Basic Study

Genetic association and epistatic interaction of the interleukin-10 signaling pathway in pediatric inflammatory bowel disease

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Abstract

AIM
To study the genetic association and epistatic interaction of the interleukin (IL)-10 and IL-10/STAT3 pathways in pediatric inflammatory bowel disease (IBD).

METHODS
A total of 159 pediatric inflammatory IBD patients (Crohn’s disease, n = 136; ulcerative colitis, n = 23) and 129 matched controls were studied for genetic association of selected single nucleotide polymorphisms (SNPs) of the IL-10 gene and the genes IL10RA, IL10RB, STAT3, and HO1, from the IL-10/STAT3 signaling pathway. As interactions between SNPs from different loci may significantly affect the associated risk for disease, additive (a) and dominant (d) modeling of SNP interactions was also performed to examine higher-order epistasis between combinations of the individual SNPs.

RESULTS
The results showed that IL-10 rs304496 was associated with pediatric IBD (P = 0.022), but no association was found for two other IL-10 SNPs, rs1800872 and rs2034498, or for SNPs in genes IL10RA, IL10RB, STAT3, and HO1. However, analysis of epistatic interaction among these genes showed significant interactions: (1) between two IL-10 SNPs rs1800872 and rs3024496 (additive-additive P = 0.00015, Bonferroni P value (Bp) = 0.003); (2) between IL-10RB rs2834167 and HO1 rs2071746 (dominant-additive, P = 0.0018, Bp = 0.039); and (3) among IL-10 rs1800872, IL10RB rs2834167, and HO1 rs2071746 (additive-dominant-additive, P = 0.00015, Bp = 0.005), as well as weak interactions among IL-10 rs1800872, IL-10 rs3024496, and IL-10RA (additive-dominant-additive, P = 0.003; Bp = 0.099), and among IL10RA, IL10RB, and HO1 genes (additive-dominant-additive, P = 0.008, Bp = 0.287).

CONCLUSION
These results indicate that both the IL-10 gene itself, and through epistatic interaction with genes within the IL-10/STAT3 signaling pathway, contribute to the risk of pediatric IBD.

Key words: Pediatric inflammatory bowel disease; Interleukin-10; HO1; Single nucleotide polymorphism; IL10-STAT3 pathway; Epistatic interaction

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Core tip: Inflammatory bowel disease (IBD) affects not only adults, but also children and newborn infants. Of the 163 genes currently associated with risk for development of IBD, only a few have been studied in pediatric patients. In this study, we found that one interleukin (IL)-10 genetic variation, rs304496, is associated with risk for pediatric IBD. IL-10 restricts excessive immune responses during intestinal inflammation. We also demonstrated epistatic interactions between genetic variants within the IL-10/STAT3 signaling pathway that contribute to a higher associated risk for pediatric IBD. These findings emphasize the importance of the IL-10 pathway in a subgroup of IBD patients.

INTRODUCTION
Pediatric inflammatory bowel disease (IBD) has a distinct clinical phenotype from adult IBD[1]. Few of the 163 genes identified to be associated with adult IBD have been identified and functionally studied in pediatric IBD. A genome-wide association study (GWAS) in the Polish population revealed that the genetic architecture is different between pediatric and adult-onset IBD[2]. Adult IBD-associated genes NOD2 (Leu1007insC) and IRGM have been shown to be associated with increased risk of Crohn’s disease (CD) and ORMDL3 variant with susceptibility to ulcerative colitis (UC) in Lithuanian early-onset IBD patients[3]. The TRIM22-NOD2 network, signaling pathways and genetic factors are associated with very early-onset (VEO) and adult IBD. Functional studies showed that variants of the tripartite motif containing 22 gene (TRIM22) disrupted its ability to regulate NOD2-dependent activity of interferon-β signaling and nuclear factor-kappa B (NF-κB)[4].

In addition, novel association of major histocompatibility complex haplotype with pediatric-onset IBD has been reported[5]. The multi-drug resistance gene MDR1 single nucleotide polymorphisms (SNPs) C1236T and G2577A/T have also been shown to be associated with CD in an Algerian pediatric CD population[6].

Mutations in IL-10 and IL-10 receptors IL10RA and IL10RB have been linked to VEO IBD[7-12]. Knockout mice lacking IL-10 develop IBD[13]. IL-10 and STAT3 have been identified as IBD-associated genes in children and adults[10,14-20]. The IL-10 gene encodes an anti-inflammatory cytokine and the IL-10/STAT3 signaling pathway plays an important role in controlling inflammation and protecting the intestine tissue from
damage\textsuperscript{[21,22]}. During the IL-10 signaling transduction, IL-10 binds to receptors IL10RA and IL10RB, and activates Jak1 and Tyk2, leading to phosphorylation of STAT3. Then, the activated STAT3 translocates into the nucleus and regulates target gene transcription to promote an anti-inflammatory response\textsuperscript{[23,24]}

Despite pronounced evidence of the role of the genes comprising IL-10 and genes within the IL-10/STAT3 signaling pathway, our knowledge about how they may interact with each other to determine IBD development is still very limited. Genetic interactions between different loci, i.e., epistasis, have been thought to be of paramount importance in complex diseases\textsuperscript{[25,26]}. Given the underlying complex pathways, it is reasonable to hypothesize that the genes detected affect IBD through a network of gene-gene interactions between genes, or SNP-SNP interactions within a gene. In this paper, we used a computational model\textsuperscript{[27]} to analyze how epistatic interactions among polymorphic loci in the IL-10 gene and IL-10/STAT3 pathways govern pediatric IBD in a case-control setting. The model cannot only estimate low-order epistasis between a pair of loci, but also detect higher-order epistasis among three loci, whereby it is equipped with a capacity to unravel etiological complexities of pediatric IBD. Furthermore, by integrating classic quantitative theory, this model dissects overall epistatic interaction into its underlying components. With this, one may better understand the genetic machinery of this disease from a mechanistic aspect.

Table 1  Study samples

| Sample     | n  | Sex, n | Race, n | Age at diagnosis |
|------------|----|--------|---------|------------------|
| IBD        | 159| Male, 85; female, 74 | White, 153; black, 6 | 13.1           |
| CD         | 136| Male, 77; female, 59 | White, 130; black, 6 | 13.1           |
| CCFA       | 118| Male, 65; female, 53 | White, 112; black, 6 | 13.0           |
| Hershey    | 18 | Male, 12; female, 6 | White, 18 | 13.4           |
| UC         | 23 | Male, 9; female, 14 | White, 23 | 13.3           |
| CCFA       | 10 | Male, 5; female, 5 | White, 10 | 12.9           |
| Hershey    | 13 | Male, 4; female, 9 | White, 13 | 13.6           |
| Control    | 129| Male, 65; female, 64 | White, 121; black, 7; unknown, 1 | 17.5           |

IBD: Inflammatory bowel disease; CD: Crohn’s disease; CCFA: Crohn’s and Colitis Foundation of America; UC: Ulcerative colitis.

**MATERIALS AND METHODS**

**Study samples**

Genomic (g)DNA samples obtained from 159 pediatric IBD patients (CD, n = 136; UC, n = 23) were studied. The age of diagnosis for all patients was < 17-years-old. The patients were Caucasian (n = 153) and African American (n = 6). gDNA samples were obtained from the Crohn’s and Colitis Foundation of America (CCFA) DNA Databank (IBD, n = 128 including 118 with CD and 10 with UC) and the Pennsylvania State University IBD Biobank (IBD, n = 31 including 18 with CD and 13 with UC)\textsuperscript{[28]}. Healthy gDNA control samples (n = 129) were obtained from CCFA (n = 70) and the Hershey Medical Center (n = 59). The race, age and sex of the controls were matched to the study cases; none of the controls were identified with gastrointestinal-related diseases (Table 1).

Informed consent was obtained for all patient samples retrieved from the Pennsylvania State University IBD Biobank and the Hershey Medical Center. All study protocols were approved by the Penn State University College of Medicine Institutional Review Board. CCFA gDNAs were collected from samples originating from the University of North Carolina at Chapel Hill, University of Chicago, Cedars-Sinai Hospital, Massachusetts General Hospital, University of Pittsburgh, and Mt. Sinai Hospital, with written informed parental or guardian consent.

**DNA isolation**

gDNA samples were obtained from CCFA as noted above. The gDNA from Hershey Medical Center was isolated from blood samples or Epstein Bar virus-immortalized B cell lines using QiaGen DNA Mini Kits (QiaGen Inc., Valencia, CA, United States). After DNA concentration was measured with a Nanodrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States), the gDNA samples were stored at -80 °C until use.

**Selection criteria and study of SNPs from IL-10, IL10RA, IL10RB, STAT3, and HO1**

Seven SNPs from these five genes were studied. These are rs1800872 (C-592>A), rs3024498 and rs3024496 from IL-10\textsuperscript{[29]}; rs3135932 from IL10RA\textsuperscript{[30]}; rs2834167 from IL10RB\textsuperscript{[7-29]}; rs744166 from STAT3; and rs2071746 from HO1\textsuperscript{[30,31]}. The criteria for SNP selection were based (1) on the potential relevance of these SNPs in the function and the regulation of genes, which have been associated with IBD and other diseases, and/or play a role in inflammatory processes; (2) on the gene location, either within the coding region that changes the encoded amino acid, or at 5’ upstream or 3’UTR potentially affecting RNA transcription, RNA stability or protein translation; and (3) being polymorphic in the study samples as tested in our preliminary study and having minor allele frequency information in existing databases. A summary of these SNPs is provided in Table 2, including genetic variation, chromosomal position, gene location, and disease implication.

**Genotype analysis**

The genotypes of all seven SNPs were determined with PCR-based RFLP/cRFLP as described previously\textsuperscript{[32]}. The PCR primers and related information are given in Table 3. Briefly, 100 ng DNA were used for PCR in a 30 µL reaction volume. The PCR cycling profile
was as follows: 95°C for 2 min, 5 cycles at 95°C for 30 s, 50°C for 1 min, and 72°C for 1 min, then 30 cycles at 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 4 min. PCR products (5 µL) were digested with an appropriate restriction enzyme (Table 3) according to manufacturer’s instructions. The digested PCR products were separated by polyacrylamide gel electrophoresis (8%), and the genotypes were scored according to the gel pattern of the digested PCR products.

**Statistical analysis**

Single SNP analysis was statistically assessed by associating each single SNP with the disease. Specifically, we calculated the genotype-based OR and P value based on Fisher’s exact test. We also calculated 95% CIs for each OR. The difference was considered as significant when P < 0.05.

**Epistatic interaction analysis of IL-10 and IL-10 pathway genes**

Epistatic analysis: Epistasis, due to the interaction between different loci, may play an important role in disease progression. By using two different SNPs simultaneously, epistasis may detect information that cannot be detected by single SNP analysis. We have developed a model of epistatic detection which allows high-order epistasis due to the interaction among more than two loci to be characterized. This model was used to test high-order epistasis between, IL-10 and IL-10 receptors, IL-10 and STAT3, IL-10 and HO1, and STAT3 and HO1. This model not only allows the testing of additive (a) and dominant (d) effects at single SNPs, but is also able to detect the epistatic effects between two or three SNPs in a case-control study.

Four types of epistatic interactions for two SNPs, namely additive-additive (aa), additive-dominant (ad), dominant-additive (da), and dominant-dominant (dd) and eight types of epistatic interactions for three SNPs, namely aaa, aad, ada, add, dda, dad, dda and ddd, were estimated and are discussed in this paper.

We estimated the pair-wise linkage disequilibria (LD) between these epistatic loci, which were detected to be non-significant, showing that these loci are segregating randomly in the population.

| Table 2 | Study single nucleotide polymorphisms for IL-10, IL10RA, IL10RB, STAT3 and HO1 genes |
|----------|-----------------------------------------------|
| Gene     | SNP ID            | Chromosomal position | Variation | Gene location | Disease implication | Ref.       |
| IL-10    | rs1800872         | 206946407            | C>50>A    | 5'-upstream   | associated with IBD | [37]       |
|          | rs3024498         | 206941529            | c>T>C     | 3’-unrelated region | associated with colorectal cancer | [41]       |
|          | rs3024496         | 206941864            | A>G       | 3’-unrelated region | associated with IBD and colorectal cancer, with decreased IL-10, with increased IgE levels | [37-39,41] |
| IL10RA   | rs3135932         | 117864063            | c.A247>G, p.Ser159Gly | coding region | mutations (other than the studied SNP) associated with pediatric IBD | [7,10,12]  |
| IL10RB   | rs2834167         | 34640788             | c.A>G, p.Lys(A)47Glu(G) | coding region | mutations (other than the studied SNP) associated with pediatric IBD | [7-11]     |
| STAT3    | rs744166          | 404514201            | A>G       | Intron 1 (closer to exon2) | associated with IBD | [20]       |
| HO1      | rs2071746         | 3577672             | A413>T    | 5'-upstream | no association with IBD, associated with asthma and allergy, anti-inflammation, anti-oxidant | [30,31]    |

**Table 3 | PCR-RFLP method for genotyping IL-10, IL10RA, IL10RB, STAT3 and HO1 genes**

| Gene | SNP ID | Variant | PCR amplification | RFLP | Recognition site |
|------|--------|---------|-------------------|------|-----------------|
| IL-10| rs1800872 | G>T     | IL-2: 5' -AACCTAGGCGATCCATGCTTAC3' | Scal | T yes; G No     |
|      | rs3024498 | T>C     | IL-5f: 5'-GCTCCGTGTTTCTCTCTAAG-3' | HpyCHV4 | C yes; T No     |
|      | rs3024496 | A>G     | IL-4f: 5'-CTATGACATGATCCATCAGG-3' | NalIII | G yes; A No     |
| IL10RA| rs3135932 | A>G     | IL-10: 5'-AAGTGAGGCTAGTGGAG-3' | Mnl | G yes; A No     |
| IL10RB | rs2834167 | A>G     | IL-12: 5'-AGTTCCCAATGGCACACAAG-3' | Carl | G yes; A No     |
| STAT3 | rs744166 | A>G     | ST2: 5'-AGTTCCCAATGGCACACAAG-3' | Alul | A yes; G No     |
| HO1   | rs2071746 | A>T     | HOM: 5'-TCAGCAGAGGATTCCACAGCAGC-3' | BfaI | A yes; T No     |

Lowercase letter indicates a mismatched nucleotide.

IBD: Inflammatory bowel disease; SNPs: Single nucleotide polymorphisms.
RESULTS

**IL-10 rs304496 is associated with pediatric IBD**

There is limited information in terms of genetic association studies for pediatric IBD. The present study of pediatric IBD builds upon and extends findings from our previous genetic association study on adult IBD. We initially wished to confirm previous findings [20] as to whether IL-10 was involved in pediatric IBD. Since published studies of IL-10 were done in adult IBD, we carried out a pilot study with adult IBD. We studied IL-10 association with 122 adult IBD (74 with CD, 48 with UC) cases (mean age of 51 years) and 172 unrelated healthy controls from Hershey Medical Center using the SNPlex Genotyping System [33,34]. The results indicated that two IL-10 SNPs are significantly associated with IBD: rs1800872 $P = 0.0056$, OR = 1.753, and 95%CI: 1.190-2.643; and rs304498 $P = 0.0008$ OR = 0.43, and 95%CI: 0.26-0.7. This pilot genetic association study as well as other association studies of adult IBD, guided our selection of genes and SNPs for the present study.

In the present study, we wished to know whether IL-10, shown previously to be associated with adult IBD is associated with pediatric IBD, and whether the IL-10/STAT3 pathway plays a role in pediatric IBD. The study samples were 159 IBD (136 with CD and 23 with UC) and the three SNPs genotyped were rs1800872, rs3024498, and rs3024496. The results indicated (Table 4) that neither of the two SNPs, rs1800872 and rs3024498, that have been previously observed to be associated with adult IBD were associated with pediatric IBD ($P = 0.71$ and $P = 0.616$, respectively). The rs3024496 was the only SNP found to significantly associate with pediatric IBD ($P = 0.022$). No association with pediatric IBD was found for the IL-10 pathway genes, IL10RA, IL10RB, STAT3, and HO1

The IL10-STAT3 signaling pathway plays an important role in controlling inflammation in intestine. The IL10RA, IL10RB and STAT3 are critical players in this pathway. IL-10 and STAT3 have previously been demonstrated to be associated with early-onset IBD. The activated STAT3 pathway regulates expression of several critical anti-inflammatory genes, including HO1, a potent anti-inflammation and anti-oxidant

| Gene     | SNP ID   | Genotype | Disease, $n$ | Control, $n$ | OR       | 95%CI         | $P$ value |
|----------|----------|----------|--------------|--------------|----------|---------------|-----------|
| IL-10    | rs1800872| CC       | 89           | 78           | 0.863    | 0.564-1.313  | 0.71      |
|          |         | CA       | 68           | 50           |          |               |           |
|          |         | AA       | 2            | 1            |          |               |           |
|          | rs3024498| C allele | 246          | 206          |          |               |           |
|          |         | A allele | 72           | 52           |          |               |           |
|          |         | CC       | 86           | 77           | 0.830    | 0.552-1.244  | 0.616     |
|          |         | CT       | 65           | 47           |          |               |           |
|          |         | TT       | 8            | 5            |          |               |           |
|          |         | C allele | 237          | 201          |          |               |           |
|          |         | T allele | 81           | 57           |          |               |           |
| IL10RA   | rs3135932| AA       | 108          | 85           | 0.925    | 0.595-1.433  | 0.160     |
|          |         | AG       | 39           | 40           |          |               |           |
|          |         | GG       | 12           | 4            |          |               |           |
|          |         | A allele | 183          | 123          |          |               |           |
|          |         | G allele | 185          | 135          |          |               |           |
| IL10RB   | rs2834167| AA       | 91           | 64           | 1.318    | 0.884-1.968  | 0.203     |
|          |         | AG       | 66           | 50           |          |               |           |
|          |         | GG       | 2            | 5            |          |               |           |
|          |         | A allele | 248          | 188          |          |               |           |
|          |         | G allele | 63           | 48           |          |               |           |
| STAT3    | rs744166 | GG       | 64           | 49           | 0.943    | 0.662-1.340  | 0.352     |
|          |         | AG       | 66           | 63           |          |               |           |
|          |         | AA       | 29           | 17           |          |               |           |
|          |         | A allele | 194          | 161          |          |               |           |
|          |         | G allele | 124          | 97           |          |               |           |
| HO1      | rs2071746| AA       | 32           | 30           | 0.957    | 0.680-1.348  | 0.634     |
|          |         | AT       | 96           | 71           |          |               |           |
|          |         | TT       | 31           | 28           |          |               |           |
|          |         | A allele | 158          | 131          |          |               |           |
|          |         | T allele | 160          | 127          |          |               |           |

SNP: Single nucleotide polymorphism.

**Table 4 Genetic association of IL-10, IL10RA, IL10RB, STAT3 and HO1 genes with pediatric inflammatory bowel disease**
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Table 5 Epistatic interaction between two single nucleotide polymorphisms in three IL-10 single nucleotide polymorphisms studied

| Epistatic model | rs3024496, P = 0.022; Bp = 0.0226 | rs3024498, P = 0.616; Bp = 1 |
|-----------------|----------------------------------|-------------------------------|
| rs1800872, P = 0.71; Bp = 1 | aa: P = 0.00015; Bp = 0.003 | P = 0.638; Bp = 1 |
|                 | ad: P = 0.057; Bp = 1           | P = 0.605; Bp = 1             |
|                 | da: P = 0.010; Bp = 0.216       | P = 0.977; Bp = 1             |
|                 | dd: P = 0.239; Bp = 1           | P = 0.049; Bp = 1             |
| rs3024496, P = 0.022, Bp = 0.0226 | aa: P = 0.371; Bp = 1 | P = 0.167; Bp = 1 |
|                 | ad: P = 0.022; Bp = 1           | P = 0.584; Bp = 1             |
|                 | da: P = 0.176; Bp = 1           | P = 0.038; Bp = 1             |

The P and Bp values for each SNP are shown next to each SNP; the P and Bp values for each of two SNP interactions are shown in the 3rd and 4th column. In the 2nd column (epistatic model), a: Additive; d: Dominant. For a two SNP interaction, four different types of interactions may occur: additive-additive (aa), additive-dominant (ad), dominant-additive (da), and dominant-dominant (dd). Bp: Bonferroni P value; SNP: Single nucleotide polymorphism.

Table 6 Gene-gene interaction between IL-10 with IL10RA, IL10RB, STAT3, or HO1 in pediatric inflammatory bowel disease

| Gene   | SNP      | Epistatic model | IL10RA rs3135932, P = 0.160; Bp = 1 | IL10RB rs2834167, P = 0.203; Bp = 1 | STAT3 rs744166, P = 0.352; Bp = 1 | HO1 rs2071746, P = 0.634; Bp = 1 |
|--------|----------|----------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| IL-10  | rs1800872| aa: P = 0.046; Bp = 1 | P = 0.029; Bp = 1 | P = 0.248; Bp = 1 | P = 0.910; Bp = 1 |
|        |          | ad: P = 0.739; Bp = 1 | P = 0.107; Bp = 1 | P = 0.954; Bp = 1 | P = 0.617; Bp = 1 |
|        |          | da: P = 0.056; Bp = 1 | P = 0.376; Bp = 1 | P = 0.117; Bp = 1 | P = 0.269; Bp = 1 |
|        |          | dd: P = 0.126; Bp = 1 | P = 0.166; Bp = 1 | P = 0.036; Bp = 1 | P = 0.671; Bp = 1 |
|        | rs3024498| aa: P = 0.330; Bp = 1 | P = 0.062; Bp = 1 | P = 0.143; Bp = 1 | P = 0.898; Bp = 1 |
|        |          | ad: P = 0.068; Bp = 1 | P = 0.629; Bp = 1 | P = 0.032; Bp = 1 | P = 0.840; Bp = 1 |
|        |          | da: P = 0.884; Bp = 1 | P = 0.307; Bp = 1 | P = 0.316; Bp = 1 | P = 0.607; Bp = 1 |
|        |          | dd: P = 0.265; Bp = 1 | P = 0.644; Bp = 1 | P = 0.029; Bp = 1 | P = 0.790; Bp = 1 |
|        | rs3024496| aa: P = 0.021; Bp = 0.433 | P = 0.425; Bp = 1 | P = 0.538; Bp = 1 | P = 0.346; Bp = 1 |
|        |          | ad: P = 0.020; Bp = 0.426 | P = 0.495; Bp = 1 | P = 0.306; Bp = 1 | P = 0.741; Bp = 1 |
|        |          | da: P = 0.081; Bp = 1 | P = 0.189; Bp = 1 | P = 0.234; Bp = 1 | P = 0.297; Bp = 1 |
|        |          | dd: P = 0.967; Bp = 1 | P = 0.570; Bp = 1 | P = 0.402; Bp = 1 | P = 0.457; Bp = 1 |
| IL10RB | rs2834167| aa: P = 0.403; Bp = 1 | P = 0.251; Bp = 1 | P = 0.128; Bp = 1 | P = 0.128; Bp = 1 |
|        |          | ad: P = 0.384; Bp = 1 | P = 0.956; Bp = 1 | P = 0.369; Bp = 1 | P = 0.369; Bp = 1 |
|        |          | da: P = 0.518; Bp = 1 | P = 0.776; Bp = 1 | P = 0.0018; Bp = 0.039 | P = 0.0018; Bp = 0.039 |
|        |          | dd: P = 0.176; Bp = 1 | P = 0.072; Bp = 1 | P = 0.289; Bp = 1 | P = 0.289; Bp = 1 |

Bp: Bonferroni P value; SNP: Single nucleotide polymorphism.

Our genetic association study results indicate that none of these genes is significantly associated with pediatric IBD (Table 4).

**Epistatic interaction of SNP-SNP (rs3024496 and rs1800872) within the IL-10 gene in pediatric IBD**

Based on previous genetic studies of the studied genes and their role in the IL-10 pathway, we speculated that some of the SNPs contribute to disease by interacting with other genes. To test this hypothesis we used our recently developed model[27] that has been demonstrated to be genetically meaningful in our previous studies on IBD susceptibility genes[30,35,36].

First, we studied SNP-SNP interaction among three SNPs (rs1800872 and rs3024496, rs1800872 and rs3024498, and rs3024496 and rs3024498) within the IL-10 gene of all possible combinations of a and d models.

A significant epistatic interaction was only observed for rs1800872 and rs3024496 (P = 0.00015; Bp = 0.003) (Table 5). A graphical depiction of this aa model is shown in Figure 1. Although the IL-10 rs1800872 was shown by itself to associate with adult IBD but not associate with pediatric IBD (P = 0.71) (Table 4), the present data indicate that it may still contribute to pediatric IBD via interaction with another IL-10 SNP, namely rs3024496.

**Epistatic interaction of the IL-10 gene with the IL-10 signaling pathway genes, IL10RB and HO1, in pediatric IBD**

We further analyzed gene-gene interactions between IL-10 and the other four genes, IL10RA, IL10RB, STAT3, and HO1, involved in the IL-10 signaling pathway. The results showed that none of the three IL-10 SNPs significantly interacted with the SNPs of the other four genes (Table 6). Although a low P-value was observed for the IL-10 rs1800872 with either the IL10RA (aa, P = 0.046), IL10RB (aa, P = 0.029), or STAT3 (dd, P = 0.036), and for the IL-10 rs3024498 with the STAT3 (ad, P = 0.032; dd P = 0.029), none of these stood as significant after Bonferroni correction was applied. Only the interaction of the IL-10 rs3024496 with the IL10RA rs3135932 showed a low Bp (aa, P = 0.021, Bp = 0.433; ad, P = 0.020, Bp = 0.426) (Table 6). A graphic depiction of the
interaction model of IL-10 with IL10RA is shown in Figure 2A. From a single association study as described above, none of the SNPs of the four genes in the IL-10 signaling pathway was associated with pediatric IBD. However, we found that SNPs of the IL-10RB and HO1 genes contribute to pediatric IBD (da, $P = 0.0018$, Bp = 0.039) (Table 6) via gene-gene interaction. Graphical depictions for the model interactions between IL10RB and HO1 are shown in Figure 2B.

**Epistatic interaction of the IL-10 pathway genes IL10RA, IL10RB, STAT3, and HO1 in pediatric IBD**

Based on the epistatic interaction of IL10RB with HO1 (Table 6, Figure 2B), we further analyzed the effect of the IL10RB and HO1 interaction in each of the four models (aa, ad, da, and dd) on the contribution to IBD in conjunction with IL-10, IL10RA and STAT3. Eight types of epistatic interactions in each set of three SNPs were studied. As shown in Table 8, a significant effect interaction of IL-10 with other genes. As shown in Table 7, in the presence of the two IL-10 SNPs, the association of IL10RA rs3135932 is increased remarkably from $P = 0.16$ (when analyzed by itself), to $P = 0.046$ (with rs1800872), $P = 0.021$ (with rs3024496) (Table 5) to $P = 0.003$ (with both rs1800872 and rs3024496) (Table 7). This epistatic interaction is an aaa model (Figure 3). However, the interaction of the two IL-10 SNPs did not exhibit any further observed effect on IL-10 interaction with the other genes, IL10RB, STAT3, and HO1 (Table 7).

**Epistatic interaction of two IL-10 single nucleotide polymorphisms, rs1800872 and rs3024496, with IL10RA, IL10RB, STAT3 and HO1 in pediatric inflammatory bowel disease**

Graphic depiction of epistatic interaction of the IL-10 SNPs rs3024496 and rs1800872 with the IL10RA rs3135932. All the interaction models analyzed for the IL-10 SNPs rs3024496 and rs1800872 with the IL10RA rs3135932 are shown. However, only one significant interaction model additive-additive-additive (a1a2a3) was observed ($P = 0.003$, Bp = 0.099). Levels of Bp values are shown in the bar as shades of blue color from none (Bp = 1) to high (Bp = 0.001). a1, d1, a2, d2, a3, d3: Letters, a and d, are for interaction model additive and dominant respectively; Numbers 1, 2, and 3 depict the combination of the two and three SNPs; such as, a1a2 for SNPs 1 and 2, and a1a2a3 for SNPs 1, 2, and 3. Bp: Bonferroni P value; SNPs: Single nucleotide polymorphisms.

**Epistatic interaction between IL-10 single nucleotide polymorphisms rs3024496 and rs1800872**

Figure 1 Epistatic interaction between IL-10 single nucleotide polymorphisms rs3024496 and rs1800872. Graphic depiction of epistatic interaction between the IL-10 SNPs rs3024496 and rs1800872. Four interaction models for rs3024496 and rs1800872 are shown. The additive-additive model is significant (Bp = 0.003), the additive-dominant model is weak (Bp = 0.216), and the other two models are not observed (Bp = 1, no color). Bp: Bonferroni P value; SNPs: Single nucleotide polymorphisms.

**Graphic depiction of gene-gene interaction between IL10 and IL10RA**

Figure 3 Epistatic interaction of two IL-10 single nucleotide polymorphisms, rs1800872 and rs3024496, with IL10RA, IL10RB, STAT3 and HO1 in pediatric inflammatory bowel disease. Graphic depiction of epistatic interaction of the IL-10 SNPs rs3024496 and rs1800872 with the IL10RA rs3135932. All the interaction models analyzed for the IL-10 SNPs rs3024496 and rs1800872 with the IL10RA rs3135932 are shown. However, only one significant interaction model additive-additive-additive (a1a2a3) was observed ($P = 0.003$, Bp = 0.099). Levels of Bp values are shown in the bar as shades of blue color from none (Bp = 1) to high (Bp = 0.001). a1, d1, a2, d2, a3, d3: Letters, a and d, are for interaction model additive and dominant respectively; Numbers 1, 2, and 3 depict the combination of the two and three SNPs; such as, a1a2 for SNPs 1 and 2, and a1a2a3 for SNPs 1, 2, and 3. Bp: Bonferroni P value; SNPs: Single nucleotide polymorphisms.
Table 7  Epistatic interaction among the two IL-10 single nucleotide polymorphisms, rs1800872 and rs3024496, and IL10RA, IL10RB, STAT3, or HO1

| Epistatic model | IL10RA rs3135932, P = 0.16; Bp = 1 | IL10RB rs2834167, P = 0.203; Bp = 1 | STAT3 rs744166, P = 0.352; Bp = 1 | HO1 rs2071746, P = 0.634; Bp = 1 |
|----------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Two IL-10 SNPs:| aaa                              | P = 0.003;                       | P = 0.080;                       | P = 0.175;                       | P = 0.216;                       |
| rs1800872 and rs3024496 |                                   | Bp = 0.099;                     | Bp = 1                           | Bp = 1                           | Bp = 1                           |
| aa: P = 0.0002; |                                   |                                  | P = 0.150;                       | P = 0.340;                       | P = 0.920;                       | P = 0.140;                       |
| Bp = 0.003     |                                   |                                  | Bp = 1                           | Bp = 1                           | Bp = 1                           | Bp = 1                           |

The additive-additive (aa) interaction model for the two IL-10 SNPs (rs1800872 and rs3024496) from Figure 1 was chosen for further analysis, in order to study the interaction of these two IL-10 SNPs with SNPs of four other genes. The two interaction models, aa and aad, were for the two IL-10 SNPs and SNPs from each of the four genes. A significant P value (P = 0.003) was observed only with the IL10RA SNP (rs3135932). Bp: Bonferroni P value; SNPs: Single nucleotide polymorphisms.

Table 8  Epistatic interaction of IL10RB and HO1 with IL-10 (three single nucleotide polymorphisms), IL10RA, or STAT3

| Epistatic model | IL-10 rs1800872, P = 0.71; Bp = 1 | IL-10 rs3024496, P = 0.616; Bp = 21 | IL-10 rs3024496, P = 0.222; Bp = 0.0226 | IL10RA rs3135932, P = 0.16; Bp = 1 | STAT3 rs744166, P = 0.352; Bp = 1 |
|----------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|
| aa IL10RB rs2834167 and HO1 rs2071746 | aaaa | P = 0.029;                       | P = 0.545;                       | P = 0.255;                       | P = 0.023;                       | P = 0.049;                       |
| aa: P = 0.127; |                                  | Bp = 1                           | Bp = 1                           | Bp = 1                           | Bp = 1                           | Bp = 1                           |
| Bp = 0.096    |                                  |                                  |                                  |                                  |                                  |                                  |
| ad IL10RB rs2834167 and HO1 rs2071746 | adda | P = 0.976;                       | P = 0.490;                       | P = 0.327;                       | P = 0.044;                       | P = 0.436;                       |
| Bp = 1        |                                  | Bp = 1                           | Bp = 1                           | Bp = 1                           | Bp = 1                           | Bp = 1                           |
| dda IL10RB rs2834167 and HO1 rs2071746 | ddda | P = 0.199;                       | P = 0.408;                       | P = 0.0015;                      | P = 0.072;                       | P = 0.140;                       |
| Bp = 1        |                                  | Bp = 1                           | Bp = 1                           | Bp = 1                           | Bp = 1                           | Bp = 1                           |

Pathway function by regulating anti-inflammatory activity in pediatric IBD.

**DISCUSSION**

In the present study, we identified a genetic association of the IL-10 gene and the IL-10 signaling pathway with pediatric IBD and demonstrated that both SNP-SNP and gene-gene epistatic interactions contribute to pediatric IBD. The specific findings include the following: (1) IL-10 rs3024496 is identified to be associated with pediatric IBD; (2) an aa interaction was found between IL-10 SNPs rs3024496 and rs1800872; (3) the SNP-SNP interaction in the IL-10 gene affects its action with the IL-10 receptor IL10RA; (4) the IL-10 signaling pathway genes IL10RB and HO1 together are significantly associated with pediatric IBD via SNP-SNP interaction; and (5) a significant association of the three genes, IL-10, IL10RB, and HO1, with pediatric IBD was identified from epistatic interaction analysis among three SNPs.

The IL-10 gene has been shown to be associated with pediatric IBD.
with adult IBD by GWASs. The most studied IL-10 SNP, rs1082432 (rs1800896), is thought of as having potential for gene transcription regulation[14,15,37]. The IL-10 SNP rs3024496 is shown to be related to inflammatory response with increased levels of IgE to dust mite[46], or decreased production of IL-10 by peripheral blood leukocytes[47,48], and with prostate[49] and colorectal[50] cancer, but has not been shown to be associated with IBD. The IL-10 rs1800872 is associated with IBD[51] and also with increased serum IL-10 levels in CD[52,53], as well as with irritable bowel syndrome[54] and cancer susceptibility[50,51,52,53]. In this study, we found that IL-10 rs3024496 is associated with pediatric IBD, and rs1800872, although by itself is not associated with pediatric IBD, appears to contribute to pediatric IBD via epistatic interaction with rs3024496.

Although currently more than 163 genes have been identified to be associated with IBD[47,50], only few of them have been studied in pediatric IBD. The estimate that a genetic contribution of the identified genes collectively represents only < 20% of the overall disease risk[47,51-55] indicates that other genetic/genomic and environmental factors may play a role in IBD pathogenesis. In the present study, we studied IL-10 gene contribution in pediatric IBD by analyzing its association with disease as well as its epistatic interaction with IL-10 pathway genes. Our results indicate, in addition to disease association of IL-10 itself, that SNP-SNP and gene-gene interactions contribute significantly to pediatric IBD.

Our results support that epistasis plays an important role in the formation and progression of human diseases[56,57]. Understanding gene-gene interaction is crucial to our understanding of the regulation of physiological function. When epistasis occurs, the presence of two or more particular loci may increase or reduce the risk of a disease more than would be expected from their independent effects[58]. A host of statistical models have been developed to analyze epistatic effects in different genetic designs[59,60].

Our recently developed model for multilocus epistatic interactions in case-control studies has proven to be genetically meaningful through the incorporation of traditional quantitative genetic principles into statistical models[27]. Using this model we have previously studied epistatic interaction between SNPs within the DLG5 gene and between IBD genes DLG5, OCTN1, IL23R and NOD2[28,35,36], and found that epistatic interaction is an important component in IBD pathogenesis. In this study, we used the same method to study gene-gene interaction in IL-10 signaling transduction pathway. IL-10 signaling transduction occurs through binding of IL-10 to its receptors IL10RA and IL10RB to form a complex, with downstream molecules, Jak1 and Tyk2, activating STAT3[24,25]. IL10RA is specific to IL-10, but IL10RB also interacts with several other cytokines. When either IL10RA or IL10RB is mutated, the signals from IL-10 cannot be received and the resulting inflammation causes tissue damage in the gastrointestinal system[23,24]. A significant epistatic interaction was observed between two IL-10 SNPs, resulting in a significant effect of IL-10 interaction with IL10RA, but not with IL10RB (Table 7). This indicates that IL-10 may interact with receptor IL10RA, which plays a role in the initiation of the signaling pathway.

We also observed a significant epistatic interaction between IL-10, IL10RB, and the downstream pathway target HO1 gene (Table 8). The key factor for interaction of these three genes is IL10RB that interacts strongly with HO1. This finding indicates that the IL-10 receptors IL10RA and IL10RB are likely to function differently in the IL-10 pathway in pediatric IBD. Although HO1 was not found to be associated with IBD[61], it has been shown to be associated with other diseases such as asthma and allergy[54,62]. HO1 is a potent enzyme of anti-inflammation, and has a very important function of the IL-10 pathway in controlling inflammation[54].
Although STAT3 is a critical component of the IL-10/STAT3 pathway, no significant interaction was observed between IL-10 and/or IL-10 receptors with STAT3, indicating that other factor(s) may play a role between these two genes in the pathway. We know that in the IL-10 pathway, upon binding of IL-10 to cell receptors IL10RA and IL10RB, the IL-10 receptor complex members Jak1 and Tyk2 are activated and catalyze phosphorylation of themselves and then of IL10RA, thereby forming a docking site for STAT3. STAT3 is phosphorylated by Jak1 and Tyk2, and this phosphorylation causes STAT3 dimerization and translocation to the nucleus where it can induce expression of its target genes including HO-1. Therefore, we speculate that Jak1 and Tyk2 play a role in pediatric IBD by their activity in the phosphorylation and activation of STAT3 in IL-10 signaling pathway.

Recently, pediatric/VEO IBD has been suggested to be a distinct form of IBD, and SNPs in IL-10 and IL-10 receptors have been associated with VEO IBD. In the present study, we identified IL-10 SNP of rs3024496 to be associated with pediatric IBD; this has not been shown to be associated with adult IBD. However, our results did not show a genetic association of the IL-10 rs1800872 and STAT3 rs744166 with pediatric IBD, which have been shown to be associated with adult IBD. Our present study also showed that epistatic interactions of IL-10 with genes IL10RA, IL10RB, STAT3, and HO1 contribute to pediatric IBD. Their physiological function in the regulation of the anti-inflammatory pathway in response to pro-inflammatory stimulation, and protection of diseased tissues from damage is currently not well studied. IBD is a major gastrointestinal disease affecting 1.4 million people in the United States. About 15%-25% of IBD patients are diagnosed in childhood. Specific investigation targeting the IL-10 signaling pathway in pediatric IBD pathogenesis will help to understand the pathogenesis of pediatric IBD, and may provide target molecules and pathways to potentially develop anti-inflammatory agents for clinical treatment of pediatric IBD.

Other cytokines are also shown to be involved in the inflammatory process of IBD. Studies on correlation between NO and IL17A, IL-23 and IL-6 levels in plasma of IBD patients indicated that the IL-23/IL17A axis and NO synthase pathway are involved in inflammation regulation in IBD.

In summary, IL-10 is associated with pediatric IBD, and the IL-10 signaling pathway that plays an important role in anti-inflammation. We propose, as depicted in Figure 5, that in pediatric IBD pathogenesis, (1) IL-10 via its interaction with receptor IL10RA, and then together with receptor IL10RB are critical for the initial step of the signaling transduction; (2) IL-10 via its interaction with receptor IL10RB plays a key role in regulating gene transcription of anti-inflammatory enzymes, such as HO1, that may lead to an anti-inflammatory response; and (3) no significant interaction was found between IL-10 and IL-10 receptors with STAT3, a key molecule in the IL-10 pathway. However, further investigation may provide insight as to whether Jak1 and Tyk2 are involved in pediatric IBD, via potential interactions with IL10RA and IL10RB where together their gene products could phosphorylate and activate STAT3.

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COMMENTS

Background

Knockout mice lacking IL-10 develop inflammatory bowel disease (IBD), and mutations in IL-10 and IL-10 receptor genes IL10RA and IL10RB have been linked to very early-onset (VEO) IBD. Both IL-10 and STAT3 have been identified as IBD-associated genes in adults, but these are not well studied in pediatric IBD.

Research frontiers

Mutations of IL-10 and genes encoding its receptors have been identified recently in VEO IBD. The authors studied genetic association of IL-10 and genes in the IL-10/STAT3 pathway with pediatric IBD. Genetic interactions between different loci, i.e., epistasis, have been thought to be of paramount importance in complex diseases. In this paper, we used a computational model...
to analyze how epistatic interactions among polymorphic loci in the IL-10 gene and IL-10/STAT3 pathway govern pediatric IBD in a case-control setting.

Innovations and breakthroughs
Despite pronounced evidence of the role of the genes comprising IL-10 and genes within the IL-10/STAT3 signaling pathway, our knowledge about how they interact with each other to determine IBD development is still very limited. In this paper, they have identified IL-10 variation associated with pediatric IBD, and found a number of epistatic interactions of IL-10 with genes in the IL-10/STAT3 pathway contributing to pediatric IBD. The contribution of interactions of the IL-10/STAT3 pathway and anti-inflammatory HO1 gene to IBD indicated that IL-10 plays a role in the control of inflammation in IBD.

Applications
The findings may help to understand the function of IL-10 and the IL-10 pathway in the control of inflammation in IBD, and identify target molecules for clinical investigation and drug discovery for controlling inflammation in IBD.

Peer-review
The authors report novel data. The obtained results indicate that both the IL-10 gene and its epistatic interaction with genes within its signaling pathway are related to pediatric IBD.

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