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FOXP3 Mutations Causing Early-Onset Insulin-Requiring Diabetes but Without Other Features of IPEX Syndrome:

FOXP3 mutations with IPEX-like Diabetes

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Abstract

Diabetes occurs in 1/90,000–1/160,000 births and when diagnosed under 6 months of age is very likely to have a primary genetic cause. *FOXP3* encodes a transcription factor critical for T regulatory cell function and mutations are known to cause “Immune dysregulation, Polyendocrinopathy (including insulin-requiring diabetes), Enteropathy, X-linked” (IPEX) syndrome. This condition is often fatal unless patients receive a bone-marrow transplant. Here we describe the phenotype of male neonates and infants who had insulin-requiring diabetes without other features of IPEX syndrome and were found to have mutations in *FOXP3*. Whole-exome or next generation sequencing of genes of interest was carried out in subjects with isolated neonatal diabetes without a known genetic cause. RT-PCR was carried out to investigate the effects on RNA splicing of a novel intronic splice-site variant. Four male subjects were found to have *FOXP3* variants in the hemizygous state: p.Arg114Trp, p.Arg347His, p.Lys393Met, and c.1044+5G>A which was detected in 2 unrelated probands and in a brother diagnosed with diabetes at 2.1 years of age. Of these, p.Arg114Trp is likely a benign rare variant found in individuals of Ashkenazi Jewish ancestry and p.Arg347His has been previously described in patients with classic IPEX syndrome. The p.Lys393Met and c.1044+5G>A variants are novel to this study. RT-PCR studies of the c.1044+5G>A splice variant confirmed it affected RNA splicing by generating both a wild type and truncated transcript. We conclude that *FOXP3* mutations can cause early-onset insulin-requiring diabetes with or without other features of IPEX syndrome.

Keywords

Monogenic diabetes; FOXP3; T Cell; autoimmunity; splice mutation

Introduction

Neonatal Diabetes occurs in every 1: 90,000–160,000 births. When diagnosed under 6 months of age, a primary genetic cause is very likely, whereas at later ages the cause is likely to be polygenic type 1 diabetes (1). *FOXP3* is one of over 20 genes (2) to be associated with neonatal diabetes and encodes a forkhead box transcription factor required for the function of CD4⁺ CD25⁺ regulatory T cells which regulate immunologic tolerance (3). Pathogenic variants in *FOXP3* most often cause the “Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked” (IPEX) syndrome, which includes not only early-onset insulin-requiring diabetes, but also severe enteropathy, eczema and other features that often lead to death by 24 months due to failure to thrive and/or sepsis unless the patient receives a bone-marrow transplant (4). More rarely, *FOXP3* mutations have been described in cases without all the features of IPEX syndrome, some without diabetes, and others with neonatal diabetes and other autoimmune features but without life-threatening enteropathy (5). Here we describe cases from our US Monogenic Diabetes Registry who had neonatal diabetes without other main features of IPEX syndrome and were found to carry *FOXP3* mutations, some of which were novel.

Subjects and Methods

Sequencing and mutation analysis

All subjects were consented for participation under a protocol approved by the institutional review board of the University of Chicago. Seventeen probands diagnosed with diabetes diagnosed at less than twelve months of age were studied. They underwent Sanger sequencing of *KCNJ11*, *INS* and *ABCC8* but no mutations were found. Whole exome or next generation sequencing of a panel of monogenic diabetes-related genes was carried out as previously described (6, 7). Variants identified in probands and family members were confirmed through single amplicon Sanger sequencing.

RNA isolation and cDNA synthesis

Lymphocytes were collected from heparinized whole blood from healthy adults and patients using erythrocyte lysis buffer (Qiagen). Total RNA was extracted from isolated leukocytes using Trizol (Invitrogen) and cDNA was synthesized using the Superscript® III First Strand Synthesis System (Invitrogen).

Analysis of FOXP3 mRNA splicing

RT-PCR spanning exons 9–11 of *FOXP3* (NM_014009.3) was carried out using the following forward and reverse primers respectively: 5′ - AATGGCACTGACCAAGGCTTC-3′ and 5′ CAAAGCACTTGTGCAGACTCAG-3′.

RT-PCR conditions consisted of an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 secs, annealing at 62 °C for 1 min, extension at 68 °C for 1 min, and a final extension step at 68 °C for 10 min. PCR products were separated on a 10% polyacrylamide gel stained with ethidium bromide and band density was measured using ImageJ. Amplified fragments were then extracted from agarose gel and sequenced to confirm the pattern of alternative splicing.

Flow Cytometry

FOXP3 protein expression from peripheral blood mononuclear cells (PBMCs) was analyzed in a clinical assay using flow cytometry (Cincinnati's Children's Hospital's Diagnostic Immunology Laboratory). Briefly, FOXP3 staining was performed using rat monoclonal anti-human FOXP3 antibody (PCH101) that reacts with the amino terminus of human FOXP3 protein. FOXP3 expression was reported as a percent of CD4⁺ CD25⁺ CD127⁻ T Cells.

Results

Whole exome or targeted sequencing identified four *FOXP3* variants in 6 cases: p.Arg114Trp (UC0075A), p.Arg347His (UC0132A), p.Lys393Met (UC1058A), and c.1044+5G>A (UC0078A, UC0078D, UC0116A). The p.Arg114Trp variant (rs200554980), located in the N-terminal proline-rich repressor domain, is rare based on population data from the Exome Aggregation Consortium (ExAC) (<http://exac.broadinstitute.org/variant/X-49113998-G-A>). The Arg114Trp variant in the proband was maternally inherited. The subject's maternal grandfather was diagnosed with diabetes at 67 years of age with no clinical information on therapy or DNA sample available for testing. The p.Arg347His variant, located in the forkhead box domain, has been previously described in patients with IPEX syndrome (8). This likely pathogenic sequence change was present in the *de novo* state in the proband. The p.Lys393Met variant, also located in the forkhead box domain, has not been identified in the general population (ExAC). This variant is predicted to be possibly damaging (Polyphen HumDiv score: 0.63) and it is situated between two previously reported *FOXP3* pathogenic variants: p.Ser390Asn and p.Arg397Trp (9, 10). DNA from the proband's mother was not available to determine inheritance. The c.1044+5G>A splice site change is adjacent to a previously reported variant c.1044+4A>G identified in one case with classic IPEX (11). We studied all available family members and identified the c.1044+5G>A variant in the unaffected mother (UC0078B) and the proband's brother (UC0078D) who was diagnosed with diabetes at 2.1 years of age (Figure 1), as well as in one other proband who was diagnosed at 8 months. Flow cytometric analysis for UC0078A showed that FOXP3 protein was expressed by 97% of CD4⁺CD25⁺ T cells in peripheral blood (reference range: 87–100) with an absolute FOXP3⁺ cell count of 77 cells/mcL (reference range: 29–303).

We then examined the effect of the c.1044+5G>A variant on mRNA splicing. RT-PCR and sequencing demonstrated abnormal splicing that generated both a wild-type and truncated transcript missing exon 10 (Figure 1). The mother's lymphocytes also showed a lower abundance of the exon 10-minus transcript (14.6 %) compared to those of her sons (32 %).

Discussion

None of the six subjects reported here had classic IPEX syndrome – enteropathy was absent and none of them were treated with immunosuppressive therapy (Table 1). Diabetes was the presenting feature in all cases, all of whom had normal birth weights. UC1058A was born prematurely at 32 weeks but his weight was appropriate for gestational age. Although each of the five subjects had variable severity of atopic dermatitis, the only other autoimmune features included beta cell antibodies in three subjects (UC0078A, UC0078D and UC1058A), thyroid antibodies in two subjects (UC0075A and UC1058A), and parietal cell antibodies in another (UC0078A). Other autoimmune features such as hemolytic anemia, thrombocytopenia, hepatitis, and nephrotic syndrome were absent. One case exhibited short stature (UC0132A) who was later treated with growth hormone and two other cases described mild chronic gastrointestinal discomfort not requiring treatment (UC0075A and UC1058A), while another had extreme failure to thrive of unknown etiology despite thorough gastrointestinal workup (UC0078D).

Despite the clinical presentation and splice variant identified in UC0078A, FOXP3-expressing CD4+T cells were readily detectable in peripheral blood. Many reported mutations in *FOXP3* cause a reduction or complete absence of protein expression and most of these cases likely had the complete IPEX syndrome or severe disease. However, a number of studies have reported normal protein expression even in cases with mutations causing the complete IPEX syndrome (3, 12, 13). Here, we demonstrate that c.1044+5G>A causes alternative splicing resulting in some cells expressing the normal transcript and some cells expressing the mutant transcript. It is thus perhaps not surprising that protein expression was within normal limits and that these cases have a milder phenotype. However, it remains uncertain whether diabetes and other features of these cases results from a reduction of normal expression, or rather may be due to a subset of dysfunctional regulatory CD4+ T cells that express a truncated protein with abnormal function.

Arg114Trp was considered a variant of unknown significance based on population data from ExAC (Population frequency in European (Non-Finnish): allele count, 12; allele number, 35194; allele frequency, 0.000341). This variant was also reported in the T2D-GENES database (<http://www.type2diabetesgenetics.org/>) in the European (8 affected and 3 unaffected) and South Asian samples (1/1,071 unaffected). It may also occur at a higher frequency in individuals of Ashkenazi Jewish ancestry (5/506 affected (3 female and 2 male) and 3/355 unaffected (3 female)). The two affected males were 76 and 50 years old at the time of ascertainment, had diabetes since age 50 and 45, respectively, and were only treated with oral agents. Neither indicated any skin or gastrointestinal disease, but these questions were not specifically asked. Antibodies were not tested. The three affected females were diagnosed with diabetes at 45, 49 and 50 years of age. Our current interpretation is that the Arg114Trp *FOXP3* variant is likely not pathogenic and not the cause of neonatal diabetes in our proband who was of Ashkenazi Jewish ancestry.

Our results show that mutations in *FOXP3* can be found and should be tested for in male patients who present with diabetes in infancy even when they lack other features of IPEX syndrome. The fact that the brother was diagnosed with diabetes at 2.1 years of age raises

the question of how to decide whether to test for *FOXP3* in patients with early onset diabetes. It also remains unclear whether cases such as those in the current study might benefit from immunomodulatory therapies if not complete bone-marrow transplant. Further research into *FOXP3*-related diabetes may provide a better understanding of the natural history and treatment of this interesting form of diabetes.

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Figure 1.
Analysis of novel *FOXP3* c.1044+5G>A splice-donor site mutation.
A) Pedigree and sequence traces of mother and affected sons. B) RT-PCR of lymphocyte RNA of mother (UC0078B), affected sons (UC0078A and UC0078D), and male and female controls showing wild-type (WT) (398 bp), abnormally spliced transcripts (321bp), and their respective band densities. C) Schematic representation and cDNA sequence of skipping of exon 10 causing a frameshift and a premature stop codon within the transcript.

Table 1

Clinical characteristics of subjects with *FOXP3* mutations.

Subject	UC0075A	UC0132A	UC1058A	UC0078A	UC0078D	UC0116A
<i>FOXP3</i> mutation	c.340C>T p.Arg114Trp	c.1040G>A p.Arg347His	c.1178A>T p.Lys393Met	c.1044+5G>A 5' splice site	c.1044+5G>A 5' splice site	c.1044+5G>A 5' splice site
Previous Report of mutation	None	Multiple reports with variable IPEX phenotype (8)	Novel	Novel. One report of classic IPEX with mutation at adjacent nucleotide: c. 1044+4 (11)		
Current age (yr)	31	19	3	7	9	28
Diabetes dx age (wk)	26.1	1.5	22.3	26.7	104.5 (2.1 yrs)	34.6
Gestational age (wk)	40	42	32	37.5	41	N/A
Birthweight (gm)	3930	3900	1927 *	3400	3520	4054
Current diabetes treatment	Glargine/lispro	Insulin pump	Insulin pump	Insulin pump	Insulin pump	Insulin
Last HbA1c (%)	7.8 (62mmol/mol)	9. (78 mmol/mol)	7.9 (63 mmol/mol)	8.4 (68 mmol/mol)	7.9 (63 mmol/mol)	9.8 (84mmol/mol)
Autoantibodies	+ anti-thyroid	N/A	+ anti-thyroid + anti-GAD65	+ anti-insulin + anti-parietal cell + anti-adrenal - anti-thyroid - anti-enterocyte - TTG/endomysial	+ anti-thyroid	-TTG
Gastrointestinal features	Mild chronic abdominal discomfort	No symptoms of enteropathy	Mild diarrhea	No symptoms of enteropathy	Extreme FTT, weight at < 5th percentile; EGD result: inactive peptic duodenitis and inactive chronic gastritis; no apparent enteropathy	No GI symptoms
Skin features	Mild eczema	Mild eczema	Mild eczema	Mild eczema	Severe eczema, vitiligo	No skin symptoms
Other medical problems	N/A	Short stature; nocturnal hypoglycemia associated with seizures	Prematurity, paralysis of the right diaphragm, multiple respiratory infections one of which was associated with cardiorespiratory failure	N/A	Paralyzed diaphragm with temporary tracheostomy; multiple pneumonias and pump site infections, one time pyelonephritis	ADHD No thyroid problems
Immunosuppression	Never	Never	Never	Never	Never	Never

FTT: failure to thrive. TTG: tissue transglutaminase. EGD: esophagogastroduodenoscopy. N/A: not available. ADHD: attention deficit hyperactivity disorder

* Appropriate for gestational age