Concurrent *FOXP3*- and *CTLA4*-associated genetic predisposition and skewed X chromosome inactivation in an autoimmune disease-prone family

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Abstract:

CLTA4 is relevant for FOXP3$^+$Treg cells, and the link between skewed X-chromosome inactivation (XCI) and autoimmunity is recognized. The observation of IPEX-syndrome and multiorgan endocrine autoimmune phenomena in various members of one family, associated with a CLTA4 polymorphism and skewed XCI provides an in vivo model of how mechanisms of immune-dysregulation may cooperate.

Introduction

Approximately 80% of patients with autoimmune diseases, which often occur in a familial setting and in genetically predisposed individuals, are women. The most prominent genetic susceptibility factors for AITD in particular are located in the HLA region and conferred by a specific CLTA4 single nucleotid polymorphism (SNP).$^{1-3}$ One of the rare X-linked syndromes that specifically predispose affected boys to the development of severe multi-organ autoimmune diseases (MOAD) with unclear genotype-phenotype correlation is the "immune dysregulation polyendocrinopathy enteropathy X-linked syndrome" (IPEX).$^4$ It is caused by frameshift and missense mutations in the fork-head DNA-binding box protein P3 (FOXP3) gene on Xp11.23 that impair the function of the encoded protein.$^{4,5}$ FOXP3 is primarily but not exclusively expressed in CD4+CD25+ regulatory T (Treg) cells that are essential for maintaining the balance between immune tolerance and suppression.$^{5-7}$ Partial FOXP3 deficiency is associated with overstimulation of T cells, which in turn is an essential component of many immune disorders and autoimmune phenomena, whereas a complete lack of the protein is the root of severe forms of the IPEX syndrome.$^{4,6,8}$ Although the majority of affected males die within the first two years of life, some of them may survive into
Despite positivity of autoantibodies in many asymptomatic patients and increasing incidence of abnormal thyroid stimulating hormone levels and thyroid disease with age in the normal population, the accumulation of autoimmune-endocrinopathies within the presented pedigree prompted detailed work-up of potential genetic predispositions, especially in the context of skewed X chromosome inactivation (XCI) in female relatives of an IPEX patient.

**Patient family presentation**

The clinical and genetic data of relevant family members are summarized in Figure 1, laboratory features in Table 1. All material from patients was obtained upon informed consent in accordance with the Declaration of Helsinki; and the study was approved by the institutional review board. We observed a FOXP3 mutation in a now 25 years old patient with a long history of typical, but mild IPEX-associated symptoms. He was born as the third child after an uneventful pregnancy. His brother, who was born 9 years earlier had severe IPEX-typical symptoms and died. The patient became symptomatic at seven months of age with diarrhea and failure to thrive. At four years of age he developed an autoimmune hepatitis that required ongoing treatment with cortison and azathioprine. Hashimoto type of autoimmune thyroid disease (AITD) was diagnosed in the boy’s mother, who also developed type 1 diabetes mellitus (DMT1) at 45 years of age. Furthermore, one of her three sisters and the maternal grandmother suffered from thyroid disease (Graves’ disease and Hashimoto, respectively) and needed according treatment, as reported by family members and the family’s primary care physician. The patient’s three years older sister is healthy, but has positive antinucleic-acid antibody titres and elevated IgE levels without symptoms or history of atopy. The index patient’s further medical course remained uneventful until he was 18, when he also developed a DMT1 as well as a marked eczema on both his upper extremities.
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and trunk. At present his disease symptoms are under control and his clinical condition remains stable.

**Results**

The *FOXP3* mutation p.R347H (c.1040G>A) in this family is located in the gene's forkhead domain and had already been reported in other IPEX patients. Subsequent screening revealed its presence also in the patient's sister, his mother and maternal grandmother (Figure 1). Because the mutation was found in two of the three females with AITD we further explored the females' XCI (see Legend). Skewing was found in the peripheral blood (PB) of the mother and the sister of the patient (both *FOXP3*+/−), but not in the aunt with AITD without a *FOXP3* mutation. Equally, XCI was skewed in sorted CD4+CD25hi T-cells of the patient’s mother and sister (not shown). Based on the repeat length of the male patient, we were able to infer that in both women, the X with the mutated *FOXP3* was preferentially inactivated. Global XCI-skewing is a rather unusual feature of female *FOXP3* mutation carriers, whereas it is quite common in women with AITD. We therefore continued to screen all family members for a potentially relevant *CTLA4* SNP (rs231775, A>G) with allele-specific oligonucleotide PCR. This autoimmunity-predisposing variant allele was found in all *FOXP3* mutation carriers as well as in the AITD patient without *FOXP3* mutation in heterozygous, and in the IPEX patient and his sister in homozygous form.

**Conclusions**

We assume that the clinical aspects and skewed XCI in this family are associated with the concurrence of a *FOXP3* and a *CTLA4* predisposing trait. The products of these two genes
FoxP3- and CTLA-4-linked familial autoimmunity interact and cooperate in the same immune regulatory signal transduction pathway\textsuperscript{17}. Taking into consideration the normal physiological function and interaction of the respective \textit{FOXP3} and \textit{CTLA4} products in the maintenance of a balanced T cell immune regulation\textsuperscript{3,5}, one is able to deduce the consequences of the concurrence of their respective mutated and variant alleles in the individual family members as follows. \textit{FOXP3} is an X-linked gene whose mutated copy will selectively be silenced in the relevant Treg cell population of female carriers\textsuperscript{14,16}. Thus, no adverse effects ensue from this specific genetic defect, and any risk of autoimmunity in these individuals is therefore most likely due to the respective \textit{CTLA4} variant. Since, on the other hand, skewed X inactivation is quite a common phenomenon in females with various forms of autoimmune diseases, the pronounced skewing in one heterozygous and one homozygous \textit{CTLA4}-positive female, who also had a \textit{FOXP3} mutation, may be associated either with the \textit{CTLA4}-predisposing variant alone or with both aberrations rather than with the \textit{FOXP3} mutation alone\textsuperscript{2,15}. Nevertheless, the widespread skewing favoring the normal \textit{FOXP3} allele in cell compartments other than the Treg subset supports the notion that X linked factors may be also important in this context as was for instance demonstrated in crossbreeding experiments of female mice that were double heterozygote knockouts for \textit{FOXP3} and the common gamma chain of the interleukin 2, 7 and 15 receptor genes\textsuperscript{8}. These experiments confirmed that two X-linked recessive genes can interact to cause disease where either alone would not, but also that additional factors are operating in modifying disease severity\textsuperscript{8}. Based on these experiments and compatible with the presented pedigree, we speculate that particular \textit{CTLA4} variants may in turn represent one of the autosomal modifiers of \textit{FOXP3} (and or other similar X-linked immune regulatory gene variants). The notion that specific \textit{CTLA4} variants may act as such potentially disease-attenuating genetic modifying factors in IPEX is perhaps further corroborated by the fact that the male \textit{FOXP3}-mutated patient with the mild clinical course even carried the \textit{CTLA4} risk allele in homozygous form. These observations imply that the \textit{CTLA4} variant has either a neutral or protective but
certainly not an aggravating effect on the \textit{FOXP3} mutation in this particular case. Naturally,
the available clinical and laboratory features of this pedigree are derived from a limited time-
segment of clinical observation, and a longitudinal life-long follow-up with regular
monitoring of many endocrinological and immunological parameters would be needed to
allow firm conclusions regarding the full impact of genes on disease development in this
pedigree.

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\textbf{Authorship} BR, ES, WDH, ND, AH, and OB were responsible for the clinical diagnosis,
took care of the patient and his family and provided the relevant clinical and laboratory data;
CI, AH, PZ and OAH performed genetic and immunologic analyses and interpreted the
ensuing results; BR and MGS designed the tables/figures; MGS and OAH wrote the
manuscript.

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written informed consent to perform non-routine genetic analyses and the scientific work-up
as well as for their permission to publish these observations.

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Tuomilehto J, Bingley P, Gillespie K, Undlien DE, Rønningen K, Guja C, Ionescu-


Legends.

Figure 1. Pedigree with clinical and genetic information.

Clinical information including status of type 1 diabetes mellitus (DMT1), autoimmune thyroid disease (AITD), or Graves’ Disease are presented underneath each symbol (additionally depicted by striped pattern). Genetic information is provided below: analyzed aberrations were $\textit{FOXP3}\ c.1040G>A\ p.R347H$ (also depicted by filled or half-filled symbols), $\textit{CTLA4}$ polymorphism rs231775 c.49A>G, and the X chromosome inactivation status, detected by methylation-specific PCR that enables the simultaneous discrimination and quantification of the unequal length and allele-specific methylation status of polymorphic triplet repeats in the $\textit{FMR1}$ and androgen receptor ($\textit{AR}$) genes. Numbers beside symbols are provided for patient identification in Table 1.
**Figure 1.**

- **1953**, healthy
  - FoxP3: wild-type
  - CTLA4: n.d.

- **1952**, DMT1, AITD
  - FoxP3: het.
  - CTLA4: +49G/A
  - X-inact.: 80/20%

- **1947**, healthy
  - FoxP3: wild-type
  - CTLA4: +49A/A
  - X-inact.: normal

- **1951**, healthy
  - FoxP3: wild-type
  - CTLA4: +49A/A
  - X-inact.: normal

- **1959**, Graves' Dis.
  - FoxP3: wild-type
  - CTLA4: +49G/A
  - X-inact.: normal

- **1923**, history of AITD
  - FoxP3: het.
  - CTLA4: +49G/A
  - X-inact.: normal

- **1977 - 1978**
  - IPEX?, intractable diarrhea, fatal sepsis; no material for genetic analysis available

- **1986**, IPEX-patient; enteropathy, hepatitis, DMT1, eczema
  - FoxP3: hemizygous
  - CTLA4: +49G/G

**analyzed mutations:**
- FoxP3 mutation: c.1040G>A
- CTLA4 SNP: rs231775 c.49A>G

*7563*
### Table 1. Laboratory features in affected family members with immune dysregulation.

<table>
<thead>
<tr>
<th>Family member</th>
<th>1&lt;sup&gt;st&lt;/sup&gt;</th>
<th>2</th>
<th>3&lt;sup&gt;rd&lt;/sup&gt;</th>
<th>4&lt;sup&gt;th&lt;/sup&gt;</th>
<th>5</th>
<th>6</th>
<th>7&lt;sup&gt;th&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH (µU/mL)</td>
<td>1.15</td>
<td>1.63</td>
<td>0.61</td>
<td>n.a.&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.34</td>
<td>1.92</td>
<td>n.a.&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>fT4 (ng/dL)</td>
<td>1.09</td>
<td>1.48</td>
<td>1.38</td>
<td>n.a.&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1.38</td>
<td>1.17</td>
<td>n.a.&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thyroid Ab’s</td>
<td>neg.</td>
<td>neg.</td>
<td>TG 84 IU/mL</td>
<td>TPO 1825 IU/mL</td>
<td>n.a.&lt;sup&gt;5&lt;/sup&gt;</td>
<td>neg.</td>
<td>neg.</td>
</tr>
<tr>
<td>Diabetes-assoc. Ab’s (U/ml)</td>
<td>GAD &gt;250↑↑ IAA 2.36 IA2 1.91</td>
<td>neg.</td>
<td>GAD 92 ↑ IAA 7.11↑ neg.</td>
<td>n.a.</td>
<td>neg.</td>
<td>neg.</td>
<td>n.a.</td>
</tr>
<tr>
<td>cardiolipin Ab’s (IgG, -M, -A)</td>
<td>neg.</td>
<td>neg.</td>
<td>borderline (IgG &amp; IgM)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.a.</td>
</tr>
<tr>
<td>IgG (mg/dL)</td>
<td>768</td>
<td>1110</td>
<td>958</td>
<td>n.a.</td>
<td>530↓</td>
<td>1080</td>
<td>n.a.</td>
</tr>
<tr>
<td>IgG subclasses</td>
<td>normal, except IgG4 (0,34)↓</td>
<td>normal, except IgG3 (180)↑</td>
<td>normal, except IgG3 (180)↑</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>IgA</td>
<td>213</td>
<td>285</td>
<td>287</td>
<td>112</td>
<td>112</td>
<td>223</td>
<td>n.a.</td>
</tr>
<tr>
<td>IgM</td>
<td>90</td>
<td>118</td>
<td>110</td>
<td>121</td>
<td>121</td>
<td>100</td>
<td>n.a.</td>
</tr>
</tbody>
</table>
### Table: Immunological Data

<table>
<thead>
<tr>
<th>Family member</th>
<th>1*</th>
<th>2</th>
<th>3*</th>
<th>4$</th>
<th>5</th>
<th>6</th>
<th>7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE (IU/ml)</td>
<td>74.7</td>
<td>174↑</td>
<td>n.d.</td>
<td>n.a.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CD3+ T cells (µL)</td>
<td>600</td>
<td>1750</td>
<td>1435</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CD4/CD8 ratio</td>
<td>1.9</td>
<td>0.95</td>
<td>3.7</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CD4+CD25+ (% CD4)</td>
<td>32</td>
<td>30</td>
<td>41</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CD4+CD25hi (% CD4)</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>gdT cells (µL)</td>
<td>15</td>
<td>100</td>
<td>30</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CD19+ B cells (µL)</td>
<td>20</td>
<td>320</td>
<td>110</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>CD56+CD3- NK cells</td>
<td>85</td>
<td>170</td>
<td>170</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>other lab chemistry abnormalities (liver, kidney, electrolytes, serum proteins, etc)</td>
<td>LFP↑</td>
<td>none</td>
<td>n.a.</td>
<td>n.a.</td>
<td>cholesterol and triglycerides↑</td>
<td>cholesterol↑</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>HbA1C↑</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*, under immunosuppressive treatment (azathioprine, low-dose prednisolone); $ under thyroid hormone (3) and insulin (1, 3) substitution therapy; $ patient not present for clinical and lab examination, but oral report from primary care physician and family members documented Graves’ disease in patient 4 and Hashimoto thyroiditis in patient 7 with need for corresponding treatment. All family members were invited for thorough endocrinology and immunologic workup, most were seen at least once at our clinics and were examined physically and by blood investigations. From one aunt (Patient 4) and the grandmother (patient 7), only one blood sample was available, which was needed for DNA analysis, and only patient 1, 2, and 3 were available for follow-up investigations.

Ab’s, antibodies; thyroid Ab’s: TG, thyreoglobulin-; TPO, thyreoperoxidase-; TSHR, thyroid stimulating hormone receptor autoantibodies; GAD, anti-glutamate-decarboxylase; IAA, anti-insulin antibodies; IA2, anti-tyrosin-phosphatase IA2; liver-autoantibodies include antibodies against smooth muscle, actin, LKM1(liver kidney microsomal type 1), soluble liver antigen; LFP, liver function parameters; HbA1C, glycosylated haemoglobin; n.d., not done; n.a., specimen not available.