WHOLE-EXOME SEQUENCING AS A DIAGNOSTIC TOOL FOR IPEX SYNDROME

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ABSTRACT

Immune dysregulation-Polyendocrinopathy-Enteropathy-X-linked (IPEX) syndrome is a life-threatening congenital autoimmune disorder caused by mutations in the forkhead box protein 3 (*FOXP3*) gene. Typical clinical manifestations of IPEX patients are early onset of intractable diarrhea, type 1 diabetes mellitus, and skin diseases. However, other autoimmune types such as severe food allergies, autoimmune cytopenias, autoimmune respiratory illness, and mesangial glomerulonephritis may complicate IPEX diagnosis. In this study, we report a Vietnamese 1-year-old boy with IPEX syndrome due to a hemizygous missense mutation, c.1190G>A (p.Arg397Gln), in exon 12 of the *FOXP3* gene (NM_014009.4). The child had dermatitis, diarrhea, respiratory infections, and splenomegaly. The patient's serum routine test results were expected, except for white blood cells and neutrophils were higher than the normal, while IgA concentration was slightly below the normal range. However, he got no signal of diabetes or failure to thrive. Whole exome sequencing was applied to identify a genetic variant, and variant validation was examined using Sanger sequencing. The patient's genetic mutation was inherited from his mother, an obligate carrier. His father had a normal genotype. This study is the first report of IPEX syndrome in a Vietnamese patient with a mutation in the *FOXP3* gene detected by WES. This study provides further evidence for the role of mutations in the *FOXP3* gene in patients with IPEX syndrome and demonstrates the need for genetic counselling and prenatal testing. Our results also show that WES sequencing is an effective tool in diagnosing genetic diseases.

Keywords: *FOXP3*, IPEX syndrome, primary immunodeficiency, Vietnamese, WES.

INTRODUCTION

The Immune dysregulation-Polyendocrinopathy-Enteropathy-X-linked (IPEX) syndrome (OMIM 304790) is a rare monogenic disorder caused by mutations in the forkhead box P3 (*FOXP3*) gene (Gambineri et al., 2018). The clinical features of the disease were first described in 1982 in a large family with 19 affected males across five generations (Powell et al., 1982), while its genetic characterization was reported in 2000 (Bennett et al., 2001). Although IPEX syndrome is a rare disease, more than 300 cases have been identified so far, with most male cases (Jamee et al., 2020). IPEX is usually lethal in infancy or childhood, with significant clinical features including watery/bloody diarrhea, failure to thrive, early type 1 diabetes mellitus, eczema, and hemolytic anaemia (Gischel. et al., 2009). However, late-onset or atypical symptoms may occur in some cases (Barzaghi et al., 2018b).

FOXP3 gene encodes for a 431 amino acid (aa) protein with a mass of 47.25 kDa. The *FOXP3* gene contains 12 exons in humans as the difference from previous reports due to counting exon -1 (the two 5' non-coding exon) as exon 1 (Kaur et al., 2010). *FOXP3* gene locates in the centromeric region of the short arm of chromosome X (Xp11.3-q13.3). It encodes an essential regulate factor required for the function of CD4+ CD25+ regulatory T cells (T$_{reg}$) (Li et al., 2015). Thymus-derived regulatory T cells are a cell subset specialized for immune suppression with a crucial role in maintaining homeostasis: unstable or impaired T$_{reg}$ in the IPEX patients is the key leading to uncontrolled autoimmunity (Passerini et al., 2014). So far, over 80 different mutations in *FOXP3* are published in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php) and mainly located on the exons of the gene. Typical clinical manifestations of IPEX patients are early onset of intractable diarrhea, type 1 diabetes mellitus, and skin diseases (Chatila et al., 2000). However, other autoimmune types such as severe food allergies, autoimmune cytopenias, autoimmune respiratory illness, and mesangial glomerulonephritis may present that complicate the diagnosis of IPEX (Barzaghi et al., 2018a).

Next-generation sequencing could be considered a promising tool for diagnosing primary immunodeficiency (PID) disorder in general and IPEX syndrome in particular. The early identification of autoimmunity in the patients increases the effectiveness of treatment and improves patients' quality of life. Indeed, next-generation sequencing has been applied in genetic analysis of unspecified IPEX cases and obtained beneficial results (Chou et al., 2012; Maffucci et al., 2016; Stoddard et al., 2014; Sun et al., 2020). These studies also proved the applicability of whole-exome sequencing (WES) for diagnosing PID disorder and its benefits in helping clinicians make the proper diagnosis based on clinical and molecular findings.

In this study, we reported a patient with IPEX syndrome caused by a mutation in the *FOXP3* gene, which was detected by whole-exome sequencing.

CASE PRESENTATION

This study conducted a genetic analysis to identify genetic mutations as the causative agent in a 1-year-old boy diagnosed with IPEX syndrome at the Vietnam National Children's Hospital. The study was performed according to the Declaration of Helsinki (2013) and approved by the Ethics Committee of the Institute of Genome Research (No. 01/QD-NCHG on 10 January 2020).

The patient, the fifth child in the family, is a full-term boy with a birth weight of 2.8 kg due to his mother's obstetric history being normal. The child was completely healthy after birth, but he admitted pneumonia at 4 and 5 months old several times. There was no family history of lung disease and known autoimmune disease. Besides, the patient had four healthy and well-being older sister siblings, but their blood samples were not collected for genetic testing.

In the first year of life, he was referred to the Vietnam National Children's Hospital with prolonged fever of unknown origin, vomiting, and bloody black diarrhea. In addition, he also had an upper respiratory tract infection, pleural effusion, and splenomegaly. The patient's routine blood test results were normal, except for eosinophil count and proportion, white blood cells, and neutrophils were higher than the normal ranges (Table 1). Laboratory tests showed that his serum IgE, IgG, and IgM concentrations were normal, while his IgA level was slightly below the normal range (Table 2). Lymphocyte subsets analysis revealed normal proportions for CD3 (57%, reference range: 55–78%), CD8 T cells (26%, reference range: 19–34%) and minimum proportion of CD4 T cells (29.00%, reference range: 29.78–39.94%) (Table 2).

Table 1. Blood routine results of hospitalization

Date	WBC ($\times 10^9$/L)	Lymph ($\times 10^9$/L)	Neut ($\times 10^9$/L)	Mono ($\times 10^9$/L)	EO ($\times 10^9$/L)	BASO ($\times 10^9$/L)	Hb (g/L)
2020-02-18	25.0	5.53	16.31	1.23	1.68	0.06	83
2020-03-06	24.0	5.76	13.63	1.75	2.92	0.04	93
2020-04-27	26.7	11.80	10.30	1.87	2.60	0.12	92

Note: WBC = white blood cell (reference range: 6–17.5 $\times 10^9$/L); Lymp = Lymphocyte (reference range: 4–12 $\times 10^9$/L); Neut = neutrophil (reference range: 1–8.5 $\times 10^9$/L); Mono = monocyte (reference range: 0.16–1.0 $\times 10^9$/L); EO = eosinophil (reference range: 0–0.8 $\times 10^9$/L); BASO = basophil (reference range: 0–0.2 $\times 10^9$/L); Hb = hemoglobin (reference range: 111–141 g/L).

Table 2. Serum immunoglobulin test and Lymphocyte subset

Date	IgA (g/L) (normal range: 0.8–3.0)	IgM (g/L) (normal range: 0.4–2.5)	IgG (g/L) (normal range: 6–16)	IgE (IU/L) (normal range: 400–600)
2020-02-04	0.64	1.24	10.49	481.0
2020-04-27	0.23	0.94	7.18	390.0
Date	CD3+ (normal range: 55–78%)	CD4+ (normal range: 29.78–39.94%)	CD8+ (normal range: 19–34%)	
2020-03-25	57%	24%	26%	

After diagnosing IPEX syndrome, DNA samples of the child and the parents were extracted and conducted for sequencing on Illumina platform sequencer (San Diego, CA, USA). WES result generated a total of 42,953,218 reads corresponding to 6,425,848,692 bases, with 93.5% of the reader having a quality score of more than 30. Of these, 42,902,055 (99.8%) reads were successfully mapped to the reference human genome. A total of 80,177 SNPs were identified, of which 11,545 missense variants. After evaluation and filtration, we found a missense mutation, NM_014009.4 (rs1057520529): c.1190G>A (p.Arg397Gln), in exon 12 in the *FOXP3* gene

in the patient. This variant has also been deposited at National Center for Biotechnology Information ClinVar under accession number VCV000379222.6.

To confirm mutation effection in the patient, the p.Arg397Gln mutation was predicted to be "probably damaging" (score: 0.997) by the Polyphen - 2 (http://genetics.bwh.harvard.edu/pph2/), Panther (Pdel: 0.85) (http://www.pantherdb.org/tools/csnp-ScoreForm.jsp) and Align GVGD (Class C35) (http://agvgd.hci.utah.edu/agvgd_input.php), "damaging" (score: -3.74) by FATHMM (http://fathmm.biocompute.org.uk/index.html), "medium effect" (FI score: 3.36) by Mutation

Assessor (http://mutationassessor.org/r3/), "deleterious" (PROVEAN score: -3.685) by PROVEAN (http://provean.jcvi.org/ seq_submit.php), "disease" (RI score: 10) by SNP&GO (https://snps.biofold.org/snps-and-go/snps-and-go.html). This mutation was predicted to be damaging, medium effect, and disease in 7 tools with high scores.
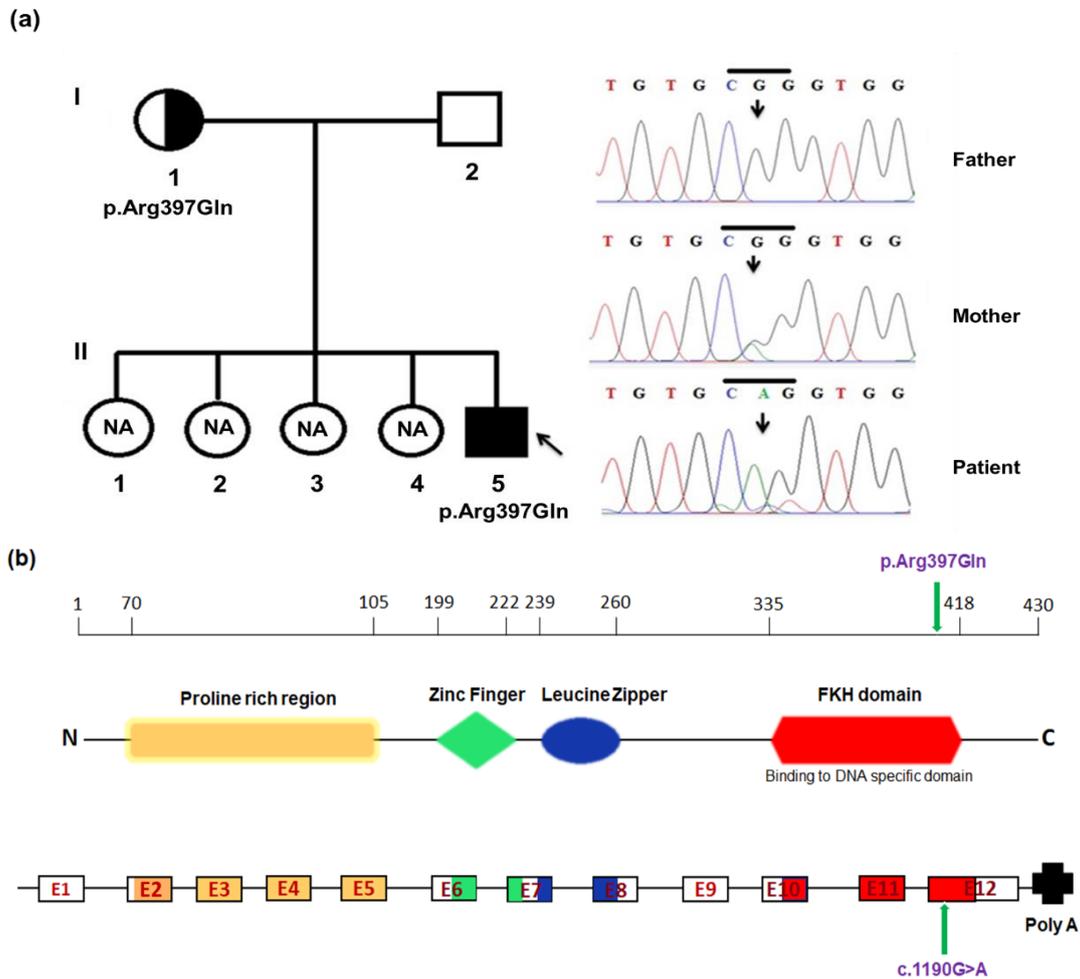

Figure 1. **(a)** Sequencing chromatogram of the *FOXP3* mutation in each family member (right panel) and patient pedigree (left panel). Square symbols are male members, and the circle is the female member of the family. Filled symbols are affected individuals; partially filled symbol is the carrier individual; empty symbol is the unaffected individual; arrow indicates the proband (patient II.5). The only male child was recruited for both exome sequencing, and Sanger validation, except his older sister siblings, had no samples for genetic testing (NA: Not Available). The patient carried a missense mutation, c.1190G>A, in exon 12 of the *FOXP3* gene in the hemizygous state. **(b)** Schematic diagram of the human *FOXP3* gene structure and mutation position. The human *FOXP3* gene includes 12 exons, within which the first exon, 5' part of exon 2, and 3' part of exon 12 belong to the non-coding region. FOXP3 protein contains four distinct parts, comprising of N-terminal proline-rich region (PRR; no color), central zinc finger (ZF; aa 199-222; green), leucine zipper (LZ; aa 239-260; blue), and forkhead domain (FKH; aa 335-418; red). These domains interact with proteins to control transcriptional activity and regulate T_{reg} cell function
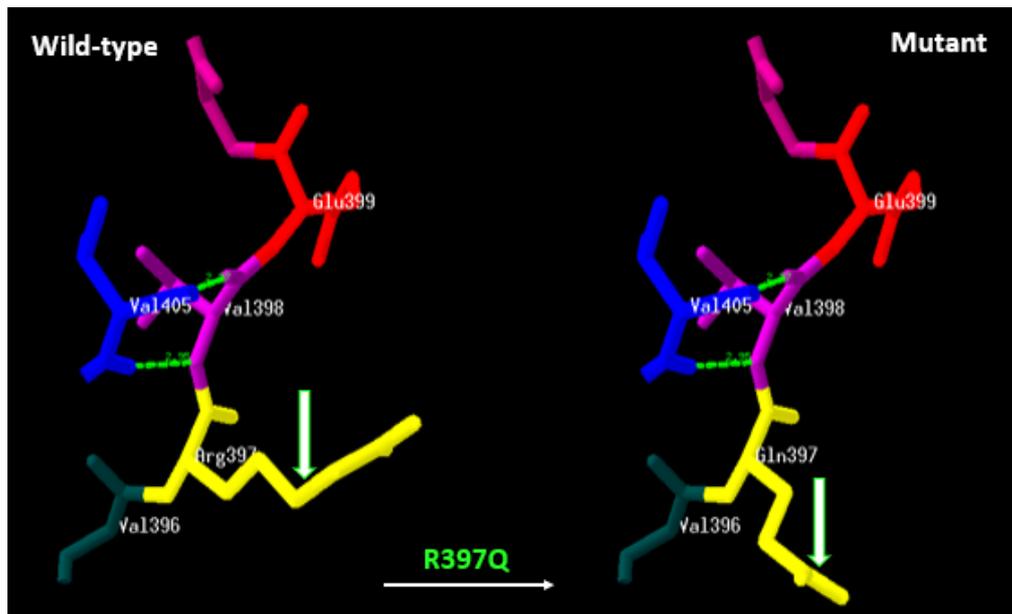
Figure 2. Prediction of possible structural consequences of FOXP3 R397Q missense mutation: Details of the 3D-model of the FOXP3 protein at the Arg397 position for the wild type (PDB ID: 3QRF; left panel) and the Gln397 position in the mutant modeled from the wild type (right panel). The figure was generated by using SPDBV (Swiss-Pdb Viewer version 4.1)

After that, Sanger sequencing was done to confirm the mutation c.1190G>A at the hemizygous state in the patient and his mother, while his father carried a normal allele on chromosome X (Fig. 1a). In addition, the three-dimensional structure of the wild type Arg397 and mutant Gln397 were constructed using Swiss Pdb Viewer v4.1 with PDB: 3QRF as a template. The result showed no change in the three-dimensional structure at the position of Arg397Gln (Fig. 1b). In other words, the substitution of amino acid Arginine by Glutamine at position 397 does not affect the spatial structure of the protein molecular.

DISCUSSION

IPEX syndrome mainly affects males due to the X-linked recessive disorder of the *FOXP3* gene. The diagnosis of IPEX syndrome mostly depends on several typical clinical symptoms and identifying a hemizygous pathogenic mutation in the *FOXP3* gene. However, some exceptions have been noted. In this study, we applied NGS technology to detect pathogenic mutations in patients with clinical phenotypes of IPEX syndrome. The 1-year-old boy presented symptoms of IPEX syndrome such as prolonged fever, bloody diarrhea, vomiting, skin abscesses, high concentration of eosinophil, and lower normal range of IgA proportion. The variant c.1190G>A (p.Arg397Gln) in the *FOXP3* gene was detected at the hemizygous state by whole-exome sequencing.

This mutation is located in exon 12 of the *FOXP3* gene and belongs to the FKH domain, reported in typical and atypical late-onset patients (Ge et al., 2017). FOXP3 protein contains four separated regions (Fig. 1b) (Mailer et al., 2009). The first three domains are vital for the interaction between proteins, and the last domain is obligatory for nuclear localization and DNA-binding activity (Ziegler, 2006). Of note, most IPEX syndrome mutations cluster within the FKH domain is responsible for DNA-binding movement (Gambineri et al., 2018). This domain is an essential active site of the

protein molecule, so mutations in this region have affected the protein's function.

According to 7 prediction tools, the candidate mutation was predicted to be pathogenic. The pathogenic variants have been reported in the *FOXP3* gene, including nonsense variants, missense variants, small in-frame amino acid deletions/insertions, and splice site variants tend to possess severe clinical presentations (An et al., 2011). However, some missense mutations lead to a typical or reduced mutant protein expression (Ziegler, 2006). Besides, Yang et al. (2020) revealed the correlation between the observed phenotypes and changes of hydrogen bond formation which means that the more hydrogen bonding changes, the more severe empirical appearances. Indeed, hydrogen bonds function in protein folding, which is a vital cellular process in its native 3D structure, and protein structure is crucial to its expression into phenotypes. Both wildtype Arg397 and mutant 397Gln do not have any hydrogen bond with any adjacent residues, suggesting no hydrogen bonding alternations in the wild type or mutant variant (Fig. 2). So, the mutation does not result in any apparent changes in the structural interface, which could explain why some typical effects were not observed in this patient. Indeed, serum IgA of the patient had a lower level despite this concentration elevating in IPEX syndrome (Patey-Mariaud de Serre et al., 2009). Besides, the patient still maintained average growth and weighted within the growth weight range (8.5 kg, reference range: 8.3-12.8 kg).

In contrast, a 1-month-old-boy case with c.1190G>A mutation had a normal immunoglobulin range, except IgE elevated concentration (Martin-Santiago et al., 2013). In comparison, the same transformation in an atypical case of Ge et al. (2017) study suggested a normal immunoglobulin A, IgG, IgM, and IgE of a 6-year-old-boy. This evidence proves that each individual in the same or different family can present with variable severity, though they have the same genotype (Seidel et al., 2016).

We have identified a mutation in the *FOXP3* gene in an IPEX patient with non-specific clinical symptoms using whole-exome sequencing. Our results contribute to the general understanding of the genetic causes of the disease and suggest that WES is a useful tool in determining genetic causes. The study also shows the need for genetic counseling and prenatal screening for genetic diseases and especially for families with a carrier of the disease gene when having more children.

CONCLUSION

A hemizygous missense mutation, c.1190G>A (p.Arg397Gln), in the *FOXP3* gene was detected for the first time by WES in a Vietnamese boy diagnosed with IPEX syndrome. This mutation in the FOXP3 protein is the cause of disease in the patient. This result concludes that WES has helpful capabilities to detect genetic causes in IPEX patients.

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