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ORIGINAL ARTICLE

Basic Study

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

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Author contributions: Lin Z and Wang Z contributed equally to this work; Lin Z designed the experimental approach and was responsible for troubleshooting, data collection and organization and the overall supervision of the project and he worked with the statistician for data analysis, manuscript writing and submission, and the response to reviewers; Wang Z and Wu R contributed to the data analysis and presentation of the results, manuscript writing and revision; Hegarty JP was involved in grant writing, preliminary data preparation and genotype analysis; Lin TR contributed to IL-10 genotype and genotyping data analysis (adult IBD and youth IBD), as well as of IL-10 gene expression in patients with different genotype; Wang Y performed genotype analysis by PCR, gel electrophoresis, genotype scoring, and prepared the data sheet for analysis; Deiling S contributed to clinical sample collection and documentation; Thomas NJ was involved in study subject recruitment, clinical sample collection and manuscript writing; Floros J was involved in experimental design, management of the research team, oversight of the project, data collection and presentation, manuscript writing, final critical reading before submission, response to reviewers and revising the manuscript for resubmission.

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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 high-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-additive-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

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.

Lin Z, Wang Z, Hegarty JP, Lin TR, Wang Y, Deiling S, Wu R, Thomas NJ, Floros J. Genetic association and epistatic interaction of the interleukin-10 signaling pathway in pediatric inflammatory bowel disease. *World J Gastroenterol* 2017; 23(27): 4897-4909 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i27/4897.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i27.4897

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 (NFκB)[4].

In addition, novel association of major histo-compatibility 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

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.

damage[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[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[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[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 high-order epistasis among three loci, thereby 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.

MATERIALS AND METHODS

Study samples

Genomic (g)DNA samples obtained from a total of 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)[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 ℃ 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*[23]; rs3135932 from *IL10RA*[7]; rs2834167 from *IL10RB*[7,29]; rs744166 from *STAT3*; and rs2071746 from *HO1*[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[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

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-592>A	5'-upstream	associated with IBD	[37]
	rs3024498	206941529	c.T>C	3'-untralated region	associated with colorectal cancer	[41]
	rs3024496	206941864	A>G	3'-untralated 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]

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

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

Gene	SNP ID	Variant	PCR amplification		RFLP	
			Primers[1]	Product, bp	Restriction enzyme	Recognition site
IL-10	rs1800872	G>T	IL-2f: 5'-AACTTAGGCAGTCACCTTAGG-3' IL-2r: 5'-CATCCTGTGACCCCTCCAGT-3'	149	ScaI	T yes; G No
	rs3024498	T>C	IL-5f: 5'-GCTCCtTGGTTTCTCTTCCTA AG-3' IL-5r: 5'-AGAAGCTTCCATTCCAAGCC TGA-3'	137	HpyCH4V	C yes; T No
	rs3024496	A>G	IL-4f: 5'-GTATCAGAGGTAATAAATATTCcAT-3' IL-4r: 5'-TAGAAGCATACATGACAATGAAG-3'	178	NlaIII	G yes; A No
IL10RA	rs3135932	A>G	RA3f: 5'-CCCGCAAATGACACATATGgA-3' RA3r: 5'-AGTTCCCAATGGCACACAAGG-3'	172	MnI	G yes; A No
IL10RB	rs2834167	A>G	RB3f: 5'-GCCATAGAGGAGAACCAAGTG-3' RB3r: 5'-GCTGTGAAAGTCAGGTTCTCTT-3'	206	CarI	G yes; A No
STAT3	rs744166	A>G	ST2f: 5'-CAGGAGTGCCAACATTGAGAG-3' ST2r: 5'-G TAATGTCTTGAGGGAATCGAG-3'	106	AluI	A yes; G No
HO1	rs2071746	A>T	HO4f: 5'-TCAGCAGAGGATTCCAGCAGG-3' TG-3' HO4r: 5'-AGGCAGCGCTGCTCAGAGCAC-3'	110	BfaI	A yes; T No

[1]Lowercase letter indicates a mismatched nucleotide.

was as follows: 95 ℃ for 2 min, 5 cycles at 95 ℃ for 30 s, 50 ℃ for 1 min, and 72 ℃ for 1 min, then 30 cycles at 95 ℃ for 30 s, 58 ℃ for 1 min, and 72 ℃ for 1 min, followed by a final extension step at 72 ℃ 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 two 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[27] 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[27].

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, daa, 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 4 Genetic association of *IL-10*, *IL10RA*, *IL10RB*, *STAT3* and *HO1* genes with pediatric inflammatory bowel disease

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			
		C allele	246	206			
		A allele	72	52			
	rs3024498	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			
	rs3024496	AA	57	27	1.487	1.055-2.099	0.022
		AG	69	69			
		GG	33	33			
		A allele	183	123			
		G allele	185	135			
IL10RA	rs3135932	AA	108	85	0.925	0.595-1.433	0.160
		AG	39	40			
		GG	12	4			
		A allele	255	210			
		G allele	63	48			
IL10RB	rs2834167	AA	91	64	1.318	0.884-1.968	0.203
		AG	66	60			
		GG	2	5			
		A allele	248	188			
		G allele	70	70			
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.

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, rs3024496, and rs3024498. 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 identified to be associated with IBD in adult, while mutations in both *IL-10 receptors A* and *B* have 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

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
	ad		*P* = 0.222; Bp = 1
	da		*P* = 0.167; Bp = 1
	dd		*P* = 0.584; 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* = 0.569; Bp = 1
		dd	*P* = 0.126; Bp = 1	*P* = 0.168; 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
		ad	*P* = 0.384; Bp = 1		*P* = 0.956; Bp = 1	*P* = 0.369; Bp = 1
		da	*P* = 0.518; Bp = 1		*P* = 0.776; Bp = 1	*P* = 0.0018; Bp = 0.039
		dd	*P* = 0.176; Bp = 1		*P* = 0.072; Bp = 1	*P* = 0.289; Bp = 1

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

enzyme. 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[23,24], 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

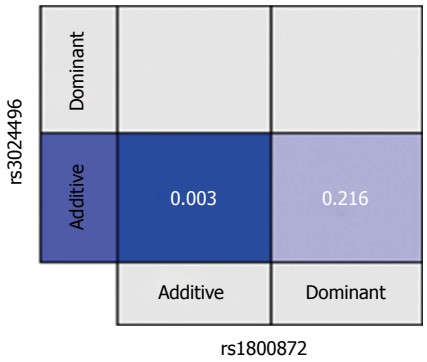

Figure 1 Epistatic interaction between *IL-10* single nucleotide polymorphisms rs3024496 and rs1800872. Graphic depiction of epistatic interaction between the *IL-10* SNPs, rs3024496 and rs1800872. Four interaction models for rs3024496 and rs1800872 are shown. The additive-additive model is significant (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.

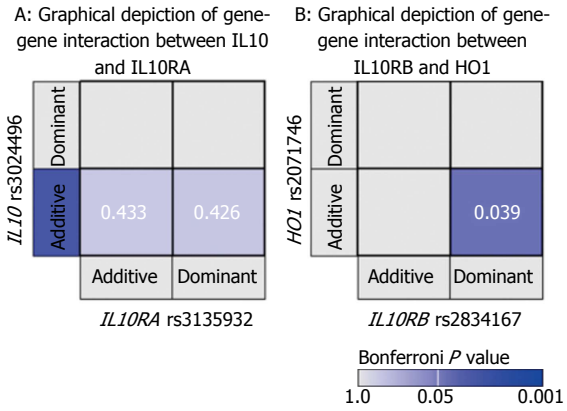

Figure 2 Gene-gene interaction between *IL-10* and *IL10RA*, *IL10RB* and *HO1* in pediatric inflammatory bowel disease. A: Graphic depiction of epistatic interaction between the *IL-10* SNP rs3024496 and the *IL10RA* rs3135932. Four interaction models for rs3024496 and rs3135932 are shown. The additive-additive and additive-dominant models are both weak (Bp = 0.433 and 0.426 respectively), and the other two models are not observed (Bp = 1, no color); B: Graphical depiction of epistatic interaction between the *HO1* rs2071746 and the *IL10RB* rs2834167. Four interaction models for rs3024496 and rs3135932 are shown. Only the additive-dominant model is significant (Bp = 0.039), and the other three models are not observed (Bp = 1, no color). The levels of Bp values are shown in the bar as shades of blue color from none (Bp = 1) to high (Bp = 0.001). Bp: Bonferroni *P* value; SNPs: Single nucleotide polymorphisms.

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. Graphic depictions for the model interactions between *IL10RB* and *HO1* are shown in Figure 2B.

Epistatic interaction of the IL-10 rs3024496 and rs1800872 with the IL10R, rs3135932 in pediatric IBD

We next investigated whether the interaction of two *IL-10* SNPs, rs1800872 and rs3024496, affects further

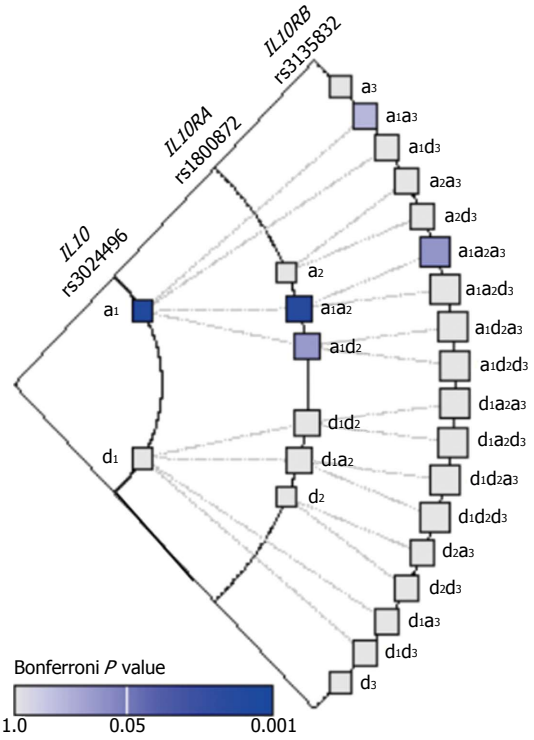

Figure 3 Epistatic interaction of two *IL-10* single nucleotide polymorphisms, rs1800872 and rs3024496, with *IL10RA*, *IL10RB*, *STAT3* and *HO1* in pediatric inflammatory bowel disease. Graphic depiction of epistatic interaction of the *IL-10* SNPs rs3024496 and rs1800872 with the *IL10RA* rs3135932. 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 *IL-10* rs3024496, *IL-10* rs1800872, and *IL10RA* rs3135932 respectively. The contribution of a single SNP in an interaction model could be either a or d (a1 and d1 for SNP #1, a2 and d2 for SNP #2, and a3 and d3 for SNP #3). The interaction models for two and three SNPs are 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.

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 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

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: rs1800872 and rs3024496 aa: *P* = 0.0002; Bp = 0.003	aaa	*P* = 0.003; Bp = 0.099	*P* = 0.080; Bp = 1	*P* = 0.175; Bp = 1	*P* = 0.216; Bp = 1
	aad	*P* = 0.130; Bp = 1	*P* = 0.340; Bp = 1	*P* = 0.920; Bp = 1	*P* = 0.140; 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, aaa 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* rs3024498, *P* = 0.616; Bp = 21	*IL-10* rs3024496, *P* = 0.022; Bp = 0.0226	*IL10RA* rs3135932, *P* = 0.16; Bp = 1	*STAT3* rs744166, *P* = 0.352; Bp = 1
aa *IL10RB* rs2834167 and *HO1* rs2071746	aaa	*P* = 0.029; Bp = 1	*P* = 0.545; Bp = 1	*P* = 0.255; Bp = 1	*P* = 0.023; Bp = 0.820	*P* = 0.049; Bp = 1
	aad	*P* = 0.127; Bp = 1	*P* = 0.240; Bp = 1	*P* = 0.928; Bp = 1	*P* = 0.193; Bp = 1	*P* = 0.554; Bp = 1
ad *IL10RB* rs2834167 and *HO1* rs2071746	ada	*P* = 0.394; Bp = 1	*P* = 0.824; Bp = 1	*P* = 0.242; Bp = 1	*P* = 0.444; Bp = 1	*P* = 0.436; Bp = 1
	add	*P* = 0.976; Bp = 1	*P* = 0.406; Bp = 1	*P* = 0.327; Bp = 1	*P* = 0.644; Bp = 1	*P* = 0.649; Bp = 1
da *IL10RB* rs2834167 and *HO1* rs2071746	daa	*P* = 0.00015; Bp = 0.005	*P* = 0.489; Bp = 1	*P* = 0.047; Bp = 1	*P* = 0.008; Bp = 0.287	*P* = 0.014; Bp = 0.491
	dad	*P* = 0.072; Bp = 1	*P* = 0.140; Bp = 1	*P* = 0.388; Bp = 1	*P* = 0.205; Bp = 1	*P* = 0.977; Bp = 1
dd *IL10RB* rs2834167 and *HO1* rs2071746	dda	*P* = 0.199; Bp = 1	*P* = 0.808; Bp = 1	*P* = 0.568; Bp = 1	*P* = 0.766; Bp = 1	*P* = 0.4731; Bp = 1
	ddd	*P* = 0.694; Bp = 1	*P* = 0.958; Bp = 1	*P* = 0.007; Bp = 0.242	*P* = 0.181; Bp = 1	*P* = 0.057; Bp = 1

was observed on the interaction of these two genes, *IL10RB* and *HO1*, with the *IL-10* gene rs1800872 (*P* = 0.00015, Bp = 0.005). The model of the three gene interaction $d_{IL10RB}a_{HO1}a_{IL\text{-}10}$ is depicted in Figure 4. Moreover, weak interactions were also observed on the interaction of these two genes with *IL10RA* rs3135932 (aaa *P* = 0.023, Bp = 0.820; daa *P* = 0.008, Bp = 0.287) and with *STAT3* rs744166 (daa *P* = 0.014, Bp = 0.491). As shown in Figure 2B, the interaction model $d_{IL10RB}a_{HO1}$ for *IL-10* and *HO1* is significant (Bp = 0.039). The three SNPs interaction analysis indicated that this model is responsible for the interaction of *IL10RB* and *HO1* with the other genes, *IL-10*, *IL10RA*, and *STAT3*, in the IL-10 pathway, as $d_{IL10RB}a_{HO1}a_{IL\text{-}10}$, $d_{IL10RB}a_{HO1}a_{IL10RA}$, and $d_{IL10RB}a_{HO1}a_{STAT3}$, respectively.

The findings indicate that the interaction between *IL10RB* and *HO1* may enhance their action with *IL-10* and *IL10RA* and elevate the pathway activity in pediatric IBD. Considering that HO1 is an important mediator of the anti-inflammatory effect of IL-10 and several mutations in *IL10RB* are associated with pediatric IBD, these gene-gene interactions may affect 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

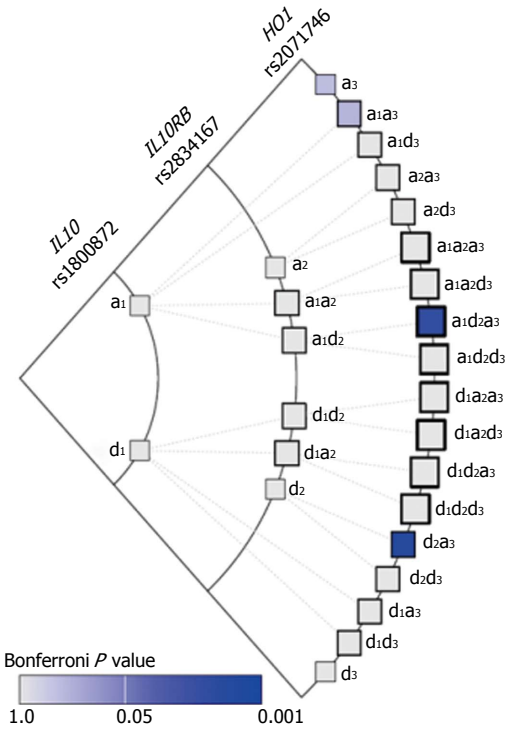

Figure 4 Epistatic interaction of *IL10RB* and *HO1* with *IL-10* (three single nucleotide polymorphisms), *IL10RA* and *STAT3* in pediatric inflammatory bowel disease. Graphic depiction of epistatic interaction of the *IL-10* rs1800872, *IL10RB* rs2834167 and *HO1* rs2071746. Of all the interaction models analyzed, a three SNP interaction (a1d2a3) for *IL-10* rs1800872, *IL10RB* rs2834167 and *HO1* rs2071746 is significant (Bp = 0.005). The 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, denote interaction models, additive and dominant respectively; Numbers 1, 2, and 3 are for *IL-10* rs1800872, *IL10RB* rs2834167, and *HO1* rs2071746, respectively. The contribution of a single SNP in an interaction model could be either a or d: a1 and d1 for SNP #1, a2 and d2 for SNP #2, and a3 and d3 for SNP #3. The interaction models for two and three SNPs are the combination of two and three SNPs (*e.g.*, a1a2 for SNPs 1 and 2, and a1a2a3 for SNPs 1, 2, and 3). Bp: Bonferroni *P* value; SNP: Single nucleotide polymorphism.

with adult IBD by GWASs. The most studied *IL-10* SNP, -1082A>G (rs1800896), is thought of as having potential for gene transcription regulation[7,14-16,37]. The *IL-10* SNP rs3024496 is shown to be related to inflammatory response with increased levels of IgE to dust mite[38], or decreased production of IL-10 by peripheral blood leukocytes[39], and with prostate[40] and colorectal[41] cancer, but has not been shown to be associated with IBD. The *IL-10* rs1800872 is associated with IBD[37] and also with increased serum IL-10 levels in CD[42,43], as well as with irritable bowel syndrome[44] and cancer susceptibility[42,43,45,46]. 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[44,61]. HO1 is a potent enzyme of anti-inflammation, and has a very important function of the IL-10 pathway in controlling inflammation[14].

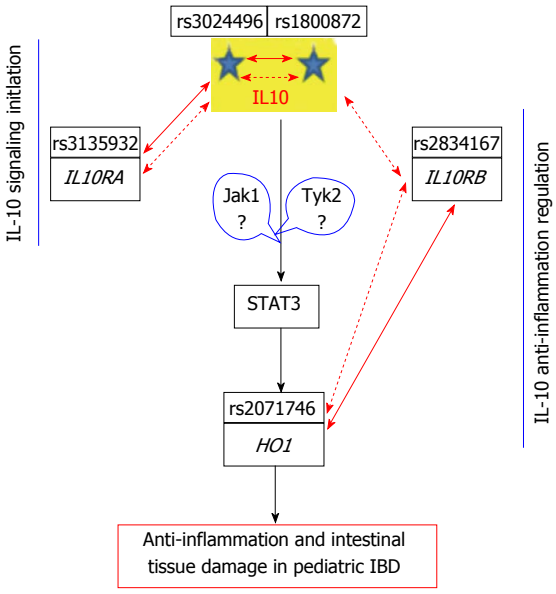

Figure 5 Summary of gene function and epistatic interaction of the IL-10/STAT3 signaling pathway in pediatric inflammatory bowel disease. In the IL-10 pathway genes, genetic variations in *IL-10* and *STAT3* are associated with adult IBD, and genetic mutations identified from *IL10RA* and *IL10RB* are associated with pediatric/very early onset inflammatory bowel disease (published data). In this study, we found SNP-SNP epistatic interaction within the *IL-10* gene, between *IL10* and *IL10RA* genes, and between *IL10RB* and *HO1* genes. We also found epistatic interaction among three SNPs between the two genes *IL-10* (two SNPs) and *IL10RA*, and among the three genes *IL-10*, *IL10RB* and *HO1*. Bp: Bonferroni *P* value.

Although STAT3 is a critical component of the IL-10/STAT3 pathway[23,24], 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[3,4], 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[11,62], and SNPs in *IL-10* and *IL-10 receptors* have been associated with VEO IBD[7-12]. 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[16,23]. 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[63].

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-inflammation 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.

ACKNOWLEDGMENTS

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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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