Significant Association of *Interleukin-10* Genotypes and Oral Cancer Susceptibility in Taiwan

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Abstract. *Interleukin-10 (IL10) is an immunosuppressive cytokine which may facilitate carcinogenesis by down-regulating interferon-gamma production and supporting tumor escape from the immune response. Polymorphisms within the promoter of IL10 gene may not only contribute to differential IL10 expression levels among individuals but also to oral cancer susceptibility. In this hospital-based study, the association of IL10 A-1082G (rs1800896), T-819C (rs3021097), and A-592C (rs1800872) polymorphisms with oral cancer risk were examined. A total of 788 cases with oral cancer risk and 956 controls were genotypes and analyzed by polymerase chain reaction and restriction fragment length polymorphism. The results showed that there were significant differential distributions among oral cancer cases and controls in the genotypic ($p=6.29\times10^{-11}$) and allelic ($p=2.80\times10^{-13}$) frequencies of IL10 A-1082G. Individuals who carried the AG or GG genotype for IL10 A-1082G had a 1.90- and 3.27-fold higher risk, respectively, of developing nasopharyngeal carcinoma compared to those who carried AA genotype (95% confidence interval=1.51-2.39 and 1.95-5.47). None of the other two polymorphisms investigated appear to affect cancer risk. In gene–lifestyle interaction analysis, we provide first evidence showing of an obvious joint effect of IL10 A-1082G genotype with individual smoking and areca chewing habits on nasopharyngeal carcinoma risk. The AG and GG genotypes of IL10 A-1082G, together with smoking and areca chewing habits, synergistically contribute to individual susceptibility for oral cancer.*

Oral cancer, which is the tenth most commonly diagnosed cancer in the world, has the highest incidence in Taiwan (1). It is the fourth cause of cancer-related death among males in Taiwan, and this has been reported to be closely associated with tobacco, alcohol and betel nut consumption habits (2-5). Compared to Western countries, the incidence rate is significantly higher in Taiwan, an island with a relative high genetic conservation. However, the genomic etiology of oral cancer and the gene–environment and –lifestyle interactions are of great interest but largely unknown. Interleukin-10 (IL10) is an immunosuppressive cytokine mainly produced by macrophages, and has been shown to inhibit various immune reactions, such as antigen presentation, cytokine production, macrophage activation and antigen-specific T-cell proliferation (6). In recent years, several lines of evidence showed that IL10 plays a critical role in tumor progression and metastasis (7, 8). Increased circulating IL10 has been reported in patients with different types of tumors, including oral cancer (9-12).

The human *IL10* gene is located on chromosome 1q31-32, and is composed of five exons and four introns. Inter-individual variations in IL10 production were genetically contributed to polymorphisms within the *IL10* promoter region. Three promoter single nucleotide polymorphisms (SNPs) exist at upstream positions A-1082G (rs1800896), T-819C (rs3021097), and A-592C (rs1800872) relative to the transcriptional start site, which were reported to influence the transcription of *IL10* mRNA and the expression of IL10 *in vitro* (13, 14). Recently, genetic polymorphisms of *IL10* gene have been implicated in the susceptibility to a range of cancer types, including hepatocellular carcinoma (15), breast cancer (16), renal cell carcinoma (17) and nasopharyngeal carcinoma (18). In 2008, Yao and colleagues found that there

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were significantly differences in the genotype and allele distribution of the A-1082G polymorphism of the *IL10* gene among an oral cancer population and age- and gender-matched healthy controls in mainland China. The A-1082G G allele carriers were associated with a significantly increased risk of oral cancer compared to the non-carriers (20). However, their sample size was relatively small (case:control=280:300), and the interaction of genetic and environmental factors was not revealed.

No studies, to date, have examined the association between genetic polymorphisms in *IL10* gene and oral cancer in Taiwan, where the oral cancer incidence in males is highest in the world. Therefore, the purpose of this study was to examine whether *IL10* gene promoter A-1082G (rs1800896), T-819C (rs3021097), and A-592C (rs1800872) polymorphisms are associated with oral cancer in Taiwan. In addition, we also investigated the joint effects of genotype with personal behaviors on oral cancer. The population of oral cancer patient (n=788) is very representative and well matched with a large healthy control population (n=956). To the best of our knowledge, this is the first study carried out to evaluate the contribution of *IL10* genotypes and their interaction with personal risky behaviors in oral oncology.

Materials and Methods

Study population and sample collection. Seven hundred and eighty-eight patients diagnosed with oral cancer were recruited at the China Medical University Hospital in central Taiwan during 1998 to 2010. All patients voluntarily participated, completed a self-administered questionnaire and provided 5 ml of their peripheral blood. The questionnaire administered to the participants included questions on history and frequency of alcohol consumption, areca chewing and smoking habits. Self-reported alcohol consumption, areca chewing and smoking habits were evaluated and classified as categorical variables. Information on these factors was obtained as more then twice a week for years as 'ever'. The 956 non-cancer healthy individuals as controls were selected by matching for age and gender after initial random sampling from the Health Examination Cohort of the hospital. The ratio of males *versus* females was 76% *versus* 24% in each group. The mean age of the patients and the controls 55.8 (SD=9.9) and 56.6 (SD=8.7) years, respectively. Our study was approved by the Institutional Review Board of the China Medical University Hospital and written-informed consents were obtained from all participants.

Genotyping conditions. Genomic DNA was prepared from peripheral blood leucocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed and stored according to our regular methodology (21-23). Briefly, the following primers were used: for *IL10* A-1082G: forward 5'-CTC GCT GCA ACC CAA CTG GC-3' and reverse 5'- TCT TAC CTA TCC CTA CTT CC-3'; for *IL10* T-819C: forward 5'-TCA TTC TAT GTG CTG GAG AT-3', and reverse 5'-TGG GGG AAG TGG GTA AGA GT-3'; and for *IL10* A-592C: forward 5'-GGT GAG CAC TAC CTG ACT AG-3', and reverse 5'-CCT AGG TCA CAG TGA CGT GG-3'. As for the polymerase chain reaction (PCR), the following cycling conditions were performed: one cycle at 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s; and then a final extension at 72˚C for 10 min. The PCR products were separated with 3% agarose gel electrophoresis after digestion with *Mnl* I, *Mae* III, and *Rsa* I restriction enzymes for *IL10* A-1082G (cut from 139 bp A genotype into 106+33 bp G genotype), T-819C (cut from 209 bp T genotype into 125+84 bp C genotype) and A-592C (cut from 412 bp C genotype into 236+176 bp A genotype), respectively. Five percent of the PCR products from each genotype were subject to direct sequence and the results matched 100% with the electrophoresis findings.

Statistical analyses. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *IL10* SNPs in the controls from those expected under the Hardy–Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's Chi-square test or Fisher's exact test (when the expected number in any cell was less than five) was used to compare the distribution of the *IL10* genotypes between cases and controls. Cancer risk associated with the genotypes was estimated as odds ratio (ORs) together with relative 95% confidence intervals (CIs) using unconditional logistic regression. Data was recognized as significant when the statistical *p*-value was less than 0.05.

Results

The demographic characteristics of 788 patients with oral cancer and 956 age- and gender-matched non-cancer controls are summarized in Table I. There were no significant differences between groups in their age or sex, as expected. There were more individuals with smoking and areca chewing habits in the patient group than the healthy group (p=0.0084 and p<0.0001, respectively), but not for those with alcohol drinking habits (p>0.05) (Table I).

The distribution analysis for the genotypic and allelic frequencies of the *IL10* A-1082G in the oral cancer and control groups are summarized in Table II. Firstly, there was a significant difference between oral cancer and control groups in the distribution of genotypic frequency ($p=6.29\times10^{-11}$), and the ORs for the AG and GG were 1.90 (95% CI=1.51-2.39) and 3.27 (95% CI=1.95-5.47) compared to the AA wild-type genotype. Secondly, we used the dominant and recessive models of carrier comparisons, finding that the ORs of the AA+AG *versus* GG and AA *versus* AG+GG were 2.82 (95% CI=1.69-4.70, p=0.0001) and 2.05 (95% CI=1.65-2.55, p=0.0001), respectively. Lastly, as for allelic frequency analysis, those carrying allele G at *IL10* A-1082G were found to have a 2-fold increased risk of oral cancer than those carrying allele A (95% CI=1.66-2.42, $p=2.80\times10^{-13}$) (Table II). As for the *IL10* T-819C (Table III) and A-592C (Table IV) SNPs, there was no difference in the distribution in either genotypic or allelic frequency between patient and control groups. The overall conclusive finding deduced from the results of Tables II~IV is that the G allele of *IL10* A-1082G may serve as a novel risky biomarker for oral cancer in Taiwanese.

Table I. *Characteristics of oral cancer patients and controls.*

Characteristics	Controls (n=956)			Patients (n=788)			*p*-Value[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			56.6 (8.7)			55.8 (9.9)	0.7951
Gender							1.0000
Male	727	76.0%		599	76.0%		
Female	229	24.0%		189	24.0%		
Indulgence							
Areca chewers	506	52.9%		661	83.9%		<0.0001*
Cigarette smokers	667	69.8%		595	75.5%		0.0084*
Alcohol drinkers	641	67.1%		560	71.1%		0.0773
Histology							
Tongue				325	41.2%		
Buccal mucosa				294	37.3%		
Mouth floor				30	3.8%		
Retromolar trigone				26	3.3%		
Alveolar ridge				18	2.3%		
Palate				18	2.3%		
Lip				39	4.9%		
Others				38	4.9%		

SD: Standard deviation; [a]Based on Chi-square test; *statistically significant at $p<0.05$.

Table II. *Distribution of interleukin-10 (IL10) A-1082G (rs1800896) genotypic and allelic frequencies among patients with oral cancer and controls. A-1082G (rs1800896).*

	Controls		Patients		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
Genotypic frequency						
AA	766	80.1%	522	66.3%	1.00 (Reference)	$6.29*10^{-11}$*
AG	168	17.6%	217	27.5%	1.90 (1.51-2.39)*	
GG	22	2.3%	49	6.2%	3.27 (1.95-5.47)*	
Carrier comparison						
AA+AG	934	97.7%	739	93.8%	1.00 (Reference)	0.0001*
GG	22	2.3%	49	6.2%	2.82 (1.69-4.70)*	
AA	766	80.1%	522	66.3%	1.00 (Reference)	0.0001*
AG+GG	190	19.9%	266	33.7%	2.05 (1.65-2.55)*	
Allelic frequency						
Allele A	1700	88.9%	1261	80.0%	1.00 (Reference)	$2.80*10^{-13}$*
Allele G	212	11.1%	315	20.0%	2.00 (1.66-2.42)*	

OR: Odds ratio, CI: confidence interval; abased on Chi-square test; *statistically significant at $p<0.05$.

After finding that the genotypes of *IL10* A-1082G, but not T-819C (Table III) or A-592C, were associated with oral cancer risk, we investigated the interaction among the genotype of *IL10* A-1082G and environmental factors, such as personal cigarette smoking, betel quid chewing, and alcohol drinking habits. The genotype of AG and GG of *IL10* A-1082G increased 2.10- and 4.24-fold the oral cancer risk among smokers (95% CI=1.61-2.75 and 2.28-7.88, respectively), but not among non-smokers (Table V). Consistent with the findings shown in Table II, the frequency of AG and GG genotypes were even higher (29.2 and 7.1%) in patients with oral cancer with smoking habit than those for smoking controls (22.1 and 3.6%). Similarly, the genotype of AG and GG of *IL10* A-1082G increased oral cancer risk by 1.89- and 3.96-fold among areca chewers (95% CI=1.48-2.63 and 2.02-7.75, respectively), but not

Table III. *Distribution of interleukin-10 (IL10) T-819C (rs3021097) genotypic and allelic frequencies among patients with oral cancer and controls. T-819C (rs3021097).*

	Controls		Patients		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
Genotypic frequency						
TT	528	55.2%	418	53.0%	1.00 (Reference)	0.6530
TC	335	35.1%	288	36.6%	1.09 (0.89-1.33)	
CC	93	9.7%	82	10.4%	1.11 (0.81-1.54)	
Carrier comparison						
TT+TC	863	90.3%	706	89.6%	1.00 (Reference)	0.6890
CC	93	9.7%	82	10.4%	1.08 (0.79-1.47)	
TT	528	55.2%	418	53.0%	1.00 (Reference)	0.3847
TC+CC	428	44.8%	370	47.0%	1.09 (0.90-1.32)	
Allelic frequency						
Allele T	1391	72.8%	1124	71.3%	1.00 (Reference)	0.3482
Allele C	521	27.2%	452	28.7%	1.07 (0.93-1.25)	

OR: Odds ratio, CI: confidence interval; [a]based on Chi-square test.

Table IV. *Distribution of interleukin-10 (IL10) A-592C (rs1800872) genotypic and allelic frequencies among oral cancer patient and control groups. A-592C (rs1800872).*

	Controls		Patients		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
Genotypic frequency						
AA	484	50.6%	408	51.8%	1.00 (Reference)	0.8921
AC	374	39.1%	301	38.2%	0.95 (0.78-1.17)	
CC	98	10.3%	79	10.0%	0.95 (0.69-1.32)	
Carrier comparison						
AA+AC	858	89.7%	709	90.0%	1.00 (Reference)	0.9365
CC	98	10.3%	79	10.0%	0.98 (0.71-1.33)	
AA	484	50.6%	408	51.8%	1.00 (Reference)	0.6649
AC+CC	472	49.4%	380	48.2%	0.96 (0.79-1.15)	
Allelic frequency						
Allele A	1342	70.2%	1117	70.9%	1.00 (Reference)	0.6578
Allele C	570	29.8%	459	29.1%	0.97 (0.84-1.12)	

OR: Odds ratio, CI: confidence interval; [a]based on Chi-square test.

among non-chewers (Table V). There was no interaction of *IL10* genotype with alcohol drinking habit (data not shown).

Discussion

Knowing that the expression levels of *IL10* may contribute to carcinogenesis of oral cancer, we selected three polymorphic sites within the promoter region of the *IL10* gene, A-1082G (rs1800896), T-819C (rs3021097), and A-592C (rs1800872), and clarified their associations with susceptibility for oral cancer in Taiwan. The results showed that the AG and GG genotypes of *IL10* A-1082G were significantly associated with a higher susceptibility for oral cancer in a Taiwanese population (Table II). This is consistent with the findings of Yao and colleagues (19) and supported by the previous literature reported that the G allele of *IL10* A-1082G not only contributed to higher IL10 expression, but with a higher frequency in undifferentiated carcinoma of nasopharyngeal-type Italian patients, compared to A allele (24). As for the SNPs of T-819C and A-592C, there was no significant differential distribution of their genotypic or allelic frequencies among patients and controls (Tables III and IV). In the current study, the minor allelic frequencies of *IL10* A-1082G, T-819C and A-592C among healthy controls were 0.111, 0.272 and

Table V. *Odds ratio (ORs) for inerleukin-10 (IL10) A-1082G genotype and oral cancer after stratified by smoking status.*

Genotypes	Non-smokers		OR (95% CI)[a]	Smokers		OR (95% CI)[a]
	Controls	Patients		Controls	Patients	
AA	230	143	1.00 (Reference)	536	379	1.00 (ref)
AG	51	43	1.36 (0.85-2.14)	117	174	2.10 (1.61-2.75)[b]
GG	8	7	1.41 (0.50-3.96)	14	42	4.24 (2.28-7.88)[b]
Total	289	193		667	595	

[a]Estimated with multivariate logistic regression analysis; [b]statistically significant.

Table VI. *Odds ratios (ORs) for interleukin-10 (IL10) A-1082G genotype and oral cancer after being stratified by areca chewing status.*

Genotypes	Non-areca chewers		OR (95% CI)[a]	Areca chewers		OR (95% CI)[a]
	Controls	Patients		Controls	Patients	
AA	360	93	1.000 (Reference)	406	429	1.000 (ref)
AG	79	31	1.52 (0.95-2.44)	89	186	1.98 (1.48-2.63)[b]
GG	11	3	1.06 (0.29-3.86)	11	46	3.96 (2.02-7.75)[b]
Total	450	127		506	661	

[a]Estimated with multivariate logistic regression analysis; [b]statistically significant.

0.298, respectively, which were similar to those observed in healthy Chinese (20), Korean (25) and Japanese (26) populations. All these frequencies were much lower than those in Italians (0.380, 0.710 and 0.710) (27, 28). This difference may be another good example for the significant genomic gap between Eastern Han Ethnics and Western European Caucasians.

To investigate the joint effects of genotypic and environmental factors on oral cancer, we firstly analyzed the gene–lifestyle interactions of *IL10* A-1082G genotype and personal risky habits for oral cancer, such as smoking, betel quid chewing and alcohol drinking. The G allele of *IL10* A-1082G indeed had joint effects with individual smoking and areca chewing habits on oral cancer susceptibility (Tables V and VI). At the same time, no obvious joint effect of *IL10* A-1082G genotype with alcohol drinking habits on NPC was found.

Consistent with our findings in oral cancer, the *IL10* A-1082G genotype seems to be associated with several types of cancer, such as nasopharyngeal carcinoma (18, 19), melanoma (29), lung cancer (30, 31), cervical cancer (32), breast cancer (33), prostate cancer (34), gastric cancer (35-37), and gastroduodenal disease (38). On the contrary, there are also a few investigations reported that show no association of this SNP with various types of cancer (25, 39-42). There is no denying that these studies would benefit from larger sample sizes. An exchange of findings between different studies would also be valuable for comparison purposes; any conclusion of the genotypic role that *IL10* plays in carcinogenesis among different populations investigated can still not be easily made.

This is to date the first study which focused on *IL10* and its synergistic effects with personal habits on oral cancer in Taiwan, where the male oral cancer incidence is the highest in the world. The AG and GG genotypes of *IL10* A-1082G, together with risky smoking and areca chewing habits, synergistically contribute to individual susceptibility to oral oncology.

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