Polymorphism rs1138272 located in *GSTP1* gene as a marker for genetic susceptibility to oral squamous cell carcinoma. App. No. 201741031364.

MOLECULAR STUDIES ON ORAL SQUAMOUS CELL CARCINOMA

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by

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Chapter IX

Summary and conclusions

- This case-control study was carried out by recruiting 100 OSCC patients and 200 healthy individuals. Genomic DNA was used for genetic analysis and formalin fixed tumor specimen was used for expression studies. Genomic DNA was prepared from the surgically resected tumor specimens of OSCC patient and from the peripheral blood of healthy individuals.

- Five experiments were carried out with the genomic DNA samples. First one, a screening study of the patient DNA sample for HPV infection followed by evaluation of all the three functional SNPs in *GSTP1* gene *viz*., Ile105Val, Ala114Val, and Arg187Trp for its association with OSCC. The third experiment was designed to evaluate the expression of GST-pi in the tumor specimen by IHC and its correlation with the three SNPs. The fourth experiment was to examine the functional SNP in *CHRNA5* gene *viz*., Asp398Asn and its association with the development of OSCC. The fifth one was to explore the tumor DNA for mutations in the *TP53* gene.

- The unique feature of this study was the predominance of women patients presenting with advanced stages of OSCC (T_3 and T_{4a}). All the patients included in the study were found to be habituated with tobacco chewing. A small fraction of the patients also used smokable tobacco and alcohol. None of the tumor samples were positive for HPV. These observations led us to the conclusion that tobacco use, mainly in the chewable form, and not HPV as the major risk factor in the development of OSCC.

- The three functional SNP analysis in *GSTP1* gene *viz.,* Ile105Val, Ala114Val, and Arg187Trp resulted in two SNPs Ala114Val and Arg187Trp associated with the predisposition to OSCC. No association was found with Ile105Val. Pair-wise linkage disequilibrium test indicated that joint association of Ala114Val and Arg187Trp were in weak linkage disequilibrium. Covariate analysis indicated that longer duration of tobacco use was associated with tumor recurrence. These observations led to the conclusion that Ala114Val and Arg187Trp were associated with the predisposition to OSCC further suggesting an important role of detoxification pathway in carcinogenesis.

- Evaluation of GST-pi enzyme in tumor tissue by immunohistochemistry indicated its expression in the tumor tissue but not in the surrounding normal tissue. The data did not yield any association between the GST-pi expression level and *GSTP1* SNPs.

- Similarly functional SNP analysis in nicotinic acetylcholine receptor gene viz., *CHRNA5* Asp398Asn resulted in a similar frequency of the minor allele in both patient and control groups indicating that this SNP might not be associated with the risk of OSCC. This observation suggested that the common variant associated with nicotinic acetylcholine receptor associated with lung cancer and smoking addiction and was not associated with smokeless tobacco linked OSCC. The lack of association may be a suggestion of differences in the mechanistic underpinnings of smokable and smokeless tobacco-related cancers.

- Mutational analysis of all the coding exons (2 – 11) of the *TP53* gene resulted in 11.7 % of the mutation frequency. All the mutations were located in the DNA-binding domain. No mutations were observed in the NLS and NES motifs.

New knowledge generated

- Reaffirm the importance of tobacco use particularly in the smokeless (chewable) form in oral carcinogenesis.

- Human papillomavirus infection may not be an important risk factor for oral carcinogenesis in rural patients.

- Functional variants in the *GSTP1* gene (Ala114Val and Arg187Trp) are associated with the predisposition to oral carcinogenesis.

- Association of *GSTP1* Arg187Trp with the predisposition to oral carcinogenesis is modified by tobacco use.

Limitations of the study

- The sample size was sufficient to obtain a power of > 80 % for the association with SNPs in the *GSTP1* gene. However, the sample size was insufficient to confirm the non-association observed in the case of SNP in the *CHRNA5* gene.

- Tobacco use was not a common risk habit in the control group. The magnitude of tobacco use among oral cancer patients was not known at the start of the study. Therefore, tobacco use was not a criterion in the selection of healthy volunteers for the control group. A second control group comprising of a healthy individual with an addiction to tobacco use could have been included to elucidate the role of addiction in a genetic association.