Autoimmune thyroid diseases (AIDs), including Hashimoto’s thyroiditis (HT) and Graves’ disease (GD), are related to environmental and genetic factors. We analyzed the association of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) gene two polymorphisms (+49 A/G, -318 C/T) with HT and GD development in Polish children, and correlated both polymorphisms with the production of thyroid autoantibodies (TPOAb and TgAb). The study involved 49 AITD patients (age 10–19) with HT (n=25) or GD (n=24) and 69 healthy controls. SNP genotyping was performed using genomic DNA and TaqMan® probes. The obtained results indicated that CTLA-4 +49 GG genotype was significantly more frequent in both HT and GD patients, whereas the AA genotype was more common in controls. CTLA-4-318 CT genotype was significantly more frequent in AITD, and the CC genotype more often occurred in controls. Significantly higher median TPOAb and TgAb values were associated with G allele in HT, and with T allele in GD patients. Concluding, both studied polymorphisms seem to be important genetic determinants of the risk of HT and GD, and appear to be associated with a predisposition to high levels of TAbs and clinical AITD. The obtained results give more information on the distribution of the CTLA-4 polymorphism in Polish AITD children, and further support the proposal that the CTLA-4 gene plays an important role in a TAb production.

Key words: Graves’ disease, Hashimoto’s thyroiditis, autoimmune thyroid disease, CTLA-4, single nucleotide polymorphism, TAb production

INTRODUCTION

Autoimmune thyroid diseases (AIDs), which include hyperthyroid Graves’ disease (GD), and Hashimoto’s (goitrous) thyroiditis (HT), are multifactorial diseases with a vital genetic background. Among many immune-related genes, which impair the self-tolerance to thyroid autoantibodies (TAbs) and determine the risk of AITD development, the role of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is highlighted. Human CTLA-4 gene, located on 2q33, encodes the immunoregulatory molecule, a major negative regulator of T-cell activity (Ban et al., 2003; Manzotti et al., 2002).

Several polymorphic regions in the CTLA-4 gene have been associated with various autoimmune disorders, including GD and HT (Kristiansen et al., 2000; Ueda et al., 2003; Petrone et al., 2005). Among them, the most clearly understood CTLA-4 single nucleotide polymorphism (SNP) associated with AITD in different populations is A to G substitution at position 49 (+49 A/G) in exon 1 (Anjos et al., 2002; Ban et al., 2003; Pastuszak-Lewandoska et al., 2012). Very little is known about the significance in AITD development of another CTLA-4 SNP, i.e., C to T substitution in the promoter region at position -318 (-318 C/T), despite several studies (Kristiansen et al., 2000; Wang et al., 2003; Zaletel et al., 2006). Similarly, not much data is available on the association between CTLA-4 polymorphisms and the thyroid autoantibody (TAb) production (Park et al., 2000; Zaletel et al., 2006). The aim of our study was to evaluate the frequency of the polymorphisms in exon 1 (+49 A/G) and the promoter (-318 C/T) of the CTLA-4 gene, as well as to assess their potential influence on the TAb production in young Polish patients with HT and GD before the treatment.

MATERIAL AND METHODS

Patients. Blood samples (5 ml, EDTA-collected) were obtained before initiation of a therapy from 49 patients with diagnosed AITD (GD and HT), and from 69 unrelated healthy individuals. In the HT group there were 20 female and 5 male patients, aged 11–19; the GD group comprised 19 female and 5 male patients, aged 10–17; the control group consisted of 53 girls and 16 boys,
aged 10–19. The blood samples were received from the Department of Pediatrics, Endocrinology, Diabetology with Cardiology Division, Medical University of Białystok, and the Department of Pediatrics and Endocrinology of the Medical University of Warsaw. Patients were diagnosed on the basis of clinical symptoms and signs, and the biochemical analysis was performed using ECL assays (Boehringer Mannheim, Germany): Elecsys Anti-Tg test (human antigens and human monoclonal antibodies against thyroglobulin, TgAb), Elecsys Anti-TPO test (recombinant antigens and human polyclonal antibodies against thyroid peroxidase, TPOAb). The upper normal limit for TgAb was set at 34.0 IU/ml, and 12.0 IU/mL for the TPOAb. Results higher than these cut-off values were considered as positive.

In each patient, the levels of TSH-Receptor antibody (TRAb), TSH, free thyroxine (fT4) and free triiodothyronine (fT3) were assessed. Ultrasonography of the thyroid gland was performed and in case of the presence of a nodule, FNAB was performed, otherwise biopsy was not carried out.

The diagnosis of GD was based on the presence of clinical and biochemical hyperthyroidism with diffuse goiter, decreased TSH value (<0.27 μIU/ml), increased levels of free thyroid hormones, and hypoechogeneity with the presence of increased vascular flow and/or the presence of TRAbs. Hashimoto’s thyroiditis was recognized when elevated serum TSH level (>5.0 μIU/ml), patients with clinical or subclinical hypothyroidism as assessed in ultrasound examination. This group involved patients with clinical or subclinical hypothyroidism as assessed when elevated serum TSH level (>5.0 μIU/ml), and low or normal fT3 and fT4 levels were observed. All patients were clinically evaluated before an implementation of the treatment (methimazole in GD and L-thyroxin in HT). The inclusion criteria for the control group were as follows: negativity for thyroid autoantibodies and euthyreosis (50 healthy children). For subsequent 19 controls, who showed symptoms of the nodular goiter, additionally routine FNAB was performed to eliminate subjects with chronic inflammation.

The Ethics Committee approved the study protocol, and also informed consents were obtained from all participants and/or their parents.

**Genotyping.** Total genomic DNA was isolated from blood samples using QIAamp DNA Mini Kit (Qiagen, Germany), according to the manufacturer’s protocol. SNP genotyping was performed using TaqMan® probes (rs231775 and rs5742909) in 7900HT Fast Real-Time PCR System using TaqMan® 5’ allelic discrimination assay (Applied Biosystems, USA). Each sample was run in duplicate for each genotype analysis. The end-point readings were analyzed according to the manufacturer’s instructions using SDS Software v. 2.4.

**Statistical analysis.** For statistical analysis, STATA version 10 (State College, TX) was used. Compliance with Hardy–Weinberg equilibrium (HWE) was assessed using the Chi-square test (χ2) and Fisher’s test. Linkage disequilibrium (LD) in the two studied polymorphic sites was evaluated using Fisher’s exact test. The risk ofAITD, associated with rs231775 and rs5742909 genotypes, was determined by unconditional logistic regression calculating odds ratios (ORs) and 95% confidence intervals (CIs). The Kruskal-Wallis test was used to analyze median Tac values in patients with different genotypes. P values <0.05 were considered as significant.

**RESULTS**

**CTLA-4 +49A/G polymorphism**

Statistical analysis confirmed the presence of the significant differences between the studied groups (AITD vs. control), concerning the distribution of CTLA-4 +49 A/G alleles. The frequency and OR value for G allele was 0.7 in AITD vs. 0.4 in the control group, OR = 3.60, CI 95% 2.10–6.19, P = 0.003 and for A allele was 0.3 in AITD vs. 0.6 in the control group, OR = 0.28, CI 95% 0.16–0.48, P = 0.003. Although without statistical significance, GG genotype was more frequent in AITD group (0.50 in AITD vs. 0.20 in control group, OR = 4.65, CI 95% 2.06–10.48, P = 0.06), whereas AA

<table>
<thead>
<tr>
<th>Table 1. CTLA-4 +49 A/G allelic and genotypic distributions in HT and GD patients in comparison with the control group.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HT (total n = 25)</strong></td>
</tr>
<tr>
<td>n/frequency</td>
</tr>
<tr>
<td><strong>Control group (total n = 69)</strong></td>
</tr>
<tr>
<td>OR (CI 95%)</td>
</tr>
<tr>
<td><strong>P value</strong></td>
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<tr>
<td><strong>GD (total n = 24)</strong></td>
</tr>
<tr>
<td><strong>Control group (total n = 69)</strong></td>
</tr>
<tr>
<td>OR (CI 95%)</td>
</tr>
<tr>
<td><strong>P value</strong></td>
</tr>
</tbody>
</table>
CTLA-4-318 C/T polymorphism

Statistical analysis confirmed the presence of significant differences between the studied groups concerning the distribution of the CTLA-4 -318 C/T genotypes and alleles. The CT genotype was significantly more frequent in the AITD group (0.40 in AITD vs. 0.20 in controls, OR = 2.97, CI 95% 1.29–6.85, P = 0.04) and the CC genotype was significantly more frequent in the control group (0.20 in AITD vs. 0.70 in controls, OR = 0.26, CI 95% 0.12–0.55, P = 0.01). The frequency and OR value for the T allele was 0.4 in AITD vs. 0.2 in the control group, OR = 2.92, CI 95% 1.64–5.21, P = 0.02 and for the C allele: 0.6 in AITD vs. 0.8 in the control group, OR = 0.34, CI 95% 0.19–0.61, P = 0.02.

Following the statistical tendency, the allelic and genotypic distributions of CTLA-4 -318 C/T SNP were analyzed in HT and GD patients in comparison with controls. It was found that T allele frequency was higher in the HT group while C allele frequency was higher in the control group (P > 0.05). For GD patients, T alleles and CT genotype were more common, although the results were also not statistically significant (P > 0.05). CC genotype frequency was insignificantly increased in controls (P > 0.05). The results are summarized in Table 2.

Thyroid autoantibody levels

For each AITD patient, TPOAb and TgAb levels were assessed and correlated with CTLA-4 genotypes. The median values of TPOAb in patients with Hashimoto’s thyroiditis carrying the G allele (AG and GG genotypes) were much higher when compared to AA patients, approaching statistical significance (P < 0.05). When comparing individual genotypes, a statistically significant difference was also seen between AG and AA patients (P < 0.05). The TgAb median value was significantly higher in GG than in AG patients (P < 0.05). In the case of the CTLA-4 promoter polymorphism, patients carrying T allele exhibited insignificantly higher median TPOAb values than CC patients. A statistically significant difference was observed between TT and CT groups, with significantly higher TPOAb median value in TT patients (P < 0.05). TgAb median values did not differ significantly between various -318 polymorphisms. The results obtained for patients with Hashimoto thyroiditis are summarized in Table 3.

In patients with Graves’ disease and CTLA-4 promoter polymorphism, TPOAb median values were higher in patients carrying the T allele (in both CT and TT genotypes) as compared with patients carrying the CC genotype (P < 0.05). The TgAb median value was significantly higher in patients carrying the T allele (in both CT and TT genotypes) as compared with patients carrying the CC genotype (P < 0.05). In the case of CTLA-4 exon 1 polymorphism, significant differences for both TPOAb and TgAb median values were observed between AG and AA patients (P < 0.05). Furthermore, patients carrying the G allele (in both AG and GG genotypes) presented significantly higher TgAb median values than patients with the AA genotype (P < 0.05). The results obtained for patients with Graves’ disease are summarized in Table 4.

Linkage disequilibrium analysis

No linkage disequilibrium (LD) was found between the two studied polymorphic sites (P > 0.05, Fisher’s exact test).

Table 2. CTLA-4-318 C/T allelic and genotypic distributions in HT and GD patients in comparison with the control group.

<table>
<thead>
<tr>
<th></th>
<th>-318 CC genotype</th>
<th>-318 CT genotype</th>
<th>-318 TT genotype</th>
<th>-318 C allele</th>
<th>-318 T allele</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HT (total n = 25)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n/frequency</td>
<td>11/0.40</td>
<td>9/0.40</td>
<td>5/0.20</td>
<td>31/0.60</td>
<td>19/0.40</td>
</tr>
<tr>
<td><strong>Control group (total n = 69)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n/frequency</td>
<td>50/0.70</td>
<td>12/0.20</td>
<td>7/0.10</td>
<td>112/0.80</td>
<td>26/0.20</td>
</tr>
<tr>
<td>OR (CI 95%)</td>
<td>0.30 (0.12–0.77)</td>
<td>2.68 (0.97–7.46)</td>
<td>2.21 (0.63–7.76)</td>
<td>0.38 (0.19–0.77)</td>
<td>2.64 (1.29–5.39)</td>
</tr>
<tr>
<td>P value</td>
<td>0.22</td>
<td>0.14</td>
<td>0.28</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td><strong>GD (total n = 24)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n/frequency</td>
<td>9/0.40</td>
<td>10/0.40</td>
<td>5/0.20</td>
<td>28/0.60</td>
<td>20/0.40</td>
</tr>
<tr>
<td><strong>Control group (total n = 69)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>n/frequency</td>
<td>50/0.70</td>
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<td>7/0.10</td>
<td>112/0.80</td>
<td>26/0.20</td>
</tr>
<tr>
<td>OR (CI 95%)</td>
<td>0.23 (0.09–0.61)</td>
<td>3.39 (1.22–9.44)</td>
<td>2.33 (0.66–8.20)</td>
<td>0.33 (0.16–0.67)</td>
<td>3.08 (1.51–6.29)</td>
</tr>
<tr>
<td>P value</td>
<td>0.12</td>
<td>0.07</td>
<td>0.25</td>
<td>0.22</td>
<td>0.22</td>
</tr>
</tbody>
</table>
The suppressive role of the CTLA-4 implies that genetic changes affecting gene expression and/or function could lead to the development of autoimmunity, increasing T-cell activation. The CTLA-4 gene is highly polymorphic and several studies have been performed to find SNP functional effects.

The best known CTLA-4 polymorphism, +49 A/G SNP, which substitutes Thr for Ala in the signal peptide, leads to misprocessing of CTLA-4 in the ER, resulting in less efficient glycosylation and diminished surface expression of the CTLA-4 protein (Anjos et al., 2002). The presence of G allele has been associated with reduced control of T-cell proliferation (Kouki et al., 2000; Ban et al., 2003). Regarding the other studied SNP, in gene promoter, the relationship between T allele and higher promoter activity has been found, although there are some controversies (Ligers et al., 2001; Wang et al., 2002; Anjos et al., 2004). On the molecular level, -318 C/T SNP may influence CTLA-4 levels by changing the binding of a transcription factor LEF-1 (lymphoid enhancing factor 1) (Chistiakov et al., 2006).

Regarding the clinical implication of the CTLA-4 polymorphisms, +49 A/G SNP is a particularly strong candidate for susceptibility to T-cell mediated autoimmune thyroid diseases (Vieland et al., 2008). Our analysis confirms the existence of a significant association between the CTLA-4 +49 GG genotype, as well as the presence of G allele, and AITD risk, i.e., both Graves’ disease and Hashimoto’s thyroiditis. This association has also been confirmed in a number of other papers (Kouki et al., 2002; Bicek et al., 2009; Yang et al., 2012) and our own previous study (Pastuszak-Lewandoska et al., 2012), although focused on an adult AITD patients. However, there are studies indicating the association between the G allele and the childhood onset of the disease (Yung et al., 2002; Chong et al., 2008). The discrepancies with the results of some studies, suggesting a lack of association between +49 A/G SNP and HT pathogenesis (Park et al., 2006), may be explained by the heterogeneity of the patient populations studied.

Table 3. Patients with Hashimoto’s thyroiditis characteristics including TPOAb and TgAb positivity and median values in relation to CTLA-4 genotypes.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>-318 C/T SNP</th>
<th>+49 A/G SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AG</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>3/6</td>
<td>1/9</td>
</tr>
<tr>
<td>Age mean ± S.D.</td>
<td>14 ± 4.2</td>
<td>13 ± 6.3</td>
</tr>
<tr>
<td>TPOAb positive</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>[&gt; 12.0 IU/ml] n = 21</td>
<td>182</td>
<td>220</td>
</tr>
<tr>
<td>TgAb positive</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>[&gt; 34.0 IU/ml] n = 18</td>
<td>182</td>
<td>997</td>
</tr>
</tbody>
</table>

The discrepancies with the results of some studies, suggesting a lack of association between +49 A/G SNP and HT pathogenesis (Park et al., 2006), may be explained by the heterogeneity of the patient populations studied.

Table 4. Patients with Graves’ disease characteristics including TPOAb and TgAb positivity and median values in relation to CTLA-4 genotypes.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>-318 C/T SNP</th>
<th>+49 A/G SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AG</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>3/6</td>
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</table>

The discrepancies with the results of some studies, suggesting a lack of association between +49 A/G SNP and HT pathogenesis (Park et al., 2006), may be explained by the heterogeneity of the patient populations studied.
higher G allele frequency is associated with higher thyrotropin (TSH) levels, whereas the presence of T allele and ATTD. This confirms some of the previously mentioned reports and shows — with regard to the small study group — trends in Polish AITD children. Considering the HT and GD groups separately, we observed a higher frequency of CT genotype in the GD group and T allele in both groups of patients. However, in some populations (Canadian, German, Slovene) an excess of CC genotype in GD or HT patients was found (Braun et al., 1998; Park et al., 2000). In our study, the C allele and CC genotype seems to have a protective effect. The discrepancies are probably due to the genetic heterogeneity of ATTD. Another factor may be the relatively small number of patients in our study, and the age of the patients. However, although the analysis of ATTD children is valuable, as it can be expected that genetic factors have a stronger influence than environmental factors on the pathogenesis of autoimmune thyroid disease in this age group, such studies are rare. There are reports focusing on Asian and South American child populations, but almost no studies have been performed on European children with ATTD (Yung et al., 2002; Chong et al., 2008; Namo Cury et al., 2008; Yesilkaya et al., 2008; Kucharska et al., 2009). With one exception (Namo Cury et al., 2008), most of them revealed an association between CTLA-4 exon 1 polymorphism and susceptibility to childhood GD and HT. The only study regarding a +49 A/G SNP in GD and HT patients on TAb production: a fluence of G-allele bearing genotypes of the +49 A/G SNP and +49 A/G SNP is suggested (Zaletel et al., 2006; Kavvoura et al. 2007). In our study, no linkage disequilibrium was found between the studied CTLA-4 exon 1 and promoter SNP genotypes.

In conclusion, the results of our preliminary study are encouraging, and it would be worth developing this line of inquiry in the nearest future, involving larger groups of young ATTD patients.

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