Diabetes occurs in 1/90 000 to 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, 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 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.
regulate immunologic tolerance. 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 within 24 months due to failure to thrive and/or sepsis unless the patient receives a bone-marrow transplant. 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. 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.

2 | METHODS

2.1 | 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 12 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. Variants identified in probands and family members were confirmed through single amplicon Sanger sequencing.

2.2 | RNA isolation and cDNA synthesis

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

2.3 | Analysis of FOXP3 mRNA splicing

RT-PCR: Reverse Transcription Polymerase Chain Reaction spanning exons 9-11 of FOXP3 (NM_014009.3) was carried out using the following forward and reverse primers, respectively: 5′-CATGGACCT-GACAAAGGCTC-3′ and 5′-GAAAAGCATTGTGCAAGAC TCAG-3′.

RT-PCR conditions consisted of an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 1 minute, extension at 68°C for 1 minute, and a final extension step at 68°C for 10 minutes. 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.

2.4 | 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.

3 | RESULTS

Whole exome or targeted sequencing identified 4 FOXP3 variants in 6 cases: p.Arg114Trp (UC0075A), p.Arg347His (UC0132A), p.Lys393-Met (UC1058A), and c.1044+5G>A (UC0078A, UC0078B, 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. 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 2 previously reported FOXP3 pathogenic variants: p.Ser390Asn and p.Arg397Trp. 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 1 case with classic IPEX syndrome. 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/μL (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%).

4 | DISCUSSION

None of the 6 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 5 subjects had variable severity of atopic dermatitis, the only other autoimmune features included
beta cell antibodies in 3 subjects (UC0078A, UC0078D, and UC1058A), thyroid antibodies in 2 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 2 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. 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 because of 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, 35 194; 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 (1/1071 unaffected). It may also occur at a higher frequency in individuals of Ashkenazi Jewish ancestry (5/506 affected [3 females and 2 males] and 3/355 unaffected [3 females]). The 2 affected males were 76 and 50 years at the time of ascertainment, had diabetes since 50 and 45 years of age, 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 3 affected females were diagnosed with diabetes at 45, 49, and 50 years of age. Our current interpretation

**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 (321 bp), 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

<table>
<thead>
<tr>
<th>Subject</th>
<th>UC0075A</th>
<th>UC0132A</th>
<th>UC1058A</th>
<th>UC0078A</th>
<th>UC0078D</th>
<th>UC0116A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous report of mutation</td>
<td>None</td>
<td>Multiple reports with variable IPEX phenotype</td>
<td>Novel</td>
<td>Novel, one report of classic IPEX with mutation at adjacent nucleotide: c.1044+4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current age (y)</td>
<td>31</td>
<td>19</td>
<td>3</td>
<td>7</td>
<td>9</td>
<td>28</td>
</tr>
<tr>
<td>Age at diabetes diagnosis (wk)</td>
<td>26.1</td>
<td>1.5</td>
<td>22.3</td>
<td>26.7</td>
<td>104.5 (2.1 y)</td>
<td>34.6</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>40</td>
<td>42</td>
<td>32</td>
<td>37.5</td>
<td>41</td>
<td>N/A</td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>3930</td>
<td>3900</td>
<td>1927&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3400</td>
<td>3520</td>
<td>4054</td>
</tr>
<tr>
<td>Current diabetes treatment</td>
<td>Glargine/lispro</td>
<td>Insulin pump</td>
<td>Insulin pump</td>
<td>Insulin pump</td>
<td>Insulin pump</td>
<td>Insulin</td>
</tr>
<tr>
<td>Last HbA1c (%)</td>
<td>7.8 (62 mmol/mol)</td>
<td>9.3 (78 mmol/mol)</td>
<td>7.9 (63 mmol/mol)</td>
<td>8.4 (68 mmol/mol)</td>
<td>7.9 (63 mmol/mol)</td>
<td>9.8 (84 mmol/mol)</td>
</tr>
<tr>
<td>Autoantibodies</td>
<td>+Anti-thyroid</td>
<td>N/A</td>
<td>+Anti-thyroid; +anti-GAD65</td>
<td>+Anti-insulin; +anti-parietal cell; +anti-adrenal; -anti-thyroid; -anti-enterocyte; -TTG/ endomysial</td>
<td>+Anti-thyroid</td>
<td>-TTG</td>
</tr>
<tr>
<td>Gastrointestinal (GI) features</td>
<td>Mild chronic abdominal discomfort</td>
<td>No symptoms of enteropathy</td>
<td>Mild diarrhea</td>
<td>No symptoms of enteropathy</td>
<td>Extreme FTT, weight at &lt;5th percentile; EGD result: inactive peptic duodenitis and inactive chronic gastritis; no apparent enteropathy</td>
<td>No GI symptoms</td>
</tr>
<tr>
<td>Skin features</td>
<td>Mild eczema</td>
<td>Mild eczema</td>
<td>Mild eczema</td>
<td>Mild eczema</td>
<td>Severe eczema, vitiligo</td>
<td>No skin symptoms</td>
</tr>
<tr>
<td>Other medical problems</td>
<td>N/A</td>
<td>Short stature; nocturnal hypoglycemia associated with seizures</td>
<td>Prematurity, paralysis of the right diaphragm, multiple respiratory infections one of which was associated with cardiorespiratory failure</td>
<td>N/A</td>
<td>Paralyzed diaphragm with temporary tracheostomy; multiple pneumonias and pump site infections, one time pyelonephritis</td>
<td>ADHD, no thyroid problems</td>
</tr>
<tr>
<td>Immunosuppression</td>
<td>Never</td>
<td>Never</td>
<td>Never</td>
<td>Never</td>
<td>Never</td>
<td>Never</td>
</tr>
</tbody>
</table>

Abbreviations: ADHD, attention deficit hyperactivity disorder; EGD, esophagogastroduodenoscopy; FTT, failure to thrive; GAD65, glutamic Acid Decarboxylase; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; TTG, tissue transglutaminase; N/A, not available.

<sup>a</sup> Appropriate for gestational age.
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.

ACKNOWLEDGEMENTS

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REFERENCES


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