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Association of *CTLA-4* gene polymorphism with oral squamous cell carcinoma

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BACKGROUND: Oral squamous cell carcinoma (OSCC) is a worldwide problem. The main mechanism of tumor immunity is the destruction of tumor cells by cytolytic T lymphocytes. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; CD152), a negative regulator of T-lymphocyte activation, plays an extremely important role in the immune tolerance and anergy. This study was designed to investigate the role of CTLA-4 polymorphism in OSCC.
METHODS: The CTLA-4 +49 A/G polymorphism was studied in 118 patients with OSCC and 147 healthy controls by using restriction fragment length polymorphism (RFLP). The genotype and phenotype frequencies were evaluated in Fisher's exact test.
RESULTS: There was no significant difference in the frequency of CTLA-4 polymorphism between the OSCC study group and healthy controls. The CTLA-4 A/A genotype was significantly associated with a younger age of onset of OSCC ($P = 0.04$). The AA genotype was associated with significantly poorer survival ($P = 0.003$).
CONCLUSION: The present study is the first to shows that the A/A polymorphism is associated with poor survival in OSCC in Taiwan.
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Keywords: cytotoxic T lymphocyte-associated antigen 4; mouth neoplasm; polymorphism

Introduction

Tumorigenesis is a sequential process involving multiple gene mutations and that affect the cell cycle and proliferation. A major step in this process is the evasion by tumor cells of immune surveillance as well as production of immunosuppressive cytokines (1). For this reason, tumor immunity is increasingly a target of research. Burnet first described the concept of immunologic surveillance in the recognition and destruction of transformed tumor cells (2). The main mechanism of the antitumor response depends on T-cell receptor engagement by major histocompatibility complex (MHC) antigens as well as CD28 ligation by B7. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; CD152) is a second receptor of the co-stimulating factors B7-1 (CD80) and B7-2 (CD86), which have structures similar to CD28. CTLA-4 inhibits T-cell activation and terminates the T-cell response by blocking signals stimulated via CD28. It is now apparent that one reason, tumors fail to elicit a protective immune response, is that they do not express co-stimulatory ligands, allowing them to escape from the immune system.

The *CTLA-4* gene is located on chromosome 2q33–q34 and comprises four exons (3, 4). There are five different *CTLA-4* genes polymorphisms (4). One is exon 1 +49 A/G polymorphism that causes an amino acid exchange (threonine to alanine) in the peptide leader sequence and is associated with several autoimmune diseases, including Hashimoto thyroiditis, rheumatoid arthritis, multiple sclerosis, diabetes mellitus, and autoimmune blood disorders (5–8). Polymorphisms of the *CTLA-4* gene have been reported to be associated with susceptibility to various cancers (4, 5, 9). In our previous study, we demonstrated that CTLA-4 polymorphism is associated with oral submucous fibrosis, a pre-cancerous condition that may lead to oral squamous cell carcinoma (OSCC; 10). However, the relationship between CTLA-4 polymorphism and OSCC has never been investigated. In this study, we investigated the genotype and phenotype of exon 1 +49 A/G CTLA-4 polymorphism in patients with OSCC.

Materials and methods

Subjects

A total of 118 patients with OSCC seen in the Oral and Maxillofacial Department of Mackay Memorial Hospital from November 1998 to December 2001 constituted the study group. In all cases, the diagnosis of OSCC was

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made by clinical examination and histopathology of biopsy specimens. These patients were followed for at least 3 years. A group of 147 control subjects were selected from among people who came to the clinic for routine physical checkups, non-neoplastic minor operations, or maxillofacial trauma. Those with autoimmune disorders, hematologic disorders, or previous malignancy were excluded. The study was approved by an ethics review committee. After informed consent was obtained, blood was drawn from the subjects and genomic DNA was extracted from fresh or frozen peripheral blood leukocytes by standard protocols.

DNA extraction

Peripheral blood samples were collected from all study and control subjects and genomic DNA was extracted by using the Pharmacia DNA isolation kit (Pharmacia Biotech, Freiburg, Germany).

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify the desired fragment of exon 1 +49 of the *CTLA-4* gene using a forward primer, 5′-AAGGCTCAGCT-GSSCCTGGT-3′, and a reverse primer, 5′-CTGCT-GAAACAAATGAAACCC-3′ (10). 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 1X 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 analysis

About 10 μl of the amplified PCR products were digested in a volume of 12.1 μl with 1 U of restriction enzyme *Bst*EII under the appropriate buffer conditions (60°C, overnight). The fragments were analyzed on 3.5% agarose gel.

Statistical analysis

Evaluation of the Hardy–Weinberg equilibrium was performed by comparing observed and expected frequencies of heterozygotes and homozygotes, as well as observed and expected genotypes, using chi-squared analysis. Genotypic frequencies of OSCC cases and controls were compared by using a Fisher's exact test with Yates' correction where appropriate (one expected number: <5). An unpaired *t*-test was used to evaluate differences in age at onset. Cumulative survival was analyzed with the Kaplan–Meier product limit method. The duration of survival was measured from the beginning of treatment to the time of death or the last follow up. Differences were considered to be statistically significant if the *P*-value was <0.05.

Results

The age of individuals with OSCC ranged from 30 to 75 years (mean: 51.4 ± 0.8) and in the control group 22 to 80 years (mean: 51.1 ± 1.8). The most common primary OSCC site was the buccal mucosa (67 cases), followed by the tongue (29), gingiva (15), palate (four), and floor of the mouth (three; Table 1). Fifty-six patients had stage IV tumors, while the remaining 62 had stages I–III disease, according to the staging system of the American Joint Committee on Cancer (Table 1). Forty-two patients had neck lymph node metastasis (LNM).

There was no significant difference in the frequency of CTLA-4 polymorphism between the OSCC study group and healthy controls (Table 2), or in the frequency of CTLA-4 polymorphism between patients with and without LNM (Table 3). However, patients with the A/G genotype presented at a significantly older age than did those with the A/A genotype (51.1 ± 1.2 vs. 45.6 ± 1.4, $P = 0.04$, unpaired *t*-test; Table 4). Subjects

Table 1 Clinical characteristics of patients with oral squamous cell carcinoma (OSCC) and controls

Characteristic	*OSCC (n = 118)*	*Control (n = 146)*
Gender		
Male	110	88
Female	8	66
Age (years)		
Mean ± SEM	51.4 ± 0.8	51.1 ± 1.8
Range	30–75	22–80
Location		
Buccal mucosa	67	
Tongue	29	
Gingiva	15	
Palate	4	
Mouth floor	3	
Stage		
I	16	
II	28	
III	18	
IV	56	
Lymph node metastases		
Absent	76	
Present	42	

Table 2 Genotyping and phenotyping of CTLA-4 alleles in patients with OSCC and controls

	OSCC		*Control*		
	n = 118	*%*	*n = 147*	*%*	*P-value*
Genotype					
A/A	12	10.1	25	17.0	ns
A/G	58	49.1	64	43.5	ns
G/G	48	40.6	58	39.5	ns
Gene frequencies					
A	82	34.7	114	38.8	ns
G	154	65.2	180	61.2	ns
Phenotype frequencies					
A	70	59.3	89	60.5	ns
G	106	89.9	122	83.0	ns

ns, not significant; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; OSCC, oral squamous cell carcinoma.

Table 3 Genotyping and phenotyping of CTLA-4 alleles in OSCC patients with and without lymph node metastases

	OSCC without LNM		*OSCC with LNM*		
	n = 76	*%*	*n = 42*	*%*	*P-value*
Genotype					
A/A	5	6.5	7	16.6	ns
A/G	40	52.6	18	42.8	ns
G/G	31	40.8	17	40.5	ns
Gene frequencies					
A	50	32.9	32	38.1	ns
G	102	67.1	52	61.9	ns
Phenotype frequencies					
A	45	59.2	25	59.5	ns
G	71	93.4	35	83.3	ns

ns, not significant; LMN, lymph node metastases; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; OSCC, oral squamous cell carcinoma.

Table 4 CTLA-4 genotypes related to age at onset of OSCC

Genotypes	*n*	*Age at onset (mean ± SEM)*	*95% CI*
A/A	12	45.6 ± 1.4	
A/G*	58	51.1 ± 1.2	0.08–10.62*
G/G	48	51.9 ± 1.5	0.29–12.75

*$P = 0.04$
CTLA-4, cytotoxic T lymphocyte-associated antigen 4; OSCC, oral squamous cell carcinoma; CI, confidence interval.

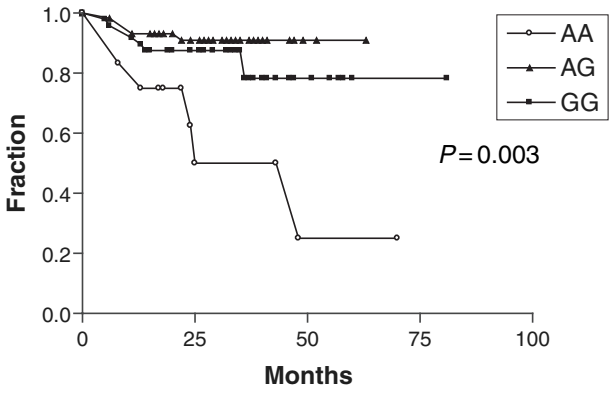

Figure 1 Survival curves in oral squamous cell carcinoma (OSCC) vis-à-vis cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) polymorphism.

with the A/A genotype had significantly worse survival than those with A/G and G/G genotypes. Kaplan–Meier analysis confirmed these results (Fig. 1, $P = 0.003$). The CTLA-4 genotype did not differ significantly with respect to the site of the tumor (detailed analysis not shown).

Discussion

The development of malignant disease may be seen as a failure of immune surveillance (11). Immune response to tumors involves the activation of T lymphocytes, antibodies, natural killer cells, and macrophages. Immunologic tolerance and anergy allow tumors to evade this response. It is therefore of crucial importance to understand the molecular events that determine how the immune system will respond to malignancy. CTLA-4 plays a significant role in regulating peripheral T-cell tolerance and attenuating T-cell response (12). CTLA-4/B7 interactions block activation and promote T-cell death or apoptosis (13–16). Recent studies demonstrate that anti-CTLA-4 monoclonal antibodies promote potent antitumor immunity (17–19). Therefore, it is not surprising that CTLA-4 polymorphism might play a role in malignant disease. Polymorphisms of the *CTLA-4* gene have been reported to be associated with susceptibility to various human cancers (4, 5, 9). The G/G genotype is significantly less common than other phenotypes in patients with breast cancer (4). The A/A genotype has been associated with larger tumor size and a greater degree of lymph node involvement. In our study, the A/A genotype was less frequent in patients with OSCC than in the control group (10.1:17%), but this was not a significant difference. It appears that the effects of CTLA-4 polymorphism vary in different cancers.

Patients with the G/G genotype, in comparison with the A/A genotype, have been shown to have increased mRNA and protein expression of interleukin (IL)-2, the primary T-cell growth factor (20). This suggests that the poor survival associated with the A/A genotype relates to IL-2 activation of T cells. Another study has reported that *CTLA-4* gene polymorphism in the leader sequence may influence the level or pattern of expression of the protein (3). Thus, T cells from G/G individuals would be expected to have reduced levels of CTLA-4 following T-cell activation when compared with T cells from A/A individuals. In individuals with OSCC, the A/A genotype was associated with an earlier onset of disease than the A/G and G/G genotypes. This suggests that the *G* allele in some way contributes to active tumor immunity. Furthermore, the A/A genotype was associated in our series with shorter survival. This, too, is consistent with the hypothesis that the lack of a G^{49} allele tends to attenuate the immune response, as well as contributing to peripheral tolerance (11, 20). Other investigations indicate that CTLA-4 deficiency or blockade induces or exacerbates autoimmunity, enhances tumor immunity, or prevents induction of immunologic tolerance (21, 22). It is interesting to speculate, therefore that if it would be possible to design treatment for patients with A/A CTLA-4 polymorphism that would enhance their antitumor response.

Surgical excision is the treatment of choice for early stage OSCC, usually combined with adjuvant radiotherapy or chemotherapy. However, the 5-year survival rate has remained approximately 50% over the past 30 years (23, 24). Improving this dismal prognosis will likely depend on combining early detection with treatments that allow organ preservation (23). Recent studies have demonstrated that anti-CTLA-4 monoclonal antibodies promote potent antitumor immunity (17–19). Huang et al. treated primary prostate tumors in transgenic (TRAMP) mice with anti-CTLA-4 and a

granulocyte colony-stimulating factor (G-CSF) expressing vaccine, resulting in a significant reduction in tumor incidence as assessed 2 months after treatment. This approach may open another avenue to treatment of prostate cancer (17). Our study raises the question of similar targeting of treatment to patients with the A/A genotype.

The present study is the first report on the association of CTLA-4 polymorphism with OSCC age of onset and survival. Further studies are needed to clarify the mechanism of the antitumor response induced by CTLA-4 blockade.

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