Association of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) gene polymorphism and non-genetic factors with Graves’ ophthalmopathy in European and Japanese populations

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Abstract

Objective: The development and severity of Graves’ ophthalmopathy (GO) may result from a complex interplay of genetic and environmental factors. The aim of this study was to investigate the association of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) gene polymorphism and non-genetic factors (age, sex, cigarette smoking) with GO in two different populations, Polish-Caucasians and Japanese.

Design: We investigated the distribution of CTLA-4 A49G polymorphism in 264 Caucasian patients with Graves’ disease (GD), of which 95 had clinically evident GO (NOSPECS class ≥ 3) and 319 Japanese patients with GD, of which 99 had ophthalmopathy. The control groups consisted of healthy Polish adults (n = 194), Polish centenarians (n = 51) and Japanese adults (n = 112).

Results: Allele G and G/G genotype were significantly increased in Caucasian patients with GD (48% and 25% respectively) and in Japanese patients with GD (69% and 47% respectively) compared with control groups. There were no significant differences in the G allele and G/G genotype frequencies in GO patients compared with GD patients without ophthalmopathy. Multiple logistic regression analysis demonstrated that cigarette smoking (P = 0.03, odds ratio (OR) = 1.7) and age of onset of GD over 42 years (P = 0.08, OR = 1.6) were contributing factors associated with susceptibility to GO in Polish patients. In Japanese patients, a younger age of onset of GD had an effect on the development of GO (P = 0.02, OR = 1.8).

Conclusions: (i) Allele G and G/G genotype confer genetic susceptibility to GD; (ii) CTLA-4 A49G polymorphism is not associated with the development of GO; (iii) different non-genetic factors may contribute to GO in different populations.

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Introduction

Graves’ disease (GD) is a heterogeneous autoimmune disorder affecting the thyroid, eyes and skin (1). While some degree of ocular involvement may be detected by sensitive imaging techniques (magnetic resonance or computed tomography) in almost all patients with Graves’ disease, clinically apparent ophthalmopathy occurs in only 30% of patients with GD (2). In its severe expression, it is a disfiguring and potentially sight threatening disorder. However, even mild to moderately severe ophthalmopathy profoundly influences and impairs the quality of life of affected individuals (3). The course of Graves’ ophthalmopathy (GO) is unpredictable and a sudden worsening of GO can occur at any time. As the treatment of GO is often unsatisfactory, there is a need to identify possible predisposing factors and establish diagnostic methods to identify GD patients at high risk of developing ophthalmopathy (4).

The etiology of GO is considered to be multifactorial. So far, cigarette smoking, advancing age, male sex and radioiodine treatment have been shown to be associated with the development and/or severity of GO (5–9). There is also an ethnic difference in the prevalence of ophthalmopathy in patients with GD, with Asians having a significantly lower risk of developing GO compared with Caucasians living in the same region (10). Recently, two studies reported an association between cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) gene polymorphism and the development of GO in UK and Italian populations (11, 12). These data pointed to the possibility of using genetic
screening to identify predisposing factors to GO (Table 1). In addition, a recent study suggested that CTLA-4 gene polymorphism may predict the remission rates of Graves' hyperthyroidism in Japanese patients treated with antithyroid drugs (13). However, several case-control studies in different populations (German, UK, North American) failed to confirm an association between CTLA-4 gene polymorphism and GO or the outcome of treatment for Graves' hyperthyroidism (14–17).

As the etiology of Graves' ophthalmopathy may be different in ethnic groups, additional studies may be required to determine the role of genetic and non-genetic factors in susceptibility to GO (18). In this study, we analyzed the association of CTLA-4 gene A/G polymorphism (Thr/Ala) at position 49 in exon 1 with GO in two different, so far not studied populations — Polish-Caucasian and Japanese. We further examined the relationship between non-genetic factors (cigarette smoking, age, sex) and the development of GO.

### Subjects and methods

#### Subjects

A total of 264 Polish-Caucasian patients with GD (204 women, 60 men), aged 13–78 (median 41) years and 319 Japanese patients with GD (246 women, 73 men), aged 13–78 (median 38) years were studied. The patients with GD were consecutive series of cases from the Department of Endocrinology, Medical University of Warsaw or the Department of Endocrinology, Centenarians Program carried out at the International Institute of Molecular and Cell Biology in Warsaw, Poland. These subjects had no personal or family history of thyroid or autoimmune disorders. In addition we studied 51 Polish subjects (41 females, 10 males) who were over 100 years old recruited from the Polish Centenarians Program carried out at the International Institute of Molecular and Cell Biology in Warsaw, Poland. These subjects had no personal or family history of thyroid disorders or autoimmune diseases, and/or increased radioiodine uptake.

#### Table 1 Association of CTLA-4 A49G gene polymorphism with Graves' ophthalmopathy.

<table>
<thead>
<tr>
<th>Author (ref.)</th>
<th>Subjects</th>
<th>Genotype (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Badenhoop et al. (14)</td>
<td>GO (n = 135)</td>
<td>AA: 24, AG: 76*, GG: 9</td>
<td>0.9</td>
</tr>
<tr>
<td>Vaidya et al. (11)</td>
<td>GH (n = 124)</td>
<td>AA: 24, AG: 76*</td>
<td></td>
</tr>
<tr>
<td>Vaidya et al. (11)</td>
<td>GO (n = 94)</td>
<td>AA: 22, AG: 53, GG: 24</td>
<td>0.012</td>
</tr>
<tr>
<td>Buzzetti et al. (12)</td>
<td>GH (n = 94)</td>
<td>AA: 40, AG: 43, GG: 17</td>
<td>0.27</td>
</tr>
<tr>
<td>Villanueva et al. (16)</td>
<td>GO (n = 85)</td>
<td>AA: 31, AG: 49, GG: 20</td>
<td>0.9</td>
</tr>
<tr>
<td>Allahabadia et al. (17)</td>
<td>GH (n = 323)</td>
<td>AA: 26, AG: 56, GG: 19</td>
<td>0.25</td>
</tr>
</tbody>
</table>

GO, Graves' ophthalmopathy; GH, Graves' hyperthyroidism without ophthalmopathy.

Submissions of AG and GG, **AA and AG.

Patients with GD were subdivided into 2 groups according to the presence of clinically evident ophthalmopathy (Table 2).

(i) **Graves' ophthalmopathy (GO)** This group consisted of 95 Polish-Caucasian and 99 Japanese patients. The severity of ophthalmopathy was assessed according to the NOSPECS classification (19). Patients with proptosis, extraocular-muscle dysfunction, exposure keratitis and optic neuropathy (NOSPECS class III and higher) were considered clinically evident. Patients were categorized according to their highest ever NOSPECS class (Caucasians: class III, 52 patients, class IV, 37 patients, class V, 2 patients, class VI, 4 patients; Japanese: class III, 76 patients, class IV, 17 patients, class V, 0 patients, class VI, 6 patients).

(ii) **Graves' hyperthyroidism without clinically evident ophthalmopathy (GH)** This group consisted of 169 Polish-Caucasian and 220 Japanese patients. The control populations comprised 194 randomly selected anonymous healthy Polish adults recruited from the Blood Transfusion Center and 112 unrelated healthy Japanese adults (74 women, 38 men) recruited from hospital staff at Kurume University School of Medicine, who had no personal or family history of thyroid or autoimmune disorders. In addition we studied 51 Polish subjects (41 females, 10 males) who were over 100 years old recruited from the Polish Centenarians Program carried out at the International Institute of Molecular and Cell Biology in Warsaw, Poland. These subjects had no personal or family history of thyroid disorders or autoimmune diseases, they were clinically and biochemically euthyroid and were negative for thyroid peroxidase antibodies.

The research program was approved by the local ethical committees and all subjects and parents of children gave written informed consent for genetic studies.

### CTLA-4 exon 1 polymorphism analysis

The CTLA-4 exon 1 position A49G dimorphism was genotyped by PCR-restriction fragment length polymorphism (RFLP) analysis, as described previously by Vaidya et al. (11). Genomic DNA extracted from peripheral blood mononuclear cells was subjected to PCR to amplify the polymorphic region in exon 1 using the following primers: 5’CCACGGCTTCTTTCTGTA3’ and 5’AGTCTCCTCACACTTGGCAG3’. PCR was performed in a 25 µl reaction mixture containing 200 ng genomic DNA, 0.3 U Taq polymerase (Life Technologies Ltd, UK), 5 pmol of each primer, 0.8 mMol/l dNTPs and 2 mMol/l MgCl2, under the following conditions: 5 min denaturation at 94 °C, followed by 35 cycles of 0.5 min at 94 °C, 0.5 min at 60 °C, 0.5 min at 72 °C and a final 7 min extension at 72 °C.

Subsequently, 2.5 µl amplified PCR products were digested in a volume of 10 µl with 0.5 U Bst7HI restriction

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enzyme (Promega Corporation, Madison, WI, USA), which cut the sequence if a G was present at position 49 resulting in 88 bp and 240 bp fragments. After incubation for 2 h at 50 °C, digested fragments were resolved on 2% agarose gel containing 10 mg/ml ethidium bromide, visualized by UV light, and compared with a 100 bp molecular size control ladder. To confirm the accuracy of the method employed, randomly selected patients were analyzed by direct sequencing. Control samples (patients with A/A, A/G and G/G genotype) were analyzed during each set of assays.

Statistical analysis

Allele and genotype frequencies were compared between groups using the Chi-square ($\chi^2$) test or Fisher’s exact probability test, where appropriate. A P value of <0.05 was considered significant. Odds ratios (OR) were calculated as a measurement of strength of association according to Woolf’s method (20). Multiple logistic regression analysis was applied in order to analyze the independent effect of genetic and environmental factors on the development of ophthalmopathy. The reference categories for the logistic regression analysis were CTLA-4 G/G genotype, cigarette smoking, and surgery.

Results

CTLA-4 exon 1 polymorphism in patients with Graves’ disease

The frequencies of alleles and genotypes of the CTLA-4 gene polymorphism are shown in Table 3. The distribution of CTLA-4 alleles differed significantly among Caucasian and Japanese control groups (genotype A/A: 40% vs 13%, $\chi^2$ test $P < 0.0001$; genotype G/G: 16% vs 30%, $P = 0.001$; G allele: 38% vs 58%, $P < 0.0001$).

Allele G was significantly increased in Caucasian patients with GD (48% vs 38%, $\chi^2$ test $P = 0.003$, OR = 1.5) and in Japanese patients with GD (69% vs 58%, $P = 0.003$, OR = 1.6), when compared with normal controls. There was also a significant increase in the G/G genotype in Polish patients with GD (25% vs 16%, $P = 0.03$, OR = 1.7) and in Japanese patients with GD (47% vs 30%, $P = 0.002$, OR = 2.1). We also compared the distribution of the CTLA-4 alleles between Caucasian patients with GD and healthy subjects. Values in parentheses are percentages of the group.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Genotype</th>
<th>Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/A</td>
<td>A/G</td>
</tr>
<tr>
<td>Caucasian</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GD total</td>
<td>264</td>
<td>75</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>194</td>
<td>77</td>
</tr>
<tr>
<td>Japanese</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GD total</td>
<td>319</td>
<td>28</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>112</td>
<td>15</td>
</tr>
</tbody>
</table>

GD total, patients with GD with or without ophthalmopathy. Odds ratio (OR) was calculated as compared with healthy subjects. *$P = 0.03$; $P = 0.003$; $P = 0.002$; $P = 0.003$ compared with healthy subjects ($\chi^2$ test).
Table 4 Genotype and allele frequencies of CTLA-4 A49G gene polymorphism in patients with Graves’ ophthalmopathy (GO) and patients with Graves’ hyperthyroidism without evident eye involvement (GH). Values in parentheses are percentages of the group.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Genotype</th>
<th>Allele</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A/A</td>
<td>A/G</td>
<td>G/G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>95</td>
<td>27 (28)</td>
<td>41 (43)</td>
<td>27 (28)*</td>
</tr>
<tr>
<td>GH</td>
<td>169</td>
<td>48 (28)</td>
<td>82 (49)</td>
<td>39 (23)</td>
</tr>
<tr>
<td>Japanese</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GO</td>
<td>99</td>
<td>12 (12)</td>
<td>41 (41)</td>
<td>46 (46)*</td>
</tr>
<tr>
<td>GH</td>
<td>220</td>
<td>16 (7)</td>
<td>99 (45)</td>
<td>105 (48)</td>
</tr>
</tbody>
</table>

Odds ratio (OR) was calculated as compared with patients with GH. aP = 0.3. bP = 0.6. cP = 0.8. dP = 0.4 compared with patients with GH (χ² test).

Association of CTLA-4 exon 1 polymorphism with Graves’ ophthalmopathy

In Caucasian patients with Graves’ ophthalmopathy, there was no significant increase in G allele (50% vs 47%, χ² test: P = 0.6, OR = 1.1) and G/G genotype (28% vs 23%, P = 0.3, OR = 1.3) compared with patients without ophthalmopathy (Table 4). There was also no correlation between the frequencies of G allele and the severity of GO: NOSPECS class I, 45%; class II, 55%; class III, 56%; class ≥ IV, 43%. In Japanese GO patients, frequencies of G allele (67% vs 70%, P = 0.4, OR = 0.9) and G/G homozygote (46% vs 48%, P = 0.8, OR = 1.0) were similar compared with patients without ophthalmopathy. There was also no association between G allele frequency and the severity of GO: NOSPECS class I, 70%; class II, 50%; class III, 66%; class ≥ IV, 69%.

Association of non-genetic factors with Graves’ ophthalmopathy

Subdividing patients with Graves’ disease according to clinical eye involvement revealed that in the Polish GO group there were significantly more cigarette smokers (χ² test: P = 0.03) and patients had an older age of onset of disease (Mann–Whitney U test: P = 0.01) (Table 2). In contrast, the number of cigarette smokers among Japanese patients with or without GO was similar. Japanese patients with GO had a younger age of onset of GD (P = 0.03) compared with patients without eye involvement. However, there were significantly more cases of severe GO (NOSPECS class ≥ IV) among patients over 32 years old compared with younger patients with GO. Polish patients without ophthalmopathy were usually treated by antithyroid drugs or radioactive iodine. In contrast, patients with GO were more frequently treated by surgery (χ² test, P = 0.002). Japanese patients with Graves’ disease were usually treated by antithyroid drugs.

We studied the independent effect of genetic factors and environmental factors on the development of GO by multiple logistic regression analysis. The reference categories for the logistic regression analysis were cigarette smoking, age of onset of GD and CTLA-4 G/G genotype (Table 5). In this model, cigarette smoking and age of onset of GD over 42 years had an effect on the development of GO in Polish patients. In Japanese patients, only age of onset of GD younger than 32 years had a significant effect on the development of GO.

Discussion

Graves’ ophthalmopathy is considered to be an autoimmune inflammatory disorder affecting the extraocular muscles and the orbital fatty/connective tissue (21).
T cells infiltrating the retroorbital tissues are likely to play an important role in the pathogenesis of GO (22). The CTLA-4 gene encodes a co-stimulatory molecule, which is expressed on activated T cells and may mediate T cell apoptosis. In vitro studies showed that CTLA-4 A49G dimorphism, resulting in an amino acid exchange (Thr/Ala) in the leading peptide, is associated with impaired function of this receptor (23). Thus CTLA-4 is a strong candidate gene for T cell-mediated autoimmune diseases.

In this population-based, case-control study, we analyzed the association of CTLA-4 A49G polymorphism with Graves’ disease in two different populations: Polish-Caucasian and Japanese. As reported previously, the distribution of CTLA-4 alleles differed significantly in the two populations studied with the G allele and G/G genotype being significantly higher and the A/A homozygote being significantly lower in Japanese subjects compared with Caucasian subjects (24). In both Caucasian and Japanese patients with GD, the frequency of G allele and G/G genotype were significantly higher compared with healthy subjects. In Caucasians, we additionally compared the frequencies of CTLA-4 alleles with subjects over 100 years old with no history of autoimmune diseases, confirming that G allele and G/G genotype are associated with susceptibility to Graves’ disease (24–27). The relative risks for developing Graves’ disease in Caucasians and Japanese conferred by G allele (1.5 and 1.6 respectively) or by G/G genotype (1.7 and 2.1 respectively) were similar to the values found in previous studies.

While the genetic predisposition to the development of Graves’ disease is well established by twin studies and whole genome linkage analysis (18, 27–29), the significance of genetic factors in susceptibility to GO remains controversial. There is some evidence for genetic susceptibility to GO: HLA alleles, polymorphisms of the CTLA-4 gene, tumor necrosis factor-α promoter and interferon-γ gene have been shown to be associated with GO (11, 12, 30–32). On the other hand, other studies were unable to confirm an association between immunoregulatory genes polymorphisms and ophthalmopathy (14–16, 33, 34). In addition, the segregation ratio for severe ophthalmopathy in families with Graves’ disease was reported to be zero, suggesting that environmental factors rather than genetic factors predispose to the development of GO (16). Our results are in accordance with reports showing that CTLA-4 gene polymorphism does not contribute to the susceptibility of clinically evident GO. Furthermore, there was no correlation between the severity of GO and the frequency of the G allele. It seems therefore unlikely that CTLA-4 A49G polymorphism analysis would be helpful in predicting the development or severity of ophthalmopathy in Caucasian and Japanese patients with Graves’ disease.

Several environmental and demographic factors, like cigarette smoking, male sex, advanced age and radioactive iodine treatment, have been directly associated with the development and/or severity of GO (5–9). In the Polish GO group, there were significantly more cigarette smokers and patients had an older age of onset of GD compared with patients with Graves’ hyperthyroidism without evident eye involvement. Sex did not affect likelihood of development of GO. In contrast, Japanese patients with GO did not differ significantly from patients without evident eye involvement in respect to smoking habits. Japanese patients with GO had a younger age of onset of GD compared with patients without eye involvement. However, the severity of GO correlated with advanced age. To compare the influence of genetic and environmental factors, we performed a logistic multiple regression analysis using the following reference categories: cigarette smoking, age of onset of GD, and CTLA-4 G/G genotype. Our model demonstrated that in Caucasians cigarette smoking and age of onset of GD over 42 years increased the risk of developing GO. In Japanese patients, only age of onset of GD younger than 32 years had an effect on the development of GO. Our results are in accordance with the notion that cigarette smoking is a definite, preventable risk factor for the development of clinically evident Graves’ ophthalmopathy in European populations (5–7, 17). The association between cigarette smoking and GO in Japanese subjects remains to be established.

In conclusion, our results indicate that: (i) CTLA-4 G allele and G/G genotype confer genetic susceptibility to Graves’ disease in Polish-Caucasian and Japanese populations; (ii) CTLA-4 G allele and G/G genotype are not associated with Graves ophthalmopathy in either Caucasian or Japanese patients; (iii) different non-genetic factors may contribute to the susceptibility to GO in different populations. Cigarette smoking and age of onset of GD over 42 years are risk factors for the development of clinically evident GO in Caucasians. The predisposing factors to GO in Japanese remain to be established.

Acknowledgements

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