ORIGINAL ARTICLE

Role of Tobacco Metabolism in the Causation of Oral Squamous Cell Carcinoma in a High-Incidence Area of South Asia

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ABSTRACT

Objective: To establish the association between *CYP1A1 Msp*I polymorphism, tobacco-habit and oral cancer.

Methodology: 150 Oral squamous cell carcinoma (OSCC) and 108 controls were enrolled, comprising of individuals without and with tobacco habits which match in frequency and duration with patients. Study subjects were divided into four groups, namely: exclusive chewers, exclusive smokers, mixed-habit and no habit. Lifetime tobacco exposure was calculated as chewing and smoking index. After age adjustment, 140 OSCC cases and 90 controls were subjected to genetic analysis. White blood cells were used for DNA isolation while *CYP1A1 Msp*I polymorphism was detected with the PCR-RFLP technique. Three polymorphisms were tested namely wild type, heterozygous variant and homozygous variants. Odds Ratios (ORs) were calculated while the precision of ORs was adjusted by 95% confidence interval (CI). The risk was determined by binary logistic regression model with *CYP1A1* m1/m1 as the reference category.

Results: Out of all 258 studied subjects, 60.85% subjects were exclusive tobacco chewers which turned out to be the most prevalent tobacco habit. Cheek was the most common site (56%) followed by tongue (21%). The frequencies *CYP1A1Msp*I wild-type, heterozygous and homozygous variants were found to be 18.57%, 62.85% and 18.57% among OSCC cases and 26.53%, 62.24% and 11.22% in controls. The homozygous (m2/m2) variant of *CYP1A1Msp*I conferred an increased risk to OSCC with an OR of 2.36 (95% CI, 1.0-6.20, p=0.05). OR further increased to 7.2 (95% CI, 1.8-27.5, p=0.003) when considered in exclusive tobacco chewer's and 26 (95% CI, 2.2-304.5, p=0.009) in the above median exposure group.

Conclusion: Present analysis showed a clear association between *CYP1A1Msp*I polymorphism and the increased risk for oral cancer and this risk seems to be tobacco modulated. Hence *CYP1A1Msp*I homozygous genotype could be a major determinant of high rates of oral cancer in the indigenous population of Karachi.

Keywords: Oral squamous cell carcinoma, Pre-cancerous lesions, Gene polymorphisms, *CYP1A1Msp*I.

INTRODUCTION:

Oral squamous cell carcinoma (OSCC) ranks as the eight most frequent malignancy globally but the incidence in developing countries like Pakistan, India and Sri Lanka are very high[1]. In Pakistan it ranks as the second most frequent cancer in adults constituting 6.69% of all malignancies[2]. Use of smokeless tobacco (SLT) products has been the most prevalent mode of consumption in our region and varies from chewing relatively pure tobacco to a mixture of tobacco with additives such as seen in products like Paan (quid), Naswar, Pan-masala, Gutka, Khaini, and Mishri, etc[3,4].

There are more than 30 known carcinogens in various tobacco products[5]. Three major classes include tobacco specific nitrosamines (TSNAs), Poly Aromatic Hydrocarbons (PAHs), and aromatic amines[6]. These compounds are procarcinogens and need metabolism by Xenobiotic Metabolizing Enzyme Systems (XMEs) for conversion into carcinogens. XMEs such as Cytochrome-P450 (CYPs) are involved in their bio-activation to carcinogenic species while Glutathione-S-transferases (GSTs) cause their detoxification[7].

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Received: 13-09-2017
Revised: 27-09-2017
Accepted: 11-10-2017

The enzyme P4501A1 or *CYP1A1* encodes for the aryl hydrocarbon hydroxylase involved in the activation of PAHs and aromatic amines. It is expressed in the oral tissue[8]. Genetic polymorphisms affect its expression levels. One base substitution of thymine by cytosine in a non-coding region of the gene at position 3801 creates an *Msp*I recognition site (*CYP1A1*2A)*, which does not exist in the wild type genotype[9]. Among all polymorphisms, *CYP1A1Msp*I is the most common and is associated with increase in enzyme activity and hence generation of more carcinogenic moieties[10,11]. This polymorphism results in three genotypes: wild-type (m1/m1), heterozygous variant (m1/m2) and homozygous (m2/m2) variant[12].

Due to variable expression profile for XMEs, polymorphisms of their respective genes can alter the cancer risk posed by tobacco-related carcinogens. Interactions between genotype and environment exposures have long been postulated and studied, however, it has assumed great significance for Pakistan in general and Karachi in particular because of the widespread tobacco use among its citizens and rising prevalence of OSCC.

A recently published meta-analysis on this association has been reviewed[13]. It comprises of 10 studies from 1999 to 2012[14-23]. The overall analysis suggested that the *CYP1A1 Msp*I gene variants (hetero and homozygous) impart an increased risk than those with wild type genotype especially among Asians. A study from the neighboring country of India, another region with a very high incidence of oral cancer associated

with smokeless tobacco (SLT) consumption, an increased risk has been reported with an OR of 3.2 among individuals having both tobacco smoking and chewing habits[24]. From northern province of Pakistan a study documented weak association of *CYP1A1* variants with OSCC [OR:1.121(0.717-1.752)]. The same study showed an increasing association if there is simultaneous presence of *GSTM1* and/or *GSTT1* null genotypes. Studied subjects were all naswar users while 30.5% were also smoking cigarettes[5].

METHODOLOGY:

A total of 150 clinically diagnosed and histologically confirmed cases of OSCC and 108 controls were initially enrolled comprising of individuals without and with tobacco habits which match in frequency and duration with patients. For the purpose of analyzing the interaction between the nature of tobacco exposure and genetic susceptibility, the study subjects were further divided into: *Exclusive chewers* – individuals who consumed tobacco only in the smokeless form either with or without additives such as betel nut and lime etc., *Exclusive smokers* – individuals who smoked tobacco in forms like cigarettes, bidis etc., *Mixed tobacco habitués* – individuals who consumed tobacco in both the smokeless form and were also smokers and *Habit free group* – individuals who reported lack of former or current consumption of tobacco in any form. Life-time exposure was calculated as chewing and smoking index among all tobacco users for both cases and controls as follows[24]:

Chewing index= Frequency of chewing events per day × Duration in years

Smoking index= Number of cigarettes/10 × Duration in years

Prior to this, age-adjustment was done so as to achieve a certain level of tobacco-habit matching by cutting down the original 150 OSCC and 108 control cases for statistical analysis to 140 and 98, respectively. Hence, mean and median lifetime exposures were determined. White blood cells were used for DNA isolation while employing the Kit-method. *CYP1A1 Msp*I polymorphism was detected with the PCR- RFLP (Restriction fragment length polymorphism) technique. A 340bp fragment of exon-7 of *CYP1A1* containing the polymorphic region was amplified by using PCR as described previously[25]. Primers sequence: Forward (5'-CAGTGAAGAGGTG TAGCCGCT-3'), Reverse (5'-TCCGTACTCTGTTCT GAGGATT-3') .

10µl reaction mixture contained 4.0 µl DNA, 1.5 µl each primer, 10X buffer 1µl Taq DNA polymerase 0.25µl, $MgCl_2$ 0.2 µl and H_2O 1.55µl.

Reaction was amplified using following thermal profile:Initial denaturation at 94ºC for 3 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 54ºC for 60 seconds and elongation at 72 ºC for 45 seconds and final elongation at 72 ºC for 5 minutes (Rotorgene Thermal Cycler). The PCR product was digested with *Msp*I (Hpall, Thermo Scientific) restriction enzyme for 12hrs at 37ºC[26].

After digestion, the *CYP1A1* amplified products were loaded on agarose gel stained with ethidium bromide and after electrophoresis observed under UV light. Presence of *Msp*I restriction site resulted in splicing of the original 340 bp *CYP1A1* fragment into two 200 bp and 140 bp fragments. On the gel each of the three polymorphisms were identified as:wild type (m1/m1) ⟶only one 340 bp band; heterozygous variant (m1/m2) ⟶three bands of 340 bp, 200 bp and 140 pb, respectively; and the homozygous variant (m2/m2)⟶ only two bands of 200 bp and 140 bp.

Odds Ratios (ORs) were calculated among cases and controls while the precision of odds ratios was adjusted by 95% confidence interval (CI). Mean age of patients was adjusted to exclude the aging factor as described previously. The risk (OR) was determined by binary logistic regression model with *CYP1A1* m1/m1 considered as the reference category.

RESULTS:

Ages of OSCC patients ranged from 20-78 years while that of controls from 15-87 years with mean ages being 47.1±12.22 and 41.6±14.58, respectively. Numbers of males/ females in cancer cases were 98/52 and in controls were 80/28, respectively (Table-1). 83.33% of OSCC patients and 92.5%% of controls gave a positive history of tobacco use. Out of all 258 studied subjects, 157 (60.85%) subjects were exclusive tobacco chewers which turned out to be the most prevalent tobacco habit. Exclusive smoking was seen in 6.2% cases while mixed habit of chewing plus smoking was present in 20.15% subjects. Cheek was the most common site for OSCC (56%) followed by tongue (21%). More than half of all OSCC cases (59%) were moderately differentiated cancers and 52% presented in advanced stages either III or IV (Figures 1& 2).

Table-1: OSCC Cases According to Age, Sex and Intraoral Sub-site

	Location within the oral cavity														
Age	Alveolar		Cheek		Floor		Lips		Palate		Retromolar		Tongue		**Total**
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	
11-20			1											1	2
21-30		1	6				3		1				2	1	14
31-40	1		20	1			3	1					5	4	35
41-50	1	1	18	11			2	4		1			4	4	46
51-60	5		10	9		1	2	1	1		1		4	2	36
61-70			3	3	1			3	1				2	1	14
71-80			1	1										1	3
Total	7	2	59	25	1	1	10	9	3	1	1	0	17	14	150

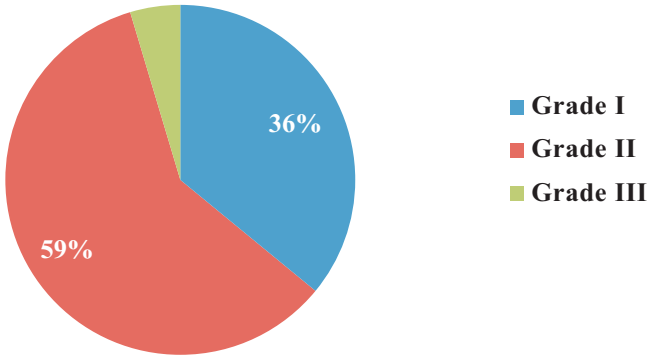

Figure-1: Proportion of OSCC cases according to histological grade

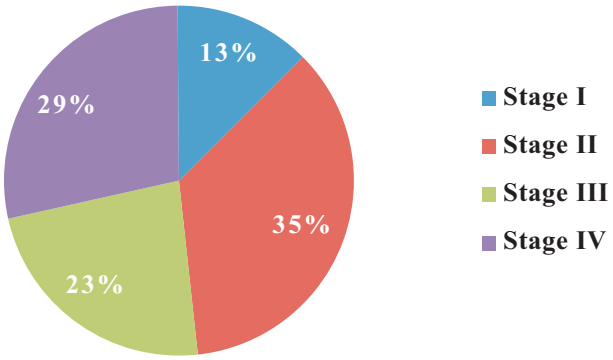

Figure-2: Distribution of OSCC cases based on clinical stage

The association between genotypes and the risk level for oral cancer was examined in individuals exposed to different tobacco habits. Table-2 and Fig.3 show tobacco indices for various tobacco habits as mean and median values. For all chewers, comprising of individuals with exclusive tobacco chewing habit plus those having chewing as a component part of mixed habit, mean and median values were comparable. However, among exclusive chewers, cancer cases reflected lower mean and median values as compared to controls. Again among all smokers, these values were comparable, while for exclusive smokers the median value was slightly higher in cancer cases.

Table-2: Tobacco indices in OSCC cases & controls

Tobacco Indices	Controls	Oral cancers
All chewers		
Mean±SE	279.01±25.65	264.27±26.96
Median	200	200
Exclusive chewers		
Mean±SE	277.46±29.0	230.17±28.01
Median	210	150
Smoking index		
All smokers		
Mean±SE	27.15±4.62	28.22±5.55
Median	21	20
Exclusive smokers		
Mean±SE	31.57±7.16	34.21±10
Median	30	40

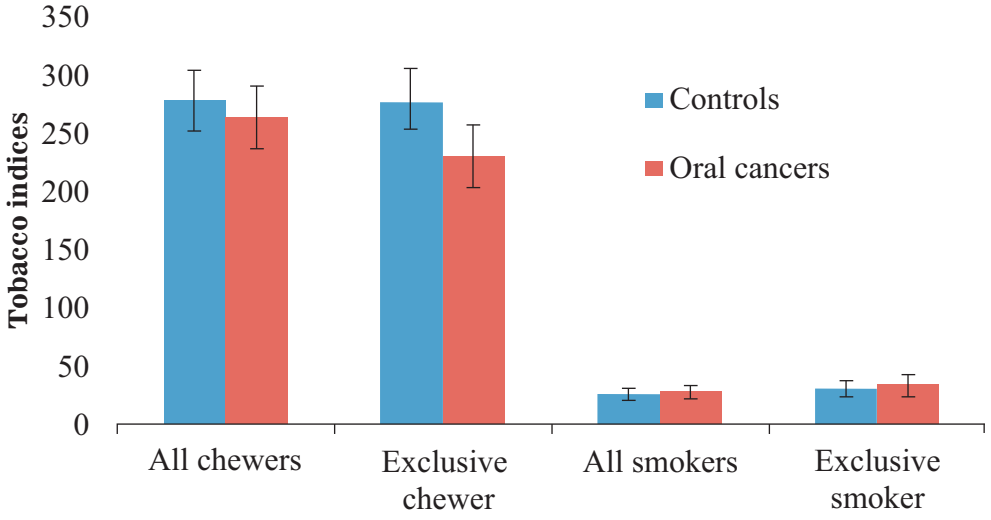

Figure 3.Mean tobacco indices in OSCC and controls

The frequency distribution of *CYP1A1Msp*I wild-type, heterozygous and homozygous variants was found to be 18.57%, 62.85% and 18.57% among OSCC cases and 26.53%, 62.24% and 11.22% in controls, respectively. The frequency of *CYP1A1* m1/m2 (heterozygous) variant in controls approximates that of cancer cases. However, the number of *CYP1A1* m2/m2 (homozygous) gene variant was appreciably higher in cancer patients as compared to controls with a significant p-value ($p<0.05$). Table-3 gives the ORs with 95% CI for this homozygous variant gene group when different tobacco habits were present.

Table-3: Risk analysis for different tobacco exposure groups

Genotype	Tobacco-habit Category	Homozygous variant in OSCC (n)	Homozygous variant in Controls (n)	Confidence Interval (CI) (95%)	Odds Ratios	P value
CYP1A1 m2/m2	Chewers	18	4	1.8-27.5	7.2	0.003
	< median exp.	5	3	0.42-13.38	2.3	0.32
	> median exp.	13	1	2.2-304.5	26	0.009
	Smokers	1	4	0.0035-1.94	0.083	0.12
	Mixed habits	4	1	0.29-39.6	3.42	0.32
	No habit	3	2	0.078-7.20	0.75	0.81
	Total	26	11	0.97-5.7	2.36	0.057

The homozygous (m2/m2) variant of *CYP1A1Msp*I conferred an increased risk to OSCC with an OR of 2.36 (95% CI, 1.0-6.20, p<0.05). When observed in the exclusive chewers Category, the OR increased to 7.2 (95% CI, 1.8-27.5, p=0.003). On dividing tobacco chewers into above and below median life-time exposure, the risk conferred was further increased to several folds for the above median exposure group (OR 26, 95% CI, 2.2-304.5, p=0.009).

DISCUSSION:

OSCC is a multi-factorial disease with evolution of tumor as an outcome of cumulative molecular events. These events are influenced by both individual's genetic predisposition as well as exposure to chemical carcinogens with a predominant role of tobacco exposure[27,28,29]. Previously contradictory reports existed about the association of *CYP1A1 Msp*I genetic aberration and oral cancer risk. Out of seven studies reviewed [14,24,30-33] , only three reported a risk association[14,24,30]. Devasena et al reported ORs of 3.2 for OSCC cases for the mixed-habit group among Indians harboring the homozygous variant of *CYP1A1Msp*I polymorphism[24]. In the current study, we have tried to evaluate the relationship between tobacco- metabolizing enzyme gene polymorphisms and oral cancer risk. This is the very first study of its kind that has been conducted on the indigenous population of Karachi which exhibits a very high incidence of this cancer. In our series, the percentages of *CYP1A1Msp*I wild-type, heterozygous and homozygous variants in the control group were quite low for the wild type genotype and high for the polymorphisms when compared to the variants reported by Zakiullah et al from naswar-consuming population from KPK province of Pakistan, i.e., 63.3% for wild type and 36.4% for polymorphisms[5]. The reported frequencies of *CYP1A1Msp*I homozygous variant allele ranged between 0-30.0% for OSCC cases and 0-10.5% for controls[13].

In the present study we observed *CYP1A1 Msp*I homozygous variant in 18.57% of cancer cases and 11.2% of controls. This is in confirmation to the previously reported data. However, the heterozygous variant (m1/m2) of the same gene we found in as much as 62.85% of our OSCC cases and in 62.24% of our controls. Both OSCC cases and controls in the present study had higher proportions of heterozygous variant than any of the ten studies included in the meta-analysis[13]. This may partly explain why our population has such a high prevalence of oral cancer especially in the presence of tobacco habit as the risk modulator, confirming the gene-environment equation. For the homozygous variant, an OR of 2.36 (95% CI, 1.0-6.20, p=0.05) was found and the result was statistically significant. A further increase in risk by homozygous variant was observed (OR=7.2) among exclusive tobacco chewers. When these individuals were stratified into above and below median exposure groups, the risk increased to several folds (OR=26) in the above median lifetime tobacco exposure category and the results were statistically significant. This observation becomes even more significant when we examine it in light of the fact that majority of OSCC cases (58%) in our series belonged to the exclusive tobacco chewer category. However, no statistically significant association was observed among exclusive smokers and mixed tobacco habitués (smoking plus chewing). There was a relatively small representation of tobacco smokers in our study population. This may partly be explained by an overall trend of reduction in cigarette smoking due to higher cost of this product in contrast to other chewable forms of tobacco.

In the current study we found grade II tumors as most rampant (59%) followed by grade I (36%). Only 5% of OSCC cases in our series were the most anaplastic grade III tumors. Other oral cancer treatment facilities from Karachi have variably reported grade-I tumors as 59.53%, 60%, 25%; grade-II tumors as 32.55%, 36%, 55% and, grade-III tumors as 7.9%, 4%, and 20%, respectively[37-39].

Metastatic involvement of neck lymph nodes determines the outcome of oral cancer patient better than any other prognostic factor. Advanced-stage OSCC cases, i.e. stage III/IV tumors, have been reported in proportions like 70%, 56.1% and 55.7% in different studies from Karachi city[39-41]. In our study clinical stage III and IV were 52% of all cases while in contrast to other studies stage II was found to be the largest group with 35% of all cases.

CONCLUSION:

In our study subjects the homozygous variant genotype of *CYP1A1Msp*I enhanced oral cancer risk only when there is a history of tobacco use, i.e., in exclusive tobacco chewers. This effect was found more pronounced among tobacco users having more than median life-time exposures, confirming the gene-environment equation.

RECOMMENDATIONS:

1) Organize awareness campaigns for general public that include genetic counseling for susceptibility genotypes in addition to tobacco-cessation drives.
2) Screening for susceptible genes should be planned by government health agencies to identify genetically vulnerable, high-risk ethnicities and sub-groups within the population.

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