ORIGINAL RESEARCH PAPER

Oral Pathology

KEY WORDS: HLA alleles-A10, HLA-B7, HLA-B8, HLA-B51/52 HLA-DR3 & HLA-Cw7 and OSF

HLA TYPING IN KERALA PATIENTS WITH ORAL SUBMUCOUS FIBROSIS

Dr Vijaykumar Biradar *
MDS Reader Department of Oral Pathology and Microbiology Vananchal Dental College and Hospital Farathiya, Gharwa, Jharkhand-822124*Corresponding Author

Dr V T Beena
(Professor and Head) Department of oral pathologyGovernment Dental College, kottayam University of Kerala

Dr R Rajendran
MDS, PhD, FRCPath Former Professor and Head Department of oral pathology Government Dental college, Trivandrum University of Kerala

Dr Moinak Banerjee
Scientist Department of Human molecular geneticsRajiv Gandhi Centre for Biotechnology Trivandrum, Kerala

ABSTRACT

BACKGROUND:A significant association of certain human leukocyte antigens (HLA) and haplotypic pairs with oral submucous fibrosis (OSF) has been reported. However, controversial result of no HLA association with OSF has also been reported. In order to determine which, if any, HLA markers were associated with an increased risk of oral submucous fibrosis, we investigated HLA antigens in 25 patients with oral submucous fibrosis and in 51 controls without oral submucous fibrosis. The main research aim was to assess the association of HLA-A10, HLA-B7, HLA-B8, HLA-B51/52 HLA-DR3 & HLA-Cw7 with OSF.

METHODS: The analysis of HLA-A10, HLA-B7, HLA-B8, HLA-B51/52 HLA-DR3 & HLA-Cw7alleles in OSF patients and healthy control subjects, was performed by serologic DNA typing using polymerase chain reaction with sequence-specific primers (PCR-SSP), respectively.

OBSERVATION AND RESULTS: 25 patients with OSF and 51 controls were considered for comparing the allele frequencies with respect to HLA-A10, HLA-B7, HLA-B8, HLA-B51/52 HLA-DR3 & HLA-Cw7. Chi-square test and Fisher's Exact Test were used to test the significance of the allelic frequencies with respect to various alleles and gender. For significance, a two-tailed 'p' value of less than 0.05 was considered. None of the alleles were significant with respect to cases and controls and also within cases, between Males and Females. One of the reasons for non-significance may be because of the inadequate number of sample in each of the categories.

SUMMARY AND CONCLUSION: To our knowledge, this is the first study on HLA-alleles association with OSF in an Indian population. Ethnic factors are considered to be a major variable for evaluating the predisposition to the disease .Further studies involving larger sample size would be helpful in elucidating the exact role of HLA antigens in the pathogenesis of OSF. Studies like this which are related to individual gene would help by contributing to the existing scientific knowledge and providing a better understanding of this condition. This will help not only the individual at risk but also help to formulate intervention strategies towards better management of these individuals.

INTRODUCTION: Oral submucous fibrosis (OSF) is an insidious chronic fibrotic condition that involves the oral mucosa and occasionally the pharynx and the upper portion of oesophagus. It is always associated with juxtaepithelial inflammatory reaction followed by progressive hyalinization of the lamina propria. Later the subepithelial and submucosal fibrosis leads to stiffness of the oral mucosa and deeper tissues with progressive limitation in opening of the mouth and protrusion of the tongue, thus causing difficulty in eating swallowing and phonation4 .The fibroelastic changes are almost entirely due to abnormal accumulation of collagen in the subepithelial tissue resulting in dense fibrous bands in the mouth[1,2].

The etiopathogenesis of OSF believed to be multifactorial includes areca-nut chewing, ingestion of chillies, genetic and immunologic processes, nutritional deficiencies and other factors. [3]

The pathogenesis of oral submucous fibrosis and the pathways that leads to progressive fibrosis, culminating in extreme difficulty of mouth functions have always aroused curiosity but still remains enigmatic. What need to be elucidated are mainly molecular pathways and also those that lead to its ultimate malignant transformation. Different hypothesis have been put forward in fully elucidating the pathogenic sequence of this disease. The hallmark of the disease is explained to be the aftermath of the stromal changes, which undergoes progressive hyalinization, decrease in vascularity and cellularity with resultant tissue ischemia? Thus the "atrophy" of the overlying epithelium was described as "ischaemic atrophy".

There is substantial evidence to support oral submucous fibrosis as an inheritable disease.[4-11] A number of genetic polymorphisms have been associated with risk for OSF in various populations.[4-11] Since host susceptibility may be defined in terms of genetic make up of the individual, a relatively recent focus in OSF has been to quantify genetic risk and identify specific role that genes play in determining host susceptibility. At present, the specific role that genes play in defining susceptibility remains largely unidentified.

The genes encoding Human Leukocyte Antigens or the Major Histocompatibility Complex have been considered as candidate marker for oral submucous fibrosis because they are involved in regulating immune responses. More than 40 diseases, most of which are autoimmune in nature have been associated with various HLA antigens.[12-17] There have been many case- control studies on HLA association and OSF but the results are inconsistent which may be due to the true heterogeneity of the disease or differences in the ethnic background of the populations studied. Most of the earlier researchers on HLA association have been done in South Africa, Taiwan and USA population. Every population differs in its ethnic make up and a genetic association found in one population may not be with other populations with a different ethnic background. As most of the studies are inconclusive and since only limited data in Indian population is available, the purpose of the present study was to assess the association of HLA-A*10, HLA-B*7, HLA-B*8, HLA-B*51/52 HLA-DR*3 & HLA-Cw7 with OSF in a sample of South Indian (Dravidian) population.

MATERIALS AND METHODS :

A study was designed to ascertain the role of HLA antigen alleles (HLA- A*10, HLA- B*7, HLA- B*8, HLA- B*51/52, HLA- DR*3, HLA- Cw*7) in the pathogenesis of oral submucous fibrosis (OSF). The study was carried out in the Department of Oral Pathology, Government Dental College Trivandrum and Department of Human Molecular Genetics, Rajiv Gandhi Centre for Biotechnology, Trivandrum.

A proforma was formulated to record the socio demographic details, history of areca nut chewing habit, clinical staging of OSF.

The study subjects all belonged to the state of Kerala, south India. The patient group contained 25 subjects with OSF who are habitués of areca nut and were recruited from outpatient department of Government Dental College Trivandrum. Healthy individuals from same geographical area who had no habits were included in the study as controls. Informed consent was obtained from all the patients. The study was approved by Institutional ethical committee, government Dental College, Trivandrum, Kerala.

Peripheral blood sample (10ml) was collected by venipuncture from patients and control groups in plastic falcon tubes containing EDTA. A modified method of standard organic extraction method was used for DNA extraction (Sambrook et al).

HLA-DNA Typing

Briefly, DNA extraction was carried out by classical phenolchloroform method from fresh whole blood samples which anticoagulated by EDTA. Polymerase chain reaction/sequence specific primer (PCR/SSP) method was used to determine HLA DNA typing. A dried primer stock solution consisting of an HLA specific primer mix, i.e., allele and group specific primers and internal positive control primer pairs, was aliquoted in 0.2 ml PCR tubes. The PCR master mix contains: DNA (50 ng/µl)-1µl, Taq buffer - 1µl ,Taq polymerase (1 unit/ µl) - 0.35µl, Forward primer -0.2 µl, Reverse primer -0.2µl, dNTPs-1 µl ,Sterile water -6.25 µl,total 10 µl with three main steps in PCR,i.e, Denaturation at 96şC, Annealing at 54şC, Extension at 72şC which are repeated for 30 or 40 cycles in an automated cycler which can heat and cool the tubes with the reaction mixture in a short time.

The primer pairs used in this study is depicted in table 1 given below:

Primer mix No	Sense Primer	Anti Sense Primer	Product Size	Antigen	Alleles
34	RT193	MB221	619bp	B7	B*0702, 0703, 0704, 0705
155	RT195	MB220	606bp	B8	B*0801, 0802
83	208	216	401 bp	B 51/ 52	B*5101, 5102, 5103, 5104, 5105, 52011, 52012
92	RT130	MB378	1062bp	Cw07	Cw*0701, 0702, 0703
-	AL6	ALC	-	A10	-

Gel pictures of PCR-SSP amplification for the following alleles;

Figure1: A 10 allele, Figure 2: B 7 allele, Figure 3: B 8 allele, Figure 4: B 51/52 allele, Figure 5: Cw 7 allele and Figure 6: DR 3 alleles are mentioned below:

Gel Pictures of PCR-SSP Amplification for the following Alleles

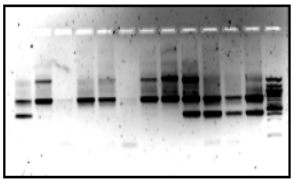

Figure 1: A*10 Allele

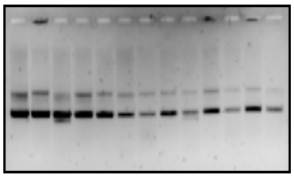

Figure 2: B*7 Allele

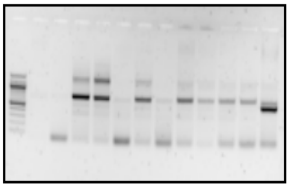

Figure 3: B*8 Allele

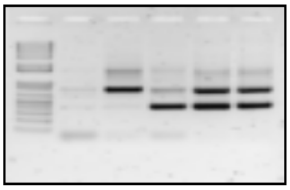

Figure 4: B*51/52 Allele

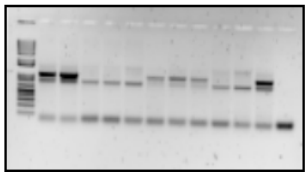

Figure 5: Cw*7 Allele

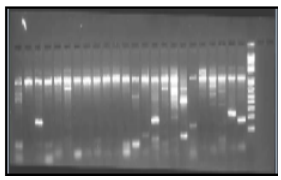

Figure 6: DR*3 Allele

Results:

Chi-square test and Fisher's Exact Test were used to test the significance of the Allelic frequencies with respect to various alleles and gender. For significance, a two-tailed 'p' value of less than 0.05 was considered. None of the alleles were significant with respect to cases and controls and also within cases, between Males and Females.

Disscusion:

Oral submucous fibrosis (OSF) is a potent pre-cancerous condition of the mouth, directly attributable, at least in a sizable percentage of cases, to the use of arecanut product to which these patients are habituated. Many recent surveys conducted in India, show an upward trend in its annual incidence and a shift in demographic pattern towards a lower age group. This could lead to an increase in the incidence of oral cancer in this country, which is already over burdened with this disease. The highest prevalence (0.36%) of OSF recorded from population- based surveys in India was in Kerala (the annual incidence per 100,00 was 13.5) this high prevalence of fibrosis, coupled with the high incidence of oral cancer (the age adjusted ratio of 24.2/100,00) poise Kerala being an ideal setting to pursue further, pathophysiology of this obscure fibrotic condition.

Because of possible immunological connection, the reporting of this disease among non- arecanut habitués and the inability to prove a dose- effect relationship in all cases, the question arose whether there is a predisposition for the disease. In this respect, the finding by Canniff et al (1985) that the human leukocyte antigen A10, B7, DR3 occurred significantly more frequently in patients with OSF (haplotypic linkage) is important. Subsequently Van Wyk et al (1990) investigated the frequency of HLA in arecanut chewers with and without OSF comparing them to a control comprising people with a similar background (ethnic group) but were unable to detect specific frequencies as was found by Canniff et al (1985). Thus the problem of a possible predilection remains unanswered. This could suggest for a strong ethnic component which predisposes certain population to risk of OSF. Recently, a familial clustering of cases of OSF from Northern Kerala, reawakening interest in genetic predisposition of this disease was reported (Rajendran et al 2004). Moreover no data available at present on the HLA haplotypic profile of OSF patients from this part of the world, where recorded the highest annual incidence of oral cancer. However, extensive study on the prevalence of HLA types in various populations of Kerala has been reported earlier (Thomas et al 2004).Because of the conflicting information on HLA frequencies reported so far, any genetic predisposition to the OSF was still worthy of further investigation. Furthermore, to the best of our knowledge, no previous study has focussed on HLA typing in OSF patients in Dravidian population or in Kerala patients. Therefore, in this study, the haplotype frequencies of HLA -A, -B, -C and DR in 25 Kerala patients with OSF were calculated and compared with those in 51 healthy control (without OSF) .

The present study was conducted at the Department of Oral Pathology, Government Dental College, Trivandrum and Human Molecular Genetics Department, Rajiv Gandhi Centre for Biotechnology, Trivandrum. The selected design was case-control study with the case control ratio of 1:2. 25 subjects seeking consultation at the outpatient wing of department of Oral Pathology and who were diagnosed as having oral sub mucous fibrosis according to the clinical and histopathological criteria. 51 healthy subjects (without OSF) among the staff of Rajiv Gandhi centre for Biotechnology served as controls.

A proforma was formulated to record the socio demographic details, history of areca nut chewing habit and clinical staging of OSF. Peripheral blood sample (10ml) was collected by venipuncture from patients and control groups in plastic falcon tubes containing EDTA. A modified method of standard organic extraction method was used for DNA extraction (Sambrook et al). HLA-A*10, HLA-B*7, HLA-B*8, HLA-B*51/52 HLA-DR*3 & HLA-Cw7 was carried out both in cases and controls using molecular methods (PCR- SSP).

Statistical analysis of 25 patients with OSF and 51 controls were considered for comparing the Allele frequencies with respect to HLA-A*10,HLA-B*7, HLA-B*8, HLA-B*51/52 HLA-DR*3 & HLA-Cw7. Chi-square test and Fisher's Exact Test were used to test the significance of the allelic frequencies with respect to various alleles, gender and severity of the disease. For significance, a two-tailed 'p' value of less than 0.05 was considered.

We found no significant increase in the phenotype frequency of HLA-A*10, HLA-B*7, HLA-B*8, HLA-B*51/52 HLA-DR*3 & HLA-Cw7 in OSF patients compared with the corresponding phenotype and haplotype frequencies in healthy control subjects. Similar findings of negative HLA association with OSF have also been reported by Van Wky et al and others. Van Wky et al were unable to demonstrate an HLA associated susceptibility in African OSF patients. Caniff et al showed significantly raised phenotype frequencies of HLA-A*10 and DR*3 as well as a significantly elevated haplotype frequency of HLA –A*10/DR*3 in 50 OSF patients. Saeed et al demonstrated significantly raised frequencies of HLA- A*24, DR B1-11 and DRB3-0202/3 in 21 OSF patients when compared to the English controls. However, when the OSF patient group was compared to the Indian controls, only the phenotype frequency of HLA – DRB1-11 was significantly raised. Higher phenotype frequencies of HLA- Cw2 and –DR-1 have also been found in OSF patients than in healthy control subjects living in Karachi, Pakistan. The significantly increased HLA phenotype and haplotype frequencies reported in three previous studies suggest a definite genetic predisposition and a positive HLA association with OSF.

Although significantly higher phenotype and haplotype frequencies have been shown in OSF patients than in healthy control subjects in these studies; but in the present study we were unable to demonstrate HLA - associated susceptibility in Kerala (Dravidian) patients. The variations and discrepancies in the results of these studies could be because of differences in the characteristics in the group studies, In OSF diagnosis methods used and in HLA typing methods used. The characteristics of the groups included sample size, race, age, sex and socioeconomic status of the study and control subjects. The study group of Canniff et al consisted of 50 OSF patients (48 Indians and 2 Pakistanis living in the UK, 37 women and 13 men, mean age 44.4years); that of Van Wyk et al consisted of 75 areca nut chewers without OSF and 47 OSF patients (all are south Africans of Indian extraction, 116 women and 6 men, mean age 52.5 years); that of saaed et al is composed of 21 OSF patients (all are Indians living in the UK, sex and mean age of the patients unknown); and that of this present study comprises 25 OSF patients (21 women and 4 men, mean age 56.04 years). It was very clear that the sample size of OSF patients in this study was small; that of Caniff et al or of Van Wyk et al was modest. Furthermore, there was a marked female predilection for the study groups of Caniff et al and Van Wyk et al and for our study group.

Various OSF diagnosis methods have been used in the previous studies and in the present study and in that of Caniff et al, all OSF cases were confirmed by histologic examination of biopsy specimens. Moreover, nearly all our OSF cases belonged to mild to moderately advanced OSF according to the histologic criteria described by Sirsat and Pindborg. In the study of Van Wyk et al, the diagnosis was based on clinical symptoms and signs especially the presence of fibrous bands at one or multiple oral mucosal sites rather than on histologic examination of biopsy specimen. About 36% (17/47) of their OSF cases belonged to early or mild OSF. As patients of various degrees of severity of OSF may have different genetic susceptibilities to OSF, the variations in HLA typing results in different groups of OSF patients could also be attributed to the use of different criteria to select OSF patients. Furthermore, the HLA typing methods used were also varied. Caniff et al and Van Wyk et al used a standard compliment dependent microlymphocytotoxicity technique to type HLA antigens. Saeed et al used a comprehensive DNA typing for HLA class I and II antigens by PCR – SSP technique. DNA typing by the use of PCR-SSP technique is supposed to have an overall resolution greater than or equivalent to good serology. Therefore, the variation in HLA typing results in different groups of OSF patients because of the use of different techniques to identify HLA antigens.

Acknowledgement:
Government Dental College, Trivandrum, Kerala Rajiv Gandhi centre for Biotecnology, Trivandrum, Kerala

Conclusion:
Oral submucous fibrosis (OSF) is a potent precancerous condition of oral mucosa, assuming epidemic proportions in Indian subcontinent. The aetiology of this condition is assumed to be multifactorial.The nature of this disease remains enigmatic and pathogenesis is obscure. This study was aimed to assess the association of HLA-A*10, HLA-B*7, HLA-B*8, HLA-B*51/52 HLA-DR*3 & HLA-Cw7 with OSF in a sample of South Indian (Dravidian) population. In the present study, none of the alleles were significant with respect to cases and controls. One of the reasons for non-significance may be because of the inadequate number of sample in each of the categories. Further studies involving larger sample size would be helpful in elucidating the exact role of HLA antigens in the pathogenesis of OSF.

Studies like this which are related to individual gene would help by contributing to the existing scientific knowledge and providing a better understanding of this condition. This will help not only the individual at risk but also help to formulate intervention strategies towards better management of these individuals.

References

1. Murti PR, Bhonsle RB, Gupta PC, Daftary DK, Pindborg JJ, Mehta FS. Etiology of oral submucous fibrosis with special reference to the role of areca nut chewing. J Oral Pathol Med 1995 Apr; 24(4):145-52.
2. Canniff JP, Harvey W, Harris M. Oral submucous fibrosis: its pathogenesis and management. Br Dent J 1986 Jun 21; 160(12):429-34.
3. Van Wyk CW, Philips VM. Collagen in submucous fibrosis: an electron microscopic study. J Oral Pathol Med 1990; 19:182-7.
4. Rajlalita P. Valis Molecular pathogenesis of oral submocous fibrosis: A collagen metabolic disorder. J oralpathol Med 2005 34: 312-8.
5. Murti PR, Bhonsle RB, Gupta PC, Daftary DK, Pindborg JJ, Mehta FS. Etiology of oral submucous fibrosis with special reference to the role of areca nut chewing. J Oral Pathol Med 1995 Apr; 24(4):145-52.
6. Canniff JP, Batchelor JR, Dodi IA, Harvey W. HLA-typing in oral submucous fibrosis. Tissue Antigens 1985 Aug; 26(2):138-42.
7. Saeed B, Haque MF, Meghji S, Harris M. HLA typing in oral submucous fibrosis. J Dent Res1997; 76:41:1024.
8. Maher R. Oral submucous fibrosis. Phil Thesis. University of London.
9. Hsin Ming Chen, Rhong Phong Hsieh, Hsiang Yang, Ying KUO, Mark Yen ping Kuo, Chun Pin Chiang. HLA typing in Taiwanese patients with oral submucous fibrosis. J Oral Pathol Med 2004; 33:191-9.
10. Chiu CJ, Chiang CP, Chang ML, Chen HM, Hahn LJ, Hsieh LL, et al. Association between genetic polymorphism of tumour necrosis factor-alpha and risk of oral submucous fibrosis, a pre-cancerous condition of oral cancer. J Dent Res 2001 Dec; 80(12):2055-9.
11. Rajendran R, Vidyarani. Familial occurrence of oral submucous fibrosis: report of eight families from northern Kerala, south India. Indian J Dent Res 2004 Oct-Dec; 15(4):139-44.
12. Yi Ning Shin, hung Ji Liu, Kuo Wei Chang. Association of CTLA 4 gene polymorphism in oral submucous fibrosis in Taiwan J Oral Pathol Med 2004; 33: 200-3.
13. Brewerton DA. Discovery: HLA and disease. Curr Opin Rheumatol 2003 Jul; 15 (4); 369-73.
14. Zanelli E, Breedveld FC, de Vries RR. HLA association with autoimmune disease: a failure to protect? Rheumatology (Oxford). 2000Oct; 39(10); 1060-6.
15. Tomlinson IP, Bodmer WF. The HLA system and the analysis of multifactorial genetic disease .Trends Genet. 1995 Dec; 11(12): 493-8.
16. Braines M, Ebringer A. HLA and disease. Mol Aspects Med. 1992; 13(14): 263-378.
17. Sveggaard A, Platz P, Ryder LP. HLA and disease 1982-a survey. Immunol Rev. 1983; 70: 193-218.
18. De Wolf Wc , Dupont B, Yunis EJ. HLA and disease: current concepts. Hum pathol. 1980 Jul; 11(4): 332-37.