IL-10R Polymorphisms Are Associated with Very-early-onset Ulcerative Colitis

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Background: Interleukin-10 (IL-10) signaling genes are attractive inflammatory bowel disease (IBD) candidate genes as IL-10 restricts intestinal inflammation. IL-10 polymorphisms have been associated with IBD in genome-wide association studies, and mutations in IL-10 and IL-10 receptor (IL-10R) genes have been reported in immunodeficient children with severe infantile-onset IBD. Our objective was to determine if IL-10R polymorphisms were associated with early-onset IBD (EO-IBD) and very-early-onset IBD (VEO-IBD).

Methods: Candidate-gene analysis of IL10RA and IL10RB was performed after initial sequencing of an infantile onset-IBD patient identified a novel homozygous mutation. The discovery cohort included 188 EO-IBD subjects and 188 healthy subjects. Polymorphisms associated with IBD in the discovery cohort were genotyped in an independent validation cohort of 422 EO-IBD subjects and 480 healthy subjects.

Results: We identified a homozygous, splice-site point mutation in IL10RA in an infantile-onset IBD patient causing a premature stop codon (P206X) and IL-10 insensitivity. IL10RA and IL10RB sequencing in the discovery cohort identified five IL10RA polymorphisms associated with ulcerative colitis (UC) and two IL10RB polymorphisms associated with Crohn’s disease (CD). Of these polymorphisms, two IL10RA single nucleotide polymorphisms, rs2228054 and rs2228055, were associated with VEO-UC in the discovery cohort and replicated in an independent validation cohort (odds ratio [OR] 3.08, combined P = 2 × 10−4; and OR 2.93, P = 6 × 10−4, respectively).

Conclusions: We identified IL10RA polymorphisms that confer risk for developing VEO-UC. Additionally, we identified the first splice site mutation in IL10RA resulting in infantile-onset IBD. This study expands the phenotype of IL10RA polymorphisms to include both severe arthritis and VEO-UC.

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*NEOPICS: interNational Early Onset Pediatric IBD Cohort Study www.NEOPICS.org; see Supporting Material for details.

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Interleukin-10 (IL-10) is an antiinflammatory cytokine secreted by a variety of cell types and is critical for maintaining immune homeostasis in the gastrointestinal (GI) tract. These immunosuppressive effects include restricting T-cell proliferation, downregulating costimulatory protein expression on antigen-presenting cells, and limiting proinflammatory cytokine production. IL-10 activates downstream signaling by binding to the IL-10 receptor (IL-10R), comprised of two α subunits (encoded by \textit{IL10RA}) and two β subunits (encoded by \textit{IL10RB}). This activates JAK1 and TYK2, leading to phosphorylation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3) and gene transcription. Intact IL-10 signaling is required for restricting inappropriate Th17 cell expansion—an effector cell type that has been associated with Crohn’s disease (CD). Consistent with the antiinflammatory role of IL-10, mice deficient in either \textit{IL-10} or \textit{IL-10R} develop enterocolitis.

Recent studies have shown that loss-of-function mutations in \textit{IL10RA} and \textit{IL10RB} and \textit{IL10} in immune-deficient patients are associated with severe, infantile-onset inflammatory bowel disease (IBD). This has stimulated renewed interest in studying IL-10 pathway genes in the pathogenesis of IBD. Genome-wide association studies (GWAS) in IBD populations (including exclusively pediatric-onset disease) have associated single nucleotide polymorphisms (SNPs) in \textit{IL10} and \textit{STAT3} loci with ulcerative colitis (UC) and CD.

Given the severe phenotype of infantile-onset IBD observed with IL-10R mutations as well as that of an infant described in this article who was found to have a homozygous, splice site mutation in \textit{IL-10RA} and \textit{IL10RB} and \textit{IL10} in immune-deficient patients are associated with severe, infantile-onset inflammatory bowel disease (IBD). This has stimulated renewed interest in studying IL-10 pathway genes in the pathogenesis of IBD. Genome-wide association studies (GWAS) in IBD populations (including exclusively pediatric-onset disease) have associated single nucleotide polymorphisms (SNPs) in \textit{IL10} and \textit{STAT3} loci with ulcerative colitis (UC) and CD.

Materials and Methods

Identification of Novel IL-10RA Mutation in Patients with Infantile Colitis

We identified an IL-10RA mutation in a Caucasian female presenting with infantile colitis who was not recognized a priori to be the product of a consanguineous marriage, but whose parents came from the same geographically isolated region of Canada. Her first year of life was complicated by recurrent fevers (initially at 6 days of life). At 3 months old, the patient developed cellulitis and scalp eczema and generalized erythroderma (Fig. 1A). Bloody diarrhea and a perianal skin tag developed at 5 months of age (Fig. 1B) that progressed to perianal and rectovaginal fistulae. Further complications included urinary tract infections, scalp pustules, and severe, effusive, large joint polyarthritis (Fig. 1C). Joint aspirations demonstrated high neutrophil counts (>100,000/mm³) despite sterile cultures. The patient did not manifest thyroid involvement. Endoscopic evaluation revealed patchy colonic ulcerations (Fig. 1D) and biopsies showed chronic colitis (Fig. 1E) with the absence of small bowel inflammation. GI symptoms were refractory to antibiotics, intravenous immunoglobulins, corticosteroids, anakinra, sulfasalazine, and azathioprine, and the patient underwent a loop ileostomy at 23 months old.

An extensive immunological evaluation included normal immunoglobulin levels and lymphocyte subsets, normal NADPH oxidase function, a normal thymic biopsy, normal karyotyping, and negative fluorescence in situ hybridization (FISH) for 22q11. Screening for JAK3, IPEX, RMRP, IL2RA, IRAK4, and TRAPS mutations and mutations associated with chronic granulomatous disease were negative.

Genomic DNA was purified from whole blood using the Puregene Blood Kit (Qiagen, Chatsworth, CA). \textit{IL10RA} and \textit{IL10RB} were amplified using intronic primers flanking each exon and sequenced with the ABI3730 DNA analyzer (Applied Biosystems, Melbourne, Australia). \textit{IL10RA} and \textit{IL10RB} variants are numbered according to GenBank accession numbers NM_001588 and NM_00628, respectively. Amino acid numbering refers to position in the immature protein (including signal peptide).

RNA was isolated from whole blood with the PAXgene Blood RNA kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA). Primers for full-length \textit{IL10RA}, full-length \textit{IL10RB}, and for exon 4 to exon 7 of \textit{IL10RA} were designed and synthesized at the Centre for Applied Genomics (Toronto, ON, Canada). Polymerase chain reaction (PCR) was performed according to standard protocol, and the purified PCR product was cloned.
into pJET cloning vector (Fermentas, Burlington, ON, Canada) and sequenced by ABI 3730 DNA analyzer (Applied Biosystems).

Functional IL-10 Testing in IL-10R-Deficient Patient

Peripheral blood mononuclear cells (PBMCs) from the patient and a healthy control were isolated using Polymorphy-Prep (Nycomed Pharma, Norway). The PBMCs were stimulated with IL-10 (40 ng/mL) or IL-6 (20 ng/mL) for 10 and 30 minutes. Cellular extracts were prepared following standard protocol. The protein concentration was measured by protein assay kit (Bio-Rad, Hercules, CA) and 50 μg protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. The membrane was incubated with antibodies to STAT3 and phosphorylated (Tyr705) STAT3 (p-STAT3) (Cell Signaling, Beverly, MA) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies. The membrane was read using a chemiluminescence system (GE Healthcare, UK). β-Actin was used as a loading control.

Flow Cytometry

Thawed PBMC were stained in 2% fetal bovine serum (FBS) with CD4-PerCP (eBioscience, San Diego, CA) and CD25-PE (Miltenyi Biotech, Auburn, CA) followed by intracellular staining with FoxP3-FITC (eBioscience). Flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA). Coexpression of CD25 and intracellular FoxP3 was assessed in cells within CD4+ lymphocyte gate.

IL-10R Sequencing in the Discovery Cohort

The discovery cohort included DNA samples from 188 EO-IBD patients from the Hospital for Sick Children (Toronto, Canada) (49 patients) including some subjects as part of the NEOPICS consortium (Supporting Table 1) and Children’s Hospital of Wisconsin (Milwaukee, WI) (139 patients). Control DNA samples were obtained from the Centre for Applied Genomics (Ontario Population Genomics Platform, plates used: 1–4); 95% of this cohort are Caucasians of European ancestry. A complete description can be found at http://www.tcag.ca/facilities/cyto_population_control_DNA.html.
The coding sequences (and intervening sequences and 3'-untranslated regions) of IL10RA and IL10RB were amplified. PCR products were sequenced by the Sanger method (Beckman-Coulter Genomics, Danvers, MA). SNPs were detected using Polyphred software and all chromatograms with reliability score <99 were manually reviewed.

Allelic frequencies were determined for SNPs identified in the discovery cohort. Testing for Hardy–Weinberg equilibrium was done by $\chi^2$ analysis. Odds ratios (ORs) were calculated by comparing the ratio of individuals with two copies of the major allele to those with one or two copies of a variant allele (DD vs. Dd/dd model) between EO-IBD and control groups. ORs were determined for UC and CD and specific age groups: children diagnosed before their 18th birthday (<18 years old) and children diagnosed before their 6th birthday (<6 years old). $P$-values were adjusted for multiple testing by Bonferroni method (based on the number of SNPs associated with IBD in discovery cohort), and an adjusted $P < 0.05$ was considered statistically significant. As quality control, allele frequencies in the discovery and validation cohorts were compared by $\chi^2$ analysis. LD analysis and haplotype testing was performed as above.

All probands had a confirmed diagnosis of IBD fulfilling standard diagnostic criteria. Phenotypic characterization was based on the Montreal Classification. Age categories reflect age at diagnosis: EO-IBD, defined as diagnosis before the 18th birthday; VEO-IBD, defined diagnosis before the 6th birthday; and infantile-onset IBD, defined as diagnosis before the 1st birthday. Institutional Review Boards at the Hospital of Sick Children, Children’s Hospital of Wisconsin, Mount Sinai Hospital (Toronto, Canada), and Massachusetts General Hospital approved these studies.

**RESULTS**

**Novel, Aberrant IL10RA Splice Site Mutation Identified in Patients with Infantile Colitis**

We identified a patient with severe, infantile-onset IBD with significant arthritis and folliculitis (Fig. 1). Sequencing of IL10RA and IL10RB in this patient identified
a homozygous mutation in the intervening sequence of intron 5 at the second base of the conserved GT splice donor site (g.IVS5+2T>C) (Fig. 2A). This novel mutation was confirmed by sequencing in an independent laboratory (data not shown) and has not been identified in the 1000 Genomes Project. This mutation was located in the 5' splice donor site, a critical position for RNA splicing. Mutation of the invariant dinucleotide GT at the 5' splice donor site impairs normal splicing and allows activation of cryptic splice site(s).17 We identified a cryptic splice donor site (CAGgtgaaa) in exon 5 of IL10RA using Human Splicing Finder (Fig. 2A).18 To confirm aberrant IL10RA splicing, RNA was isolated from whole blood and IL10RA transcripts were analyzed by reverse transcription (RT)-PCR. The expected 433-bp PCR fragment was detected in five healthy donors. However, only a smaller fragment was observed in the patient (Fig. 2B) and sequencing of this fragment revealed a 76-bp deletion (c.690_765del) from the 3' end of exon 5 (Fig. 2C). The resulting reading frame-shift mutation led to incorporation of three aberrant amino acids of exon 6 (p.204ISP206) and a premature stop codon at P206X (Fig. 2C). The truncated protein contained only the extracellular domain of IL10RA (Fig. 3A). The patient’s asymptomatic parents were g.IVS5+2T>C carriers displaying wildtype and altered splicing variants (Fig. 2B).

**IL10RA Splice Mutation Prevents IL-10-dependent STAT3 Phosphorylation**

In order to determine the functional effects of P206X IL10R1, we assessed in vitro STAT3 phosphorylation, a required step in the IL-10 signaling cascade for downstream immunosuppressive effects.19 PBMCs were stimulated with IL-10 and STAT3 phosphorylation was measured by western blot analysis. IL-10-induced STAT3 phosphorylation was defective in the patient. IL-6-induced STAT3 phosphorylation was intact (Fig. 3B). Thus, P206X IL-10R1 leads to defective IL-10 downstream signaling.

**FoxP3+ Regulatory T Cells Are Present in the Absence of IL-10 Signaling**

Regulatory T cells (Tregs) that express the transcription factor FoxP3 possess suppressive ability, and defects in the FOXP3 gene (in mice and humans) cause systemic autoimmunity.20,21 As some but not all studies have suggested that IL-10 signaling is critical for the stability of FoxP3 expression,3,22 we sought to determine whether FoxP3+ Tregs were present in the absence of IL-10 signaling. FoxP3+ Tregs were identified in the peripheral blood of the affected patient (Fig. 3C). This provides evidence in humans that FoxP3+ Tregs can develop in the absence of intact IL-10 signaling.
IL10RA and IL10RB Polymorphisms Are Associated with IBD

In order to determine whether IL10RA and IL10RB mutations are associated more generally with IBD and extend beyond the rare patients with infantile-onset IBD that have homozygous, loss-of-function mutations, we performed deep sequencing of coding and flanking intronic regions of IL10RA and IL10RB in a cohort of 188 EO-IBD patients and 188 controls (Supporting Table 2). The EO-IBD group included 121 children with CD, two with IBD-unclassified, and 65 with UC. The ages of children ranged from 6.5 months to 11 years old, with 68 VEO-IBD subjects (including two infants).

In this discovery cohort, 59 SNPs (28 novel) were found in IL10RA and 39 SNPs (15 novel) in IL10RB (Supporting Table 3). There were 14 (six novel) nonsynonymous SNPs (12 in IL10RA and two in IL10RB). Each novel, nonsynonymous SNP was identified only in the heterozygous state, and none of the resulting amino acid changes were predicted to be deleterious by SIFT23 or Polyphen.24 Additionally, we found five SNPs in IL10RA were associated with EO-UC and two SNPs in IL-10RB were associated with EO-CD (Table 1; Supporting Table 3).

In subgroup analysis of these five IL10RA SNPs, four of the SNPs were found to be associated with VEO-UC, while there was a positive trend for VEO-UC in rs2228055 (Table 2). Of the IL10RB SNPs that were associated with EO-CD, rs1058867 was associated with increased CD risk and rs8178561 was protective for EO-CD. The minor allelic frequencies of the SNPs described above did not differ between the IBD cohorts from Wisconsin and Toronto (data not shown).

Linkage analysis of these SNPs showed that rs2228054 and rs2228055 were in LD (P < 0.0001) (Haplotype Block 1 AG) as were rs10892202, rs4252249, and rs4252270) were associated with increased risk for EO-UC and VEO-IBD. In subgroup analysis of these five IL10RA SNPs, four of the SNPs were found to be associated with VEO-UC, while there was a positive trend for VEO-UC in rs2228055 (Table 2). Of the IL10RB SNPs that were associated with EO-CD, rs1058867 was associated with increased CD risk and rs8178561 was protective for EO-CD. The minor allelic frequencies of the SNPs described above did not differ between the IBD cohorts from Wisconsin and Toronto (data not shown).
Haplotype Block 2 had a protective effect for VEO-IBD (OR 0.40, \(P = 0.025\)), UC (OR 0.29, \(P = 0.007\)) and VEO-UC (OR 0.28, \(P = 0.025\)), EO-IBD (OR 0.57, \(P = 0.030\)) but had no significant effect in EO-CD.

Validation Study Confirms IL10RA SNP Associations with VEO-UC

We determined the frequency of the SNPs (found in the discovery cohort) in an independent cohort of 422 children with EO-IBD and 480 healthy controls (validation cohort) by Taqman genotyping. This EO-IBD cohort was comprised of 122 children with UC and 300 children with CD (including 18 VEO-UC and 10 VEO-CD). \(\chi^2\) analysis of discovery and validation controls showed that they were similar with respect to the studied SNPs (data not shown).

Although the association with the seven IL10R SNPs and EO-IBD did not replicate in the validation cohort, combined analysis of the discovery and showed nominal association with all the IL10RA SNPs (Table 1) and EO-UC. Furthermore, the association with VEO-UC with both rs2228054 and rs2228055 was replicated in the validation VEO-UC cohort, (rs2228054 OR 3.86 [95% CI 1.39–10.71, \(P = 0.005\), adjusted \(P = 0.044\)] and rs2228055 OR 4.21 [95% CI 1.51-11.69, \(P = 0.003\), adjusted \(P = 0.024\)]. Table 2) and remained significant in a combined analysis of the discovery and validation cohorts (OR 3.08, combined \(P = 2 \times 10^{-4}\); and OR 2.93, \(P = 6 \times 10^{-4}\), respectively).

Linkage analysis of Haplotype Block 1 AG and Haplotype Block 2 CAT showed that these SNPs were also in LD in the validation cohort (\(P < 0.0001\)). The Haplotype Block 1 AG was also associated with VEO-IBD (OR 2.43, \(P = 0.023\)), and VEO-UC (OR 3.40, \(P = 0.006\) but not for overall IBD or EO-UC (Supporting Table 4). Haplotype Block 2 CAT was not associated with VEO-IBD (\(P = 0.89\)) or VEO-UC (\(P = 0.51\)).

**DISCUSSION**

Homozygous, loss-of-function mutations in *IL10RA* and *IL10RB* have been reported to cause severe, infantile-onset IBD.\(^5^7\) In this report, we identify a novel splice site mutation in *IL10RA* resulting in defective IL-10 signaling. In addition to the colitis, severe perianal disease, and

| Table 2. IL-10R Polymorphisms Associated with Very Early Onset IBD |
|---------------------------------|----------------|----------------|----------------|
| **IL10RA SNP**                  | **Discovery Cohort** | **Validation Cohort** | **Combined Cohorts** |
|                                | OR   | \(P\)-value | OR   | \(P\)-value | OR   | \(P\)-value |
| rs10892202                     | 0.41 | 0.044       | 1.17 | 0.701       | 0.6  | 0.086       |
| VEO-IBD                        | 0.28 | 0.03        | 0.71 | 0.585       | 0.4  | 0.032       |
| VEO-UC                         | 0.62 | 0.23        | 2.35 | 0.179       | 0.98 | 0.951       |
| rs2228054                      | 2.69 | 0.016       | 2.58 | 0.024       | 2.49 | 3x10^-3     |
| VEO-IBD                        | 3.29 | 0.013       | 3.86 | 0.005       | 3.08 | 2x10^-4     |
| VEO-UC                         | 1.85 | 0.14        | 1.93 | 0.404       | 1.66 | 0.273       |
| rs2228055                      | 2.38 | 0.032       | 2.8  | 0.014       | 2.56 | 2x10^-3     |
| VEO-IBD                        | 2.58 | 0.058       | 4.21 | 0.003       | 2.93 | 6x10^-4     |
| VEO-UC                         | 2.08 | 0.057       | 2.1  | 0.345       | 2.01 | 0.106       |
| rs4252249                      | 0.41 | 0.045       | 1.18 | 0.701       | 0.61 | 0.882       |
| VEO-IBD                        | 0.28 | 0.046       | 0.71 | 0.585       | 0.4  | 0.033       |
| VEO-UC                         | 0.63 | 0.24        | 2.35 | 0.179       | 0.98 | 0.958       |
| rs4252270                      | 0.41 | 0.045       | 1.16 | 0.722       | 0.6  | 0.081       |
| VEO-IBD                        | 0.28 | 0.046       | 0.7  | 0.572       | 0.4  | 0.031       |
| VEO-UC                         | 0.62 | 0.24        | 2.32 | 0.186       | 0.97 | 0.938       |
| rs1058867                      | 1.55 | 0.186       | 0.94 | 0.868       | 1.16 | 0.515       |
| VEO-IBD                        | 1.28 | 0.596       | 1.13 | 0.814       | 1.1  | 0.748       |
| VEO-UC                         | 2.11 | 0.058       | 0.56 | 0.364       | 1.26 | 0.519       |
| rs8178561                      | 0.7  | 0.528       | 1.23 | 0.687       | 0.92 | 0.804       |
| VEO-IBD                        | 1.04 | 1           | 1.89 | 0.267       | 1.32 | 0.447       |
| VEO-UC                         | 0.23 | 0.212       | 0.74 | 0.26        | 0.37 | 0.160       |

Odds ratios using DD vs. Dd/dd model. Discovery cohort: VEO-IBD (\(n=65\)), VEO-UC (\(n=41\)), VEO-CD (\(n=24\)), and controls (\(n=188\)). Validation cohort: VEO-IBD (\(n=28\)), VEO-UC (\(n=18\)), VEO-CD (\(n=10\)), and controls (\(n=480\)). Threshold for significance in validation cohort was 0.007 to correct for multiple testing.
recurrent infections that have been previously described in patients with IL-10R mutations, this patient also suffered from severe arthritis.

The contribution of IL10R variants to IBD susceptibility more generally in children was heretofore unclear. Our study is the first to specifically examine by deep sequencing the role of IL-10R SNPs in EO-IBD and VEO-IBD. We identified two IL10RA SNPs associated with VEO-UC and these results have been validated in an independent replication cohort with OR of 2.9 and 3.1. These two SNPs, rs2228054 and rs2228055, were frequently found in the heterozygous state among IBD patients and inherited as a haplotype. The conferred risk may be due to one or both SNPs. Alternatively, the increased risk may reside in a regulatory region (e.g., promoter) in LD with these SNPs. We propose that this risk haplotype exerts a mild phenotype in the general population resulting in disease only in the presence of another genetic variant(s) or environmental trigger. In contrast to the complete loss-of-function IL-10R mutations (described here and previously6,7) which present with symptoms more typical of CD, these SNPs are associated with UC, which is consistent with the association of SNPs in IL-10 signaling genes and UC.10,12 Although this study points to an important role of the IL-10 pathway in VEO-UC, due to the limited number of VEO-IBD patients, this association with VEO-UC will require further functional investigations and replication.

Although GWAS of adult and pediatric IBD have identified novel pathways important in the pathogenesis of IBD, the SNPs detected by GWAS only account for ∼25% of the assumed genetic heritability.11 The risk for IBD associated with SNPs in IL10, TYK2, and STAT3 loci demonstrate the importance of IL-10 in IBD pathogenesis.9,12 However, GWAS have not identified an association between SNPs in the IL10RA or IL10RB loci and IBD risk in the pediatric population.13,25 Similarly, the recently published meta-analysis of GWAS of UC patients failed to show a statistically significant increase in risk for rs2228054.26 This is most likely due to the unique subset of patients examined in our study, and highlights the importance of candidate gene studies examining rare presentations of IBD. GWAS evaluating exclusively pediatric IBD focus primarily on EO-IBD patients, which has been shown to be phenotypically more similar to adult-onset IBD than VEO-IBD.14 Therefore, the contribution of IL-10R SNPs to the susceptibility of IBD in very young children has been underestimated in recent adult and late-onset pediatric GWAS, as VEO-IBD represents a unique population that is poorly represented even in GWAS focusing on pediatric IBD.13

Although defective IL-10 signaling causes a strong inflammatory phenotype in the GI tracts of both humans and mice, it is still unclear which is the most critical cell types(s) that require IL-10 signaling. While Murai et al22 suggested that an IL-10-dependent signal was required for the maintenance of FoxP3+ Tregs, Chaudhry et al23 demonstrated in mice with a Treg lineage-specific deletion of IL-10R that IL-10-dependent signals were required for the function but not the maintenance of Tregs. Peripheral blood FoxP3+ Tregs were present in the patient studied in this report with defective IL-10 signaling, demonstrating that this signal is not required for the development of FoxP3+ Tregs.

In summary, we have described a novel splice site mutation in IL10RA that broadens the existing phenotype of IL-10R deficiency to include severe arthritis. The discovery of this patient in a cohort of VEO-IBD patients reinforces that IL-10R deficiency should be considered in all infantile-onset IBD cases, especially those with severe perianal disease and now arthritis, and that this diagnosis should prompt consideration of stem cell transplantation. Additionally, the association of SNPs in IL10RA with VEO-UC implicates a role for IL-10R in the pathogenesis of IBD in a broader population of patients than recently appreciated. Such information may permit stratification of patients for clinical trials that target the IL-10 pathway.

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