J Oral Pathol Med (2004) 33: 191–9

www.blackwellmunksgaard.com/jopm

HLA typing in Taiwanese patients with oral submucous fibrosis

Hsin-Ming Chen[1,2], Rhong-Phong Hsieh[3], Hsiang Yang[2], Ying-Shiung Kuo[1,2], Mark Yen-Ping Kuo[1,2,*], Chun-Pin Chiang[1,2,*]

[1]*School of Dentistry, College of Medicine, National Taiwan University,* [2]*Department of Dentistry, and* [3]*Department of Laboratory Medicine, National Taiwan University Hospital, Taipei, Taiwan*

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 this study, the phenotype and haplotype frequencies of HLA-A, -B, -C, -DRB1, and -DQB1 in 135 Taiwanese OSF patients were calculated and compared with those in 540 healthy control Taiwanese.
METHODS: The analysis of HLA-A, -B, and -C antigens, and of HLA-DRB1 and -DQB1 alleles in OSF patients and healthy control subjects, was performed by serologic typing and DNA typing using polymerase chain reaction with sequence-specific primers (PCR-SSP), respectively.
RESULTS: We found that the phenotype frequency of HLA-B76 (3.0%) in OSF patients was significantly greater than that (0%) in healthy control subjects (corrected *P* (*Pc*) = 0.000). In addition, the haplotype frequencies of HLA-B48/Cw7 (3.0%), -B51/Cw7 (6.7%), and -B62/Cw7 (8.2%) in OSF patients were significantly greater than the corresponding haplotype frequencies (0, 0.7, and 1.9%, respectively) in healthy control subjects (*Pc* = 0.000). The relative risk (RR) values of haplotypes B51/Cw7 (9.57) and B62/Cw7 (4.7) were greater than the RR values of phenotypes B51 (1.81), B62 (2.31), and Cw7 (1.91) in OSF patients. In addition, the etiologic fraction (EF) value of haplotype B51/Cw7 (0.63) was higher than the EF values of phenotypes B51 (0.2) and Cw7 (0.59).
CONCLUSIONS: We conclude that some Taiwanese areca quid (AQ) chewers with particular HLA phenotypes and haplotypes are prone to have OSF. In addition, some particular HLA haplotypes may play more important roles than the individual HLA phenotypes for the genetic susceptibility to OSF. However, the significantly increased HLA phenotype B76 and three of the common HLA haplotypes detected are present in only about 20% of incident cases of OSF.
J Oral Pathol Med (2004) **33**: 191–9

Keywords: HLA typing; oral submucous fibrosis

Introduction

Oral submucous fibrosis (OSF) is an oral precancerous condition (1) characterized by symptoms such as intolerance to spicy food, altered salivation, progressive difficulty in opening the mouth, and signs like vesiculation, ulceration, blanching, rigidity, and stiffening of the oral mucosa and decreased mobility of the tongue (2). It is seen predominantly in Indians and people of South Asian origin and is a chronic progressive and scaring disease of the oral cavity characterized by juxtaepithelial inflammatory cell infiltration followed by fibrosis in the lamina propria and submucosa of the oral mucosa (3). Epidemiologic studies have suggested the habit of areca quid (AQ) chewing as a major etiologic factor of OSF (4–6). In Taiwan, there are two million people who have the AQ chewing habit (7). Recent studies have demonstrated that all the OSF patients in Taiwan have the AQ chewing habit (8–10).

OSF has rarely been described in non-AQ chewing subjects (11), and severe forms of OSF can be associated with a very short history of chewing (2, 12). In addition, only 0.1–11% of the AQ chewers develop OSF (5, 13, 14), and the majority of the AQ chewers show no signs and symptoms of OSF. Furthermore, the occurrence of OSF is not necessarily dose-dependent, and there is no relationship between the onset of the OSF and the frequency of the AQ chewing habit (11). This inconsistency in disease association can be indicative of genetic predisposition.

The earlier investigations show a female predilection for OSF patients (3). OSF frequently occurs in patients of middle-aged group (3, 8–10, 15). Increases in serum levels of immunoglobulins (15, 16) and in circulating immune complexes and their immunoglobulin contents (17, 18) have been reported in OSF patients. The defects in cellular

Correspondence: Chun-Pin Chiang, Department of Oral Pathology and Oral Diagnosis, National Taiwan University Hospital, 1 Chang-Te Street, Taipei 100, Taiwan. Tel.: +886 2 23123456/ext. 6855. Fax: +886 2 23893853. E-mail: cpchiang@ha.mc.ntu.edu.tw
*These two authors equally contributed to this paper.
Accepted for publication August 11, 2003

immunity have also been observed in patients with OSF (17, 19, 20). Furthermore, the high incidence of autoantibodies, including anti-nuclear, anti-smooth muscle, anti-gastric parietal cell, anti-thyroid microsomal, and anti-reticulin antibodies, has been observed in patients with OSF (10, 15). A genetic predisposition, involving the human leukocyte antigens (HLA) A10, DR3, DR7, and haplotypic pairs A10/DR3, B8/DR3, and A10/B8 has also been demonstrated in OSF patients (21). Recent study by use of the polymerase chain reaction (PCR) also shows significantly raised frequencies of HLA-A24, DRB1-11, and DRB3-0202/3 in OSF patients when compared with the English controls (22, 23). Higher frequencies of Cw2 and DR1 have also been reported in OSF patients than in healthy controls in Karachi, Pakistan (24). The female predilection, the onset of disease in the middle life, alterations in humoral and cellular immunities, the high incidence of autoantibodies, and the involvement of the DR locus in the genetic predisposition suggest that OSF may be an autoimmune disease (15). In addition, certain HLA alleles are associated with an increased risk for autoimmune diseases such as scleroderma and rheumatoid arthritis (25, 26). However, controversial result of no HLA-associated susceptibility to OSF has been reported in African OSF patients (27). 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 focused on HLA typing in OSF patients in Taiwan or in South-east Asia. Therefore, in this study, the phenotype and haplotype frequencies of HLA-A, -B, -C, -DRB1, and -DQB1 in 135 Taiwanese patients with OSF were calculated and compared with those in 540 healthy control Taiwanese. We tried to find out whether some Taiwanese AQ chewers with specific HLA phenotypes or haplotypes are prone to have OSF and whether some specific HLA haplotypic pairs are more important than the individual HLA phenotypes for the genetic susceptibility to OSF.

Materials and methods

Patients and control subjects

One hundred and thirty-five OSF patients (aged 17–60 years, mean age 35.7 ± 9.8 years; 132 men and 3 women) were included in this study. For the ethnicity of 135 OSF patients, there were 121 Fukienese, 4 Hakkas, 8 mainlanders, and 2 aborigines. Clinical diagnosis of OSF was made when patients showed characteristic features of OSF, including intolerance to spicy foods, blanching and stiffness of the oral mucosa, fibrous bands in the buccal or labial mucosa, and progressive inability to open the mouth. Some OSF patients might also have a burning sensation in the mouth, xerostomia, the presence of vesicles or ulcers on the oral mucosa, depapillation of the tongue, and impaired tongue mobility. The maximum mouth opening (MMO) and the oral mucosal sites (soft palate, retromolar area, buccal mucosa, labial mucosa, floor of the mouth, and tongue) of involvement in OSF patients were recorded. The mean MMO of 135 OSF patients was 28.8 ± 9.3 mm; 55 had MMO $\leq$25 mm, 65 had MMO between 26 and 40 mm, and 15 had MMO $\geq$41 mm. The mean number of site of involvement of 135 OSF patients was 4.5 ± 1.1. Of 135 OSF cases, the disease involved 2 sites in 5 cases, 3 sites in 25 cases, 4 sites in 34 cases, 5 sites in 40 cases, and 6 sites in 31 cases. The buccal mucosa and retromolar area were the two sites that were involved by OSF in every case, with extra involvement of soft palate in 130 cases (96.3%), of labial mucosa in 95 cases (70.4%), of floor of the mouth in 56 cases (41.5%), and of tongue in 56 cases (41.5%).

For each patient, clinical diagnosis was confirmed by histopathologic examination of a biopsy specimen taken from the buccal mucosa. The histologic criteria for a diagnosis of OSF were: (i) atrophic epithelium with parakeratosis or hyperkeratosis; and (ii) mild, moderate, or marked fibrosis or hyalinization in the lamina propria, submucosa, and superficial muscle layer. These 135 OSF specimens were further divided into early ($n = 6$), moderately advanced ($n = 63$), and advanced ($n = 66$) OSF subgroups according to the histologic criteria described by Sirsat and Pindborg (28).

All the 135 OSF patients had AQ chewing habit; they chewed 2–300 (mean, 29.2 ± 32.5) quids per day for 0.5–36 (mean, 10.5 ± 7.1) years. One hundred and eighteen of the 135 (87.4%) OSF patients were smokers; they smoked 1–60 (mean, 21.0 ± 11.2) cigarettes per day for 3–40 (mean, 14.9 ± 7.9) years. Sixty-nine of the 135 (51.1%) OSF patients were alcohol drinkers; they drank 15.9–6450.3 (mean, 563.6 ± 955.0) grams of pure alcohol per week for 0.5–40 (mean, 11.9 ± 9.2) years.

For each OSF patient, four healthy control subjects of the same gender were selected with the age of these four subjects being within ± 2 years of the age of the corresponding OSF patient. Thus, 540 age- and sex-matched healthy control subjects (aged 18–61 years, mean age 36.1 ± 9.5 years; 528 men and 12 women; 497 Fukienese, 11 Hakkas, 25 mainlanders, and 7 aborigines) were included in this study. These control subjects were selected from the dental patients with dental caries, pulpal diseases, malocclusion, or missing of teeth but without any oral mucosal diseases. All the OSF patients and selected control subjects did not have any systemic and autoimmune diseases. They were all treated at the Department of Dentistry, National Taiwan University Hospital (NTUH), Taipei, Taiwan during the period from 1997 to 2002. In addition, all of them were unrelated to avoid overlapped calculation of some specific HLA antigens or haplotypes. A verbal explanation of the HLA typing and risks was given to each subject participating in this study, and 15 ml of peripheral venous blood was collected from each subject after informed consent being obtained. This study was approved by the Ethic Committee on Use of Humans as Experimental Subjects in NTUH.

Serologic typing

This study began in 1997; at that stage, only serologic typing for MHC class I (HLA-A, -B, and -C) antigens was available in our laboratory. Although DNA typing for MHC class I antigens has been developed in our laboratory since 2001, for the consistency throughout the entire study we used only serologic typing for the analysis of MHC class I antigens in all subjects. The analyzed lymphocytes were isolated by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation from 10 ml of peripheral blood. Determination of antigens was performed by a standard

complement-dependent microlymphocytotoxicity technique (29) in Terasaki Chinese HLA-ABC 72-well trays (One Lambda Inc., Canoga Park, CA, USA).

DNA typing

The DNA typing for HLA-DRB1 and -DQB1 polymorphisms has been established in our laboratory since 1996. Therefore, the identification of HLA-DRB1 and -DQB1 alleles in all subjects was performed by DNA typing using PCR with sequence-specific primers (PCR-SSP) as previously described (30–32). Briefly, genomic DNA was obtained from Ficoll-Hypaque-purified lymphocytes, and 80 ng of the DNA was amplified by PCR (Perkin Elmer Thermocycler 9600; PE Applied Biosystems, Foster City, CA, USA) in 30 cycles. A total of 48 sequence-specific oligonucleotide primers were used to identify 14 DRB1 and 13 DQB1 alleles. The detailed sequences of the PCR primers have been shown in the previous study (30).

Statistical analysis

Our previous studies have shown a significant correlation between MMO and the number of site of involvement in Taiwanese OSF patients (8–10). However, no significant correlation has been found between MMO, the number of site of involvement, or histologic staging of OSF and the daily/weekly or total consumption of AQs, cigarettes and alcohol or the duration of these oral habits in Taiwanese OSF patients. In addition, there is no significant association between histologic staging of OSF and MMO or the number of site of involvement in Taiwanese OSF patients (8–10). Therefore, in this study, we did not attempt to analyze the relationship between any two of the clinical and histologic parameters.

In order to evaluate the association of HLA antigens with OSF, chi-square with Yates' correction or, in the expected frequencies being too small ($n < 5$), Fisher's exact test was used to calculate the *P*-value. The value was then corrected by multiplying with the number of antigens tested ($n = 85$). Corrected *P*-value (*P*c) was considered to be significant when *P*c was less than 0.05 (33). For measuring the strength of an association of a specific antigen with OSF, relative risk (RR) was estimated by the following formula:

$$RR = (P^+ \times C^-)/(P^- \times C^+)$$

P^+ or P^- denotes the number of OSF patients with positive or negative expressions for a specific antigen, and C^+ or C^- denotes the number of control subjects having positive or negative results for the same antigen, respectively (34). If there was zero number in any one cell, the *P*-value and 95% confidence interval of RR were calculated by Cornfield's iterative approximation (35). A RR value greater than 1.0 implies that subjects carrying the antigen are at an increased risk of a disease (a positive association), while a RR value less than 1.0 implies that subjects carrying the antigen are at a lower risk than those not carrying the antigen (a negative association). Furthermore, the etiologic fraction (EF), which may be a more informative estimate of the strength of association, was calculated as follows: EF = (RR − 1/RR) × frequency in patients (36). The EF value of 1.0 means complete association.

Results

The phenotype frequencies of HLA-A, -B, -C, -DRB1, and -DQB1 antigens in 135 OSF patients and 540 healthy control subjects are showed in Tables 1–4. We found that the phenotype frequency of HLA-B48 (7.4%, $P = 0.032$), -B51 (15.6%, $P = 0.048$), -B62 (20.7%, $P = 0.001$), -B76 (3.0%, $P = 0.000$), -Cw4 (14.1%, $P = 0.042$), -Cw7 (43%, $P = 0.001$), or -DQ5.3 antigen (12.6%, $P = 0.005$) in OSF patients was significantly greater than the corresponding phenotype frequency in healthy control subjects. However, the phenotype frequency of HLA-B55 (0.7%, $P = 0.048$), -B58 (9.6%, $P = 0.019$), -DR17 (5.2%, $P = 0.002$), -DQ02 (9.6%, $P = 0.002$), or -DQ6.1 antigen (15.6%, $P = 0.002$) in OSF patients was significantly lower than the corresponding

Table 1 Phenotype frequencies of HLA-A antigens in patients with OSF and in healthy control subjects

	OSF patients (n = 135)						*Control subjects (n = 540)*	
Antigens	*No.*	*%*	*P*	*Pc*	*RR*	*EF*	*No.*	*%*
A1	3	2.2	0.690	–	–	–	7	1.3
A2	68	50.4	0.985	–	–	–	270	50
A3	0	0	0.707	–	–	–	4	0.7
A11	78	57.8	0.922	–	–	–	312	57.8
A23	0	0	0.707	–	–	–	4	0.7
A24	41	30.4	0.849	–	–	–	157	29.1
A25	0	0	0.575	–	–	–	5	0.9
A26	8	5.9	0.618	–	–	–	24	4.4
A29	0	0	0.473	–	–	–	6	1.1
A30	4	3	0.957	–	–	–	18	3.3
A31	9	6.7	0.578	–	–	–	27	5
A32	0	0	0.707	–	–	–	4	0.7
A33	19	14.1	0.278	–	–	–	100	18.5
A34	0	0	0.859	–	–	–	2	0.4
A66	0	0	–	–	–	–	0	0
A68	0	0	–	–	–	–	0	0
A74	0	0	–	–	–	–	0	0

P, *P*-value; *P*c, corrected *P*-value; RR, relative risk; EF, etiologic fraction.

Table 2 Phenotype frequencies of HLA-B antigens in patients with OSF and in healthy control subjects

	OSF patients (n = 135)						*Control subjects (n = 540)*	
Antigens	*No.*	*%*	*P*	*Pc*	*RR*	*EF*	*No.*	*%*
B7	2	1.5	0.839	–	–	–	12	2.2
B8	1	0.7	0.885	–	–	–	2	0.4
B13	16	11.9	0.252	–	–	–	88	16.3
B18	1	0.7	0.859	–	–	–	1	0.2
B27	6	4.4	0.541	–	–	–	34	6.3
B35	10	7.4	0.600	–	–	–	31	5.7
B37	1	0.7	0.575	–	–	–	4	0.7
B38	8	5.9	0.969	–	–	–	35	6.5
B39	9	6.7	0.243	–	–	–	21	3.9
B42	0	0	–	–	–	–	0	0
B44	0	0	0.275	–	–	–	9	1.7
B45	0	0	0.859	–	–	–	2	0.4
B46	25	18.5	0.179	–	–	–	132	24.4
B47	0	0	0.859	–	–	–	2	0.4
B48	10	7.4	0.032	2.72	2.62	0.17	16	3
B49	0	0	–	–	–	–	0	0
B50	0	0	0.453	–	–	–	1	0.2
B51	21	15.6	0.048	4.08	1.81	0.2	50	9.3
B52	1	0.7	0.116	–	–	–	21	3.9
B53	1	0.7	0.885	–	–	–	2	0.4
B54	11	8.2	0.104	–	–	–	23	4.3
B55	1	0.7	0.048	4.08	0.14	0	27	5
B56	3	2.2	0.951	–	–	–	14	2.6
B57	0	0	0.473	–	–	–	6	1.1
B58	13	9.6	0.019	1.615	0.47	−0.2	100	18.5
B59	0	0	–	–	–	–	0	0
B60	62	45.9	0.164	–	–	–	210	48.9
B61	6	4.4	0.964	–	–	–	27	5
B62	28	20.7	0.001	0.085	2.31	0.39	55	10.2
B63	0	0	–	–	–	–	1	0.2
B67	0	0	0.710	–	–	–	4	0.7
B71	0	0	–	–	–	–	0	0
B75	8	5.9	0.261	–	–	–	51	9.4
B76	4	3	0.000	0.000	∞	–	0	0
B77	0	0	–	–	–	–	0	0

P, *P*-value; *Pc*, corrected *P*-value; RR, relative risk; EF, etiologic fraction.

phenotype frequency in healthy control subjects. After correction of the *P*-value, only the phenotype frequency of HLA-B76 (3.0%) in OSF patients was still significantly greater than that (0%) in healthy control subjects ($Pc = 0.000$). As none of the healthy control subjects bore HLA-B76 antigen, 95% confidence interval of RR was between 4.25 and infinite calculated by Cornfield's iterative approximation (35) (Table 2).

Further analysis of the haplotype frequencies in OSF patients and healthy control subjects discovered that there was a significant increase in the haplotype frequencies of B62/Cw4 (11.1%, $P = 0.004$), B48/Cw7 (3.0%, $P = 0.000$), B51/Cw7 (6.7%, $P = 0.000$), B62/Cw7 (8.2%, $P = 0.000$), B48/DQ5.3 (3%, $P = 0.046$), and B51/DQ5.3 (2.2%, $P = 0.006$) in OSF patients as well as a significant decrease in the haplotype frequencies of DR17/DQ02 (5.2%,

Table 3 Phenotype frequencies of HLA-C antigens in patients with OSF and in healthy control subjects

	OSF patients (n = 135)						*Control subjects (n = 540)*	
Antigens	*No.*	*%*	*P*	*Pc*	*RR*	*EF*	*No.*	*%*
Cw1	42	31.1	0.555	–	–	–	185	34.3
Cw4	19	14.1	0.042	3.57	1.89	0.19	43	8
Cw6	9	6.7	0.847	–	–	–	36	6.7
Cw7	58	43	0.001	0.085	1.91	0.59	153	28.3
Cw9	13	9.6	0.826	–	–	–	58	10.7
Cw10	37	27.4	0.088	7.48	1.49	0.23	109	20.2

P, *P*-value; *Pc*, corrected *P*-value; RR, relative risk; EF, etiologic fraction.

Table 4 Phenotype frequencies of HLA-DR and -DQ antigens in patients with OSF and in healthy control subjects

	OSF patients (n = 135)						*Control subjects (n = 540)*	
Antigens (alleles)	*No.*	*%*	*P*	*Pc*	*RR*	*EF*	*No.*	*%*
DR01 (DRB1*01)	0	0	0.575	–	–	–	5	0.9
DR04 (DRB1*04)	43	31.9	0.082	–	–	–	130	24.1
DR07 (DRB1*07)	8	5.9	0.839	–	–	–	32	5.9
DR08 (DRB1*08)	21	15.6	0.060	–			127	23.5
DR09 (DRB1*09)	40	29.6	0.950	–	–	–	164	30.4
DR10 (DRB1*10)	2	1.5	0.265	–	–	–	21	3.9
DR11 (DRB1*11)	33	24.4	0.125	–	–	–	98	18.2
DR12 (DRB1*12)	36	26.7	0.461	–	–	–	164	30.4
DR13 (DRB1*13)	6	4.4	0.303	–	–	–	40	7.4
DR14 (DRB1*14)	23	17	0.062	–			58	10.7
DR15 (DRB1*15)	22	16.3	0.536	–	–	–	103	19.1
DR16 (DRB1*16)	14	10.4	0.875	–	–	–	56	10.4
DR17 (DRB1*0301)	7	5.2	0.002	0.17	0.29	−0.16	85	15.7
DRB1*0302; 0303	0	0	–	–	–	–	0	0
DQ02 (DQB1*02)	13	9.6	0.002	0.17	0.39	−0.21	117	21.7
DQ07 (DQB1*0301)	65	48.1	0.893	–	–	–	254	47
DQ08 (DQB1*0302)	28	20.7	0.152	–	–	–	82	15.2
DQ09 (DQB1*0303)	42	31.1	0.950	–	–	–	164	30.4
DQ4.1 (DQB1*0401)	17	12.6	0.318	–	–	–	50	9.3
DQ4.2 (DQB1*0402)	5	3.7	0.411	–	–	–	11	2
DQ5.1 (DQB1*0501)	3	2.2	0.099	–	–	–	34	6.3
DQ5.2 (DQB1*0502)	25	18.5	0.980	–	–	–	103	19.1
DQ5.3 (DQB1*0503)	17	12.6	0.005	0.425	2.54	0.27	29	5.4
DQ6.1 (DQB1*0601)	21	15.6	0.002	0.17	0.45	−0.27	156	28.9
DQ6.2 (DQB1*0602)	7	5.2	0.473	–	–	–	40	7.4
DQ6.3 (DQB1*0603)	0	0	0.328	–	–	–	8	1.5
DQ6.459 (DQB1*060459)	6	4.4	0.646	–	–	–	32	5.9

P, *P*-value; *P*c, corrected *P*-value; RR, relative risk; EF, etiologic fraction.

$P = 0.005$), B58/DR17 (4.4%, $P = 0.014$), and B58/DQ02 (5.2%, $P = 0.013$) in OSF patients, compared with the corresponding haplotype frequencies in healthy control subjects (Tables 5 and 6). After correction of the *P*-value, only the haplotype frequencies of B48/Cw7 (3.0%, $Pc = 0.000$), B51/Cw7 (6.7%, $Pc = 0.000$), and B62/Cw7 (8.2%, $Pc = 0.000$) in OSF patients were still significantly higher than the corresponding haplotype frequencies (0, 0.7, and 1.9%, respectively) in healthy control subjects. The RR value for OSF patients with B51/Cw7 or B62/Cw7 haplotype was 9.57 or 4.7, respectively, suggesting that patients with HLA-B51/Cw7 and -B62/Cw7 haplotypes had 9.57- and 4.7-fold risk to contract OSF compared to healthy control subjects. In addition, the EF value for OSF patients with B51/Cw7 or B62/Cw7 haplotype was 0.63 or 0.37, respectively, indicating that the genes coding for HLA-B51/

Table 5 Haplotype frequencies of HLA-B/C and DR/DQ antigens in patients with OSF and in healthy control subjects

	OSF patients (n = 135)						*Control subjects (n = 540)*	
Antigens	*No.*	*%*	*P*	*Pc*	*RR*	*EF*	*No.*	*%*
B48/Cw4	0	0	–	–	–	–	0	0
B51/Cw4	1	0.7	0.885	–	–	–	2	0.4
B55/Cw4	0	0	0.859	–	–	–	2	0.4
B58/Cw4	0	0	0.859	–	–	–	2	0.4
B62/Cw4	15	11.1	0.004	0.34	2.81	0.27	23	4.3
B76/Cw4	1	0.7	0.453	–	–	–	0	0
B48/Cw7	4	3	0.000	0.000	–	–	0	0
B51/Cw7	9	6.7	0.000	0.000	9.57	0.63	4	0.7
B55/Cw7	0	0	0.885	–	–	–	3	0.6
B58/Cw7	6	4.4	0.961	–	–	–	21	3.9
B62/Cw7	11	8.2	0.000	0.000	4.7	0.37	10	1.9
B76/Cw7	1	0.7	0.453	–	–	–	0	0
DR17/DQ02	7	5.2	0.005	0.425	0.32	−0.1	79	14.6
DR17/DQ5.3	1	0.7	0.453	–	–	–	0	0
DR17/DQ6.1	1	0.7	0.380	–	–	–	13	2.4

P, *P*-value; *P*c, corrected *P*-value; RR, relative risk; EF, etiologic fraction.

Table 6 Haplotype frequencies of HLA-B/DR, Cw/DR, B/DQ, and Cw/DQ antigens in patients with OSF and in healthy control subjects

	OSF patients (n = 135)						*Control subjects (n = 540)*	
Antigens	*No.*	*%*	*P*	*Pc*	*RR*	*EF*	*No.*	*%*
B48/DR17	0	0	0.575	–	–	–	5	0.9
B51/DR17	0	0	0.885	–	–	–	3	0.5
B55/DR17	0	0	–	–	–	–	0	0
B58/DR17	6	4.4	0.014	1.19	0.33	−0.12	66	12.2
B62/DR17	0	0	0.088	–	–	–	16	3
B76/DR17	0	0	–	–	–	–	0	0
Cw4/DR17	0	0	0.328	–	–	–	8	1.5
Cw7/DR17	4	3	0.958	–	–	–	19	3.5
B48/DQ02	0	0	0.575	–	–	–	5	0.9
B51/DQ02	2	1.5	0.924	–	–	–	5	0.9
B55/DQ02	0	0	–	–	–	–	0	0
B58/DQ02	7	5.2	0.013	1.105	0.36	−0.13	72	13.3
B62/DQ02	0	0	0.055	–	–	–	19	3.5
B76/DQ02	0	0	–	–	–	–	0	0
Cw4/DQ02	0	0	0.141	–	–	–	13	2.4
Cw7/DQ02	5	3.7	0.887	–	–	–	24	4.4
B48/DQ5.3	4	3	0.046	3.91	5.47	0.16	3	0.6
B51/DQ5.3	3	2.2	0.006	0.51	–	–	0	0
B55/DQ5.3	0	0	–	–	–	–	0	0
B58/DQ5.3	1	0.7	0.453	–	–	–	0	0
B62/DQ5.3	2	1.5	0.575	–	–	–	3	0.6
B76/DQ5.3	0	0	–	–	–	–	0	0
Cw4/DQ5.3	1	0.7	0.453	–	–	–	0	0
Cw7/DQ5.3	8	5.9	0.302	–	–	–	19	3.5
B48/DQ6.1	2	1.5	0.69	–	–	–	8	1.5
B51/DQ6.1	3	2.2	0.839	–	–	–	11	2
B55/DQ6.1	0	0	0.196	–	–	–	11	2
B58/DQ6.1	2	1.5	0.346	–	–	–	19	3.5
B62/DQ6.1	3	2.2	0.424	–	–	–	5	0.9
B76/DQ6.1	0	0	–	–	–	–	0	0
Cw4/DQ6.1	2	1.5	0.575	–	–	–	3	0.6
Cw7/DQ6.1	11	8.2	0.238	–	–	–	66	12.2

P, *P*-value; *Pc*, corrected *P*-value; RR, relative risk; EF, etiologic fraction.

Cw7 and -B62/Cw7 haplotypes in AQ chewers were partially (63 and 37%, respectively) responsible for the susceptibility to OSF (Tables 5 and 6).

Discussion

In this study, the HLA phenotype and haplotype frequencies in OSF patients were determined and compared with those in healthy control subjects. We found a significant increase in the phenotype frequency of HLA-B76 and in haplotype frequencies of HLA-B48/Cw7, B51/Cw7, and B62/Cw7 in OSF patients compared with the corresponding phenotype and haplotype frequencies in healthy control subjects. Similar findings of a positive HLA association with OSF have also been reported by others (21–24). Canniff et al. (21) showed significantly raised phenotype frequencies of HLA-A10 and -DR3 as well as a significantly elevated haplotype frequency of HLA-A10/DR3 in 50 OSF patients. Saeed et al. (22) demonstrated significantly raised frequencies of HLA-A24, -DRB1-11, 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 (22). Higher phenotype frequencies of HLA-Cw2 and -DR1 have also been found in OSF patients than in healthy control subjects living in Karachi, Pakistan (24). The significantly increased HLA phenotype and haplotype frequencies reported in three previous and the present 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 (21–24), van Wky et al. (27) were unable to demonstrate an HLA-associated susceptibility in African OSF patients. The variations and discrepancies in the results of these five studies could be because of differences in characteristics of the groups studied, in OSF diagnosis methods used, and in HLA typing methods used. The characteristics of the groups included sample size, race, age, sex, and socio-economic status of the study and control subjects. The study group of Canniff et al. (21) consists of 50 OSF patients (48 Indians and 2 Pakistanis living in the UK, 37 women and 13 men, mean age 44.4 years); that of van Wyk et al. (27) consists 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 Saeed et al. (22) 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 study comprises 135 OSF patients (121 Fukienese, 4 Hakkas, 8 mainlanders, and 2 aborigines;

3 women and 132 men, mean age 35.7 years). The characteristic features of Maher's study group are not available (24). It was very clear that the sample size of OSF patients in this study was the largest; that of Canniff et al. (21) or of van Wyk et al. (27) was modest, and that of Saeed et al. (22) was small. Furthermore, there was a marked female predilection for the study groups of Canniff et al. (21) and van Wyk et al. (27) and a prominent male predisposition for our study group. In addition, the majority of study subjects of van Wyk et al. (27) are non-economically active, elderly women, and they form a relatively small proportion of the whole population. However, most of our study subjects are economically active, young or middle-aged adult men, and they are more evenly distributed in the whole population. As the sample size, race, sex, and age may have influence on genetic susceptibilities to a specific disease like OSF, differences in sample size, race, age, sex, and socio-economic status of the study and control subjects could partially explain the variations and discrepancies in the HLA typing results in different groups of OSF patients.

Various OSF diagnosis methods have been used in the previous and present studies. In the present study and in that of Canniff et al. (21), all OSF cases were confirmed by histologic examination of biopsy specimens. Moreover, nearly all of our OSF cases (95.6%) belonged to moderately advanced and advanced OSF according to the histologic criteria described by Sirsat and Pindborg (28). In the study of van Wky et al. (27), the diagnosis of OSF is based on clinical symptoms and signs especially the presence of fibrous band in one or multiple oral mucosal sites rather than on histologic examination of biopsy specimens. About 36% (17/47) of their OSF cases belong to early or mild OSF. As patients with 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. Canniff et al. (21) and van Wky et al. (27) used a standard complement-dependent microlymphocytotoxicity technique to type HLA antigens. Saeed et al. (22, 23) used a comprehensive DNA typing for HLA class I and II antigens by PCR-SSP technique. We used a serologic typing for assessing the HLA-A, -B, and -C antigens and a genotyping with PCR-SSP technique for determining the HLA-DRB1 and -DQB1 antigens. DNA typing by use of PCR-SSP technique is supposed to have an overall resolution greater than or equivalent to good serology (23). Therefore, the variations in HLA typing results in different groups of OSF patients could also be because of the use of different techniques to identify HLA antigens.

In this study, assumed haplotype frequencies were determined for OSF patients. Haplotypic pairs of HLA-B51/Cw7 and B62/Cw7 showed significantly higher frequencies in OSF patients than in healthy control subjects even after the *P*-value had been corrected. The RR values of haplotypes B51/Cw7 (9.57) and B62/Cw7 (4.7) were greater than the RR values of phenotypes B51 (1.81), B62 (2.31), and Cw7 (1.91). In addition, the EF value of haplotype B51/Cw7 (0.63) was higher than the EF values of phenotypes B51 (0.2) and Cw7 (0.59). These findings suggest that some particular HLA haplotypes are more important than individual HLA antigens for the genetic susceptibility to OSF. Similar results of greater importance of HLA haplotypes than phenotypes have also been reported in other systemic diseases, like rheumatoid arthritis, insulin-dependent diabetes mellitus (37), and gold-induced nephropathy (38). Recently, we also reported that some specific HLA-DR/DQ haplotypes are found to be more important than individual HLA-DR and -DQ phenotypes for the development of mucocutaneous type of Behcet's disease and for the disease shift from recurrent aphthous stomatitis to mucocutaneous type of Behcet's disease (39).

In addition to MHC class I- and class II-related genes, there may be other susceptible genes that are not in the MHC class I and class II regions but are responsible for the development of OSF. Our recent study (40) demonstrated that the genetic polymorphism of the genes coding for tumor necrosis factor-α (TNF-α) is significantly associated with the risk of OSF. The peripheral blood mononuclear cells from OSF patients secrete increased levels of stimulated TNF-α compared to the control subjects (41). TNF-α can stimulate fibroblast proliferation *in vitro* (42) and has been demonstrated to up-regulate mRNA expression of collagen types I and III (43). Intradermal injections of TNF-α stimulate the accumulation of fibroblasts and collagen (44). Therefore, TNF-α is a cytokine that plays a positive role in the pathogenesis of OSF. As the TNF-α gene locates in the MHC class III region, it is beyond the detection of this study. Therefore, it is highly possible that there may exist other genes in the MHC class III region or other non-MHC associated genes that are responsible for the susceptibility to OSF. Further investigations are needed to verify this possibility.

Although a significant increase in phenotype frequency of HLA-B76 and in haplotype frequencies of HLA-B48/Cw7, -B51/Cw7, and -B62/Cw7 was shown in our OSF patients, the percentages OSF patients carrying HLA-B76, -B48/Cw7, -B51/Cw7, and -B62/Cw7 were only 3, 3, 6.7, and 8.2%, respectively. In the study of Canniff et al. (15, 21), significantly elevated phenotype frequencies of HLA-A10 and -DR3 and significantly raised haplotype frequencies of HLA-A10/DR3 are found. However, the percentages OSF patients bearing A10, DR3, and A10/DR3 are only 23, 34, and 16%, respectively. These findings indicate that HLA class I and class II genes only play a minor role in the pathogenesis of OSF. There must be other factors that play more important roles in the pathogenesis of OSF.

Several factors have been involved in the etiology of OSF. Epidemiologic studies have shown the intimate relationship between the AQ chewing habit and OSF (4–6). Our recent studies have also demonstrated that all the OSF patients in Taiwan have the AQ chewing habit (8–10). The areca nut alkaloids can stimulate the fibroblast proliferation and collagen synthesis (45). Cytokines and growth factors produced by inflammatory cells within the OSF tissues may promote fibrosis by inducing proliferation of fibroblasts, up-regulating collagen synthesis and down-regulating collagenase production (46). The areca nut polyphenols, (+)-catechin and tannins, can stabilize the collagen structure (47, 48). OSF fibroblasts have been shown to produce the collagen with more stable structure such as collagen type I trimer (49) and secrete more lysyl oxidase, which causes an increase in

collagen cross-linkages (50, 51). In addition, the OSF fibroblasts secrete less amount of collagenase (52) and have less collagen phagocytosis activity than the normal oral mucosal fibroblasts (53). These previous studies support that, in addition to genetic susceptibility, the mechanisms leading to increased production of collagen and decreased degradation of collagen also play very important roles in the pathogenesis of OSF.

To the best of our knowledge, this is the first paper reporting the HLA typing results in Taiwanese patients with OSF. This study found significantly greater phenotype frequency of HLA-B76 and haplotype frequencies of HLA-B48/Cw7, -B51/Cw7, and -B62/Cw7 in Taiwanese OSF patients than in healthy control subjects. These findings suggest that some Taiwanese AQ chewers with specific HLA phenotypes or haplotypes are prone to have OSF. Further analysis of the RR and EF values for OSF patients with these specific HLA phenotypes and haplotypes indicates that some particular HLA haplotypes play more important roles than the individual HLA phenotypes for the genetic susceptibility to OSF.

References

1. Pindborg JJ. *Oral Cancer and Precancer.* Bristol: John Wright, 1980; 108–11.
2. Warnakulasuriya S. Semi-quantitative clinical description of oral submucous fibrosis. *Ann Dent* 1987; **46**: 18–21.
3. Pindborg JJ, Sirsat SM. Oral submucous fibrosis. *Oral Surg Oral Med Oral Pathol* 1966; **22**: 764–79.
4. Maher R, Lee AJ, Warnakulasuriya KAAS, Lewis JA, Johnson NW. Role of areca nut in the causation of oral submucous fibrosis: a case-control study in Pakistan. *J Oral Pathol Med* 1994; **23**: 65–9.
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; **24**: 145–52.
6. Shah N, Sharma PP. Role of chewing and smoking habits in the etiology of oral submucous fibrosis (OSF): a case-control study. *J Oral Pathol Med* 1998; **27**: 475–9.
7. Ko YC, Chiang TA, Chang SJ, Hsieh SF. Prevalence of betel quid chewing habit in Taiwan and related sociodemographic factors. *J Oral Pathol Med* 1992; **21**: 261–4.
8. Chen HM, Wang CY, Chen CT, et al. Autofluorescence spectra of oral submucous fibrosis. *J Oral Pathol Med* 2003; **32**: 337–43.
9. Chiang CP, Wu HY, Liu BY, Wang JT, Kuo MY. Quantitative analysis of immunocompetent cells in oral submucous fibrosis in Taiwan. *Oral Oncol* 2002; **38**: 56–63.
10. Chiang CP, Hsieh RP, Chen TH, et al. High incidence of autoantibodies in Taiwanese patients with oral submucous fibrosis. *J Oral Pathol Med* 2002; **31**: 402–9.
11. Seedat HA, van Wyk CW. Submucous fibrosis (SF) in ex-betel nut chewers: a report of 14 cases. *J Oral Pathol* 1988; **17**: 226–9.
12. Warnakulasuriya S. Clinical and pathological criteria for diagnosis of oral submucous fibrosis. *The First Asia-Pacific Workshop for Oral Mucosal Lesions*. Nagoya, Japan, 1992.
13. Bhonsle RB, Murti PR, Daftary DK, et al. Regional variations in oral submucous fibrosis in India. *Community Dent Oral Epidemiol* 1987; **15**: 225–9.
14. Rajendran R. Oral submucous fibrosis: etiology, pathogenesis, and future research. *Bull World Health Organ* 1994; **72**: 985–96.
15. Canniff JP, Harvey W, Harris M. Oral submucous fibrosis. its pathogenesis and management. *Br Dent J* 1986; **160**: 429–34.
16. Rajendran R, Sugathan CK, Remani P, Ankathil R, Vijayakumar T. Cell mediated and humoral immune responses in oral submucous fibrosis. *Cancer* 1986; **58**: 2628–31.
17. Balaram P, Pillai MR, Abraham T. Immunology of premalignant and malignant conditions of the oral cavity. Part II. Circulating immune complexes. *J Oral Pathol Med* 1987; **16**: 389–91.
18. Remani P, Ankathil R, Vijayan KK, Haseena Beevi VM, Rajendran R, Vijayakumar T. Circulating immune complexes as an immunological marker in premalignant and malignant lesions of the oral cavity. *Cancer Lett* 1988; **40**: 185–91.
19. Pillai MR, Balaram P, Abraham T, Nair MK. Lymphocyte population in premalignant and malignant lesions of the oral cavity. *Neoplasm* 1987; **34**: 469–79.
20. Pillai MR, Balaram P, Kannan S, et al. Interferon activation of latent natural killer cells and alteration in kinetics of target cell lysis: clinical implication for oral precancerous lesion. *Oral Surg Oral Med Oral Pathol* 1990; **70**: 458–61.
21. Canniff JP, Batchelor JR, Dodi IA, Harvey W. HLA-typing in oral submucous fibrosis. *Tissue Antigens* 1985; **26**: 138–42.
22. Saeed B, Haque MF, Meghji S, Harris M. HLA-typing in oral submucous fibrosis. *J Dent Res* 1997; **76**: 1024 (abstr).
23. Bunce M, O'Neill CM, Barnardo MCNM, et al. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, CRB5 & DQB1 by PSR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens* 1995; **46**: 355–67.
24. Maher R. Oral submucous fibrosis. MPhil Thesis, University of London, 1997.
25. Vlachoyiannopoulos PG, Dafni UG, Pakas I, Spyropoulou-Vlachou M, Stavropoulos-Giokas C, Moutsopoulos HM. Systemic scleroderma in Greece: low mortality and strong linkage with HLA-DRB1*1104 allele. *Ann Rheum Dis* 2000; **59**: 359–67.
26. Fries JF, Wolfe F, Apple R, et al. HLA-DRB1 genotype associations in 793 white patients from a rheumatoid arthritis inception cohort: frequency, severity, and treatment bias. *Arthritis Rheum* 2002; **46**:2320–9.
27. van Wyk CW, Grobler-Rabie AF, Martell RW, Hammond MG. HLA-antigens in oral submucous fibrosis. *J Oral Pathol Med* 1994; **23**: 23–7.
28. Sirsat SM, Pindborg JJ. Subepithelial changes in oral submucous fibrosis. *Acta Pathol Microbiol Scand* 1967; **70**: 161–73.
29. Terasaki PI, McClelland JD. Microdroplet assay of human serum cytotoxins. *Nature* 1964; **204**: 998–1000.
30. Shen SW, Hu CY, Lin CY, Yang YC, Hsieh RP. Human leukocyte antigen polymorphisms in the Taiwanese population. *J Formos Med Assoc* 1999; **98**: 11–8.
31. Olerup O, Zetterquist H. HLA-DR tying by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantations. *Tissue Antigens* 1992; **39**: 225–35.
32. Olerup O, Aldener A, Fogdell A. HLA-DQB1 and DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. *Tissue Antigens* 1993; **41**:119–34.
33. Svejgaard A, Jersild C, Nielson LS, Bodmer WF. HLA antigens and disease: statistical and general considerations. *Tissue Antigens* 1974; **4**: 95–105.
34. Grumet FC. HLA and disease association. *Transplant Proc* 1977; **9**: 1839–44.
35. Rothman KJ. *Modern Epidermiology.* Boston: Little, Brown and Company, 1986; pp. 168–9.

36. Green A. The epidemilogical approach to studies of association between HLA and disease. Part II. Estimation of absolute risks, etiologic and preventive fraction. *Tissue Antigens* 1982; **19**: 259–68.
37. McCluskey J, Kay PH, Dawkins RL, Lomori K, Christiansen FT, McCann UJ. Association of specific MHC supratypes with rheumatoid arthritis and insulin dependent diabetes mellitus. *Dis Marker* 1983; **1**: 197–212.
38. Batchelor JR, Dodi IA, Woo P, Panayi GS, Ansell B, Williams PL. Family study of gold induced nephritis in patients with rheumatoid arthritis. In: Albert E, Baur M, eds. *Histocompatibility Testing 1984*. Berlin: Springer–Verlag, 1985; 375–8.
39. Sun A, Hsieh RP, Chu CT, Wang JT, Liu BY, Chiang CP. Some specific human leukocyte antigen (HLA)-DR/DQ haplotypes are more important than individual HLA-DR and -DQ phenotypes for the development of mucocutaneous type of Behcet's disease and for disease shift from recurrent aphthous stomatitis to mucocutaneous type of Behcet's disease. *J Oral Pathol Med* 2001; **30**: 402–7.
40. Chiu CJ, Chiang CP, Chang ML, et al. Association between genetic polymorphism of tumor necrosis factor-alpha and risk of oral submucous fibrosis, a pre-cancerous condition of oral cancer. *J Dent Res* 2001; **80**: 2055–9.
41. Haque MF, Meghji S, Khitab U, Harris M. Oral submucous fibrosis patients have altered levels of cytokine production. *J Oral Pathol Med* 2000; **29**: 123–8.
42. Vilcek J, Palombella VJ, Henriksen-DeStefano D, et al. Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. *J Exp Med* 1986; **163**: 632–43.
43. Zhang Y, Lee TC, Guillemin B, Yu MC, Rom WN. Enhanced IL-1 beta and tumor necrosis factor-alpha release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure. *J Immunol* 1993; **150**: 4188–96.
44. Piguet PF, Grau GE, Vassalli P. Subcutaneous perfusion of tumour necrosis factor induces local proliferation of fibroblasts, capillaries, and epidermal cells, or massive tissue necrosis. *Am J Pathol* 1990; **136**: 103–10.
45. Harvey W, Scutt A, Meghji S, Canniff JP. Stimulation of human buccal mucosa fibroblasts *in vitro* by betel-nut alkaloids. *Arch Oral Biol* 1986; **31**: 45–9.
46. Haque MF, Harris M, Meghji S, Barrett AW. Immunolocalization of cytokines and growth factors in oral submucous fibrosis. *Cytokine* 1998; **10**: 713–9.
47. Kuttan R, Donnely PV, Di Ferrante N. Collagen treated with (+)-catechin becomes resistant to the action of mammalian collagenase. *Experientia* 1981; **37**: 221–3.
48. Scutt A, Meghji S, Canniff JP, Harvey W. Stabilisation of collagen by betel nut polyphenols as a mechanism in oral submucous fibrosis. *Experientia* 1987; **43**: 391–3.
49. Kuo MYP, Chen HM, Hahn LJ, Hsieh CC, Chiang CP. Collagen biosynthesis in human oral submucous fibrosis fibroblast cultures. *J Dent Res* 1995; **74**: 1783–8.
50. Ma RH, Tsai CC, Shieh TY. Increased lysyl oxidase activity in fibroblasts cultured from oral submucous fibrosis associated with betel nut chewing in Taiwan. *J Oral Pathol Med* 1995; **24**: 407–12.
51. Trivedy C, Warnakulasuriya KAAS, Hazarey VK, Tavassoli M, Sommer P, Johnson NW. The upregulation of lysyl oxidase in oral submucous fibrosis and squamous cell carcinoma. *J Oral Pathol Med* 1999; **28**: 246–51.
52. Shieh TY, Yang JF. Collagenase activity in oral submucous fibrosis. *Proc Natl Sci Counc ROC(B)* 1992; **16**: 106–10.
53. Tsai CC, Ma RH, Shieh TY. Deficiency in collagen and fibronectin phagocytosis by human buccal mucosa fibroblasts *in vitro* as a possible mechanism for oral submucous fibrosis. *J Oral Pathol Med* 1999; **28**: 59–63.

Acknowledgements

This study was supported by the research grant of NHRI-CN-9002S from National Health Research Institute, Taipei, Taiwan.