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Association of CTLA-4 gene polymorphism with oral submucous fibrosis in Taiwan

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BACKGROUND: Oral submucous fibrosis (OSF) is an insidious, pre-cancerous, chronic disease that may affect the entire oral cavity and sometimes extend to the pharynx. It has been reported to be associated with immune function. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4; CD (cluster of differentiation) 152) is a negative regulator of T-lymphocyte activation. Particular genotypes of the locus encoding the CTLA-4 glycoprotein have been associated with susceptibility to various autoimmune diseases. This study was designed to investigate the role of CTLA-4 polymorphism in susceptibility to OSF.
METHODS: We genotyped 62 patients with OSF and 147 healthy controls for allelic determinants at the exon 1 +49 polymorphism site by restriction fragment length polymorphism. Genotype and phenotype frequencies were evaluated with Chi-squared test.
RESULTS: The G allele at position +49 of exon 1 was significantly associated with OSF. The frequency of A/A homozygotes was higher in controls than in patients (17.0% vs. 3.2%; $\chi^2 = 7.65$, $P = 0.02$); the G phenotype was more frequent in patients than in controls (96.8% vs. 83.0%; $\chi^2 = 9.31$, $P = 0.002$). Compared with controls, the G allele genotype and phenotype frequencies were increased in patients with OSF.
CONCLUSION: This is the first report that the CTLA-4 +49 G allele confers an increased risk of OSF in Taiwan.
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Keywords: cytotoxic T-lymphocyte-associated antigen 4; oral submucous fibrosis; polymorphism; RFLP

Introduction

Oral submucous fibrosis (OSF) is an insidious, pre-cancerous chronic disease that may affect the entire oral cavity; it sometimes extends to the pharynx (1, 2). OSF is characterized by mucosal rigidity of varying intensity because of fibro-elastic transformation of the juxta-epithelial layer. A subepithelial inflammatory reaction is followed by fibro-elastic changes of the lamina propria accompanied by epithelial atrophy (3). This leads to restricted mouth opening and inability to eat (2). The presence of palpable fibrous bands is a requisite diagnostic criterion for this condition.

OSF is predominately seen in people in south Asian countries (4), or in south Asian immigrants to other parts of the world (5, 6). It is now a public health issue in many parts of the world, including the UK (7), South Africa (8), and many south-east Asian countries (6, 9, 10).

Although the available epidemiological evidence indicates that the chewing of areca nut is an important risk for development of OSF (9, 11–13), not all chewers develop the disease. To date, no conclusive etiologic agent has been identified, despite investigation of factors implicated in the development of oral cancer, including genetic factors, infectious agents, carcinogens, and nutritional, immunologic, and autoimmune factors (2, 14–19). Haque et al. (20) studied OSF with immunohistochemical methods and found immunocompetent cells to be present, with a high ratio of CD4 to CD8 cells. Therefore, they suggested an ongoing cellular immune response leading to a possible imbalance of immunoregulation with eventual local alterations in tissue architecture.

The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4; CD152) located on chromosome 2q33 plays an important role in the maintenance of immune tolerance. It is a homolog of CD28 on the T-cell surface. They share common ligands – B7-1 (CD80) and B7-2 (CD86) – on antigen-presenting cells (APC) and thus constitute the B7-CD28/CTLA-4 costimulatory pathway of T-cell activation (21). CD28–B7 interaction activates T cells and induces antigen-specific T-cell clone proliferation, whereas binding of CTLA-4 to the same ligand down-regulates T-cell activation and might contribute to peripheral tolerance (22–26).

The human CTLA-4 gene contains a polymorphism at position −318 in the promoter region (27), an A/G transition at position +49 in exon 1 (28), and a dinucleotide $(AT)_n$ repeat sequence in the 3′ untranslated region of exon 4 at position 642 (28–30). The A/G polymorphism at exon 1

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position +49 results in an amino acid exchange, threonine to alanine, in the peptide leader sequence. In addition, several *in vitro* studies have demonstrated that +49 polymorphism alters CTLA-4 function vis-à-vis T-cell activation (31). Certain autoimmune diseases, including systemic lupus erythematosus (32, 33), insulin-dependent diabetes mellitus (34–36), Graves' disease (37, 38), Hashimoto thyroiditis (38), multiple sclerosis (39, 40), and rheumatoid arthritis (41) have been shown to be associated with CTLA-4 +49 polymorphism. We designed this study to investigate an association between CTLA-4 polymorphism at position +49 and OSF.

Materials and methods

Subjects

Between November 2000 and December 2002, we recruited 62 consecutive male patients with OSF from the Oral and Maxillofacial Department at the Taipei Mackay Memorial Hospital. All patients were diagnosed by clinical examination and histopathology of biopsy specimens. None of the patients had oral cancer. One hundred and forty-seven control subjects were selected from among people who came for routine physical checkups, non-neoplastic minor operations, or maxillofacial trauma. Those with autoimmune disorders, blood diseases, and previous malignancy were excluded.

DNA extraction

Peripheral blood samples were drawn from all study subjects. Genomic DNA was extracted from fresh or frozen peripheral blood leukocytes using the Pharmacia DNA isolation kit (Pharmacia Biotech, Germany).

Polymerase chain reaction (PCR)

The A/G polymorphisms at exon 1 position +49 of CTLA-4 were amplified using a forward primer, 5′-AAGGCT-CAGCTGAACCTGGT-3′, and a reverse primer, 5′-CTGCTGAAACAAATGAAACCC-3′ (36). Amplification was carried out using 1 μg of genomic DNA, 100 ng of forward and reverse primers, 200 μM dNTP, and 1 U Tag polymerase in 30 μl of reaction mixture containing 1× PCR buffer (10 mM Tris–HCl, pH 8.4, 50 mM KCl, 0.01% gelatin). Samples were subjected to initial denaturation at 95°C for 3 min, followed by 35 cycles of 30 s at 94°C for denaturing, 30 s at 56°C for annealing and 1 min at 72°C for extension, and a final extension at 72°C for 7 min in a DNA Thermal Cycler (Perkin-Elmer Corporation, Foster City, CA, USA). The amplified PCR products were analyzed on 2.0% agarose gel.

Restriction fragment length polymorphism (RFLP) analysis

Ten microliters of the amplified PCR products was digested in a volume of 12.1 μl with 1 U restriction enzyme *Bst*EII under the appropriate buffer conditions (60°C, overnight). The fragments were analyzed on 3.5% agarose gel.

Statistical analysis

Genotype was treated as a 2×3 contingency with Chi-squared test for comparison. Statistical significance was defined as $P_c < 0.05$.

Results

The amplified PCR products consisted of 153-bp fragments. *Bst*EII digestion yielded fragments of 131 and 22 bp (Fig. 1).

Table 1 shows that the +49 genotypes in the patients and 147 controls were patients (17.0% vs. 3.2%; $\chi^2 = 7.65$,

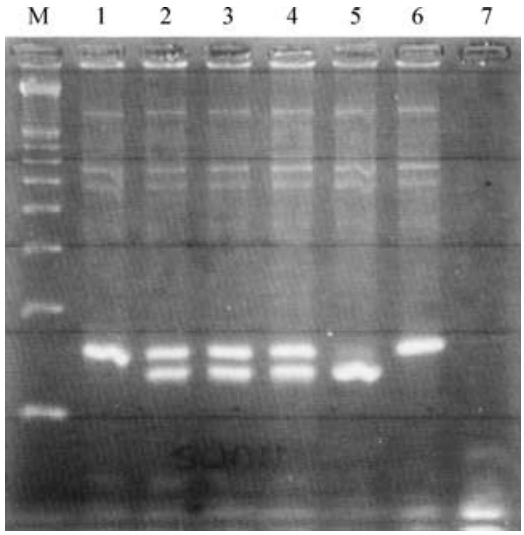

Figure 1 *Bst*EII-digested PCR products analyzed on 3.5% agarose gel . Lane M denotes the 100-bp protein marker; lanes 1 and 6 are homozygous

Table 1 Comparison of CTLA-4 exon 1 +49 genotype polymorphism between OSF patients and controls

	OSF		*Control*			
	N = 62	%	*N* = 147	%	χ^2	*P-value*
Genotype						
A/A	2	3.2	25	17.0	7.652	0.02
A/G	29	46.8	64	43.5		
G/G	31	50.0	58	39.5		
Gene frequencies						
A	33	26.6	114	38.8	5.66	0.01
G	91	73.4	180	61.2	5.66	0.01
Phenotype frequencies						
A	31	50.0	89	60.5	0.8	0.37
G	60	96.8	122	83.0	9.31	0.002

N, number of cases; χ^2, Chi-squared.

$P = 0.02$). Compared with controls, the G allele genotype and phenotype frequencies were increased in patients with OSF (73.4% vs. 61.2%; $\chi^2 = 5.66$, $P = 0.01$ and 96.8% vs. 83.0%, $\chi^2 = 9.31$, $P = 0.002$).

Discussion

Our case-control study demonstrated that patients with OSF had a higher frequency of the G allele at position +49 in exon 1 of CTLA-4 compared with controls. This is the first study to demonstrate such an association with OSF. OSF was originally called idiopathic scleroderma of the mouth (17). The defects in cellular immunity in the disorder are suggestive of an autoimmune phenomenon. Therefore, it is not surprising that CTLA-4 polymorphism might play a role in susceptibility to OSF. In functional studies, Ligers et al. (42), demonstrated that the exon 1 allele is associated with a lower expression of the CTLA-4 protein. This might cause dysregulation of CTLA-4-driven down-regulation of T-cell activation, reduced immune tolerance, and development of autoimmune disease.

Several factors have been suggested in the etiology of OSF. Some authors have suggested that molecular mimicry may explain the association between human leukocyte antigen (HLA) and autoimmune disease (14). Canniff et al. (7) performed HLA tissue typing of OSF and observed that the frequencies of HLA A10, DR3, and DR7 were significantly different from that of a control group, with a higher incidence of DR3 in OSF and also the presence of serum immunoglobulins and autoantibodies. In addition, other antibody studies and HLA-typing in individuals with OSF have led to the conclusion that the origin of the disease is multifactorial and that it may be an autoimmune condition with a genetic predisposition (7, 8, 14). We previously investigated the allelic distribution of microsatellite polymorphism in the transmembrane region of the MHC class I Chain-related gene A (MICA) gene among OSF patients in Taiwan. In that study, we provided novel evidence, indicating a higher frequency of the MICA A6 allele in OSF patients in comparison with normal individuals (43).

Expression of the G/G allele of CTLA-4 correlates with increased T-cell proliferation after stimulation with an allogenic cell line and demonstrates significantly increased CTLA-4 surface expression in cells from donors homozygous for adenine at position +49 (39, 44). According to recent studies, the majority of CTLA-4 protein is found intracellularly and can be rapidly mobilized from these intracellular stores to the site of T-cell receptor engagement on the cell surface (45). These data suggest that CTLA-4 surface expression is dynamically regulated with transition of the molecules between intracellular stores and the cell surface in response to environmental stimuli. Therefore, differences in the amount of intracellular CTLA-4 might result in variations in CTLA-4 cell-surface expression. Exon 1 of the CTLA-4 gene encodes for the hydrophobic 37 amino acid leader sequence of the protein (46). The leader peptide serves as a signal peptide that directs the secreted protein to the endoplasmic reticulum, which may result in an 'altered address' from the CTLA-4 protein, and might interfere with the intracellular storage pool (31, 47). This could result in an altered transition between intracellular stores and the cell surface.

In order to substantiate the functional relevance of the CTLA-4 +49 polymorphism in not only a qualitative but also a quantitative way. CD28/B7 interaction results in increased IL-2 production and subsequent arrest of cell cycle progression, rather than induction of apoptotic cell death (48, 49). This reversal of CD28-dependent T-cell activation suggests that CTLA-4 acts as a competitive antagonist of CD28. Cells with the G/G genotype showed an increased mRNA protein expression of the primary T-cell growth factor IL-2 in comparison with cells with the A/A genotype. This antagonism is of particular importance early in the immune response under suboptimal stimulation conditions (often present in autoimmune reactions), because CTLA-4 can attenuate weak signals delivered by the T-cell receptor and CD28, and might in that way contribute to peripheral tolerance.

Genetic factors are thought to be responsible for immune abnormalities in many autoimmune diseases. Immune response genes may be linked to the HLA-DR locus of the major histocompatibility complex (MHC) in humans, and association between this locus and autoimmune diseases have been sought. The DR antigens are associated with susceptibility to diseases with an autoimmune aspect in their pathogenesis; this may be because the immune response genes are situated at or near the D locus on chromosome 6. As many of the connective tissue disorders, including rheumatoid arthritis and systemic lupus erythematous, have been reported to be associated with unique HLA-DR antigens, a similar association has been sought for OSF. We are planning further investigation to define the role of genetic abnormalities apart from MHC variability in OSF in Taiwan.

References

1. Pindborg JJ, Murti PR, Bhonsle RB, Gupta PC, Daftary DK, Mehta FS. Oral submucous fibrosis as a precancerous condition. *Scand J Dent Res* 1984; **92**(3): 224–9.
2. Pillai R, Balaram P, Reddiar KS. Pathogenesis of oral submucous fibrosis. Relationship to risk factors associated with oral cancer. *Cancer* 1992; **69**(8): 2011–20.
3. Jian XC, Liu SF, Shen ZH, Yang YH. Histomorphology of oral submucous fibrosis. Report of 24 cases. *Chin Med J (Engl)* 1988; **101**(7): 505–9.
4. Anuradha CD, Devi CS. Serum protein, ascorbic acid & iron & tissue collagen in oral submucous fibrosis – a preliminary study. *Indian J Med Res* 1993; **98**: 147–51.
5. van Wyk CW, Grobler-Rabie AF, Martell RW, Hammond MG, HLA-antigens in oral submucous fibrosis. *J Oral Pathol Med* 1994; **23**(1): 23–7.
6. Yang YH, Lee HY, Tung S, Shieh TY. Epidemiological survey of oral submucous fibrosis and leukoplakia in aborigines of Taiwan. *J Oral Pathol Med* 2001; **30**(4): 213–9.
7. Canniff JP, Harvey W, Harris M. Oral submucous fibrosis. its pathogenesis and management. *Br Dent J* 1986; **160**(12): 429–34.
8. Seedat HA, van Wyk CW. Betel-nut chewing and submucous fibrosis in Durban. *S Afr Med J* 1988; **74**(11): 568–71.
9. Maher R, Lee AJ, Warnakulasuriya KA, 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**(2): 65–9.

10. Zain RB, Ikeda N, Razak IA, et al. A national epidemiological survey of oral mucosal lesions in Malaysia. *Community Dent Oral Epidemiol* 1997; **25**(5): 377–83.
11. Sinor PN, Gupta PC, Murti PR, et al. A case-control study of oral submucous fibrosis with special reference to the etiologic role of areca nut. *J Oral Pathol Med* 1990; **19**(2): 94–8.
12. Tang JG, Jian XF, Gao ML, Ling TY, Zhang KH. Epidemiological survey of oral submucous fibrosis in Xiangtan City, Hunan Province, China. *Community Dent Oral Epidemiol* 1997; **25**(2): 177–80.
13. Gupta PC, Hebert JR, Bhonsle RB, Sinor PN, Mehta H, Mehta FS. Dietary factors in oral leukoplakia and submucous fibrosis in a population-based case control study in Gujarat, India. *Oral Dis* 1998; **4**(3): 200–6.
14. Canniff JP, Batchelor JR, Dodi IA, Harvey W. HLA-typing in oral submucous fibrosis. *Tissue Antigens* 1985; **26**(2): 138–42.
15. Rajendran R, Vijayakumar T, Vasudevan DM. An. alternative pathogenetic pathway for oral submucous fibrosis (OSMF). *Med Hypotheses* 1989; **30**(1): 35–7.
16. 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**(1): 45–9.
17. Ip S. Idiopathic scleroderma of mouth. *Arch Otolaryngol* 1954; **59**: 330–2.
18. Canniff JP, Harvey W. The aetiology of oral submucous fibrosis: the stimulation of collagen synthesis by extracts of areca nut. *Int J Oral Surg* 1981; **10**(Suppl. 1): 163–7.
19. Gupta SC, Yadav YC. 'MISI' an aetiologic factor in oral submucous fibrosis. *Ind J Otolaryngol* 1978; **30**: 5–6.
20. Haque MF, Harris M, Meghji S, Speight PM. An immunohistochemical study of oral submucous fibrosis. *J Oral Pathol Med* 1997; **26**(2): 75–82.
21. Linsley PS, Greene JL, Tan P, et al. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J Exp Med* 1992; **176**(6): 1595–604.
22. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 1996; **14**: 233–58.
23. Brunner MC, Chambers CA, Chan FK, Hanke J, Winoto A, Allison JP. CTLA-4-mediatedinhibition of early events of T cell proliferation. *J Immunol* 1999; **162**(10): 5813–20.
24. Oosterwegel MA, Greenwald RJ, Mandelbrot DA, Lorsbach RB, Sharpe AH. CTLA-4, T cell activation. *Curr Opin Immunol* 1999; **11**(3): 294–300.
25. Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 1995; **182**(2): 459–65.
26. Walunas TL, Lenschow DJ, Bakker CY, et al. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1994; **1**(5): 405–13.
27. Deichmann K, Heinzmann A, Bruggenolte E, Forster J, Kuehr J. An Mse I RFLP in the human CTLA4 promotor. *Biochem Biophys Res Commun* 1996; **225**(3): 817–8.
28. Harper K, Balzano C, Rouvier E, Mattei MG, Luciani MF, Golstein P. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J Immunol* 1991; **147**(3): 1037–44.
29. Dariavach P, Mattei MG, Golstein P, Lefranc MP. Human Ig superfamily CTLA-4 gene: chromosomal localization and identity of protein sequence between murine and human CTLA-4 cytoplasmic domains. *Eur J Immunol* 1988; **18**(12): 1901–5.
30. Polymeropoulos MH, Xiao H, Rath DS, Merril CR. Dinucleotide repeat polymorphism at the human CTLA4 gene. *Nucl Acids Res* 1991; **19**(14): 4018.
31. Maurer M, Loserth S, Kolb-Maurer A, et al. A polymorphism in the human cytotoxic T-lymphocyte antigen 4 (CTLA4) gene (exon 1 +49) alters T-cell activation. *Immunogenetics* 2002; **54**(1): 1–8.
32. Pullmann R, Jr, Lukac J, Skerenova M, et al. Cytotoxic T lymphocyte antigen 4 (CTLA-4) dimorphism in patients with systemic lupus erythematosus. *Clin Exp Rheumatol* 1999; **17**(6): 725–9.
33. Ahmed S, Ihara K, Kanemitsu S, et al. Association of CTLA-4 but not CD28 gene polymorphisms with systemic lupus erythematosus in the Japanese population. *Rheumatology (Oxford)* 2001; **40**(6): 662–7.
34. Lee YJ, Huang FY, Lo FS, et al. Association of CTLA4 gene A-G polymorphism with type 1 diabetes in Chinese children. *Clin Endocrinol (Oxf)* 2000; **52**(2): 153–7.
35. Nistico L, Buzzetti R, Pritchard LE, et al. The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry. *Hum Mol Genet* 1996; **5**(7): 1075–80.
36. Marron MP, Raffel LJ, Garchon HJ, et al. Insulin-dependent diabetes mellitus (IDDM) is associated with CTLA4 polymorphisms in multiple ethnic groups. *Hum Mol Genet* 1997; **6**(8): 1275–82.
37. Yanagawa T, Hidaka Y, Guimaraes V, Soliman M, DeGroot LJ. CTLA-4 gene polymorphism associated with Graves' disease in a Caucasian population. *J Clin Endocrinol Metab* 1995; **80**(1): 41–5.
38. Braun J, Donner H, Siegmund T, Walfish PG, Usadel KH, Badenhoop K. CTLA-4 promoter variants in patients with Graves' disease and Hashimoto's thyroiditis. *Tissue Antigens* 1998; **51**(5): 563–6.
39. Ligers A, Xu C, Saarinen S, Hillert J, Olerup O. The CTLA-4 gene is associated with multiple sclerosis. *J Neuroimmunol* 1999; **97**(1,2): 182–90.
40. Harbo HF, Celius EG, Vartdal F, Spurkland A. CTLA4 promoter and exon 1 dimorphisms in multiple sclerosis. *Tissue Antigens* 1999; **53**(1): 106–10.
41. Seidl C, Donner H, Fischer B, et al. CTLA4 codon 17 dimorphism in patients with rheumatoid arthritis. *Tissue Antigens* 1998; **51**(1): 62–6.
42. Ligers A, Teleshova N, Masterman T, Huang WX, Hillert J. CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. *Genes Immun* 2001; **2**(3): 145–52.
43. Liu CJ, Chang KW, Lee YJ, Shih YN, Liu HF, Dang CW. Polymorphism of the MICA gene and risk for oral submucous fibrosis. *J Oral Pathol Med.* 2004; **33**(1): 1–6.
44. Kouki T, Sawai Y, Gardine CA, Fidfalen ME, Alegre ML, DeGroot LJ. CTLA-4 gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves' disease. *J Immunol* 2000; **165**: 6606–11.
45. Linsley PS, Bradshaw J, Greene JL, Peach R, Bennett KL, Mittler RS. Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity* 1996; **4**: 535–43.
46. Khatlani TS, Ohno K, Ma Z, Inokuma H, Onishi T. Cloning and sequencing of dog cDNA encoding the T-cell costimulatory molecules (CTLA-4). *Immunogenetics* 2000; **51**: 79–81.
47. Nicchitta CV, Blobel G. Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. *Cell* 1993; **73**: 989–98.
48. Krummel MF, Allison JP. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* 1996; **183**: 2533–40.
49. Walunas TL, Bakker CY, Bluestone JA. CTLA-4 ligation blocks CD28-dependent T cell activation. *J Exp Med* 1996; **183**: 2541–50.