J Oral Pathol Med (2005) 34: 608–12

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Tumor necrosis factor-α promoter polymorphism is associated with susceptibility to oral squamous cell carcinoma

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BACKGROUND: Oral squamous cell carcinoma (OSCC) is one of the leading cancers in most Asian countries. Alterations of immune function have been detected in OSCC patients. The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is a central mediator of the immune response involved in a wide range of immuno-inflammatory and infectious diseases. Polymorphism of the TNF-α gene has been intensively studied as a potential determinant of susceptibility to numerous cancers.

METHODS: We genotyped 192 patients with OSCC and 146 healthy case controls by using polymerase chain reaction-double restriction fragment length polymorphism with amplification-created restriction sites to assess allelic determinants at the TNF-α polymorphic sites −308 and −238 in the promoter region. Genotype frequencies were evaluated with Fisher's test.

RESULTS: The −308 TNFG (tumor necrosis factor G) allele genotype was higher in patients with OSCC (91.2% vs. 82.2%; $P = 0.02$) and TNFG/A was lower (8.3% vs. 11.8%; $P = 0.02$); the −238 TNFG/A allele genotype was lower in patient with OSCC (2.1% vs. 6.9%; $P = 0.02$).

CONCLUSION: This is the first report that the TNF-α polymorphism is associated with the risk for OSCC in Taiwan.

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Keywords: mouth neoplasms; polymorphism; RFLP; TNF-α

Introduction

Oral squamous cell carcinoma (OSCC) is one of the leading cancers in most Asian countries. It is the fifth most common malignancy in males in Taiwan (1–3). It is believed to develop through accumulated gene alterations (4, 5). Alterations of immune function have been detected in patients with OSCC (6, 7), as have polymorphous autoimmune reactions to various tissue antigens (8). To date, no single etiologic agent has been identified, despite investigation of factors implicated in the development of oral cancer, including infectious agents; carcinogens; and genetic, nutritional, immunologic, and autoimmune factors (9–15). It has been suggested that an ongoing cellular immune response may lead to an imbalance of immunoregulation with eventual changes in tissue architecture.

The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is a central mediator of the immune response involved in a wide range of immuno-inflammatory and infectious diseases (16). Although TNF-α has a beneficial function in host defense, uncontrolled, excessive production can in itself contribute to disease (17).

Tumor necrosis factor-α was originally discovered as a serum factor causing necrosis of transplanted tumors in mice (18). A large number of preclinical and clinical studies have demonstrated its therapeutic antitumor effects (19, 20). However, there is also increasing evidence that TNF-α may promote the development and spread of cancer. Increased serum TNF-α levels have been measured in cancer patients (21, 22) and are associated with an adverse disease outcome (23). TNF-α expression levels may thus be a determinant of pathogenesis and disease progression in cancer (24, 25).

Tumor necrosis factor-α expression is mostly regulated at the transcriptional level (26), and polymorphism in the TNF-α gene has been intensively studied as a potential determinant of disease susceptibility in a number of diseases where TNF-α levels are thought to be important. A G-to-A substitution at position −308 in the TNF-α promoter (27), which is associated with increased TNF-α production (28), has been a subject of

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Accepted for publication March 14, 2005

particular interest. Associations between the rare TNF-α A allele and a variety of inflammatory and infectious diseases have been reported (29–31). A G-to-A substitution at position −238 is a common polymorphism in the TNF-α promoter (32). The functional significance of the polymorphism product is not yet clear, but a putative repressor site is located in a 25 bps stretch including position −238 (33). It is therefore reasonable to speculate that the TNF-α A allele may influence TNF-α expression.

In this study, we investigated whether polymorphism in the −308 and −238 TNF-α promoter region are associated with susceptibility to OSCC in Taiwan.

Materials and methods

Subjects

One hundred and ninety-two patients with OSCC seen between November 2000 and March 2004 at the Oral and Maxillofacial Department at the Taipei Mackay Memorial Hospital comprised the study group. One hundred and forty-six case control subjects (matched by gender and age) were recruited from people who came for routine physical checkups and had minor oral operations for non-neoplastic lesions or maxillofacial trauma. Individuals with autoimmune disorders, blood diseases, or previous malignancy were excluded from the control group.

DNA extraction

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

Polymerase chain reaction

Both polymorphic sites in the promoter region, −308 and −238, were amplified using two modified primers designed to create restriction sites specific for the variations at these positions (34). The polymorphism at position −308 was identified by using the 5′-primer 1, which creates a *Nco*I restriction site for G as described previously (13) and the 5′-primer 2 that creates a *Rca*I site that types A. To analyze the variations at position −238, 3′-primer 1 and 2 were introduced restriction sites for *Ava*II and *Bgl*II respectively. Primer sequence were listed in Table 1. Polymerase chain reaction (PCR) amplification was carried out using 1 μg of genomic DNA, two units Taq polymerase, 10X buffer and 0.2 M of each primer, 200 M dNTP and 1 mM $MgCl_2$. Samples were subjected to initial denaturation at 94°C for 5 min, followed by 35 cycles of 45 s at 94°C for denaturing, 30 s at 58°C for annealing, and 45 s at 72°C for extension, with a final extension at 72°C for 7 min in a DNA Thermal Cycler (Perkin-Elmer Corporation, Foster City, CA, USA).

Table 1 Primer sequence

	5′ primer1	*5′ primer 2*
−308 polymorphism	aggcaataggttttgagggCcat	aggcaataggttttgagggTcat
	3′ primer 1	*3′ primer 2*
−238 polymorphism	ctGgtccctcctacccctcaca	ctAgAccctcctacccctcaca

Table 2 Predicted sizes of the fragment obtained for TNF-α alleles

	NcoI			*RcaI*		
	117 bp	*97 bp*	*20 bp*	*117 bp*	*97 bp*	*20 bp*
−308 alleles						
GG	−	+	+	+	−	−
AA	+	−	−	−	+	+
AG	+	+	+	+	+	+
	AvaI			*BglI*		
	71 bp	*49 bp*	*46 bp*	*22 bp*	*117 bp*	*97 bp*	*20 bp*
−238 alleles							
GG	−	+		−	+	−	−
AA	+	+	−	−	−	+	+
AG	+	+	+	+	+	+	+

Restriction fragment length polymorphism (RFLP) analysis

Ten microliters of the amplified PCR products (117 bp) were subjected to digestion with *Nco*I, *Rca*I, *Ava*II and *Bgl*II under the appropriate buffer conditions (60°C, overnight). The fragments obtained were analyzed by electrophoresis on a 4% agarose gel and stained with ethidium bromide. The predicted sizes of the fragments generated after restriction enzyme digestion are shown in Table 2.

Statistical analysis

The genotypes were analyzed in a 2×2 contingency table, using Fisher's exact test for comparison. Statistical significance was defined as a P-value of < 0.05.

Results

The clinical characteristics of patients with OSCC and controls, including age, gender, are described in Table 3. The amplified PCR products of the −308 and −238 promoter sites consisted of 117-bp fragments. The patterns yielded by restriction enzyme digestion are shown in Fig. 1. Comparison of the TNF-α genotype in study patients and controls is shown in Table 4. The frequency of the −308 TNFG allele genotype was higher in patients with OSCC (91.2% vs. 82.2%, $P = 0.02$) and TNFA/G was lower (8.3% vs. 11.8%, $P = 0.02$); the frequency of the −238 TNFG/A allele genotype was lower in the study group (2.1% vs. 6.9%, $P = 0.02$).

Table 3 Clinical characteristics of patients with OSCC and healthy controls

	OSCC (n = 192)	*Controls (n = 146)*
Age	54.12 ± 9.78	52.04 ± 10.97
Sex (♂/♀)	173/19	130/16
Clinical Stage		
Early	71	
Late	121	

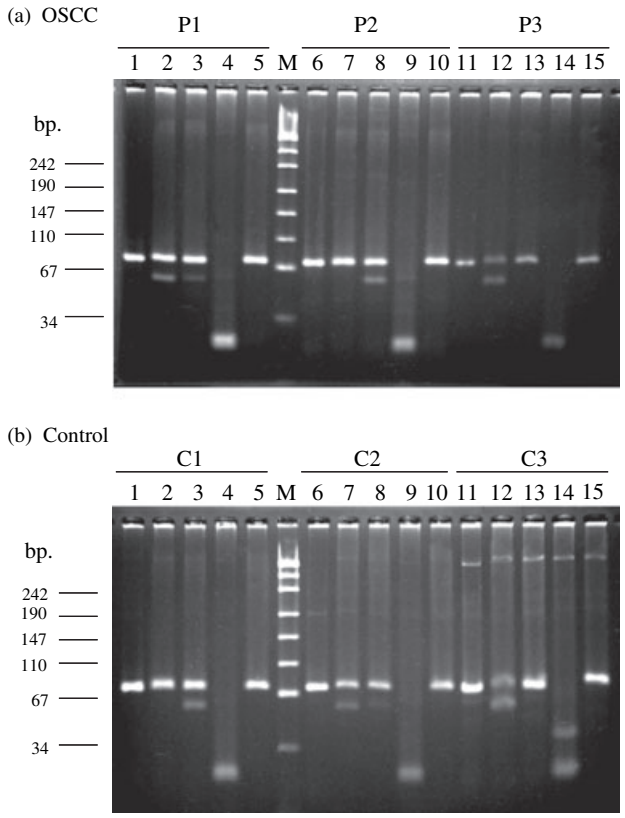

Figure 1 PCR-RFLP + ACRS strategy for TNF-α allele typing. The PCR-amplified product was 117 bp (lane 1, 6 and 11). The fragments after digestion with *Nco*I (lanes 2, 7 and 12), *Rca*I (lanes 3, 8 and 13), *Ava*II (lanes 4, 9 and 14) and *Bgl*II (lanes 5, 10 and 15) were obtained in patients (P) with OSCC (Fig. 1a) and controls (C; Fig. 1b). In the −308 polymorphic site, P3, and C3 are TNF1; P2, and C1 are TNF2; P1, and C2 are TNF1/2. In the −238 polymorphic site, C3 is TNF G/A, and the others are TNFG. No homozygosity for TNFA was observed. M denotes a pUC19/MspI marker.

Table 4 Distribution of tumor necrosis factor alpha (TNF-α) genotype in patients with OSCC and healthy controls

	OSCC [n = 192] (%)	*Control [n = 146] (%)*	*P-value*	*OR*	*95% CI*
−308 polymorphism					
TNFG	175 (91.2)	120 (82.2)	0.02	2.23	1.16–4.29
TNFA/G	16 (8.3)	24 (16.4)	0.02	2.16	1.10–4.24
TNFA	1 (0.5)	2 (1.4)	Ns		
−238 polymorphism					
TNFG	188 (97.9)	136 (93.2)	Ns		
TNFG/A	4 (2.1)	10 (6.9)	0.02	0.26	0.08–0.8
TNFA	0 (0)	0 (0)	Ns		

Ns, not significant.

Discussion

The TNF-α gene is particularly difficult to study in isolation because, being part of the major histocompatibility complex (MHC) class III cluster, it is an integral component of this human leukocyte antigen (HLA) gene complex. More pertinently, polymorphism at TNF-α −308 is linked with HLA haplotype (35). Compared with the techniques previously used to study TNF-α polymorphisms (27, 29, 30, 36–38) we analyzed TNF-α gene −308 and −238 polymorphism in the promoter region by PCR-double restriction fragment length polymorphism (RFLP) with amplification-created restriction sites (ACRS). Oligonucleotides spanning both sites were constructed and two primers were designed for each polymorphic site to create restriction sites specific for the known variations at these positions (34). There were variable results in different populations (Table 5). In this study we using the case control model (that matched with age and sex distribution) that may be make difference between our results and other study.

Table 5 TNF-α polymorphism in different populations

	Taiwanese[a] [n = 146] (%)	*Taiwanese[b] [n = 220] (%)*	*Korean[c] [n = 92] (%)*	*Spain[d] [n = 102] (%)*	*Germany[e] [n = 200] (%)*
−308 polymorphism					
TNFG	82.2	81.8	92.4	82.4	71
TNFA/G	16.4	12.3	7.6	15.7	28
TNFA	1.4	5.8	0	1.9	0.5
−238 polymorphism					
TNFG	93.2	95.7	86.9	81.4	88.5
TNFG/A	6.9	3.3	12	18.6	11.5
TNFA	0	1.1	1.1	0	0

[a]Present study.
References [b]41; [c]38; [d]40; [e]39.

Compared with the techniques previously used to study TNF-α polymorphisms (27, 29, 30, 36–41) the strategy we used had several advantages: restriction enzymes are a much more potent tool to detect polymorphism, making false positive results very unlikely; and interpretation of the results is not ambiguous as both variations of each polymorphic site is positively tested. Consequently, the results obtained for TNF-α alleles allows totally reliable assignment of the genotypes of the samples studied.

In this study, we investigated the possibility that polymorphisms in the TNF-α promoter may contribute to disease susceptibility to OSCC. This study is the first to show that the −238 and −308 polymorphisms are associated with OSCC. The frequency of the rare TNF −238 AG allele was significantly lower in patients with OSCC compared with controls. The functional consequences of the TNF −238A allele are unclear, despite its known association with a variety of diseases (42, 43). Kaluza el al. (44) reported that the TNF −238A allele caused a significant decrease in the transcription of the TNF-α gene in human T and B cells. Thus, the protective effect of the TNF −238A allele against cancer may be conferred by a decrease in TNF-α production. This finding supports the proposed role for TNF-α as an endogenous tumor promoter (45). In our study, heterozygosity for this allele, that is, a −238AG genotype, appears to offer a protective effect against OSCC. It may also be that the TNF-α alleles are associated with some other polymorphism affecting TNF regulation, as the

TNF receptor genes are in a closely linked locus within the MHC.

The fact that the TNF −308A allele is known to upregulate transcription of the TNF-α gene (28) is also in line with a critical role for the TNF-α in tumor promotion (45). The work of Cabrera and others showed that cells containing the −308A allele produced up to six times more mRNA than those containing the −308G allele (28, 30, 46–48). In our study, patients with OSCC had a higher frequency of homozygosity for TNF −308G. This again suggests that the −308A allele confers a protective effect against cancer, possibly increasing of TNF-α production.

The biologic activity, and therefore sensitivity to the growth regulatory properties of TNF-α, is mediated by two distinct cell surface receptors. Lack of expression of both these has been reported in a human cervical cancer cell line (49). Our study of polymorphisms that influence the production of TNF-α did not address the role of its receptors. It would be instructive to investigate TNF-α receptor genes in OSCC as well.

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Acknowledgments

The authors thank Dr M.J. Buttrey for her kin review and instructive criticism of this paper. The study is supported by Grant MMH- E – 93007 from Taipei Mackay Memorial Hospital.