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# Remission of Graves' Hyperthyroidism and A/G Polymorphism at Position 49 in Exon 1 of Cytotoxic T Lymphocyte-Associated Molecule-4 Gene

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**We studied whether a patient with Graves' disease will go into remission during antithyroid drug (ATD) treatment. Remission of Graves' hyperthyroidism is predicted by a smooth decrease in TSH receptor antibody (TRAb) during ATD treatment. Cytotoxic T cell lymphocyte-associated molecule-4 (CTLA-4) may play an important role in the development of Graves' hyperthyroidism and in its remission. We studied A/G polymorphism at position 49 in exon 1 of the CTLA-4 gene in 144 Japanese Graves' patients. We intended to reveal the possible association of CTLA-4 gene polymorphism with the remission of Graves' hyperthyroidism. All patients with Graves' disease were treated with ATD. Thyroid-stimulating antibody and TSH binding inhibitory Ig were measured as TRAb. We analyzed CTLA-4 genotypes and alleles with PCR. We calculated the frequencies of CTLA-4 genotypes and alleles. A significant increase in the frequency of the G allele was seen in Graves' patients compared with controls ($P = 0.0095$). Graves' patients were divided into three groups (A, B, and C) according to time of TRAb disappearance after the start of ATD treatment. In group A patients TRAb had disappeared within 1 yr after the start of ATD treatment, in group B TRAb had disappeared between the beginning of the second year and the end of the fifth year of treatment, and in group C TRAb continued to be positive after 5 yr of ATD treatment. The frequencies of the GG genotype and the G allele were significantly higher in group C patients with persistently positive TRAb over 5 yr of ATD treatment than in the other groups ($P < 0.0001$). Group C patients did not have the AA genotype. The periods of time until remission were significantly shorter in the AA genotype. Graves' patients with the G allele need to continue ATD treatment for longer periods. (*J Clin Endocrinol Metab* 87: 2593–2596, 2002)**

GRAVES' DISEASE IS an organ-specific autoimmune disorder associated with T lymphocyte abnormality (1, 2). Graves' disease is a multifactorial disease that develops as the result of a complex interaction between genetic susceptibility genes and environmental factors (3). Human leukocyte antigen (4), GD-1, GD-2, GD-3 (4, 5), and cytotoxic T cell lymphocyte-associated molecule-4 (CTLA-4) (6–12) are susceptibility gene candidates. CTLA-4 plays an important role in the development of Graves' hyperthyroidism (8, 9, 11, 12). CTLA-4 gene polymorphisms play a role in the development of autoimmune thyroid diseases (8, 10–12). The activity of T cells requires a costimulatory signal mediated by CD28/B7 interaction (13). The CTLA-4 gene product delivers a negative signal to T cells and mediates apoptosis. This CTLA-4 gene product is a T cell surface molecule that binds to the B7 molecule on the antigen-presenting cells. The expression of CTLA-4 on T cells may affect the course of an ongoing immune process (14–16). TSH-receptor antibody (TRAb) causes Graves' hyperthyroidism. TRAb has been measured as thyroid-stimulating antibody (TSAb) and TSH binding inhibitory immunoglobulin (TBII). TRAb has been used to diagnose Graves' disease and to follow Graves' patients.

We questioned whether a patient with Graves' disease will go into remission during antithyroid drug (ATD) treatment. A parameter to detect the remission of Graves' disease has been sought for years (17–19). Remission of Graves' hyperthyroidism is predicted by a smooth decrease in TRAb during ATD treatment (20). However, the association of CTLA-4 gene polymorphism with the remission of Graves' hyperthyroidism has not been studied.

We studied A/G polymorphism at position 49 in exon 1 of CTLA-4 gene in Japanese patients with Graves' hyperthyroidism. This report supports the hypothesis that the A/G polymorphism at position 49 in exon 1 of the CTLA-4 gene is associated with the remission of Graves' hyperthyroidism in ATD-treated patients.

Abbreviations: ATD, Antithyroid drug; CTLA-4, cytotoxic T cell lymphocyte-associated molecule-4; TBII, TSH binding inhibitory Ig; TRAb, TSH receptor antibody; TSAb, thyroid-stimulating antibody.

## Subjects and Methods

### *Subjects*

We studied 144 patients with Graves' disease (119 females and 25 males, 15–75 yr old) and 110 healthy controls (78 females and 32 males, 19–59 yr old). Hyperthyroidism due to Graves' disease was diagnosed on the basis of history and signs of hyperthyroidism with diffuse goiter and the laboratory findings, including elevated serum $T_4$ and $T_3$ concentrations, undetectable serum TSH, and positive TRAb. TRAb was measured as TBII or TSAb. The patients were treated with methimazole (or propylthiouracil) at an initial dose of 20–30 (or 200–300) mg/d, which was reduced gradually as serum thyroid hormone concentrations declined. Thereafter, they received the minimum doses of ATD to maintain the normal serum $T_4$, $T_3$, and TSH levels. Patients were seen every 2–4 wk until their serum $T_4$ and $T_3$ concentrations had become normal. They were then followed every 4 wk. TBII and TSAb were measured every 1–3 months. ATD was given for at least 2 yr in each case and was discontinued 4–6 months after the disappearance of TRAb (TSAb and TBII). Then the patients were seen every 1–3 months. If the patients

continued to be in a euthyroid state and to have negative TSAb and negative TBII for more than 1 yr after ATD discontinuation, they were considered to be in remission.

Graves' patients were divided into three groups (A, B, and C) according to TRAb disappearance time after the start of ATD treatment; in 59 group A patients, TRAb (TSAb and TBII) disappeared within 1 yr after the start of ATD treatment; in 64 group B patients, TRAb disappeared between the beginning of the second and the end of the fifth year after the start of treatment; and, in 21 group C patients, TRAb continued to be positive after 5 yr of ATD treatment.

We studied the frequencies of AA, AG, and GG genotypes and A and G alleles of the A/G polymorphism in exon 1 and analyzed whether the CTLA-4 exon 1 polymorphism was associated with the remission of Graves' hyperthyroidism. Healthy control subjects had no clinical evidence of autoimmune thyroid disease or other autoimmune disorders.

The study plan was reviewed and approved by our institutional review committee, and informed consent was given by all patients and control subjects. All subjects in this study were Japanese.

### *Laboratory analysis*

Serum concentrations of $T_4$, $T_3$, and TSH were determined by RIA using commercially available kits. The normal ranges for serum $T_4$ and $T_3$ were 77–155 nmol/liter (6–12 $\mu$g/dl) and 1.2–2.8 nmol/liter (78–182 ng/dl), respectively. The normal range for serum TSH was 0.3–4.0 mU/liter.

TSAb was determined as described previously (14), with minor modification (21), using porcine thyroid cells. The cells were incubated with crude IgG in Hanks' solution without NaCl, pH 7.5, containing 1.5% BSA, 20 mM HEPES, and 0.5 mM 3-isobutyl-1-methylxanthine. cAMP production during 2-h incubation with 3 mg IgG/300 $\mu$l was measured by RIA using a commercially available kit (Yamasa, Chiba, Japan). The activity was expressed as the percentage of cAMP production compared with the mean value for 20 normal controls. Crude IgG preparations purified with 12.5% polyethylene glycol 6000 were used for TSAb. Normal IgG was obtained from normal pooled serum. The cut-off value for TSAb was 180%.

TBII was measured by RRA with a commercially available kit (R.S.R. Ltd., Cardiff, UK). Assay results were expressed as the percent inhibition of [$^{125}$I]TSH binding to thyroid plasma membranes as previously reported (7). The cut-off value for TBII was 10%.

### *Genotype*

Genomic DNA was prepared from peripheral white blood cells using the DNA purification kit (QIAGEN, Hilden, Germany). We analyzed CTLA-4 genotypes and allele with PCR. Analyses with PCR were performed in duplicate in each sample. PCR was performed with oligonucleotide primers (forward, 5′-GCTCTATTCCTGAAGACCT-3′; reverse, 5′-AGTCTCACTCACCTTTGCAG-3′) using premix *Taq* (Takara, Shiga, Japan). PCR was performed by initial denaturation for 4 min at 94 C, annealing for 45 sec at 57 C, extension for 45 sec at 72 C, denaturation for 45 sec at 94 C (for 35 cycles), and a final extension for 4 min at 72 C. The presence of G alleles was determined in each subject by PCR amplification of CTLA-4, followed by digestion with *Bbv*1, which acts on the G variation, but not on the A variation. If a G allele was at position 49, 88/74-bp fragments were obtained. PCR products were detected by electrophoresis in a 3% agarose gel.

### *Statistical analyses*

Statistical analyses of the differences between groups were made using $\chi^2$ test with Yate's correction, Fisher's exact probability test, or two-tailed unpaired *t* test. Either $3 \times 2$ or $3 \times 3$ contingency tables were used to analyze the allele or genotype, respectively. Two-tailed unpaired *t* test was used to compare the duration of ATD treatment until remission. *P* less than 0.05 was considered statistically significant.

## Results

### *Genotype frequencies at position 49 in exon 1 of CTLA-4 gene in Graves' patients*

We studied genotype frequencies at position 49 in exon 1 of the CTLA-4 gene in Graves' patients and normal control subjects (Table 1). The frequencies of AA, AG, and GG genotypes were significantly different between Graves' patients and controls; the frequency of GG was higher in Graves' patients than in controls, and that of AA was lower in the former than in the latter. A decrease in the AA genotype and an increase in the GG genotype were seen in Graves' patients. We calculated the frequencies of A and G alleles in Graves' patients and controls. A significant increase in the frequency of G allele was seen in Graves' patients compared with controls ($P = 0.0095$).

### *A/G polymorphism in exon 1 of the CTLA-4 gene and TRAb changes in Graves' patients during ATD treatment*

We followed the serial changes in TRAb (TSAb and/or TBII) in Graves' patients during ATD treatment. We studied the association of CTLA-4 exon 1 polymorphism with the patterns of TRAb changes (Table 2). Graves' patients were divided into three groups (A, B, and C) according to TRAb disappearance time after the start of ATD treatment. In 59 group A patients, TRAb had disappeared within 1 yr after the start of ATD treatment. In 64 group B patients, TRAb had disappeared between the beginning of the second year and the end of the fifth year after the start of treatment, and, in 21 group C patients, TRAb continued to be positive after 5 yr of ATD treatment. We determined the frequencies of genotype and allele at the position 49 A/G polymorphism in exon 1 of the CTLA-4 gene. The frequencies of GG genotype and G allele were significantly higher in the patients with persistently positive TRAb (group C) than in the others (groups A and B; $P < 0.0001$). Graves' patients who continued to have positive TRAb after 5 yr of ATD treatment did not have the AA genotype.

### *Remission of Graves' hyperthyroidism and A/G polymorphism at position 49 in exon 1 of the CTLA-4 gene*

We studied the association of A/G polymorphism at position 49 in exon 1 of CTLA-4 with the remission of Graves' hyperthyroidism (Table 3). All patients (n = 47) in remission had been in the euthyroid state. Mean serum concentrations of $T_4$, $T_3$, and TSH in remission were 103.0 ± 23.2 nmol/liter (8.0 ± 1.8 $\mu$g/dl), 1.3 ± 0.3 nmol/liter (84.0 ± 18.0 ng/dl), and 1.4 ± 0.9 mU/liter, respectively. We analyzed genotypes at position 49 in exon 1 of the CTLA-4 gene. We calculated the frequencies of CTLA-4 genotypes and alleles. The genotypes were associated with the durations of ATD treatment needed to achieve remission; the duration was significantly

**TABLE 1.** Frequencies of genotypes and alleles of A/G polymorphism at position 49 in exon 1 of CTLA-4 gene in Graves' patients and controls

| | Graves' disease (n = 144) | Controls (n = 110) | $\chi^2$ | *P* |
|---|---|---|---|---|
| Genotype | | | | |
| G/G | 50 (34.7) | 26 (23.6) | | |
| A/G | 62 (43.1) | 46 (41.8) | | |
| A/A | 32 (22.2) | 38 (34.6) | 6.0 | 0.0493 |
| Allele | | | | |
| G | 162 (56.3) | 98 (44.5) | | |
| A | 126 (43.7) | 122 (55.5) | 6.4 | 0.0095 |

Data are reported as number (%).

**TABLE 2.** CTLA-4 exon 1 polymorphism and TRAb disappearance

| Group | Genotypes | | | | Alleles | | |
|---|---|---|---|---|---|---|---|
| | G/G | A/G | A/A | $P^a$ | G | A | $P^b$ |
| Group A | 18 | 26 | 15 | | 62 | 56 | |
| Group B | 14 | 33 | 17 | | 61 | 67 | |
| Group C | 18 | 3 | 0 | <0.0001 | 39 | 3 | <0.0001 |

Data are reported as number. Group A, TRAb had disappeared within 1 yr after the start of ATD treatment; group B, TRAb had disappeared between the beginning of the second and the end of the fifth year after the start of ATD treatment; group C, TRAb continued to be positive after 5 yr of ATD treatment.

[a] Statistical analysis with 3 × 3 contingency tables.

[b] Statistical analysis with 3 × 2 contingency tables.

**TABLE 3.** CTLA-4 exon 1 polymorphism and duration of ATD treatment and remission rate

| Genotypes | Duration of ATD treatment yr (mean ± SD) | Remission rate (%) |
|---|---|---|
| G/G | 5.8 ± 3.6 | 32.3 |
| A/G | 5.0 ± 2.3 | 36.2 |
| A/A | 3.3 ± 1.1[a,b] | 30.9 |

Graves' patients were divided into three subgroups according to the genotypes.

[a] $P = 0.0414$, compared with G/G genotype Graves' patients.

[b] $P = 0.0496$, compared with A/G genotype Graves' patients.

shorter in the A/A genotype than in the G/G genotype ($P = 0.0414$) or the A/G genotype ($P = 0.0496$). Patients with G alleles in exon 1 of the CTLA-4 gene were required to take ATD for longer durations than the others to achieve remission. The remission rate did not differ among G/G, G/A, and A/A genotypes.

## Discussion

We studied the A/G polymorphism at position 49 in exon 1 of the CTLA-4 gene in Japanese patients with Graves' hyperthyroidism. The frequencies of the GG genotype and G allele were significantly higher in the patients with persistently positive TRAb than in the other two groups. Graves' patients who continued to have positive TRAb after 5 yr of ATD treatment did not have the AA genotype. Patients with the G allele in exon 1 of the CTLA-4 gene are required to continue ATD treatment for longer periods to achieve remission. This is the first report to suggest the association of the A/G polymorphism at position 49 in exon 1 of the CTLA-4 gene with the remission of Graves' hyperthyroidism.

G allele frequency has been reported to be high in Graves' patients (7, 8, 12). We demonstrated that Graves' patients had higher frequencies of G allele (GG genotype) and lower frequencies (or absence) of A allele (AA genotype) than the controls. Susceptibility to Graves' disease has significant genetic components. CTLA-4 gene polymorphisms have been reported to be associated with Graves' disease (6–9, 11). The CTLA-4 molecule is a member of the same family of cell surface molecule as CD28 and can bind to B7. The CTLA-4/B7 complex competes with the CD 28/B7 complex and delivers negative signals to the T cells, affecting T cell expansion, cytokine production, and immune responses (13, 14, 21). However, we do not know how CTLA-4 gene polymorphisms may contribute to the development of Graves' hyperthyroidism. Three polymorphic sites (A/G polymorphism in exon 1, C/T polymorphism in the promoter, and microsatellite repeat in the 3′-untranslated region of exon 4) in the CTLA-4 gene have been reported to be associated with autoimmune endocrine disorders (10–12, 22–25). Heward *et al.* (7) reported the relationship between the CTLA-4 genotype and the severity of thyroid dysfunction at diagnosis; free $T_4$ concentrations were highest in patients with the GG genotype and lowest in patients with the AA genotype. Graves' patients have more G alleles than the controls, suggesting that the CTLA-4 GG genotype might induce down-regulation of T cell activation.

Remission of Graves' hyperthyroidism is predicted by a smooth decrease in TRAb during ATD treatment (20). Graves' patients have high frequencies of the G allele (GG genotype) and lower frequencies (or absence) of the A allele (AA genotype). We studied the A/G polymorphism at position 49 in exon 1 of the CTLA-4 gene in Japanese patients with Graves' hyperthyroidism and demonstrated an association of CTLA-4 gene polymorphism with the remission of Graves' hyperthyroidism. We demonstrated an association of the CTLA-4 gene polymorphism with the remission of Graves' hyperthyroidism. The frequencies of GG genotype and G allele were higher in the Graves' patients. Group C patients, who continued to have persistently positive TRAb after over 5 yr of ATD treatment, did not have the AA genotype. The A/G polymorphism at position 49 in exon 1 of CTLA-4 gene is associated with the remission of Graves' hyperthyroidism. However, we do not know how CTLA-4 contributes to the remission of Graves' hyperthyroidism with ATD treatment. CTLA-4 signal induces down-regulation of T cell activation. If the function of CTLA-4 with the G alleles at position 49 in exon 1 was impaired, the Graves' patients with the impaired CTLA-4 function might have difficulty achieving remission.

The number of patients studied was small. We performed the power calculation using the method of Cohen *et al.* (26), assuming that the $\alpha$ level was set at 0.05 (two-sided). When the patients were divided into 3 subgroups, the power to check the association was weak [0.27 (group A *vs.* group B), 0.66 (group A *vs.* group C), and 0.69 (group B *vs.* group C); >280 patients were required to get the power values above 0.8]. Further studies will be required to determine a clear association of the CTLA-4 gene polymorphism with the remission of Graves' hyperthyroidism.

The frequencies of GG genotype and G allele were higher in Graves' patients. Group C patients with persistently positive TRAb over 5 yr of ATD treatment did not have the AA genotype. Graves' patients with the G allele in exon 1 of the CTLA-4 gene were required to continue ATD treatment for longer periods to achieve remission.

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