Journal of Clinical Laboratory Analysis 19:93–98 (2005)

Interleukin-4 Gene, but not the Interleukin-1 Beta Gene Polymorphism, Is Associated With Oral Cancer

Ming-Hsui Tsai,[1] Wen-Chi Chen,[2,3] Chang-Hai, Tsai,[4] Liang-Wen Hang,[5] and Fuu-Jen Tsai[2,6]*

[1]*Department of Otolaryngology, China Medical University Hospital, Taichung, Taiwan*
[2]*Department of Medical Genetics, China Medical University Hospital, Taichung, Taiwan*
[3]*Department of Urology, China Medical University Hospital, Taichung, Taiwan*
[4]*Graduate Institute of Bioinformatics, Taichung Healthcare and Management University, Taichung, Taiwan*
[5]*Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan*
[6]*Department of Pediatrics, China Medical University Hospital, Taichung, Taiwan*

We aimed to evaluate whether polymorphisms of the interleukin-4 (IL-4) gene promoter and intron 3 regions, and polymorphisms of the IL-1 beta gene promoter and exon 5 regions are associated with oral cancer. This study included 130 patients with oral cancer and 105 age-matched healthy controls who lived in the same area as the patients. Each genetic polymorphism was typed by polymerase chain reaction (PCR)-based restriction analysis. We then compared the genotype distribution and allelic frequencies of each polymorphism between the oral cancer patients and the controls. The CC homozygote genotype of the IL-4 gene promoter −590 region differed significantly between the patients with oral cancer and the controls (odds ratio (OR)=6.0, 95% confidence interval (CI)=1.2−30.7, chi-square test, $P=0.044$). No significant difference in either the genotype distribution or the allelic frequencies of the IL-1 beta gene polymorphisms was observed between patients with oral cancer and controls. The IL-4 gene −590 C/T polymorphism is associated with oral cancer and is a suitable genetic marker for screening for oral cancer. However, whether the −590 C/T polymorphism of the IL4 gene plays a role in oral cancer remains unclear. Further substantiation based on larger patient samples is needed. J. Clin. Lab. Anal. 19:93–98, 2005.

Key words: IL-1 gene polymorphism; IL4 gene polymorphism; oral cancer

INTRODUCTION

Oral cancer is a malignancy that is commonly seen in Taiwanese who habitually chew betel quid and/or smoke cigarettes (1). Betel quid chewing causes oral mucosal inflammatory responses, oral submucosal fibrosis, DNA damage, and malignant dysplasia (2). The most common chemical constituent of the betel nut is arecholine, an alkaloid that induces interleukin-1 (IL-1) secretion in cell cultures (3). High doses of arecholine have been shown to have cytotoxic effects on cultured human gingival fibroblasts (4). In addition to betel quid chewing and cigarette smoking, cytokines have also been reported to be associated with cancer (5–8). Therefore, an interaction between environmental and genetic factors is thought to be involved in the onset of the disease (9).

Researchers have recently used single nucleotide polymorphisms (SNPs) to identify oral cancer disease genes, and early results indicate that polymorphisms of the p53 gene, cytochrome p450 gene, and the glutathione S-transferase gene are associated with an increased risk of developing oral cancer (9,10). IL-l beta, located at chromosome 2q12, is a potent proinflammatory agent that plays a central role in immunoregulation, fever, inflammation, and cancer formation (11). Polymorphisms of the IL-1 beta gene promoter and exon 5 regions have been used to screen for the

Grant sponsor: China Medical University Hospital; Grant number: DMR-90-050.

*Correspondence to: Fuu-Jen Tsai, M.D., Ph.D., Department of Medical Genetics and Pediatrics, China Medical University Hospital, No. 2 Yu-Der Road, Taichung, 404, Taiwan.
E-mail: d0704@www.cmuh.org.tw

Received 22 December 2004; Accepted 14 January 2005
DOI 10.1002/jcla.20060
Published online in Wiley InterScience (www.interscience.wiley.com).

relationship between the occurrence and severity of rheumatoid arthritis and osteoporosis (12,13). In this study we chose two cytokines to study SNPs of the IL-1 beta and IL-4 genes, both because they have been reported to be associated with cancer and because they represent different inflammatory pathways of tissue response to environmental factors.

IL-4 is a key cytokine that induces the activation and differentiation of B cells, as well as the development of the Th2 subset of lymphocytes. Th2 cytokines, such as IL-4, IL-6, and IL-10, primarily support antibody production, and many studies have confirmed that cancer patients have high levels of such cytokines in serum (8,14–16). The gene for IL-4 has been mapped to the q arm (q23-31) of chromosome 5 (17), in a cluster of cytokine genes (IL-3, IL-5, IL-9, IL-13, IL-15, GM-CSF, and interferon regulatory factor). A functional polymorphism representing a C-to-T substitution at position −590 was recently described in the promoter region of the IL4 gene (18). This polymorphism is located upstream of all of the known control elements of the IL4 gene, and has been shown to affect its transcriptional activity. Another polymorphism is located in the third intron and is composed of a variable number of tandem repeats (VNTR) of a 70-bp sequence (19). To elucidate whether these polymorphisms play a role in susceptibility to oral cancer, we investigated their distribution in the control group and oral cancer patients by analyzing the PCR results.

MATERIALS AND METHODS

The patient group consisted of 130 patients (10 women and 120 men) who were diagnosed in this hospital as having oral cancer (average age = 53.27 ± 12.27 years, range = 22–79 years). The diagnosis was based on pathological specimens from total excision or oral biopsy. All of the tumor cell types were squamous cell carcinoma. There were 41 well differentiated, 68 moderately differentiated, and 21 poorly differentiated cell types. Ninety-six patients (73.8%) had chewed betel quid for more than 10 years, 116 patients (89.2%) had smoked cigarettes for more than 10 years, and 95 patients (73.1%) had smoked cigarettes and chewed betel quid. Eleven patients (8.5%) had not smoked cigarettes or chewed betel quid. The patients were followed for at least 3 years. The control group consisted of 105 unrelated healthy individuals from the same area as the patients (60 men and 45 women, age range = 40–87 years, average age = 53.02 ± 10.08 years). Informed consent was obtained from each individual enrolled in this study.

The genomic DNA was extracted from anticoagulated peripheral blood by a standard proteinase K digestion and phenol/chloroform extraction method. PCR was performed in a total reaction volume of 25μl with 2.5–10 *p*mole of each primer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate, and 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, Forster City, CA). The primers for the IL-4 polymorphism at position −590 were upstream: 5′-ACTAGGCCTCACCTGATACG-3′ and downstream: 5′-GTTGTAATGCAGTCCTCCTG-3′. The following cycling conditions were used: one cycle at 94°C for 5 min; 35 cycles of 94°C for 20 sec, 58°C for 20 sec, and 72°C for 20 sec; and a final extension at 72°C for 10 min. The cycles were performed in a Perkin-Elmer 2400 thermal cycler. The PCR products were studied after *Bsm* FI restriction enzyme digestion (New England Biolabs, Beverly, MA, USA). Two alleles exist at the −590 position. *Bsm* FI causes cleavage of a restriction site at the −590 position when the "C" allele is present, giving rise to fragments of 60 and 192 bp in length, or 252 bp when "T" is present. The digested fragments were analyzed by electrophoresis on 3% agarose gels (Fig. 1). The primers for the IL-4 gene intron 3 polymorphism were upstream 5′-AGGCTGAAAGGGGGAAAGC-3′ and downstream 5′-CTGTTCACCTCAACTGCTCC-3′. The IL-4 gene intron 3 polymorphism PCR products, including the 70-bp VNTR region, were directly analyzed by 3% agarose gel electrophoresis, and each allele was recognized according to its size. The RP1 and RP2 alleles were 183 bp and 253 bp, respectively.

The primers for the IL-1 beta gene promoter region −511 C/T polymorphism were upstream 5′-TGGCATTGATCTGGTTCATC-3′ and downstream 5′-GTTTAGGAATCTGGACCAGA-3′. The cycling conditions were one cycle at 95°C for 5 min, 35 cycles of 95°C for

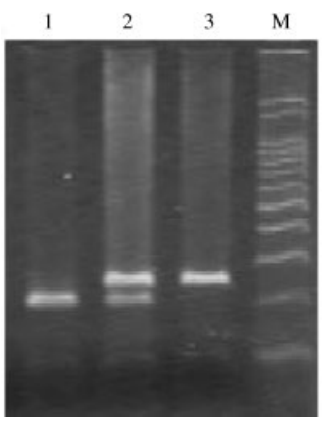

Fig. 1. Product of PCR-based restriction analysis of the IL-4 gene −590 C/T polymorphism shown on agarose electrophoresis. Lane 1: CC homozygote 192-bp + 6o-bp (invisible from gel). Lane 2: C/T heterozygote. Lane 3: TT homozygote, undigested, 252-bp. Lane 4: marker, 100-bp ladder.

30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The restriction enzyme for the analysis was *Ava* I (New England Biolabs, Beverly, MA); 304 bp of PCR product was digested into 190bp + 114 bp if the restriction site was present ("C" allele). The polymorphism of IL-1 beta gene exon 5 was detected by restriction analysis following Cantagrel et al. (12). The primers for the IL-1 beta gene exon 5 polymorphism were upstream 5′-GTTGTCATCA-GACTTTGACC-3′, and downstream 5′-TTCAGTT-CATATGG ACCAGA-3′. The cycling conditions were one cycle at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. The region containing the polymorphic site within exon 5 of the IL-1 beta gene was amplified and then digested by *Taq* I (New England Biolabs). Class "E1" was 135bp + 114bp and "E2" was 249 bp as shown on electrophoresis.

The software used for the calculation was the statistical package for social science (SPSS®) system. The allelic frequencies and genotype distributions of these polymorphisms in both groups were analyzed by means of the chi-square test. When the assumption of the chi-square test was violated (i.e., when one cell had an expected count of <1, or $>20\%$ of the cells had an expected count of <5), Fisher's exact test was performed. A P-value less than 0.05 was considered statistically significant. Odds ratios (ORs) were calculated from allelic frequencies and carriage rates with a 95% confidence interval (CI).

RESULTS

The genotype distributions and allelic frequencies for the promoter and intron 3 regions in the IL-4 gene obtained from the patients and controls are shown in Table 1. The genotype frequencies for the promoter and exon 5 regions of the IL-1 beta gene in the oral cancer and control groups are shown in Table 2. There were significant differences in the genotype distribution of the IL-4 gene promoter region −590 C/T polymorphism between patients and controls ($P = 0.044$; Table 1). The distribution of the CC homozygote in oral cancer patients (6.9%) was higher than in the controls (1.9%). The OR for the CC homozygote was significantly higher than that for the CT heterozygote (OR = 6.0, 95% CI = 1.2–30.7, $P = 0.0315$). However, the allelic frequency of −590C was not significantly different between the oral cancer patients and the controls (chi-square test, $P = 0.94$). No significant differences in genotype distributions or allelic frequencies of the IL-4 gene intron3 polymorphism were observed between the oral cancer patients and controls. There were no significant differences in the genotype distributions or allelic frequencies of the IL-1 beta gene promoter and exon 5 polymorphism between the oral cancer patients and controls.

DISCUSSION

The prominence of the CC homozygote of the IL-4 gene −590 C/T polymorphism in cancer patients

TABLE 1. Comparison of the IL-4 gene promoter and intron 3 region genotype distributions and allele frequencies observed in oral cancer patients and healthy control subjects

	Controls n = 105 (%)	Cancer patients n = 130 (%)	OR		
			P	(95% CI)	*P*
IL-4 promoter					
Genotype-590			0.044[a]		
C/C	2 (1.9)	9 (6.9)		6.0 (1.2 ~ 30.7)[a]	0.032
C/T	28 (26.7)	21 (16.2)		1 (referent)	
T/T	75 (71.4)	100 (76.9)		1.8 (0.9 ~ 3.4)	0.078
Allelic frequencies					
C	32 (15.2)	39 (15.0)	0.94[a]		
T	178 (84.8)	221 (85.0)			
IL-4 intron3	n = 105 (%)	n = 130 (%)			
Genotype			0.168[b]		
RP1/RP1	67 (63.8)	97 (74.6)			
RP1/RP2	33 (31.4)	30 (23.1)			
RP2/RP2	5 (4.8)	3 (2.3)			
Allelic frequencies			0.063[a]		
RP1	167 (80.1)	224 (86.2)			
RP2	43 (19.9)	36 (13.8)			

[a]Chi-square test.
[b]Fisher's exact test.

TABLE 2. Comparison of the IL-1 beta gene promoter and exon 5 genotype distributions and allele frequencies observed in oral cancer patients and healthy control subjects

	Controls n = 105 (%)	Cancer patients n = 130 (%)	*P*
IL-1 promoter			
Genotype-511			0.420[a]
C/C	28 (26.7)	31 (23.8)	
C/T	50 (47.6)	73 (56.1)	
T/T	27 (25.7)	26 (20.1)	
Allelic frequencies			0.781[a]
C	106 (50.5)	135 (51.9)	
T	104 (49.5)	125 (48.1)	
IL-1 exon 5	n = 105 (%)	n = 130 (%)	
Genotype			0.812[b]
E1E1	102 (97.1)	127 (97.7)	
E1E2	3 (2.9)	2 (1.5)	
E2E2	0 (0)	1 (0.8)	
Alleleic frequencies			1.0[b]
E1	207 (98.6)	256 (98.5)	
E2	3 (1.4)	4 (1.5)	

[a]Chi-square test.
[b]Fisher's exact test.

indicates that the −590 C/T polymorphism is associated with oral cancer. Further studies on this relationship are warranted. However, no significant difference in the IL-4 gene intron 3 VNTR was found between the oral cancer patients and normal controls. This lack of statistical significance may be attributable to linkage disequilibrium, but it is also possible that the intron 3 in the IL-4 gene is located in a noncoding region that does not influence transcriptional activity. Furthermore, no association between the IL-1 gene and oral cancer was observed in this study, indicating that the IL-1 gene and its inflammatory response are not associated with the development of oral cancer.

Complex diseases such as diabetes, cancer, asthma, and arthritis probably result from subtle changes in multiple genes caused by environmental and lifestyle factors. Dissecting the genetics of common and complex disorders, such as cancer, remains one of the great challenges in human genetics. Oral cancer is considered to be a composite of multigenic diseases involving several overlapping autoimmune responses, each of which is mediated by a distinct genetic profile. Therefore, studying the genetic polymorphisms of different genes will further clarify the relationship between genetics and cancer formation. Knowledge of the association between oral cancer and various genetic markers has helped increase our understanding of the genetics of the immune response and pathogenesis of oral cancer.

The IL-4 −590 "T" allele has been reported to be associated with higher IgE levels in American and Japanese populations (20,21). In the present analysis we chose to screen two polymorphisms in the IL4 gene: the −590T/C in the promoter region, and the RP1/RP2 in intron 3. It is thought that the C-to-T exchange at position −590 enhances either IL-4 production or T-cell activity (20,21). In this study, the allelic frequency of −590T significantly differed between the oral cancer patients and controls. Therefore, IL-4 may influence the formation of cancer either through the IgE pathway or by its transcription activity.

Over 70% of the oral cancer patients in this study smoked cigarettes and chewed betel quid, which indicates a strong association between the two habits and oral cancer. Smoking is a common worldwide risk factor for oral cancer (22). However, betel quid chewing is common only in Asian countries, including Taiwan. The high coincidence of cancer patients who smoke cigarettes and chew betel quid suggests that their interaction has carcinogenic effects. Further studies to clarify the interaction between smoking and betel quid chewing may help us understand the role such risk factors play in oral cancer.

One epidemiological study revealed that the chemical constituents of betel quid are possible carcinogens (1); however, the exact mechanism remains unclear. Sundqvist et al. (23) found that betel nut alkaloids have cytotoxic and genotoxic effects on cultured human buccal epithelial cells. Arecholine, the most common chemical constituent, is known to cause inflammatory responses and may be a possible pathway. Hsu et al. (24) found that arecholine inhibits the secretion of cytokines from mononuclear cells, such as IL-2, tumor necrosis factor-alpha (TNF), transforming growth factor-beta (TGF), and interferon-gamma. A study of human oral epidermoid carcinoma cell lines found that IL-1 secretion was elevated when the cells were treated with nicotine and/or arecoline (3). Chang et al. (25) found a synergistic effect of nicotine on arecholine-induced cytoxicity in human bucosal fibroblasts, which may explain why a high proportion of oral cancer patients both smoke cigarettes and chew betel quid. Therefore, cytokines may play an important role play in the formation of oral cancer in patients with both habits.

Cytokines may modulate the severity of cancer (5). Yamamoto et al. (5) studied serum levels of cytokines in several mucous disorders, and found that IL-3, IL-4, and IL-6 appear to reflect a characteristic pathophysiology of oral disorders, such as oral cancer. Mehrotra et al. (7) found no effect of IL-4 on the proliferation of SCC cell lines in head and neck malignancies. However, expression of the IL-4 receptor observed in these

malignant cell lines indicated that the IL-4 receptor might be more useful than the IL-4 gene for diagnosing oral cancer. The fact that our results are not compatible with the above reports may be due to the incomplete match between the serum level and its expression in tissue. Further studies should be done to clarify the discrepancy.

IL-1 and IL-6 may be responsible for tissue destruction in chronic inflammatory periodontal disease and periapical inflammation (8). An immunohistochemical study of oral SCC specimens reported that the level of coexpression of IL-6 and its receptor IL-6R may influence patient survival (15). IL-1 beta has also been reported to be correlated with oral cancer cell transformation. Tsai et al. (6) detected a significantly higher level of expression of IL-1 in oral cancer tissue than in normal controls. Jablonska et al. (16) also found significantly increased serum levels of IL-1 in oral cancer patients, which correlated with both the clinical stages of the disease and the serum levels of c-reactive protein. The reasons for the discrepancies between our results and the above reports remain unclear.

The polymorphisms in the IL4 gene studied here appear to be associated with an increased risk of developing oral cancer when compared with the control group. However, based on the statistical analysis, there were no significant differences in the genotype distributions of the IL-1 gene polymorphism between the patients and controls. Our results indicate that −590T/C of the IL4 gene is a suitable genetic marker of susceptibility to or severity of oral cancer. The relationship between cytokines and oral cancer is clear from this study. However, whether the release of cytokines due to environmental factors is related to the formation of oral cancer remains to be determined by further investigation.

REFERENCES

1. Ko YC, Huang YL, Lee CH, Chen MJ, Lin LM, Tsai CC. Betel quid chewing, cigarette smoking, and alcohol consumption related to oral cancer in Taiwan. J Oral Pathol Med 1995;24: 450–453.
2. Jayant K, Balakrishnan V, Sanghvi LD, Jussawala DJ. Quantification of the role of smoking and chewing habits in oral, pharynx, and esophageal cancer. Br J Cancer 1977;35: 232–235.
3. Cheng YA, Tsai CC. Nicotine- and arecoline-induced interleukin-1 secretion and intercellular adhesion molecular-1 expression in human oral epidermoid carcinoma cells in vitro. Arch Oral Biol 1999;44:843–851.
4. Jeng JH, Kuo ML, Hahn LJ, Kuo MYP. Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. J Dent Res 1994;73:1043–1049.
5. Yamamoto T, Yoneda K, Ueta E, Hirota J, Osaki T. Serum cytokine levels in patients with oral mucous membrane disorders. J Oral Pathol Med 1991;20:275–279.
6. Tsai CC, Chen CC, Lin CC, Chen CH, Lin TS, Shieh TY. Interleukin-1 beta in oral submucous fibrosis, mucous hyperplasia and squamous cell carcinoma tissues. Kaohsiung J Med Sci 1999;15:513–519.
7. Mehrotra R, Varricchio F, Husain SR, Puri RK. Head and neck cancers, but not benign lesions, express interleukin-4 receptors in situ. Oncol Rep 1998;5:45–48.
8. Sosroseno W, Herminajeng E, Goeno S. The interleukin network in the immunopathogenesis of oral diseases. Asian Pac J Allergy Immunol 1994;12:161–168.
9. Das BR, Nagpal JK. Understanding the biology of oral cancer. Med Sci Monit 2002;8:258–267.
10. Nagpal JK, Patnaik S, Das BR. Prevalence of high-risk human papilloma virus types and its association with p53 codon-72 polymorphism in tobacco addicted oral squamous cell carcinoma (OSCC) patients of eastern India. Int J Cancer 2002;97: 649–653.
11. Steinkasserer A, Spurr NK, Cox S, Jeggo P, Sim RB. The human IL-1 receptor antagonist gene (IL1RN) maps to chromosome 2q14-q21, in the region of the IL-1alpha and IL-1beta loci. Genomics 1992;13:654–657.
12. Cantagrel A, Navaux F, Loubet-Lescoulie P, et al. Interleukin-1beta, interleukin-1 receptor antagonist, interleukin-4, and interleukin-10 gene polymorphisms: relationship to occurrence and severity of rheumatoid arthritis. Arthritis Rheum 1999;42:1093–1100.
13. Langdahl BL, Lokke E, Carstens M, Stenkjaer LL, Eriksen EF. Osteoporotic fractures are associated with an 86-base pair repeat polymorphism in the interleukin-1-receptor antagonist gene but not with polymorphisms in the interleukin-1beta gene. J Bone Miner Res 2000;15:402–414.
14. Chandler SW, Rassekh CH, Rodman SM, Ducatman BS. Immunohistochemical localization of interleukin-10 in human oral and pharyngeal carcinomas. Laryngoscope 2002;112: 808–815.
15. Wang YF, Chang SY, Tai SK, Li WY, Wang LS. Clinical significance of interleukin-6 and interleukin-6 receptor expressions in oral squamous cell carcinoma. Head Neck 2002;24: 850–858.
16. Jablonska E, Piotrowski L, Grabowska Z. Serum levels of IL-1b,IL-6, TNFa, sTNF-RI and CRP in patients with oral cavity cancer. Pathol Oncol Res 1997;3:126–129.
17. Rosenwasser LJ, Klemm DJ, Dresback JK, et al. Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. Clin Exp Allergy 1995;2(S):74–78.
18. Le Beau MM, Lemons RS, Espinosa R, Larson RA, Arai N, Rowley JD. Interleukin-4 and interleukin-5 map to human chromosome 5 in a region encoding growth factors and receptors and are deleted in myeloid leukemias with a del (5q). Blood 1989;73:647–650.
19. Mout R, Willemzze R, Landegent JE. Repeat polymorphisms in the interleukin-4 gene. Nucleic Acids Res 1991;19: 3763.
20. Rosenwasser LJ, Borish L. Genetics of atopy and asthma: the rationale behind promoter-based candidate gene studies (IL-4 and IL-10). Am J Respir Crit Care Med 1997;156: S152–S155.
21. Noguchi E, Shibasaki M, Arinami T, et al. Evidence for linkage between asthma/atopy in childhood and chromosome 5q31-q33 in a Japanese population. Am J Respir Crit Care Med 1997;156:1390–1393.
22. Epstein JB, Zhang L, Rosin M. Advances in the diagnosis of oral premalignant and malignant lesions. J Can Dent Assoc 2002;68:617–621.

23. Sundqvist K, Liu Y, Nair J, Bartsch H, Arvidson K, Grafstrom RC. Cytotoxic and genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells. Cancer Res 1989;49:5294–5298.
24. Hsu HJ, Chang KL, Yang YH, Shieh TY. The effects of arecoline on the release of cytokines using cultured peripheral blood mononuclear cells from patients with oral mucous diseases. Kaoshiung J Med Sci 2001;17:175–182.
25. Chang YC, Hu CC, Tseng TH, Tai KW, Li CK, Chou MY. Synergistic effects of nicotine on arecholine-induced cytoxicity in human bucosal fibroblasts. J Oral Pathol Med 2001;30: 458–464.