

Full length Research paper

Study of susceptibility to oral submucous fibrosis (OSF) in the population of West Bengal at XRCC 1 (Arg399Gln) by RFLP

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Oral submucous fibrosis (OSF) is a chronic insidious disease characterized by irreversible generalized fibrosis of the oral soft tissues. It is a debilitating disease of the oral cavity characterized by inflammation and progressive fibrosis of the sub mucosal tissues (lamina propria and deeper connective tissues). It results in marked rigidity and an eventual inability to open the mouth. 10% of the cases resulted to cancer. Associated disorders include leukoplakia and lichen planus. A thorough understanding of the etiopathogenesis of OSF is fundamental to the prevention and control of this dreadful disease. In the present study, attempts have been made to find out the association of polymorphic X-ray repair cross complementing group 1 (XRCC1) Arg399Gln DNA repair genes with the disease. OSF, a precancerous lesion, is attributable to oral chewing habits like chewing of areca nuts, betel quid, paan masala, gutkha and other forms of smokeless tobacco, a significant percent of which resulted to malignancy. Although, this disease is a significant public health burden, particularly in West Bengal, as well as India, no studies have yet investigated the impact of genetic determinants on host susceptibility to this oral disease in Indian population. To address this question, we have examined the frequency of XRCC1 399 genotypes in a cross-section of an asymptomatic population of West Bengal compared with the prevalence in OSF patients. In this study, 52 OSF patients were selected and 126 asymptomatic individuals were selected as control. From patient and control group, DNA was isolated and PCR was done for amplification of DNA. Then, restriction fragment length polymorphism (RFLP) was done to calculate and observe the association of different polymorphic forms of XRCC1 399 DNA repair gene {heterozygous mutant (Arg/Gln), homozygous mutant (Gln/Gln) or wild type (Arg/Arg) } with respect to OSF.

Key words: X-ray repair cross complementing group 1 (XRCC1), fragment length polymorphism (RFLP), polymorphic forms, oral submucous fibrosis (OSF).

INTRODUCTION

The term oral submucous fibrosis (OSF) means oral (meaning mouth), submucous (meaning below the mucosa of the mouth) and fibrosis (meaning hardening and scarring) (Pindborg, 1985). OSF is a chronic insidious disease characterized by irreversible generalized fibrosis of the oral soft tissues. It is a

debilitating disease of the oral cavity characterized by inflammation and progressive fibrosis of the sub mucosal tissues (lamina propria and deeper connective tissues). It results in marked rigidity and an eventual inability to open the mouth. 10% of the cases resulted to cancer. Associated disorders include leukoplakia and lichen planus (Murti et al., 1985). The main causes behind this disease is chewable agents, primarily, areca nuts (*Areca catechu*), contain substances that irritate the oral mucosa, making it to lose its elasticity. This irreversible precancerous condition is also strongly associated with the habit of

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chewing betel quid (combined areca nut, betel leaf, tobacco and slack lime) (Boyland, 1968). In very early stage, fine fibrillar collagen, marked edema, large fibroblasts, dilated and congested blood vessels and inflammatory infiltrates are found. In the early stages, it may be preceded by, or associated with, vesicle formation and a burning sensation. Early hyalinization is characterized by thickened collagen bundles, moderate numbers of fibroblasts and inflammatory cells (Sinor et al., 1990). Dense bundles and sheets of collagen and thick bands of sub epithelial hyalinization extend into the submucous tissues. Patient experience a burning sensation of the oral mucosa, occasional mucosal ulceration, a peculiar marble-like blanching of the mucosa and palpable fibrous bands of the buccal mucosa, soft palate and lips. This malady is characterized by a sub epithelial inflammatory reaction, eventually followed by the formation of dense fibrous bands, which leads to mucosal rigidity, trismus and inability to eat.

Arecoline, an alkaloid found in the areca nut, promotes salivation, stains saliva red and stimulates fibroblasts to increase production of collagen by 150% (Cox and Walker, 1996). Lime acts to keep the active ingredient in its freebase or alkaline form, enabling it to enter the bloodstream via sublingual absorption. Chewing areca quid may also activate NF-kappaB expression, thereby stimulating collagen fibroblasts and leading to further fibrosis in persons with OSF (Ni et al., 2006). Areca nuts have also been shown to have a high copper content, and chewing areca nuts for 5 to 30 min significantly increases soluble copper levels in oral fluids. This increased level of soluble copper supports the hypothesis that copper acts as an initiating factor that stimulates fibrogenesis through up-regulation of copper-dependent lysyl oxidase activity (Trivedy et al., 2000). Flavanoid, catechin and tannin in areca nuts cause collagen fibers to cross-link, making them less susceptible to collagenase degradation. This results in increased fibrosis by causing both increased collagen production and decreased collagen breakdown (Aziz, 1997). A chemical and toxicological evaluation of *pan masala* revealed the presence of well-established carcinogens like polyaromatic hydrocarbons; nitrosamine; toxic metals, such as lead, cadmium and nickel; residual pesticides like dichlorodiphenyltrichloroethane (DDT) and benzene hexa chloride (BHC); various types of fungi like *Aspergillus*, which is known to produce aflatoxin, a potent liver carcinogen. Arecolidine, tannin in catechu and super oxide ions generated due to auto-oxidation of polyphenols and interaction of catechin with lime are all suspected to have carcinogenic potential.

XRCC1 with its function

X-ray repair cross complementing group 1 (XRCC1) is a DNA repair protein. The protein encoded by this gene is

involved in the efficient repair of DNA single-strand breaks formed by exposure to ionizing radiation and alkylating agents. This protein interacts with DNA ligase III, polymerase beta and poly (ADP-ribose) polymerase to participate in the base excision repair pathway. A rare microsatellite polymorphism in this gene is associated with cancer in patients of varying radiosensitivity. Human XRCC1 is composed of 17 exons and spans a genomic distance of 32 kb. It is located on chromosome 19q13.2 to 13.3 (Kubota et al., 1996). XRCC1 has 633 amino acids. There are three coding polymorphisms at codons 194, 280 and 399 in XRCC1 (Thompson et al., 1990).

The codon 399 polymorphic site is located within the BRCT-1 domain, and is associated with significant reduction in the repair capacity. It has been suggested that polymorphism of active sites involved in protein-protein interactions may result in reduced efficiency to repair DNA damage and confer an increased risk to cancer. The *Gln* allele (codon 399) was distinguished from the *Arg* allele as an undigested fragment (615 bp) when compared with the 221 and 374 bp digested fragments of the *Arg* allele.

An important role for XRCC1 399 is base excision repair. Mammalian XRCC1 399 proteins show a high level of conservation and have interaction regions for other proteins involved in Base excision repair (BER), such as DNA ligase III, DNA polymerase β , AP endonuclease and poly (AD ribose) polymerase1 and polymerase2 (Caldecott et al., 1996). BER is the major pathway involved in removal of endogenous and mutagen induced DNA damage.

OSF is a significant public health burden, particularly in India, yet very few study have investigated the impact of genetic determinants on host susceptibility to OSF in Indian populations. To address this question, we have examined the frequency of XRCC1 399 genotypes in a cross-section of an asymptomatic Indian population and compared these with the prevalence in OSF patients in West Bengal.

MATERIALS AND METHODS

Patients and controls

Unrelated patients diagnosed with OSF in the oral cavity were selected in the Dr. R. Ahmed Dental College and Hospital (Kolkata, India). For all patients, the department of oral pathology conducted histopathologic confirmation of OSF. Unrelated controls came for treatment of dental ailments, but without any previous and present lesions in oral cavity were selected from OPD of Dr. R. Ahmed Dental College and Hospital (Kolkata, India). XRCC1 399 polymorphism might have effects on the incidence of some diseases, so to avoid selection bias, individuals with any prior or present diagnosis of lung, colon, gastric and bladder cancer or respiratory ailments were also excluded from the controls. After obtaining informed written consent, all individuals were personally interviewed using a questionnaire. Information on age, sex, occupation, alcohol consumption, type of tobacco habit, daily tobacco use frequency, duration of habits and economic status was

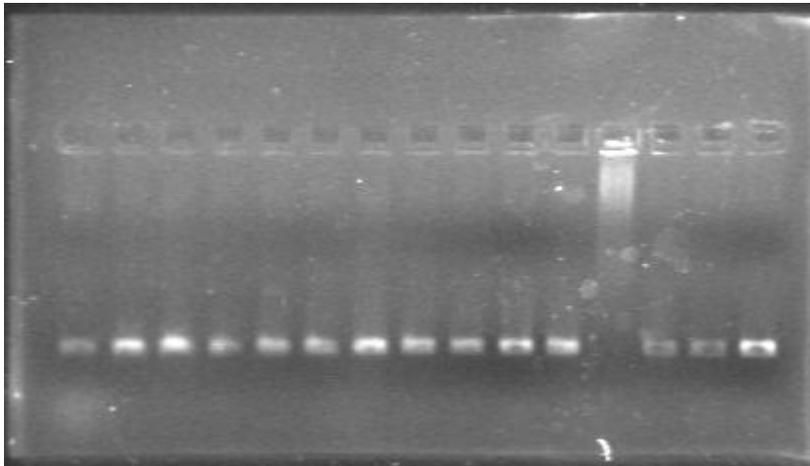


Figure 1. Image showing PCR amplicons in 1% agarose gel.

recorded. Data pertaining to histopathologic diagnosis and clinical staging were obtained from the pathologic reports of the biopsy materials. Most of the patients and controls belonged to low-income group, and this is one of the reasons for which they visited government hospital for treatment. Both patients and controls had occupation in diverse areas, such as agriculture, industry, car driving, private sector office and small business. Most of the females were housewives and doing only household jobs.

Patients and controls reported tobacco habits, such as smoking of beedi and/or cigarette and chewing of tobacco in different forms. In India, the prevalent tobacco chewing habits involve use of betel quid (betel leaf with tobacco, areca nut and lime), gutkha (dried mixture of betel quid and tobacco sold in attractive pouches), mawa and zarda (flavored tobacco) or Khaini (crude form of dried and ground tobacco lime). Some patients and controls reported dual habits comprising both smoking and chewing of tobacco, while the majority had single habit. Information provided by the tobacco chewers regarding the amount of tobacco used per chew, was not reliable. In this present study, total of 52 individuals with OSF and 126 healthy individuals (controls) were selected and evaluated.

Collection of samples and DNA isolation

Clinical blood samples were collected from all patients and control individuals by vein puncture from Dr. R. Ahmed Dental College and Hospital (Kolkata, India). For all patients, in the department of pathology from the same performed histopathologic confirmation of lesions, about 5 ml blood samples were collected by adding ethylenediaminetetraacetic acid (EDTA) as anticoagulant and stored at 20°C until DNA isolation. Genomic DNA was isolated from whole blood according to standard procedure standardized in the laboratory after series of experiments.

Isolation of DNA was performed by addition of 0.9% NaCl with heparinised blood and was mixed by inversion. Serum was collected by centrifugation at 2000 g × 10 min. Solution A (Sucrose 109.5 g; 1 M MgCl₂ 5.0 ml; TritonX-10 ml for 1 L solution) was added four times greater volume of serum by slow mixing. Supernatant was discarded after centrifugation of 3,500 g × 5 min. Later, 2 ml Solution B (1 M Tris HCl 40 ml; 0.5 M EDTA 12 ml; 1 M NaCl 15 ml; 20% SDS 5 ml and water for 100 ml solution), 500 µl Solution C (5 M sodium perchlorate 70.23 g; water 100 ml) and 2 ml of chilled chloroform were added and mixed well. Supernatant was collected after centrifugation at 2,800 g × 5 min and equal

volume of chilled isopropanol was added and centrifuged at 13,000 g × 5 min. DNA was observed as bunch of threads. Then, DNA was collected after washing with 70% alcohol and was dried. Pure DNA was dissolved in TE buffer and was stored at 4°C for further use.

Experimental PCR-RFLP approach

The polymorphism XRCC1 399 was determined using a modified multiplex polymerase chain reaction (PCR) approach. PCR reaction was carried out by following the protocol, and the reaction was performed after standardization of annealing temperature, concentration of MgCl₂, template DNA, Taq DNA polymerase, dNTP's mixture and primers. Primer used for identification are XR1 F – 5' TTG TGC TTT CTC TGT GTC CA 3' and XR1 R – 5' TCC TCC AGC CTT TTC TGA TA 3' for forward and reverse reaction, respectively. The PCR reaction components consists of 2 µl (2.5 N) dNTPs mixture; 3 µl reaction buffer; 1 µl forward primer; 1 µl reverse primer; 0.3 µl Taq polymerase; 21.7 µl water and 1 µl DNA (20 ng) in assay volume of 30 µl.

PCR reaction was performed with Perkin-Elmer PCR system. Each cycle of initial denaturation at 94°C × 5 min, followed by 35 cycles at 94°C × 30 s, 62°C × 1 min and 72°C × 45 s and final extension at 72°C × 5 min. PCR amplified products were analyzed on 1% agarose gel (Figure 1).

Restriction enzyme required for the digestion of XRCC1 399 gene is MspI, an *Escherichia coli* strain cloned gene from *Moraxella* species (5'↓CGG3' and 3'GGC↑C5'). 6% polyacrylamide gel electrophoresis (Native PAGE) was done for the analysis of restriction digestion pattern of XRCC1 399 gene. Query has been done between 126 (54 male and 72 female) normal population as control and 52 (30 male and 22 female) OSF affected population.

RESULTS AND DISCUSSION

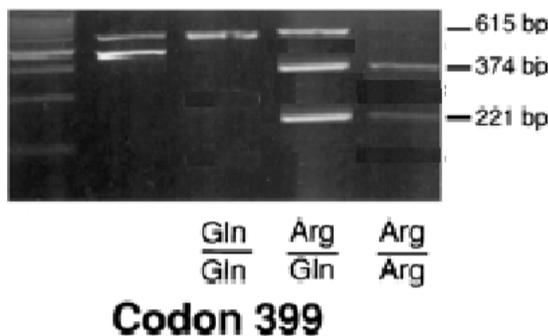
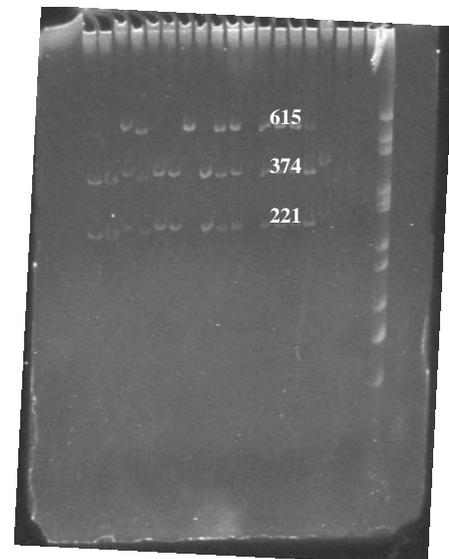
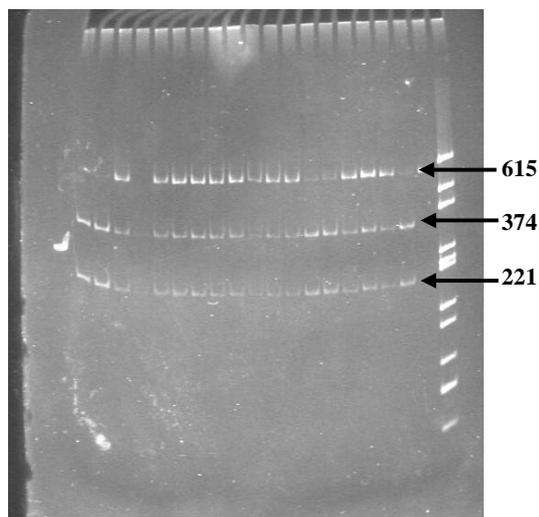
A thorough understanding of the etiopathogenesis of OSF is the fundamental in the prevention and control of this dreadful disease. In the present study, attempts have been made to find out influence of genetic variants of XRCC1 Arg399Gln DNA repair genes with involvement of risk of OSF among the tobacco chewers. Thus, we

Table 1. Percentage of male and female in control and OSF patients.

Gender	126 normal population	52 OSF affected population
	Control (%)	OSF (%)
Male	54 (42.86)	30 (57.69)
Female	72 (57.14)	22 (42.31)

Table 2. Age of control and OSF patients with its mean and range.

Age	Control	OSF
Range	19 - 75	14 - 73
Mean \pm SD	38 \pm 12.87	37 \pm 13.51

**Figure 2.** Three digested product after RFLP.**Figure 4.** Image showing 6% PAGE of OSF-patients.**Figure 3.** Image showing 6% PAGE of controls.

examined different characteristics, such as sex and age of cases and controls (Tables 1 and 2). Mean age was 37 years for cases and 38 for controls. After restriction

enzyme digestion, we found 3 different bands of different size, such as 615, 374 and 221 bp, as shown in Figure 2. Picture of 6% PAGE of control and OSF patients are as shown in Figures 3 and 4. Tables 3 and 4 show the genotype frequencies, odds ratios (ORs) with 95% confidence intervals (CIs) and P value (Pearson's value) for the association between XRCC1 codon 399 variant was analyzed (Table 3 and 4) and the data shows a positive association of heterozygous mutant (Arg/Gln), with OR = 3.0329, 95% confidence interval -1.5548 – 5.9163 along with P value = 0.000911 when compared with XRCC1 Arg/Arg genotype (OR = 0.3838, 95% confidence interval -0.1902 – 0.7746) and XRCC1 Gln/Gln genotype (OR = 0.5261, 95% confidence interval -0.1985 – 1.3944). Thus, in conclusion, our result suggests that risk of OSF is significantly elevated among

Table 3. Genotype of XRCC1 399 of control and OSF patients.

Genotype XRCC1 399	Control (%) of 126 population	OSF affected (%) of 52 population
Arg/Arg	26 (20.63)	21 (40.38)
Arg/Gln	89 (70.64)	23 (44.24)
Gln/Gln	11 (8.73)	8 (15.38)

Table 4. Odd's ratio (OR, 95% confidence interval and Pearson's Value of XRCC1 codon 399 genotypic variants of control and OSF patients.

Genotype	Control (n=126)	Cases (n=52)	OR (95% confidence interval)	P – value (Pearson's value)
	Absent/present			
Arg/Arg	100/26	31/21	0.3838 (0.1902 – 0.7746)	0.006559
Arg/Gln	37/89	29/23	3.0329 (1.5548 – 5.9163)	0.000911
Gln/Gln	115/11	44/8	0.5261 (0.1985 – 1.3944)	0.190985

current/light tobacco chewers with XRCC1 codon 399.

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