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The Predictive Value of CTLA-4 and Tg Polymorphisms in the Recurrence of Graves' Disease after Antithyroid Withdrawal

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Graves' disease (GD) is a multifactorial disease that develops as a result of complex interactions between genetic and environmental factors. The aim of our study is to determine the frequency of cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) A/G and TG C/T exon 33 SNPs (Tg E33SNP) in GD and to evaluate the relation between recurrence and these polymorphisms. A total of 187 subjects, including 97 previously treated GD patients and 90 age and gender matched control subjects were studied. We examined the relationship between the A/G and C/T polymorphism and various clinical and laboratory variables among patients with GD. TT genotype frequency in the GD patients was significantly higher than the controls. Number of recurrent patients was significantly higher in AG and GG carriers in comparison to AA carriers (57% and 45% vs 14%, $p = 0.0001$). CTLA-4 AG genotype had an eightfold (OR: 8.050; 95% CI: 2.87–22.5; $p = 0.0001$) and GG genotype had a sevenfold (OR: 7.025; 95% CI: 1.67–29.4; $p = 0.007$) increase in the risk of recurrence in the patients with GD. In conclusion, early interpretation for definitive treatment procedures (i.e., radioactive iodine or surgery) may be considered in the patients with G allele and E33SNP of Tg gene is conformed the susceptibility to GD in a Turkish population and having TT genotype increases the susceptibility to GD.

Key Words: Graves' disease; CTLA-4; Tg E33SNP; polymorphisms.

Introduction

Graves' disease (GD) is an autoimmune disease; it is characterized by hyperthyroidism, diffuse goiter, Graves' ophthalmopathy, and the presence of thyroid-stimulating hormone (TSH) receptor autoantibodies. GD is a multifactorial disease that develops as a result of complex interaction between genetic and environmental factors. Multiple genetic factors are thought to contribute the distinct autoimmunity seen in GD, but a particular genetic factor has not been established *(1)*.

The cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) gene is located on chromosome 2q33 and consists of four exons. The CTLA-4 receptor protein is expressed on activated T lymphocytes and counteracts their stimulation by the CD28 molecule. A product of the CTLA-4 gene provides a negative signal to the T cell, thereby restricting immune response *(2)*. In addition, CTLA-4 confers protection against autoimmunity by mediating the antigen-specific apoptosis of T cells and prevents autoreactive proliferation of T lymphocytes *(3)*. Previous studies strongly suggest that molecular variants of CTLA-4 are involved in T-cell–mediated autoimmune and inflammatory diseases *(4–10)*. One of these variants is expressed due to A→G substitution at position 49 of exon 1 of CTLA-4 gene (CTLA-4 A/G polymorphism) and has a significant association with some autoimmune endocrine diseases like GD, hypothyroidism, and type 1 diabetes mellitus *(4,10,11)*. Some cohort studies in different populations and in vitro studies suggest that the presence of polymorphic G allele has been especially associated with GD *(11–15)*.

More recently, the 8q24 locus, which contains the thyroglobulin (Tg) gene, has been shown to be strongly linked with autoimmune thyroid disease *(6)*. All 48 exons of the Tg gene have been sequenced and 14 single-nucleotide polymorphisms (SNPs) have been identified. It has been shown that among these SNPs the exon 10–12 cluster and an exon 33 SNP are significantly associated with autoimmune thyroid disease *(6)*. However, studies of different populations gave conflicting results *(6–8)*.

The aim of our study is to determine the frequency of CTLA-4 A/G and TG C/T exon 33 SNPs (Tg E33SNP) in GD and to evaluate the relation between recurrence and these polymorphisms.

Results

The frequencies of CTLA-4 A/G genotypes for controls and patients are shown in Table 1. AA, AG, and GG geno-

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Table 1
Genotype and Allele Distribution of CTLA-4 A/G
in the Patients with Graves' Disease and Control Subjects

Genotype and allelic distribution of CTLA-4	Graves disease (*n*)	%	Control subjects (*n*)	%	OR	95% CI*
AA	48	49.5	42	46.67	1.12	0.630–1.898
AG	38	39.2	34	37.78	1.06	0.588–1.913
GG	11	11.3	14	15.56	0.69	0.297–1.621
A allele	134	69.07	118	65.56	1.17	0.761–1.809
G allele	60	30.93	62	34.44	0.85	0.552–1.314

*$p > 0.05$.

Table 2
Genotype and Allele Distribution of Tg E33SNP
in the Patients with Graves' Disease and Control Subjects

Genotype and allelic distribution of TG	Graves disease (*n*)	%	Control subjects (*n*)	%	OR	95% CI*
CC	40	41.24	38	42.22	0.96	0.536–1.718
CT	38	39.18	44	48.89	0.67	0.376–1.203
TT**	19	19.59	8	8.89	2.49	1.033–6.035
C allele	118	60.82	120	66.67	0.79	0.521–1.214
T allele	76	39.18	60	33.33	1.256	0.823–1.917

*$p > 0.05$ for all except TT genotype.
**$p = 0.01$.

type frequencies in GD patients were 49%, 39%, and 11%, respectively. The frequencies observed for the controls were not different from the GD patients.

The frequencies of TG C/T genotypes for controls and patients are shown in Table 2. TT genotype frequency in the GD patients was significantly higher than the controls (19% vs 8%; $p = 0.01$).

The distribution of the genotypes was compatible with the Hardy–Weinberg equilibrium in both the patients and the controls. It was $\chi^2 = 0.66$, $p > 0.05$ for CTLA-4 A/G and $\chi^2 = 3.07$, $p > 0.05$ for TG C/T in the GD patients, and $\chi^2 = 2.40$, $p > 0.05$ for CTLA-4 A/G and $\chi^2 = 0.90$, $p > 0.05$ for TG C/T in the controls.

In the patients with GD, age, gender, goiter size, T3, TSH, anti-TPO, anti-Tg concentrations, presence of ophthalmopathy, type of ATD received, duration of antithyroid drug treatment, duration of hyperthyroidic symptoms before treatment, and number of patients relapsed after 1 yr follow-up showed no difference with respect to TG C/T gene polymorphism. Serum T4 concentration was significantly lower in CC genotype carriers than the TT and CC carriers ($p = 0.03$) (Table 3).

For CTLA-4 A/G gene polymorphism no difference was observed with respect to age, goiter size, T3, T4, TSH, anti-TPO, anti-Tg concentrations, presence of ophthalmopathy, type of ATD received, duration of antithyroid drug treatment, duration of hyperthyroidic symptoms before treatment in patients with GD. Female AA carrier patients were significantly higher than the AG and GG carriers ($p = 0.05$). No significant differences were observed between female and male patients with respect to clinical and hormonal parameters (data not shown). Number of the recurrent patients was significantly higher in AG and GG carriers in comparison to AA carriers (57% and 45% vs 14%, $p = 0.0001$) (Table 4).

Patients with recurrence were also evaluated for their family history, rate of grade 3 goiter, or the choice of ATD (either PTU or MMI). We did not find any significant difference between recurrent and non-recurrent patients for family history and rate of grade 3 goiter (17.1% and 8.5% vs 16.1% and 8%). When we compare the patients with recurrence with the patient without recurrence , there were no significant differences between the PTU or MMI usage (57% and 43% vs 51% and 49%).

The effects of independent parameters on the recurrence were also determined in a logistic regression analysis model. For this model age, gender, goiter size, choice of ATD, serum TSH, T4, T3, thyroid antibody concentrations, ophthalmopathy, duration of ATD, duration of hyperthyroidic symptoms before treatment, CTLA-4 A/G and TG C/T genotypes were used as independent variables and recurrence as the dependent variable. CTLA-4 AG genotype had an eightfold (OR: 8.050; 95% CI: 2.87–22.5; $p = 0.0001$) and GG genotype had a sevenfold (OR: 7.025; 95% CI: 1.67–29.4;

Table 3
Comparison of Clinical and Laboratory Parameters
of Patients with Graves' Disease with Respect to Tg E33SNP Genotype

Genotype	CC	CT	TT
Age (yr)	36.60 ± 10.83	36.60 ± 11.73	41.05 ± 8.38
Gender F/M	32/8	26/12	14/5
T3 (ng/mL)	2.37 ± 0.84	2.01 ± 0.63	2.19 ± 0.68
T4 (μg/dL)*	9.10 ± 2.10	10.36 ± 2.52	10.25 ± 2.43
TSH (mIU/L)	0.98 ± 1.00	1.08 ± 1.40	0.97 ± 1.61
anti-TPO (IU/mL)	195.63 ± 103	212.54 ± 110	235.52 ± 109
Anti-Tg (IU/mL)	155.97 ± 75	131.65 ± 64	144.11 ± 72
Goiter size 1 (*n* = 51)	22/40 (55%)	19/38 (50%)	10/19 (52.6%)
Goiter size 2 (*n* = 38)	15/40 (37.5%)	15/38 (39.4%)	8/19 (42.1%)
Goiter size 3 (*n* = 8)	3/40 (7.5%)	4/38 (10.5%)	1/19 (5.2%)
Opthalmopathy (*n*)	13/40 (32.5%)	14/38 (35.0%)	4/19 (21.0%)
PTU treatment (*n* = 52)	24/40 (60%)	20/38 (52.6%)	8/19 (42.1%)
MMI treatment (*n* = 45)	16/40 (40%)	18/38 (47.3%)	11/19 (57.8%)
Duration of ATD 9 treatment (mo)	15.85 ± 10	19.65 ± 13	16.47 ± 11
Duration of hyperthyroidic symptoms (mo)	22.15 ± 14	21.81 ± 11	19.73 ± 10
Patients with recurrence (*n*)	14/40 (32.5%)	13/38 (34.2%)	8/19 (42.1%)

*$p = 0.03$.

Table 4
Comparison of Clinical and Laboratory Parameters
of Patients with Graves' Disease with Respect to CTLA-4A/G Genotype

Genotype	AA	AG	GG
Age (yr)*	39.00 ± 10.74	38.28 ± 11.99	38.90 ± 6.97
Gender F/M	36/12	31/7	5/6
T3 (ng/mL)	2.21 ± 0.79	2.11 ± 0.69	2.39 ± 0.69
T4 (μg/dL)	9.72 ± 2.58	9.46 ± 2.21	11.11 ± 1.92
TSH (mIU/L)	1.01 ± 1.31	1.14 ± 1.36	0.46 ± 0.68
anti-TPO (IU/mL)	212.45 ± 108	205.55 ± 108	212.62 ± 106
Anti-Tg (IU/mL)	145.93 ± 73	155.34 ± 71	166.54 ± 85
Goiter size 1 (*n* = 51)	28/48 (58.3%)	19/38 (50%)	4/11 (36.6%)
Goiter size 2 (*n* = 38)	17/48 (35.4%)	15/38 (39.4%)	6/11 (54.5%)
Goiter size 3 (*n* = 8)	3/48 (6.2%)	4/38 (10.5%)	1/11 (9%)
Opthalmopathy (*n*)	17/48 (35.4%)	9/38 (23.7%)	5/11 (45.4%)
PTU treatment (*n* = 52)	26/48 (54.1%)	20/38 (52.6%)	6/11 (54.5%)
MMI treatment (*n* = 45)	22/48 (45.8%)	18/38 (47.3%)	5/11 (45.5%)
Duration of ATD treatment (mo)	16.72 ± 13	18.65 ± 11	16.54 ± 9.2
Duration of hyperthyroidic symptoms (mo)	24.22 ± 13	20.19 ± 19	22.82 ± 13
Patients with recurrence (*n*)**	7/48 (14.65)	22/38 (57.9%)	6/11 (45.5%)

*$p = 0.05$.
**$p = 0.0001$.

$p = 0.007$) increase in the risk of recurrence in the patients with GD (Table 5). We did not find any relation in these parameters for TG C/T genotypes.

Discussion

In a study Donner et al. *(16)* showed that in CTLA-4 A/G polymorphism the frequency of GG and AG genotypes are significantly high in patients with GD in comparison to controls. In this study they also showed that the patients with and without recurrence or ophthalmopathy had similar CTLA-4 allele distribution. Recently in a Turkish population, it has been shown that the frequency of GG genotype is significantly higher in GD patients and it is associated with increased recurrence rates *(5)*. In this study the frequencies of GG genotype were 19.5 % in Turkish patients and 7.1 % in controls. In our study, the frequencies of GG genotype were 11.3 % in the patients and 15.3 % in the controls, showing no significant difference. The results of these two studies both carried in Turkey are not compatible. Because our study group consisted of previously treated GD patients, the difference may be due to the patients enrolled to the study. In our study, AG, AA, and GG genotypes of the patients with

Table 5
Results of Multiple Logistic Regression Analysis for Recurrent Hyperthyroidism

Variable	B	S.E.	df	OR	95 %CI	*p*
AG	2.09	0.52	1	8.050	2.879–22.50	0.0001
GG	1.95	0.73	1	7.025	1.678–29.41	0.0076
Constant	−1.77	0.41	1	0.171		0.0000

OR, odds ratio; CI, confidence interval.

Independent variables: age, sex, goiter size, serum TSH, T4, T3, and thyroid antibody concentrations, ophtalmopathy, type of ATD received, duration of ATD treatment, duration of hyperthyroidic symptoms before treatment, AA, AG, GG and CC, CT, TT genotypes. Dependent variable: recurrent hyperthyroidism.

GD also showed no significant difference with respect to the studied parameters; however, AG and GG genotypes exhibited an increased risk of recurrence in the multivariate analysis model.

The results of Heward et al.'s study demonstrate *(7)* higher GG genotype in patients with GD in comparison to controls, and they also showed that patients with GG genotype have the highest serum FT4 concentrations. As a result they suggested that there is a strong relation between G allele and GD, and GG genotype is associated with more severe disease at presentation. In our study we neither showed higher GG genotype in patients with GD in comparison to controls nor a significant relation between G allele and recurrence of the disease.

On the other hand, Ban et al. *(19)* reported an association between E33SNP of Tg gene and increased susceptibility to autoimmune thyroid disease. They suggest that the substitution of a hydrophobic amino acid (tryptophane) to a hydrophilic amino acid (arginine) due to Tg C/T polymorphism changes the structure of Tg at that region, and this may cause increased susceptibility to autoimmune thyroid disease. However, the results of Collins et al.'s study *(17)* was not compatible with Ban et al.'s *(19)*. Collins et al. *(17)* showed no significant differences in allele frequencies of the four Tg SNPs between autoimmune thyroid disease patients and controls. The results of our study are compatible with Ban et al.'s. In our study the frequency of TT genotype was significantly high in patients with GD in comparison to controls. In addition, patients with CT and TT genotypes had higher T4 concentrations even if they were in a euthyroid state in comparison with patients with CC genotype.

In addition to these conflicting results, Gough *(18)* has a more discreet approach for the subject. Gough reported that the inability to replicate apparent association results is due to inadequately sized datasets, sampling bias, and the misclassification of phenotypes, and more studies are required before the Tg gene can be confirmed as the third genetic locus for autoimmune thyroid disease. Our study might provide for more information about the Tg gene polymorphism. In our study patients selection and classification of phenotypes are appropriate.

In conclusion, our findings show that (1) the CTLA-4 A/G polymorphism is not related with susceptibility to GD but is associated with recurrence of the disease in a Turkish population with GD. Therefore, early interpretation for definitive treatment procedures (i.e., radioactive iodine or surgery) may be considered in the patients with G allele. (2) E33SNP of Tg gene is conformed the susceptibility to GD in a Turkish population and having TT genotype increases the susceptibility to GD.

Patients and Method

Patients

A total of 187 subjects, including 97 previously treated GD patients (25 male, 72 female, aged 17–65 yr, mean 38.7 ± 10) and 90 age and gender matched control subjects (28 male, 62 female, aged 19–70 yr, mean 41.3 ± 9), were studied. No predominance of any ethnic groups was observed in our patients. None of the controls had personal or family history of thyroid disease and goiter on examination (goiter size was classified according to WHO); they had normal thyroid functions and were negative for thyroid autoantibodies. Sixteen persons (17.7%) of the control subjects were smokers. GD was diagnosed on the basis of clinical and laboratory evidence of thyrotoxicosis, palpable diffuse goiter, diffuse thyroidal uptake on radioactive scan. In addition, patients had at least one of the following findings: ophthalmopathy (class 2–6 according to the NOSPECS classification), positive antithyroid peroxidase (antiTPO), and/or antithyroglobulin (antiTg) antibodies. Of 97 GD patients, 16 (16.9%) had familial history of autoimmune thyroid disease and 18 (18.5%) were smokers. Of 97 GD patients, 37 patients have been treated surgically. The indications for surgical treatment were severe ophthalmopathy ($n = 18$), large goiter ($n = 8$), suspicion of malignancy ($n = 6$), and recurrence after antithyroid drug (ATD) treatment ($n = 5$). Thirty one of 37 patients received ATD prior to surgery for 12–18 mo and 6 patients with suspicion of malignancy received ATD for 3–5 mo. Sixty patients with small goiter and no ophthalmopathy were suitable for nonsurgical treatment. Of 60 patients, 45 received ATD treatment and 15 RAI. There were 23 and 7 recurrences in patients treated with ATD and RAI, respectively. In the study group patients, there were a total of 35 patients with recurrent GD.

Patients with Graves' disease were treated with an initial dosage of propranolol (40–60 mg/d), PTU (300–400 mg/d) or MMI (10–30 mg/d), which were reduced gradually to maintain euthyroidism as serum thyroid hormone concentrations declined. Graves' patients remained on this treatment for a minimum of 6 mo and were followed in our clinic for at least 1 yr after cessation of the treatment. Relapse was confirmed by clinical presentation and laboratory data. Recurrence was diagnosed when serum T4 and/or T3 levels

exceeded the upper limit of the normal range of our laboratory. The mean duration of antithyroid treatment was 17.46 mo (3–44 mo). The duration of hyperthyroidic symptoms before antithyroid treatment started ranged from 4 to 72 mo for the patients (mean ± SD, 21.54 ± 12 mo). All patients were euthyroid at the time of evaluation for genetic polymorphism.

Thyroid hormone and thyroid antibody concentrations were determined by chemiluminescent assay using DPP Modular System, Roche Diagnostics F. Hoffmann-La Roche Ltd, Basel, Switzerland. Normal ranges of the measured parameters as follows: triiodothyronine (T3): 0.8–2 ng/mL; thyroxine (T4): 5–12 μg/dL; free thyroxine (fT4): 10–25 pmol/L; thyrotropin (TSH): 0–4.2 mIU/L; anti-TPO: 0–35 IU/mL; and antiTg (antiTg 20–300 IU/mL). The study plan was reviewed and approved by our institutional ethical committee, and informed consent was obtained for all patients and control subjects.

Genotyping

Genomic DNA was isolated from peripheral blood by High Pure PCR Template Preparation Kit (Roche Applied Science, Roche Diagnostics GmbH, Mannheim, Germany). For the determination of CTLA-4 polymorphism, 162 bp fragments of CTLA-4 gene were amplified by polymerase chain reaction (PCR). The oligonucleotide primers used for amplification were: forward 5'-AGTCTCACTCACCTTT GCAG-3' and reverse 5'-GCTCTACTTCCTGAAGAC CT-3'. For amplifications, about 200 ng of genomic DNA was used in a final volume of 50 μL containing 1 μL of each 40 pmol primer, 200 μ*M* of each dNTP, 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 8.0, 50 m*M* KCl, and 0.5 unit of *Taq* DNA polymerase. The PCR conditions were: denaturation at 94°C for 4 min one cycle, followed by 30 cycles (94°C for 45 s, 57°C for 45 s, and 72°C for 45 s) and finally by an extension at 72°C for 10 min. The PCR product was digested with Bbv I (New England BioLabs, UK); separated by gel electrophoresis on a 12% polyacrylamide, and visualized by silver staining. The resulting fragments were 88 and 74 bp for the G allele (presence of the restriction site) and 162 bp for the A allele (absence of the restriction site) *(12)*.

For the determination of E33SNP of Tg gene, 375 bp fragments of Tg gene was amplified by PCR. The oligonucleotide primers used for amplification were: forward 5'-ATTAGCCAGTTG CCCTCTCC-3' and reverse 5'-ATAT TGACCAAAGCACCCCC-3'. For amplifications, about 200 ng of genomic DNA was used in a final volume of 50 μL containing 1 μL of each 40 pmol primer, 200 μ*M* of each dNTP, 1.5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 8.0, 50 m*M* KCl, and 1 unit of *Taq* DNA polymerase. The PCR conditions were: denaturation at 94°C for 4 min one cycle, followed by 30 cycles (94°C for 30 s, 55°C for 45 s, and 72°C for 45 s) and finally by an extension at 72°C for 10 min. The PCR product was digested with Hpy 99I (New England BioLabs, UK), separated by gel electrophoresis on a 3% agarose, and visualized by ethidium bromide. The resulting fragments were 208 and 167 bp for the C allele (presence of the restriction site) and 375 bp for the T allele (absence of the restriction site) *(19)*.

Statistical Analyses

Data were analyzed using SPSS 11.0 for Windows. Results were expressed as mean ± SD. The genotype and alleles of patients and controls were compared using the χ^2 or Fisher's exact test, odds ratios (OR) were calculated by Woolf's approximation method. Comparison of individual clinical and laboratory variables between genotypes were assessed with one-way ANOVA and Kruskall–Wallis one-way ANOVA, χ^2, or Fisher's exact test. To assess the strength of the association between hyperthyroidism recurrence and the clinical and laboratory variables, multivariate regression analysis was carried out. A p value less than 0.05 was considered statistically significant.

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