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Research Article

CYP1A1 genotypes and haplotypes and risk of oral cancer: A case-control study in South Indians

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Abstract

The *CYP1A1* gene encodes for the enzyme, aryl hydrocarbon hydroxylase, which is involved in the biotransformation of various aromatic tobacco precarcinogens. In the present study, the association between *CYP1A1* gene polymorphisms (IVS1-728G > A, Thr461Asn and Ile462Val), and the risk of oral cancer, was examined among 157 patients with oral cancer and 132 age-matched controls, in a south Indian population. The strength of the association between *CYP1A1* variants and oral cancer was estimated by logistic regression. It was found that Thr461Asn was not polymorphic. Both IVS1-728G > A and Ile462Val frequencies were consistent with Hardy-Weinberg equilibrium in the control group. There were no significant differences in genotype or haplotype frequencies between controls and cases with oral cancer. Hence, *CYP1A1* SNPs can be considered as not being associated with oral cancer at either the genotype or haplotype levels in the population studied.

Key words: CYP1A1, haplotypes, allele, oral cancer.

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Introduction

Oral squamous cell carcinoma (OSCC) is a significant global health problem with increasing incidence and mortality rates. Annually, approximately 500,000 patients are diagnosed with oral cancer worldwide (Parkin *et al.*, 2005). Smoking is by far the major risk factor for developing oral cancer. The use of alcohol and smoking further increases the risk of oral cancer, along with cancer of the larynx and esophagus. About 300 carcinogens in tobacco smoke or components are known to leach from smokeless tobacco into saliva (International Agency for Research on Cancer, 1987). The most outstanding are the aromatic hydrocarbon benzpyrene, and the tobacco-specific nitrosamines (TSNs), nitroso-nor-nicotine (NNN), nitrosopyrrollidine (NPYR), nitrosodimethylamine (NDMA), and 4-(methylnitrosamino)-1-(3-pyridyl)- 1-butanone (NNK). Their metabolism usually involves oxygenation by p450 enzymes in cytochromes, followed by conjugation with the enzyme glutathione S transferase (GST) (Nakajima *et al.*, 1995). The *CYP1A1* gene encodes for the enzyme aryl hydrocarbon hydroxylase, involved in the biotransformation of various aromatic tobacco precarcinogens (Nebert, 1991).

The *CYP1A1* gene has seven exons and is located on chromosome 15q22-24 (Hildebrand *et al.*, 1985). It is well established that both *CYP1A1* expression and activity are inducible by PAHs through the activation of an *aryl hydrocarbon receptor,* a ligand activated transcription factor (Whitlock, 1999; Ma and Lu, 2007). Certain polymorphic variants of *CYP1A1* genes, besides causing enhanced enzyme activity, are also known to play a major role in the pathogenesis of several cancers (Singh *et al.*, 2007; Wright *et al.*, 2010; Kristiansen *et al.*, 2011). These *CYP1A1* polymorphisms have been extensively studied with regard to oral cancer risk. Although some studies report increased risk in the presence of some of the mutations (Park *et al.*, 1997; Oude Ophuis *et al.*, 1998; Xie *et al.*, 2004), there are many contradictory results apparently due to ethnic differences (Leichsenring *et al.*, 2006; Chatterjee *et al.*, 2009), whereby the perception of inconsistency in findings (Zhuo *et al.*, 2009). Since one of the major risk factors for oral cancer is tobacco use, we hypothesized that polymorphisms in genes coding for tobacco carcinogen-metabolizing enzymes may play a role in oral cancer susceptibility. Therefore, a case-control study was undertaken to extend investigations on specific genetic risk factors in a south Indian

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population, hence the evaluation of the effect of *CYP1A1* gene polymorphisms in developing this risk.

Materials and Methods

Subjects

Three ml blood samples were obtained from 157 oral-cancer patients receiving care at the Kanchipuram cancer hospital. The mean age was 55.07 ± 10.59 years, and included 54.8% men. Diagnosis of the oral cancer as squamous cell carcinoma was confirmed histopathologically. The anonymous donors, with no personal history of cancer of any organ, and which formed the control group of subjects, were recruited from the Sri Ramachandra Medical College. On routine questioning and examination, all the 132 control donors, with a mean age of 53.08 ± 10.72 years and including 34.8% men, appeared healthy. For cases and controls, information regarding age, gender, occupation and details about duration, frequency, nature of the tobacco habit (smoking or smokeless), and alcohol consumption was noted through a detailed questionnaire. 54 (34.4%) of the oral-cancer patients were habitual smokers, whereas only 23 individuals (17.4%) were so in the control group. Prior to the study, all the patient-participants gave informed written-consent. The study was approved by the Ethics Committee of Sri Ramachandra University, Chennai, under the guidelines of the Ministry of Education, Culture, Science and Technology.

Genotyping

Genomic DNA was extracted from all the participants, according to a published protocol (Thangaraj *et al.*, 2002). Three SNPs of the *CYP1A1* gene rs4646421 (Intron 1), rs1799814 (Thr461Asn) and rs1048943 (Ile462Val) were genotyped. The primers and probes for all the SNPs (Table 1) used in this study were purchased from Applied Biosystems (Foster City, CA, USA). Each reaction contained 2.5 μL of TaqMan Universal PCR Master Mix, 0.125 μL of TaqMan SNP Genotyping Assay, 1.375 μL of distilled water and 1 μL of DNA (10 ng/μL), thus reaching a final volume of 5 μL. At least two non-template controls without DNA were included in each of the 384 well-plates. These plates also contained a positive control for wild-type and variant genotypes. Before DNA analysis, a pilot test was run to confirm assay-accuracy. After successful pilot testing, sample analysis was carried out with 384-well optical reaction microplates (Applied Biosystem). Fluorescence was measured with an *Applied Biosystems 7900HT* Fast *Real-Time PCR System,* and analyzed with its System SDS 2.3 software version.

Statistical analysis

Allele frequencies were determined by direct gene counting. Genotype distribution of each site per sample was evaluated for Hardy-Weinberg equilibrium by using a Monte Carlo permutation test implemented in the *HWSIM* program (Cubells *et al.*, 1997). Besides computing expected genotype distribution according to Hardy-Weinberg proportions, HWSIM also tests for agreement between observed and expected distribution, through the usual chi-square test. In order to estimate the strength of association between *CYP1A1* variant alleles and smoking status, their interaction in causing oral cancer was assayed through logistic regression analysis. Genotype indicator variables were created by using the wild-type genotype as the reference category in regression models. As the homozygous Ile462Val variant trait is infrequent in the study subjects, cancer risk associated with the combined hetero-

Table 1 - Primers and probes used for genotyping *CYP1A1* gene polymorphisms.

Gene/Polymorphism	Primers/Probe	Sequence
rs4646421	Forward	CCTTCATTGATCTGACCACTCTTCA
	Reverse	ACTCAGACTCCTTAGGGACACTTC
	Probe 1 (VIC)[1]	CTGTCACAT**G**TACCTCC
	Probe 2 (FAM)	CTGTCACAT**A**TACCTCC
rs1799814 (Thr461Asn; m4)	Forward	GGGCAAGCGGAAGTGT
	Reverse	GAGAAAGACCTCCCAGCGG
	Probe 1 (VIC)	TCGGTGAGA**A**CATTGC
	Probe 2 (FAM)	CGGTGAGA**C**CATTGC
rs1048943(Ile462Val; m2)	Forward	GCAAGCGGAAGTGTATCGG
	Reverse	AAGAGAAAGACCTCCCAGC
	Probe 1 (VIC)	TGAGACC**A**TTGCCC
	Probe 2 (FAM)	TGAGACC**G**TTGCCC

[1]Probes corresponding to different alleles were labeled with VIC and FAM fluorescent dyes (Applied Biosystems).
[2]Polymorphic bases are underlined.

zygous and homozygous variants in our analysis (wild-type individuals versus 'any variant allele') was examined. The statistical package SPSS 14.0 was used for computing percentages, odds ratios (OR) with a 95% confidence interval and chi square tests. Linkage disequilibrium (LD) values of D' and r^2 were estimated using HaploView 3.12 (Barrett *et al.*, 2005). The THESIAS program was also used for haplotype-phenotype analysis (Tregouet and Garelle, 2007).

Results

The difference between the mean ages of control and oral cancer subjects (53.08 ± 10.72 *vs.* 55.07 ± 10.59; p = 0.113) was insignificant. On the other hand, the percentage of men included was 54.8% and 34.8% in oral cancer and control groups, respectively. 54 (34.4%) of oral cancer patients of the study admitted to habitual smoking, whereas in the control group only 23 (17.4%) subjects did so. The distribution of smokers was significantly different between cases and controls (p = 0.001) with OR (CI) 2.485 (1.375-4.512). According to genotype analysis, rs1799814 was not polymorphic. Minor allele frequencies of the two polymorphisms in the control group was 35.2% and 13.6% for IVS1-728G > A and Ile462Val, respectively. For both, frequencies were consistent with Hardy-Weinberg equilibrium in the control group (Table 2). There were no significant differences in genotype or allele frequencies of two SNPs (IVS1-728G > A and Ile462Val) between controls and cases with oral cancer (Table 2). Following stratification of the study subjects according to smoking status (non-smokers and smokers), genotype groups of both SNPS proved not to be significantly associated to oral cancer risk. In non-smokers, observed ORs (CI) for subjects carrying IVS1-728G > A, and Ile462Val polymorphisms were 1.529 (0.844-2.772) and 0.966 (0.483-1.930), respectively. As to smokers, OR (CI) values for the same genotype groups were 1.558 (0.520-4.689) and 0.992 (0.289-3.491), respectively. Furthermore, stratified analysis by smoking status provided no support to either interaction or confounding, thereby indicating observed association to be the most precise and unbiased (Table 3). IVS1-728G > A and Ile462Val were in weak linkage disequilibrium (LD), D' = 0.678; r^2 = 0.116. According to haplotype phenotype association analysis, there was no evidence of association (Table 4).

Discussion

It is well known that the induction of *CYP1A1* plays a major role in the development of oral cancer and other smoking-related respiratory-tract cancers (Rendic and Guengerich, 2010). The PAHs present in tobacco smoke activate *CYP1A1* gene transcription (Omiecinski *et al.*, 1990), whereas in turn, *CYP1A1* enzymes convert PAHs into active carcinogens (Hecht *et al.*, 1993). There are contradictory reports on the role of *CYP1A1* variants in altering the levels of gene expression or mRNA stability. Ile462Val polymorphism revealed higher levels of induced or basal *CYP1A1* mRNA, with an increase in Val variants, whereas in purified *Escherichia coli*, there was no difference in benzo[*a*]pyrene metabolism in Ile and Val variants (Crofts *et al.*, 1994; Zhang *et al.*, 1996). In the present study, investigation of IVS1-728G > A (rs4646421), Thr461Asn (rs1799814) and Ile462Val (rs1048943) in the *CYP1A1* gene revealed only IVS1-728G > A and Ile462Val to be polymorphic in this south Indian population. Although smoking was established as a risk factor in the present

Table 2 - Distribution of genotypes and the relationship between polymorphisms of the CYP1A1 gene and the risk of oral cancer.

Genotype	Control	Oral cancer	OR (95% CI)	p value
IVS1-728G > A (rs4646421)				
GG	59 (44.70%)	55 (35.03%)	Reference	
GA	53 (40.15%)	80 (50.96%)	1.619 (0.947-2.773)	0.073
AA	20 (15.15%)	22 (14.01%)	1.180 (0.548-2.544)	0.719
GA+AA	73 (55.30%)	102 (64.97)	1.499 (0.907-2.479)	1.160
MAF	35.2	39.5		
HWE p	0.167	0.407		
Ile462Val (rs1048943)				
AA	101 (76.52%)	120 (76.43%)	Reference	
AG	26 (19.70%)	34 (21.66%)	1.101 (0.596-2.036)	0.772
GG	5 (3.78%)	3 (1.91%)	0.505 (0.093-2.50)	0.476
AG+GG	31(23.48%)	37 (23.57%)	1.005 (0.562-1.797)	1.000
MAF	13.6	12.7		
HWE p	0.060	0.745		

MAF: Minor allele frequency; OR: odds ratio; HWE p: Hardy-Weinberg equilibrium p value.

Table 3 - Joint effects between genotypes and smoking status.

Genotype	Smoking	Cases n (%)	Control n (%)	Strata specific OR (95% CI)	Strata specific p value	Interaction OR (95% CI)	$p_{-interaction}$
Rs4646421							
GG	No	35 (22.3)	48 (36.4)	Reference			
GA+AA	No	68 (43.3)	61 (46.2)	1.53 (0.84-2.77)	0.134		
GG	Yes	20 (12.7)	11 (8.3)	Reference			
GA+AA	Yes	34 (27.7)	12 (9.1)	1.56 (0.52-4.69)	0.377	1.54 (0.92-2.57)	0.107
rs1048943							
AA	No	80 (51.0)	84 (63.6)	Reference			
AG+GG	No	23 (14.6)	25 (18.9)	0.97 (0.48-1.93)	0.916		
AA	Yes	40 (25.5)	17 (12.9)	Reference			
AG+GG	Yes	14 (8.9)	6 (4.5)	0.99 (0.29-3.70)	0.988	0.97 (0.54-1.76)	0.965

Abbreviations: CI, confidence interval; OR, odds ratio.

Table 4 - *CYP1A1* gene haplotypes and oral cancer.

rs4646421 and rs1048943				
Haplotype	Control	Case	OR (95% CI)	p value
GA	0.613	0.585	Reference	
GG	0.034	0.02	0.511 (0.142-1.842)	0.305
AA	0.25	0.287	1.185 (0.794-1.767)	0.407
AG	0.102	0.108	1.131 (0.665-1.923)	0.651

study, IVS1-728G > A and Ile462Val polymorphisms of the *CYP1A1* gene were not associated with the risk of oral cancer. Furthermore, *CYP1A1* SNPs could not even be associated with oral cancer at either the genotype or haplotype levels.

Although Thr461Asn (m4), specific for Caucasians and with a frequency ranging from 3 to 6%, has also been related to greater enzyme catalytic efficiency (Schwarz *et al.*, 2001), none of the subjects in our sample carried this variant. The famous and well studied Ile462Val (m2) variant in *CYP1A1*, present in both Caucasians (7%) and Asians (35%), is mostly detected in linkage disequilibrium with m1 (MspI site) (Georgiadis *et al.*, 2005). A study from North India revealed 51% and 17% of the *CYP1A1* m2 allele (462Val carriers), respectively, in oral cancer and controls, thus associated with increased risk (Sreelekha *et al.*, 2001). In another study, also of a north Indian population, the frequencies of genotypes were similar in patient and control groups, with no indication of any significant association (Sikdar *et al.*, 2003). An analysis of Caucasian oral-cancer patients revealed almost identical heterozygous genotype distribution between cases and controls (6.5% oral cancer, 4.3% controls), with no association between m2 and oral cancer (Hahn *et al.*, 2002). The Ile462Val heterozygous genotype is significantly associated with the earlier age of onset of OSCC than in the wild homozygous genotype (Kao *et al.*, 2002). Two independent case-control studies from Puerto Rico and south Brazil, revealed that genotypes of the *CYP1A1* (462Val) variant were not associated with oral-cancer risk (Xie *et al.*, 2004; Leichsenring *et al.*, 2006). Chilean individuals carrying a T to C substitution at the 3' untranslated region (*CYP1A1**2A/ m1 allele/ rs4646903), are reported to be more susceptible to oral cancer induced by environmental tobacco smoking (Cordero *et al.*, 2010).

Although no association between *CYP1A1* genotypes and oral cancer has been reported from various geographic regions, such as Japan (Sugimura *et al.*, 2006), Brazil (Leichsenring *et al.*, 2006; Marques *et al.*, 2006; Losi-Guembarovski *et al.*, 2008), the USA (using whites) (Buch *et al.*, 2008), and Jakarta (Amtha *et al.*, 2009), the *CYP1A1* (m2/m2) genotype did present this association with increased risk, in Koreans (Cha *et al.*, 2007). Furthermore, gene to gene interaction studies revealed that, besides oral cancer (Anantharaman *et al.*, 2007), *GSTM1* null and *CYP1A1* polymorphism increased the risk of head and neck cancers (Gattas *et al.*, 2006).

In the present study, the prevalence of IVS1-728G > A, and Ile462Val polymorphisms in the *CYP1A1* gene in south Indian oral-cancer patients and controls, was presented. Apparently there was no association between this gene and oral cancer. Although the whole range of polymorphic genes involved in detoxification metabolism have been the base of intensive epidemiological studies over the past few years, further investigation is still called for in order to determine whether these or additional gene-gene interactions are crucial in determining the possibility of carriers of *CYP1A1* alleles presenting an increased or reduced risk of cancer.

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