ACTA ABP BIOCHIMICA POLONICA

Vol. 60, No 4/2013
641–646
on-line at: www.actabp.pl

Regular paper

CTLA-4 polymorphisms (+49 A/G and -318 C/T) are important genetic determinants of AITD susceptibility and predisposition to high levels of thyroid autoantibodies in Polish children — preliminary study

Dorota Pastuszak-Lewandoska[1], Daria Domańska[1], Magdalena Rudzińska[2], Artur Bossowski[3], Anna Kucharska[4], Ewa Sewerynek[5], Karolina Czarnecka[1], Monika Migdalska-Sęk[1] and Barbara Czarnocka[2]

[1]Department of Molecular Bases of Medicine, 1st Chair of Internal Diseases, Medical University of Lodz, Łódź, Poland; [2]Department of Biochemistry & Molecular Biology, Medical Center of Postgraduate Education, Warsaw, Poland; [3]Department of Pediatrics, Endocrinology, Diabetology with Cardiology Division, Medical University, Bialystok, Białystok, Poland; [4]Department of Pediatrics and Endocrinology, Medical University of Warsaw, Warsaw, Poland; [5]Department of Endocrine Disorders and Bone Metabolism, 1st Chair of Endocrinology, Medical University of Lodz, Łódź, Poland

Autoimmune thyroid diseases (AITDs), 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

Received: 28 August, 2013; revised: 02 December, accepted: 03 December, 2013; available on-line: 20 December, 2013

INTRODUCTION

Autoimmune thyroid diseases (AITDs), 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*., 2002; 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,

✉e-mail: dorota.pastuszak-lewandoska@umed.lodz.pl

Abbreviations: Ab, autoantibody; AITD, autoimmune thyroid disease; CTLA4, cytotoxic T-lymphocyte-associated antigen 4; ER, endoplasmic reticulum; FNAB, fine needle aspiration biopsy; fT4, free thyroxine; fT3, free triiodothyronine; GD, Graves' disease; GO, Graves' ophthalmopathy; HT, Hashimoto's thyroiditis; OR, odds ratio; SNP, single nucleotide polymorphism; TAb, thyroid autoantibody; Tg, thyroglobulin; TPO, thyroid peroxidase; TSH, thyroid-stimulating hormone; TR, TSH-receptor.

aged 10–19. The blood samples were received from the Department of Pediatrics, Endocrinology, Diabetology with Cardiology Division, Medical University of Bialystok, 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 levels of TPOAb and/or TgAb were observed (> 12.0 IU/mL and > 34.0 IU/ml, respectively), and hypoechogeneity of the thyroid gland was described 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 ($\chi 2$) and Fisher's test. Linkage disequilibrium (LD) in the two studied polymorphic sites was evaluated using Fisher's exact test. The risk of AITD, 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 TAb 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 1. ***CTLA-4*** **+49 A/G allelic and genotypic distributions in HT and GD patients in comparison with the control group.**

	+49 AA genotype	+49 AG genotype	+49 GG genotype	+49 A allele	+49 G allele
HT (total n = 25)					
n/frequency	3/0.10	8/0.30	14/0.60	14/0.30	36/0.70
Control group (total n = 69)					
n/frequency	29/0.40	27/0.40	13/0.20	85/0.62	53/0.38
OR (CI 95%)	0.19 (0.05–0.69)	0.73 (0.28–1.96)	1.20 (0.37–3.91)	0.24 (0.12–0.49)	4.12 (2.045–8.36)
P value	0.04	0.67	0.01	0.16	0.16
GD (total n = 24)					
n/frequency	3/0.10	9/0.21	12/0.79	15/0.11	33/0.89
Control group (total n = 69)					
n/frequency	29/0.40	27/0.40	13/0.20	85/0.62	53/0.38
OR (CI 95%)	0.20 (0.05–0.72)	0.93 (0.36–2.43)	4.31 (1.58–11.74)	0.28 (0.14–0.57)	3.53 (1.75–7.12)
P value	0.05	0.92	0.03	0.04	0.04

genotype frequency was increased in control group (0.10 AITD *vs.* 0.40 control group, OR = 0.22, CI 95% 0.09–0.54, $P = 0.08$).

Following the statistical tendency, the allelic and genotypic distributions of +49 A/G SNP were analyzed in HT and GD patients and compared with controls. The results are summarized in Table 1. In the HT group, the frequency of the G allele was higher compared to the control group, although not significantly ($P > 0.05$). The AA genotype was seen significantly more frequently ($P < 0.05$) in controls. In the GD group, statistically significant results were observed for G allele distribution ($P < 0.05$). Moreover, the GG genotype was significantly more frequent in this group of patients ($P < 0.05$). The presence of the A allele was significantly more common in controls ($P < 0.05$).

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, although without statistical significance ($P > 0.05$). CC genotype frequency was insignificantly increased in controls ($P > 0.05$). 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: patients with the CT genotype demonstrated significantly higher median values than those with 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.

	-318 CC genotype	-318 CT genotype	-318 TT genotype	-318 C allele	-318 T allele
HT (total n = 25)					
n/frequency	11/0.40	9/0.40	5/0.20	31/0.60	19/0.40
Control group (total n = 69)					
n/frequency	50/0.70	12/0.20	7/0.10	112/0.80	26/0.20
OR (CI 95%)	0.30 (0.12–0.77)	2.68 (0.97–7.46)	2.21 (0.63–7.76)	0.38 (0.19–0.77)	2.64 (1.29–5.39)
P value	0.22	0.14	0.28	0.31	0.31
GD (total n = 24)					
n/frequency	9/0.40	10/0.40	5/0.20	28/0.60	20/0.40
Control group (total n = 69)					
n/frequency	50/0.70	12/0.20	7/0.10	112/0.80	26/0.20
OR (CI 95%)	0.23 (0.09–0.61)	3.39 (1.22–9.44)	2.33 (0.66–8.20)	0.33 (0.16–0.67)	3.08 (1.51–6.29)
P value	0.12	0.07	0.25	0.22	0.22

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

Patient characteristics	+49 A/G SNP			-318 C/T SNP		
	AA	AG	GG	CC	CT	TT
Sex (M/F)	1/2	1/7	3/11	3/8	1/8	1/4
Age mean ±S.D.	12 ± 3.8	14 ± 6.8	13 ± 6.6	14 ± 5.8	12 ± 4.5	15 ± 3.2
TPOAb positive [> 12.0 IU/ml] n = 21	2	6	13	11	6	4
TPOAb median	150	305[a] / 420[b]	473	288	429	600[d]
TgAb positive [> 34.0 IU/ml] n = 18	2	7	9	6	7	5
TgAb median	341	368	472[c]	295	356	445

[a]$P<0.004$ compared with AA genotype; [b]$P<0.04$ compared with AA genotype; [c]$P<0.03$ compared with AG genotype; [d]$P<0.01$ compared with CT genotype (Chi-square test).

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

Patient characteristics	-318 C/T SNP			+49 A/G SNP		
	CC	CT	TT	AA	AG	GG
Sex (M/F)	3/6	1/9	1/4	0/3	2/7	3/9
Age mean ± SD	14 ± 4.2	13 ± 6.3	11 ± 3.1	11 ± 2.8	13 ± 5.5	15 ± 2.2
TPOAb positive [> 12.0 IU/ml] n = 21	9	8	4	3	9	9
TPOAb median	182	220[a] / 738[b]	440	566	850[c]	219
TgAb positive [> 34.0 IU/ml] n = 18	8	6	3	1	7	9
TgAb median	182	997	220	369	600[d] / 824[e]	998

[a]$P<0.01$ compared with CC genotype; [b]$P<.03$ compared with CC genotype; [c]$P<0.002$ compared with AA genotype; [d]$P<0.04$ compared with AA genotype; [e]$P<0.02$ compared with AA genotype (Chi-square test).

DISCUSSION

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 out 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., 2000; Petrone *et al*., 2001), could be explained by different genetic background, including geographical differences in allele frequencies, or differences in phenotype classification. It should be stressed, however, that the studies focusing on *CTLA-4* polymorphism in HT are few in number and, in this regard, our preliminary study and the obtained results seem to be valuable, and worth continuing.

Concerning *CTLA-4* promoter polymorphism, there is no consensus on its role in AITD development. Some populations show an association between this SNP and Graves' disease (Park *et al*., 2000), while others do not (Kouki *et al*., 2002; Vaidya *et al*., 2003; Zhang *et al*., 2006). Similarly, in HT development, the results are opposite (Braun *et al*. 1998; Park *et al*., 2000). In our study, a statistically significant association was found between -318 CT genotype, as well as the presence of T allele, and AITD. 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; Zaletel *et al*., 2006). 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 AITD. Another factor may be the relatively small number of patients in our study, and the age of the patients. However, although the analysis of AITD 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 regarding Asian and South American child populations, but almost no studies have been performed on European children with AITD (Yung *et al*., 2002; Chong *et al*., 2008; Namo Cury *et al*., 2008; Yeşilkaya *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 -318 polymorphism in children with HT (Yeşilkaya *et al*., 2008) did not find significantly higher SNP frequency in patients than in controls. The trend demonstrated in our study requires confirmation in a larger number of young patients before they can acquire statistical significance.

One of the hallmarks of the AITD is the production of high levels of thyroid autoantibodies (TAbs), i.e., autoantibodies against thyroglobulin (TgAb), and thyroid peroxidase (TPOAb), and it has been suggested that this tendency has a genetic basis. Our results indicate the influence of G-allele bearing genotypes of the +49 A/G SNP in GD and HT patients on TAb production: a higher G allele frequency is associated with higher thyroid autoantibody levels. It is in agreement with the results obtained by others (Tomer *et al*., 2001; Zaletel *et al*., 2002; Zaletel *et al*., 2006). Regarding -318 C/T SNP, the genotype distribution in our AITD group highlighted the role of the T allele, and it was found to be linked with TPOAb production in patients with Hashimoto's thyroiditis. The opposite results, indicating the role of CC genotype, were reported in one study (Zaletel *et al*., 2006). In patients with Graves' disease, we found the association between T allele and significantly higher levels of TPOAb and TgAb. It should be stressed that our study is one of very few such analyses of a *CTLA-4* promoter polymorphism in GD. In general, there is a paucity of studies focusing on the relationship between a *CTLA-4* gene and thyroid autoantibody production, and especially in children with AITD.

The present study demonstrates the association of both SNPs with a predisposition to high levels of TAbs and clinical AITD, i.e. both GD and HT. The contribution of +49 A/G SNP seems to be in stronger relationship with AITD risk, in that a statistically significant association was found between a *CTLA-4* exon 1 polymorphism and the development of HT and GD. The obtained results, although preliminary and involving a relatively small group of patients, give more information on a *CTLA-4* polymorphism distribution in Polish AITD patients, and add further support that the *CTLA-4* gene plays an important role in a TAb production. In particular, our study group comprising the children and adolescents, represents a valuable group in the genetic analysis. However, further studies are needed to assess the causative role of the described polymorphisms, or other SNPs remaining in linkage disequilibrium with them. In some studies, an LD value between 60 C/T 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 AITD patients.

REFERENCES

Anjos S, Nguyen A, Ounissi-Benkalha H, Tessier MC, Polychronakos C (2002) A common autoimmunity predisposing signal peptide variant of the cytotoxic T-lymphocyte antigen 4 results in inefficient glycosylation of the susceptibility allele. *J Biol Chem* **277**: 46478–46486.

Anjos SM, Tessier MC, Polychronakos C (2004) Association of the cytotoxic T lymphocyte-associated antigen 4 gene with type 1 diabetes: evidence for independent effects of two polymorphisms on the same haplotype block. *J Clin Endocrinol Metab* **89**: 6257–6265.

Ban Y, Davies TF, Greenberg DA, Kissin A, Marder B, Murphy B, Concepcion ES, Villanueva RB, Barbesino G, Ling V, Tomer Y (2003) Analysis of the CTLA-4, CD28, and inducible costimulator (ICOS) genes in autoimmune thyroid disease. *Genes Immun* **4**: 586–593.

Bicek A, Zaletel K, Gaberscek S, Pirnat E, Krhin B, Stopar TG, Hojker S (2009) 49A/G and CT60 polymorphisms of the cytotoxic T-lymphocyte-associated antigen 4 gene associated with autoimmune thyroid disease. *Hum Immunol* **70**: 820–824.

Braun J, Donner H, Siegmund T, Walfish PG, Usadel KH, Badenhoop K (1998) CTLA-4 promoter variants in patients with Graves' disease and Hashimoto's thyroiditis. Tissue *Antigens* **51**: 563–566.

Chistiakov DA, Savost'anov KV, Turakulov RI, Efremov IA, Demurov LM (2006) Genetic analysis and functional evaluation of the C/T (-318) and A/G(-1661) polymorphisms of the CTLA-4 gene in patients affected with Graves' disease. *Clin Immunol* **118**: 233–242.

Chong KK, Chiang SW, Wong GW, Tam PO, Ng TK, Hu YJ, Yam GH, Lam DS, Pang CP (2008) Association of CTLA-4 and IL-13 gene polymorphisms with Graves' disease and ophthalmopathy in Chinese children. *Invest Ophthalmol Vis Sci* **49**: 2409–2415.

Kavvoura FK, Akamizu T, Awata T, Ban Y, Chistiakov DA, Frydecka I *et al*. (2007) Cytotoxic T-lymphocyte associated antigen 4 gene polymorphisms and autoimmune thyroid disease: a meta-analysis. *J Clin Endocrinol Metab* **92**: 3162–3170.

Kouki T, Sawai Y, Gardine CA, Fisfalen ME, Alegre ML, DeGroot LJ (2000) CTLA-4 gene polymorphism at position 49 in exon 1 reduces the inhibitory function of CTLA-4 and contributes to the pathogenesis of Graves' disease. *J Imunol* **165**: 6606–6611.

Kouki T, Gardine CA, Yanagawa T, Degroot LJ (2002) Relation of three polymorphisms of the CTLA-4 gene in patients with Graves' disease. *J Endocrinol Invest* **25**: 208–213.

Kristiansen OP, Larsen ZM, Pociot F (2000) CTLA4 in autoimmune diseases — a general susceptibility gene to autoimmunity? *Genes Immun* **1**: 170–184.

Kucharska AM, Gorska E, Wasik M, Pyrzak B, Demkow U (2009) Expression of CD152 (CTLA-4) in children with autoimmune thyroid-

itis and +49 A/G polymorphism of exon 1 of the CTLA-4 gene. *J Physiol Pharmacol* **60**: 77–80.
Ligers A, Teleshova N, Masterman T, Huang WX, Hillert J (2001) CTLA-4 gene expression is influenced by promoter and exon 1 polymorphisms. *Genes Immun* **2**: 145–152.
Manzotti CN, Tipping H, Perry LC, Mead KI, Blair PJ, Zheng Y, Sansom DM (2002) Inhibition of human T cell proliferation by CTLA-4 utilizes CD80 and requires CD25+ regulatory T cells. *Eur J Immunol* **32**: 2888–2896.
Namo Cury A, Longui CA, Kochi C, Calliari LE, Scalissi N, Salles JE, Neves Rocha M, Barbosa de Melo M, Rezende Melo M, Monte O (2008) Graves' disease in Brazilian children and adults: lack of genetic association with CTLA-4 +49A>G polymorphism. *Horm Res* **70**: 36–41.
Park YJ, Chung HK, Park DJ, Kim WB, Kim SW, Koh JJ, Cho BY (2000) Polymorphism in the promoter and exon 1 of the cytotoxic T lymphocyte antigen-4 gene associated with autoimmune thyroid disease in Koreans. *Thyroid* **10**: 453–459.
Pastuszak-Lewandoska D, Sewerynek E, Domańska D, Gladyś A, Skrzypczak R, Brzeziańska E (2012) CTLA-4 gene polymorphisms and their influence on the predisposition to the autoimmune thyroid diseases (Graves' disease and Hashimoto's thyroiditis). *Arch Med Sci* **8**: 415–421.
Petrone A, Giorgi G, Mesturino CA, Capizzi M, Cascino I, Nistico L, Osborn J, Di Mario U, Buzzetti R (2001) Association of DRB1*04-DQB1*0301 haplotype and lack of association of two polymorphic sites at CTLA-4 gene with Hashimoto's thyroiditis in an Italian population. *Thyroid* **11**: 171–175.
Petrone A, Giorgi G, Galgani A, Alemanno I, Corsello SM, Signore A, Di Mario U, Nisticò L, Cascino I, Buzzetti R (2005) CT60 single nucleotide polymorphisms of the cytotoxic T-lymphocyte associated antigen-4 gene region is associated with Graves' disease in an Italian population. *Thyroid* **15**: 232–238.
Tomer Y, Greenberg DA, Barbesino G, Concepcion E, Davies TF (2001) CTLA-4 and not CD28 is a susceptibility gene for thyroid autoantibody production. *J Clin Endocrinol Metab* **86**: 1687–1693.
Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, et al. (2003) Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* **423**: 506–511.
Vaidya B, Oakes EJ, Imrie H, Dickinson AJ, Perros P, Kendall-Taylor P, Pearce SH (2003) CTLA4 gene and Graves' disease: association of Graves' disease with the CTLA4 exon 1 and intron 1 polymorphisms, but not with the promoter polymorphism. *Clin Endocrinol (Oxf)* **58**: 732–735.
Vieland VJ, Huang Y, Bartlett C, Davies TF, Tomer Y (2008) A multilocus model of the genetic architecture of autoimmune thyroid disorder, with clinical implications. *Am J Hum Genet* **82**: 1349–1356.
Wang XB, Zhao X, Giscombe R, Lefvert AK (2002) A CTLA-4 gene polymorphism at position -318 in the promoter region affects the expression of protein. *Genes Immun* **3**: 233–234.
Yang J, Qin Q, Yan N, Zhu YF, Li C, Yang XJ, Wang X, Pandey M, Hou P, Zhang JA (2012) CD40 C/T(-1) and CTLA-4 A/G(49) SNPs are associated with autoimmune thyroid diseases in the Chinese population. *Endocrine* **41**: 111–115.
Yeşilkaya E, Koç A, Bideci A, Camurdan O, Boyraz M, Erkal O, Ergun MA, Cinaz P (2008) CTLA4 Gene Polymorphisms in Children and Adolescents with Autoimmune Thyroid Diseases. *Genet Test* **12**: 461–464.
Yung E, Cheng PS, Fok TF, Wong GW (2002) CTLA-4 gene A-G polymorphism and childhood Graves' disease. *Clin Endocrinol (Oxf)* **56**: 649–653.
Zaletel K, Krhin B, Gaberscek S, Pirnat E, Hojker S (2002) The influence of the exon 1 polymorphism of the cytotoxic T lymphocyte antigen 4 gene on thyroid antibody production in patients with newly diagnosed Graves' disease. *Thyroid* **12**: 373–376.
Zaletel K, Krhin B, Gaberscek S, Hojker S (2006) Thyroid autoantibody production is influenced by exon 1 and promoter CTLA-4 polymorphisms in patients with Hashimoto's thyroiditis. *Int J Immunogenet* **33**: 87–91.
Zhang Q, Yang YM, Lv XY (2006) Association of Graves' disease and Graves' ophthalmopathy with the polymorphisms in promoter and exon 1 of cytotoxic T lymphocyte associated antigen-4 gene. *J Zhejiang Univ Sci B* **7**: 887–891.