# Genetic polymorphisms of Xenobiotic Metabolizing Enzymes (XMEs), tobacco habit and susceptibility to oral cancer among citizens of Karachi.

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**ABSTRACT**

**Objective:** To detect polymorphisms of CYP1A1, GSTM1 and GSTT1 gene loci among various tobacco habit groups and to relate these with susceptibility of individuals to oral squamous cell carcinoma.
**Study Design:** A cross-sectional case-control study.
**Place and Duration:** Department of Pathology, Ziauddin University, Karachi from 5th January 2012 to 1st January 2017.
**Methodology:** The study comprises of 140 histologically confirmed oral squamous cell carcinoma cases and 98 habit-matched controls. Life-time tobacco-exposures were calculated as tobacco chewing and smoking indices for evaluation between cases and controls. Routine histopathology was followed by molecular analysis by employing Polymerase Chain Reaction (PCR) and PCR-Restriction Fragment Length Polymorphism (RFLP) techniques.
**Results:** The CYP1A1MspI heterozygous variant showed no significant association with oral cancer. The homozygous variant contributed an enhanced risk (OR=2.36, 95% CI, 1.0-6.20). When detected in exclusive chewer's category, the risk and hence the OR increased to 7.2, 95% CI, 1.8-27.5. The risk conferred was further increased in the above median tobacco intake group, OR=26, 95% CI, 2.2-304.5. For GSTM1 null genotype, no significant association was observed. However, the GSTT1 null polymorphism revealed an overall OR=6.63, 95% CI, 1.49-29.4, independent of presence or absence of tobacco as an environmental insult.
**Conclusion:** Two out of three xenobiotic metabolizing enzyme genes evaluated in this study, namely CYP1A1 MspI and GSTT1, significantly affected oral cancer risk and particular tobacco exposure seems to modify this risk in the former.
**Keywords:** Polymerase chain reaction, Restriction fragment length polymorphism, Habit-matched controls, Tobacco indices, Xenobiotic metabolizing enzyme, Oral squamous cell carcinoma.

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## INTRODUCTION

By virtue of their genotype for enzymes involved in tobacco metabolism, certain individuals are more susceptible to cancer formation when exposed to tobacco products[1]. This could possibly explain why only a small number of persons among those addicted to tobacco develop cancer in their lifetime. There is well documented inter-individual variation in toxicity responses connected to enzyme activity and expression of genes involved in xenobiotic metabolism by theses xenobiotic metabolism enzymes (XMEs)[2].

Cytochrome P450 (CYP) family of genes especially CYP1A1 and Glutathione S-transferases (GSTs) have been the most implicated enzyme systems that are involved in tobacco metabolism and hence generation of related potential carcinogens. Although, a sizable number of studies have analyzed mutational polymorphisms in CYP1A1, GSTM1 and GSTT1 genes, either singly or in various combinations, and their association with oral squamous cell carcinoma (OSCC) but the results documented therein were quite variable[3,4].

Enzymes coded by CYP1A1 gene are involved in the activation of pro-carcinogens in several human tissues[5]. Studies have evaluated the association between genetic aberrations of CYP1A1 gene, especially its MspI polymorphism, and oral cancer risk. The stated frequencies of CYP1A1 MspI homozygous genotype remained between 0-30% for oral cancer and 0-10.5% for matched-controls[4]. The risk has been reported among

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Indians for the mixed tobacco-habit group as depicted by Odds Ratios (ORs) of 3.2 for Oral squamous cell carcinoma (OSCC) cases and 4.29 for oral pre-cancerous lesions (PCLs) carrying the homozygous variant[6]. Meta-analysis on oral cancers reported an overall OR of 1.45, 95% CI = 1.15 – 1.83, P<0.05 for this gene variant[7].

Glutathione-S-Transferases (GSTs) are phase II biotransformation enzymes responsible for detoxification of phase I-derived substrates by conjugation. Null genotypes for GSTs results in reduced detoxification and increases susceptibility to cancers[8]. An increased risk to OSCC has been found among Iranian population carrying GSTM1 null genotype by an OR of 2.6, 95% CI = 1.04-6.5; p=0.01[9]. Contrary to this, a reduced risk by an OR of 0.31, 95% CI = 0.07-1.27, to hypopharyngeal cancer among heavy smokers by this gene variant has been reported from Japan[10]. Zakiullah and co-researchers, documented in the Pashto-speaking population of Pakistan an increased risk conferred by an adjusted OR of 3.019, 95%CI = 1.861-4.898[3].

For GSTT1 null allele an increased risk to OSCC has been found among Iranian population carrying this genotype by an OR of 11, 95% CI = 9-31; p = 0.001[9]. The same null allele posed a reduced risk by an OR of 0.42, 95% CI = 0.10-1.82, to hypopharyngeal cancer among heavy smokers in Japanese study[10]. The study by Zakiullah and co-researchers demonstrated an increased risk by an adjusted OR of 3.011, 95%CI = 1.865-4.862.[3] The study from north-east India by Ihsan and co-researchers interestingly showed that smoking conferred a reduced risk to lung cancer in those having GSTT1 null genotype, OR=0.44, 95% CI = 0.25-0.79, as compared to normal genotype[11].

In our study we aimed to detect polymorphisms of CYP1A1, GSTM1 and GSTT1 gene loci among various tobacco habit groups and to relate these with susceptibility of individuals to oral squamous cell carcinoma. Ours is the very first study of its kind that has been conducted on citizens of Karachi, one of the highest reported incidence areas for oral cancer in the World, as regards to genes involved in tobacco metabolism. Here there is a strong association with much prevalent chewable forms of tobacco. Identification of "at risk genotypes" will have substantial preventive implications in terms of persuading these individuals to quit tobacco use. At the level of society, this type of categorization will save already meagre resources by directing preventive programs towards smaller groups rather than involving large population requiring huge financial resources to be successful. This study was conducted with an objective to detect polymorphisms of CYP1A1, GSTM1 and GSTT1 gene loci among various tobacco habit groups and to relate these with susceptibility of individuals to oral squamous cell carcinoma.

## METHODOLOGY

The cross-sectional case-control study was conducted at the Department of Pathology, Ziauddin University (ZU), Karachi, from 5th January 2012 to 1st January 2017. The subjects were enrolled from the Oncology Wards in Ziauddin University Hospitals Karachi, the Ear-Nose-Throat (ENT) Wards of Ruth Pfau Civil Hospital Karachi and by setting up camps (for controls) in diverse localities of the city.

Approval from the ethics review boards of Ziauddin University (ZU) and Dow University of Health Sciences (DUHS), Ref#: IRB-517/DUHS/-14; ZU-ERC dated 15.8.2010, has been achieved. The sample size was calculated using Epi-Info calculator version 6.0.[12] at 10% precision and design effect is 2. Non-probability purposive sampling was done including histologically-confirmed cases of OSCC and tobacco habit-matched controls. Inclusion criteria was age >10 years, histologically confirmed OSCC (for cases), no prior history of any cancer, including oral cancer (for controls), and the tobacco habit that approximately matches in duration and frequency among both cases and controls. Exclusion criteria was age <10 years, cancer of any site other than oral cavity, any other serious disease and subjects who did not give consent.

For exploring the interaction between the type/mode of tobacco consumed and gene polymorphisms, both patients and controls were segregated into groups as; exclusive chewers, exclusive smokers, mixed habit (smokers plus chewers), and no-habit (absence of tobacco use). Life-time tobacco exposure to the oral mucosa was calculated as chewing/ smoking indices in all studied subjects.

After signing of consent form, 5 ml blood sample was collected in sterile glass tubes containing ethylene-diaminetetra acetic acid (EDTA). Deoxyribonucleic acid (DNA) was retrieved from the blood using the Kit-method (PureLink® Genomic DNA Kits for DNA purification manufactured by Invitrogen Life Technologies, Carlsbad USA. Cat. # K182001) from whole blood-lymphocytes samples and was stored at -80º Celsius.

Polymorphisms of target genes were tested as under.

(a) CYP1A1MspI polymorphism (Gene location 15q24.1):

A 340bp fragment was amplified via PCR from exon-7 of CYPIAI containing the polymorphic region as defined formerly[13].

MspI (Hpall, Thermo Scientific) restriction enzyme was used to digest the PCR product for 12hrs at 37ºC.[7] Subsequently, the digested product was loaded on agarose gel stained with ethidium bromide and subjected to UV analysis. In the presence of MspI restriction site the original 340 bp fragment was spliced into 200 bp and 140 bp fragments. On the gel electrophoresis polymorphisms were identified as under:

1. If only one unsliced 340 bp band → Wild type (m1/m1).
2. If three bands of 340 bp, 200 bp & 140 pb → Heterozygous variant (m1/m2).
3. If only two bands of 200 bp and 140 bp → Homozygous variant (m2/m2).

(b) GSTM1 null polymorphism (Gene location 1p13.3):

The GSTM1 deletion polymorphism was identified by amplification of a 219bp fragment with specific primers as described previously[13].

(c) GSTT1 null polymorphism (Gene location 22q11.23):

The GSTT1 deletion polymorphism was identified by amplification of 480bp fragment with specific primers as described previously[13].

After PCR amplification, the products of GSTM1 and GSTT1 were subjected to agarose gel electrophoresis which was stained with

ethidium bromide for identification under UV-light. The presence or absence of a band at specified region corresponded to the presence or absence of GSTM1 and GSTT1 alleles.

**Data Analysis:** Demographic data was analyzed by descriptive statistics. Genotypes variants of CYP1A1, GSTM1 and GSTT1 were distributed among different habit groups and results are expressed as percentage of total number of cases from each category. Odds ratios were calculated while the precision of odds ratios was adjusted by 95% Confidence Interval (CI). The risk of oral cancer due to studied genes was determined by binary logistic regression model with CYP1A1 MspI wild type (m1/m1), GSTM1 not null and GSTT1 not null considered as the reference category. Multi-variant analyses were performed to understand gene–environment interactions. SPSS (Statistical program for social sciences) software version 20 was used for the statistical analysis of data. Chi square test was applied for the determination of significance. The significance level was considered as $p<0.05$.

## RESULTS

The study comprises of 238 subjects among them 140 histologically confirmed oral squamous cell carcinoma cases and 98 tobacco-habit-matched controls. Table-I gives the distribution of CYP1A1 MspI, GSTM1 and GSTT1 gene variants in oral cancer cases and controls. The frequency distribution of CYP1A1 MspI m1m2 and m2m2 variants was found to be 62.85% and 18.57% among OSCC cases and 62.24% and 11.22% in controls, respectively. The distribution of GSTM1 null variant was found to be 30.71% and 31.63% in cases and controls, respectively. The GSTT1 null variant was seen in 12.14% of cancer cases and 2.04% of controls. The numbers of GSTT1 null and CYP1A1 m2/m2 (homozygous) gene variants were appreciably higher in cancer patients as compared to controls with p-Values ≤ 0.05. The frequency of GSTM1 null and CYP1A1 m1/m2 (heterozygous) variants in controls approximates that of cancer cases.

The overall distribution of these genetic variants, as depicted by respective ORs, points towards a possible influence on the occurrence of OSCC (Figure-1). The CYP1A1 m2m2 variant and GSTT1 null genotypes amplified oral cancer risk by revealing ORs of 2.36 and 6.63, respectively. On the contrary, GSTM1 showed a trend towards protection with an OR of 0.95. However, for this gene results remained statistically insignificant (p-Values > 0.05).

The study also evaluated the risk conferred by these genes according to the nature of tobacco exposure. Table-II and figure-2 summarize results of genotype-tobacco interactions by giving the exact number of cases for each of the tested polymorphisms in subjects, both cases and controls, which were engaged in different types of tobacco habits. The presence of CYP1A1 MspI heterozygous (m1/m2) genotype and oral cancer showed no significant association. However, the homozygous (m2/m2) variant contributed an overall increased risk of oral cancer among all habit groups with an OR of 2.36 (95% CI, 1.0-6.2).

However, the GSTT1 null polymorphism revealed an enhanced risk for OSCC with an overall OR of 6.63 (95% CI, 1.49-29.4) independent of any type of tobacco exposure (Table-I).When the same observed in the exclusive chewer's category, the risk and hence the OR increased to 7.2 (95% CI, 1.8-27.5). Upon further dividing the tobacco chewer's group into above and below median exposure categories, the risk was further increased as an OR=26 (95% CI, 2.2-304.5) for the above median exposure group (Table-II). For GSTM1 null genotype, no significant association was observed with OSCC in any of the tobacco exposure category.

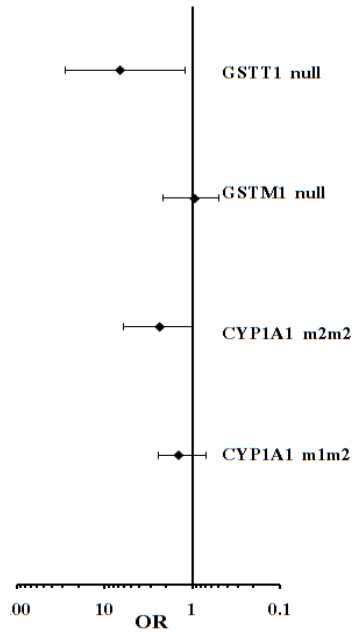


**Figure-1: Odds Ratios (ORs) with 95% CI for CYP1A1 MspI, GSTM1 and GSTT1 genotype variants among OSCC patients (n=140) and controls (n=98) with significance. (N=238)**

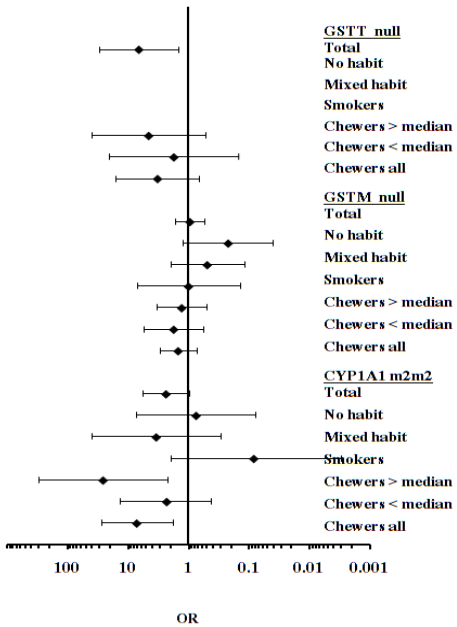


**Figure-2: Odds Ratios (ORs) with 95% CI for genotypes among OSCC patients (n=140) and controls (n=98) divided into different tobacco- exposure groups with significance. (N=238)**

**Table-I: Genotype variants in OSCC cases and controls (N=238)**

| Genotype | | Cancer (n = 140) | Control (n = 98) | CI (95%) | OR | p-Value |
|---|---|---|---|---|---|---|
| CYP1A1 | m1/m1 | 26 (18.57%) | 26 (26.53%) | | | |
| | m1/m2 | 88 (62.85%) | 61 (62.24%) | (0.71-2.52) | 1.44 | 0.26 |
| | m2/m2 | 26 (18.57%) | 11 (11.22%) | (1.0-6.20) | 2.36 | 0.05 |
| GSTM1 | Not null | 97 (69.28%) | 67 (68.36%) | | | |
| | Null | 43 (30.71%) | 31 (31.63%) | (0.51-2.22) | 0.95 | 0.91 |
| GSTT1 | Not null | 123 (87.85%) | 96 (97.95%) | | | |
| | Null | 17 (12.14%) | 2 (2.04%) | (1.49-29.4) | 6.63 | 0.01 |

Abbreviations: OSCC Oral Squamous cell carcinoma; n Number of cases; CI Confidence interval

**Table-II: Genotype distribution among different tobacco exposure groups for oral cancer and controls (N=238)**

| Genotype | Tobacco exposure | Genotype/ Ref. | Control (n = 98) | Cancer (n = 140) | CI (95%) | OR | p- Value |
|---|---|---|---|---|---|---|---|
| CYP1A1 m2/m2 | Chewers | m2/m2 | 4 | 18 | | | |
| | Ref. | m1/m1 | 16 | 10 | 1.8-27.5 | 7.2 | 0.003 |
| | < median | m2/m2 | 3 | 5 | | | |
| | Ref. | m1/m1 | 10 | 7 | 0.42-13.38 | 2.3 | 0.32 |
| | > median | m2/m2 | 1 | 13 | | | |
| | Ref. | m1/m1 | 6 | 3 | 2.2-304.5 | 26 | 0.009 |
| | Smokers | m2/m2 | 4 | 1 | | | |
| | Ref. | m1/m1 | 1 | 3 | 0.0035-1.94 | 0.08 | 0.12 |
| | Mixed habits | m2/m2 | 1 | 4 | | | |
| | Ref. | m1/m1 | 6 | 7 | 0.29-39.6 | 3.42 | 0.32 |
| | No habit | m2/m2 | 2 | 3 | | | |
| | Ref. | m1/m1 | 3 | 6 | 0.078-7.20 | 0.75 | 0.81 |
| | Total | m2/m2 | 11 | 26 | | | |
| | Ref. | m1/m1 | 26 | 26 | 0.97-5.7 | 2.36 | 0.057 |
| GSTM1 null | Chewers | Null | 16 | 28 | | | |
| | | Not-null | 47 | 55 | 0.72-3.09 | 1.49 | 0.28 |
| | < median | Null | 6 | 11 | | | |
| | | Not-null | 24 | 25 | 0.56-5.51 | 1.76 | 0.33 |
| | > median | Null | 10 | 17 | | | |
| | | Not-null | 23 | 30 | 0.50-3.37 | 1.30 | 0.59 |
| | Smokers | Null | 4 | 4 | | | |
| | | Not-null | 4 | 4 | 0.14-7.0 | 1 | 1 |
| | Mixed habits | Null | 6 | 5 | | | |
| | | Not-null | 13 | 22 | 0.12-1.93 | 0.49 | 0.31 |
| | No habit | Null | 5 | 6 | | | |
| | | Not-null | 3 | 16 | 0.04-1.24 | 0.22 | 0.08 |
| | Total | Null | 31 | 43 | | | |
| | | Not-null | 67 | 97 | 0.54-1.67 | 0.95 | 0.88 |
| GSTT1 null | Chewers | Null | 2 | 8 | | | |
| | | Not-null | 61 | 75 | 0.66-15.88 | 3.25 | 0.14 |
| | < median | Null | 1 | 2 | | | |
| | | Not-null | 29 | 33 | 0.15-20.39 | 1.75 | 0.66 |
| | > median | Null | 1 | 6 | | | |
| | | Not-null | 32 | 42 | 0.52-39.87 | 4.57 | 0.16 |
| | Smokers | Null | 0 | 1 | | | |
| | | Not-null | 8 | 7 | N/R | | |
| | Mixed habits | Null | 0 | 7 | | | |
| | | Not-null | 19 | 20 | N/R | | |
| | No habit | Null | 0 | 1 | | | |
| | | Not-null | 8 | 21 | N/R | | |
| | Total | Null | 2 | 17 | | | |
| | | Not-null | 96 | 123 | 1.49-29.4 | 6.63 | 0.01 |

Abbreviations: n Number of cases, CI Confidence interval, OR Odds Ratio.

## DISCUSSION

Studies conducted on head and neck (HNC) and lung cancers have been consistently documenting the interaction between genotypes of carcinogen-metabolizing enzymes and environmental factors like tobacco in modulating cancer risk[14-16]. Our results indicate that CYP1A1MspI homozygous variant enhance risk to oral cancer in the presence of tobacco habit as

an environmental trigger, GSTM1 null genotype does not separately enhance risk to OSCC, while GSTT1 null polymorphism surfaced as an independent risk factor conferring a significant increased risk to oral cancer and this risk was not associated with tobacco exposure in any of its forms.

In the current study we observed for the CYP1A1 MspI gene a lower percentage of wild-type genotype and higher percentages for polymorphisms, both heterozygous and homozygous forms, when compared to the variants reported by Zakiullah and co-researchers from KPK province of Pakistan mainly involved in the tobacco habit of naswar (a chewable tobacco product containing a mixture of raw tobacco with slacked lime which causes erosion of oral mucosa thereby enhancing tobacco absorption).[3] Proportions of homozygous variant for the CYP1A1 MspI gene in our series among both oral cancer cases and controls remains in confirmation with ten studies included in the meta-analysis by Xie and colleagues[4]. The same variant imparted enhanced oral cancer risk particularly among tobacco chewers which is even higher to the one reported in the north-east Indian population[16]. However, heterozygous variant of the same gene we observed in higher frequency than studies included in the meta-analysis[4].

These observations of ours put some light on the high prevalence of OSCC in our study population especially in individuals using tobacco which acts as a risk modulator and hence suggesting the gene-environment interaction. Secondly, our data also partly explains increased frequency of oral cancer out of different genetic inheritance of the Urdu-speaking ethnicity of Karachi comprising mostly of immigrants from northern and central parts of Indian Subcontinent and has been engaged in smokeless tobacco (SLT) consumption. In contrast the KPK province population has been traditionally linked to the Central Asian and European descent[17].

In our series, the frequency of GSTM1 null genotype among control subjects was found to be almost half of what has been reported previously for Pakistani and Indonesian Malay population[3,18]. However, these still remained within the range of 17-38% as reported from neighboring India[14,19]. We further observed a decreased overall OR for cancers for this polymorphism, meaning a protective effect of this genotype, but the results lacked statistical power. The GSTM1 null polymorphism resulting from homozygous deletion causes functional loss of GSTM1 enzyme[20]. This deletion leads to the loss of detoxification and may thus result in a higher oral cancer risk, especially in tobacco users. Previously studies have reported for the GSTM1 (null) genotype to be significantly associated with an increased risk of oral squamous cell carcinoma[21]. However, we do not found any statistically significant relationship between GSTM1 null polymorphism and OSCC risk not only in our overall cancer cases but also in neither of tobacco habit groups.

GSTT1 null polymorphism has been either reported to pose no risk association with nasopharyngeal carcinoma or is documented as a protective genotype variant[6,22]. In the present series, the percentage of GSTT1 null polymorphism among control subjects was observed to be remarkably lower than what has been reported previously from Pakistan, India and Far-East[3,14,18,19]. However, in the current study this genotype among OSCC patients is not only much higher as compared to controls but also appeared as an independent risk factor conferring a significant risk to OSCC. The risk posed is almost double of what has been reported in tobacco chewers from the KPK province[3]. This can probably be explained by the different ethnic descent of our studied subjects in contrast to the above mentioned studies.

Our results of all three gene variants that are related to tobacco metabolism contribute to the existing information on the variability of CYP1A1 MspI, GSTM1 null and GSTT1 null polymorphism worldwide, emphasizing the relationship between ethnic origin, tobacco use and oral cancer.

## CONCLUSION

Two out of three xenobiotic metabolizing enzyme genes evaluated in this study, namely CYP1A1 MspI homozygous variant and GSTT1, significantly affected oral cancer risk and particular tobacco exposure seems to modify this risk in the former.

**Recommondations:**

1. Ban on the sale, manufacture, storage, advertising, sponsorships and promotion of all tobacco based chewable products.
2. Identification of at risk population through genotyping followed by vigorous tobacco cessation campaigns to reduce oral cancer mortality with carefully planning, new research projects and targeted utilization of financial resources.
3. Confounding effects of additives that are mixed with tobacco in various tobacco products needs to be evaluated in future researches over similar population.

## AUTHOR'S CONTRIBUTION

**Alamgir MM**: Conceived idea, Designed research methodology, Data collection, Molecular analysis, Data interpretation, Statistical analysis, Manuscript writing.
**Mirza T:** Data interpretation, Proof reading of manuscript
**Jamal Q:** Data collection, Statistical analysis, Proof reading of manuscript.

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