CTLA-4 gene polymorphisms predispose to autoimmune endocrinopathies but not to celiac disease

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Abstract

OBJECTIVES: The aim of the study was to determine the association of two CTLA-4 gene polymorphisms (CT60, +49 A/G) with Hashimoto thyroiditis (HT), type 1 diabetes mellitus (T1DM) and celiac disease (CD) as well as with the occurrence of multi-organ involvement by autoimmunity in children.

METHODS: Genotyping was done by RFLP analysis in Slovak children with HT (n=63) and CD (n=120) and both Slovak and Slovene children with T1DM (n=320) and healthy controls (n=231).

RESULTS: We found a significant association of the G allele of the CT60 polymorphism with HT (p<0.0005) in the Slovak population and T1DM in both Slovak (p<0.01) and Slovene populations (p<0.005). The G allele of the +49A/G polymorphism was significantly, though less strongly, associated with T1DM (p<0.05) and HT (p<0.05). Distribution of genotypes of CTLA-4 gene polymorphisms in CD patients did not differ significantly from controls. None of the polymorphisms was associated with multi-organ involvement by autoimmunity.

CONCLUSION: The G allele of both examined CTLA-4 gene polymorphisms predisposes to HT and T1DM, but not to CD. No association with multi-organ involvement was found. The GG genotype of the CT60 polymorphism may identify CD patients at an increased risk for concomitant T1DM and HT. Further studies to assess the predictive value of CTLA-4 polymorphisms for the co-occurrence of HT and T1DM in CD patients are needed.
INTRODUCTION

Type 1 diabetes mellitus (T1DM), Hashimoto thyroiditis (HT) and celiac disease (CD) are among the most common autoimmune diseases of childhood.

The cytotoxic T lymphocyte associated antigen 4 (CTLA-4) is a high-affinity co-stimulatory molecule expressed by activated T and B lymphocytes. Interaction of CTLA-4 with its ligands B7.1 and B7.2 leads to inhibition of the ongoing T lymphocyte activation. (Krummel et al., 1996) CTLA-4 action is a key immune mechanism that limits exaggerated immune reactions against self-antigens and thus an important check-point of immune tolerance. (Masteller et al., 2000) A fully functional native soluble form of CTLA-4 (sCTLA-4) is elevated in autoimmune diseases. (Magistrelli et al., 1999)

Polymorphisms of the CTLA-4 gene region (2q33) have been associated with T1DM and HT. (Braun et al., 1998; Kikuoka et al., 2001) The exon 1 +49 A/G polymorphism has been shown to affect CTLA-4 function. Recently, polymorphisms in the 3′ terminal untranslated region (possibly affecting mRNA stability and sCTLA-4 expression) were found to be significantly associated with T1DM, Graves-Basedow disease and HT. (Ueda et al., 2003)

HT and CD are known to occur frequently in T1DM patients, however little is known about predictors of the development of HT and CD in T1DM patients. In a number of studies, association of T1DM with CTLA-4 SNPs was limited to patients with an associated autoimmune disorder. (Blomhoff et al., 2005; Djilali-Saia et al., 1998; Ikegami et al., 2006; Vaidya et al., 2002) Hence, genetic factors may be responsible for the accumulation of organ-specific autoimmune disorders in some patients. Because of the central role of CTLA-4 in immune regulations and associations of its gene polymorphisms with pathophysiologically distinct autoimmune disorders we hypothesized that CTLA-4 gene SNPs may be a risk factor for multi-organ involvement by autoimmunity.

To test this hypothesis, we examined two polymorphic markers of the CTLA-4 gene in cohorts of pediatric T1DM patients with and without concomitant HT from two different ethnically related Slavic (Slovak and Slovene) populations. Additionally, cohorts of Slovak HT and CD patients were genotyped.
used. (Bittencourt et al., 2003) The 50 µl PCR reaction was set up to contain 5 µl 10x PCR buffer, 25 pmol of each primer, 0.02 mmol of each dNTP (Invitrogen, Brazil), 0.05 mmol MgCl₂ and 1.5 units of Taq polymerase (Invitrogen, Brazil). The PCR was run for 35 cycles (60 s denaturation at 94°C, 60 s annealing at 59°C, 60 s elongation at 72°C) followed by 10 minutes final extension at 72°C. The resulting 328 bp product was digested by 2 units of BbvI (NEB BioLabs, New England) for 3 hours at 37°C, which resulted in 244 bp and a 84 bp fragments in the presence of the G allele. The A allele yielded an intact 328 bp fragment.

For the amplification of a 328 bp gene fragment comprising the CT60 polymorphism oligonucleotide primers were designed (5'-atctgtggtggtcgttt-3' forward, 5'-agggaaggaacaagttct-3' reverse) using the published human CTLA-4 and ICOS gene sequence (GeneBank, NT_005403.16). Amplification was performed in a mixture of genomic DNA, 5 µl 10x PCR buffer, 25 pmol of each primer, 0.01 mmol of each dNTP (Invitrogen, Brasil), 0.05 mmol MgCl₂ and 1.25 units of Taq polymerase (Invitrogen, Brasil) in a final volume of 50 µl. The mixture was subjected to 35 cycles of 60 s denaturation at 94°C, 60 s annealing at 59°C, 60 s polymerization at 72°C followed by 10 minutes final extension at 72°C. The PCR product was subjected to digestion by 2.5 units of HpyCH4 IV (NEB BioLabs, New England) for 3 hours at 37°C. Digestion resulted in 252 bp and 76 bp fragments in the presence of the G allele. In the case of the A allele, no digestion occurred.

All PCR reactions were performed on a TECHNE® GENE TC-312 thermal cycler. In every run of PCR reactions a negative control reaction with no DNA added was included. PCR and digestion products were run for 20 min at 10V/cm on an ethidium bromide stained 3% agarose (3:1) gel in 1x TBE buffer.

Table 1: Characteristics of examined subgroups of patients.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Slovak</th>
<th>Slovene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease</td>
<td>T1DM</td>
<td>T1DM+HT</td>
</tr>
<tr>
<td>N</td>
<td>70</td>
<td>56</td>
</tr>
<tr>
<td>M / F %</td>
<td>44 / 56</td>
<td>34 / 66</td>
</tr>
<tr>
<td>age of onset (SEM±SD)</td>
<td>7.5±3.6</td>
<td>7.3±4.3</td>
</tr>
</tbody>
</table>

Table 2: Allele and genotype frequencies of CT60 A/G and +49 A/G polymorphisms of the CTLA-4 gene in examined groups of Slovak and Slovene patients and results of the χ²-test for deviation from HWE (χ², p) for each subgroup.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Slovak</th>
<th>Slovene</th>
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<tbody>
<tr>
<td>Disease</td>
<td>T1DM</td>
<td>T1DM+HT</td>
</tr>
<tr>
<td>Genotype %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT60 A/G</td>
<td>AA</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>35.7</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>40.0</td>
</tr>
<tr>
<td>Allele frequency</td>
<td>A</td>
<td>0.422</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.578</td>
</tr>
<tr>
<td>HWE deviation</td>
<td>χ²</td>
<td>5.10</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.03</td>
</tr>
<tr>
<td>+49 A/G</td>
<td>AA</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>44.3</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>21.4</td>
</tr>
<tr>
<td>Allele frequency</td>
<td>A</td>
<td>0.564</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>0.436</td>
</tr>
<tr>
<td>HWE deviation</td>
<td>χ²</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.41</td>
</tr>
</tbody>
</table>
Statistical analysis. All patients with T1DM, HT and CD were included in “all T1DM”, “all HT” and “CD” groups, respectively. Only patients with T1DM and HT with no other concomitant autoimmune disease were included in “T1DM” and “HT” groups. To increase accuracy, only T1DM patients with no proven HT or CD during a minimum 48 month follow-up period were included in the T1DM group. Patients with T1DM and concomitant CD (n=18) were included in the “all T1DM” group when this was compared to controls, however were excluded when “all T1DM” and “CD” groups were compared. Patients with T1DM and both HT and CD (n=3) were included in both “T1DM +HT” and “CD” groups, since these were not compared (Table 1). Allele and genotype frequencies of each CTLA-4 gene polymorphism were determined by direct counting and calculation in each group of patients and controls. Genotype frequencies were checked for deviation from Hardy-Weinberg equilibrium by the goodness of fit \( \chi^2 \) test with one degree of freedom (Table 2). Allele frequencies in patients and controls were compared using Fisher’s exact test. The significance of differences in genotype distributions was assessed using the \( \chi^2 \) test with two degrees of freedom. For cohorts with significantly different genotype distributions, odds ratios (OR) at a 95% confidence interval (CI) were calculated using the approximation of Woolf. For all statistical tests a \( p \) value <0.05 was considered significant.

RESULTS

The distribution of CT60 genotypes in Slovak HT (“all HT”) and T1DM (“all T1DM”) patients was significantly different from controls (\( p<0.005 \) and \( p<0.01 \), respectively). The association of the G allele was more significant in HT patients (OR 3.1 [95% CI 1.4–7.0] for homozygosity and OR 1.7 [95% CI 0.6–2.9] for a single dose) than in T1DM patients (OR 2.2 [95% CI 1.1–4.3] for homozygosity). To eliminate bias caused by the strong association of CT60 with HT in T1DM patients, and vice versa, T1DM patients with no HT and HT patients with no T1DM were analyzed separately (“T1DM” and “HT” groups). The genotype distribution in T1DM patients remained significantly different (\( p<0.05 \)), however a significant deviation from HWE was noted. In patients with HT only, the significance of the association remained unaffected (\( p<0.005 \), OR 5.8 [95% CI 1.8–18.7] for homozygosity and OR 2.4 [95% CI 0.8–7.5] for a single dose).

Genotype distribution and allele frequencies in the Slovene cohort were not different at direct comparison with the Slovak cohort. The increased frequency of the G allele and GG genotype in Slovene T1DM patients reached a similar level of statistical significance (\( p<0.005 \), OR 3.9 [95% CI 1.8–8.3] for homozygosity and OR 2.0 [95% CI 1.0–3.9] for a single dose), however decreased if patients with accompanying autoimmune diseases were excluded from analysis (\( p<0.05 \), OR 3.0 [95% CI 1.3–6.8] for homozygosity).

As to the +49 A/G polymorphism, alleles and genotypes were similarly distributed in both populations. The only significant differences were observed in the Slovene cohort of T1DM patients (\( p<0.01 \), OR 3.9 [95% CI 1.5–10.3] for homozygosity and OR 2.3 [95% CI 1.2–5.0] for single dose), however only in the “all T1DM” group.

To increase the power of our study and to account for the significant deviation from HWE in the Slovak T1DM group, patients and sex- and geographically-matched controls from both populations were combined where appropriate to create larger cohorts. Deviation from HWE in all resulting groups including the T1DM group (\( \chi^2 = 2.69, p = 0.1 \) for the CT60 and \( \chi^2 = 0.33, p = 0.57 \) for the +49A/G polymorphism) was not significant. The association of T1DM with the G allele of the CT60 polymorphism remained significant (“all T1DM” \( p<0.0001 \), OR 2.8 [95% CI 1.7–4.7] for CT60 G homozygosity and OR 1.3 [95% CI 0.9–2.1] for CT60 G single dose and for T1DM with no associated HT \( p<0.001 \), OR 2.2 [95% CI 1.2–3.9] for homozygosity). Interestingly, with the increased number of patients a significant association for the +49A/G polymorphism with T1DM could be observed (\( p<0.05 \), OR 1.9 [95% CI 1.1–3.4] for the GG genotype). No significant differences could be observed between T1DM patients with and without concomitant HT.

As to CD, distribution of CT60 genotypes and allele frequencies was not significantly different from healthy controls. After exclusion of all patients with accompanying autoimmune disorders (T1DM, thyroiditis, juvenile chronic arthritis, dermatitis herpetiformis Dühring, autoimmune cardiomyopathy) (n=28) from the CD group as well as of all HT patients from the T1DM group (n=56) and T1DM patients from the HT group, we analyzed a possible difference in genotype distribution and allele frequencies between both T1DM and HT patients on one side and CD patients on the other. In HT, the predisposing genotypes of the CT60 polymorphism were more frequent (\( p<0.01 \)) with significant risk conferred by the G allele (OR 6.2 [95% CI 1.8–21.2] for homozygosity and OR 4.87 [95% CI 1.4–16.6] for a single dose). For T1DM, the difference in genotype distribution to CD patients did not reach statistical significance. We did not find any significant differences in the distribution of alleles or genotypes of the polymorphism +49 A/G in CD patients.

Genotypes were examined also in conjunction with clinical data of patients. The GG genotype of the +49 A/G polymorphism was weakly associated with an earlier onset of disease in T1DM patients (GG vs. AA 90.5 ± 45.4 months vs. 115.25 ± 50.6 months, n=171, \( p<0.05 \)). The highest documented titre of aTPO and aTG antibodies in HT and total villous atrophy upon
DISCUSSION

We confirmed a significant association of the G allele of the CTLA-4 CT60 polymorphism with T1DM in two related Slavic populations. Also, we found an extremely significant association of the CT60 GG genotype with HT in the Slovak cohort. These results are in line with reported data concerning the association of the CT60 G allele with HT (Ban et al., 2005; Ikegami et al., 2006; Ueda et al., 2003) and T1DM (Zhernakova et al., 2005) in other populations.

HT has been found to occur in up to 21–28% of T1DM patients (Fernandez-Castaner et al., 1999, Kordonouri et al., 2002) and thyroid function monitoring has become the standard of care for T1DM patients. Such association fulfills the clinical criteria of a type 2 autoimmune polyendocrine syndrome (at least one endocrine autoimmune disease in association with another autoimmune disease not fulfilling type 1 APS criteria) and some authors consider it a separate defect of immune tolerance. (Eisenbarth et al., 2004) At the same time, in some studies, associations of CTLA-4 gene polymorphisms were limited to subgroups of patients with a concomitant autoimmune disease; T1DM and HT (Djilali-Saiah et al., 1998, Ikegami et al., 2006, Vaidya et al., 2002), T1DM and Graves-Basedow disease (Mochizuki et al., 2003), vitiligo and other autoimmune diseases (Blomhoff et al., 2005). Such observations may be due to either the association of the polymorphism with multi-organ involvement by autoimmune i.e. predisposition to a type 2 APS or a strong effect of the concomitant autoimmune disorder.

We explored a possible effect of the CTLA-4 gene polymorphism on the occurrence of HT in T1DM by comparing subgroups of T1DM patients with and without concomitant HT. Association of the CT60 G allele with T1DM and HT was significant in both groups when compared to controls and direct comparison of T1DM and T1DM+HT groups did not yield significant results. This is in line with a similar study of Asian populations. (Djilali-Saiah et al., 1998) This result speaks for the association of the CT60 polymorphism with both T1DM and HT and against the possibility that the association with T1DM is due to the effect of concomitant occurrence of latent HT. (Ikegami et al., 2006) Nevertheless, we have to critically state, that the exclusion of concomitant HT in T1DM patients may not have been reliable in all cases. In up to 46% of our T1DM patients, HT was diagnosed later than 48 months after T1DM manifestation. Elevated aTPO and aTG antibodies are considered to indicate thyroid autoimmunity in euthyroid subjects (Holowell et al., 2002) and may precede HT manifestation by years (Kordonouri et al., 2002). We may not have recognized all cases of thyroid autoimmunity in our cohort of T1DM patients, since thyroid autoantibody testing had not been carried out in all patients. Euthyroid T1DM patients with unrecognized elevated thyroid antibody titers may have affected our analysis, since aTPO and aTG antibody levels are associated with the genotype of CTLA-4 gene polymorphisms. (Howson et al., 2007)

Based on our data, we propose, that the G allele of the CT60 polymorphism predisposes to both T1DM and HT, though more strongly to HT. This is in line with the central role of CTLA-4 in immune responses and the published associations of polymorphisms of its gene with a wide range of autoimmune diseases. Thus CTLA-4 gene polymorphisms may act as one of a number of universal predisposition loci for autoimmune diseases.

Studies on the association of CTLA-4 gene polymorphisms with CD published so far have yielded controversial results; the G allele of the CT60 polymorphism showed borderline (van Belzen et al., 2004) or no association with CD (Rueda et al., 2005) and was part of predisposing (Hunt et al., 2005) as well as protective haplotypes (Amundsen et al., 2004). We did not find an association of the CT60 polymorphism with CD in our cohort. Hence, CTLA-4 may not play a crucial role in CD pathogenesis. Nonetheless, an up to 10-fold increase in the occurrence of autoimmune thyroiditis and T1DM has been reported in CD patients. (Cuoco et al., 1999; Lorini et al., 1996; Talal et al., 1997) The increased prevalence of autoimmune diseases in CD patients is often explained by the sharing of common genetic susceptibility factors. In the case of T1DM and HT, this may not be true with respect to the examined CTLA-4 gene polymorphisms. An alternative explanation may be that celiac disease may start off a sequence of immunopathologic events that result in the initiation of organ-specific autoimmune phenomena in genetically predisposed individuals. This is supported by the fact that autoimmune complications of celiac disease are in relation to the duration of gluten-exposure and dietary intervention can prevent the development of autoimmunity in CD patients (Cuoco et al., 1999; Ventura et al., 1999) as well as decrease levels of organ-specific autoantibodies (Sategna-Guidetti et al., 2001)

As a novel observation, we suggest that the GG genotype of the CT60 CTLA-4 gene polymorphism (present in 34.7% of our CD patients) may modify the risk of T1DM and HT in CD patients and may have the potential to identify CD patients at an increased risk for the development of concomitant T1DM and HT. Identified early, these patients might benefit from regular selective sonographic and autoantibody screening. Reduction of hidden morbidity from undiagnosed disease and individualization of the strict-
ness of gluten-free diet might be based on the patient’s genotype. This hypothesis should be confirmed in studies comparing a representative cohort of CD patients with and without concomitant T1DM and HT. A similar approach was applied in a study of T1DM patients with and without CD, in which no association of the +49 A/G CTLA-4 gene polymorphism with the occurrence of CD in T1DM was found. (Sumnik et al., 2006) However, polymorphisms of the 3’ terminal region of the CTLA-4 gene were not examined and comparison of CD patients with and without T1DM was not done in that study. The small number of T1DM patients with CD in our study (n=19) did not allow us to analyze such associations. Interestingly, however, none of the CD patients with concomitant T1DM carried the protective AA genotype, and 9 patients (47.4%) carried the predisposing GG genotype. In conclusion, we confirmed that the CT60 polymorphism predisposes to the development of T1DM and HT in children of Slavic origin. This association is stronger than that of the exon 1 polymorphisms in position +49. No role of any of the examined polymorphisms in predisposition to HT in T1DM or in celiac disease could be demonstrated. Genotyp- ing of CD patients for the CT60 polymorphism may be a useful clinical tool to identify patients at an increased risk for the development of concomitant HT and T1DM.

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