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# In children with autoimmune thyroiditis *CTLA4* and *FCRL3* genes – but not *PTPN22* – are overexpressed when compared to adults

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**Abstract**

**BACKGROUND:** Numerous genetic studies revealed several susceptibility genes of autoimmune thyroid diseases (AITD), including *CTLA4*, *PTPN22* and *FCRL3*. These immune-modulating genes are involved in genetic background of AITD among children and adult patients. However, possible age-related differences in overexpression of these genes remain unclear.
**PURPOSE:** The goal of this single centre cohort study was evaluation of expression levels of three (3) genes *CTLA4*, *PTPN22* and *FCRL3* in adult patients and children with autoimmune thyroiditis.
**METHODS:** A total of 47 patients – 24 adults (mean age – 47.7 years) and 23 children (mean age – 12.4 years) with autoimmune thyroiditis were assessed for the level of expression of *CTLA4*, *PTPN22* and *FCRL3* genes, utilizing ABI PRISM® 7500 Sequence Detection System (Applied Biosystem, Foster City, CA, USA).
**RESULTS:** The overexpression of *PTPN22* (mean RQ=2.988) and *FCRL3* (mean RQ=2.544) genes were confirmed in adult patients with autoimmune thyroiditis, at the same time the expression level of *CTLA4* gene was significantly decreased (mean RQ=0.899) ($p<0.05$). Similar discrepancies were not observed in children with autoimmune thyroiditis in whom overexpression of all three genes – *CTLA4*, *PTPN22* and *FCRL3* – was observed. Differences in *CTLA4* and *FCRL3* genes expression levels in patients with autoimmune thyroiditis were found depending on the age, with increased expression levels of *CTLA4* (mean RQ=3.451) and FCRL3 (mean RQ=7.410) in children when compared to adults ($p<0.05$) (Mann-Whitney's U-test). There were moderate negative linear correlations between two genes in question (*CTLA4* and *FCRL3*) expression level and patients' age [correlation coefficient (r)=–0.529 ($p<0.0002$) and –0.423 ($p<0.0032$), respectively; Spearman's rank correlation test].
**CONCLUSION:** Our results are consistent with the hypothesis that there are few age-dependent genetic differences as regards autoimmune thyroiditis in adults and children. Accordingly, *CTLA4* and *FCRL3* genes overexpression may play an important role in children suffering from autoimmune thyroiditis.

ORIGINAL ARTICLE

## INTRODUCTION

Autoimmune thyroid diseases (AITD) are multifactorial disorders in which autoimmunity toward thyroid autoantigens has a specific genetic background and is facilitated by exposure to environmental factors. Among many immune-related genes, which impair the self-tolerance to thyroid autoantibodies and determine the risk of AITD development, the role of *CTLA4*, *PTPN22* and *FCRL3* genes is especially highlighted.

Cytotoxic T lymphocyte-associated factor 4 (CTLA4, also known as CD152) has been shown to confer susceptibility to autoimmune diseases (Gough *et al.* 2005), including AITD (Hou *et al.* 2015). This protein is a negative regulator of T cell response and acts by delivering an inhibitory signal which can reverse the T cell receptor (TCR)-induced stop signal needed for interaction between T cell and antigen presenting cell (APC), thus reducing adhesion periods between these cells, which in – turn – decreases cytokine production and proliferation (Schneider *et al.* 2006; Downey *et al.* 2007, Schneider *et al.* 2008).

PTPN22 has already been recognized as a risk factor of autoimmunity and it is associated with Graves' disease (GD). It is a non-receptor type protein tyrosine phosphatase, expressed mainly in hematopoetic cells. PTPN22 has been shown to attenuate the strength of TCR signals (Wu *et al.* 2006). There are reports indicating that human PTPN22 also inhibits the activity of B cell antigen receptor (Rieck *et al.* 2007; Arechiga *et al.* 2009). Polymorphism of *PTPN22* resulted both in the gain and loss of PTPN22 protein function in T cells in some reports (Rieck *et al.* 2007; Zhang *et al.* 2011).

*Fc receptor-like 3* (*FCRL3*) gene is also involved in the pathogenesis of AITD, particularly in GD. It is an orphan cell surface receptor of unknown function with structural homology to classical receptors for immunoglobulin constant chains (Fc receptors). FCRL3 plays a key role in the development, maturation and function of B-lymphocytes (Matesanz-Isabel *et al.* 2011). The pathogenic activation of FCRL3 expression leads to down-regulation of B-cell receptor-mediated signalling, incomplete anergy and deletion in autoreactive B-cells, and finally to breakdown of B-cell tolerance (Kochi *et al.* 2009). Presence of FCRL3 was also demonstrated on the surface of a subset of Treg cells, characterized by lower relative response to antigenic stimulation and reduced suppressor activity (Swainson *et al.* 2010).

Emerging evidence suggests that these immune-modulating susceptibility genes are involved in pathogenesis of AITD in children (Pastuszak-Lewandoska *et al.* 2013), as well as in adult patients (Lee *et al.* 2015). However, any possible association between *CTLA4*, *PTPN22* and *FCRL3* genes overexpression and patients' age remains unknown. In contrast to other genetic studies evaluating children and adults patients with AITD separately, in this study, for the first time, a representative number of samples from patients of all ages with positive concentrations of antibodies against thyroperoxidase (anti-TPO) have been collected. Data have been analyzed and compared among others as regards the expression of these genes.

## METHODS

Peripheral blood samples from patients with autoimmune thyroiditis, hospitalized in Department of Endocrinology and Metabolic Disease Medical University of Lodz, were obtained. Inclusion criteria for autoimmune thyroiditis were the presence of positive values of antibodies against thyroid autoantigens (anti-TPO and antithyroglobulin or anti-TPO alone) and negative value of antibodies against thyrotropin receptor (anti-TSHR). Patients were divided into two groups based on age: 23 children (aged 6–18 years; mean age 12.4) and 24 adults (aged 19–75 years; mean age 47.7). The study procedures were approved by the Local Ethical Committee of Medical University of Lodz and written informed consent was obtained from all participating adults individuals or – in case of children – from parents or legal guardians. Blood samples from the patients without thyroid autoimmunity served as a control for real-time PCR experiment (calibrator). Total RNA from the blood was extracted according to modi-

**Tab. 1.** Basic measures characterizing gene expressions in selected groups.

| | | N | Mean | SD | SEM | 95% CI | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower CB | Upper CB | | |
| CTLA4 | children | 23 | 3.451 | 4.584 | 0.955 | 1.468 | 5.433 | 0.263 | 17.168 |
| | adults | 24 | 0.899 | 1.364 | 0.278 | 0.322 | 1.475 | 0.039 | 6.305 |
| | both | 47 | 2.147 | 3.558 | 0.518 | 1.103 | 3.192 | 0.039 | 17.168 |
| PTPN22 | children | 23 | 3.353 | 4.076 | 0.850 | 1.591 | 5.116 | 0.304 | 18.762 |
| | adults | 24 | 2.988 | 5.332 | 1.088 | 0.736 | 5.239 | 0.215 | 25.291 |
| | both | 47 | 3.167 | 4.711 | 0.687 | 1.783 | 4.550 | 0.215 | 25.291 |
| FCRL3 | children | 23 | 7.410 | 9.751 | 2.033 | 3.193 | 11.626 | 0.949 | 37.045 |
| | adults | 24 | 2.544 | 2.979 | 0.608 | 1.286 | 3.803 | 0.337 | 14.569 |
| | both | 47 | 4.925 | 7.480 | 1.091 | 2.729 | 7.122 | 0.337 | 37.045 |

(SD – standard deviation; SEM – standard error of the mean; CI - confidence interval; CB – confidence bound).

fied Chomczynski and Sacchi's method. The purity of total RNA was assessed by NanoDrop® ND-100 spectrophotometr (data not presented). Total RNA was used in the first strand cDNA synthesis with TaqMan® Reverse Transcription Reagents (Applied Biosystem, Branchburg, New Jersey, USA) according to manufacturers' instruction. Real-time PCR was performed on the ABI PRISM® 7500 Sequence Detection System (Applied Biosystem, Foster City, CA, USA) by using *Taq*Man® Universal PCR Master Mix (Applied Biosystem) and *Taq*Man® Gene Expression Assays probe and primer mix (Applied Biosystem) according to the manufacturer's specification. The Assays Identification numbers were: CTLA4: Hs03044418_m1; PTPN22: Hs01587518_m1, FCRL3: Hs00364720_m1. Thermal cycler conditions were as follows: hold for 10 min. at 95 °C, followed by two-step PCR for 50 cycles of 95 °C for 15 s followed by 60 °C for 1 min. Amplification reactions, in triplicate for each sample, were performed and the results were normalized to the ACTB gene expression level. An analysis of relative gene expression data was performed, using the $2^{-\Delta\Delta CT}$ method on an ABI PRISM® 7500 Sequence Detection System Software. The calibrator was prepared as a cDNA mix from all cDNA samples (separately, 5 healthy adult controls and 2 healthy children controls). The fold change in studied gene expression, normalised to endogenous control, was calculated using: $RQ=2^{-\Delta\Delta CT}$ (Table 1).

All statistical calculations were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp. Armonk, NY) with the level of statistical significance $p<0.05$. Basic measures of location (i.e. mean), measures of dispersion (SD, SEM), and 95% confidence interval for the mean and minimum and maximum values were calculated to provide detailed descriptions of gene expressions in selected groups (Table 1). A *t*-test was used to evaluate *CTLA4, PTPN22* and *FCRL3* gene expression levels in children and adult groups. Subsequently, the data were statistically analyzed, using non-parametric Mann-Whitney's *U* test in order to compare expression level values (RQ) *CTLA4*, *FCRL3* and *PTPN22* between studied independent groups (adults vs. children). Furthermore, Spearman's rank correlation coefficient was used to describe the correlation between *CTLA4*, *FCRL3* and *PTPN22* genes expression levels and patient's age.

## RESULTS

The overexpression of *PTPN22* (mean RQ=2.988) and *FCRL3* (mean RQ=2.544) genes were confirmed in adult patients with autoimmune thyroiditis, at the same time the expression level of *CTLA4* gene was significantly decreased (mean RQ=0.899) ($p<0.05$). Similar discrepancies were not observed in children with autoimmune thyroiditis in whom the overexpression of all three genes – *CTLA4*, *PTPN22* and *FCRL3* – was observed.

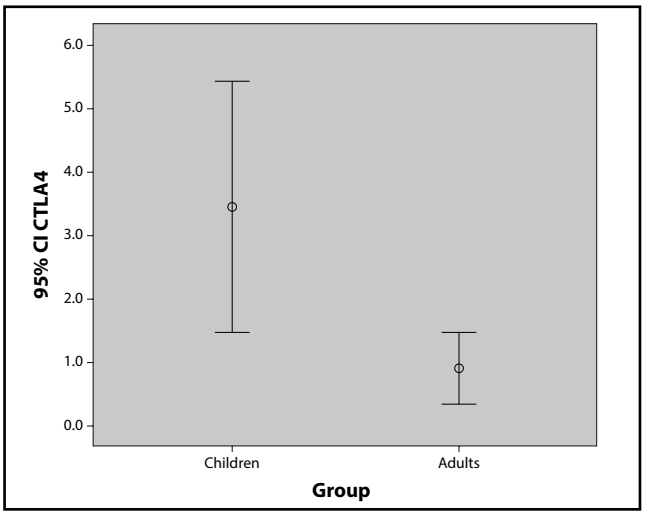


**Fig. 1.** The box-and-whisker plot diagram representing the different expression levels of *CTLA4* in adults and children (data represent means ± and 95% confidence interval).

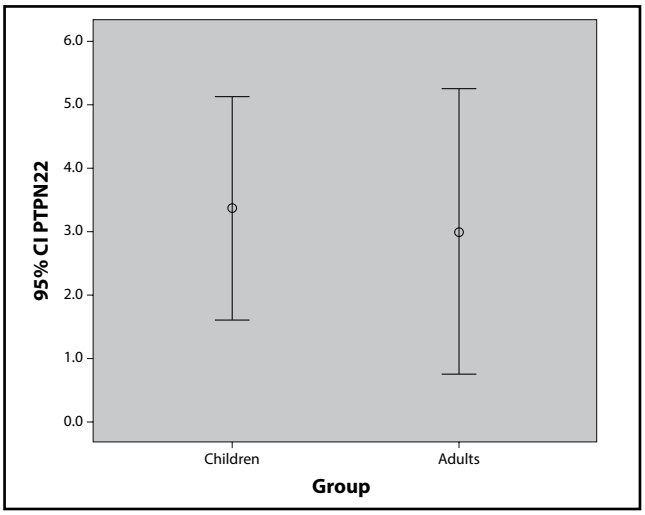


**Fig. 2.** The box-and-whisker plot diagram representing the different expression levels of *PTPN22* in adults and children (data represent means ± and 95% confidence interval).

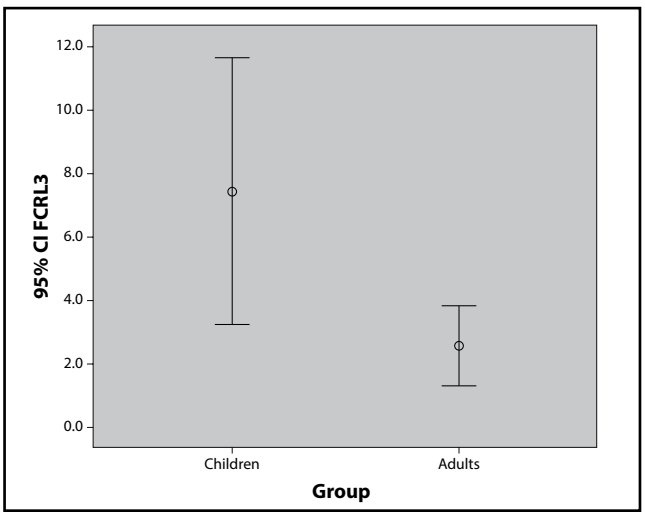


**Fig. 3.** The box-and-whisker plot diagram representing the different expression levels of *FCRL3* in adults and children (data represent means ± and 95% confidence interval).

Differences in *CTLA4* and *FCRL3* genes expression levels in patients with autoimmune thyroiditis were found depending on the age, with increased expression levels of *CTLA4* (mean RQ=3.451) and *FCRL3* (mean RQ=7.410) in children when compared to adults ($p<0.05$) (Mann-Whitney's U test). The box-and-whisker plot diagrams, representing the different expression levels of *CTLA4*, *PTPN22* and *FCRL3* genes in adults and children, are shown in Figures 1–3, respectively.

Accordingly, there were moderate negative linear correlations between two genes in question (*CTLA4* and *FCRL3*) expression level and patients' age [correlation coefficient (r)=–0.529 ($p<0.0002$) and –0.423 ($p<0.0032$), respectively; Spearman's rank correlation test]. Such relationship was not recorded for *PTPN22* gene (r=–0.154, $p>0.3$).

## DISCUSSION

Autoimmune thyroid diseases arise due to complex interactions between environmental and genetic factors (Effraimidis & Wiersinga 2014). Possible involvement of environmental insults, endocrine disruptors and duration of exposure are unquestioned risk factors for the development of diseases. That is confirmed by far less prevalence of AITD in children than in adults (Wiersinga 2014). However, latest studies demonstrate and highlight a strong genetic influence on the development of AITD, especially in children and adolescence (Pastuszak-Lewandoska *et al.* 2013; Wiersinga 2014). Accordingly, female dominance as regards autoimmune diseases – commonly observed in adulthood – is less marked in children and this phenomenon cannot be explained exclusively by shorter exposure to environmental factors. This fact possibly results from specific age-related genetic background in this group of patients (Wiersinga 2014). In accordance, AITD in children is quite frequently related to various genetic syndroms (Glick *et al.* 2013). Identification of AITD in childhood enhances risk of developing other autoimmune disorders with genetic background, including diabetes, celiac disease, etc.(Tolone *et al.* 2009; Glick *et al.* 2013; Riquetto *et al.* 2015). Significant progress has been made in finding the AITD susceptibility genes and understanding the mechanisms by which they confer risk for disease in both adults and children. On the other hand, little is known about particular, risk genetic factors involved in pathogenesis of autoimmune thyroiditis in childhood and adolescence.

In the present study, the increased expression of *CTLA4*, *PTPN22* and *FCRL3* genes in children with autoimmune thyroiditis has been proved. Comparing children and adults with autoimmune thyroiditis, significant differences in expression of *CTLA4* and *FCRL3* have been observed, with higher values in children (Figures 1–3). This relation has not been recorded for *PTPN22* gene. Our further statistical analysis has confirmed existence of moderate negative correlation between *CTLA4* or *FCRL3* gene expression level and patients' age. This observation speaks in favour of the hypothesis on age-specific genetic risk factors acting in childhood and precipitating autoimmune thyroiditis in children.

To our knowledge, there are only scarce reported data on the expression of *CTLA4*, *PTPN22* and *FCRL3* in pathogenesis of autoimmune thyroiditis in children. In contrast, the significant role of above genes in the pathogenesis of not only AITD, but also in several other autoimmune processes in adults has been demonstrated in many reports. According to the previous studies, a polymorphism in *FCRL3* is predisposing to rheumatoid arthritis, systemic lupus erythemathosus and biliary cirrhosis (Kochi *et al.* 2005; Effraimidis & Wiersinga 2014; Zhao *et al.* 2013) and interestingly, the increased expression of *FCRL3* by real-time PCR has also been confirmed in GD (Zhao *et al.* 2013) and endometriosis (Szczepańska *et al.* 2013).

Noteworthy, *PTPN22* gene was originally proved to be associated with type 1 diabetes mellitus (DM1) in children (Liu *et al.* 2015), subsequently it was shown to increase the risk of other autoimmune diseases, including GD, rheumatoid arthritis, juvenile idiopathic arthritis and autoimmune primary adrenal insufficiency (Lee *et al.* 2006), but it reduced the risk of Crohn's disease (Spalinger *et al.* 2013).

*CTLA4* has been identified as the most important genetic factor in both GD and Hashimoto thyroiditis (HT) in adults (Hou *et al.* 2015). Results of studies by Kucharska *et al.* (2010, 2013) demonstrated that surface expression of CTLA4 on T-cells is decreased in children with HT. Our present results are consistent with above cited data in group of adult persons, but not in children in whom expression of *CTLA4* has been increased. Our data speak for an essential role of *CTLA4* gene in development of childhood autoimmune thyroiditis.

Autoimmune thyroid diseases are often associated with DM1. It has been shown that both *PTPN22* and *CTLA4* genes are associated with co-occurrence of HT and DM1 in the same individual (Tomer *et al.* 2015). It is to be emphasized that increased expression of these genes in patients involved in our study could predispose them to another autoimmune disease in future.

Summing up, the pathogenesis of autoimmune thyroiditis is much more complex than formerly thought and relations gene-gene and gene-environment have hardly been researched. It is possible that there are many more still unknown susceptibility genes, each variant contributing just a little to the development of autoimmune thyroiditis. In our opinion, the application of knowledge about susceptibility genes is slowly entering clinical practice.

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***Competing interests:*** *The authors declare that they have no competing interests.*

***Authors' contributions:*** *KW-D designed the study, carried out the molecular genetic studies, participated in coordination of the study and in preparation of manuscript. KK-R, RS, AZ provided blood samples for analysis, collected from their patients. AL senior author, supervised the study and wrote the final version of manuscript. All authors have read and approved the final manuscript.*

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