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# Association of Graves' disease and Graves' ophthalmopathy with the polymorphisms in promoter and exon 1 of cytotoxic T lymphocyte associated antigen-4 gene

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**Abstract:** Objective: To investigate the association of Graves' disease and Graves' ophthalmopathy with the C/T transition polymorphism at position –318 of promoter and the A/G transition polymorphism at position 49 of exon 1 within cytotoxic T lymphocyte associated antigen-4 (CTLA-4) gene. Methods: Thirty-three patients with ophthalmopathy of Graves' disease, fifty-six Graves' patients without ophthalmopathy and sixty normal subjects as control were involved in the present case-control study. The polymorphisms were evaluated by polymerase chain reaction fragment length polymorphism (PCR-RFLP). Comparisons were made of gene frequencies and allele frequencies between the groups. Results: The gene frequencies of CT and allele frequencies of T were much higher in Graves' patients with ophthalmopathy than that in the group without ophthalmopathy ($P$=0.020, $P$=0.019). The gene frequencies of GG and allele frequencies of G in patients with Graves' disease were significantly increased as compared with control group ($P$=0.008, $P$=0.007). The data suggest that smokers with Graves' disease seemed to be more predisposed to ophthalmopathy than non-smokers ($P$=0.018). Conclusion: Our results suggest that an allele of T at position –318 of promoter is associated with genetic susceptibility to Graves' ophthalmopathy while an allele of G at position 49 of exon 1 is associated with genetic susceptibility to Graves' disease instead. Smoking is believed to be a major risk factor for ophthalmopathy.

**Key words:** Graves' ophthalmopathy, Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) gene, Gene frequency, Susceptibility gene

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## INTRODUCTION

Graves' disease (GD) is an autoimmune disease, characterized clinically by hyperthyroidism, diffuse goiter, Graves' ophthalmopathy (GO) and the presence of TSH (thyroid-stimulating hormone) receptor autoantibodies. GD is a multifactorial disease that develops as the result of a complex interaction between genetic susceptibility genes and environmental factors. Multiple genetic factors are believed to influence the autoimmunity evident in GD, but a firm genetic basis for GD has not been established. The cytotoxic T lymphocyte associated antigen-4 (CTLA-4) gene is one of the susceptibility gene candidates. Recently, reports about the influences of CTLA-4 gene on GD and GO in different countries are not consistent; this may be due to the genetic heterogeneity. Few researches on Chinese were reported (Jiang *et al*., 1999; Wang *et al*., 2001). In our study, we analyzed the polymorphism of the promoter and the exon 1 within CTLA-4 gene in the patients of GD and GO by polymerase chain reaction fragment length polymorphism (PCR-RFLP); and evaluated the contribution of CTLA-4 gene to genetic susceptibility of GD and GO in Chinese patients.

## MATERIALS AND METHODS

### Patients

We studied a total of 89 Chinese patients from

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two groups: 33 patients with GO (15 men and 18 women), defined by American Thyroid Association (ATA) class 3 or a higher class; 56 GD patients without GO (19 men and 37 women); defined by no manifestation of eyes. GD was diagnosed on the basis of history, signs and the laboratory findings, including elevated serum free $T_4$ and $T_3$ concentrations and positive TRAB (TSH receptor antibodies). The mean age of GO group was (38.2±13.4) years, compared with (41.4±13.3) years of GD group without GO. The time between the first manifestation and assessment was 1 month to 13 years in GO group versus 0.5 month to 12 years in GD group without GO. Data gathered from the patients were compared to those obtained from a control population of 60 unrelated healthy subjects (27 men and 33 women) with no clinical evidence or family history of autoimmune diseases.

**Methods**

Genomic DNA was prepared from peripheral white blood cells using the DNA extraction kit (TaKaRa Biotechnology Dalian Co., Ltd.).

**Polymerase chain reaction**

The CTLA-4 promoter polymorphism at position –318 was defined employing the polymerase chain reaction (PCR) with primers 5'-AAATGAATT GGACTGGATGGT-3', 5'-TTACGAGAAAGGAAG CCGTG-3'. A 247 bp fragment was amplified using 1 μl genomic DNA, 0.3 μmol/L of each primer, 0.2 mmol/L dNTP, 1 U *Taq* polymerase with appropriate buffer. PCR was performed by initial denaturation for 3 min at 94 °C, annealing for 30 s at 58 °C, extension for 30 s at 72 °C, denaturation for 30 s at 94 °C (for 35 cycles), and a final extension for 4 min at 72 °C. The products were detected by electrophoresis in a 1.5% agarose gel. Amplification of target DNA in exon 1 of CTLA-4 gene was carried out using PCR with primers 5'-GCTCTACTTCCTGAAGACCT-3', 5'-AGTC TCACTCACCTTTGCAG-3'. The reaction was performed in a final volume of 25 μl containing 0.5 μl genomic DNA, 0.3 μmol/L of each primer, 0.2 mmol/L dNTP, 1 U *Taq* polymerase with appropriate buffer. The following conditions were applied: initial denaturation for 3 min at 94 °C, following 30 cycles (30 s 94 °C, 30 s 59 °C, 30 s 72 °C) and a final extension for 4 min at 72 °C. The amplicon was 162 bp in length and was visualized on a 2% agarose gel.

**Fragment length polymorphism analysis**

PCR products were further subjected to restriction fragment length polymorphism (RFLP) analysis. The 247 bp products were digested with the restriction enzyme *Mse* I. PCR fragments with T at position –318 in promoter were cut into three fragments (21, 96 and 130 bp), whereas fragments with C at the same position only had the restriction site at 21 bp. The 21 bp fragment was not visible on the gel. The 162 bp products were digested with the restriction enzyme *Bbv* I. The G allele at position 49 in exon 1 corresponds to the presence of two fragments (88 and 74 bp), and the A allele corresponds to the 162 bp uncleaved fragments.

**Statistical analysis**

The gene frequencies and allele frequencies were determined by Hardy-Weinberg equilibrium. The statistical software SPSS 10.0 was used. The differences were made using $\chi^2$ test and logistic regression analysis; and odds ratios (OR) were calculated. $P<0.05$ was considered significant.

RESULTS

1. The distribution of genotypes CC, CT and TT among patients and control subjects is summarized in Table 1 and the distribution of the C and T alleles is shown in Table 2. The distribution of the C/T genotypes and alleles did not differ between GD group without GO and control group. The gene frequencies of CT and allele frequencies of T were much higher in GO group than those in GD group without GO ($P$=0.020, $P$=0.019).

2. The distribution of genotypes AA, AG and GG among patients and control subjects is summarized in Table 3 and the distribution of the A and G alleles is summarized in Table 4. The gene frequencies of GG and allele frequencies of G in patients with GD were significantly increased as compared with control group ($P$=0.008, $P$=0.007). No significant differences of A/G genotypes and alleles were found between the GD group without GO and the GO group.

3. Calculation of the delta value revealed linkage

disequilibrium between position −318 in promoter and position 49 in exon 1 of CTLA-4 gene. The promoter polymorphism was not linked to the exon 1 polymorphism (*Delta*=0.01439, *P*=0.097).

4. No difference was found between male and female in their distribution of the promoter and exon 1 genotypes in CTLA-4 gene (*P*=0.554, *P*=0.564).

5. Comparisons of the risk factors of GO including smoking and sex were made between the GO group and GD group without GO. The comparison of smoking between the two groups is shown in Tables 5 and 6. The proportion of smokers in GO group was higher than that in GD group without GO (*P*=0.018). The same analysis was done on male patients and significance was also found (*P*=0.016). The distribution of male and female in GO group was not similar to GD group without GO, but the difference was not significant (1:1.2 vs 1:2.0, *P*=0.280).

**Table 1 Distribution of the genotypes at position –318 in promoter of the CTLA-4 gene in patients and control subjects**

| Group | *n* | CC (%) | CT (%) | TT (%) | $\chi^2$ | *P* | *OR* |
|---|---|---|---|---|---|---|---|
| Control | 60 | 46 (76.7) | 12 (20.0) | 2 (3.3) | − | − | − |
| GD without GO | 56 | 47 (83.9) | 7 (12.5) | 2 (3.6) | 0.572* | 0.449 | 0.750 |
| GO | 33 | 18 (54.5) | 15 (45.5) | − | 5.434** | 0.020 | 2.761 |

* Compared to control group, 95% confidence interval=0.355~1.583; ** Compared to GD group without GO, 95% confidence interval=1.143~6.671

**Table 2 Frequencies of the alleles of the C/T polymorphism in promoter of the CTLA-4 gene in patients and control subjects**

| Group | *n* | C (%) | T (%) | $\chi^2$ | *P* | *OR* |
|---|---|---|---|---|---|---|
| Control | 120 | 104 (86.7) | 16 (13.3) | − | − | − |
| GD without GO | 112 | 101 (90.2) | 11 (9.8) | 0.695* | 0.405 | 0.708 |
| GO | 66 | 51 (77.3) | 15 (22.7) | 5.545** | 0.019 | 2.700 |

* Compared to control group, 95% confidence interval=0.313~1.599; ** Compared to GD group without GO, 95% confidence interval=1.157~6.303

**Table 3 Distribution of the genotypes at position 49 in exon 1 of the CTLA-4 gene in patients and control subjects**

| Group | *n* | AA (%) | AG (%) | GG (%) | $\chi^2$ | *P* | *OR* |
|---|---|---|---|---|---|---|---|
| Control | 60 | 7 (11.7) | 26 (43.3) | 27 (45.0) | − | − | − |
| GD without GO | 56 | 1 (1.8) | 18 (32.1) | 37 (66.1) | 7.097* | 0.008 | 2.340 |
| GO | 33 | 1 (3.0) | 11 (33.3) | 21 (63.6) | 0.100** | 0.752 | 0.877 |

* Compared to control group, 95% confidence interval=1.235~4.433; ** Compared to GD group without GO, 95% confidence interval=0.390~1.971

**Table 4 Frequencies of the alleles of the A/G polymorphism in exon 1 of the CTLA-4 gene in patients and control subjects**

| Group | *n* | A (%) | G (%) | $\chi^2$ | *P* | *OR* |
|---|---|---|---|---|---|---|
| Control | 120 | 40 (33.3) | 80 (66.7) | − | − | − |
| GD without GO | 112 | 20 (17.9) | 92 (82.1) | 7.237* | 0.007 | 2.300 |
| GO | 66 | 13 (19.7) | 53 (80.3) | 0.093** | 0.760 | 0.886 |

* Compared to control group, 95% confidence interval=1.244~4.253; ** Compared to GD group without GO, 95% confidence interval=0.408~1.925

**Table 5 Comparison of the proportion of smokers between GD group without GO and GO group**

| Group | non-smoker (%) | smoker (%) | $\chi^2$ | *P* | *OR* |
|---|---|---|---|---|---|
| GD without GO | 49 (87.5) | 7 (12.5) | − | − | − |
| GO | 22 (66.7) | 11 (33.3) | 5.586 | 0.018 | 3.500 |

95% confidence interval=1.197~10.233

**Table 6 Comparison of the proportion of smokers in male patients between GD group without GO and GO group**

| Group | non-smoker (%) | smoker (%) | $\chi^2$ | *P* | *OR* |
|---|---|---|---|---|---|
| GD without GO | 13 (68.4) | 6 (31.6) | – | – | – |
| GO | 4 (26.7) | 11 (73.3) | 5.846 | 0.016 | 5.958 |

95% confidence interval=1.402~25.316

## DISCUSSION

CTLA-4 is an important costimulatory molecule that participates in the interaction between T cells and antigen-presenting cells (APC). APC activate T cells by presenting to the T cell receptor an antigenic peptide bound to an HLA (human leukocyte antigen) class II protein on the cell surface. However, a second signal is also required for T cell activation. The binding of B7 to CD28 on T cells costimulates T cell activation, the presence of CTLA-4, which has a higher affinity for B7, down-regulates T cell activation by competing for the binding of B7 to CD28. The suppressive effects of CTLA-4 on T cell activation have raised the possibility that mutations altering CTLA-4 expression and/or function could result in an exaggerated T cell activation and lead to the development of autoimmunity. Three different polymorphisms are described in the CTLA-4 gene: one in the promoter region at position –318 from the ATG start codon consisting of a C/T change; a second in position 49 of exon 1, which lies in an A/G transition; and a third in the 3' untranslated region with variant lengths of a dinucleotide $(AT)_n$ repeat.

GD is an autoimmune disorder of multifactorial etiology with a polygenic mode of inheritance. GO is a subgroup of GD, but only 3%~5% of GD patients develop severe GO. One possible reason is that they are two different disorders with different genetic backgrounds. Recent reports have demonstrated that there is a linkage and association between the genetic markers of the CTLA-4 gene on chromosome 2q33 and GD. In order to confirm this association in Chinese, two polymorphisms of the CTLA-4 gene were analyzed by PCR-RFLP in a case-control study.

CTLA-4 –318 promoter polymorphism analysis showed no difference between GD patients and control subjects. The same results were found in the researches of Heward *et al*.(1998) and Hadj Kacem *et al*.(2001). When the allelic and genotypic frequencies were compared in GD patients, the frequencies of CT genotype and T allele were significantly higher in GO patients than in without GO patients (*P*=0.020, *OR*=2.761, 95% *CI*=1.143~6.671; *P*=0.019, *OR*=2.700, 95% *CI*=1.157~6.303). The allelic frequency of T in control group was 13.3%, which was similar to previous reports (Deichmann *et al*., 1996). The delta value between promoter and exon 1 was 0.01439 in our study (*P*=0.097), and suggested that the promoter polymorphism was not linked to the exon 1 polymorphism and that CTLA-4 promoter variants were independent genetic risk markers. But whether the –318 promoter polymorphism affects the transcriptional regulation of CTLA-4 remains to be clarified. Differing from our population, analysis of the CTLA-4 C/T polymorphism in Canadian and German showed an excess of the genotype CC in GD and found that the associations were based on linkage to the exon 1 (Braun *et al*., 1998). These differences could be explained by genetic heterogeneity of GD.

Our study showed that the gene frequencies of GG and allele frequencies of G in patients with GD without GO were significantly increased as compared with control group (*P*=0.008, *OR*=2.340, 95% *CI*=1.235~4.433; *P*=0.007, *OR*=2.300, 95% *CI*=1.244~4.253). As previously described, the A/G polymorphism was linked to GD. No significant differences of A/G genotypes and alleles were found between the GD group without GO and with GO group, which suggested that A/G polymorphism did not have a significant role in clinical expression of GO. The results were in accordance with the researches of Donner *et al*.(1997) and Allahabadia *et al*.(2001), but were different from others (Vaidya *et al*., 1999); this may be due to the different genetic background. The A/G substitution is not expected to affect the function of the leader peptide; on the contrary, it is in linkage disequilibrium with the CTLA-4 $(AT)_n$ repeat in the 3' untranslated region and could affect mRNA stability (Shaw and Kamen, 1986), down-regulation of T cell function, and subsequent development of disease. Equally, it may be in linkage disequilibrium with another, as yet unknown, disease-causing mutation.

Risk factors analysis revealed that cigarette smoking was a significant contributing factor to the development of GO. After the initial report by Hägg

and Asplund (1987), several reports documented a close association between cigarette smoking and GD. Furthermore, among patients with GO, smokers tended to have more severe ocular involvement than non-smokers (Prummel and Wiersinga, 1993). In addition, cigarette smoking has been documented to have a negative influence on the effectiveness of orbital radiotherapy and high-dose systemic glucocorticoids (Bartalena *et al*., 1998). Our research showed that the proportion of smokers in GO group was higher than that in GD group without GO ($P$=0.018, $OR$=3.500, 95% $CI$=1.197~10.233); with the same result being found in male patients ($P$=0.016, $OR$=5.958, 95% $CI$=1.402~25.316). The mechanisms whereby cigarette smoking affects the GO remain to be clarified. In addition to direct irritant effects, smoking may enhance the formation of superoxide radicals by causing hypoxia in the retrobulbar space (Burch *et al*., 1997). Smoking may enhance IL-1 secretion and activity, which has pro-inflammatory and fibrogenic effects (Metcalfe and Weetman, 1994). It has also been found that antibodies to heat shock protein 72 are present in smokers (Prummel *et al*., 1997). In summary, cigarette smoking appears to be a definite risk factor for the occurrence of GO.

In conclusion, our findings showed that the T allele at position –318 of CTLA-4 gene is linked to GO in Chinese, while the G allele at position 49 is associated with GD. These results indicate that CTLA-4 may play an important role in regulating self-tolerance by the immune system and hence in the pathogenesis of autoimmune disorders. However, further genetic and functional immunological studies are needed to discover the true identity and mechanism of action of the etiological mutation at this locus.

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