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## CTLA-4 Gene Polymorphism in +49 A/G Position: A Case Control Study on Patients with Oral Lichen Planus

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### Abstract:

**Background:** Oral lichen planus (OLP) is a premalignant mucocutaneous disease in which genetic factors and immune responses play a major role. Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) is a co-inhibitory molecule that down-regulates immune responses to prevent autoimmunity. This study aimed to investigate the relationship between polymorphism of the CTLA-4 gene in +49 A/G position and OLP.

**Materials and Methods:** Thirty-five patients with OLP (16 males, 19 females, with a mean age of [ $\pm$  standard deviation (SD)] 55.92 [ $\pm$  12.83]) and 105 sex- and aged-matched healthy subjects (48 males, 57 females, with a mean age of [ $\pm$  SD] 56.82 [ $\pm$  14.71]) were recruited in this study. Genomic DNA of both groups was extracted from white blood cells and then CTLA-4 genotypes and allele frequencies were investigated using polymerase chain reaction-restriction fragment length polymorphism methods. The data were collected and examined using Pearson's Chi square test (SPSS version 11.5).

**Results:** In the patient group, AA, AG, and GG genotypes occurred in position 49 A/G in the CTLA-4 gene with the frequency of 19 (55.9%), 11 (31.4%), and 3 (8.8%), respectively. With respect to the control group, they occurred with the frequency of 58 (55.2%), 39 (37.1%), and 8 (7.6%), respectively. As far as the frequency of A and G alleles in this position was concerned, we had, respectively, 49 (74.24%) and 17 (25.75%) for patients and, respectively, 155 (73.80%) and 55 (26.19%) for the control group.

The calculated values were not significantly different between these groups ( $P > 0.05$ ).

**Conclusion:** Polymorphisms of CTLA-4 genes in position +49 A/G did not show any significant relationship with each other in OLP patients in Shiraz, Iran.

**Key Words:** +49 A/G, CTLA-4, lichen planus, polymorphism

### Introduction

As a disease of unknown etiology, oral lichen planus (OLP) is a chronic inflammatory mucocutaneous disease. This oral lesion is the most common non-infectious soft tissue disease that exerts an impact on 1-2% of adult patients in oral medicine clinics. OLP which has various forms include white striae, white papules, white plaques, erythema, erosions, or bullae. The most common site of the involvement is buccal mucosa.<sup>1,2</sup>

Female adults aged over 40 are affected more than males of all age ranges (1.4:1). Younger adults and children may experience it as well. OLPs are typically bilateral, and often appear as a combination of clinical subtypes. Approximately, all cases of OLP are considered as reticular keratotic streaks in the oral mucosa. Accordingly, all oral mucosal lesions should be carefully examined for fine keratotic lines proximal to atrophic and/or erosive areas in the buccal mucosa, ventral or lateral surface of the tongue, gingiva, or other sites. OLP gingival lesions often appear as fiery red erythema that involves the entire width of the attached gingiva. OLP lesions may occasionally be accompanied with melanin deposition.<sup>3</sup> Lichen planus skin lesions are considered as pruritic flat-topped violaceous papules and plaques, mostly on the flexor sites of the wrists or ankles, or extensor aspects of the lower legs. Furthermore, some authors have reported the genital involvement similar to skin lesions.<sup>4</sup>

Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) also known as CD152 is expressed on activated T-cells to down-regulate effector function through competition with CD28 molecule for binding to their ligands, B7.1 and B7.2, on antigen presenting cells.<sup>5</sup> Interaction between CTLA-4 protein and cognate ligands makes an inhibitory signal which results in the reduction of T-cell proliferation and cytokine production and subsequently plays a critical role in the maintenance of immune tolerance and prevention of autoimmunity.<sup>6</sup> CTLA-4 deficiency, in a mouse model, is reported to be associated with lymphocyte

infiltration, whereas transgenic expression of this molecule into activated conventional T-cells is capable of the delay in manifestations of autoimmunity.<sup>7,8</sup> Regulatory T-cells (Tregs) restrict immune responses to self-antigens and, thereby, they also could prevent autoimmune disorders. Previous findings revealed that expression of CTLA-4 on Tregs contributes to the inhibitory function of these subsets in the immune system.<sup>9,10</sup> Human CTLA-4 gene (Gene ID: 1493) is mapped on chromosome 2q33.<sup>11</sup> Variation in CTLA-4 gene, especially in +49A/G position is observed to affect expression and/or function of the CTLA-4 protein.<sup>12</sup> Previous studies on different ethnic groups indicate that CTLA-4 gene polymorphism in +49A/G position (rs231775) may predispose individuals to several autoimmune diseases such as systemic lupus erythematosus, RA, MS, and Type 1 diabetes.<sup>13</sup> Moreover, our previous study revealed a strong association between CTLA-4 haplotypes and genetic susceptibility of Iranian people to head and neck cancers.<sup>14</sup> Due to immunologic origin of OLP, the current study aimed to examine the presence of any correlation on CTLA-4 polymorphism in 49A/G position (rs231775) in patients with OLP versus control group.

## Materials and Methods

### Methods

Thirty-five ethylenediaminetetraacetic acid (EDTA)-blood samples from known cases of OLP patient, including 16 males and 19 females aged between 20 and 58 and 105 sex- and age-matched healthy subjects, including 48 males and 57 females aged between 19 and 65 were enrolled in this case-control study.

All selected patients in the patient group were those patients whose OLP was clinically and histopathologically diagnosed in Oral Medicine Department of Shiraz University of Medical Sciences. Information on OLP patients suffering from any systemic disease, cancer, autoimmunity (themselves or their first-degree relatives) as well as on any medication-administering that might produce a lichenoid reaction in the last 3 months were collected from their profiles.

Participants in the control group were sex- and age-matched healthy blood donors who were selected among persons attending blood transfusion center.

All participants were informed about the study and agreed to participate by signing a consent form. OLP lesions were divided into two forms according to their clinical pattern, reticular and/or plaque lesions (21) and erosive atrophic lesions (14). Both patient group and healthy control groups were living in south of Iran. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences.

### DNA extraction and concentration

As previously described by Miller, 7 ml of EDTA-peripheral blood was taken from each donor and genomic DNA was

extracted from white blood cells using salting-out process.<sup>15</sup> After DNA extraction, in order to determine the amount and the purity of extracted DNA, the absorbance of each sample at 260 nm and 280 nm wavelengths was measured by spectrophotometry (Eppendorf, Germany). For each DNA sample, the final concentration of 0.3 µg/µl was calculated and then they were stored at -20°C until they were ready to be used.

### Genotype analysis

Employing polymerase chain reaction-restriction fragment length polymorphism methods (PCR-RFLP), CTLA-4 polymorphism in +49A/G position (rs231775) were investigated. Using a forward Primer: (5-GCTCTACTTCCTGAAGACCT-3 and a reverse primer: 5-AGTCTCACTCACCTTTGCAG-3 in Eppendorf thermo cycler (Mastercycler, Bochum, Germany), amplification of a 162-bp fragment were performed. PCR program was begun with an initial denaturation (94°C, 3 min), followed by 4 cycles, including denaturation (45 s), annealing (45 s), extension (45 s), and completed with a final extension (72°C, 5 min). The information on cycles is given in Table 1.

The reaction volume contained 100 ng of genomic DNA, 1 pm of each primer (Takapozist, Iran), 2 U of Taq DNA polymerase (CinnaGen, Iran), 0.75 mM of dNTP (CinnaGen, Iran), 2.5 µL of 10 × PCR buffer, 0.5 mM MgCl<sub>2</sub>, and double-distilled water. 2 U of *Bbv1* (*BseXI*) restriction enzyme (Fermentas, Lithuania) was incubated with 10 µL of amplified products at 37°C overnight. The products were stained with GelRed (Biotium, USA) and at the same time, run on an agarose gel (2.5%). The enzyme could cut mutant allele (G allele) into two fragments (88 bp and 74 bp) but failed to digest the wild allele (A allele). Heterozygote sample contained three bands of 160 bp, 88 bp, and 74 bp.

### Statistical analysis

Statistical analyses were performed using SPSS software (version 11.5; SPSS Inc, Chicago, IL, USA) as well as EPI Info 2002 (Centers for Disease Control, Atlanta, GA) software packages. Pearson's Chi square test was applied to determine the differences in genotype and allele frequency between the two study groups. The level of significance was  $P < 0.05$ .

## Results

Information on age, sex, frequency of different genotypes and alleles in +49A/G position in both patient group and control one is given in Table 2. There was no deviation from

Table 1: Four cycles of PCR program.

Cycle	Number of cycles	D	A	E
I	11	94°	70°	72°
II	20	94°	60°	72°
III	2	94°	59°	72°
IV	2	94°	58°	72°

D: Denaturation, A: Annealing, E: Extension, PCR: Polymerase chain reaction

Table 2: Information regarding age, sex, frequency of different genotypes and alleles in +49A/G position in both patient and control group.

+49 position	Sex		Mean age±SD	Genotype (frequency)%			P value (Chi square)	Allele (frequency)%		P value (Chi square)
	Males	Females		AA	AG	GG		A	G	
Patient	16 (45.7)	19 (54.3)	55.92±12.83	19 (55.9)	11 (31.4)	3 (8.8)	0.90	49 (74.24)	17 (25.75)	0.92
Control	48 (45.7)	57 (54.3)	56.82±14.71	58 (55.2)	39 (37.1)	8 (7.6)	0.19	155 (73.80)	55 (26.19)	0.01

SD: Standard deviation

Hardy-Weinberg equilibrium in these loci among patients and control subjects.

The mean age ( $\pm$  standard deviation) of OLP patients and participants in the control group were 55.92 ( $\pm$  12.83) and 56.82 ( $\pm$  14.71), respectively. The most common OLP site was the buccal mucosa (17 cases), followed by the tongue (11 cases) gingiva (7 cases).

AA, AG, and GG genotypes occurred in position 49A/G in the CTLA-4 gene with frequency of 19 (55.9%), 11 (31.4%), and 3 (8.8%), respectively, in patients and with a frequency of 58 (55.2%), 39 (37.1%), and 8 (7.6%), respectively, in the control group. The genotypes did not show any significant difference in the distribution at this locus between patients and participants in the control group ( $P = 0.9$ ). Besides, A and G alleles occurred in this position with the frequency of 49 (74.24%) and 17 (25.75%), respectively, in patients and the frequency of 155 (73.80%) and 55 (26.19%), respectively, in the control group. There was no statistical significant difference between these two groups ( $P = 0.92$ ).

## Discussion

OLP is a T-cell mediated chronic inflammatory disease in which interaction between inflammatory cells, chemokines, cytokines, mast cell degranulation, and matrix metalloproteinase activation has a significant role in the pathogenesis of the disease.<sup>16</sup> Antigen-specific CD8+ CTLs are dominant cells in sub epithelial and intra epithelial cells of OLP lesions and trigger keratinocyte apoptosis.<sup>17,18</sup> It appears that the expression of lichen planus antigen at the lesion site results in OLP lesion formation through local keratinocytes.<sup>16</sup> Beside T-lymphocytes, lichen planus lesions contain B-lymphocytes, plasma cells, and deposits of immunoglobulin or complement.<sup>16</sup>

Since co-inhibitory molecules like CTLA-4 attenuates immune responses after T-cell activation, it is obvious that the deficiency or mutation in these regulatory molecules results in autoimmune disorders in both mice and humans.<sup>19</sup> Therefore, the level of tolerance in autoimmune diseases seems to increase through administering CTLA4-Ig against such co-inhibitory molecules.<sup>20,21</sup>

In recent years, the bulk of research has been conducted on the role of genes which encodes immune regulatory proteins such as CTLA4 polymorphism in malignancies, autoimmune diseases as well as hepatitis B.<sup>13,22-28</sup>

However, there is a dearth of research on oral lesions and its association with CTLA-4 polymorphism. While in Wong *et al.* studies, no significant relationship was found between the frequency of CTLA-4 polymorphism and oral squamous cell carcinoma (SCC), it was demonstrated that CTLA-4 A/A genotype may be highly related to a younger age SCC.<sup>29</sup>

Erfani *et al.* (2006) examined 80 Iranian patients with head and neck cancer genotype and found that whenever ACG, GTA and especially GCA haplotypes have predisposition to it, CT60 A allele as well as ACA and GTG haplotypes in CTLA-4 gene might have protective roles against head and neck cancer.<sup>25</sup>

In their study, on 62 patients with oral submucous fibrosis in Taiwan, Shin *et al.* (2004) reported a significant relationship between CTLA-4 G allele polymorphism and the disease.<sup>30</sup> Our findings are not consistent with those of the study by Shin *et al.* (2004) in this regard.

So far, few studies have been conducted on the relationship between lichen planus and CTLA-4 alleles or other gene polymorphism. Stanimirovic *et al.* (2013) studied 101 OLP patients and 104 healthy blood donors. Their results revealed that the risk of the disease increased in cases with rs5743312 toll-like receptor 3 (TLR3) gene polymorphism, while there was no significant relationship between TLR2, TLR4, and CD14 gene with respect to the polymorphisms.<sup>31</sup> The present study also did not indicate any relationship between CTLA-4 gene polymorphism and OLP.

The study by Chauhan *et al.* (2013) in India revealed a close relationship between A allele in the tumor necrosis factor alpha-308 gene and susceptibility to OLP.<sup>32</sup>

Wu *et al.* (2013) examined 42 OLP patients and 86 healthy persons in China. This case control study showed that polymorphisms of CIITA rs4774 and rs6498122 gene are associated with OLP.<sup>33</sup> As far as OLP patients in Iran were concerned, in the study of Ghabanchi *et al.*,<sup>34</sup> there was no significant correlation between polymorphism of tumor protein p53 codon 72 and OLP. Our findings were in agreement with this result; however, we studied only CTLA-4 allele polymorphism.

Alaibac *et al.* (2000) investigated the expression of CTLA-4 in some cases of inflammatory and neoplastic skin disease and reported their occurrence in three patients out of five ones with lichen planus.<sup>35</sup> Our investigation demonstrated correlation

with respect to CTLA-4 polymorphism in OLP lesion in the control and patient groups.

Pekiner *et al.* (2012) studied 30 patients with OLP and 30 other patients in which they reported a higher percentage of CD8+CD154+ and granzyme-B+, while a lower percentage of CD8+, CD8+CD184+, and apoptotic cells in OLP. It should be noted that no statistical difference was found between these two groups in terms of CD8+CD152+ (CTLA-4) marker expression.<sup>36</sup> However, our findings were to some extent consistent with their findings. On the other hand, our findings were in contradiction with those obtained from the study by Alaibac *et al.* (2000).

### Conclusion

We observed that the polymorphism of CTLA-4 genes in position +49A/G, did not have a significant relationship with OLP patients in Shiraz. However, in order to generalize the obtained results, it is suggested that further studies be conducted on a larger population.

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