Journal of Crohn's and Colitis, 2016, 1366–1371
doi:10.1093/ecco-jcc/jjw102
Advance Access publication May 13, 2016
Short Report

Short Report

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

Seak Hee Oh,[a*] Jiwon Baek,[b*] Herty Liany,[c] Jia Nee Foo,[c] Kyung Mo Kim,[a] Stephen Chang-Oh Yang,[b] Jianjun Liu,[c] Kyuyoung Song[b]

[a]Department of Pediatrics, Asan Medical Center Children's Hospital, University of Ulsan College of Medicine, Seoul, Korea [b]Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, Seoul, Korea [c]Human Genetics Group, Genome Institute of Singapore, Singapore

*These authors contributed equally to this work.

Corresponding author: Kyuyoung Song, PhD, Department of Biochemistry and Molecular Biology, University of Ulsan College of Medicine, 88 Olympic-ro, 43-gil Songpa-Gu, Seoul 138–736, Korea. Tel.: 822-3010-4277; fax: 822-3010-4248; email: kysong@amc.seoul.kr

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].[1,2,3,4,5,6,7,8,9,10,11] As a cytokine, IL10 plays an important anti-inflammatory role in mucosal homeostasis,[12,13] and deleterious mutations in genes encoding IL10 and its receptors have been shown to cause severe bowel inflammation among infantile IBD patients.[13] 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.[1,2,3] 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.[2]

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

Analytical pipeline	Family A	Family B	Common between the two families
All coding variants	35088	34580	26202
Filtering by quality	26656	26346	19743
Filtering by quality and 1000 genomes frequencies [< 1% in each population]	1872	1773	520
Filtering by family data[a]			
de novo	1	0	0
simple recessive	2	0	0
compound heterozygote	12[6][b]	6[3][b]	2[1][b]

[a]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].

[b]Number of genes.

Table 2. Variant profiles of *IL10RA*.

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

SNP, single nucleotide polymorphism.

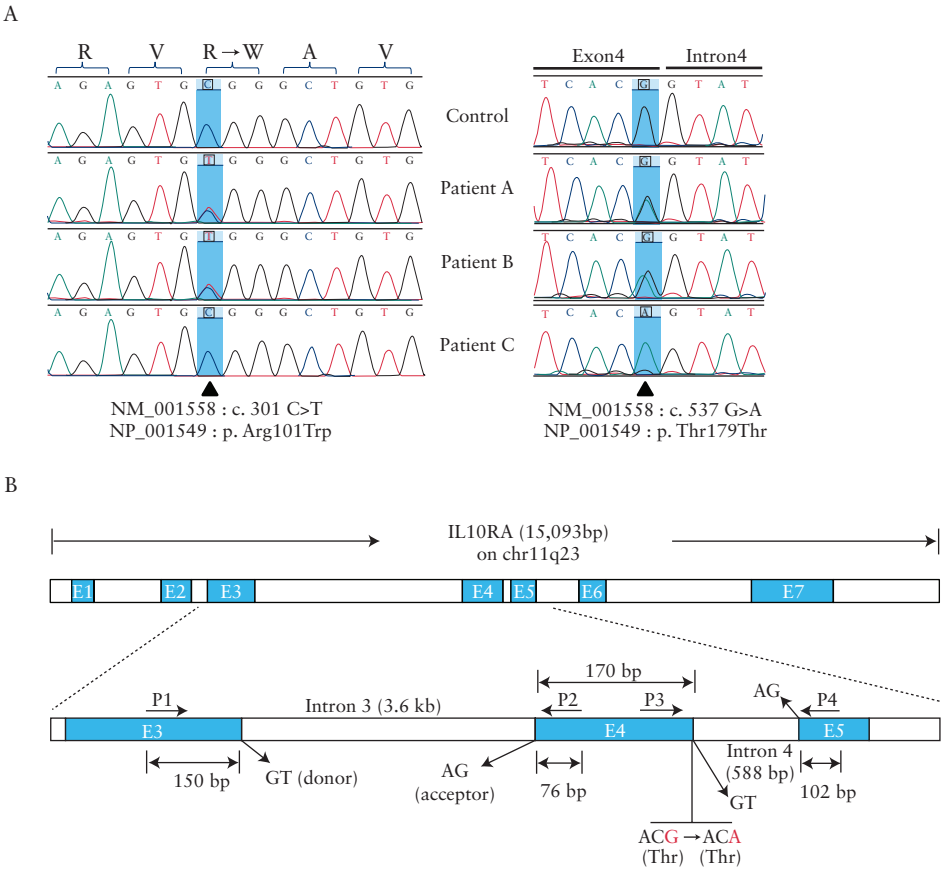

Figure 1. Identification of *IL10RA* mutations. [A] Identification of *IL10RA* p.R101W [c. C301T] and p.T179T [c. G537A] mutations in patients A, B, and C and a healthy control. [B] Location of the p.T179T mutation.

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.[3] 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, diarrhoea, 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 families of patient A or B. She was healthy and did not present with any gastrointestinal manifestations until the age of 6 years when she experienced episodes abdominal pain and diarrhoea. At the age of 9 years, 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 but became refractory later on. No response was observed to azathioprine, infliximab, or adalimumab. She experienced five episodes of severe flares with high fever for 8 months. At the age of 10 years, ileostomy was performed; however, her colitis remained refractory to long-term adalimumab therapy, and her growth failure persisted. Thus, she underwent total colectomy at the age of 12 years. She did not have any perianal complications during the course of her disease.

WES revealed that patient C was homozygous for p.T179T [Table 2]. Both her parents were heterozygous for the synonymous p.T179T variant, and she had a healthy sibling. However, her disease was not infantile onset and she did not present with perianal disease, which were not typical for IL10RA deficiency.[7,8,9,10,11,12,13,14,15,16,17,18] The synonymous amino acid change [p.T179T] was present at the exon/intron boundary [Figure 1B], which carried a G→A transition in exon 4 one base before the splice donor in intron 4 and suggested that the base substitution leads to splicing errors resulting in protein truncation. To assess the effect of the synonymous amino acid change of p.T179T variant on splicing, *in silico* prediction tools were used [Table 3].

To address the clinical relevance of a synonymous amino acid change allele, reverse transcription-polymerase chain reaction [RT-PCR] was performed using blood cell mRNA from patient C, her asymptomatic parents, and patient B. RT-PCR was performed using primers to exons flanking exon 4: forward CCAACTGTAGCCAGACCCTG [P1] and reverse ACTTCGGGAAGCGACAGATG [P4] primers. The expected full-length RT-PCR product was 422 bp, whereas the predicted product from mRNA that excluded exon 4 was 252 bp. Figure 2A shows the results of RT-PCR using wild-type *IL10RA* in a healthy donor, producing only a 422-bp fragment, whereas the same reaction using mRNA from the parents of the patient yielded both the full-length and truncated fragments.

The truncated fragment was a major product in patient C; however, a faint band of 422 bp was also observed [Figure 2A]. To confirm that the patient's mRNA could generate normal splicing, the presence of exon 4 in *IL10RA* transcripts was analysed by RT-PCR using the forward P1 primer in exon 3 and reverse GGCCTGGGTAGCTGAATCTT [P2] primer in exon 4, and forward CCTCGGGAAGATTCAGCTAC [P3] primer in exon 4 and reverse P4 primer in exon 5. The expected full-length RT-PCR products were 226 bp and 223 bp for exons 3–4 and 4–5, respectively. As shown in Figure 2B, the expected RT-PCR products of exon 3–4 and exon 4–5 were observed in patient C, suggesting normal splicing.

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

In silico prediction of splicing-altering SNV	Wild-type allele	mutant allele
	G	A
1. Position Weight Matrix	75.4	63.48
2. MaxEntScan score	8.47	1.94
3. NNSplice	0.97	0.07
4. Human Splicing Finder[a]	83.56	72.98
Position Weight Matrix [http://ccg.vital-it.ch/pwmtools/, accessed May 25, 2016]		
MaxEntScan score [http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html, accessed May 25, 2016]		
NNSplice [http://www.fruitfly.org/seq_tools/splice.html, accessed May 25, 2016]		
Human Splicing Finder 3.0 [http://www.umd.be/HSF3/index.html, accessed May 25, 2016]		

SNV, single nucleotide variant.

1. Position Weight Matrix: the web interface Splice-Site Analyzer Tool [http://ibis.tau.ac.il/ssat/SpliceSiteFrame.htm, accessed May 25, 2016].

2. MaxEntScan score; score, [–20]–[+20]; higher score implies higher probability of the sequence being a true splice site; threshold is defined at 3.

3. NNSplice; score, 0–1; higher score implies greater potential for splice site.

4. Human Splicing Finder; score, 0–100; higher score implies greater potential for splice site, threshold is defined at 65, signal with a score above the threshold is considered to be a splice site.

[a]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 break splice site. In this case, the difference is -12.66, supporting the presence of splicing mutation.

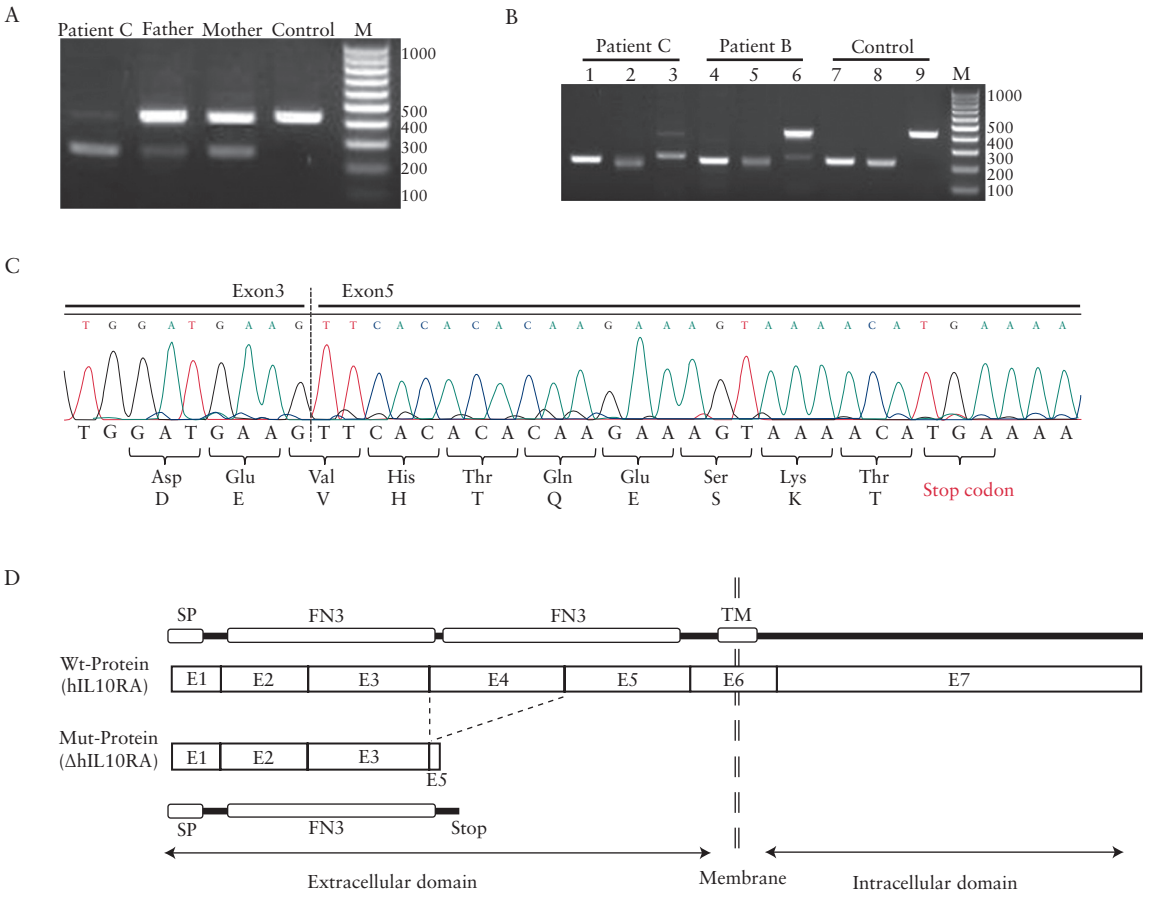

Figure 2. Identification of a splicing error caused by *IL10RA* p.T179T mutation. [A] Reverse transcription-polymerase chain reaction [RT-PCR] of *IL10RA* [exon 3–5] from RNA isolated from patient C, her parents, and a healthy control. [B] RT-PCR of exon 3 [lane 1, 4, 7], exon 4 [lane 2, 5, 8], and exon 3–5 [lane 3, 6, 9] from patients C, B, and a healthy control. [C] Sequence of the truncated 252-bp RT-PCR product shows deletion of exon 4 leading to a premature stop codon. [D] Predicted structure of the altered protein by p.T179T mutation. Signalling peptide [SP], fibronectin 3 [FN3], and transmembrane [TM] domains are shown.

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 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 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.[15,16] Deleterious *IL10RA* mutations have been shown to induce severe colitis, perianal fistulae, and recurrent sepsis during infancy.[1,2,3,4,5,6,7,8,9,10,11] 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.[3] 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

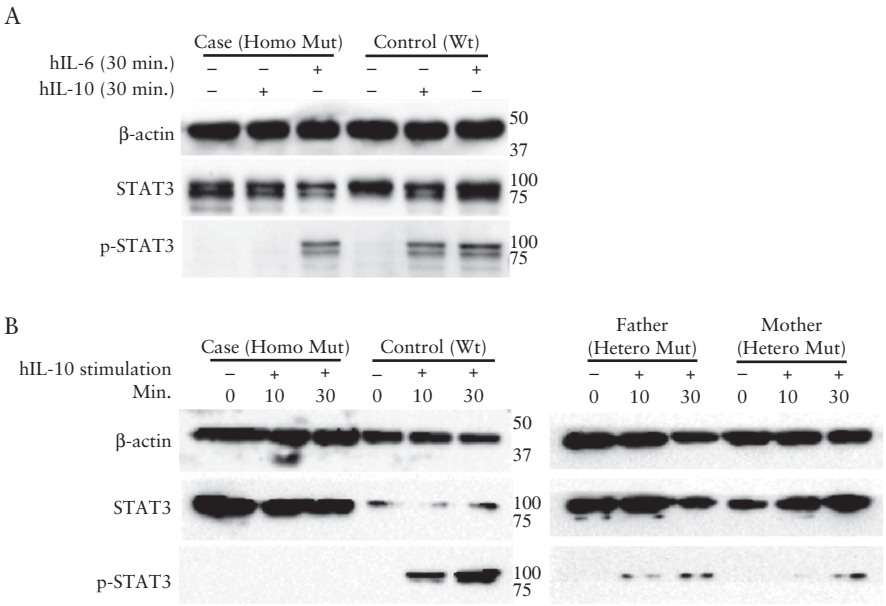

Figure 3. IL10 signalling in patient C homozygous for p.T179T. [A] Intact IL6 signalling versus defective IL10 signalling in patient C homozygous for p.T179T. Western blot analysis of IL10- and IL6-induced STAT3 phosphorylation in peripheral blood mononuclear cells [PBMCs] from patient C and a healthy 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.

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.[2,3,4,5,6,7,8,9,10,11] During the review period of this manuscript, there has been a description of p.T179T variant in a infantile-onset IBD patient.[17] 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 paediatric 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.[11] An EX3del mutation in *IL10RA*, identified in a heterozygote carrier, caused a truncation mutation among Semitic populations.[18] 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.

Funding

This work was supported by a Mid-career Researcher Program grant through the National Research Foundation of Korea to K. Song [2014R1A2A1A09005824] funded by the Ministry of Science, Information & Communication Technology and Future Planning, the Republic of Korea.

Conflict of Interest

All authors ensure the integrity of the work and disclose no conflicts.
Ethical approval was obtained from the institutional review board of the University of Ulsan College of Medicine and the Asian Medical Center.

Acknowledgments

We thank all participating patients who provided DNA and clinical information necessary for this study. J. Liu and other authors of Genome Institute of Singapore are supported by the Agency for Science, Technology and Research [A*STAR], Singapore.

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.

References

1. Begue B, Verdier J, Rieux-Laucat F, *et al*. Defective IL10 signaling defining a subgroup of patients with inflammatory bowel disease. *Am J Gastroenterol* 2011;**106**:1544–55.
2. Glocker EO, Kotlarz D, Boztug K, *et al*. Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *N Engl J Med* 2009;**361**:2033–45.
3. Kotlarz D, Beier R, Murugan D, *et al*. *Loss of interleukin-10 signaling and infantile inflammatory bowel disease: implications for diagnosis and therapy*. *Gastroenterology* 2012;**143**:347–55.
4. Engelhardt KR, Shah N, Faizura-Yeop I, *et al*. Clinical outcome in IL-10- and IL-10 receptor-deficient patients with or without hematopoietic stem cell transplantation. *J Allergy Clin Immunol* 2013;**131**:825–30.
5. Beser OF, Conde CD, Serwas NK, *et al*. Clinical features of interleukin 10 receptor gene mutations in children with very early-onset inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2015;**60**:332–8.
6. Shim JO, Seo JK. Very early-onset inflammatory bowel disease [IBD] in infancy is a different disease entity from adult-onset IBD; one form of interleukin-10 receptor mutations. *J Hum Genet* 2014;**59**:337–41.
7. Mao H, Yang W, Lee PP, *et al*. Exome sequencing identifies novel compound heterozygous mutations of IL-10 receptor 1 in neonatal-onset Crohn's disease. *Genes Immun* 2012;**13**:437–42.
8. Dinwiddie DL, Bracken JM, Bass JA, *et al*. Molecular diagnosis of infantile onset inflammatory bowel disease by exome sequencing. *Genomics* 2013;**102**:442–7.
9. Murugan D, Albert MH, Langemeier J, *et al*. Very early onset inflammatory bowel disease associated with aberrant trafficking of IL-10R1 and cure by T cell replete haploidentical bone marrow transplantation. *J Clin Immunol* 2014;**34**:331–9.
10. Pigneur B, Escher J, Elawad M, *et al*. Phenotypic characterization of very early-onset IBD due to mutations in the IL10, IL10 receptor alpha or beta gene: a survey of the Genius Working Group. *Inflamm Bowel Dis* 2013;**19**:2820–8.
11. Moran CJ, Walters TD, Guo CH, *et al*. IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. *Inflamm Bowel Dis* 2013;**19**:115–23.
12. Moore KW, de Waal Malefyt R, Coffman RL, *et al*. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;**19**:683–765.
13. Shouval DS, Ouahed J, Biswas A, *et al*. Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. *Adv Immunol* 2014;**122**:177–210.
14. Williams L, Bradley L, Smith A, *et al*. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. *J Immunol* 2004;**172**:567–76.
15. Kuhn R, Lohler J, Rennick D, *et al*. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;**75**:263–74.
16. Spencer SD, Di Marco F, Hooley J, *et al*. The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. *J Exp Med* 1998;**187**:571–8.
17. Yanagi T, Mizuochi T, Takaki Y, *et al*. Novel exonic mutation inducing aberrant splicing in the IL10RA gene and resulting in infantile-onset inflammatory bowel disease: a case report. *BMC Gastroenterol* 2016;**16**:10.
18. Gasche C, Reinisch W, Huber WD, *et al*. IL-10RA truncation mutations and Semite populations. *Inflamm Bowel Dis* 2011;**17**:1438.