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Clinical and structural impact of mutations affecting the residue Phe367 of FOXP3 in patients with IPEX syndrome

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ABSTRACT

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is a monogenic autoimmune disease characterized by early-onset life-threatening multisystemic autoimmunity. This rare hereditary disorder is caused by loss-of-function mutations in the gene encoding the forkhead box P3 (FOXP3) transcription factor, which plays a key role in the differentiation and function of $CD4^+CD25^+$ natural regulatory T cells (Tregs), essential for the establishment and maintenance of natural tolerance. We identified a novel mutation in the *FOXP3* gene affecting the Phe367 residue of the protein (F367V) in a family with three male siblings affected by IPEX. Two other mutations affecting the FOXP3 Phe367 residue (F367L and F367C) have been described previously. This unique situation of three mutations affecting the same residue in FOXP3 led us to study the molecular impact of these mutations on the structure of FOXP3 protein. Structure analysis showed that Phe367 is involved in a rich interaction network related to both monomer and dimer structure stabilization, and is crucial for FOXP3 regulatory activity. The relevance of this location is confirmed by the results of SIFT and PolyPhen-2 pathogenicity predictions for F367V mutation. In summary, as assessment of the pathogenicity of a novel mutation is crucial to achieve a proper molecular diagnosis, we analysed the impact of mutations affecting the Phe367 residue using a combined approach that provides a mechanistic view of their pathogenic effect.

1. Introduction

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (OMIM #304790) is a rare monogenic primary immunodeficiency characterized by multiorgan autoimmunity, including severe diarrhoea due to enteropathy, chronic dermatitis, endocrinopathy (e.g., type 1 diabetes mellitus, hypothyroidism) and other organ-specific diseases such as anaemia, thrombocytopenia, hepatitis, and nephritis [1]. IPEX is an X-linked recessive disorder with onset in infancy, and is often fatal by the age of 2 years if aggressive treatment is not used [2,3]. Long-term therapeutic options include immunosuppression and haematopoietic stem cell transplantation [4,5].

The immunopathogenesis of IPEX is explained by a loss of functional $CD4^+CD25^+$ T regulatory cells (Tregs), which are critical for maintaining immune tolerance [6]. This loss is caused by mutations in the transcription factor, forkhead box p3 (FOXP3), the master gene for Treg differentiation [7]. More than 60 *FOXP3* mutations have been identified and characterized in IPEX, each affecting Treg development or function [8]. FOXP3 deficiency results in a paucity of these cells, leading to a severe disruption of immunological tolerance and ultimately, aggressive multiorgan autoimmune disease.

The *FOXP3* genotype/clinical phenotype correlation in IPEX is not straightforward, but some general principles have emerged. For example, a relatively mild clinical phenotype, with compromised regulatory function but near-normal protein levels and normal Treg counts is associated with four mutation types: missense mutations and small in-frame deletions that do not destroy the functional domain of any protein, and deletions and mutations in the promoter and 5′ untranslated region [9,10]. In contrast, mutations abrogating expression of functional FOXP3 (certain missense variants, as well as nonsense and frameshift mutations or splicing defects resulting in premature stop codons) tend to produce a more severe phenotype [8]. Nonetheless, disease severity is not always related to the absence of protein expression, and most individuals that carry missense mutations express the mutated protein at levels ranging from low to normal. This, together with the fact that

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disease manifestations may differ considerably between patients with the same mutation, suggests that severity can be modulated by modifying genes affecting Treg function. As always when comparing the immune system between individuals, it should be kept in mind that phenotypic variability may also result from differences in the HLA haplotype and the lymphocyte repertoires [11].

Another factor that increases the complexity of the genotype/clinical phenotype relationship in IPEX is that other genetic defects affecting Treg function can originate an IPEX-like phenotype. These include loss-of-function mutations in *CD25*, *STAT5B*, and *ITCH* and gain-of-function mutations in *STAT1* [12]. Furthermore, despite the progress attained in identifying the molecular basis of IPEX-like diseases, the underlying defects in a large percentage of individuals affected with these conditions remain obscure.

Here, we present a study in which IPEX was suspected in three siblings, based on the clinical history of the family, starting 20 years ago. Identification of a novel mutation in the Phe367 residue of FOXP3 in the family led us to study the molecular impact of this and other mutations in the same residue on the structure of the FOXP3 protein. Functionally, this protein acts as a component of a dynamic multisubunit complex involved in histone modification and chromatin remodelling after T-cell receptor stimulation [13,14]. Its three-dimensional structure, in complex with NFAT1 (the DNA-binding domain of the nuclear factor of activated T cells-1) and a DNA fragment from interleukin-2 promoter, has been recently resolved experimentally [15]. Based on this structure, we analysed the spatial neighbourhood of Phe367 and its relationship with other *FOXP3* mutations. Our results show that Phe367 is part of a rich network of hydrophobic interactions that are crucial for stabilizing both monomer and dimer structures. Many known IPEX-causing mutations are clustered in this region, suggesting a high sensitivity to sequence variations. The structural information provided here may be of value for future characterization of new mutations in this *FOXP3* region.

2. Material and methods

2.1. Study sample

The family reported here attended the Paediatric Infectious Diseases and Immunodeficiencies Unit of Hospital Universitari Vall d'Hebron (Barcelona, Spain). Informed consent was obtained from the family members studied, following the procedure of the Ethics Committee of Hospital Universitari Vall d'Hebron.

2.2. Mutation analysis

Genomic DNA was extracted from EDTA-containing whole blood samples using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) to amplify the 12 exons of *FOXP3* and their flanking regions was carried out using the primers and PCR conditions specified in Supplementary Table 1. Purified PCR products were sequenced on an ABI 3100 DNA Sequencer (Applied Biosystems, Foster, VA, USA).

2.3. Structure analyses and modelling

All the structure analyses performed, such as the location of known mutations and contact computations, were done using the experimentally resolved structure of FOXP3 as reference (PDB code: 3QRF) [15].

To identify intradomain and interdomain residue-residue interactions, two residues were considered to be in contact when at least two of their atoms were located at a distance of 5 Å or less.

Visual inspection to assess the impact of the mutations and creation of the structure images in the figures was done with the PyMol software package (PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC).

3. Results

3.1. Family with suspected IPEX

We report a non-consanguineous Spanish family with five siblings (three boys and two girls) from two different fathers (Fig. 1A). The family history included a maternal uncle who died due to hepatitis and diarrhoea at the age of three months. The three boys experienced early-onset diarrhoea (clinical data in Table 1). The first boy, born in 1970, died at the age of two months because of probable infectious meningoencephalitis. He showed thymic hypoplasia, thrombocytopenia, pulmonary haemorrhage, and eosinophilic infiltrate in the gastrointestinal tract and adrenal glands. The second boy, born of a second marriage in 1986, had growth retardation, persistent diarrhoea, severe skin lesions and leucocytosis with eosinophilia in the first months of life. The infant died at the age of 13 months due to *Klebsiella* spp. sepsis. The third boy, born in 1988, showed growth retardation, hepatosplenomegaly, diarrhoea and severe eczema, leucocytosis with eosinophilia and had high IgE levels (>2000 kU/L). Several sepsis episodes caused by bacteria and fungi led to the child's death at the age of 13 months. Available hematologic and immunologic data of the patients are shown in Table 2.

3.2. Identification of the FOXP3 p.Phe367Val mutation

Since the three affected siblings died more than 20 years ago, we were only able to trace DNA from the post mortem study of case 2. Unfortunately, the DNA was too degraded to successfully amplify all *FOXP3* exons; hence, we resorted to study of the mother. Complete sequencing of the 12 exons of *FOXP3* in the mother revealed a heterozygous nucleotide change (T > G) in exon 11, affecting the first position of codon 367 and leading to an amino acid change (phenylalanine to valine). This mutation had not been reported in the literature or databases, and, following the recommendations of the Human Genome Variation Society (HGVS), we named it c.1099 T > G/p.Phe367Val (Fig. 1B). To confirm that the mutation was present in at least one of the three affected siblings, we designed a specific pair of primers to amplify a short fragment of DNA containing the mutation from the sibling who had undergone post-mortem study. We successfully amplified and sequenced the *FOXP3* fragment containing the mutation and confirmed that the boy had been a hemizygous carrier of the p.Phe367Val mutation. On study of the other family members, we found that the older sister was also a carrier, and genetic counselling was indicated (Fig. 1A).

3.3. Molecular impact of the F367V mutation

To understand the molecular repercussions of the F367V mutation on FOXP3 function, we analysed its impact on the structure and predicted interactions of the protein. We found that the mutated residue (F367) locates within the experimentally determined structure of the DNA-binding domain (forkhead) of FOXP3, available in complex with NFAT1, the DNA-binding domain of nuclear factor of activated T cells-1, and a DNA fragment from the promoter of interleukin 2 [15].

We analysed this structural information to provide a mechanistic view of the pathogenic effect of mutations at position Phe367. Subsequently, we sought additional support for this view by mapping the known *FOXP3* pathological mutations to the forkhead structure [8].

3.3.1. F367 is a key structural residue of the DNA-binding domain of FOXP3

The mutation described in this study (F367V) is not the only one occurring at position 367; two others, F367L and F367C, have been described. F367L has been reported in two separate IPEX patients: a Japanese infant with neonatal diabetes mellitus, intractable diarrhoea, liver dysfunction, thrombocytopenia, and sepsis who died at 16 weeks of age [16], and a French child with similar clinical manifestations [17] (Table 1). The group that performed the second study later reported the F367C mutation in a patient with severe protracted diarrhoea and