CLINICAL STUDY

Soluble CTLA-4 receptor an immunological marker of Graves’ disease and severity of ophthalmopathy is associated with CTLA-4 Jo31 and CT60 gene polymorphisms

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(J Daroszewski and E Pawlak contributed equally to this work)

Abstract

Objective: Graves’ disease (GD) is an autoimmune disorder with genetic and environmental background. CTLA-4 is a candidate gene for thyroid autoimmunity. Increased serum levels of soluble CTLA-4 (sCTLA-4) were found in some autoimmune diseases.

Aim: The aim of the study was to evaluate the relation between sCTLA-4 level and clinical manifestation of Graves’ ophthalmopathy (GO), thyroid status, and CTLA-4 gene polymorphisms.

Design: Serum sCTLA-4 concentrations were determined in 93 GO patients and 93 healthy controls. In the GO group, CTLA-4 gene was genotyped in five polymorphic sites: g.319C>T, c.49A>G, CT60 by means of PRC-RFLP, Jo31, and g.*642AT(8_33) by means of minisequencing assay.

Results: Serum sCTLA-4 level was significantly higher in the GO group than in controls (median: 7.94 vs 0.00 ng/ml, P=0.000001). This level was higher in severe than in nonsevere GO (median: 10.3 vs 5.6 ng/ml, P=0.01). sCTLA-4 concentration was related neither to the activity of GO nor to thyroid function. Elevated sCTLA-4 levels were observed in carriers Jo31[G] allele (genotype GG GT) as compared with subjects with an absence of the [G] allele (TT genotype; median: 9.18 vs 4.0 ng/ml, P=0.02). Also patients possessing CT60[G] allele (genotype GG GA) had higher serum sCTLA-4 levels than subjects who lack the [G] allele (AA genotype; median: 8.73 vs 2.28 ng/ml, P=0.03).

Conclusions: It was shown for the first time that increased serum concentration of sCTLA-4 correlate with the severity of GO. Genetic variation in the CTLA-4 gene region in GD patients at least partially determines the level of sCTLA-4.

Introduction

Graves’ disease (GD) is an organ-specific autoimmune multifactorial disease that develops as a result of complex interaction between genetic susceptibility genes and environmental factors (1–6). GD is characterized immunologically by a lymphocytic infiltrate of the thyroid and by evidence of immune system activation, with an increased number of circulating T lymphocytes and levels of thyroid-specific antibodies, mimicking TSH action and thus causing hyperthyroidism (1–4). Graves’ ophthalmopathy (GO) is an extrathyroidal manifestation of GD. In 5–10% of patients, eye symptoms occur in severe stage and require an intensive treatment.

The CD80/CD86/CD28/CTLA-4 co-stimulatory pathway is important in the pathogenesis of autoimmune diseases (7). The cytotoxic T lymphocyte antigen-4 (CTLA-4) molecule plays a key role in the maintenance of peripheral tolerance as well as termination of T-cell responses (8, 9). Apart from the membrane CTLA-4 molecule, an alternate transcript of CTLA-4 mRNA that encodes a protein lacking a transmembrane region, which likely represents a native soluble form of CTLA-4 (sCTLA-4), has recently been described (10–12). Native sCTLA-4 possesses binding ability to CD80/CD86, but the immunological effects mediated by endogenous sCTLA-4 have not yet been clarified (11–13). It is suggested that sCTLA-4 can act as a competitor of CD28 to bind CD80 or CD86, thereby interfering with T-lymphocyte activation in the initiation of immune response (14).

Studies of serum sCTLA-4 levels in autoimmune disease are still very limited. The CTLA-4 isoform is
observed significantly more often in patients with
generalized autoimmune disorders, such as systemic
lupus erythematosus (15), systemic sclerosis (16),
multiple sclerosis (17), and organ-specific diseases
such as type 1 diabetes (18–20). Studies performed for
sCTLA-4 isofrom level in sera in myasthenia gravis gave
inconsistent results (21–23). In Hashimoto’s thyroiditis,
a high sCTLA-4 concentration seems to be firmly
confirmed, but data regarding GD are limited (11, 13).
Recently, some data regarding the association of
CTLA-4 gene polymorphisms with circulating sCTLA-4 level in
serum, were presented (15, 18–21, 24–25).

In this study, we reported data on sCTLA-4
centrations in patients with different stages of GD
accompanied by ophthalmopathy. The results were
compared with regard to thyroid status and the clinical
manifestation of GO. We also assessed the correlation
between the serum sCTLA-4 levels and CTLA-4 gene
polymorphisms. To the best of our knowledge, no data
describing the association of serum sCTLA-4 levels and
thyroid function, clinical presentation of GO in the large
cohort as well as the association of CTLA-4 gene
polymorphisms g.319C>T, c.49A>G, CT60, Jo31, and g.642AT(8_33) and sCTLA-4 in GD and GO were
reported to date.

Materials and methods

Subjects

Ninety-three patients suffering from GO (76 females and
17 males, mean age: 50.0±11.7 years, range: 20–82
years) were enrolled into the study. The initial diagnosis
of GD was based on clinical and laboratory tests,
including a history of thyrotoxicosis, diffuse goiter,
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reported to date.

Assessment of ocular changes

Ophthalmological examination was performed at the
time of blood collection. The severity of ophthalmo-
pathy was assessed using criteria given by Bartalena
et al. (26, 27) taking into account the degree of
proptosis, diplopia, and optic neuropathy (Table 1).
Twenty-one patients presented a mild, 21 patients a
moderate, and 51 patients a severe degree of eye
symptoms. The patients were divided into two groups:
with nonsevere eye symptoms (mild and moderate GO)
and with severe GO (Table 2). Ophthalmopathy was also
scored using an ophthalmopathy index based on the
NOSPECS classification (28). The activity of GO was
assessed by means of a numerical clinical activity score
(CAS), originally described by Mourits et al. (29).

DNA preparation

Genomic DNA was prepared from peripheral white
blood cells using a QIAamp DNA Blood Mini Kit
(Qiagen GmbH, Hilden, Germany).

Genotyping/determination of polymorphisms

The polymorphisms g.319C>T (rs5742909) in the
promoter region, c.49A>G (rs231775) in exon 1, and
CT60 (g.*6230G>A, rs3087243) in the 3’UTR of the
CTLA-4 gene were examined by PCR-restriction frag-
ment length polymorphism (PCR-RFLP) using TruI,
BseXI, and TailI enzymes (Fermentas, Burlington,
Ontario, Canada). The conditions for digestion with
the restriction enzymes are listed in Table 3. The Jo31
(g.*10223G>T: rs11571302) polymorphism in the
3’UTR region of the CTLA-4 gene was genotyped using
PCR followed by single-nucleotide primer extension
reactions with dideoxy-NTPs labeled with different
fluorochromes corresponding to each allele (SNAPshot
Multiplex kit, Applied Biosystems, Warrington, UK). The
CTLA-4 g.642AT(8_33) polymorphism in 3’UTR
containing an (AT)n repeat was amplified with the
pair of primers where the 5’ end of the forward primers
was labeled with JOE (Bionovo, Legnica, Poland).
Genotyping was performed in a mixture of amplified
products with the internal size standard Gene Scan–350
ROX Size Standard (Applied Biosystems, Foster City, CA).

Table 1 Assessment of severity of Graves’ ophthalmopathy
according to Baralena et al. (26, 27).

<table>
<thead>
<tr>
<th>Degree of involvement</th>
<th>Proptosis (mm)</th>
<th>Diplopia</th>
<th>Optic neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>19–20</td>
<td>Intermittent</td>
<td>Subclinical</td>
</tr>
<tr>
<td>Moderate</td>
<td>21–23</td>
<td>Inconstant</td>
<td>Visual acuity 8/10–5/10</td>
</tr>
<tr>
<td>Marked</td>
<td>&gt; 23</td>
<td>Constant</td>
<td>Visual acuity &lt;5/10</td>
</tr>
</tbody>
</table>

Severe ophthalmopathy: at least one marked, or two moderate, or one moderate and two mild manifestations.
USA) using an ABI PRISM 310 capillary electrophoresis system. The PCR reaction condition as well as single-nucleotide primer extension reactions and fluorescence-based technique was carried out as described by Suwalska et al. (30). The primers were designed according to the complete CTLA-4 gene sequence derived from the NCBI Sequence Viewer (http://www.ncbi.nlm.nih.gov/).

**Assay of sCTLA-4, TSH, fT4, fT3, anti-TPO, and TBII**

Serum sCTLA-4 concentrations in GD patients and control subjects were measured by ELISA using specific reagent kits for human sCTLA-4 (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer’s protocol. This assay has a linear range between 2.0 and 10 ng/ml. Each sample was analyzed in duplicate. The results below the lower range of test are considered as negative with value 0.0.

**Statistical analysis**

Statistical significance between means of sCTLA-4, fT3, and fT4 for different groups was calculated by one-way ANOVA, alternatively using the Kruskal–Wallis test, when the variances in groups were not homogeneous (the homogeneity of variance was determined by the Bartlett’s test) or when the number of cases was too small. Correlations of sCTLA-4 level and clinical parameters were assessed using the Spearman or Pearson correlation coefficient. Association of sCTLA-4 level and the pattern of CTLA-4 gene polymorphisms were assessed using one-way ANOVA, alternatively using the Kruskal–Wallis test. Comparison between groups in regards of an occurrence of a particular parameter was assessed by means of odds ratio (OR). A P value of <0.05 was considered significant.

Statistical analyses were made using the EPIINFO Version 3.3.2 program (version 09-02-2005).

**Results**

Serum sCTLA-4 concentrations were measurable in 77.4% (72/93) of the samples taken from the GD patients and in 45.2% (42/93) of the samples from the healthy subjects (odds ratio (OR) = 3.82, 95% confidence interval (CI): 2.03–7.20, P = 0.00001). Serum sCTLA-4 concentrations were significantly higher in the GD subjects compared with the controls (median: 7.94 vs 0.00 ng/ml, P = 0.000001; Fig. 1).

An increased level of sCTLA-4 was found in the patients with severe GO compared with the controls (median: 10.3 vs 0.00 ng/ml, P = 0.000001) and in

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Clinical and hormonal characteristics of patients with different stages of Graves' ophthalmopathy (GO) severity.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonsevere GO</td>
</tr>
<tr>
<td>Number of cases</td>
<td>42</td>
</tr>
<tr>
<td>Age (range)</td>
<td>49.0 (20–82)</td>
</tr>
<tr>
<td>Female/male</td>
<td>34/8</td>
</tr>
<tr>
<td>Smoking/ nonsmoking</td>
<td>20/22</td>
</tr>
<tr>
<td>fT4 (pmol/l)</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>15.7</td>
</tr>
<tr>
<td>fT3 (pmol/l)</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>9.0</td>
</tr>
<tr>
<td>Orbitopathy index</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>5.00</td>
</tr>
<tr>
<td>CAS</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>3.00</td>
</tr>
<tr>
<td>TSH</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>0.09</td>
</tr>
<tr>
<td>Anti-TPO</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>1575.5</td>
</tr>
<tr>
<td>TG</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>80.5</td>
</tr>
<tr>
<td>ATG</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>13.6</td>
</tr>
<tr>
<td>TBII</td>
<td>Mean ± s.d.</td>
</tr>
<tr>
<td>Median</td>
<td>5.1</td>
</tr>
</tbody>
</table>

P <0.05 are in bold.

**Table 3** Conditions for PCR product digestion with restriction enzymes.

<table>
<thead>
<tr>
<th>Amplicon (bp)</th>
<th>SNP</th>
<th>Enzyme</th>
<th>Temperature and duration of digestion</th>
<th>Products of digestion visible on gel (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>814</td>
<td>CTLA-4g.319C &gt; T</td>
<td>Trul (Msel)</td>
<td>65 °C, 4 h</td>
<td>C: 51, 101, 100, 562</td>
</tr>
<tr>
<td>814</td>
<td>CTLA-4c.49A &gt; G</td>
<td>BseXI (BbvI)</td>
<td>65 °C, 4 h</td>
<td>T: 51, 101, 100, 94, 468</td>
</tr>
<tr>
<td>806</td>
<td>CT60G &gt; A</td>
<td>TaII (Msell)</td>
<td>65 °C, 4 h</td>
<td>A: 207, 607</td>
</tr>
</tbody>
</table>

http://www.eje-online.org
The patients with nonsevere GO (median: 10.3 vs 5.6 ng/ml, \(P=0.01\); Fig. 1).

Values higher than the mean plus doubled s.d. of the control serum samples (16.62 ng/ml) were considered to be substantially elevated in this study. Elevated sCTLA-4 levels were noted in 31.2% (29/93) of all GD patients. The elevated soluble isofrom of CTLA-4 was more frequently observed in the group of patients with severe symptoms of GO (41.2%, 21/51), while only in 19% (8/42) patients with nonsevere GO (OR = 2.98, 95% CI: 1.15–7.70, \(P<0.02\)). By contrast, in the control group, elevated sCTLA-4 level was observed only in six persons.

We did not observe any correlation of the ophthalmopathy index and CAS, parameters reflecting the clinical index and CAS, parameters reflecting the clinical severity of GD. The elevation of serum sCTLA-4 levels was noted in 31.2% (29/93) of all GD patients. The elevated soluble isoform of CTLA-4 was more frequently observed in the group of patients with severe symptoms of GO (41.2%, 21/51), while only in 19% (8/42) patients with nonsevere GO (OR = 2.98, 95% CI: 1.15–7.70, \(P<0.02\)). By contrast, in the control group, elevated sCTLA-4 level was observed only in six persons.

The study of an association of the CTLA-4 gene polymorphisms: g.319C>T, c.49A>G, CT60, Jo31, and g.*642AT(8_33) with serum sCTLA-4 concentration showed a link between Jo31 and CT60 polymorphic variants and serum sCTLA-4 level. Patients possessing Jo31[G] allele (genotype GG+GT, 74/92) had elevated serum sCTLA-4 compared with subjects with a lack of the [G] allele (carriers of the TT genotype, 18/92; median: 9.18 vs 4.0 ng/ml, \(P=0.02\); Table 4, Fig. 2). Elevated serum sCTLA-4 was observed in carriers of CT60[G] allele (genotype GG+GA, 81/93) compared with subjects with an absence of the G allele (carriers of the AA genotype, 12/93; median: 8.73 vs 2.28 ng/ml, \(P=0.03\); Table 4, Fig. 2). Moreover, carriers of [G] allele at both polymorphic sites CT60 and Jo31 (72/93, 77.4%) have significantly higher serum sCTLA-4 level than patients double-homozygous Jo31[TT]&CT60[AA] (11/93, 11.8%; median: 9.32 vs 1.39 ng/ml, \(P=0.01\)). We also noted a significant difference between the level of sCTLA-4 in sera in patients double [GG] homozygous at studied polymorphic sites (34/93, 36.6%) in comparison with counter partners [AA]&[TT] (11/93, 11.8%; median: 7.35 vs 1.39 ng/ml, \(P=0.02\)). The other CTLA-4 gene polymorphisms studied were not related to serum sCTLA-4 levels (Table 4).

### Discussion

GD is a common autoimmune disorder of the thyroid in which stimulatory antibodies bind to the TSH receptor and activate glandular function, resulting in hyperthyroidism.

In the present study, we observed significantly increased serum sCTLA-4 levels in GD patients compared with controls. It should be noted that despite using a commercial kit with possibly less sensitivity than in very initial studies (10), the presence of the sCTLA-4 molecule was found in a vast majority of the study and in nearly half of the control group. Our results are in agreement with those of Oaks & Hallet (11), and Saverino et al. (13) performed in much smaller groups of subjects.

Since there is a need to find a biochemical parameter with the power to recognize the active phase of GD or identify GD patients at risk of severe eye symptoms, we

### Table 4: Effect of CTLA-4 gene polymorphism variant on sCTLA-4 levels in Graves’ disease patients.

<table>
<thead>
<tr>
<th>Polymorphic site</th>
<th>Genotype</th>
<th>sCTLA-4 (ng/ml)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>g.319C&gt;T</td>
<td>AA + CT</td>
<td>11.58</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>7.76</td>
<td></td>
</tr>
<tr>
<td>c.49A&gt;G</td>
<td>GG + AG</td>
<td>9.12</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>8.15</td>
<td></td>
</tr>
<tr>
<td>Jo31</td>
<td>GG + GT</td>
<td>9.80</td>
<td>0.02</td>
</tr>
<tr>
<td>CT60</td>
<td>GG + GA</td>
<td>8.73</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>2.28</td>
<td></td>
</tr>
<tr>
<td>g.*642AT(8_33)</td>
<td>(AT)&lt;8/(AT)&lt;8</td>
<td>8.39</td>
<td>0.12</td>
</tr>
<tr>
<td>Jo31/CT60</td>
<td>(AT)&lt;8/(AT)&lt;8</td>
<td>(AT)&lt;8/(AT)&lt;8</td>
<td>7.60</td>
</tr>
<tr>
<td></td>
<td>GG + GT &amp; (GG + GA)</td>
<td>9.32</td>
<td>0.01</td>
</tr>
<tr>
<td>Jo31/CT60</td>
<td>TT &amp; AA</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GG &amp; GG</td>
<td>7.35</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>TT &amp; AA</td>
<td>1.39</td>
<td></td>
</tr>
</tbody>
</table>

\(P<0.05\) are marked in bold.
also analyzed sCTLA-4 in patients with different stages of GO. We found an association of sCTLA-4 concentrations with GO status, as patients with severe GO had statistically higher sCTLA-4 levels than both controls and patients with nonsevere eye symptoms. It is worth noting that the high sCTLA-4 level was observed significantly more often in patients with severe GO than in the nonsevere group. Saverino et al. reported no relationship between sCTLA-4 level and severity of GO (13). However, this observation was based on data from a small group of 31 patients.

There was no link between sCTLA-4 concentration and clinical symptoms of active GO. Studies of correlations of sCTLA-4 levels with disease activity in other autoimmune diseases gave inconsistent results. sCTLA-4 was found to correlate with autoantibodies against nicotinic acetylcholine receptors in myasthenia gravis (21) and area of skin lesions and severity of index values in psoriasis vulgaris (24), but not with disease activity in systemic lupus erythematosus (15). Our observations refute a potential utility of sCTLA-4 as a biochemical marker of ocular change activity, but may testify in favor of a predominantly genetic over an environmental regulation of sCTLA-4 synthesis. It has been hypothesized that increased sCTLA-4 levels in autoimmune thyroid disease are related more to genetic than to environmental factors (11, 31).

There was no association between thyroid function and serum sCTLA-4 level, as was reported also by others (13). No relationships between sCTLA-4 concentration and factors known to aggravate GO, such as smoking habit (32), age, and gender (33), were found in our study, similarly to Saverino et al. (13). The different sex ratio in GO subjects and control group does not seem to influence results of sCTLA-4 estimation and comparison since the molecule level did not differ between both genders.

The influence of the polymorphisms within CTLA-4 gene on the GD (31, 34–42) as well as on GO (43–45) has been widely studied. The CT60 in the 3′UTR region of this gene was the most promising locus for the autoimmune thyroid disease (31, 40–42). Notwithstanding, the present study has been focused on the possible influence of tested polymorphic sites on the serum sCTLA-4 concentration.

We found a significant correlation of the two SNPs in 3′UTR of the CTLA-4 gene, which were shown to be in strong linkage disequilibrium (32), and levels of soluble isoform of CTLA-4 protein. We observed for the first time a statistically higher serum sCTLA-4 concentration in patients possessing the Jo31[G] allele (genotype GG + GT) than in patients possessing the [TT] genotype. Moreover, carriers of CT60[G] allele (genotype GG + GA) also had higher sCTLA-4 levels in serum than other patients. In contrast, others (20, 25) did not observe any relation between CT60 polymorphism and serum sCTLA-4 level. We did not report any significant correlation of other CTLA-4 gene polymorphisms studied with sCTLA-4 level in serum; however, we noted that promoter polymorphism (g.319C>T) tended to be associated with higher sCTLA-4 levels. On the contrary, no association of this polymorphism in SLE patients (15) and in psoriasis vulgaris (22) was found. Similarly to us, Luszczek et al. (22) failed to find an association of c.49A>G and sCTLA-4 serum level in psoriasis vulgaris, while the latest study in type 1 diabetes suggests a possible gene dosage effect for the concentrations of sCTLA-4 among the different c.49A>G genotypes of CT60 (25). Wang et al. also did not observe any association between the g.642AT(8,33) polymorphism in the 3′UTR and sCTLA-4 level in myasthenia gravis (21). We have provided preliminary data to support the hypothesis that the Jo31[G] and CT60[G] alleles influence the concentrations of sCTLA-4.

The results of the present study may suggest that the ability to produce sCTLA-4 is under genetic control and that after immune activation individuals possessing [G] allele at position Jo31 and CT60 are eager producers of sCTLA-4. If this should turn out to be true, our finding of higher sCTLA-4 levels in severe GO subjects could imply therapeutic consequences. It would suggest a need for an especially careful follow-up of GD patients with high sCTLA-4 levels regarding eye symptoms, including a tight metabolic control of hormonal disturbances, avoidance of harmful environmental factors, as well as early immunosuppressive treatment of GO. This idea must be strengthened by studies on longitudinal measurements of sCTLA-4 serum concentration at different stages of GD and by setting a threshold serum sCTLA-4 level with a sufficient specificity.

To summarize, our study revealed that serum sCTLA-4 concentration seems to be more genetically determined than influenced by environmental or hormonal factors and appears to be related to the severity of eye changes. Serum sCTLA-4 level might be considered a useful biochemical marker of the occurrence of severe GO.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References


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