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Association of the haem oxygenase-1 gene with inflammatory bowel disease

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Summary

The anti-inflammatory genes, haem oxygenase 1 (HO-1, HMOX1) rs2071746 (unrestricted model: $p = 9.07 \times 10^{-4}$; recessive model: $p = 4.99 \times 10^{-4}$; multiplicative model: $p = 0.0009$; and additive model: $p = 1.87 \times 10^{-4}$) and interleukin-10 (IL-10) rs1800872 (dominant model: $p = 0.0277$) have been associated with paediatric inflammatory bowel disease. The present family-based case-trio study (n = 52) examined HO-1 gene expression in the presence of proinflammatory lipopolysaccharide and tumour necrosis factor-alpha in four B lymphocyte cell lines established from children with inflammatory bowel disease and demonstrated that mutations in IL-10 and IL-10 receptor B reduced HO-1 messenger RNA expression. This observation supports our hypothesis that HO-1 is regulated by the IL-10/STAT3 pathway and that both genes (IL10 and STAT3) could be involved in the pathogenesis of inflammatory bowel disease. We also compared HO-1 expression in diseased intestinal tissues with adjacent normal tissues from adults with inflammatory bowel disease. Of the 17 Crohn's disease patients, HO-1 expression in diseased tissues was downregulated in 9 patients (53%) and of the 10 ulcerative colitis patients HO-1 was downregulated in 7 patients (70%), compared with adjacent normal tissues. The downregulation of HO-1 gene expression may lower anti-inflammatory effects and worsen tissue injury in affected areas by inflammatory bowel disease.

Key words: HO-1, paediatric IBD, genetic association, anti-inflammation, case-trio study

Author contributions
ZL and HH contributed to this work equally.
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Introduction

Haem oxygenase 1 (HO-1) catalyses the degradation of haem to biliverdin and carbon monoxide. HO-1 plays an important role in regulating intestinal homeostasis through anti-apoptosis and angiogenesis in cancer [1]. HO-1 also has anti-inflammatory properties and thus it has been suggested that it may have a role in the recovery from inflammatory bowel disease (IBD). However, in previous genetic association studies, HO-1 single nucleotide polymorphisms (SNPs) have not been shown to associate with adult IBD [2, 3] and paediatric Crohn's disease [2] based on the (GT)n dinucleotide repeat within the HO-1 promoter region, or adult IBD based on the SNP rs2071746 upstream of the HO-1 gene [4]. Furthermore, in our case-control study (submitted for publication) we did not observe association of the HO-1 rs2071746 with paediatric IBD, which is consistent with the available literature.

The HO-1 gene is known to be regulated by the interleukin-10 / signal transducer and activator of transcription 3 (IL-10/STAT3) signalling pathway [5, 6,]. Of interest, IL-10 and STAT3 SNPs have been shown to associate with IBD [7–10]. Recently, mutations in IL-10 and IL-10 receptors (IL-10RA and IL-10RB) were shown to be associated with paediatric IBD [7, 11–14], and our recent case-control study (submitted) showed association of IL-10 SNP rs3024496 with paediatric IBD ($p = 0.022$), as well as an epistatic interaction (dominant-additive $p = 0.0018$) of IL-10RB (rs2834167) and HO-1 (rs2071746).

In the present family based case-trio study, we hypothesised that HO-1 SNPs would be associated with paediatric IBD. We also studied HO-1 gene expression in B lymphocyte cell lines from paediatric IBD patients carrying IBD-associated gene variants and their response to the inflammatory inducers, lipopolysaccharide (LPS) and tumour necrosis factor alpha (TNFα), as well as HO-1 gene expression in diseased and nondiseased tissues from adult IBD patients.

Materials and methods

Study samples

For the case-trio study, 52 trio families (51 triad and 1 dyad families) were studied. Genomic DNA samples were obtained from the Crohn's and Colitis Foundation of America (CCFA) DNA Databank. CCFA genomic DNAs were collected from samples originating from: the University of North Carolina at Chapel Hill; University of Chicago, IL; Cedars-Sinai Hospital, CA; Massachusetts General Hospital; University of Pittsburgh, PA; and Mount Sinai Hos-

pital, NY. Informed written consent was obtained from parents or guardians of the patients. The genomic DNA samples were stored at -80ºC until use.
The study protocol was approved by the ethics committee of Penn State University College of Medicine Institutional Review Board (IRB HY98-057) [15].

Diseased tissues from adult IBD patients
Intestinal tissues were obtained from 17 Crohn's disease and 10 ulcerative colitis patients undergoing surgery at the Penn State Hershey Medical Center. All use of human tissues was approved by the Human Subjects Protection Office of The Pennsylvania State University College of Medicine, and was undertaken with the understanding and written consent of each subject. Macroscopically normal areas of intestine and areas of intestine with obvious disease were classified by a pathologist [15]. Nondiseased tissue samples were excised from noninflamed, macroscopically normal tissue that was adjacent to, but a minimum of 5 cm away from, the diseased tissue. The intestinal tissues were immediately submerged in RNAlater Solution (Ambon, CA, USA) and stored at 4°C overnight. Tissues were stored frozen at -70°C until total RNA extraction.

SNP selection criteria for IL-10, IL-10RA, IL-10RB, STAT3 and HO-1
Seven SNPs from these five genes were studied, including: rs1800872 (C-592>A), rs3024498 and rs3024496 from IL-10 [16]; rs3135932 from IL-10RA [11]; rs2834167 from IL-10RB [11, 16]; rs744166 from STAT3; and rs2071746 from HO-1 [4, 17]. The criteria for SNP selection and a summary of these SNPs, including genetic variation, chromosomal position, gene location, and disease implication were described in our previous paper [18]. Briefly, the criteria for SNP selection were based on: (a) 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 have a known functional role in inflammatory processes; (b) the gene location, either within the coding region that changes the encoded amino acid, or at 5′ upstream or 3′ untranslated region (UTR) potentially affecting RNA transcription, RNA stability, or protein translation; and (c) being polymorphic in the study samples as tested in our preliminary study and having minor allele frequency (MAF) information in existing databases.

B cell line culture and treatment with LPS and TNFα
B cell lines were established from blood samples from IBD patients by means of infection with Epstein–Barr virus (EBV) [15]. Informed parental or guardian written consent documentation was obtained for all patient samples received from the Pennsylvania State University IBD Biobank and the Hershey Medical Centre.
Cell lines were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum and incubated at 37°C in an atmosphere of 5% CO_2 [15]. The cells were treated with LPS (1 μg/μl) and TNFα (0.1 μg/μl) for 24 hours. The cells were collected and cell pellets were stored at -80ºC until used for RNA isolation. All the experiments were repeated three times.

Genotype analysis
The genotype of all seven SNPs was determined with polymerase chain-reaction (PCR)-based RFLP/cRFLP as described previously (RFLP: restriction fragment length polymorphism) [19]. The methods for PCR amplification, gel electrophoresis, and genotype score were as previously described [18]. In brief, 100 ng DNAs were used for PCR in a 30 μl reaction volume. The PCR cycling profile was 95ºC for 2 min, 5 cycles at 95ºC for 30 sec, 50ºC for 1 min, and 72ºC for 1 min, then 30 cycles at 95ºC for 30 sec, 58ºC for 1 min, and 72ºC for 1 min, followed by a final extension step at 72ºC for 4 min. PCR products were digested with an appropriate restriction enzyme, separated with polyacrylamide gel electrophoresis (8%), and the genotypes scored according to the gel pattern of digested PCR products.

Case-trio analysis
Suppose the case-trio study involves n triad families (each containing one affected offspring and two parents, the parents either affected or unaffected), m dyad families (each containing one affected offspring and one parent, either father or mother, with the parent affected or unaffected), and s affected singleton monads (each containing only one affected offspring). At each SNP location, suppose there are two alleles A and a, with allele frequencies p and q, respectively. Let

$$\psi_1 = \frac{prob(disease|Aa)}{prob(disease|aa)}$$

and

$$\psi_2 = \frac{prob(disease|AA)}{prob(disease|aa)}$$

be the relative risk parameters,

$$R = p^2\psi_2 + 2pq\psi_1 + q^2.$$

For the triad families, table 1 summarises the conditional probabilities of parental genotype and offspring genotype given that the offspring is affected. The likelihood function corresponding to the triad families is given by:

$$\log L_{Triad} =$$

$$\left[4n_1 + 3n_2 + 3n_3 + 2n_4 + 2n_5 + 2n_6 + 2n_7 + n_8 + n_9\right]\log p$$

$$+\left[n_2 + n_3 + 2n_4 + 2n_5 + 2n_6 + 2n_7 + 3n_8 + 3n_9 + 4n_{10}\right]\log q$$

$$+\left[n_3 + n_4 + n_6 + n_8\right]\log\psi_1 + \left[n_1 + n_2 + n_5\right]\log\psi_2 - n\log R$$

(1)

For the dyad families, table 2 summarises the conditional probabilities of parental genotype and offspring genotype given that the offspring is affected. The likelihood function corresponding to the dyad families is given by:

$$\log L_{dyad} =$$

$$\left[3m_1 + 2m_2 + 2m_3 + m_4 + m_5 + m_6\right]\log p$$

$$+\left[m_2 + m_3 + m_4 + 2m_5 + 2m_6 + 3m_7\right]\log q$$

$$+\left[m_2 + m_4 + m_6\right]\log\psi_1 + \left[m_1 + m_3\right]\log\psi_2 - m\log R$$

(2)

For the singleton monads, suppose the number of genotype *AA, Aa, aa* are s_1, s_2, s_3, respectively. The conditional probabilities of the genotypes given IBD affection are

$$prob(AA|disease) = p^2\psi_2 \,/\, R,$$

$$prob(Aa|disease) = 2pq\psi_1 \,/\, R,$$

and

$$prob(aa|disease) = q^2 \,/\, R.$$

The likelihood function corresponding to the singleton monads is given by:

$$\log L_{monad} = \left[2s_1 + s_2\right]\log p + \left[s_2 + 2s_3\right]\log q + s_2\log\psi_1 + s_1\log\psi_2 - s\log R \quad (3)$$

The final likelihood function is the summation of two parts corresponding to the triad families and the dyad families, respectively:

$$\log L = \log L_{Triad} + \log L_{dyad} + \log L_{monad} \quad (4)$$

Using the likelihood ratio test, we can test the association between the genotype at each SNP location and the risk of IBD, with the following 5 types of hypotheses tested against the null hypothesis H_0: $\psi_1 = 1$ *and* $\psi_2 = 1$:

1. Unrestricted model (no restriction on the risk parameters ψ_1 and ψ_2)
2. Dominant model with alternative hypothesis H_a: $\psi_1 = \psi_2$
3. Recessive model with alternative hypothesis H_a: $\psi_1 = 1$
4. Multiplicative model with alternative hypothesis H_a: $\psi_2 = \psi_1^2$
5. Additive model with alternative hypothesis H_a: $\psi_2 = 2\psi_1 - 1$

Reverse transciptase PCR

Total RNA was isolated from B cells and intestine tissues with a Qiagen RNA isolation Mini kit. First strand copy DNA was synthesised with Invitrogen Superscript III Kit, according to the manufacturer's manual. The reverse transcriptase (RT)-PCR primers for HO-1 are given in table 3. PCR amplification was carried out at 95ºC for 2 min, 30 cycles at 95ºC for 30 sec, 58ºC for 1 min, and 72ºC for 1 min, followed by a final extension step at 72ºC for 4 min. PCR products were separated by 1.5% agarose gel electrophoresis and photographed under ultraviolet light.

The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene, and the gene relative mRNA expression level of the gene under study was noted as the ratio of the RT-PCR products of each target gene to GAPDH [20].

Table 1: Conditional probabilities of parental genotype and offspring genotype given that offspring is affected (for the triad families).

Parental genotype	Offspring genotype	Conditional probability	Number of obs.
AA × AA	AA	$p^4\psi_2 \,/\, R$	n_1
AA × Aa	AA	$2p^3q\psi_2 \,/\, R$	n_2
	Aa	$2p^3q\psi_1 \,/\, R$	n_3
AA × aa	Aa	$2p^2q^2\psi_1 \,/\, R$	n_4
Aa × Aa	AA	$p^2q^2\psi_2 \,/\, R$	n_5
	Aa	$2p^2q^2\psi_1 \,/\, R$	n_6
	aa	$p^2q^2 \,/\, R$	n_7
Aa × aa	Aa	$2pq^3\psi_1 \,/\, R$	n_8
	aa	$2pq^3 \,/\, R$	n_9
aa × aa	aa	$q^4 \,/\, R$	n_{10}

Table 2: Conditional probabilities of parental genotype and the offspring genotype given that the offspring is affected (for the dyad families).

Parental genotype	Offspring genotype	Conditional probability	Number of obs.
AA	AA	$p^3\psi_2 \,/\, R$	m_1
	Aa	$p^2q\psi_1 \,/\, R$	m_2
Aa	AA	$p^2q\psi_2 \,/\, R$	m_3
	Aa	$pq\psi_1 \,/\, R$	m_4
	aa	$pq^2 \,/\, R$	m_5
aa	Aa	$pq^2\psi_1 \,/\, R$	m_6
	aa	$q^3 \,/\, R$	m_7

Gel image analysis with Quantity One

Digital gel images were converted to TIF format and then analysed using Bio-Rad Quantity One software v4.5.0. The trace quantity was normalised against the reference gene, GAPDH. The ratio of HO-1 to GAPDH was used as relative gene expression level of HO-1 for the study.

Statistical analysis

Pearson's chi-squared tests with two degrees of freedom for allelic association were performed using Haploview software to analyse genetic patterns of linkage disequilibrium. We also calculated the genotype-based odds ratio (OR) using Fisher's contingency tables and tested association similarly to analyse genetic association. The difference was considered as significant when $p < 0.05$.

Results

Association of HO-1 gene with paediatric inflammatory bowel disease

The genotype of the HO-1 rs2071746, and of the other six SNPs of IL-10 (rs1800872, rs3024498, and rs3024496), IL-10RA (rs3135932), IL-10RB (rs2834167), and STAT3 (rs744166) were analysed for 52 trio families with one affected offspring in each family.

We used a likelihood approach to analyse the genetic association with paediatric IBD using data from a case-trio study [21]. As shown in table 4, HO-1 rs2071746 was highly associated with paediatric IBD in different models: $p = 0.0009$ in an unrestricted model, $p = 0.0005$ in a recessive model, $p = 0.0010$ in a multiplicative model, and $p = 0.0002$ in an additive model.

IL10 rs1800872 was also found to be associated with paediatric IBD ($p = 0.0277$ in a dominant model).

The other genes, IL10-RA, IL-10RB, and STAT3, did not show a significant association with paediatric IBD, although we observed a likelihood ratio of 3.57 in an unrestricted model and 3.21 in a dominant model for IL-10RB, and a likelihood ratio of 3.82 in an unrestricted model, a likelihood ratio of 3.77 in a multiplicative model and a likelihood ratio of 3.70 in an additive model for STAT3. A full list of the maximum likelihood estimations of allele frequencies and risk parameters, and likelihood ratio test statistics and p-values for all models 1–5 are shown in table 4.

HO-1 gene expression in B cell lines from paediatric IBD patients

We aimed to study the potential function of disease-associated alleles in HO-1 gene expression and their effect on cellular response to inflammation stimulated by LPS or TNFα compared with untreated cells. Four paediatric IBD B cell lines (genotypes of the five studied genes indicated in figure 1) were treated with LPS (1 μg/μl) or TNFα (0.1 μg/μl). The results are shown in fig 1.

Effect of HO-1 rs207146 on HO-1 gene expression

Of the four cell lines, only the 777-27-1 cell line carried a homologous wild type HO-1 genotype, the other three cell lines were heterozygous for rs207146. Compared with the HO-1 expression in control cells, the relative HO-1 expression varied among cell lines, with 777-27-1 expression be-

Figure 1: Effect of lipopolysaccharide (LPS) and tumour necrosis factor-alpha (TNFα) on haem oxidase-1 (HO-1) expression in B cell lines generated from blood samples from paediatric inflammatory bowel disease patients with genetic variations in genes interleukin-10 (IL-10), IL-10 receptor B (IL-10RB) and HO-1. For C1of 777-27, n = 2.Genotype of IL10 rs1800872 (G/T, Mut T) was GG for 888-58-1, GT for 888-118-1, GT 95-09-1, and GG for 777-27-1. Genotype of IL10 rs3024496 (A/G, Mut G) was AA for 888-58-1, AA for 888-118-1, GG 95-09-1, and AG for 777-27-1. Genotype of IL10RB rs2834167 (A/G, Mut G) was AA for 888-58-1, AG for 888-118-1, GG 95-09-1, and AG for 777-27-1. Genotype of HO-1 rs2071746 (A/T, Mut T) was AT for 888-58-1, AT for 888-118-1, AT 95-09-1, and AA for 777-27-1.Bar graphs show gene expression as a ratio to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in four B cell lines (888-58-1, 888-118-1, 95-09-1, and 777-27-1). Cells treated with TNFα, LPS, and control (without treatment) were indicated within the figure as TNF1, LPS1, and C1, respectively. All experiments were repeated three times.

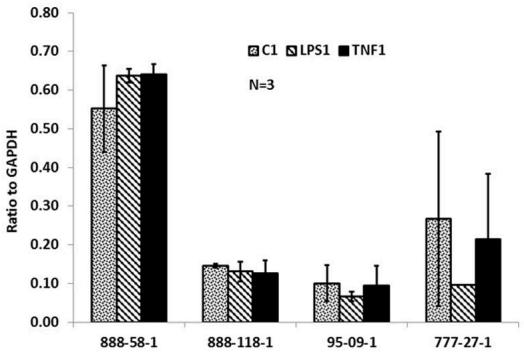


Table 3: PCR primers for seven SNPs from genes IL-10, IL-10RA, IL-10RB, STAT3 and HO-1.

Gene	SNP ID	Variant	PCR amplication	
			Primers*	Product (bp)
IL-10	rs1800872	G>T	IL2f: 5'-AAC TTA GGC AGT CAC CTT AGG-3' IL2r: 5'-CA TCC TGT GAC CCC TCC AGT-3'	149
	rs3024498	T>C	IL5f: 5'-GCT CCt TGG TTT CTC TTC CTA AG-3' IL5r: 5'-AG AAG CTT CCA TTC CAA GCC TGA-3'	137
	rs3024496	A>G	IL4f: 5'-GTA TCA GAG G TAA TAA ATA TTC cAT-3' IL4r: 5'-TA GAA GCA TAC ATG ACA ATG AAG-3'	178
IL-10RA	rs3135932	A>G	RA3f: 5'-CCC GCA AAT GAC ACA TAT GgA-3' RA3r: 5'-AGT TCC CAA TGG CAC ACA AGG-3'	172
IL-10RB	rs2834167	A>G	RB3f: 5'-GCC ATA GAG GAG AAC CAA GTG-3' RB3r: 5'-G CTG TGA AAG TCA GGT TCT CTT-3'	206
STAT3	rs744166	A>G	ST2f: 5'-CAG GAG TGC CAA CAT TGA GAG-3' ST2r: 5'-G TAA TGT CTT GAG GGA ATC GAG-3'	106
HO-1	rs2071746	A>T	HO4f: 5'-TCA GCA GAG GAT TCC AGC AGG TG-3' HO4r: 5'-AGG CAG CGC TGC TCA GAG CAC-3'	110

HO-1 = haem oxidase-1; IL-10 = interleukin-10; IL-10R = interlukin-10 receptor; PCR = polymerase chain-reaction; SNP = single nucleotide polymorphism; STAT3 = signal transducer and activator of transcription 3 * Lower case letter: mismatched nucleotide.

ing at a middle level, much lower than the 888-58-1 cell line. Clearly the HO-1 rs2071746 has no significant effect on HO-1 gene expression in B cells.

IL10 and IL10RB variants and HO-1 gene expression

The highest HO-1 gene expression level was observed for the cell line 888-58-1, the only cell line without any mutation in IL-10 and IL-10RB (both homozygous for the wild type allele). In contrast, the cell lines 95-09-1 (heterozygous at IL10 rs1800872, homozygous at both IL10 rs3024496 and IL10RB rs2834167; 888-118-1 (heterozygous at both IL10 rs1800872 and IL10RB rs2834167); and 777-27-1 (heterozygous at both IL10 rs3024496 and IL10RB rs2834167) exhibited much lower HO-1 expression than the cell line 888-58-1 (fig 1). These results support our speculation that IL10 and IL10RB may have a functional role in the regulation of HO-1 gene expression, although the number of the cell lines analysed is small to reach a firm conclusion.

Response of mutated alleles in IL-10, IL-10RB and HO-1 to LPS and TNFα stimulation

To study the regulation of HO-1 expression in response to inflammation via the IL10/STAT3 pathway, B cells were treated with LPS and TNFα. LPS can induce inflammation, and TNFα can mediate inflammation via its primary role in the regulation of immune cells.

Overall changes in HO-1 expression level in response to LPS and TNFα were not significant (fig. 1). A trend of an increase of HO-1 gene expression was observed in the 888-58-1 B cell line that has no mutated alleles in IL-10 and IL-10RB; however in the other three cell lines with mutations in IL-10 and/or IL-10RB, HO-1 expression was reduced or showed no clear change in the presence of LPS

Table 4: Parameter estimation and hypothesis testing summary.

SNP	Model	MLE			LRT	p-value
rs1800872 (IL-10)	Unr	0.7643	7408.7300	8321.8940	4.9623	0.0836
	Dom	0.7729	1363.6760	1363.6760	4.8478	**0.0277**
	Rec	0.7866	1.0000	1.1300	0.0949	0.7580
	Mult	0.7634	1.4460	2.0910	1.0767	0.2994
	Add	0.7223	1218.2120	2435.4250	2.7805	0.0954
	Null	0.7949	1.0000	1.0000	N/A	N/A
rs3024498 (IL-10)	Unr	0.8202	0.8037	0.4003	3.1121	0.2110
	Dom	0.7703	0.8726	0.8726	0.0400	0.8414
	Rec	0.8151	1.0000	0.5050	3.0110	0.0827
	Mult	0.8202	0.5636	0.3177	2.6809	0.1016
	Add	0.8235	0.6682	0.3364	3.0314	0.0817
	Null	0.7672	1.0000	1.0000	N/A	N/A
rs3024496 (IL-10)	Unr	0.5761	0.8208	0.4165	2.8984	0.2348
	Dom	0.5314	0.8441	0.8441	0.1770	0.6739
	Rec	0.5626	1.0000	0.5142	2.6513	0.1035
	Mult	0.5761	0.6556	0.4298	2.1585	0.1418
	Add	0.5842	0.6864	0.3729	2.6565	0.1031
	Null	0.5204	1.0000	1.0000	N/A	N/A
rs3135932 (IL-10RA)	Unr	0.8352	0.6908	0.6818	0.1961	0.9066
	Dom	0.8344	0.6912	0.6912	0.1951	0.6587
	Rec	0.8296	1.0000	0.9912	0.0004	0.9831
	Mult	0.8352	0.9211	0.8484	0.0460	0.8302
	Add	0.8345	0.9329	0.8657	0.0412	0.8392
	Null	0.8290	1.0000	1.0000	N/A	N/A
rs2834167 (IL-10RB)	Unr	0.7973	1152.0260	1442.2480	3.5749	0.1674
	Dom	0.8123	2472.0810	2472.0810	3.2078	0.0733
	Rec	0.8120	1.0000	1.2920	0.3607	0.5481
	Mult	0.7955	1.5429	2.3805	1.2379	0.2659
	Add	0.7665	733.4084	1465.8170	2.4439	0.1180
	Null	0.8280	1.0000	1.0000	N/A	N/A
rs744166 (STAT3)	Unr	0.4607	0.5854	0.2980	3.8179	0.1482
	Dom	0.4305	0.5842	0.5842	2.0819	0.1491
	Rec	0.4156	1.0000	0.5206	1.6602	0.1976
	Mult	0.4607	0.5599	0.3135	3.7708	0.0522
	Add	0.4541	0.6546	0.3092	3.7030	0.0543
	Null	0.3874	1.0000	1.0000	N/A	N/A
rs2071746 (HO-1)	Unr	0.6559	0.5808	0.1179	14.0109	**0.0009**
	Dom	0.5641	0.6199	0.6199	1.4015	**0.2365**
	Rec	0.6192	1.0000	0.2144	12.1203	**0.0005**
	Mult	0.6559	0.3823	0.1462	10.8485	**0.0010**
	Add	0.6586	0.5567	0.1135	13.9988	**0.0002**
	Null	0.5333	1.0000	1.0000	N/A	N/A

Add = additive; Dom = dominant; HO-1 = haem oxidase-1; IL-10 = interleukin-10; IL-10R = interlukin-10 receptor; LRT = likelihood ratio test; MLE = maximum likelihood estimation; Mult = multiplicative; Rec = recessive; STAT3 = signal transducer and activator of transcription 3; Unr = unrestricted

and TNFα. Although changes in the expression of the wild type for HO-1 and IL-10 cell line were small, the cell lines with mutation in IL-10 and IL-10RB had a negative or no effect on HO-1 expression in response to inflammation induced by LPS and TNFα.

HO-1 expression in diseased intestinal tissues from adult IBD patients

We further studied HO-1 expression and its association with pro- or anti-inflammatory activity in IBD affected intestinal tissues. As Crohn's disease can occur in both the small intestine and large intestine, but ulcerative colitis affects only the large intestine, the tissues selected from the 27 adult IBD patients included three IBD subtypes: 17 Crohn's disease tissues of which 12 were located in the small intestine and 5 in the large intestine, and 10 ulcerative colitis tissues located in the large intestine. We studied HO-1 expression and its association with inflammatory activity by comparing diseased tissues to nondiseased (noninflamed) surgical tissue from the same patients. To examine differences in gene expression and inflammation between Crohn's disease and ulcerative colitis we compared 12 Crohn's disease tissues from both the small and large intestine with 10 ulcerative colitis tissues from the large intestine. To examine gene expression between disease locations, we compared 12 Crohn's disease tissues from the small intestine with 5 Crohn's disease tissues from the large intestine. The results are shown in fig. 2.

The HO-1 expression level varied greatly among the 27 IBD patients. The variation may be due to many different factors, such as disease severity, genetic variation, clinical treatment, drug therapy, etc. To study the anti-inflammatory function of the IL-10 pathway via HO regulation, we compared the HO-1 fold-change mRNA expression of diseased with nondiseased tissues. The results are given in fig. 2 and table 5.

In the 17 patients with Crohn's disease (n = 12 in the small intestine and n = 5 in the large intestine), HO-1 expression in diseased tissue was downregulated in 9 patients (53%, varying from 0.06- to 0.81-fold) and upregulated in 7 patients (41%, varying from 1.39- to 9.45-fold); in one (6%) there was no clear change (1.14-fold) (fig. 2A and table 5).

In the 10 patients with ulcerative colitis (in the large intestine), HO-1 expression was downregulated in 7 patients (70%, varying from 0.23- to 0.81-fold), and upregulated in only 2 (20%, 1.7-1 and 2.02-fold, respectively); in one there was no change (10%, 0.99-fold) (fig. 2B and table 5). This similarity between Crohn's disease and ulcerative colitis suggests a common regulation of HO-1 expression in the human large intestine.

In 15 patients with IBD affecting the large intestine, HO-1 was downregulated in 10 (67%) and upregulated in 3 (20%); there was no clear change in 2 (13%). The differential expression of HO-1 in this group was similar to that in ulcerative colitis, not Crohn's disease, raising a question of tissue-specific regulation of HO-1 expression in the small intestine and large intestine responses to intestinal inflammation.

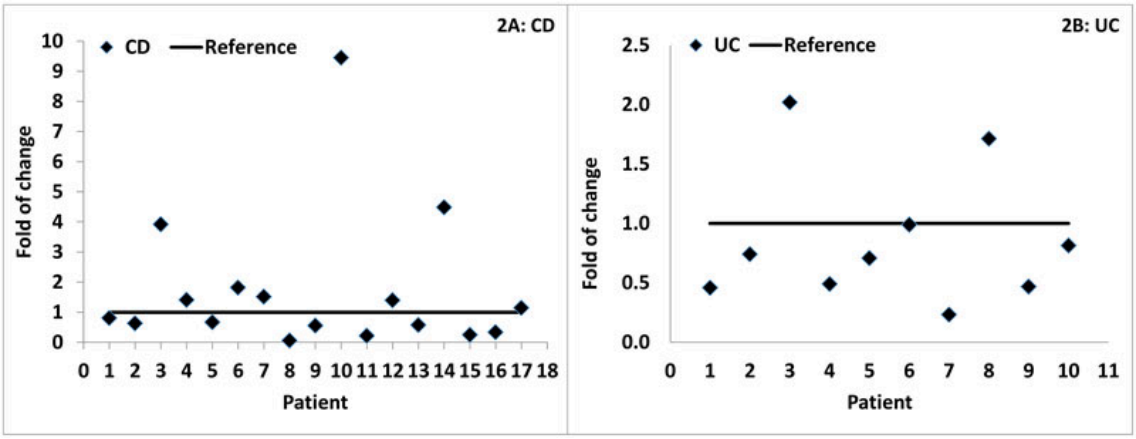
Discussion

Association of HO-1 gene with paediatric IBD

IBD is a chronic and recurrent intestinal inflammatory disorder. HO-1 has been shown to be a protective and anti-inflammatory protein. A role of the HO-1 gene in IBD has been demonstrated in an IBD animal model [22, 23]. However, a genetic association of HO-1 with adult IBD and paediatric IBD has not been observed. In various association studies [2], in which HO-1 (GT)n variants were used as markers, no association was found with IBD (Crohn's disease n = 179, ulcerative colitis n = 110, control n = 56) [3], with Crohn's disease (n = 504, controls n = 370) [2], or with paediatric Crohn's disease (n = 119 [2]). A genetic association study with another HO-1 SNP rs2071746 upstream of the gene also found no association with adult IBD (Danish cohort 336 Crohn's, 498 ulcerative colitis, 779 controls) [4]. Moreover, in a case-control study we did not observe an association of HO-1 rs2071746 with paediatric IBD.

However, in the present case-trio study we showed an association of HO-1 with paediatric IBD. Utilisation of case-trio data from triad and dyad families benefits genetic association studies by reducing both false negative and false positive rates [21]. The integration of additional genotypic information from the parent(s) has the potential to increase

Figure 2: Comparison of haem oxidase-1 (HO-1) expression of diseased tissues with normal tissues in adult inflammatory bowel disease patients. Study samples were surgical tissues from 17 Crohn's disease (CD) patients (small intestine n = 12; large intestine n = 5) (fig 2A) and 10 ulcerative colitis (UC) patients (fig. 2B). Gene expression fold-change (normalised to glyceraldehyde 3-phosphate dehydrogenase) in diseased tissue/adjacent normal tissue All experiments were repeated three times.



the statistical power. Also, with the genotypes of the parent(s) included in the likelihood model, the analysis result is less affected by population stratification and more robust in terms of false positive rates. Though our case-trio study does not contain singleton monads, the likelihood approach is general enough to be applied to a traditional case-control study where only the genotypic information of the child is available.

HO-1 gene expression and function in intestinal inflammation

HO-1 and its enzymatic product carbon monoxide regulate both acute and chronic inflammation and ameliorate intestinal inflammation by promoting bacterial clearance. An early investigation of HO-1 expression in clinical and experimental colitis demonstrated a scattered expression of HO-1 in the epithelium of severely inflamed colonic mucosa of patients with IBD compared with control specimens from patients with, for example, diverticulitis, suggesting dysregulated expression in IBD. Therefore, a protective role of HO-1 was suggested, if it is induced before the onset of inflammation [24].

HO-1 is a protein with a wide range of anti-inflammatory and immune regulatory functions, and with significant protective roles in various T cell-mediated diseases. Cellular localisation of HO-1 gene expression indicated that in patients with active ulcerative colitis, HO-1 staining is positive in mononuclear cells in the submucosa of the colon but negligible in the epithelial cells [25].

In dextran sulphate sodium (DSS)-induced colitis mice, DSS markedly induced HO-1 expression in the colon epithelium. Inhibition of HO-1 expression aggravated the colitis. The anti-inflammatory mechanism involved the induction of regulatory T cells, IL-17 and apoptosis [21, 23, 26]. In DSS-induced mouse colitis, the anti-inflammatory effects of grapefruit-derived nanovesicles were mediated by upregulation of HO-1 and inhibition of IL-1β and TNFα in intestinal macrophages [27]. In a 2, 4, 6 trinitrobenzene sulphonic acid (TNSB)-induced colitis animal model, significantly increased colonic HO-1 expression was observed in comparison with the control group. Octreotide treatment increased HO-1 expression, but decreased expression of nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) [22]. In biopsy specimens from 18 ulcerative colitis patients and 13 colon cancer patients, expression of HO-1 mRNA and protein was significantly increased in the colonic mucosa of patients with active ulcerative colitis compared with normal mucosa [25]. These results collectively indicate that there may be an induction of HO-1 in the colon of ulcerative colitis patients

In the present study, we observed a downregulation of HO-1 expression in diseased intestinal tissues compared to adjacent normal tissues from 9 out of 17 Crohn's disease patients (53%) and 7 out of 10 ulcerative colitis patients

Table 5: Ho-1 gene expression in diseased tissues from adult patients with inflammatory bowel disease.

Patient ID	Relative expression		Fold	Downregulation	Upregulation
Tissues	Normal	Diseased	Diseased/normal		
Crohn's disease					
777-75-1	15.72	12.67	0.81	Down	
777-39-1	10.31	6.51	0.63	Down	
105-03-1	0.62	2.43	3.92		Up
132-01-1	2.42	3.40	1.41		Up
130-03-1	0.95	0.64	0.67	Down	
117-01-1	2.91	5.28	1.82		Up
777-76-1	3.00	4.55	1.52		Up
777-78-1	6.40	0.37	0.06	Down	
777-86-1	4.69	2.60	0.55	Down	
162-01-1	0.32	3.03	9.45	Down	
161-01-1	1.31	0.29	0.22	Down	
888-53-1	1.12	1.56	1.39		Up
777-16-1	45.13	25.83	0.57	Down	
777-59-1	3.35	15.01	4.48		Up
159-08-1	1.03	0.26	0.25	Down	
132-01-1	11.93	4.04	0.34	Down	
777-78-1	2.67	3.05	1.14		Up
Total (n = 17)	*6.70*	*5.38*	*0.80*	*10 (59%)*	*7 (41%)*
Ulcerative colitis					
888-90-1	12.57	5.77	0.46	Down	
888-128-1	10.11	7.50	0.74	Down	
888-139-1	0.63	1.27	2.02		Up
777-60-1	4.67	2.29	0.49	Down	
157-01-1	1.96	1.38	0.71	Down	
125-01-1	1.94	1.92	0.99		
120-01-1	1.10	0.26	0.23	Down	
115-1-1	3.12	5.34	1.71		Up
888-49-1	0.52	0.24	0.47	Down	
888-51-1	1.36	1.11	0.81	Down	
Total (n = 10)	*3.80*	*2.71*	*0.71*	*7 (70%)*	*2 (20%)*

(70%). Evidence indicated that HO-1 / carbon monoxide pathway ameliorates intestinal inflammation [1]. Down-regulation of HO-1 expression will probably weaken endogenous defensives, shifting the pro/anti-inflammation balance towards inflammation, potentially resulting in tissue injury in IBD.

The observations of an increased HO-1 expression in IBD by Kokurs et al. [25] as well as an upregulation and down regulation of HO-1 expression in different individuals in this study, suggest that the activation and maintenance of HO-1 expression is controlled by many factors, including genetic factors (as discussed above), oxidative stress, inflammatory processes and disease stage. A further study of the underlying mechanisms, on clinical samples, is necessary.

Impact of IL-10/STAT3 pathway on HO expression

The IL-10/STAT3 pathway is involved in regulation of inflammation. In this pathway, IL-10 signalling transduction occurs through binding of IL-10 to its receptors IL110RA and IL110RB to form a complex, with downstream molecules janus kinase-1 (Jak1) and tyrosine kinase 2 (Tyk2) activating STAT3 to regulate HO-1 transcription. Previous studies indicated that I-10 and Tyk2 SNPs were genetically associated with IBD, and mutations in IL-10RA and IL-10RB have been found to be associated with paediatric and very early onset IBD. In this study, we showed that SNPs rs1800872 of IL-10 and rs2071746 of HO-1 are associated with paediatric IBD. In our previous study we also observed a significant epistatic interaction between IL-10RB gene in the IL-10/STAT3 signalling pathway and target HO-1 gene (table 5). These findings indicate that the IL-10/STAT3 pathway plays a role in anti-inflammation in paediatric IBD. These studies help in the discovery of genetic contributors to intestinal inflammation and provide specific targets in personalised medicine.

Induction of HO-1 gene expression by proinflammatory LPS and TNFα

Although HO-1 possesses anti-inflammatory properties, we did not, in this study, observe a significant effect of proinflammatory LPS and TNFα on HO-1 expression in human B cells. This may be due to the small number of samples studied, and/or related to the patients' treatment (information that was not available). A further investigation of HO-1 expression in response to LPS and TNFα treatment, with a larger number of samples with clinical treatment information, is needed for understanding the role of HO-1 in the control of inflammation in IBD.

Dorresteijn et al. [28] showed in a recent study that LPS downregulates HO-1 expression in primary human mononuclear blood cells. They further indicated that LPS-dependent HO-1 regulation is cell type- and species-specific. In that study, human B cells (as opposed to mouse B cells), unlike mononuclear blood cells, lacked expression of TLR4 and were not activated by treatment with LPS. Furthermore, the B lymphocyte cell lines used in the present study were transformed by Epstein Barr virus and were not primary B cells. These issues may contribute to the observed differences between the results of the present study and those in the previous literature [28].

HO-1 as a potential target for therapy

HO-1 has anti-inflammatory properties and has been postulated to have a role in the amelioration of IBD. This was demonstrated with grapefruit-derived nanovesicles (GDNs) that can induce HO-1 activity and produce endogenous antioxidant and anti-inflammatory moieties such as bilirubin and carbon monoxide in DSS-induced mouse colitis [29]. This indicates that HO-1 may act as an immune modulator molecule, attenuating inflammatory response in IBD.

Conclusion

Our previous results and present case-trio study suggest: that genes IL-10 and HO-1 are involved in paediatric IBD; that gene-gene interaction of IL-10RB with HO-1 is involved in paediatric IBD; and that mutations in IL10 and IL10RB reduced HO-1 expression. These results indicate that the IL-10/STAT3 pathway and the IL-10 receptor IL10RB may regulate HO-1 activity, which in turn may lead to protective and/or anti-inflammatory effects in IBD therapy.

Disclosure statement

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