CTLA-4 gene is considered to be one of the most important regions, except HLA, determining the genetic susceptibility to autoimmune diseases, especially thyroid autoimmune disease (1). The association between its polymorphisms and autoimmune disease was found in many populations, irrespectively of the ethnic origin (2). CTLA-4 gene is located on 2q33, close to genes of other regulatory molecules: CD28 and ICOS (3). It consists of 4 exons and 3 introns. The first exon encodes the leader peptide sequence, the second the immunoglobulin domain, with a key motive MYPPPY which binds the ligand, the third encodes the hydrophobic transmembrane domain, and the fourth the cytoplasmic domain (4, 5). Polymorphism leading to substitution of alanine by guanine at position +49 of CTLA-4 gene (+49 A/G) is present in exon 1 and causes an alteration of the leader peptide sequence of the CTLA-4, which makes it an attractive candidate for the functional polymorphism of the CTLA-4 gene.

The aim of the present study was to investigate surface expression of CTLA-4 on peripheral T cells in homozygotes AA and GG at position +49 of CTLA-4 gene in children with Hashimoto's thyroiditis and in healthy controls. Blood samples were obtained from 100 children: 45 with Hashimoto's thyroiditis and 55 controls. CTLA-4 exon 1 polymorphism was defined by SSCP and RFLP with BbvI enzyme. T cells were analyzed with three color flow cytometry by Coulter EPICS XL. We found that CTLA-4 expression was significantly lower in the thyroiditis patients than in controls, but CTLA-4 expression in homozygotes GG and AA was comparable. We therefore conclude that decreased expression of CTLA-4 on T cells in children with Hashimoto's thyroiditis is not dependent on polymorphic changes at position +49 of CTLA-4 gene.

Key words: CTLA-4, Hashimoto's thyroiditis, polymorphism, T cell, homozygotes
5'-GCCTACTTCTGAGACCT-3' and reverse 5'-AGTCTC
ACTCACCTTTGGAGCAG-3', according to the sequence of human
cDNA of CTLA-4 described by Harper and Balzano (5).
Polyorphism at the position +49 of exon 1 of the CTLA-4 gene
was examined by the method of Single Strand Conformation
Polyorphism (SSCP) followed by confirmation test of
restriction fragment- length polymorphism (RFLP) using Bbv I
enzyme (New England BioLabs, Beverly, MA, USA): 10 µl of a
mixture of 1x Neubuffer 2 and 1.5 µl of a restriction enzyme Bbv
I (New England BioLabs, Beverly, MA, USA) was added to 15
µl of a product of PCR. The samples, after covering with mineral
oil, were incubated for 24 h at 37°C. Then, they were
electrophoresed on 2% agarose (60 min, 500mA, 40W, 120V) at
25°C. Electrophoregrams were visualized with ethidium
bromide. The laboratory evaluation was performed by a
laboratory technician, blinded to the information of the sample
origin (from patient or control).

**Cytometric analysis**

Heparinized blood samples from HT children and healthy
controls were diluted three times with saline, and centrifuged for
30 min at 400 g on Histopaque 1077-1 density gradient (Sigma
Diagnostics, St. Louis, MO). Isolated peripheral blood
mononuclear cells (PBMC) were washed three times with saline
and suspended in PBS. PBMC were then incubated with
monoclonal antibodies for 30 min at 25°C in darkness. An
analysis was performed with the use of a combination of
monoclonal antibodies: CD4- FITC/CD28 -PC5/CD152 -PE and
CD8 -FITC/CD82-PC5/CD152-PE obtained from Immunotech
Beckman Coulter Company (Beckman Coulter S.A. Paris Nord,
France). After incubation, samples were fixed and lysed by the
reagent set Uti-Lyse (Dako Cytomation, Gdynia, Poland). The T
cell phenotype was evaluated using the flow cytometer Beckman
Coulter EPICS XL 4C (EPICS XL/XL-MCL, version 2.0
(Beckman Coulter Company, Paris Nord, France).
The results were statistically analyzed by a t-test, using
STATISTICA XL7.0. Genotype frequency analysis was
performed by a Fisher-Snedecor test.

**RESULTS**

In the group of 45 children with Hashimoto's thyroiditis,
13 (29%) patients were homozygous for A allele and 14 (31%)
patients were for G allele. In the control group, 12 (22%)
subjects were homozygous for A allele and 8 (15%) subjects
were for G allele. The statistically significant difference in
Fisher-Snedecor test showed the increased frequency of GG
homozygotes in patients with Hashimoto's thyroiditis (P<0.04,
odds ratio, OR=2.65; confidence interval, CI: 0.99-7.06)
(Table 1).

The surface expression of the CTLA-4 (CD152) on T cells
was compared between AA and GG homozygotes in following
subgroups:

- Homozygotes AA with Hashimoto's disease vs. homozygotes
  AA from controls;
- Homozygotes GG with Hashimoto's disease vs. homozygotes
  GG from controls;
- Homozygotes AA with Hashimoto's disease vs. homozygotes
  AA from controls;
- Homozygotes AA with Hashimoto's disease vs. homozygotes
  GG from controls;
- Homozygotes AA vs. homozygotes GG in the Hashimoto
disease;
- Homozygotes AA vs. homozygotes GG in the control group.

There was no significant difference in the CD152 expression
between AA and GG homozygotes among the Hashimoto
patients (Table 2). In the control group, expression of CTLA-4
on T cells did not differ between AA and GG homozygotes
either. A statistically significant difference in the expression of
CD152 was found only between the children with Hashimoto's
thyroiditis and the healthy controls (P<0.05) (Table 2).

**DISCUSSION**

The finding of an association between polymorphic changes
and a disease is one of the first steps to be followed by an
analysis of genetic predisposition to the disease (1, 11). An
increased frequency of the examined in the present study
polyorphism +49 A/G in patients with autoimmune thyroid
diseases is well confirmed in the literature (2) and our results are
consistent with these observations. Nevertheless, the strongest
evidence for association with the disease is the influence of the
polyorphism on the biological function of the gene product,
involved in the disease development (1). One can speculate that
the polymorphic changes of the CTLA-4 gene could impair the
peptide function in several ways. On the one hand, by decreased
expression of CTLA-4 on T cells, lower affinity for the ligand,
or disturbances in extra-/intracellular signal transduction.
According to this consideration in our laboratory were evaluated
physiological consequences of gene polymorphisms in obesity
or leukemia (12, 13). The polymorphism at the position +49 of
exon 1 of the CTLA-4 gene changes the sequence of leather
peptide of CTLA-4 and, theoretically, can change only the
CTLA-4 expression, but not the affinity to ligands or signaling
pathway. Polymorphic homozygotes GG (Ala/Ala) should
potentially have decreased CTLA-4 expression and impaired
inhibitory function of CTLA-4 compared with wild
homozygotes AA (Thr/Thr). Such hypothesis has been
confirmed by Kouki et al. (14). The authors stated that G allele
at the position +49 of the CTLA-4 gene is associated with
impaired control of T cell proliferation. Maurer et al. (15) also
have suggested that the surface expression of the CTLA-4 and its
intracellular distribution correlates with the genotype at position
+49. Homozygotes GG present with decreased surface
expression of the CTLA-4, whereas activated T cells in

**Table 1. Frequency of particular alleles at position +49 of the CTLA-4 gene.**

<table>
<thead>
<tr>
<th></th>
<th>Homozygotes AA (%)</th>
<th>Homozygotes GG (%)</th>
<th>Heterozygotes AG (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children with Hashimoto's disease (n=45)</td>
<td>29</td>
<td>31*</td>
<td>40</td>
</tr>
<tr>
<td>Healthy children (n=55)</td>
<td>22</td>
<td>15*</td>
<td>64</td>
</tr>
</tbody>
</table>

*Significant difference (P<0.05)
Table 2: T cell antigen expression in patients compared with controls in relation to the frequency of alleles at position +49 of CTLA-4 gene.

<table>
<thead>
<tr>
<th>GROUPS FOR COMPARISON</th>
<th>CD4+CD152+</th>
<th>CD8+CD152+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygotes AA with Hashimoto’s disease vs. homozygotes AA from controls</td>
<td>P=0.004</td>
<td>NS</td>
</tr>
<tr>
<td>Homozygotes GG with Hashimoto’s disease vs. homozygotes GG from controls</td>
<td>P=0.02</td>
<td>P=0.02</td>
</tr>
<tr>
<td>Homozygotes GG with Hashimoto’s disease vs. homozygotes AA from controls</td>
<td>P=0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Homozygotes AA with Hashimoto’s disease vs. homozygotes GG from controls</td>
<td>P=0.01</td>
<td>P=0.005</td>
</tr>
<tr>
<td>Hashimoto’s homozygotes AA vs. homozygotes GG</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Control homozygotes AA vs. homozygotes GG</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Groups compared statistically with a t-test; NS- no statistical difference.

homozygotes AA show another pattern of intracellular distribution (14). The association between polymorphism at position +49 and CTLA-4 expression has also been confirmed by Ligers et al. (16). These authors showed that homozygotes AA present with a higher surface expression CTLA-4 after activation and with a higher level of mRNA for CTLA-4 in unstimulated T cells. In the present study, the baseline percentage of peripheral T lymphocytes with the surface expression of CTLA-4 was evaluated in children with Hashimoto’s thyroiditis and compared with healthy controls. In Hashimoto patients, the percentage of T cells with the surface expression of CTLA-4 was significantly decreased. This difference was not dependent on the presence of the polymorphism at position +49 of exon 1 of the CTLA-4 gene, as in both examined groups, the expression of CTLA-4 did not differ between AA and GG homozygotes. The finding suggests that a lower expression of CTLA-4 in children with Hashimoto's thyroiditis is associated with the presence of disease, but it is not related to the examined polymorphism. In our previous paper, we reported that patients with Hashimoto's thyroiditis have a decreased expression of CTLA-4 on CD4+ and CD8+ T cells (17). Accordingly, one can conclude that this observation is not a simple consequence of the presence of +49 A/G polymorphism. The association of the frequency of the polymorphic G allele with Hashimoto's disease and lack of its influence on CTLA-4 expression may suggest that the +49 A/G polymorphism is probably in linkage disequilibrium with other unknown genetic changes responsible for this effect. Thus, decreased surface expression of CTLA-4 observed in patient with Hashimoto's thyroiditis is not dependent on the genotype at position +49 of exon 1 of the CTLA-4 gene.

Conflict of interests: None declared.

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