

RESEARCH ARTICLE

Roles of CYP1A1 and CYP2E1 Gene Polymorphisms in Oral Submucous Fibrosis

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Abstract

Background: Oral submucous fibrosis (OSF) is a precancerous condition with a 4 to 13% malignant transformation rate. Related to the habit of areca nut chewing it is mainly prevalent in South-east Asian countries where the habit of betel quid chewing is frequently practised. On chewing, alkaloids and polyphenols are released which undergo nitrosation and give rise to N-nitrosamines which are cytotoxic agents. CYP450 is a microsomal enzyme group which metabolizes various endogenous and exogenous chemicals including those released by areca nut chewing. CYP1A1 plays a central role in metabolic activation of these xenobiotics, whereas CYP2E1 metabolizes nitrosamines and tannins. Polymorphisms in genes that code for these enzymes may alter their expression or function and may therefore affect an individuals susceptibility regarding OSF and oral cancer. The present study was therefore undertaken to investigate the association of polymorphisms in CYP1A1 m2 and CYP2E1 (RsaI/PstI) sites with risk of OSF among areca nut chewers in the Northern India population. A total of 95 histopathologically confirmed cases of OSF with history of areca nut chewing not less than 1 year and 80, age and sex matched controls without any clinical signs and symptoms of OSF with areca nut chewing habit not less than 1 year were enrolled. DNA was extracted from peripheral blood samples and polymorphisms were analyzed by PCR-RFLP method. Gene polymorphism of CYP1A1 at NcoI site was observed to be significantly higher ($p = 0.016$) in cases of OSF when compared to controls. Association of CYP1A1 gene polymorphism at NcoI site and the risk of OSF (Odd's Ratio = 2.275) was also observed to be significant. However, no such association was observed for the CYP2E1 gene polymorphism (Odd's Ratio = 0.815). Our results suggest that the CYP1A1 gene polymorphism at the NcoI site confers an increased risk for OSF.

Keywords: Oral submucous fibrosis - genetic polymorphism - CYP1A1 - CYP2E1 - risk factor

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Introduction

Oral cancer is the fifth most common tumor or malignancy diagnosed worldwide. Approximately 90 % of oral cancer are oral squamous cell carcinoma (OSCC). Several factors like tobacco, areca nut, alcohol, genetic predisposition, hormonal and nutritional factors are suspected as possible risk factors. In addition, OSCC can also arise from various potentially malignant disorders (PMDs) (Vargas et al., 2012; Joshi et al., 2014).

PMDs is defined by World Health Organization (WHO) as the risk of malignancy being present in a lesion or condition either during the time of initial diagnosis or at a future date. It has been well established that nearly all oral carcinomas are preceded by potentially malignant disorders. Therefore, correct and timely treatment of PMDs may help prevent malignant transformation in oral lesions as well help in early detection of oral cancer (Tanwir et al., 2011; Sudarshan et al., 2012; Mortazavi et al., 2014)

Oral submucous fibrosis (OSF), is a potentially

malignant condition with a malignant transformation rate of 4 to 13%. It was first described by Schwartz in 1952, as a chronic debilitating disease of the oral cavity characterized by inflammation and progressive fibrosis of the submucosal tissues leading to reduced mouth opening (Sudarshan et al., 2012). The disease is mainly prevalent in the Indian subcontinent and in many South-east Asian countries where habit of betel quid chewing is frequently practised. In India, the prevalence has increased over the past four decades from 0.03% to 6.42% (Hebbar et al., 2014, Nigam et al., 2014). Various local and systemic factors such as arecanut chewing, chilli consumption, autoimmunity, and genetic predisposition have been implicated in the pathogenesis of OSF. Amongst these, arecanut chewing either alone or as a component of betel quid is believed to be the main etiological factor (Pindborg et al., 1968; Hazarey et al., 2007)

On chewing, arecanut releases alkaloids and polyphenols (flavonols, tannins) which stimulates proliferation of fibroblasts, increased collagen synthesis and decreased breakdown leading to accumulation of

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excessive amount of collagen. These alkaloids and polyphenols undergoes nitrosation and give rise to N-nitrosamines, which have a cytotoxic effect on cells. During metabolism of these procarcinogens, they undergo bioactivation and deactivation via different phases of detoxification carried out by microsomal enzymes cytochrome P450 (Mukherjee et al., 2012; Mathew et al., 2014).

Cytochrome P450 enzymes are a family of constitutive and inducible mono-oxygenase enzymes that metabolize many lipophilic, biologically active endogenous and xenobiotic substrates including a large number of therapeutic drugs and toxic environmental chemicals (Sudarshan et al., 2012). They are involved in phase I of biotransformation, catalyzes the addition of an atom of oxygen to lipophilic toxic compounds, converts them into more hydrophilic compounds and facilitates their easy excretion (Mathew et al., 2014). Among the cytochrome P450 family, cytochrome P4501A1 (CYP1A1) enzyme plays central role in the metabolic activation of these xenobiotics, whereas CYP2E1 metabolizes the nitrosamines and tannins (Mukherjee et al., 2012).

Numerous polymorphisms have been described in this gene. CYP1A1 Ile462Val (rs1048943) is one of the most studied polymorphisms of CYP1A1, caused by adenine to guanine substitution at exon 7, which results in an isoleucine for valine aminoacidic substitution (Ile462Val; rs1048943) resulting in loss of NcoI site and increased catalytic action of microsomal enzymes (Rodriguez et al., 2012). Several studies have reported significant association of Ile462Val polymorphism and increased risk of oral precancer (Kao et al., 2002; Chaudhuri et al., 2013) and HNSCC (Liu et al., 2001).

The most commonly studied genetic polymorphisms for CYP2E1 gene are two strongly linked SNP located in the 5' flanking region: -1293 G>C (rs3813867), a restriction site for PstI enzyme; and 1053 C>T (rs2031920), a restriction site for RsaI enzyme (Liu et al., 2001). Studies have analyzed both alleles in HNSCC patients and have observed a significant association between these polymorphisms and the risk of head and neck cancer (Bouchardy et al., 2000; Liu et al., 2001).

Studies have shown that polymorphisms in genes coding for cytochrome P450 enzymes may alter their expression or function of activation or detoxification of carcinogenic compounds and influence an individual's genetic susceptibility to cancer (Rodriguez et al., 2012).

This phenomenon increases the susceptibility to cancer because the genetic polymorphism aids in increasing the concentration of carcinogenic tobacco specific nitrosamines and harmful reactive oxygen species in oral cavity (Rodriguez et al., 2012; Chaudhuri et al., 2013). Therefore, identification of CYP polymorphism can help in screening individuals at genetically higher risk of developing OSF and oral cancer. It may also help in improving and guiding the treatment regimens for the patients since CYP are also involved in the metabolism of various drugs.

Hence, this study was undertaken to evaluate the role of gene polymorphism of CYP1A1 at NcoI site, CYP2E1

at RsaI/PstI site in OSF patients among North Indian population, which may serve as a genetic marker for patients at risk for OSF and oral cancer.

Materials and Methods

Study Population

Patients visiting Out Patient Department (OPD) in Maulana Azad Institute of Dental Sciences were selected, and written informed consent was obtained from each individual selected for the study. Patients (N=95) with histopathologically confirmed OSF with history of arecanut chewing habit not less than 1 year were selected as study samples. Age and sex matched patients (N=80) without any clinical signs and symptoms of OSF with arecanut chewing habit not less than 1 year and genetically unrelated to the cases from the same area were selected as controls. Patients and controls with any other history of oral lesions, any systemic disorders contradicting biopsy were excluded. After obtaining patients consent, 3ml of blood was collected from the antecubital vein of all the subjects selected for the study. The study was approved by the Institutional Ethical Committee Board for research purpose.

Clinical staging of OSF patients

The lesion was thoroughly examined clinically. A detailed history was recorded including duration of lesion, habit history of arecanut chewing. Interincisal distance was measured using vernier calliper and lesion was then graded clinically based on classification by Lai et al. (1995) Group A: >35mm, Group B: Between 30-35mm, Group C: Between 20-30mm, Group D: <20mm

Obtaining biopsied tissue samples from OSF patients

Informed consent was obtained from the patient. Routine blood investigations including complete hemogram, blood sugar, clotting time, bleeding time were performed. Incisional biopsy tissue was obtained, fixed in 10% formalin and stained with haematoxylin and eosin for histopathological assessment. The tissue sections were studied by two pathologists independently and classified according to Pindborg JJ and Sirsat SM (1966) as: Very early stage - Finely fibrillar collagen dispersed with marked edema. Plump young fibroblast containing abundant cytoplasm. Blood vessels are dilated and congested. Inflammatory cells, mainly polymorphonuclear leucocyte with occasional eosinophils are found.

Early stage - Juxta - epithelial area shows early hyalinization. Collagen fibers are still in separate thick bundles. Moderate numbers of plump young fibroblasts are present. Dilated and congested blood vessels. Inflammatory cells are primarily lymphocytes, eosinophils and occasional plasma cells.

Moderately advanced stage - Collagen is moderately hyalinized. Thickened collagen bundles are separated by slight residual edema. Fibroblast response is less marked. Blood vessels are either normal or compressed. Inflammatory exudate consists of lymphocytes and plasma cells.

Advanced stage - Collagen is completely hyalinized.

Smooth sheets with no separate bundles of collagen are seen. Edema is absent. Hyalinized area is devoid of fibroblasts. Blood vessels are completely obliterated or narrowed. Inflammatory cells are lymphocytes and plasma cells.

Collection of blood sample

Bilingual informed consent was obtained from each patient and controls who were willing to participate in the study. 3 ml of peripheral blood was withdrawn from each patient and controls under aseptic conditions in sterile

Table 1. Demographic Status of OSF Patients and Controls

	OSF	CONTROLS
Mean age	34.4	34.03
Sex		
Male	76	69
Female	19	11
Clinical stages		
Group A ^a	6	
Group B ^b	12	
Group C ^c	48	
Group D ^d	29	
Histological grades		
Very early	2	
Early	39	
Moderately advanced	47	
Advanced	7	

^a Group A: >35mm, ^b Group B:30-35mm, ^c Group C:20-30mm, ^d Group D:<20mm

Table 2. Distribution of CYP1A1 and CYP2E1 Genotypes among OSF and Controls

	OSF		CONTROLS		
	N	a (%) ^b	N	a (%) ^b	
CYP1A1 Wild type (+)	51	(53.7)	58	(72.5%)	p = .016 ^c
CYP1A1 Polymorphism (-)	44	(46.3)	22	(27.5%)	
CYP2E1 Wild type c1(RsaI+/PstI-)	85	(89.5)	73	(91.3)	
CYP2E1 Polymorphism c2(RsaI-/PstI+)	7	(7.4)	6	(7.5)	
CYP2E1 Polymorphism c3(RsaI+/PstI+)	1	(1.1)	0	(0)	p = .791 ^d
CYP2E1 Polymorphism c4(RsaI-/PstI-)	2	(2.1)	1	(1.3)	

N^a – Number of cases, (%)^b - Percentage, ^cGene polymorphism of CYP1A1 at NcoI site was significantly higher in cases of OSF as compared to controls, ^dNo significant difference was found for gene polymorphism of CYP2E1 (c1, c2, c3, c4) between cases of OSF and controls

Table 3. Odds Ratio for CYP1A1 Gene Polymorphism at NcoI site and CYP2E1 Gene Polymorphism

	Cases	Controls		
CYP1A1 Wild Type	51	58	“OR (odds ratio) = 2.27451”	“95% Confidence Interval =1.209204 - 4.276206”
CYP1A1 Polymorphism	44	22		
“CYP2E1 Wild Type c1”	85	73	“OR (Odds ratio) = 0.8150685”	95% Confidence Interval p=0.692 ^b
“CYP2E1 Polymorphism c2, c3, c4”	10	7		=0.304904 -2.183629

^aOdds Ratio was calculated for CYP1A1 gene polymorphism at NcoI site between cases of OSF and controls which was found to be significant with p value = 0.0105; ^bOdds Ratio was calculated for CYP2E1 gene polymorphism which was not significant

tubes containing EDTA anticoagulant. The blood samples were stored at - 80° C until used.

DNA Extraction

Genomic DNA was isolated from venous blood samples of patient and control by using geneaid extraction kit. Extracted DNA was stored at -20°C until used.

Quantification of extracted genomic DNA

Quantification of extracted genomic DNA was done using Nanodrop. Minimum DNA concentration of 60 nano gram/ micro liter was used for PCR.

CYP1A1 NcoI genotyping

Polymorphism of CYP1A1 at NcoI restriction site was determined by polymerase chain reaction (PCR) and RFLP. The DNA samples were amplified with the primers: forward: 5'-GAAAGGCTGGGTCCACCCTCT-3' and reverse: 5'-CCAGGAAGAGAAAGACCTCCCAGCGGGGCA-3'. PCR was performed in 25 µl volume containing 4 µl extracted genomic DNA, 12 µl Dream Taq master mix (containing Tris-HCl, KCl, MgCl, Deoxyribonucleotide triphosphates and Taq polymerase) 0.3 µl of Forward Primer (25 pm), 0.3 µl Reverse Primer (25 pm) and 8.4 µl of Nucleas free water. The PCR conditions were initialized at 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 1 min and final extension at 72°C for 7 min. PCR products (370 bp) were digested with 0.5 µl NcoI for 1 h at 37°C. The fragments were separated on 2% agarose gel containing ethidium bromide and visualized under UV illuminator. Wild type DNA is cut by NcoI enzyme resulting in fragments 263 and 107 bp. The DNA carrying the polymorphic variant is not cut resulting in 370 bp band.

CYP2E1 RsaI and PstI genotyping

Polymorphism of CYP2E1 at RsaI and PstI restriction site was determined by polymerase chain reaction (PCR) and RFLP. The DNA samples were amplified with the primers: forward: 2Els': 5-CCAGTCGAGTCTA-CATTGTCA-3' and reverse: 2Elas': 5'TTCATTC-TGTCTTCTAACTGG-3'. PCR was performed in 25 µl volume containing 4 µl extracted genomic DNA, 12 µl Dream Taq master mix (containing Tris-HCl, KCl, MgCl, Deoxyribonucleotide triphosphates and Taq polymerase) 0.3 µl of Forward Primer (25 pm), 0.3 µl Reverse Primer (25 pm) and 8.4 µl of Nucleas free water. The PCR conditions were initialized at 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 55.8°C for 30 s, 72°C for 1 min and final extension at 72°C for 7 min. PCR products (412 bp) were digested with 1 µl of RsaI and 1 µl of enzymes for 30 min at 37°C. The fragments

were separated on 2% agarose gel containing ethidium bromide and visualized under UV illuminator. Wild type c1 (RsaI +/PstI -) genotype showed 351 bp and 61 bp, other genotypes c2 (RsaI - / PstI +) showed 294 bp and 118 bp, c3 (RsaI + / PstI +) genotype showed 233 bp, 118 bp and 61 bp, c4 (RsaI - / PstI -) genotype showed 412 bp uncleaved DNA.

Statistical Analysis

The mean values, standard deviation and ranges (maximum and minimum) were calculated for each variable. The resulting data was analyzed using SPSS software. Data was expressed as mean \pm standard deviation. Differences between different variables were analyzed using non-parametric chi-square test. Odds ratio was calculated to assess the association of gene polymorphism as a risk factor for OSF. P value \leq 0.05 was considered to be significant.

Results

Distribution of patients and controls according to age, gender, clinical staging and histopathological grading was studied as shown in Table 1. Percentage of CYP1A1 gene polymorphism at NcoI site was determined amongst cases (46.3%) and controls (27.5%). The comparison of test groups and controls was done using chi - square test as shown in Table 2. Gene polymorphism of CYP1A1 at NcoI site was found to be significantly higher ($p=0.016$) in cases of OSF as compared to controls. But no significant difference ($P=0.0791$) was obtained for CYP2E1 gene polymorphism among OSF cases and controls as shown in Table 2. Gene polymorphism of CYP1A1 genotype was also compared with clinical stage (Table 3) and histopathological grade (Table 4) using chi - square test. No significant correlation could be determined. Odd's ratio was used to determine the association of CYP1A1 and CYP2E1 gene polymorphism with the risk of development of OSF in the population (Table 5). A significant association for CYP1A1 polymorphism at NcoI site and the risk of OSF development (Odd's Ratio=2.275; $p=0.01$) was observed. While no such association could be determined with CYP2E1 gene polymorphism (Odd's Ratio=0.815; $p=0.69$).

Discussion

As studies have established that arecanut chewing is the main etiological factor for OSF, we included 95 cases of OSF and 80 age and sex matched controls with habit of arecanut chewing for not less than one year. The mean age of OSF patient in our study was 34.4 years which is within the age group as reported in literature (Table 1). Higher male to female ratio of 4:1 was observed in our study which is similar with other studies of Indian population (Table 1) (Hazarey et al., 2007; Pandya et al., 2009; Angadi et al., 2011).

Distribution of polymorphic genotype of CYP1A1 at NcoI site in our study was observed to be significantly higher in patients of OSF (46.3%) when compared to controls (27.5%) (Table 2). Polymorphism of CYP1A1

at m2/NcoI site occurs due to A to G transition mutation which results in an amino acid substitution of isoleucine to valine at 462 codon in exon 7. This transition leads to loss of site recognized by NcoI restriction enzyme and increases the microsomal enzyme activity. As CYP1A1 enzyme plays a central role in metabolic activation of xenobiotics during phase I of metabolism, increase in CYP1A1 enzyme activity caused by polymorphism at NcoI site will lead to increased production of intermediate metabolites and thereby increasing an individual's risk for OSF. The result of our study was similar to Chaudhari SR et al who observed significantly ($p=0.00001$) higher distribution of polymorphic genotype of CYP1A1 at NcoI site in OSF patients (57%) compared to control (14%) in an eastern Indian population (Chaudhari SR et al., 2013). However, our results were in contrast to the other study in which higher frequency of CYP1A1 (m1 and m2) genotypes were observed in controls (Ghosh T et al., 2012).

Various studies on association of CYP1A1 gene polymorphism and oral cancer have been carried out in the past. Some studies have reported higher risk of cancer in individuals with CYP1A1 gene polymorphism (Kao et al., 2002; Wang et al., 2002; Anantharaman et al., 2007). Studies conducted on gene polymorphism of CYP1A1 in the Indian subcontinent have shown conflicting results. A study conducted by Sreelekha TT et al showed higher distribution of CYP1A1 m2 gene among oral cancer patients (17%) when compared to controls (5%) in a North Indian population (Sreelekha et al., 2001). However Sikder N et al observed no significant difference in distribution of CYP1A1 between patients of oral precancer and controls in North Indian population (Sikder et al., 2003).

CYP2E1 is an ethanol-inducible cytochrome P-450 isoenzyme that metabolically activates various carcinogens, such as benzene, vinyl chloride, and low-molecular weight nitrosamines. The CYP2E1 gene contains two SNP polymorphisms at nucleotides -1259 caused by G>C transition and -1053 C>T upstream of the CYP2E1 transcriptional start site which are detectable by PstI and RsaI restriction enzyme digestion, respectively. This polymorphism is correlated with the higher transcriptional activity of the gene compared with the wild phenotype. Based upon the presence or absence of RsaI or PstI recognition sequences at these polymorphic sites, four alleles have been postulated to exist, the common "wild-type" allele (RsaI[+]/PstI[-]) referred to as c1, (RsaI[-]/PstI[+]) known as c2, c3 (RsaI[+]/PstI[+]) and c4 (RsaI[-]/PstI[-]) (Gonzalez et al., 1998; Liu et al., 2001). c2 allele is observed to be the most common polymorphic variant of CYP2E1 found in the Caucasian and Asian populations (Liu et al., 2001).

In the present study, c1 (RsaI+/PstI-) was the most prevalent CYP2E1 allele found in both study group (89.5%) and controls (91.3%) (Table 2) which was similar to the previous studies (Liu et al., 2001). In contrast to our study, Lu D et al and Tang K et al have shown c2 (Rsa-/PstI+) allele as the most common polymorphic allele when compared to c3 (RsaI+/PstI+) and c4 (RsaI+/PstI+) (Lu et al., 2001; Tang et al., 2010). No significant difference in distribution of CYP2E1 wild type (c1) and polymorphic

variants (c2, c3, c4) were observed between study group and controls in this study (Table 2). Lower frequency of polymorphic variants (c2, c3, c4) which did not reach a statistically significant level in our study may be due to lower prevalence of CYP2E1 c2 allele.

Although CYP2E1 gene polymorphism has been extensively studied in oral cancer, only one such study has been conducted till date in OSF at PstI site by Chaudhuri SR et al, in which significantly higher frequency of heterozygous (+/-) CYP2E1 PstI genotype in patients of OSF (13%) was observed when compared to control (5%) (Chaudhuri et al., 2013). However, the current study has not shown any association, probably because the combination of RsaI and PstI sites were studied for the presence of c1, c2, c3 and c4 alleles with c1 allele predominating over the rest. There was no significant difference in any of the alleles between OSF and controls. Hence, it can be hypothesized that CYP2E1 gene polymorphism is not contributing as a risk factor for developing OSF.

Various studies on association of gene polymorphism of CYP2E1 at RsaI and PstI site and HNSCC has shown conflicting results. Ruwali et al found 3 fold increased risk for HNSCC in individuals of Indian population with CYP2E1*5b (RsaI) and CYP2E1*6 (DraI) polymorphism (Ruwali et al., 2009). In contrast, Balaji et al, Buch et al and Gonzalez et al found no such association between CYP2E1 gene polymorphism and HNSCC (Gonzalez et al., 1998; Buch et al., 2008; Balaji et al., 2011).

Correlation of gene polymorphism of CYP1A1 at NcoI site and CYP2E1 at PstI site has also been correlated with the mouth opening of OSF patients in previous studies. The frequency of heterozygous (+/-) genotype of CYP1A1 at NcoI site was reported to be higher (69%) in patients of the advanced stage compared to those belonging to the less advanced stage (47%) (Chaudhuri et al., 2013). No such correlation between CYP1A1 gene polymorphism at NcoI site and clinical mouth opening and histopathological grade of OSF was observed in our study. This could probably be due to the skewed distribution of cases in the subgroup of cases showing CYP1A1 polymorphism at NcoI site. Such correlation for CYP2E1 was not ascertained because no significant correlation of CYP2E1 gene polymorphism between cases and controls was observed in our study.

The odds ratio was calculated for assessing the risk of developing OSF at 95% confidence interval. There was a significantly increased risk of developing OSF when CYP1A1 polymorphism is observed at NcoI site (m2) (Table 3). However, there was no significant risk when any allele variant (c1, c2, c3, c4) of CYP2E1 gene polymorphism was assessed (Table 3). The results of CYP2E1 gene polymorphism in the current study were in contrast to that of Chaudhuri et al. (2013). This could be attributed to the population under consideration as the current study was carried out on North Indian population versus a Eastern Indian population as assessed by Chaudhuri et al., 2013.

The present study has shown a significantly increased risk of developing OSF when gene polymorphism is present in CYP1A1 m2 site (NcoI site). Further studies

on larger sample size and different populations should be carried out to assess the accurate role of CYP2E1 gene polymorphism as a risk factor for developing OSF. Other cytochrome P450 enzymes like CYP3A5 which was found to be among the top two down regulated genes in OSF in microarray analysis study needs to be assessed (Li N et al., 2008). Various xenobiotic metabolic activators of CYP450 families such as CYP2D6, CYP1A2, CYP1B1 which are associated with increased susceptibility to HNSCC can also be studied to further elucidate genetic interplay and susceptibility to OSF (Li N et al., 2008; Lin et al., 2013).

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