A Synonymous Variant in IL10RA Affects RNA Splicing in Paediatric Patients with Refractory Inflammatory Bowel Disease

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Abstract

Interleukin-10 receptor [IL10R] mutations are associated with severe childhood inflammatory bowel disease [IBD]. Two unrelated patients who died of very early-onset severe IBD and sepsis were identified as harbouring the same compound heterozygous mutations in IL10RA [p.R101W; p.T179T]. A third patient was found to be homozygous for p.T179T. The missense change of p.R101W has been reported. The synonymous change of p.T179T, with a minor allele frequency of 0.035% in the population, was novel. The p.T179T mutation was located before the 5′ splice donor site, leading to exon skipping and out-of-frame fusion of exons 3 and 5, causing altered STAT3 phosphorylation in IL10-induced peripheral blood mononuclear cells. The patient developed colitis at 6 years of age, the oldest reported age of onset among patients with IL10RA mutations, and did not suffer from perianal disease. We report three paediatric patients with a rare, synonymous p.T179T variant causing a splicing error in IL10RA.

Key Words: Crohn’s disease; exome sequencing; IL10RA; synonymous variant

1. Introduction

Defects in the interleukin [IL]-10 pathway underlie the pathology of an important subgroup of very early-onset inflammatory bowel diseases [IBD]. As a cytokine, IL10 plays an important anti-inflammatory role in mucosal homeostasis, and deleterious mutations in genes encoding IL10 and its receptors have been shown to cause severe bowel inflammation among infantile IBD patients. The onset of this monogenic IBD type occurs at typically less than 1 year of age, and the main clinical phenotypes are severe colitis, recurrent infections and perianal disease refractory to conventional IBD treatments such as azathioprine, infliximab, antibiotics, and surgery. Most IBD patients with defects in the IL10 pathway undergo colectomy or ileostomy for the temporary resolution of disease activity. Haematopoietic stem cell transplantation is the only curative approach in these patients.

Recently, we experienced the deaths of two patients with very early-onset IBD at our IBD centre. Family-based whole-exome sequencing [WES] analyses revealed that these patients carried identical compound heterozygous mutations in IL10RA [p.R101W; p.T179T]. We also identified a third patient with IBD onset at the age of 6 years, who carried a homozygous mutation of p.T179T in IL10RA. In this study, we demonstrate that the synonymous p.T179T variant causes a splicing error in IL10RA, resulting in a defect in the IL10 pathway.
2. Case Presentations

2.1. Patient A

Patient A, a female, was born at 41 weeks of gestation with a birthweight of 2.6 kg to non-consanguineous Korean parents. At the age of 2 months, she was referred to paediatric surgery due to rectovaginal fistula and anal fissure, and had recurrent febrile illnesses thereafter. When she was 4 months old, she was noted to have growth failure [fifth percentile for height age; < third percentile for weight age], persistent anaemia, and hypoalbuminaemia. The patient underwent sigmoid loop colostomy due to recurrent rectovaginal fistula. Colonoscopy conducted at the age of 9 months showed linear ulcerations and a cobblestone appearance extending from the hepatic flexure to the distal colon, which were compatible with findings of Crohn's disease [CD] [A1a, L2, B3, P1, G1, according to the Paris classification]. Colitis was refractory to corticosteroids, azathioprine, and infliximab, and the patient experienced frequent flares. At the age of 13 months, she died of sepsis. Her parents and sister have been healthy.

The family-based WES analysis [Supplementary Text 1, available as Supplementary data at ECCO-JCC online] for family A revealed that patient A carried one de novo variant, two homozygous variants, Table 1.

Table 1. Analytical step of family-based exome sequencing for families A and B.

<table>
<thead>
<tr>
<th>Analytical pipeline</th>
<th>Family A</th>
<th>Family B</th>
<th>Common between the two families</th>
</tr>
</thead>
<tbody>
<tr>
<td>All coding variants</td>
<td>35088</td>
<td>34580</td>
<td>26202</td>
</tr>
<tr>
<td>Filtering by quality</td>
<td>26656</td>
<td>26346</td>
<td>19743</td>
</tr>
<tr>
<td>Filtering by quality and 1000 genomes frequencies [% 1% in each population]</td>
<td>1872</td>
<td>1773</td>
<td>520</td>
</tr>
<tr>
<td>Filtering by family data&lt;sup&gt;a&lt;/sup&gt; de novo</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>simple recessive</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Counting only filtered [by quality and 1000 genomes frequencies] non-synonymous [including nonsense, frameshifts, and splice] variants with complete genotype calls in all family members. These variant calls were also confirmed by visual inspection of the read alignments on the integrative genomics viewer [http://www.broadinstitute.org/igv/, accessed May 25, 2016].

<sup>b</sup>Number of genes.

Table 2. Variant profiles of IL10RA.

<table>
<thead>
<tr>
<th>Location</th>
<th>Exon 3</th>
<th>Exon 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosome position</td>
<td>chr11: 117,860,269</td>
<td>chr11: 117,864,125</td>
</tr>
<tr>
<td>SNP</td>
<td>rs368287711</td>
<td>NA</td>
</tr>
<tr>
<td>Change of nucleotide</td>
<td>C301T</td>
<td>G537A</td>
</tr>
<tr>
<td>Change of amino acid</td>
<td>p.R101W</td>
<td>p.T179T</td>
</tr>
<tr>
<td>Patient A</td>
<td>C/T</td>
<td>A/G</td>
</tr>
<tr>
<td>Patient B</td>
<td>C/T</td>
<td>A/G</td>
</tr>
<tr>
<td>Patient C</td>
<td>C/C</td>
<td>A/A</td>
</tr>
</tbody>
</table>

SNP, single nucleotide polymorphism.

and six genes with compound heterozygous variants [Table 1]. Among them, patient A carried one missense mutation [p.R101W] and one synonymous amino acid change [p.T179T] in the IL10RA gene [Table 2]. The two variants were confirmed by Sanger sequencing [Figure 1A]. A missense mutation [p.R101W] of IL10RA was reported as a causative allele in the homozygous state for refractory infantile IBD.1 This mutation was demonstrated to abrogate IL10-induced signalling, as shown by deficient signal transducer and activator of transcription-3 [STAT3] phosphorylation following IL10 stimulation. However, p.T179T was neither reported in public databases nor was its functional impact examined.

2.2. Patient B

Patient B, a female, was born at 39 weeks of gestation with a birthweight of 3.9 kg to non consanguineous Korean parents who were not related to the family of patient A. Recurrent febrile illnesses, diarrhea, and oral ulcers were noted during her infancy. At the age of 10 months, the patient underwent surgery for perianal fistula. At the age of 15 months, she was diagnosed with CD [A1a, L2, B1, P1, G1, by the Paris classification] of the entire colon. The initial Paediatric Crohn’s Disease Activity Index [PCDAI] was 30 and failure to thrive [third to fifth percentile for height age; < third percentile for weight age] was noted. Azathioprine and infliximab were used; however, she developed an intra-abdominal abscess with general worsening of the perianal fistula. Due to recurrent flares, the patient received an ileostomy at the age of 3.5 years and adalimumab therapy. At the age of 6.5 years, total colectomy was recommended. However, her parents refused to follow up until she was admitted with septic shock at the age of 7.5 years, which was the cause of death. Her parents and sister have been healthy.

Family B did not show any de novo or recessive homozygous variants except for three genes with compound heterozygous variants found in patient B. The only gene with compound heterozygous variants in both patients A and B was IL10RA [p.R101W and p.T179T]. Patient B inherited a missense mutation from her father [p.R101W] and a synonymous amino acid change from her mother [p.T179T].

2.3. Patient C

Patient C, a female, was born at 41 weeks of gestation with a birthweight of 3.2 kg to non-consanguineous Korean parents who were not related to the family of patient A. Recurrent febrile illnesses, diarrhea, and oral ulcers were noted during her infancy. At the age of 10 months, she was referred to our centre where she was diagnosed with CD [A1a, L3, B1, P0, G1, by the Paris classification]. The initial PCDAI was 40, and growth delay was noted. Her disease had initially been ameliorated by corticosteroid therapy. The initial PCDAI was 40, and growth delay was noted. Azathioprine and infliximab were used; however, she developed an intra-abdominal abscess with general worsening of the perianal fistula. Due to recurrent flares, the patient received an ileostomy at the age of 3.5 years and adalimumab therapy. At the age of 6.5 years, total colectomy was recommended. However, her parents refused to follow up until she was admitted with septic shock at the age of 7.5 years, which was the cause of death. Her parents and sister have been healthy.

Family C did not show any de novo or recessive homozygous variants except for three genes with compound heterozygous variants found in patient C. The only gene with compound heterozygous variants in both patients A and B was IL10RA [p.R101W and p.T179T]. Patient C inherited a missense mutation from her father [p.R101W] and a synonymous amino acid change from her mother [p.T179T].

Table 3. Splicing prediction scores for IL10RA p.T179T [NM_001558: c. 537 G>A].

<table>
<thead>
<tr>
<th>In silico prediction of splicing-altering SNV</th>
<th>Wild-type allele</th>
<th>mutant allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Position Weight Matrix</td>
<td>75.4</td>
<td>63.49</td>
</tr>
<tr>
<td>2. MaxEntScan score</td>
<td>8.47</td>
<td>1.94</td>
</tr>
<tr>
<td>3. NNSplice</td>
<td>0.97</td>
<td>0.07</td>
</tr>
<tr>
<td>4. Human Splicing Finder</td>
<td>83.56</td>
<td>72.98</td>
</tr>
</tbody>
</table>

SNV, single nucleotide variant.
2. MaxEntScan score; score, [-20]:[+20]; higher score implies higher probability of the sequence being a true splice site; threshold is defined as 3.
3. NNSplice; score, 0–1; higher score implies greater potential for splice site.

In the Human Splicing Finder prediction model, if wild-type score is above the threshold value and the difference between wild-type and mutant score is lower than -10%, the mutation is considered to be a splice site. In this case, the difference is -12.66, supporting the presence of splicing mutation.
The same reactions using mRNA from patient B with compound heterozygous variants [p.R101W; p.T179T] yielded both the truncated and the major full-length fragments [exon 3–5], as expected. To demonstrate that the 252-bp fragment represented an exon 4-skipping event, the fragment was purified and sequenced in both directions. The sequence contained the predicted splice junction between IL10RA exons 3 and 5 [Figure 2C]. The 3′ end of exon 3 falls between the first and second positions of codon 123, and the 5′ end of exon 5 begins with codon 180. The out-of-frame splice junction between exons 3 and 5 is predicted to add 7 novel codons to the 3′ end of codon 123, followed by a stop codon. The predicted truncation removes 448 wild-type amino acids from the carboxy terminus of the IL10RA protein. This shortened mRNA has a premature stop codon in exon 5, causing the loss of one of the two fibronectin type III, transmembrane, and intracellular domains of the mature stop codon in exon 5, causing the loss of one of the two fibronectin type III, transmembrane, and intracellular domains of the IL10 receptor [Figure 2D].

Functional validation was performed only in patient C [p.T179T homozygote], given that patients A and B were both deceased. The anti-inflammatory effects of IL10 is predominantly mediated by STAT3 signalling.14 Thus, to assess IL10-mediated signalling, peripheral blood mononuclear cells [PBMC] from patient C, her parents, and a healthy control were stimulated using IL10, and STAT3 phosphorylation at tyrosine 705 was measured by western blot analysis. IL6 was used in parallel as a control. IL10-induced STAT3 phosphorylation was defective in PBMCs from patient C, whereas IL6-induced STAT3 phosphorylation was intact [Figure 3]. However, IL10 was able to induce STAT3 phosphorylation in PBMCs of patient C’s parents and of the healthy control.

To estimate how rare this allele was in the Korean population, genotyping for p.T179T variant of IL10RA was performed in 2885 unrelated healthy controls using the TaqMan® SNP genotyping assay. Only two subjects were found to carry a heterozygous p.T179T variant, and the minor allele frequency of the p.T179T variant was determined as 0.035%.

3. Discussion
A critical role of IL10 in intestinal mucosal homeostasis has already been shown in animal knock-out models lacking IL10 or IL10 receptors.13,14 Deleterious IL10RA mutations have been shown to induce severe colitis, perianal fistulae, and recurrent sepsis during infancy.12,3,4,5,6,7,9,10,11 Our compound heterozygote [p.R101W; p.T179T] patients A and B also had IBD with similar clinical presentations. A previously reported patient with the p.R101W mutation in IL10RA suffered from severe colitis during infancy and underwent colectomy.1 Interestingly, patient C, who was homozygous for the synonymous p.T179T variant, did not have perianal disease. She underwent colectomy for refractory severe colitis, resulting in temporary improvement. Unlike other reported cases, her disease
manifested at the age of 6 years, which is the oldest reported age of onset among patients with IL10RA mutations. This relatively delayed onset was unique among IBD cases with IL10RA mutations.\(^3\)\(^,\)\(^4\)\(^,\)\(^5\)\(^,\)\(^6\)\(^,\)\(^7\)\(^,\)\(^8\)\(^,\)\(^9\)\(^,\)\(^10\) During the review period of this manuscript, there has been a description of p.T179T variant in a infantile-onset IBD patient.\(^10\) All patients with IL10RA mutations had symptoms at less than 2 years of age; further, only one patient with IL10RB mutations had a delayed age of onset at 3.5 years.\(^4\)

A faint band of a full-length splicing product, in addition to the major band of truncated product, was observed on RT-PCR of PBMCs from patient C, suggesting that a relatively smaller amount of normal IL10RA transcript was also produced. Although the IL10-induced STAT3 phosphorylation was not detected by western blotting in this patient, we could not rule out the possibility of ‘leakage’ of the normal IL10RA transcript, which might explain her relatively delayed onset of disease.

A total of 18 mutations in IL10RA were reported from 52 pediatric patients with IBD to date [Supplementary Table 1, available as Supplementary data at ECCO-JCC online]. Among them, 12 allelic variants in IL10RA were functionally validated to cause pathogenicity in IL10 pathways. IVS5+2T>C mutation in the 5’ splice donor site led a premature stop codon [p.P206X] on exon 5, and the resultant truncated IL10RA protein lacked the intracellular domain. The identification of the new splice site mutation in the 5’ intron of IL10RA was functionally validated to cause a truncation mutation among Semitic populations.\(^11\) An EX3del mutation in IL10RA, identified in a heterozygote carrier, caused a truncation mutation among Semitic populations.\(^12\) This variant led to the loss of a 179-bp fragment and a frameshift, resulting in an IL10RA mutant lacking the intracellular domain. The identification of the new splice site mutation with p.T179T variant highlights that synonymous changes must be meticulously investigated among children suspected of having an IL10RA deficiency.

By family-based WES, we managed to identify the identical compound heterozygous mutations in patients A and B. Family-based analysis might be superior to WES for a single exome, with improved accuracy of variant calling, enhanced ability to make calls in low-coverage regions, and the ability to directly observe the inheritance of variants. In addition, our data have suggested that synonymous variants located near splice sites should also be meticulously investigated during the WES analytical pipeline for children suspected of an IL10R deficiency. Considering the population frequency of 0.035%, CD patients with early-onset and refractory clinical phenotypes should be screened for the p.T179T variant.

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**Conflict of Interest**

All authors ensure the integrity of the work and disclose no conflicts.

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**Author Contributions**

Guarantor of the article, KS; designed the study, KS; participated in diagnostic evaluation and recruited subjects, SHO, KK; prepared DNA samples, performed genotyping, in vitro experiments, and data analysis, JB, SCY; performed whole-exome sequencing data analyses, JB, HL, JNF; supervised the exome sequencing data analysis, JL; drafted the manuscript, KS, KK; revised the manuscript, KS, SHO.

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**Figure 3.** IL10 signalling in patient C homozygous for p.T179T. [A] Intact IL6 signalling versus defective IL10 signalling in patient C homozygous for p.T179T. Western blot analysis of IL10- and IL6-induced STAT3 phosphorylation in peripheral blood mononuclear cells (PBMCs) from patient C and a healthy control. [B] Defective IL10 signalling in patient C homozygous for p.T179T. Western blot analysis of IL10-induced STAT3 phosphorylation in PBMCs from patient C, her parents, and a healthy control.
References