Gene 513 (2013) 268–271

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

CYP1AI and *CYP2E1* gene polymorphisms may increase susceptibility to Oral Submucous Fibrosis among betel quid chewers of Eastern India

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ARTICLE INFO

Article history:
Accepted 16 October 2012
Available online 30 October 2012

Keywords:
Betel quid chewing
Oral Submucous Fibrosis
CYP1A1
CYP2E1
Genetic polymorphism

ABSTRACT

Chewing betel quid may release chemical carcinogens including xenobiotics resulting in oral malignancy cases preceded by potential malignant lesions and conditions — Oral Submucous Fibrosis (OSF) being one of them. The cytochrome P4501A1 (CYP1A1) enzyme is central to the metabolic activation of these xenobiotics, whereas CYP2E1 metabolizes the nitrosamines and tannins. The present study investigated the association of polymorphisms at *CYP1A1*m1 (T3801C), m2 (A2455G), and *CYP2E1 Pst*I site (nucleotide 21259) with the risk of OSF. The study was conducted on 75 OSF patients and 150 controls from an eastern Indian population. The above polymorphisms were analyzed by PCR-RFLP method. Analyses of data show that polymorphisms in *CYP1A1*m2 [OR = 8.25 (4.31–15.80)]; *CYP1A1*m1 [OR = 2.88 (1.57–5.24)] and *CYP2E1 Pst*I site [OR = 3.16 (1.10–9.04)] revealed significant association with OSF. Our results suggest that polymorphism in *CYP1A1* and *CYP2E1* may confer an increased risk for Oral Submucous Fibrosis.

1. Introduction

Oral cancer including lip and oral cavity (ICD-10: C00-08) is the most frequent cancer of the head and neck region, with squamous cell carcinoma being by far the commonest single entity, accounting alone for about 90% of all malignancies of the oral cavity. While the global estimate of oral cancer is around 275,000 cases (Ferlay et al., 2004) the number of cases registered every year in India alone is over 100,000 (Parkin et al., 2002) of which the incidence of lip and oral cavity cancer according to a recent report is 69820 (http://globocan.iarc.fr/factsheet.asp). It has been well established that nearly all oral cancers are preceded by potentially malignant disorders (Warnakulasuriya et al., 2007). Oral Submucous Fibrosis (OSF, ICD-10-CM K13.5) is among one such potentially malignant condition characterized by excessive deposition of collagen starting within the lamina propria and with time extending to submucosa, to muscle and beyond and associated with juxtaepithelial inflammation. The oral mucosa becomes stiff causing trismus and eventually leading to difficulty in mouth opening (Angadi and Rao, 2011; Pindborg and Sirsat, 1966). The disease is predominantly seen in India, Bangladesh, Sri Lanka, Pakistan, Taiwan, Southern China, Polynesia and Micronesia (Lee et al., 2011). Several case-series are reported among Asian immigrants to the UK and South and East Africa (Johnson et al., 2012). Ironically, in India, the etiology of this condition is well established that includes risk habits such as chewing areca nut in the form of '*Pan Masala*' and '*Gutkha*' — a form of prepared smokeless tobacco with areca nut and other condiments, commonly called betel quid, in addition alcoholism may also be associated. The majority of patients present with an intolerance to spicy food, rigidity of lip, tongue and palate leading to varying degrees of limitation of opening of the mouth and tongue movement. Recent studies have mounting evidences that genetic susceptibility (Mukherjee et al., 2012) and dietary factors may enhance the effect of the procarcinogens released from chewing of tobacco with betel quid that increases the concentrations of carcinogenic tobacco specific nitrosamines and reactive oxygen species in mouth (Nair et al., 1992). As an early sign of damage to oral mucosa, tobacco smokers and chewers often develop potentially malignant lesions such as leukoplakia and submucosal fibrosis, which are easily accessible to diagnosis and can be considered as indicators of oral cancer risk, the transition to malignancy being about 7–14% within several years (Reichart and Nguyen, 2008).

Cytochrome P450 enzymes are involved in phase I of the metabolism of xenobiotic compounds catalyzing the addition of an atom of molecular oxygen to lipophilic toxic compounds which, in turn, converts them into more hydrophilic compounds helping in easy excretion.

Abbreviations: ICD-10-CM, International Classification of Diseases, Tenth Revision, Clinical Modification the International Classification of Diseases (ICD); OSF, Oral Submucous Fibrosis; CYP, cytochrome P450; SCC, squamous cell carcinoma; IID, interincisal distance; bp, base pair; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; pMol, pico Mol; U, units; P, probability; OR, odds ratio; CI, confidence interval; IARC, International Agency for Research on Cancer; ICMR, Indian Council of Medical Research; SNP, single nucleotide polymorphism; SD, standard deviation.

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http://dx.doi.org/10.1016/j.gene.2012.10.043

The identification of polymorphic enzyme genotypes in the population at risk may serve as a genetic biomarker and indicative of individual risk for cancer (Zhou et al., 2009). The polymorphic *Msp*I restriction enzyme site in *CYP1A1* gene, at the 264th base downstream from additional poly (A) signal in the 3′-flanking region, could modulate expression of gene and has been shown to be associated with susceptibility to lung and oral squamous cell carcinoma (SCC) (Le Marchand et al., 1998; Sato et al., 1999; Tanimoto et al., 1999). *CYP2E1* is expressed in cultured human oral epithelial cells and metabolizes potentially important carcinogens such as benzene, butadiene, carbon tetrachloride, vinyl chloride and low molecular weight nitrosamines (Lieber, 1997). Polymorphism in this gene, at nucleotides −1370 to −1349 is located in the transcription regulation region and detectable by *Pst*I restriction enzyme digestion. Polymorphisms in *CYP2E1* have been shown to be associated with head and neck cancer in Asians (Tang et al., 2010), colorectal cancer in Caucasians (Zhou et al., 2010) etc. Thus from previous studies of association of *CYP* polymorphism on oral carcinogenesis (Hernando-Rodriguez et al., 2012) it will now be informative to study the role played by these polymorphisms in susceptibility to Oral Submucous Fibrosis.

2. Materials and methods

2.1. Study populations

Patients diagnosed with and histopathologically confirmed Oral Submucous Fibrosis attending at Guru Nanak Institute of Dental Science and Research, Panihati and Chittaranjan National Medical College, both located in Kolkata, during the years 2005–2007 were enrolled as cases (N = 75) in the present study with written consent. The volunteers having a history of chewing habit not less than 6 months and genetically unrelated to the cases from the same area were included as controls (N = 150) with written consent. The study was duly approved by the institutional ethics committee.

A thorough questionnaire was performed which included parameters like age, sex, oral habits and any other systemic medical problems. Patients or controls having any other history of oral lesions or undergoing medication were excluded from the present study. The control population had a minimum history of six months of chewing and this could extent longer. Also each of the control subjects was clinically examined for presence of any blanched mucosa/fibrotic bands/any other early symptoms of OSF. About 2–5 ml of blood was collected from the antecubital vein from all of the study participants.

2.2. Clinical classification of OSF stages

The classification/grading of OSF was carried out according to the degree of mouth opening, which directly correlates with the degree of OSF and progression of the disease. This was assessed by measuring the incisal distance (IID) and graded as less advanced (IID, >1.9 but <3.5 cm) and advanced (IID <1.9 cm), considering IID >3.5 as normal.

2.3. DNA isolation and genotyping

Genomic DNA was isolated from venous blood samples of patient and control individuals by proteinase-K treatment and salt extraction procedure (Miller et al., 1988).

2.4. CYP1A1 Msp1genotyping (CYP1A1m1)

The *CYP1A1 Msp*1 (or M_1 allele) involves a T>C substitution at 3081 (rs#4646093) of the 3′ untranslated region which creates an *Msp*I restriction site. The DNA samples were amplified with the primers: Forward: 5′-CAGTGAAGAGGTGTAGCCGC-3′ and reverse 5′-TAGGAGTCTTGTCTCATGCC-3′ (downstream) (Perkin-Elmer). The PCR amplification was carried out with 1 μg DNA in 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.3 mM deoxyribonucleotide triphosphates (Perkin-Elmer), 0.2 μM of each primer and 1.5 U of Taq polymerase (AmpliTaq; Perkin-Elmer) in a total volume of 30 μl. Amplification was performed with an initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 61 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplification product of 340 bp (10 μl) was digested with 3 U *Msp*I (New England Biolabs, Beverly, MA) in 50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT) at 37 °C and incubation for 4 h. Then fragment lengths were analyzed on a 6% poly acrylamide gel electrophoresis. When an *Msp*I restriction site was present, the fragment of 340 base pairs (bp) was digested into two fragments: 140 and 200 bp. Homozygous wild type yielded a 340 bp fragment while heterozygotes showed three bands of 340, 200 and 140 bp; homozygous rare allele lacked 340 bp band and had 200 and 140 bp bands.

2.5. CYP1A1 NcoI genotyping (CYP1A1m2)

An isoleucine ^{462} valine (rs#1048943) substitution in exon7, which results in a loss of *Nco*I restriction site at the heme binding region, was determined by polymerase chain reaction (PCR) and RFLP. The DNA samples were amplified with the primers: forward: 5′-GAAAGGCTGGGTCCACCCTCT-3′ and reverse: 5′-CCAGGAAGAGAA AGACCTCCCAGCGGGGCCA-3′. PCR was performed in 30 μl volume containing 1 μg DNA in 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.3 mM deoxyribonucleotide triphosphates (Perkin-Elmer), 0.2 μM of each primer and 1.5 U of Taq polymerase (AmpliTaq; Perkin-Elmer). The PCR conditions were initialized at 94 °C for 5 min followed by 35 cycles of 94 °C, 30 s — 65 °C, 30 s — 72 °C, 1 min and final extension at 72 °C for 7 min. PCR products (370 bp) were digested with 0.5 μl *Nco*I for 1 h at 37 °C. The fragments were separated on 6% acrylamide gel and visualized after staining by ethidium bromide. Wild type DNA is cut by enzyme *Nco*I resulting in fragments 263 and 107 bp. The DNA carrying the variant is not cut resulting in 370 bp band.

2.6. CYP2E1 PstI genotyping

The *CYP2E1* polymorphism was detected by restriction fragment length polymorphism (RFLP) analysis as described above. The transcription regulation region of *CYP2E1* was amplified in a 30 μl polymerase chain reaction (PCR) reaction mixture containing 25 pmol of each *CYP2E1* primers forward: 5′-CCAGTCGAGTCTACATTGTCA-3′ and reverse: 5′-TTCATTCTGTCTTCTA ACTGG-3′ and 1.5 mM of $MgSO_4$. An initial denaturation step (95 °C, 1 min) was followed by 25 cycles of amplification at 95 °C, 1 min — 55 °C, 1 min — 72 °C, and 1 min with a final extension at 72 °C for 4 min. After amplification, 25 μl of the PCR product was digested overnight at 37 °C with 10 U of *Pst*I enzyme (Invitrogen, Carlsbad, CA, USA). The presence of a rare allele restriction site was shown by two fragments of 120 and 290 bp analyzed on an ethidium bromide-stained 6% polyacrylamide gel. The presence of a restriction site resulted in only two fragments (120 and 290 bp) indicating rare allele, and in case of heterozygotes three fragments of 410, 120 and 290 bp were observed.

2.7. Statistical analysis

The calculation of genotypic and allele frequencies for cases and controls was carried out using the POPGENE v1.32 software available online at http://www.ualberta.ca/~fyeh/popgene_download.html. A detailed power analysis was performed and with the present sample size. The association between polymorphisms of respective genes with the risk of OSF was estimated by computing the odds ratio (OR) and calculating the 95% confidence interval (CI) using a chi-square table analysis, and