# Strong Association of CTLA-4 Variation (CT60A/G) and CTLA-4 Haplotypes with Predisposition of Iranians to Head and Neck Cancer

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## ABSTRACT

**Background:** Variations in Cytotoxic T Lymphocyte Antigen-4 (*CTLA-4*) affect the expression and function of this protein. **Objective:** We aimed to investigate the association of +49 A/G (rs231775), +1822 C/T (rs231779) and +6230 A/G (CT60, rs3087243) genetic variations, as well as the merged haplotypes in CTLA-4 gene with susceptibility to, or progression of head and neck cancer. **Methods:** Eighty patients with confirmed head and neck (HN) cancer (age $54.9 \pm 16.1$ years) and 85 healthy age/sex-matched controls (age $56.3 \pm 12.4$ years) were enrolled in the study. Genotypes were investigated by the PCR-RFLP method. Arlequin software package was used to check for Hardy-Weinberg equilibration, and to estimate the haplotypes. **Results:** At position +6230 A/G (CT60), AA genotype, as well as A allele was significantly decreased in patients with HN cancers than controls (18.8% vs. 40.7%, p=0.004; odds ratio=0.34, and 46.3% vs. 61.7, p=0.007; odds ratio=0.53%, respectively). Nearly the same results were obtained when we compared the subgroup of patients with squamous cell carcinoma of the HN (SCC-HN) with control subjects. The frequencies of genotypes and alleles at other positions were not significantly different between patients and controls, however ACG, GTA and GCA haplotypes emerged from three investigated loci occurred with significantly more frequencies in patients (p<0.0001), while ACA and GTG haplotypes were more frequent among controls (p<0.0001). Significant differences of haplotypes, genotypes and alleles frequencies resisted the Bonferroni correction. **Conclusion:** Our results suggest that CT60 A allele, as well as ACA and GTG haplotypes in *CTLA-4* gene may have protective roles against HN cancer in Iranian population, while ACG, GTA and specially GCA haplotypes may render susceptibility.

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## INTRODUCTION

Cytotoxic T Lymphocyte-Associated Antigen- 4 (CTLA-4; CD152) is a CD28 homologue (1) which is well known to participate in the attenuation of T cell activation and clonal expansion (2). While CD28 is expressed constitutively on the surface of resting and activated T cells and acts as a co-stimulatory molecule, CTLA-4 is only expressed on the surface of activated T cells, down-regulates the stimulatory signals from CD28 and competes for binding to B7 family members i.e. B7.1 and B7.2 (3-5). This co-inhibitory molecule also interferes with signals delivered by T cell receptor (TCR) (6,7). Investigations indicated that regulatory T cells constitutively express CTLA-4 and the molecule has a crucial impact on the development and function of these inhibitory cells (8,9). The function of immune suppressive molecules, including CTLA-4, may attenuate tumor immunity in cancer patients (10). CTLA-4 blockade with anti CTLA-4 mAb has been reported to augment the tumor immunity both at early stages of tumor growth and in vaccinated cancer patients (11-13). CTLA-4 blockade has been demonstrated to increase the expression of inducible costimulator (ICOS) as well as IFN-γ, but diminishes FoxP3 expression in tumor tissues of the patients with localized bladder cancer (14).

The CTLA-4 gene (Gene ID: 1493) has been mapped on chromosome 2q33 in humans, and is closely linked with the CD28 gene (1). The CTLA-4 gene polymorphisms are believed to influence the expression pattern of the protein and/or the functional activity of CTLA-4 (15-19). Among all known polymorphisms of CTLA-4 gene, we investigated in a group of patients with head and neck cancer, the distribution of three more known single nucleotide polymorphisms (SNPs), including an A to G transition in exon 1, encoding a threonine (Thr) to alanine (Ala) substitution at codon 17 (+49 A/G, rs231775) (19), a C to T transition in intron 1 (+1822 C/T, rs231779) (17) and an A to G exchange at the 3'-untranslated region of the gene (+6230 A/G; known as a CT60 polymorphism, rs3087243) (18). The genotypic data, as well as the merged haplotypes were compared with healthy control subjects to assess the association of these genetic variations with cancer incidence and progression.

## MATERIALS AND METHODS

**Subjects.** Eighty histopathologically confirmed patients, 57 (70%) males and 23 (30%) females with a mean age of 54.9 ± 16.1 years having head and neck cancer were recruited in the present study. A group of 85 age and sex-matched volunteers with a mean age of 56.3 ± 12.4 including 59 (69.4%) males and 26 (30.6%) females from the same area and with no personal or familial (first degree relatives) history of cancer or autoimmune diseases were enrolled as the control group. All of the participants were from Shiraz hospitals in the south of Iran. Patients' clinicopathological information was obtained from their medical files. This study was approved by the ethics committee of Shiraz University of medical sciences and informed consent was obtained before sampling.

PCR Amplification of SNP Genotyping. Genomic DNA was extracted from peripheral blood by salting out method (20). All of three polymorphisms were genotyped by the means of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) assays as previously reported by others (17,21,22) with minor changes.

The amplified products were digested overnight with restriction enzymes mentioned in Table 2 (Fermantas, Lithuania), followed by 3% agarose gel (Invitrogen, UK) electrophoresis. CTLA-4 gene-specific primers (Takapouzist, Iran), restriction enzymes, RFLP incubation temperatures and the length of digested fragments are illustrated in Table 1. Figures 1, 2 and 3 illustrate the PCR-RFLP results for +49 A/G, +1822 C/T and +6230 A/G (CT60) polymorphisms, respectively.
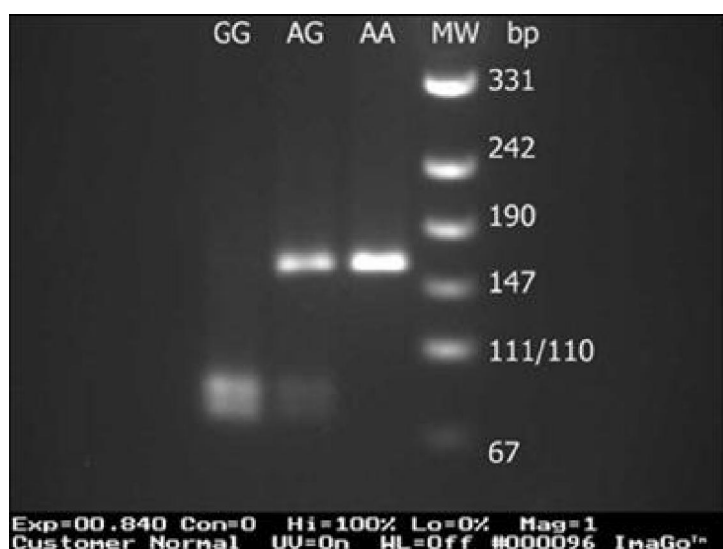


**Figure 1.** Genotyping of +49 A/G in *CTLA4* exon 1 using PCR-RFLP technique and *BbV*1(*Bse*XI) enzyme. AA: 162 bp, AG:162 ,88 and 74 bp, GG: 88 and 74 bp.
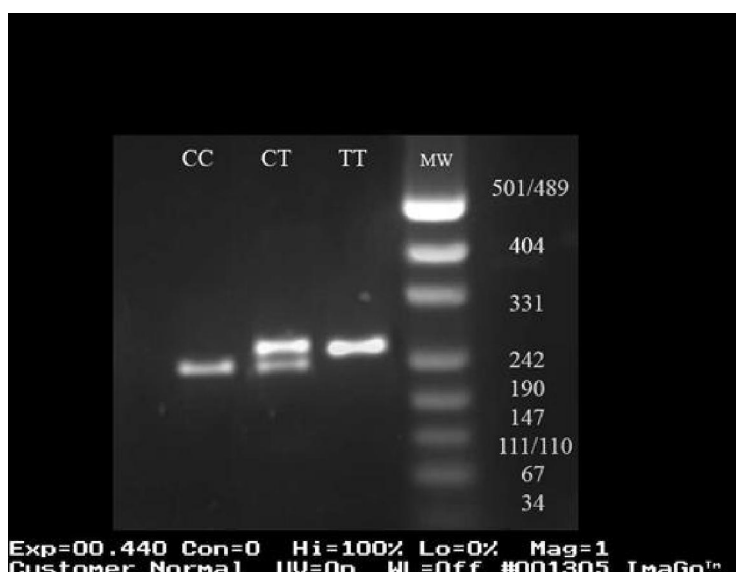


**Figure 2.** Genotyping of +1822 C/T SNP in *CTLA4* intron 1 using PCR-RFLP technique and *Hae*III enzyme. TT: 257 bp, CT: 257, 237 and 20 bp, CC: 237 bp.
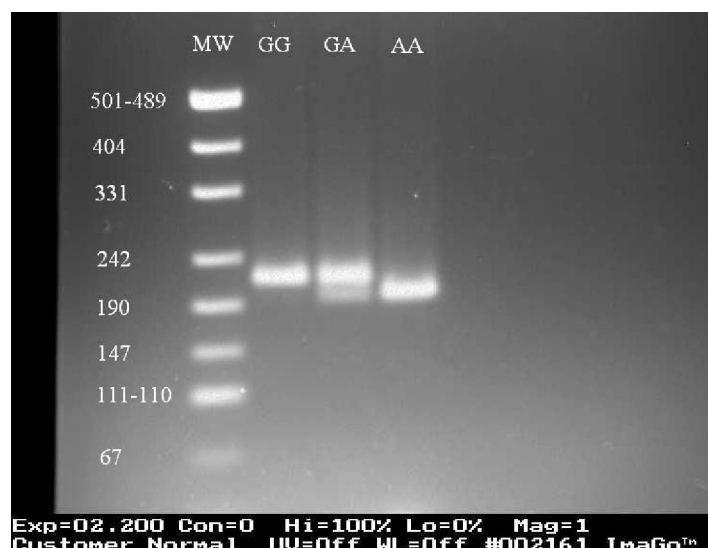
**Figure 3.** Genotyping of CT60 SNP (+6230 A/G) in *CTLA4* 3'UTR using PCR-RFLP technique and *Nco*I enzyme. AA: 196 and 20 bp, AG: 216, 196 and 20 bp, GG: 216 bp.

**Statistical Analysis.** All statistical analyses were done by the SPSS software package (version 11.5; SPSS Inc, Chicago, IL, USA). Hardy-Weinberg equilibrium test and haplotype analysis were by Arlequin software package version 3.1 (L. Excoffier, CMPG, University of Berne, Switzerland). P value less than 0.05 was considered statistically significance. Bonferroni correction was used to find out the threshold of significance for multiple statistical analyses of the genotypes, alleles and haplotypes.

## RESULTS

Patients' clinicopathological information is illustrated in Table 1. As indicated; fifty six out of 80 patients (70%) had squamous carcinoma of the head and neck (SCC-HN), 4 (5%) had salivary gland carcinoma, 15 (18.8%) had other types of head and neck cancer and for 5 (6.3%) cases known as head and neck cancer cases tumor types were unknown. The clinicopathological information of the patients with SCC-HN is also shown in a separate column along with the total number of cancer patients in Table 1.

The frequencies of genotypes and alleles corresponding to three SNPs for both patients with head and neck cancer and the control group are illustrated in Table 3. All three SNPs were in Hardy-Weinberg equilibrium when the observed data were compared with the expected values by Arlequin software package (p>0.05). As illustrated in Table 3, at position +6230 (CT60) AA genotype was observed to be significantly decreased in patients with head and neck carcinoma in comparison to healthy control subjects (15/80 (18.8%) vs. 33/85 (40.7%), p=0.004, Odds ratio 0.34, 95% CI for Odds ratio 0.14-0.73). Consistant with this finding, A allele frequency at this position was observed to be 46.3% in the patients and 61.7% in the control subjects, demonstrating a significant decrease in the prevalence of A allele in the patients (p=0.007, Odds ratio 0.53, 95% CI 0.33-0.85). All these significant values resisted Bonferroni correction which lessened the level of significance to a p value of 0.01. Similar results were obtained when we

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compared the patients with squamous cell carcinoma of the head and neck (SCC-HN) with the control group (Table 3).

**Table 1. Clinicopathological features of patients with head and neck cancer, as well as patients with Squamous cell carcinoma of the head and neck (SCC-HN).**

| Feature | Head and neck cancer (HN), N=80 | | Squamous cell carcinoma (SCC)-HN, N=56 | |
|---|---|---|---|---|
| | No. out of 80 | Statistics" | No. out of 56 | Statistics" |
| Age (years) | 80 | Mean ± SD: 54.9±16.1, Minimum: 15, Maximum: 88 | 56 | Mean ± SD: 57.3 ± 14.9, Minimum: 15, Maximum: 88 |
| Tumor Types | 80 | (SCC)-HN: 56 (70%) Salivary gland carcinoma: 4 (5%) Other types: 15 (18.8%) Unknown tumor type: 5 (6.3%) | 56 | (SCC)-HN: 56 (100%) |
| Tumor stage | 68 | Stage 2 : 22.4% Stage 3: 31.6% Stage 4: 46.1% | 56 | Stage 2 : 25.0% Stage 3: 25.0% Stage 4: 50.0% |
| Lymph node (LN) involvement | 73 | Neg: 64.4% Pos: 35.6% | 52 | Neg: 63.5% Pos: 36.5% |
| Tumor size (cm) | 37 | Size ≤2: 32.7%, 2 < Size ≤ 5: 59.3% Size >5: 8.0% | 22 | Size ≤2: 18.2%, 2 < Size ≤ 5: 81.8% Size >5: 0.0% |
| Lymphovascular (LVI) invasion | 67 | Neg:59.7% Pos: 40.3% | 49 | Neg:51.0% Pos: 49.0% |
| Distant metastasis | 68 | Neg: 85.3% Pos: 14.7% | 49 | Neg: 89.8% Pos: 10.2% |

"Except for tumor type, all other percentages in this table are valid percentages, i.e. excluding missing data.

The genotype and allele frequencies at +49 position were not significantly different between patients and controls (p=0.43 and p=0.65 for genotypes and alleles, respectively). Similarly, there were no significant differences in the frequencies of genotypes and alleles at position +1822 between the patients and the controls (p=0.09 and p=0.52 for genotypes and alleles, respectively).

Investigation of the genotype association with clinicopathological characteristics of the patients indicated no association with the cancer progression factors including tumor type, TNM stage, T stage, N stage, M stage, LN involvement status, tumor size, distant metastasis and lymphovascular invasion.

Haplotype analysis revealed the presence of eight haplotypes in patients and seven in the control group (Table 4). Statistical analysis indicated that ACG, GTA and GCA haplotypes emerging from the three loci occurred with significantly more frequencies in patients than the controls (50% vs. 13.8%; 13.8% vs. 0.6%; and 8.8% vs. 0% respectively (p<0.0001). In contrast, ACA and GTG haplotypes were more frequent in

**Table 2. Region specific primers, annealing temperatures, restriction enzymes and length of digested fragments used for genotyping of SNPs in *CTLA-4* in the present study.**

| CTLA4 Locus | Primer | Primer sequences | Ref. | Restriction Enzyme | RFLP Temperature C° | Length of digested fragments |
|---|---|---|---|---|---|---|
| +49 A/G | Forward | 5'- GCTCTACTTCCTGAAGACCT -3' | (21) | *Bbv*1(*Bse* XI) | 65 | A:162bp |
| | Reverse | 5'- AGTCTCACTCACCTTTGCAG -3' | | | | G: 88bp,74bp |
| +1822 C/T | Forward | 5'- CACTATTTTTGAGTTGATGCAG -3' | (17) | *Hae*III (*Bsu* RI) | 37 | T:257bp |
| | Reverse | 5'- CCCTGGCATTGTTGTAGAGTG -3' | | | | C:237bp,20bp |
| +6230A/G (CT60) | Forward | 5'- CACCACTATTTGGGATATACC -3' | (22) | *NCO* I | 37 | G:216 bp |
| | Reverse | 5'- AGGTCTATATTTCAGGAAGGC -3' | | | | A: 196bp,20bp |

the controls than the patients (16.2% in patients vs. 58.1% in controls and 1.2% in patients vs. 20.6% in controls, respectively ($p<0.0001$). All the significant values resisted Bonferroni correction which decreased the level of significance to a p value of 0.005.

**Table 3. The frequencies of genotypes and alleles of *CTLA-4* SNPs in patients with head and neck (HN) cancer as well as squamous cell carcinoma of the head and neck (SSC-HN) in comparison to healthy control group.**

| *CTLA-4* Locus | | | Control group (n=85, 2n=170) | Head and neck (HN) cancer (n=80, 2n=160) | | Squamous cell carcinoma (SSC)-HN (n=56, 2n=112) | |
|---|---|---|---|---|---|---|---|
| | | | Frequency | Frequency | P Value[¥] | Frequency | P Value[¥] |
| +49 | Genotype | AA | 50(58.8%) | 41(51.3%) | 0.41 | 27 | 0.28 |
| A/G | | AG | 29(34.1%) | 35(43.8%) | 0.27 | 26 | 0.20 |
| | | GG | 6(7.1%) | 4(5.0%) | 0.75 | 3 | 1.0 |
| | Missing | | 0 | 0 | - | 0 | - |
| | Allele | A | 129(75.9%) | 117(73.1%) | 0.65 | | |
| | | G | 41(24.1%) | 43(26.9%) | | | |
| | Missing | | 0 | 0 | | | - |
| +1822 | Genotype | CC | 47(55.9%) | 45(56.3%) | 0.90 | 33 | 0.86 |
| (C/T) | | CT | 30(35.7%) | 34(42.5% | 0.46 | 22 | 0.80 |
| | | TT | 7(8.4%) | 1(1.3%) | 0.06[#] | 1 | 0.14 |
| | Missing | | 1 | 0 | | 0 | - |
| | Allele | C | 124(73.8%) | 124(77.5%) | 0.52 | | |
| | | T | 44(26.2%) | 36(22.5%) | | | |
| | Missing | | 2 | 0 | | | |
| +6230 | Genotype | AA | 33(40.7%) | 15(18.8%) | 0.004* | 12 | 0.03 |
| A/G | | AG | 34(42%) | 44(55.0%) | 0.13 | 31 | 0.21 |
| (CT60) | | GG | 14(17.3%) | 21(26.3%) | 0.23 | 13 | 0.52 |
| | Missing | | 4 | 0 | | 0 | |
| | Allele | A | 100(61.7%) | 74(46.3%) | 0.007* | 55 | 0.05 |
| | | G | 62(38.3%) | 86(53.7%) | | 57 | |
| | Missing | | 8 | 0 | | 0 | |

¥P value in comparison to the control group.

* Considered significant even after Bonferroni correction (P values less than 0.01)

# Result of Fisher exact test. The rest are the results of Yates corrected Chi-square test.

**Table 4. Frequency of haplotypes emerging from three *CTLA-4* SNPs in patients with head and neck cancer (HN) in comparison to healthy control group.**

| *CTLA-4* Haplotype (+49 A/G, +1822 C/T, +6230 (CT60) A/G) | Head and neck cancer(2n=160) | Control group (2n=160)[#] | P Value | Odds ratio | 95% CI for odds ratio |
|---|---|---|---|---|---|
| A C G | 80 (50.0%) | 22(13.8%) | <0.0001* | 6.27 | 3.52-11.26 |
| A C A | 26(16.2%) | 93(58.1%) | <0.0001* | 0.14 | 0.08-0.25 |
| G T A | 22(13.8%) | 1(0.6%) | <0.0001* | 25.35 | 3.57-511.65 |
| G T G | 2(1.2%) | 33(20.6%) | <0.0001* | 0.05 | 0.01-0.21 |
| G C A | 14(8.8%) | 0 (0%) | <0.0001* | NA[¥] | NA |
| A T A | 11(6.9%) | 4(2.5%) | 0.11 | NA | NA |
| G C G | 4(2.5%) | 4(2.5%) | 1.0 | NA | NA |
| A T G | 1(0.6%) | 3(1.9%) | 0.28 | NA | NA |

#Five cases, out of 85 control subjects, had no complete genotyping data and were excluded from haplotype analysis.*Considered significant, even after Bonferroni correction, with a P value less than 0.005 ¥NA: not applicable.

## DISSCUSION

It has already been established that the variations in CTLA-4 gene may alter gene expression and function, and consequently the strength of anti tumor immunity, in such a way that affect cancer susceptibility and/or cancer progression (15-19). We examined the possible association of three polymorphisms in CTLA-4 gene (+49 A/G, +1822 C/T and +6230 (CT60) A/G), which, to our knowledge, have not been previously investigated in head and neck cancer. Our results indicated a significant decrease, in comparison to controls, in the frequencies of AA genotype and A allele at position +6230 A/G (CT60) in patients with head and neck cancers (p=0.004; odds ratio 0.34, and p=0.007; odds ratio 0.53), as well as the subgroup of patients with squamous cell carcinoma of the head and neck (SCC-HN). +6230 A/G (CT 60) SNP in the 3' untranslated region of CTLA-4 gene is supposed to affect the proportion of soluble isoform of CTLA-4 (sCTLA-4) to membrane bound (m)CTLA-4 (18). Soluble isoform of CTLA-4 is generated through translation of alternative spliced mRNA and could either inhibit or exacerbate the immune responses in different manners (23,24). It has been previously suggested the G allele at position +6230 (CT60 G) may decrease sCTLA-4 transcript up to 50% (18). Therefore, it is most likely that a decrease in the frequencies of CT60 A allele or AA genotype in our patients, changes the balance between sCTLA-4/mCTLA-4 in favor of lower sCTLA-4, and consequently, higher

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mCTLA-4 expression. Consistent with our data, the frequencies of CT60 G allele has been reported to be increased in patients with breast cancer in Chinese population (25,26). In spite of such studies, others could not find an association of CT60 variants with susceptibility to lung cancer in Iranian patients (27), cervical squamous cell carcinoma in polish patients (28) and HPV-16-associated cervical squamous cell carcinoma in Taiwanese women (29). Furthermore, CT60 AA genotype has been reported with a higher significant incidence in Spanish patients with renal cell cancer (30), which may imply differences among different ethnic groups, variation in molecular pathology of different cancer types, or may be due to minor differences in sample size and study design. In consistent with the findings of the present study, a more recent work on Caucasians of New Hampshire with an acceptable sample size indicated that CT60 G allele decreases the risk of non-melanoma skin cancer (NMSC) (31).

The results of the present study did not show an association between genotypes and alleles at position +49 A/G in head and neck cancer. The A to G variation at +49 position within exon 1 of CTLA-4 leads to an amino acid exchange (threonine to alanine) in the leading sequence of CTLA-4 molecule and may affect the expression pattern of this molecule (19). Although, the +49 A/G SNP has been reported to be associated with predisposition of individuals to breast cancer in Iranian patients (32), non-Hodgkin's lymphoma (NHL) in Italian Caucasian patients (33) , and several other types of cancer (recently reviewed in ref. 34), no association was reported in some other carcinomas including lung (27), colorectal (35) and gastric (35) cancers in Iranian population, oral squamous cell carcinoma in Taiwanese women (36), cervical squamous cell carcinoma in polish patients (28), and colorectal cancer in Italian Caucasians patients (37),and in Turkish population (38). We also did not observe any significant differences in the frequencies of genotypes or alleles at position +1822 between patients with head and neck cancer and the control group. Similarly, no significant association was reported between the mentioned SNP and the risk of lung cancer in Iranian population (27).

Despite observing no association between +49 A/G and +188 22 C/T SNPs with head and neck cancer, the haplotype analysis of the three investigated SNPs (+49 A/G, +1822 C/T, +6230 (CT60) A/G) revealed a significant increase in ACG, GTA and GCA haplotype frequencies and a significant decrease in ACA and GTG haplotype frequencies among patients (All with p<0.0001). Interestingly, GCA haplotype which was absent in controls occurred with a considerable frequency in patients (14 out of 160 haplotypes (8.8%), p<0.0001). Although a functional study is required to assess the significance of the observed haplotypes, these findings suggest that the occurrence of ACG, GTA and specially GCA haplotypes in Iranian population is associated with the risk of head and neck cancer. On the other hand, the increase of ACA and GTG haplotypes in our population might be associated with protection against this cancer. Consistent with our study, the TACG haplotype (-1722T,-1661A,-318C and +49G) in Iranian patients with colorectal and gastric cancers (35) and GAT and AGT haplotypes

(+49,-1661 and -1722) in Chinese patients with gastric cancer (39) have already been reported to increase the risk of cancer predisposition.

Conclusively, our results suggest a strong genetic association of *CTLA-4* gene variants and haplotypes with susceptibility to head and neck carcinoma in Iranian population. Incidence of CT60 A allele, as well as ACA and GTG haplotypes may have protective roles against cancer, while ACG, GTA and specially GCA haplotypes may render susceptibility.

## ACKNOWLEDGEMENT

## REFRENCES

1   Harper K, Balzano C, Rouvier E, Mattei MG, Luciani MF, Golstein P. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. J Immunol. 1991;147:1037-44.
2   Walunas TL, Lenschow DJ, Bakker CY, Linsley PS, Freeman GJ, Green JM, et al. CTLA-4 can function as a negative regulator of T cell activation. Immunity. 1994; 1:405-13.
3   Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity. 1995;3:541-7.
4   Lee KM, Chuang E, Griffin M, Khattri R, Hong DK, Zhang W, et al. Molecular basis of T cell inactivation by CTLA-4. Science. 1998; 282:2263-6.
5   Lindsten T, Lee KP, Harris ES, Petryniak B, Craighead N, Reynolds PJ, et al. Characterization of CTLA-4 structure and expression on human T cells. J Immunol. 1993; 151:3489-99.
6   Chikuma S, Imboden JB, Bluestone JA. Negative regulation of T cell receptor-lipid raft interaction by cytotoxic T lymphocyte-associated antigen 4. J Exp Med. 2003; 197:129-35.
7   Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J Exp Med. 1995; 182:459-65.
8   Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, et al. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. J Exp Med. 2000; 192:303-10.
9   Tang Q, Boden EK, Henriksen KJ, Bour-Jordan H, Bi M, Bluestone JA. Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function. Eur J Immunol. 2004; 34:2996-3005.
10  Chen L, Ashe S, Brady WA, Hellstrom I, Hellstrom KE, Ledbetter JA, et al. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. Cell. 1992; 71:1093-102.
11  Hodi FS, Mihm MC, Soiffer RJ, Haluska FG, Butler M, Seiden MV, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. Proc Natl Acad Sci USA. 2003; 100:4712-7.
12  Yang YF, Zou JP, Mu J, Wijesuriya R, Ono S, Walunas T, et al. Enhanced induction of antitumor T-cell responses by cytotoxic T lymphocyte-associated molecule-4 blockade: the effect is manifested only at the restricted tumor-bearing stages. Cancer Res. 1997; 57:4036-41.
13  Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science. 1996; 271:1734-6.
14  Liakou CI, Kamat A, Tang DN, Chen H, Sun J, Troncoso P, et al. CTLA-4 blockade increases IFNgamma-producing CD4+ICOShi cells to shift the ratio of effector to regulatory T cells in cancer patients. Proc Natl Acad Sci U S A. 2008; 105:14987-92.
15  Maurer M, Loserth S, Kolb-Maurer A, Ponath A, Wiese S, Kruse N, et al. A polymorphism in the human cytotoxic T-lymphocyte antigen 4 ( CTLA4) gene (exon 1 +49) alters T-cell activation. Immunogenetics. 2002; 54:1-8.
16  Sun T, Zhou Y, Yang M, Hu Z, Tan W, Han X, et al. Functional genetic variations in cytotoxic T-lymphocyte antigen 4 and susceptibility to multiple types of cancer. Cancer Res. 2008; 68:7025-34.
17  Marron MP, Zeidler A, Raffel LJ, Eckenrode SE, Yang JJ, Hopkins DI, et al. Genetic and physical mapping of a type 1 diabetes susceptibility gene (IDDM12) to a 100-kb phagemid artificial chromosome clone containing D2S72-CTLA4-D2S105 on chromosome 2q33. Diabetes. 2000; 49:492-9.

18  Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature. 2003; 423:506-11.

19  Donner H, Rau H, Walfish PG, Braun J, Siegmund T, Finke R, et al. CTLA4 alanine-17 confers genetic susceptibility to Graves' disease and to type 1 diabetes mellitus. J Clin Endocrinol Metab. 1997; 82:143-6.

20  Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16:1215.

21  Donner H, Braun J, Seidl C, Rau H, Finke R, Ventz M, et al. Codon 17 polymorphism of the cytotoxic T lymphocyte antigen 4 gene in Hashimoto's thyroiditis and Addison's disease. J Clin Endocrinol Metab. 1997; 82:4130-2.

22  Torres B, Aguilar F, Franco E, Sanchez E, Sanchez-Roman J, Jimenez Alonso J, et al. Association of the CT60 marker of the CTLA4 gene with systemic lupus erythematosus. Arthritis Rheum. 2004; 50:2211-5.

23  Magistrelli G, Jeannin P, Herbault N, Benoit De Coignac A, Gauchat JF, Bonnefoy JY, et al. A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. Eur J Immunol. 1999;29:3596-602.

24  Oaks MK, Hallett KM, Penwell RT, Stauber EC, Warren SJ, Tector AJ. A native soluble form of CTLA-4. Cell Immunol. 2000; 201:144-53.

25  Li H, Fu ZK, Wang LH, Li DL, Wu N, Zhang J, et al. [Association of cytotoxic T lymphocyte antigen-4 gene polymorphisms with susceptibility to breast cancer]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2008; 24:282-4.

26  Wang L, Li D, Fu Z, Li H, Jiang W. Association of CTLA-4 gene polymorphisms with sporadic breast cancer in Chinese Han population. BMC Cancer. 2007; 7:173.

27  Khaghanzadeh N, Erfani N, Ghayumi MA, Ghaderi A. CTLA4 gene variations and haplotypes in patients with lung cancer. Cancer Genet Cytogenet. 2010; 196:171-4.

28  Pawlak E, Karabon L, Wlodarska-Polinska I ,Jedynak A, Jonkisz A, Tomkiewicz A, et al. Influence of CTLA-4/CD28/ICOS gene polymorphisms on the susceptibility to cervical squamous cell carcinoma and stage of differentiation in the Polish population. Hum Immunol. 2010; 71:195-200.

29  Su TH, Chang TY, Lee YJ, Chen CK, Liu HF, Chu CC, et al. CTLA-4 gene and susceptibility to human papillomavirus-16-associated cervical squamous cell carcinoma in Taiwanese women. Carcinogenesis. 2007; 28:1237-40.

30  Cozar JM, Romero JM, Aptsiauri N, Vazquez F ,Vilchez JR, Tallada M, et al. High incidence of CTLA-4 AA (CT60) polymorphism in renal cell cancer. Hum Immunol. 2007; 68:698-704.

31  Welsh MM, Applebaum KM, Spencer SK, Perry AE, Karagas MR, Nelson HH. CTLA4 variants, UV-induced tolerance, and risk of non-melanoma skin cancer. Cancer Res. 2009; 69:6158-63.

32  Ghaderi A, Yeganeh F, Kalantari T, Talei AR, Pezeshki AM, Doroudchi M, et al. Cytotoxic T lymphocyte antigen-4 gene in breast cancer. Breast Cancer ResTreat. 2004; 86:1-7.

33  Monne M ,Piras G, Palmas A, Arru L, Murineddu M, Latte G, et al. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene polymorphism and susceptibility to non-Hodgkin's lymphoma. Am J Hematol. 2004; 76:14-8.

34  Ghaderi A. CTLA4 Gene Variants in Autoimmunity and Cancer :a Comparative Review. Iranian J Immunol. 2011; 8:127-49.

35  Hadinia A, Hossieni SV, Erfani N, Saberi-Firozi M, Fattahi MJ, Ghaderi A. CTLA-4 gene promoter and exon 1 polymorphisms in Iranian patients with gastric and colorectal cancers. J Gastroenterol Hepatol. 2007; 22:2283-7.

36  Wong YK, Chang KW, Cheng CY, Liu CJ. Association of CTLA-4 gene polymorphism with oral squamous cell carcinoma. J Oral Pathol Med. 2006; 35:51-4.

37  Solerio E, Tappero G, Iannace L, Matullo G, Ayoubi M, Parziale A, et al. CTLA4 gene polymorphism in Italian patients with colorectal adenoma and cancer. Dig Liver Dis. 2005; 37:170-5.

38  Dilmec F, Ozgonul A, Uzunkoy A, Akkafa F. Investigation of CTLA-4 and CD28 gene polymorphisms in a group of Turkish patients with colorectal cancer. Int J Immunogenet. 2008; 35:317-21.

39  Hou R, Cao B, Chen Z, Li Y, Ning T, Li C, et al. Association of cytotoxic T lymphocyte-associated antigen-4 gene haplotype with the susceptibility to gastric cancer. Mol Biol Rep. 2010; 37:515-20.