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ORIGINAL ARTICLE

TGF-β1 and IL-10 single nucleotide polymorphisms as risk factors for oral cancer in Taiwanese

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Abstract Cytokine production capacity varies among individuals and depends on cytokine gene polymorphisms. Transforming growth factor-beta 1 (TGF-β1) plays a significant role in regulating the proliferation and apoptosis of epithelial cells. Interleukin 10 (IL-10) is an immunoregulatory cytokine with biological functions of anti-inflammation, immunosuppression, allergy, and anti-agenesis. The two cytokines are supposed to play an important role in carcinogenesis. The association between cytokine gene polymorphisms with oral cancer (OC) was investigated. We studied the association between the polymorphism in TGF-β1 (G to C polymorphism at codon 25 <+915>) and IL-10 (−1082 G/A, -819 C/T, and −592 C/A) and the risk of OC in patients ($n = 162$) and healthy controls ($n = 118$) in Taiwan. All genotyping experiments were performed using the polymerase chain reaction sequence-specific primer (PCR-SSP) method. It was found that the codon 25 GC genotype of TGF-β1 is significantly higher in frequency in patients with OC compared with a healthy control group ($p < 0.0001$). People with the GC genotype in codon 25 had an 11.09-fold increased risk of OC [odds ratio (OR) = 11.09; 95% confidence interval (CI) = 6.16–113.23]. IL-10 polymorphisms in −819 and −592 positions correlated with the risk of OC ($p < 0.0001$). The IL-10 -592 C allele-containing genotypes posed an increased risk of OC (OR = 1.79, 95% CI = 1.11–2.91). People with the CT genotype in IL-10 -819 had a 3.32-fold increased risk of OC (OR = 3.32; 95%

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CI = 1.64—6.94). The results suggest that polymorphisms in TGF-β1 and IL-10 may have a significant influence on the development of OC.

Introduction

Oral cancer (OC) is the sixth most common cancer in the world with approximately 300,000 new cases diagnosed annually. Taiwan has the most rapidly increasing incidence of this disease, and OC is the major cause of cancer-related death in Taiwanese males aged 25—44 years [1]. Although the available epidemiological evidence indicates that smoking and areca nut chewing are major risk factors for OC, there is a low prevalence rate to development of the disease [2]. Genetic predisposition might explain this individual variability.

Virchow hypothesized that cancer results from inflammation caused by prolonged irritation, tissue injury, and activation of a local host response that favors cell proliferation [3]. Cytokines may have important roles in the carcinogenesis of various malignant solid tumors.

Transforming growth factor-β (TGF-β) has important roles in regulating cell growth, differentiation, apoptosis, adhesion, and motility. The TGF-β pathway regulates many of these cellular processes, and alterations in the signaling cascade in cancer patients may promote tumor development [4]. All three isoforms (TGF-β1, -β2, and -β3) of TGF-β have tissue-specific expressions and each is encoded by a distinct gene. The inhibitory effects of TGF-β1 in head and neck squamous tumors have been determined mainly in cultured cell lines [5]. *In vivo* studies indicate that TGF-β1 induces tumor regression through Smad4-independent pathways that sensitize keratinocytes to mitochondrial-mediated apoptosis [6]. The TGF-β1 gene is located on chromosome 19q13. Single nucleotide polymorphisms (SNPs) in cytokine genes may affect cytokine expression or function via the cytokine network. Many SNPs have been evaluated for possible roles in inflammatory diseases and cancer predisposition. A TGF-β1 polymorphism located on codon 25 (G/C) of exon 1 encodes the leader sequence of the protein and has an importance functional role in modulating its transmembrane transport.

Interleukin 10 (IL-10) is an immunoregulatory cytokine with biological functions such as anti-inflammation, immunosuppression, allergy, and anti-agenesis. It operates through the Janus kinase—signal transducer and activator of transcription (JAK—STAT) signaling pathway by blocking nuclear factor-kappa-B (NF-κB) nuclear translocation [7]. Although macrophages are the major source of IL-10 in normal tissues, IL-10 can also be produced by T lymphocytes, B lymphocytes, mast cells, eosinophils, or even keratinocytes [8]. The IL-10 gene spans about 4.7 kb on chromosome 1q21-32 and contains five exons and four introns [8]. In head and neck carcinomas, higher than normal IL-10 expression in serum or tumor tissue is associated with poor prognosis [9].

The cytokine IL-10 also has an important role in tumorigenesis. Promoter polymorphisms may be subject to the effects of gene transcription and protein production. The IL-10 -1082 SNP and −1082, -819, -592 haplotype is reportedly associated with differential IL-10 expression *in vitro*, and the −1082 A,-819 T, -592 A haplotype is associated with lower IL-10 expression in comparison with the −1082 G, -819 C, -592 C haplotype [10]. Polymorphisms in the IL10 promoter are associated with increased risk of lung and gastric cancers, lymphoma, and hepatocellular carcinoma [11—15].

To the best of our knowledge, whether TGF-β1 and IL-10 gene polymorphisms are associated with OC risk has not been studied in the Taiwanese population. Therefore, the aim of this study was to examine whether gene polymorphisms of TGF-β1 (codon 25: G/C) and IL-10 (−1082 G/A, -819 C/T, and −592 C/A) may affect susceptibility to OC.

Methods

Recruitment of study participants

The participants were OC patients recruited between April 2003 and November 2006. The male controls were recruited between March 2005 and November 2006. The final population included 162 OC patients who were treated at the Department of Oral and Maxillo-facial Surgery of Chung-Ho Memorial Hospital, Kaohsiung Medical University, Kaohsiung, Taiwan and who gave informed consent to this study. The healthy male control group comprised 118 consecutive volunteers who attended an annual health check-up. All OC patients were diagnosed histologically with incisional biopsy or surgical specimen. The clinical stages were categorized according to the Tumor-Node-Metastasis (TNM) system. In cases who had received radical surgery, the final pathological stage was applied. In cases who had not received surgery, the clinical stage was applied. Pathological grades of the OC were classified using Broder's criteria as follows: Grade I, tumors showing a marked tendency to differentiation, with at least three-fourths of their cells differentiated; Grade II, tumors with three-fourths to one-half of their cells differentiated; Grade III, tumors with one-half to one-fourth of their cells differentiated; Grade IV, tumors with one-fourth to none of their cells differentiated. Trained interviewers used a standard questionnaire to collect demographic data from each participant, including history of exposure to risk factors such as betel quid chewing, cigarette smoking, and alcohol drinking. After obtaining written informed consent, 10-mL blood samples were drawn into coded ethylene diamine tetraacetate (EDTA) tubes. Participants who smoked at least

once a week were defined as smokers. The same rule was applied to drinkers and betel quid chewers. The experimental protocol was approved by the Institutional Review Board of Kaohsiung Medical University Hospital.

DNA isolation and cytokine genotyping

Genomic DNA was extracted from whole EDTA-treated peripheral blood using a QIAamp Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. SNPs were analyzed in TGF-β1 and IL-10 for genotype assignment. Single nucleotide mutations in the coding region were surveyed for TGF-β1: codon 25 rs1800471, either C or G. Three different polymorphisms were examined for the IL-10 promoter region: position −1082 rs1800896 (G vs. A), position −819 rs1800871 (C vs. T), and position −592 rs1800872 (A vs. C). Cytokine genotyping was performed by the polymerase chain reaction sequence-specific primer (PCR-SSP) method by using a commercially available kit (One Lambda, Inc., Canoga Park, CA, USA) in accordance with the manufacturer's instructions. All PCR products were visualized after electrophoresis on a 2% agarose gel and ethidium bromide staging. The DNA extractions and PCR amplifications were performed by a technician blinded to the study groups.

Statistical analysis

JMP software Version 9 (SAS Institute Inc., Cary, NC, USA) for Microsoft Windows was used for all statistical analysis, including determinations of the mean and standard error of numeric variables. The Hardy–Weinberg equilibrium of each genetic polymorphism was tested using a goodness-of-fit χ^2 test to compare observed genotypes frequencies with those in healthy controls. Two-sample t tests or χ^2 tests were used as needed to compare means and proportions between the control group and the OC group as needed. To determine whether cytokine genotype and allele frequency were associated with cancer risk, the significance of the difference in distributions of genotypes and alleles between patients and control participants was calculated by the χ^2 statistic or Fisher's exact test and presented as a p value. All p values were two-sided. A p value < 0.05 was considered statistically significant. Associations between TGF-β1 genotypes and diseases were analyzed by logistic regression of patients versus controls. Independent variables were adjusted to control for potentially confounding effects of age, smoking, drinking status, and duration of betel quid chewing. Logistic regression was performed by JMP statistical software.

Results

Table 1 shows the clinicopathological features of the patients. Of the 162 patients in this study, 125 (77.16%) were male and 37 (22.84%) were female. The two groups significantly differed in mean age, sex distribution, and use of betel quid, alcohol, and tobacco. The age range of the patients with OC was 23–85 years old [median age, 52.04 years old; 95% confidence interval (CI), 40.41–63.67 years]. The age range of the healthy controls was 23–83 years (median age, 59.2 years; 95% CI, 46.29–72.11 years). More than 73% patients had a history of habitual betel nut chewing.

The buccal mucosa (45.68%) was the most commonly affected site of OC (Table 1), and 91.36% of patients had squamous cell carcinoma of the oral cavity. Ten patients (6.17%) had verrucous carcinoma. The remainder (2.47%) had other malignancies. Most (62.96%) had advanced disease (Stage III/IV), and 37.04% of cases had Stage I or Stage II tumors. Histological differentiation in all patients revealed that the tumors were well differentiated in 87.04% and moderately differentiated in 4.32%. No patients had poorly differentiated tumors.

The control group genotype data for all SNPs were analyzed for fitness in Hardy–Weinberg equilibrium. No cases showed significant deviation.

Association between TGF-β1 and OC risk

The high producer GG (Arg/Arg) genotype at codon 25 was found in 53% (84/158) of OC patients and in 92.74% of the healthy controls (Table 2). The intermediate producer GC (Arg/Pro) genotype was present in 74/158 (46.84%) of the OC patients and in 8/124 (6.45%) of the healthy controls. Only one person in the control group and no patients in the OC group had the low producer CC (Pro/Pro) genotype. The percentage of participants with the high producer G (Arg) allele significantly differed between the OC group (76.58%) and the control group (95.97%) ($p < 0.0001$). The frequencies of the C allele were 23.42% in the OC group and 4.03% in the control group. The groups significantly differed in polymorphisms at codon 25 of TGF-β1 gene ($p < 0.0001$). Participants with TGF-β1 codon 25 C-allele containing genotypes GC or CC had a higher OC risk [adjusted odds ratio (OR) = 11.09, 95% CI = 6.16–113.23, $p < 0.0001$] compared to those with the GG genotype.

Association between IL-10 and OC risk

The −1082 A/G polymorphism of the IL-10 gene did not significantly differ between the OC and control groups. However, the −819 C/T and −592 C/A polymorphic sites significantly differed (Tables 2,3). The 'T' allele was found more frequently in both the OC and control groups (0.58 and 0.70, respectively) compared to the 'C' allele at −819. However, the frequency of the 'C' allele, known as the high producing allele, was more common in the OC group (OR 1.79, 95% CI 1.11–2.91, $p = 0.0158$) and also the TC genotype was more frequent whereas the TT genotype was statistically less common in the OC group compared to the control group (OR 3.32, 95% CI 1.64–6.94, $p = 0.0008$). The 'A' allele was found more frequently in both the OC and control groups (0.58 and 0.70, respectively) compared to the 'C' allele at −592. However, the 'C' allele, known as the high producing allele, occurred more frequently in the OC group (OR 1.79, 95% CI 1.11–2.91, $p = 0.0158$) and also the TC genotype was more frequent, whereas the TT genotype was statistically less common in the OC group compared to the control group (OR 3.32, 95% CI 1.64–6.94, $p = 0.0008$).

Table 1 Characteristics of the study population.

Characteristic	Cases ($n = 162$)	Value	p
		Controls ($n = 118$)	
Age (y)			
Range	23–85	23–83	
Average	52.70 ± 12.38	59.20 ± 12.91	0.0018 *
≤ 55	106 (65.43%)	49 (38.28%)	
> 55	56 (34.57%)	79 (61.72%)	
Sex			< 0.0001 *
Male	125 (77.16%)	77 (60.16%)	
Female	37 (22.84%)	51 (39.84%)	
Betel quid chewing status (%)			< 0.0001 *
Ever	119 (73.46%)	22 (17.19%)	
Never	43 (26.54%)	106 (82.81%)	
Alcohol drinking consumption status (%)			< 0.0001 *
Ever	112 (69.14%)	32 (25%)	
Never	50 (30.85%)	96 (75%)	
Smoking status (%)			< 0.0001 *
Ever	116 (71.60%)	38 (29.69%)	
Never	46 (28.42%)	90 (70.31%)	
Site			
Tongue	38 (23.46%)		
Buccal mucosa	74 (45.68%)		
Floor of mouth	4 (2.47%)		
Alveolus	17 (10.49%)		
Lips	16 (9.88%)		
Retromolar trigone	7 (4.32%)		
Palate	6 (3.70%)		
Tumor size			
T1 + T2	111 (68.52%)		
T3 + T4	51 (31.48%)		
Lymph node involvement			
N0	79 (48.77%)		
N1	56 (34.57%)		
Distant metastasis			
M0	162 (100%)		
M1	0 (0%)		
Clinical stage			
Early stage (I + II)	60 (37.04%)		
Late stage (III + IV)	102 (62.96%)		
Pathological diagnosis			
Squamous cell carcinoma			
Well differentiated	141 (87.04%)		
Moderate differentiation	7 (4.32%)		
Poor differentiation	0 (0%)		
Verrucous carcinoma	10 (6.17%)		
Other malignancy	4 (2.47%)		

* Statistically significant difference between patients and control subjects ($p < 0.05$).

As previously described by Edwards-Smith et al, All individuals were further divided into low (ATA/ATA, ACC/ATA, ACC/ACC), intermediate (GCC/ACC, GCC/ATA), and high (GCC/GCC) producer genotypes. The frequency of high/intermediate producer genotypes did not significantly differ in either the OC group or in the control group (10.34%, 14.29%, respectively, $p = 0.3381$). The low producing genotype also showed no significant difference between the two groups (Table 4).

Discussion

Betel quid chewing and smoking are known to be important risk factors for oral squamous cell carcinoma and precancerous lesions, including oral submucous fibrosis (OSF) and leukoplakia (OL) [16]. The data in our study were consistent with the literature. Betel quid chewing and smoking were high risk factors and males were more susceptible than females. After nominal logistic regression analysis of the data,

Table 2 Distribution and allele frequency of TGF-β1 (codon 25), IL-10 (−1082, −819, −592) among OC patients and healthy controls.

Cytokine gene	Genotype frequency (%)	Cases	Controls	*p*	Adjusted OR (age, sex, habits)	95% CI	*p*
TGF-β1 (codon 25)							
	CC	0 (%)	1 (0.81%)	< 0.0001 *	5.074E-07	0.00−4.90	n.s.
	GC	74 (46.84%)	8 (6.45%)		11.09	6.16−113.23	< 0.0001 *
	GG	84 (53.16%)	115 (92.74%)		1.00		
	C	74 (23.42%)	10 (4.03%)	< 0.0001 *	5.76	2.66−13.61	< 0.0001 *
	G	242 (76.58%)	238 (95.97%)		1.00		
IL-10 (−1082)							
	GG	1 (0.69%)	0 (0%)				
	GA	14 (9.66%)	16 (14.29%)	n.s.			
	AA	130 (89.66%)	96 (85.71%)				
	G	16 (5.52%)	16 (7.14%)	n.s.	0.76	0.31−1.87	n.s.
	A	274 (94.48%)	208 (92.86%)		1.00		
IL-10 (−819)							
	CC	11 (7.59%)	8 (7.14%)	< 0.0001 *	2.46	0.69−9.10	n.s.
	CT	101 (69.66%)	51 (45.54%)		3.32	1.64−6.94	0.0008 *
	TT	33 (22.76%)	53 (47.32%)		1.00		
	C	123 (42.41%)	67 (29.91%)	0.0034 *	1.79	1.11−2.91	0.0158 *
	T	167 (57.59%)	157 (70.09%)		1.00		
IL-10 (−592)							
	CC	11 (7.59%)	8 (7.14%)	< 0.0001 *	2.46	0.69−9.10	n.s.
	CA	101 (69.66%)	51 (45.54%)		3.32	1.64−6.94	0.0008 *
	AA	33 (22.76%)	53 (47.32%)		1.00		
	C	123 (42.41%)	67 (29.91%)	0.0034 *	1.79	1.11−2.91	0.0158 *
	A	167 (57.59%)	157 (70.09%)		1.00		

* Statistically significant difference between patients and control subjects ($p < 0.05$).
CI = confidence interval; IL-10 = interleukin 10; n.s. = not significant; OC = oral cancer; OR = odds ratio; TGF-β1 = transforming growth factor-beta 1.

Table 3 Distributions of combined polymorphisms at different positions of IL-10.

Cytokine gene polymorphism	Cases, *n* (%)	Controls, *n* (%)	*p*
IL-10 (−1082, −819, −592) [a]			
GCC/GCC	1 (0.69%)	0 (0.0)	
GCC/ACC	2 (1.38%)	5 (4.46%)	
GCC/ATA	12 (8.28%)	11 (9.82%)	
ACC/ACC	8 (5.52%)	3 (2.68%)	0.0002 *
ACC/ATA	89 (61.38%)	40 (35.71%)	
ATA/ATA	33 (22.76%)	53 (47.32%)	
ACA/ATA	0 (0.0)	0 (0.0)	
ATC/ATA	0 (0.0)	0 (0.0)	
GCC/ATC	0 (0.0)	0 (0.0)	

* Statistically significant difference between patients and control subjects ($p < 0.05$).
CI = confidence interval; IL-10 = interleukin 10; n.s. = not statistically significant ($p > 0.05$); OR = odds ratio.
[a] Due to some technical problems, the genotyping results of 165 patients and 112 controls were included.

Table 4 Cytokine gene polymorphisms and their associated phenotypes.

Cytokine gene polymorphism	Cases, *n* (%)	Controls, *n* (%)	*p* *
IL-10 (−1082, −819, −592) [a]			
High/intermediate	15 (10.34%)	16 (14.29%)	n.s. (0.3381)
Low	130 (89.66%)	96 (85.71%)	

* Statistically significant difference between patients and control subjects ($p < 0.05$).
CI = confidence interval; IL-10 = interleukin 10; n.s. = not statistically significant ($p > 0.05$); OR = odds ratio.
[a] Due to some technical problems, the genotyping results of 165 patients and 112 controls were included.

adjusted for sex, age, smoking, drinking, and betel quid chewing, a protective factor was identified in males (OR = 0.12; 95% CI = 0.07–0.42). In the absence of betel quid chewing, females are more susceptible to oral diseases compared to males. In males, the higher incidence of oral mucosa diseases results from the stronger tendency to consume substances such as tobacco, alcohol, and betel quid.

Interestingly, not all betel quid chewers develop oral mucosal lesions [2]. Inherited differences in the effectiveness of the disease may play an important role in host susceptibility. Acrolein in cigarette smoke inhibits the T cell response [17]. The interaction between genetic factors of the immune system and oral consumption of tobacco, alcohol, and betel quid is interesting and needs further study.

This study also compared the TGF-β1 allele frequencies at codon 25 in Taiwan with those reported in other ethnic groups. In Hong Kong Chinese, the prevalence of codon 25 G appeared to be lower, and that of codon 25 C appeared to be higher. In Caucasians, the prevalence was similar [18]. The differences may be related to the governance of Taiwan by many different countries.

This study revealed an increased risk of the C allele at codon 25 with respect to the G allele in patients with OC. The GC genotype was significantly higher in the OC group than in the controls and appeared to be associated with an 11.9-fold increased risk as compared to the wild type GG genotype.

TGF-β is a potent anti-inflammatory cytokine that regulates cell proliferation, cell differentiation, intercellular adhesion, apoptosis, wound repair, and tissue recycling. The TGF-β signal transduction cascade from the cytoplasm to the nucleus involves a novel family of proteins called Smads [4,19,20]. The associations between TGF-β1 expression and cancer development and progression are complex. Mutations resulting in the Smad signaling proteins decrease overall survival [21]. Overexpression of TGF-β1 confers resistance to induced carcinogenesis, which suggests a tumor-suppressant function of TGF-β [22]. Absence of TGF-β1 expression is associated with a high risk of malignant conversion of skin tumors in mice [23].

To our knowledge, this study is the first to report prevalence data for polymorphisms of the TGF-β1 gene in a Taiwan population and is the first to investigate an association between such polymorphisms and OC. Since codon 25 of TGF-β1 increases serum levels of TGF-β1 in the presence of the G allele, it might contribute to long-term suppression of epithelium proliferation and might decrease OC risk. The C allele at codon 25 may reduce suppression of the epithelium proliferation, which increases OC risk. TGF-β1 low-production genotypes not only increase the risk of breast cancer [24], but are also associated with an increased risk of breast cancer relapse [25] and a reduced 5-year disease-free survival rate [26]. TGF-β1 inhibits the expression of the early viral transforming regions E6 and E7, which are key oncoproteins. Studies suggest that squamous cell carcinomas are devoid of TGF-β1, which raises the possibility that elevated levels of this growth factor could protect against cervical cancer [27].

The findings of this study suggest that IL-10 polymorphism might have an important role in increasing OC risk. Compared to the control group, the OC group had more heterozygote genotypes (C/T and C/A) at positions −819 and −592, respectively. The results published by Vairaktaris et al [28] regarding the role of IL-10 -1082 A/G polymorphism in the pathogenesis of oral squamous cell carcinoma revealed a strong association with increased OC risk. The discordance between these two studies might be explained by different patient characteristics. For example, Vairaktaris et al included 144 cases and 141 controls from Greek and German populations whereas all patients in our study were Asian. Since polymorphisms are known to vary by ethnicity, data from studies of different ethnic populations must be interpreted cautiously. In another report published by Yao et al [29], analysis of the role of polymorphisms of the IL-10 promoter gene in the −1082, -819, and −592 positions, revealed that the −1082 G allele carriers are associated with a significantly increased risk of OC as compared with the −1082 A allele. However, genotype and allele frequencies of the IL-10 promotor −819 T/C and −592 A/C polymorphisms in OC patients did not significantly differ from those in healthy controls in that study. In our study, the IL-10 promoter gene in the −1082 position showed no association with OC risk. However, the IL-10 -819 C allele and −592 C allele were associated with a higher risk as compared to the −819 T allele and the −592 A allele. The distribution of the grade of tumor differentiation also differed. Histological differentiation of the cancers was available in all patients analyzed in our study. Well-differentiated tumors were present in 87.04% of the samples; the remainders were moderately differentiated (4.32%) and no patients had poorly differentiated tumors. Yao et al [29] reported well-differentiated tumors in 14.6% of cases. The tumors were moderately differentiated in 23.9% of cases and poorly differentiated in 61.4% of cases. Differentiation often affects outcome of treatment and prognosis. The differences are interesting and need further study. Yao et al did not report betel quid chewing status. However, betel quit chewing status was one of the most important etiologies in our OC patients, and 73.46% previously chewed betel quid. Possible explanations are differences in the ethnicity, allelic distribution, and clinical features of the patients. Further studies are needed to clarify the relations between betel quid chewing habit and IL-10.

For development of OC, environmental factors are more important than SNPs. Analyses of the role of SNP in OC patients who used alcohol, tobacco, and betel quid showed no interaction between polymorphisms of IL-10 and TGF-β1 and oral habits such as smoking, alcohol consumption, and betel quid chewing (data not shown). This may explain why some patients who did not have the risk factors of smoking, betel nut chewing, and alcohol drinking still developed OC.

The analytical results suggest that the intrinsic, genetically determined ability to produce high or low levels of TGF-β1 as represented by G/C polymorphisms at codon 25 and polymorphisms of the IL-10 gene (−819 T/C and −592 A/C) is the critical factor in the development of OC.

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