ORIGINAL ARTICLE

Familial autoimmune thyroid disease and *PTPN-22*

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ABSTRACT

Aim Autoimmune thyroid disease (AITD) is a multifactorial disease with a genetic predisposition. The protein tyrosine phosphatase-22 (*PTPN-22*) gene is a powerful inhibitor of T-cell activation. The aim of this study was to compare messenger RNA (mRNA) *PTPN22* expression between healthy persons and patients with hypothyroidism and with their affected relatives.

Methods This was a cross-sectional, prospective and descriptive study. DNA was extracted from leukocytes (4,000-10,000 cells) using the Magna Pure LC 2.0 Instrument and MagNA Pure LC RNA Isolation Kit I (Roche, Germany). A real-time polymerase reaction (qPCR) was performed utilizing the primer sets specific for the *PTPN-22* gene, and the succinate dehydrogenase complex, the subunit A, Flavoprotein (Fp) (*SDHA*) constitutive gene. All reactions were performed with the 7500 Fast Real Time PCR System (Applied Biosystems, Applera International, Inc. Cheshire, UK) employing the SYBR Advantage qPCR Premix Kit (Clontech, USA).

Results Twenty five patients with AITD (hypothyroidism), all females (mean age 39.6 ± 11.8 years) and 23 control subjects (mean age 24.4 ± 4.2 years) were included in the study. There was no statistical difference between both groups in *PTPN-22* mRNA expression (p = 0.125).

Conclusion There is no clear difference in mRNA *PTPN-22* expression. The ideal genes for a systematic screening for familial AITD are yet to be found.

Key words: genetic screening, hypothyroidism, qPCR

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INTRODUCTION

Autoimmune thyroid disease (AITD) is the most common autoimmune condition, affecting approximately 2% of the female population and 0.2% of the male population (1). Although the exact etiology is not yet known, AITD is multifactorial in that a genetic predisposition combines with environmental risk factors to promote disease (2,3).

Up to 90% of patients with AITD-related hypothyroidism are anti-thyroid peroxidase (TPO) antibody-positive. It should be noted that 10–15% of the general population are positive for anti-TPO antibodies and that low titers are less specific for AITD (2).

Symptoms of hypothyroidism may be subtle, even with marked biochemical derangement, but as the disease progresses, subclinical and then clinical hypothyroidism appears. In the early stages of hypothyroidism, the thyroid stimulating hormone (TSH) may be normal and anti-thyroperoxidase (TPO) antibodies may be positive with or without goiter. Later, TSH elevation becomes modest (5–10 IU/ml) with a normal FT4 (biochemical or subclinical hypothyroidism).

Some studies have reported that siblings of persons affected by Graves disease or hypothyroidism have a 33% chance of developing the disease (4). In the casuistry of Ciprés Grupo Médico (CGM), Toluca, Mexico, the percentage of mothers with AITD and with an affected daughter is 13% (5).

The first gene found to be associated with both Graves disease and hypothyroidism was *HLA-DR3*. Since this discovery, significant progress has been made in the genetic contributions and the mechanisms underlying thyroid autoimmunity. To date, several loci have been associated with AITD. In addition to *HLA-DR* subtypes, these include two groups of the non-major histocompatibility complex (MHC) genes, e. g., immunoregulatory genes (*CD40*, *CTLA-4*, *PTPN-22*, *FOXP3*, and *CD25*), and thyroid-specific genes (*Tg* and *TSHR*) (6,7). Polymorphic variations of all these genes have been identified and linked with AITD susceptibility, but the existing studies have often given inconsistent results, with some showing associations and others not (8,9). One of the many unexpected findings of these genetic studies is that the majority of the genes identified exert very minor effects (10). Indeed, with the exception of the DRb1-Arg74 HLA variant, which resulted in an Odds ratio (OR) for Graves' disease of >5, all of the remaining AITD genes gave very low OR of <1.5 (11); on the other hand, family history is positive in about 50% of patients with AITD. It is usually supposed that a strong genetic effect in the disease is related with the inheritance of many genes with small effect (12).

Lymphoid tyrosine phosphatase (Lyp) encoded by protein tyrosine phosphatase-22 (*PTPN-22*) gene locus on chromosome 1p13.3–13.1, such as *CTLA-4*, is a powerful inhibitor of T-cell activation (13). A single nucleotide polymorphism in *PTPN-22* has been reported as an autoimmune susceptibility locus that associates with type 1 diabetes (14), rheumatoid arthritis (15), systemic lupus erythematosus (16), Hashimoto's thyroiditis (17), Graves' disease (18), Addison's disease (19), Myasthenia gravis (20), vitiligo (21), systemic sclerosis (22), and juvenile idiopathic arthritis (23), suggesting that allelic variants of *PTPN-22* could predispose to a more general autoimmune diathesis.

Protein tyrosine phosphatase and protein tyrosine kinases (PTKs) are enzymes that specifically catalyze the reversible addition or release of phosphate groups from tyrosine residues on signaling intermediates. Broadly speaking, PTK amplify signals, while the mode, tempo and duration of the signal are governed by PTP. Protein tyrosine phosphatase and PTK are divided into two groups: receptor (membrane bound-RPTP or RPTK) or non-receptor (cytoplasmic-NRPTP or NRPTK) (24).

Aim of this study was to compare mRNA *PTPN-22* expression between healthy persons and patients with AITD.

PATIENTS AND METHODS

Study population

This was a cross-sectional prospective and descriptive study conducted at the Medical Sciences Research Center (CICMED), Autonomous University of the State of Mexico (UAEMex) and at the Ciprés Grupo Médico (CGM), both in Toluca, Mexico, from August 2013 to July 2014. Diagnosis of thyroidopathy was made based on the

presence of a thyroid profile with TSH ≥ 10 IU together with a significant titer of autoantibodies (anti-thyroglobulin > 40 IU or anti-TPO > 35 IU).

The study was approved by the Institutional Review Board of the Medical Sciences Research Center (CICMED), UAEMex (25/09/13) and was performed according to the ethical standards of the Helsinki Declaration (Fortaleza, Brazil). Written informed consents were obtained from all patients and their relatives who participated in this project.

Sample calculation

Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test, 25 subjects per group were required for the recognition as statistically significant of a difference ± 4 relative units (RU). The common standard deviation was assumed to be 5.

Clinical measurements

Weight (kg), height (m) (Seca, GmbH, Germany) and waist circumference (cm) were measured in all participants. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared.

RNA extraction

A blood sample (vacutainer tubes) was taken from each patient. Leukocytes were obtained according to the ACK-lysing buffer (LONZA) protocol. Briefly, a peripheral blood sample was placed in an EDTA tube and then centrifuged at 2,500 rpm for 10 min. All samples were maintained at -80°C until further analysis.

The RNA was extracted from leukocytes (4,000-10,000 cells) in the Magna Pure LC 2.0 Instrument using the MagNA Pure LC RNA Isolation Kit I (Roche, Germany). After extraction, the RNA was quantified using a Nano Photometer (Implen GmbH, Germany), reporting concentration (in µg/mL) and purity (as 260/280 absorbance).

Gene expression

A total of 200–400 ng total mRNA was reverse-transcribed into complementary RNA (cDNA) utilizing a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science). The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was assessed according to the ratio of the absorbance values at 260 and 280 nm; purity ranged between 1.8 and 2.1, demonstrating a high RNA quality. The samples were measured with a NanoPhotometer (Implen GmbH, Germany), and the extracts were then adjusted to a concentration of 20 µg RNA L^{-1} for the PCR reaction. A real-time Polymerase chain reaction (qPCR) was performed using the primer sets specific for the *PTPN-22* (NM_001193431.1) as follows: forward: 5'-AGGCAGACAAAACCTATCCTACA-3', reverse: 5'-TGGGTGGCAATATAAGCCTTG-3' and the succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*) constitutive gene (NM_004168) as follows:, forward: 5'-AGAGGGAGGCATTCTCATTAAC-3', reverse: 5'-ACCGAGACACCACATCTCTA-3'. All reactions were performed with the 7500 Fast Real Time PCR System (Applied Biosystems, Applera International, Inc., Cheshire, UK) using the SYBR Advantage qPCR Premix Kit (Clontech). The expression levels of the genes were examined by placing 4 µL of the reverse transcription mix for each PCR reaction in a total volume of 20 µL. The thermal cycling conditions were as follows: 10 min at 95°C followed by 45 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. The comparative threshold cycle (CT) method was used to calculate fold amplification, as specified by the manufacturer.

The Taguchi method was employed to set the best conditions for primer amplification. The fold change in *PTPN-22* was normalized against the constitutively expressed reference gene and then compared with the controls (healthy volunteers) as follows: $2^{-\Delta\Delta CT}$, where ΔΔCT=(CT-target − CT-reference) treated-sample − (CT-target − CT-reference) calibrator-sample. Calibrator-sample refers to the expression level (1×) of the target gene normalized to the constitutive gene. The calibrator was chosen from healthy volunteers and was given a relative expression value of 1.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney U-test after performing the Levene test. The normality hypothesis was tested using the Kolmogorov-–Smirnov test. Statistical significance was tested at the $p \leq 0.05$ level.

RESULTS

Twenty five patients with AITD (hypothyroidism), all females (mean age, 39.6 ± 11.8 years) and 23 control subjects (mean age, 24.4 ± 4.2 years) were included in the study. In Table 1, we depict the affected relatives of the patients, which included one case for a grandmother, nine cases for a mother, one for a father, seven for a sister, one for a brother, four for an aunt, one for an uncle, and six for a cousin. Average time of disease evolution was 18.4 ± 24.2 months prior to participation in this study with a mean Levothyroxine dose of 66.2 ± 23.3 μg per day.

Table 1. Characteristics of thirty three affected relatives in 25 patients with autoimmune hypothyroidism

Case	Grand-mother	Mother	Father	Sister	Brother	Aunt	Uncle	Cousin
1						X*		
2								X*
3								X*
4		X						
5		X						
6						X*,†		X† (3)
7		X						
8					X			
9			X					
10		X						
11		X		X				X*
12						X†		X†
13				X				
14	X*							
15		X						
16							X†	X† (2)
17				X				
18				X				
19		X						
20				X				
21		X						
22		X						
23				X				
24				X				
25						X*		

*maternal, †paternal

Anthropometrically, while the patients showed a BMI of 24.7 ± 2.4, the control group showed one of 25.1 ± 1.7.

According to the place of residence there were 12 patients from Toluca, five from Mexico City, four from Metepec, and one patient from each Otzolotepec, Naucalpan, Zinacantepec, and Guadalajara.

In relation to antibodies, six patients were positive for anti-thyroglobulin, five for anti-TPO, and 14 for both.

In the qPCR analysis there was no statistically significant difference in *PTPN-22* expression between both groups (p=0.125). In the subgroup of patients with affected relatives, the 50th percentile of *PTPN-22* expression was 1.13 RU (Figure 1).

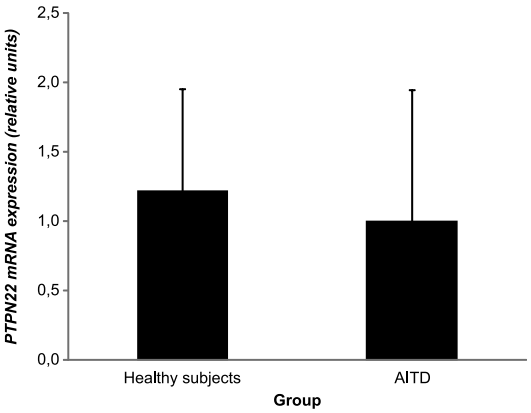

Figure 1.

DISCUSSION

The comparable prevalence and incidence of AITD in geographically different populations suggest a significant genetic effect (19,20). In this regard, the purpose of this study was not to associate a geographical location with AITD, due to lack of information related with this topic in Latin America. In another line, obesity has been postulated as a factor linked to the cause of AITD (21). Although this is a strong argument, in our sample population this possible link does not play a critical role as the patients maintained a normal BMI.

In all patients with associated diseases, AITD is usually detected in its initial phase when thyroid function is preserved, with normal or only slightly elevated TSH levels. At this stage, signs and symptoms of thyroid disease are usually absent, but because worsening of thyroid function is a possibility, early recognition of thyroid dysfunction is necessary to prevent the negative effects of hypothyroidism on growth and metabolic function (16). In our study, we evaluated patients who had been already diagnosed.

Subclinical thyroid disease is a common clinical problem, and because the majority of patients are asymptomatic, screening is the only way to detect the condition in most patients (22).

In our study, we performed an initial familial survey without finding a clear difference in *PTPN-22* mRNA expression. This is in accordance with Inoue et al. whose authors only found an association of *CD40* and *FCRL3* gene polymorphisms with Graves' disease intractability, and a *ZFAT*

polymorphism association with Hashimoto's disease severity, but neither *PTPN-22* -1123C/G nor *PTPN22* SNP37 were associated with any option (23). Contrariwise, Ichimura et al. found an association of the SNP37 of the *PTPN-22* gene with susceptibility to Graves' disease in a Japanese population (24).

In an initial attempt, a difference in *PTPN-22* expression between familiar hypothyroidism and healthy subjects cannot be concluded from our results. In contrast, several other genes have been tested without a definitive conclusion (25-26) but of these genes taken together probably do not explain more than about 10% of the heritability of AITD (27).

We cannot exclude two kinds of bias in our study: recall bias and 'lead-time' bias. In the first case, we registered four patients with affected relatives who initially denied knowing another relative suffering from hypothyroidism until a second clinical interview. In the second type of bias, it is possible that, with a different evolution time with biochemical hypothyroidism, *PTPN-22* expression could exhibit different patterns. Unfortunately, our sample size limits us to perform a stratified analysis. Expecting a lower RU difference between healthy subjects and families affected with AITD implies a higher number of persons per group to be analyzed.

The question pertaining to how to explain the familial heritability may be more straightforward. First, the female preponderance is explained partially by fetal microchimerism and X-chromosome inactivation (27). Second, imprinted genes and epistasis, the modification of expression of one gene by one or several other genes, are believed to be important genetic contributors to complex diseases. We could not forget that in addition to the gene–gene interactions, the clinical phenotype of affected individuals is also influenced by gene–environment interactions (28).

In summary, the results of our study indicate that the ideal genes for systematic AITD screening continue to be missing, but these must be defined in order to be carried out in relatives of patients with AITD, including the offspring of these patients. *PTPN-22* remains a possible gene candidate for further study including its promiscuous association with familial AITD, but it would be better to study a set of genes. Such a determination would have huge implications in our current screening strategies for diagnosing earlier novel thyroidopathies.

Admittedly, the sample size of our family collection remained insufficiently large for conclusion of and exclusion of the participation of *PTPN-22* in the genesis of AITDs in Mexican population, but we must take into account that studies in different geographic regions revealed ethnic differences in associations most probably due to founder effects and/or to the presence or absence of certain variants in specific ethnic groups (4-8). Further studies including proteomic analyses are required.

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TRANSPARENCY DECLARATIONS

Conflict of interest: none to declare.

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