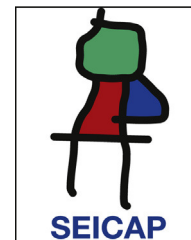




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## ORIGINAL ARTICLE

# Analysis of *FOXP3* gene in children with allergy and autoimmune diseases



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Treg

## Abstract

**Background:** Allergy and autoimmunity are important immunological entities underlying chronic diseases in children. In some cases both entities develop simultaneously in the same patient. *FOXP3* gene codes for a transcription factor involved in regulation of the immune system.

Considering that regulatory T cells are involved in controlling immunological disease development, and the relevant role of *FOXP3* in this kind of T cells, the objective of this study was to analyse the *FOXP3* gene in the most prevalent autoimmune diseases and/or allergies in childhood in a European population.

**Methods:** A total of 255 Caucasian individuals, 95 controls and 160 patients diagnosed with allergic, autoimmune or both diseases were included in this study. The molecular analysis of *FOXP3* was performed by DNA sequencing following the recommendations for quality of the European Molecular Genetics Quality Network. Genomic DNA was extracted from peripheral blood of all participants and was amplified using the polymerase chain reaction. After the visualisation of the amplified fragments by agarose gel-electrophoresis, they were sequenced.

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**Results:** Thirteen different polymorphisms in *FOXP3* gene were found, seven of which had not been previously described. The mutated allele of SNP 7340C>T was observed more frequently in the group of male children suffering from both allergic and autoimmune diseases simultaneously ( $p = 0.004$ , OR = 16.2 [1.34–195.15]).

**Conclusions:** In this study we identified for first time genetic variants of *FOXP3* that are significantly more frequent in children who share allergic and autoimmune diseases. These variants mainly affect regulatory sequences that could alter the expression levels of *FOXP3* modifying its function including its role in Treg cells.

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## Introduction

Chronic diseases affecting children produce a significant impairment in their quality of life, an alteration in familial and social relationships, and an increase in morbidity and mortality. Many of them are of immune origin, such as allergic and autoimmune diseases. Allergies are due to an immunological response to a usually innocuous antigen (allergen) resulting in the proliferation of T-helper type 2 (Th2) lymphocytes. Autoimmune diseases are produced by an immune response with proliferation of Th1 lymphocytes, which react against own antigens recognised as foreign (autoantigens). Genetic and environmental factors participate in their pathogenic mechanisms. Asthma, rhinoconjunctivitis (RC) and atopic dermatitis (AD) are the most prevalent allergic diseases, and type 1 diabetes mellitus (DM1), coeliac disease (CD) and autoimmune thyroiditis (AT) stand out among autoimmune diseases.

The IPEX syndrome (immunodysregulation, polyendocrinopathy, enteropathy, X-linked), also called XLAAD (X-linked autoimmunity-allergic dysregulation), encompasses both immune pathologies. Forkhead Box P3 (*FOXP3*) was identified as a gene involved in this syndrome.<sup>1</sup> However, in some patients with IPEX symptoms no mutations have been identified in the *FOXP3* coding region being diagnosed as IPEX-like syndrome.<sup>2,3</sup> Mutations have been described in the regulatory regions in some of them.<sup>2</sup>

*FOXP3* gene, also called *JM2*, is located in Xp11.23 and has eleven coding and three non-coding exons (Fig. 1). It is expressed mainly in a subgroup of CD4+ cells, called T regulatory cells (Treg).<sup>4</sup> The *FOXP3* protein is the most important marker of this cell subtype. Its function is not completely established, but it is involved in Treg cell activation.<sup>5,6</sup>

Treg cells represent 5–10% of CD4+ T cells.<sup>7</sup> They also express the alpha chain of the IL-2 receptor, CD25. In addition, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid induced tumour necrosis factor receptor family related gene (GITR) are also important for Treg function.<sup>4,5</sup> These Treg cells regulate the innate and adaptive immunity, thus contributing to the maintenance of immune tolerance.<sup>6,7</sup> When their function is affected, autoimmune and allergic diseases can appear. Moreover, Treg cells regulate the immune system involved in host defence against infection and tumour proliferation.<sup>7,8</sup> The mechanisms involved in these immunosuppressive effects are not well elucidated; some studies suggest they are cell contact-dependent,<sup>9,10</sup> while others report them to be cell contact-independent.<sup>10</sup> *FOXP3* is thought to interact to

DNA assisted by *FOXP3* cofactors orchestrating a transcription network by multimerisation. Thus *FOXP3* functions may change depending on the partners forming the so-called *FOXP3* interactome. *FOXP3* is considered a multifaceted transcription factor that regulates immune homeostasis.<sup>11</sup> It stimulates the production of inhibitory cytokines, like interleukin-10 (IL-10) and tumour growth factor- $\beta$  (TGF- $\beta$ ), suppressing IL-2 and IL-4. When this regulation is not present, autoimmunity and allergy can emerge.<sup>5,7</sup>

Considering that Treg cells are involved in controlling the development of autoimmune and allergic diseases, and the relevant role of *FOXP3* in these cells, our hypothesis was that *FOXP3* could be involved in the development of immune diseases and the objective of this study was to analyse the *FOXP3* gene for polymorphisms associated with the most prevalent autoimmune and/or allergic diseases in children.

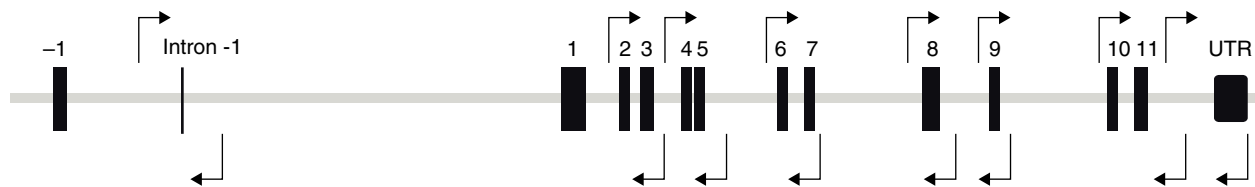
## Materials and methods

### Participants

This is an observational, case-control study in which 255 non-related Caucasian individuals were included: 95 controls and 160 patients that were recruited at the Allergy and Paediatric Departments of the University Hospital of Salamanca. The study was performed following the recommendations of the Hospital's Ethics Committee. An informed written consent was obtained from the children's parents or caregivers.

All patients were under 18 years old, and were affected by one or more autoimmune diseases (DM1, AT and/or CD) and/or atopy. Diagnosis was according to World Health Organization and American Diabetes Association criteria for DM1;<sup>12</sup> thyrotropin up to 10  $\mu$ U/mL plus thyroid autoantibodies for AT;<sup>13</sup> the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) criteria for CD;<sup>14</sup> atopy was defined by a positive skin prick test to at least one of a battery of common aeroallergens previously described;<sup>15</sup> in addition, patients had rhinoconjunctivitis, and/or asthma and/or atopic dermatitis diagnosed by an allergologist.

Controls were selected from adults, confirming the absence of atopy or autoimmune disease during childhood and adulthood.<sup>16</sup> The following inclusion criteria were required: (i) absence of autoimmune diseases; (ii) absence of clinical symptoms compatible with bronchial asthma or other respiratory diseases; (iii) absence of rhinoconjunctivitis or atopic dermatitis; (iv) absence of other allergic



**Figure 1** *FOXP3* gene scheme. Numbers indicate exonic regions and arrows represent primers employed in this study.

diseases; and (v) negative skin prick tests to the same battery of common aeroallergens employed with patients.

### Molecular analysis of *FOXP3* gene

A molecular analysis of *FOXP3* was carried out in all subjects included in the study. All laboratory procedures were developed following the recommendations of quality of European Molecular Genetics Quality Network.<sup>17</sup> Genomic DNA was extracted from peripheral blood with MagNA Pure Compact System (Roche, Barcelona, Spain). *FOXP3* regions were amplified using the polymerase chain reaction (PCR) (Fig. 1).

The pair of primers ATCTCCATGAGCCTCAGTTTC and CGT-TTTTGGAGGGTGGG were used to amplify exons 2 and 3, AGTCAAATGTCAGCACCTG and ATGGGGTAAATAACAGCAC for exons 4 and 5, AGAGGGAGACTGAGGTAGAGAG and TGAATGTGAGGTTAGGTTCC for exons 6 and 7, AGGCT-GAGGCAGAAGACTTG and CCAAGGGAGTCAGGGCTA for exon 8, CTGGACACGGGTGTTGACGG and GCGACTCAGAGGGA-GACAGG for exon 9, ATGTCAGATGGCTCGGGGTAG and TCAGGGCTGTGCTTGTGTG for exons 10 and 11, GCCAAACA-GAGCCTTCACAAC and GTGATGGGGGCTGGTGAG for 3'UTR region and TCGATTCTGCAGGCTTAGCA and GGAAATGGAG-GTATGGAGAGGT to amplify intron -1. The PCR was carried out in an MWG-BIOTECH thermocycler (Ebersberg, Germany). The amplification programme included one cycle of 95°C for 5 min; 35 cycles at 94°C for 30s, annealing temperature (60–67.5°C) of each reaction for 1 min and 72°C for 1 min; and an extension period at 72°C for 5 min. The annealing temperature was established depending on annealing temperature of the pair of primers used: 60°C for exons 4, 5, 6 and 7; 61°C for exons 2 and 3; 63°C for exon 8; 64°C for intron -1; 65°C for exon 9; and 67.5°C for exons 10 and 11 and 3'UTR region. Following PCR, the amplified fragments were visualised by horizontal agarose gel-electrophoresis using Redsafe (iNtRON Biotechnology Inc., Korea). The PCR products were purified using ExoSAP-IT (Affymetrix, Barcelona, Spain) or IllustraExoSTAR 1-step or 2-steps (VWR International Eurolab, Barcelona, Spain) and sequenced in a 3100 Genetic Analyzer Sequencer using the same PCR primers (Applied Biosystems, CA, USA).

### Bioinformatic analysis

NG\_007392.1, the reference sequence of *FOXP3*, was identified by OMIM platform (<http://www.ncbi.nlm.nih.gov/omim>) and polymorphisms were sought in an on-line platform NCBI (<http://www.ncbi.nlm.nih.gov/snp>). Primers were designed with VectorNTI 10.3.0© 2006 Invitrogen Corporation (Carlsbad, California, USA) and they were

optimised with the on-line programmes Beacon Designer (<http://www.premierbiosoft.com/qpcr/index.html>) and Netprimer (<http://premierbiosoft.com/netprimer/index.html>). Chromas 2.3 (Technelysium. Pty. Ltd. 1998–2004, South Brisbane, Australia) was used to visualise chromatograms of each sequenced fragment; and sequence alignments were then performed using Vector NTI to compare with the reference sequence of *FOXP3*, NG\_007392.1. ExPASy software platform (<http://web.expasy.org/translate>) was used for protein predictions.

### Statistical analysis

A descriptive analysis was performed using percentages for qualitative variables, and measures of central tendency (mean) and dispersion measures (standard deviation) for quantitative variables. The allele, genotype and haplotype frequencies were compared in patients and controls. As the *FOXP3* gene is located on chromosome X males and females were separately analysed. These frequencies were compared between groups using  $\chi^2$ , Pearson and Fisher's exact test. Odds ratios (95% Confidence Interval) were calculated. The Hardy-Weinberg equilibrium was tested by a  $\chi^2$  test (Pearson's *P*-value). To avoid type I error, the statistical power and the false positive report probability (FPRP) were calculated, and a more stringent *p* value of 0.01 was considered statistically significant instead of 0.05. Bonferroni was applied when proceeded. To analyse the data the software package SPSS version 18.0 (Chicago, Illinois, USA) and SHEsis software platform (<http://analysis.bio-x.cn/myAnalysis.php>) were employed.

### Results

As stated, 255 unrelated Caucasian individuals were included, 95 controls and 160 patients, 43.2% of controls (mean age of  $53.7 \pm 16.9$ ) and 55.6% of patients (mean age  $8.6 \pm 3.9$ ) were males; 76 (47.5%) patients were atopic, 72 (45%) had an autoimmune disorder and 12 (7.5%) had both atopy and autoimmunity.

In the *FOXP3* gene, 13 different polymorphisms were found (Table 1), seven of which had not been previously described. Gene frequencies of these SNPs, separated by gender, were calculated in both control and case groups (Tables 2 and 3). Hardy-Weinberg equilibrium was accomplished in all groups. Statistically significant differences were observed, especially in the male children with both autoimmune and atopic diseases. There was an increase in the presence of the mutated allele in the position 7340C>T, specifically in those patients that suffered from both atopic and autoimmune diseases ( $p=0.004$ , OR=16.2

**Table 1** Polymorphisms observed in the molecular analysis of the *FOXP3* gene.

Number	Nomenclature	Reference number	Gene location	Effect
SNP1	–47040T	rs6609859	Intron –1	–
SNP2	9260T	Not described	Intron 2	–
SNP3	16510T	rs2232367	Exon 5	Synonymous mutation
SNP4	1855A>G	Not described	Intron 5	–
SNP5	2680G>A	rs2232368	Intron 7	–
SNP6	4572G>A	Not described	Exon 8	Synonymous mutation
SNP7	4640T>C	rs 11465476	Intron 8	–
SNP8	5234G>T	Not described	Intron 8	–
SNP9	7093G>A	Not described	Exon 11	Synonymous mutation
SNP10	7340C>T	rs56066773	3'UTR	–
SNP11	7841G>A	rs148013438	3'UTR	–
SNP12	7872G>A	Not described	3'UTR	–
SNP13	8150C>T	Not described	3'UTR	–

[1.34–195.15]), statistical power of 75%, with a false positive report probability (FPRP) of 7%. Meanwhile, the mutated allele in position 1651C>T was more frequent in controls ( $p=0.01$ , OR=0.16 [0.03–0.79]) than in patients who had only one disease, i.e. atopy or autoimmunity (statistical power of 77.7% and FPRP of 6.2%) (Table 2). No statistically significant differences were observed in females (Table 3).

Haplotype combinations and their frequencies were also determined (Table 4). In males, haplotype H8 was only described in patients with both diseases ( $p=0.006$ ) in contrast to haplotype H1 that was only identified in controls ( $p=0.005$ ).

## Discussion

We studied the *FOXP3* gene and evaluated allele, genotype and haplotype frequencies in a paediatric population of atopic patients and patients with autoimmune diseases. We identified 13 SNPs, including seven previously undescribed. We found different associations according to gender. These differences between genders have been previously described in other genetic association studies for SNPs in genes located on chromosome X.<sup>18</sup>

The most relevant difference was detected in the group of participants that simultaneously suffered atopic and autoimmune diseases. The mutated allele of 7340C>T was significantly associated with the simultaneous presence of autoimmunity and atopy in male children, but not those that only suffered from one disease. This polymorphism is located in the 3' untranslated region, which is described as a regulatory region, since it could modify mRNA stability and translation efficiency.<sup>19</sup> In addition, this polymorphism could modify cellular localisation signals affecting protein location, and consequently, *FOXP3* function.

We also detected a significant increase in the frequency of the mutated allele of 1651C>T in the male control group. This SNP is located in the coding region (Exon 5) and it is a synonymous one, with no change in protein sequence. Traditionally these changes were considered silent mutations. Nowadays it is thought that it could be involved in

transcriptional regulation.<sup>20</sup> Polymorphisms may reside in exonic splicing enhancers, near the exonic extreme that defines the point where the splicing 'machinery' has to work.<sup>21</sup> Another described mechanism is related to the mRNA structure that can be different according to the mutation. The mRNA structure determines its stability, altering translation speed and protein folding.<sup>22</sup>

Haplotype analyses provide more complete information in association studies.<sup>16</sup> In our work, different haplotype combinations were observed between males and females. In males, there was a statistically significant difference in the global distribution of haplotypes between the control group and the group of patients with both autoimmune and atopic disease. The haplotype H8 was only detected in patients (statistical power for this sample size 70%); whereas H1 was identified only in controls (statistical power 82%). These results again highlight that in males the group of patients that simultaneously developed both disorders seems to shape a well-defined group with a genetic background that appears to be unique to this clinical subgroup and is not seen in the groups of patients that have only one of the diseases.

The group of children that simultaneously present both immunological disorders is uncommon and there can be difficulties in finding sufficient subjects. For this reason we calculated the statistical power for our sample size and only associations with statistical significance higher than 70% are shown. Considering that 80% should be used, these results should be confirmed in larger populations although, as mentioned above, the group of children that simultaneously present both immunological disorders is uncommon. Another possible limitation of this study is the probability of reporting a false positive result due to multiple comparisons. Because of this, we calculated in all cases the probability of reporting a false positive result and only when this probability was less than 10% were the results considered. In addition, only associations with a significant  $p$  level less than 0.01 were considered instead of the 0.05.

It has been speculated that mutations in *FOXP3* could be involved in each of the immunological pathologies included in this study (DM1, AT and CD). Several studies have examined the association of *FOXP3* polymorphisms with these

**Table 2** Distribution of allelic frequencies in males.

4640A>G				4572G>A			
	<i>n</i>	A	G		<i>n</i>	G	A
Controls	41	1	–	Controls	41	1	–
Total patients	89	1	–	Total patients	89	0.99	0.01
Atopy	58	1	–	Atopy	58	0.98	0.02
Autoimmunity	25	1	–	Autoimmunity	25	1	–
Both disorders <sup>a</sup>	6	1	–	Both disorders	6	1	–
7841G>A				7093G>A			
		G	A			G	A
Controls		0.98	0.02	Controls		1	–
Total patients		0.99	0.01	Total patients		0.99	0.01
Atopy		1	–	Atopy		0.98	0.02
Autoimmunity		0.96	0.04	Autoimmunity		1	–
Both disorders		1	–	Both disorders		1	–
1651C>T				926C>T			
		C	T			C	T
Controls		0.93	0.07	Controls		1	–
Total patients		0.98	0.02	Total patients		0.99	0.01
Atopy		0.98	0.02	Atopy		0.98	0.02
Autoimmunity		1	–	Autoimmunity		1	–
Both disorders		0.83	0.17	Both disorders		1	–
2680G>A				7872G>A			
		G	A			G	A
Controls		1	–	Controls		1	–
Total patients		0.99	0.01	Total patients		1	–
Atopy		1	–	Atopy		1	–
Autoimmunity		0.96	0.04	Autoimmunity		1	–
Both disorders		1	–	Both disorders		1	–
7340C>T				5234G>T			
		C	T			G	T
Controls		0.99	0.01	Controls		0.98	0.02
Total patients		0.99	0.01	Total patients		1	–
Atopy		1	–	Atopy		1	–
Autoimmunity		1	–	Autoimmunity		1	–
Both disorders <sup>*</sup>		0.83	0.17	Both disorders		1	–
–4704C>T				8150C>T			
		C	T			C	T
Controls		1	–	Controls		1	–
Total patients		1	–	Total patients		1	–
Atopy		1	–	Atopy		1	–
Autoimmunity		1	–	Autoimmunity		1	–
Both disorders		1	–	Both disorders		1	–
1855A>G							
		A	G				
Controls		1	–				
Total patients		1	–				
Atopy		1	–				
Autoimmunity		1	–				
Both disorders		1	–				

(–) Not detected.

<sup>a</sup> Patients who suffer atopy and autoimmunity simultaneously.

<sup>\*</sup> *p*-value = 0.004.

diseases and only one of our SNPs was reported. The 2680G>A polymorphism was identified in a study of rhinitis in an adult Chinese population but no association was observed.<sup>23</sup> We did not find a relationship in our population either. In addition several studies analysed the *FOXP3* gene in diabetic populations but only two found associations with some polymorphisms.<sup>24–29</sup> No association has been found between *FOXP3* polymorphisms and AT<sup>30,31</sup> or CD.<sup>24,32</sup>

The *FOXP3* polymorphisms have been analysed in patients with allergy in several studies and all of them found a

statistical association.<sup>23,33–38</sup> However, only five of them included children,<sup>24,28,29,33,34</sup> with one of them including a Spanish population<sup>32</sup> although none of them studied the entire coding region of *FOXP3*. In the present study we found an association between *FOXP3* polymorphisms and the simultaneous development of allergic and autoimmune disorders.

To the best of our knowledge this is the first study to identify an association between *FOXP3* polymorphisms in patients simultaneously suffering from Th1 and Th2 related disorders, suggesting that the genetic background of the

**Table 3** Distribution of genotypic and allelic frequencies in females.

Phenotype		Genotype			Allele	
<b>4640A&gt;G</b>	<i>n</i>	<b>AA</b>	<b>AG</b>	<b>GG</b>	<b>A</b>	<b>G</b>
Controls	54	1	-	-	1	-
Total patients	71	0.99	0.01	-	0.99	0.01
Atopy	18	0.94	0.06	-	0.97	0.03
Autoimmunity	47	1	-	-	1	-
Both disorders <sup>a</sup>	6	1	-	-	1	-
<b>7841G&gt;A</b>	<i>n</i>	<b>GG</b>	<b>GA</b>	<b>AA</b>	<b>G</b>	<b>A</b>
Controls	54	0.98	0.02	-	0.99	0.01
Total patients	71	0.97	0.03	-	0.99	0.01
Atopy	18	1	-	-	1	-
Autoimmunity	47	0.98	0.02	-	0.99	0.01
Both disorders	6	0.83	0.17	-	0.92	0.08
<b>1651C&gt;T</b>	<i>n</i>	<b>CC</b>	<b>CT</b>	<b>TT</b>	<b>C</b>	<b>T</b>
Controls	54	0.91	0.09	-	0.95	0.05
Total patients	71	0.90	0.09	0.01	0.94	0.06
Atopy	18	0.94	0.06	-	0.97	0.03
Autoimmunity	47	0.91	0.09	-	0.94	0.06
Both disorders	6	0.83	0.17	-	0.92	0.08
<b>2680G&gt;A</b>	<i>n</i>	<b>GG</b>	<b>GA</b>	<b>AA</b>	<b>G</b>	<b>A</b>
Controls	54	0.96	0.04	-	0.98	0.02
Total patients	71	1	-	-	1	-
Atopy	18	1	-	-	1	-
Autoimmunity	47	1	-	-	1	-
Both disorders	6	1	-	-	1	-
<b>7340C&gt;T</b>	<i>n</i>	<b>CC</b>	<b>CT</b>	<b>TT</b>	<b>C</b>	<b>T</b>
Controls	54	0.93	0.07	-	0.96	0.04
Total patients	71	0.97	0.03	0.01	0.99	0.01
Atopy	18	0.94	0.06	-	0.97	0.03
Autoimmunity	47	0.98	0.02	-	0.99	0.01
Both disorders	6	1	-	-	1	-
<b>-4704C&gt;T</b>	<i>n</i>	<b>CC</b>	<b>CT</b>	<b>TT</b>	<b>C</b>	<b>T</b>
Controls	54	1	-	-	1	-
Total patients	71	0.97	0.03	-	0.99	0.01
Atopy	18	0.94	0.06	-	0.97	0.03
Autoimmunity	47	0.98	0.02	-	0.99	0.01
Both disorders	6	1	-	-	1	-
<b>1855A&gt;G</b>	<i>n</i>	<b>AA</b>	<b>AG</b>	<b>GG</b>	<b>A</b>	<b>G</b>
Controls	54	1	-	-	1	-
Total patients	71	0.99	0.01	-	0.99	0.01
Atopy	18	1	-	-	1	-
Autoimmunity	47	0.98	0.02	-	0.99	0.01
Both disorders	6	1	-	-	1	-
<b>4572G&gt;A</b>	<i>n</i>	<b>GG</b>	<b>GA</b>	<b>AA</b>	<b>G</b>	<b>A</b>
Controls	54	1	-	-	1	-
Total patients	71	1	-	-	1	-
Atopy	18	1	-	-	1	-
Autoimmunity	47	1	-	-	1	-
Both disorders	6	1	-	-	1	-
<b>7093G&gt;A</b>	<i>n</i>	<b>GG</b>	<b>GA</b>	<b>AA</b>	<b>G</b>	<b>A</b>
Controls	54	1	-	-	1	-
Total patients	71	1	-	-	1	-
Atopy	18	1	-	-	1	-
Autoimmunity	47	1	-	-	1	-
Both disorders	6	1	-	-	1	-

Table 3 (Continuación)

Phenotype	<i>n</i>	Genotype			Allele	
		CC	CT	TT	C	T
<b>926C&gt;T</b>						
Controls	54	1	-	-	1	-
Total patients	71	1	-	-	1	-
Atopy	18	1	-	-	1	-
Autoimmunity	47	1	-	-	1	-
Both disorders	6	1	-	-	1	-
<b>7872G&gt;A</b>						
Controls	54	0.98	0.02	-	0.99	0.01
Total patients	71	1	-	-	1	-
Atopy	18	1	-	-	1	-
Autoimmunity	47	1	-	-	1	-
Both disorders	6	1	-	-	1	-
<b>5234G&gt;T</b>						
Controls	54	0.98	0.02	-	0.98	0.02
Total patients	71	1	-	-	1	-
Atopy	18	1	-	-	1	-
Autoimmunity	47	1	-	-	1	-
Both disorders	6	1	-	-	1	-
<b>8150C&gt;T</b>						
Controls	54	0.98	0.02	-	0.99	0.01
Total patients	71	0.99	0.01	-	0.99	0.01
Atopy	18	1	-	-	1	-
Autoimmunity	47	1	-	-	1	-
Both disorders	6	0.83	0.17	-	0.92	0.08

(-) Not detected.

<sup>a</sup> Patients who suffer atopy and autoimmunity simultaneously.**Table 4** Haplotype frequencies. Data represent the percentage of each haplotype in each phenotypic group. Haplotypes with a frequency >1% among either controls or patients are included.

Haplotypes		Controls	Patients	Atopy	Autoimmunity	Both <sup>a</sup> disorders
<i>Males</i>						
CCCAGGAGGC	H0	0.92	0.92	0.92	0.92	0.66
CCCAGGATGCGGC	H1	0.04*	0.00	0.00	0.00	0.00
CCTAGGAGGC	H2	0.04	0.02	0.02	0.00	0.17
CCCAGGAGGCAGC	H3	0.00	0.01	0.00	0.04	0.00
CCCAAGAGGC	H4	0.00	0.01	0.00	0.04	0.00
CCCAGAAGGC	H5	0.00	0.01	0.02	0.00	0.00
CCCAGGAGACGGC	H6	0.00	0.01	0.02	0.00	0.00
CTCAGGAGGC	H7	0.00	0.01	0.02	0.00	0.00
CCCAGGAGGTGGC	H8	0.00	0.01	0.00	0.00	0.17**
<i>Females</i>						
CCCAGGAGGC	H0	0.90	0.89	0.91	0.91	0.76
CCTAGGAGGC	H2	0.03	0.05	0.03	0.05	0.08
CCCAGGAGGCAGC	H3	0.00	0.01	0.00	0.01	0.08
CCCAAGAGGC	H4	0.02	0.00	0.00	0.00	0.00
CCCAGGAGGTGGC	H8	0.05	0.01	0.03	0.01	0.00
TCCAGGGACGGC	H9	0.00	0.01	0.03	0.00	0.00
CCCGGAGGC	H10	0.00	0.01	0.00	0.01	0.00
TCTAGGAGGC	H11	0.00	0.01	0.00	0.01	0.00
CCCAGGAGGC	H12	0.00	0.01	0.00	0.00	0.08

The order of the SNPs is -4704C>T, 926C>T, 1651C>T, 1855A>G, 2680G>A, 4572G>A, 4640A>G, 5234G>T, 7093G>A, 7340C>T, 7841G>A, 7872G>A and 8150C>T (this is the order in the gene sequence). Bold letters indicate the mutated allele.

<sup>a</sup> Patients who suffer atopy and autoimmunity simultaneously\*  $p = 0.005$ .\*\*  $p = 0.006$ .

*FOXP3* gene may be involved in the development of several immunological diseases in the same patient, as occurs in the IPEX syndrome.<sup>2</sup> Furthermore, these *FOXP3* modifications could be involved in regulatory mechanisms implicated in these diseases. The critical role of *FOXP3* in regulatory T cells by controlling the development of autoimmune and allergic diseases is clear,<sup>4,6</sup> therefore *FOXP3* mutations could be involved in the development of these diseases. The majority of SNPs detected in this study were located in regulatory regions. In fact, some authors have reported several mutations in the non-coding regions of *FOXP3*, such as UTR in patients with IPEX syndrome<sup>2,19</sup> and others have confirmed epigenetic controls, such as histone methylation and acetylation, thus regulating transcription factor binding to the *FOXP3* promoter region.<sup>4,5</sup>

To summarise, we have identified novel genetic variants of *FOXP3* that are specifically present in children that simultaneously suffer from Th2 and Th1 disorders, atopic and autoimmune disease located mainly in regulatory regions. These variants could affect the expression levels of *FOXP3*, modifying its function. Further studies that analyse other regulatory sequences in this gene, as well as functional analysis, are required to better explain the role that *FOXP3* polymorphisms play in these immunological disorders.

## Conflict of interest

The authors have no conflict of interest to declare.

## Ethical disclosures

**Patients' data protection.** Confidentiality of data. The authors declare that no patient data appears in this article.

**Right to privacy and informed consent.** The authors declare that no patient data appears in this article.

**Protection of human subjects and animals in research.** The authors declare that the procedures followed were in accordance with the regulations of the responsible Clinical Research Ethics Committee and in accordance with those of the World Medical Association and the Helsinki Declaration.

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## References

1. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, et al. JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic dysregulation syndrome. *J Clin Invest.* 2000;106:R75–81.
2. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Genet.* 2002;39:537–45.
3. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. *Blood.* 2009;114:3727–35.
4. González Parias JL, Duque Giraldo VE, Velásques-Lopera MM. *FOXP3*: master gene controlling the development and function of regulatory cells. *Inmunología Revisión.* 2010;29:74–84.
5. Van der Vliet HJ, Nieuwenhuis EE. IPEX as a result of mutations in *FOXP3*. *Clin Dev Immunol.* 2007;2007:89017.
6. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev.* 2011;241:260–8.
7. Sakaguchi S. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol.* 2005;6:345–52.
8. Waterfield M, Anderson MS. Clues to immune tolerance: the monogenic autoimmune syndromes. *Ann N Y Acad Sci.* 2010;1214:138–55.
9. Kronenberg M, Rudensky A. Regulation of immunity by self-reactive T cells. *Nature.* 2005;435:598–604.
10. Von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol.* 2005;6:338–44.
11. Lozano T, Casares N, Lasarte JJ. Searching for the Achilles Heel of *FOXP3*. *Front Oncol.* 2013;3:294.
12. American Diabetes Association. Standards of medical care in diabetes – 2010. *Diabetes Care.* 2014;33:S11–61.
13. Slatosky J, Shipton B, Wahba H. Thyroiditis: differential diagnosis and management. *Am Fam Phys.* 2000;61:1047–52, 1054.
14. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child.* 1990;65:909–11.
15. Sanz C, Isidoro-García M, Davila I, Moreno E, Laffond E, Avila C, et al. Promoter genetic variants of prostanoid DP receptor (PTGDR) gene in patients with asthma. *Allergy.* 2006;61:543–8.
16. Sanz C, Isidoro-García M, Davila I, de Pedro MP, Méndez SdeA, Padrón J, et al. A new PTGDR promoter polymorphism in a population of children with asthma. *Pediatr Allergy Immunol.* 2009;20:151–6.
17. Muller CR. Quality control in mutation analysis: the European Molecular Genetics Quality Network (EMQN). *Eur J Pediatr.* 2001;160:464–7.
18. Arriba-Mendez S, Sanz C, Isidoro-García M, Davila I, Laffond E, Moreno E, et al. 927T>C polymorphism of the cysteinyl-leukotriene type-1 receptor (CYSLTR1) gene in children with asthma and atopic dermatitis. *Pediatr Allergy Immunol.* 2006;17:323–8.
19. Bennett CL, Brunkow ME, Ramsdell F, O'Briant KC, Zhu Q, Fuleihan RL, et al. A rare polyadenylation signal mutation of the *FOXP3* gene (AAUAAA→AAUGAA) leads to the IPEX syndrome. *Immunogenetics.* 2001;53:435–9.
20. Czech A, Fedyunin I, Zhang G, Ignatova Z. Silent mutations in sight: co-variations in tRNA abundance as a key to unravel consequences of silent mutations. *Mol Biosyst.* 2010;6:1767–72.
21. Lalonde E, Ha KC, Wang Z, Ignatova Z. RNA sequencing reveals the role of splicing polymorphisms in regulating human gene expression. *Genome Res.* 2011;21:545–54.
22. Saunders R, Deane CM. Synonymous codon usage influences the local protein structure observed. *Nucleic Acids Res.* 2010;38:6719–28.
23. Zhang L, Zhang Y, Desrosiers M, Wang C, Zhao Y, Han D. Genetic association study of *FOXP3* polymorphisms in allergic rhinitis in a Chinese population. *Hum Immunol.* 2009;70:930–4.
24. Bjornvold M, Amundsen SS, Stene LC, Joner G, Dahl-Jørgensen K, Njølstad PR, et al. *FOXP3* polymorphisms in type 1 diabetes and coeliac disease. *J Autoimmun.* 2006;27:140–4.
25. Howson JM, Walker NM, Smyth DJ, Todd JA, Type I Diabetes Genetics Consortium. Analysis of 19 genes for association with type I diabetes in the Type I Diabetes Genetics Consortium families. *Genes Immun.* 2009;10 Suppl. 1:S74–84.



26. Iwase K, Shimada A, Kawai T, Okubo Y, Kanazawa Y, Irie J, et al. FOXP3/Scurfin gene polymorphism is associated with adult onset type 1 diabetes in Japanese, especially in women and slowly progressive-type patients. *Autoimmunity*. 2009;42:159–67.
27. Nakanishi K, Shima Y. No contribution of a GT microsatellite polymorphism in the promoter region of the FOXP3 gene to susceptibility to type 1 diabetes in the Japanese population. *Clin Chim Acta*. 2007;384(1–2):171–3.
28. Zavattari P, Deidda E, Pitzalis M, Zoa B, Moi L, Lampis R, et al. No association between variation of the FOXP3 gene and common type 1 diabetes in the Sardinian population. *Diabetes*. 2004;53:1911–4.
29. Bassuny WM, Ihara K, Sasaki Y, Kuromaru R, Kohno H, Matsuura N, et al. A functional polymorphism in the promoter/enhancer region of the FOXP3/Scurfin gene associated with type 1 diabetes. *Immunogenetics*. 2003;55:149–56.
30. Ban Y, Tozaki T, Tobe T, Ban Y, Jacobson EM, Concepcion ES, et al. The regulatory T cell gene FOXP3 and genetic susceptibility to thyroid autoimmunity: an association analysis in Caucasian and Japanese cohorts. *J Autoimmun*. 2007;28:201–7.
31. Inoue N, Watanabe M, Morita M, Tomizawa R, Akamizu T, Tatsumi K, et al. Association of functional polymorphisms related to the transcriptional level of FOXP3 with prognosis of autoimmune thyroid diseases. *Clin Exp Immunol*. 2010;162:402–6.
32. Sanchez E, Rueda B, Orozco G, Oliver J, Vilchez JR, Paco L, et al. Analysis of a GT microsatellite in the promoter of the foxp3/scurfin gene in autoimmune diseases. *Hum Immunol*. 2005;66:869–73.
33. Bottema RW, Kerkhof M, Reijmerink NE, Thijs C, Smit HA, van Schayck CP, et al. Gene-gene interaction in regulatory T-cell function in atopy and asthma development in childhood. *J Allergy Clin Immunol*. 2010;126:338–46.
34. Bottema RW, Kerkhof M, Reijmerink NE, Koppelman GH, Thijs C, Stelma FF, et al. X-chromosome Forkhead Box P3 polymorphisms associate with atopy in girls in three Dutch birth cohorts. *Allergy*. 2010;65:865–74.
35. Fodor E, Garaczi E, Polyanka H, Koreck A, Kemény L, Széll M. The rs3761548 polymorphism of FOXP3 is a protective genetic factor against allergic rhinitis in the Hungarian female population. *Hum Immunol*. 2011;72:926–9.
36. Hassannia H, Abediankenari S, Ghaffari J. FOXP3 and TGF-beta gene polymorphisms in allergic rhinitis. *Iran J Immunol*. 2011;8:218–25.
37. Lee SM, Gao B, Dahl M, Calhoun K, Fang D. Decreased FoxP3 gene expression in the nasal secretions from patients with allergic rhinitis. *Otolaryngol Head Neck Surg*. 2009;140:197–201.
38. Zhang Y, Wang CS, Zhang L. Correlation between FOXP3 gene polymorphisms and allergic rhinitis. *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi*. 2010;45:397–400.