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Loci on 20q13 and 21q22 are associated with pediatric-onset inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is a common inflammatory disorder with complex etiology that involves both genetic and environmental triggers, including but not limited to defects in bacterial clearance, defective mucosal barrier and persistent dysregulation of the immune response to commensal intestinal bacteria. IBD is characterized by two distinct phenotypes: Crohn’s disease (CD) and ulcerative colitis (UC). Previously reported GWA studies have identified genetic variation accounting for a small portion of the overall genetic susceptibility to CD and an even smaller contribution to UC pathogenesis. We hypothesized that stratification of IBD by age of onset might identify additional genes associated with IBD. To that end, we carried out a GWA analysis in a cohort of 1,011 individuals with pediatric-onset IBD and 4,250 matched controls. We identified and replicated significantly associated, previously unreported loci on chromosomes 20q13 (rs2315008 [T] and rs4809330[A]; $P = 6.30 \times 10^{-8}$ and 6.95×10^{-8}, respectively; odds ratio (OR) = 0.74 for both) and 21q22 (rs2836878[A]; $P = 6.01 \times 10^{-8}$; OR = 0.73), located close to the *TNFRSF6B* and *PSMG1* genes, respectively.

CD is an inflammatory disorder that can affect any part of the intestine, whereas UC is restricted to the colon. CD is characterized by discontinuous penetrating lesions with full thickness (transmural) inflammation leading to stricturing and fistulization, whereas UC presents with confluent mucosal inflammation ranging from proctitis to pancolitis[1]. CD is twice as common as UC among children. The incidence of CD is 100–250/100,000 in the UK and the United States, compared to incidence of 80–100/100,000 for UC. There is strong evidence in support of CD and UC being complex genetic diseases with significant overlap, based on recurrence rates among families, twin studies and phenotype concordance studies.

Linkage studies facilitated the ‘positional cloning’ of the first two genes involved in the pathogenesis of IBD[2], including *NOD2* (also known as *CARD15*), the first and most widely replicated CD susceptibility gene, and the IBD5 locus on chromosome 5q31. More recently, GWA studies have identified several genes involved in the pathogenesis of IBD. Duerr *et al.* were the first to report a highly significant association between CD and *IL23R* in non-Jewish individuals of European ancestry with ileal CD[3]. A coding variant, rs11209026 (R381Q), was shown to confer a strong protective effect against the disease and was then replicated in the same study in separate cohorts of individuals with CD or UC. Others have replicated this finding, including our own laboratory in a cohort with pediatric-onset CD[4]. Hampe *et al.*[5] subsequently reported an independent association of a nonsynonymous SNP in *ATG16L1* on chromosome 2q37.1 (ref. [5]) and confirmed the previously reported variants in the *SLC22A4* and *NOD2* genes. Rioux *et al.*[6] also reported strong association with the coding variant in *ATG16L1* as well as independent replication to variation within an intergenic region on 10q21.1, in the genomic regions encoding *PHOX2B*, *NCF4* and *FAM92B*.

In a joint GWA study in the British population that examined 2,000 individuals for each of seven major diseases, including CD, against a shared set of approximately 3,000 controls[7], the Wellcome Trust Case Control Consortium identified nine independent association signals for CD at $P < 5 \times 10^{-7}$ corroborating the *ATG16L1*, 5q31, *IL23R*, 10q21 and 5p13.1 loci[8]. Their study also identified four previously unreported association signals located on chromosomes 3p21, 5q33, 10q24 and 18p11, respectively. Parkes *et al.* later reported replication for the

signals in the *ATG16L1* and *IRGM* genes[9]. We have also successfully shown the association of *ATG16L1* variation in our cohort of individuals with pediatric-onset CD[10].

Given that these genetic variants associated with CD do not account for the entire genetic risk, further efforts are necessary to identify and characterize additional genes associated with IBD. GWA studies have confirmed that genetic variants associated with IBD are indeed common and individually contribute only modestly to overall disease risk. As such, a barrier to performing further studies is the need for large sample sizes necessary to identify additional variants with smaller effect size; however, an alternative strategy is to ascertain individuals with a younger age of disease onset, as has been successfully carried out with asthma studies[11]. Such a tactic is attractive for IBD studies for several reasons. First, CD-affected children are more likely to have colonic CD than adults[12,13]. Second, UC-affected children are more likely to have extensive colitis than adults, and a young age of IBD onset is associated with a stronger family history of IBD.

As such, childhood-onset IBD demonstrates unique characteristics in phenotype, severity and family history; all of which justify ascertaining children with IBD for GWA studies seeking to identify previously unreported genes associated with IBD. However, we must emphasize that this approach was not undertaken to search for association signals that are exclusive to pediatric IBD, but rather to search for signals that are more apparent in children and that reach the bar of genome-wide significance but that may have been missed in the counterpart GWA studies in adults, where the results may have been clouded to a certain degree by environmental exposures. Here we report the results of an ongoing IBD GWA study in which we are testing an alternative hypothesis of stratifying cases by age of onset. We genotyped approximately 550,000 SNPs on the Illumina Human Hap550 Genotyping BeadChip in a discovery cohort of 1,011 IBD cases (including 647 CD and 317 UC, with the remainder being indeterminate colitis) of European ancestry and 4,250 ancestry-matched controls.

In the IBD discovery cohort, we compared single-marker allele frequencies using χ^2 statistics for all markers. Twelve markers were above the threshold for significance after correction (Table 1), most of which were either previously reported or resided within the MHC locus (driven by UC); however, two markers on chromosome 20q13, rs2315008 and rs4809330, and one marker on chromosome 21q22, rs2836878, had not been previously identified. The two noncoding variants on 20q13 are in strong linkage disequilibrium (LD) (rs2315008[T] and rs4809330[A]) and yield *P* values of 6.30×10^{-8} (corrected $P = 0.032$) and 6.95×10^{-8} (corrected $P = 0.036$), respectively, with protective odds ratios (OR) of 0.74 for both (Table 1). In addition, the one noncoding variant on 21q22 (rs2836878[A]) yields a *P* value of 6.01×10^{-8} (corrected $P = 0.031$), with a protective OR of 0.73. As all previously discovered genes associated with IBD are primarily associated with CD, except for the notable exception of *IRF5* in UC[14], it is important to note that the contribution to these newly identified signals comes from both UC and CD (Table 2). In addition, these signals replicate in an independent cohort of 173 IBD cases and 3,481 controls collected according to the same definitions as the discovery cohort after recruitment and genotyping of the discovery cohort was completed, as well as in the IBD cohort from the WTCCC[7] study, which includes individuals with CD (Table 3). The LD structures for the 20q13 and 21q22 loci pinpointing the associated SNPs and genes within these regions are shown in Figure 1 and Supplementary Figure 1 online, respectively. These significant SNP alleles confer protection from IBD.

The 20q13 signal resides in a complex telomeric region of LD (Fig. 1); the central block of LD harbors the genes for *STMN3* (stathmin-like 3), *RTEL1* (regulator of telomere elongation helicase 1), *TNFRSF6B* (tumor necrosis factor receptor superfamily member 6B), *ARFRP1* (ADP-ribosylation factor related protein 1), *ZGPAT* (zinc finger CCCH-type with G patch domain), *LIME1* (Lck interacting transmembrane adaptor 1), *SLC2A4RG* (solute carrier family

2 member 4 regulator) and *ZBTB46* (zinc finger and BTB domain containing 46; formerly *BTBD4)*. Although we were unable to pinpoint the exact gene associated with the disease in this region based on LD or the fact that all the SNPs occurred in noncoding regions, we considered the *TNFRSF6B* gene the most compelling candidate on the basis of what is already known about the critical role of specific polymorphisms within genes involved in the TNF pathway in the pathogenesis of IBD[15]. Moreover, our observation that the mRNA expression of *TNFRSF6B* is markedly different in colonic biopsies obtained from individuals with IBD compared to disease-free controls lends further support to the potential role of *TNFRSF6B* and the TNF-pathway in IBD (Fig. 2a). Furthermore, we found that *TNFRSF6B* mRNA expression correlated with the degree of mucosal inflammation within the colon ($n = 31$, $r^2 = 0.29$, $P = 0.002$ for linear regression for the Crohn's disease histological index of severity (CDHIS)[16, 17] and *TNFRSF6B* expression). Although we did not observe any difference in colonic mRNA expression of *TNFRSF6B* between IBD subjects with and without the key alleles of these two identified SNPs, this may have been confounded by a greater degree of mucosal inflammation, and therefore different cell populations, in the colon biopsies for the subjects who did not carry the associated alleles (mean ± s.e.m.) CDHIS for minor allele SNP: 3.7 ± 1 versus major allele SNP: 7 ± 1.2, $P = 0.05$. By comparison, we observed no significant differences in colon mRNA expression between individuals with IBD and disease-free controls for the other genes at or immediately neighboring this locus, including *RTEL1*, *ARFRP1*, *ZGPAT*, *LIME1*, *SLC2ARG*, *BTBD4*, *TPD52L2*, *c20orf135* or *DNAJC5* (Supplementary Fig. 2a online).

It is of interest that the protein product for *TNFRSF6B* acts as a decoy receptor (DCR3) in preventing FasL-induced cell death, and a resistance to FasL-dependent apoptosis has previously been shown for T lymphocytes in CD[18]. We therefore asked whether serum DCR3 concentration would differ between individuals with IBD and controls and, within the IBD group, between those with and without the identified at-risk variants captured by the *TNFRSF6B* tagging SNPs. We found that mean ± s.e.m. serum DCR3 concentration increased from 84 ± 37 pg/ml in healthy controls to 4,333 ± 1,637 pg/ml in individuals with IBD carrying the major allelic variants, and 11,793 ± 2,452 pg/ml in individuals with IBD carrying the minor allelic variants (Fig. 2b, $P < 0.05$ for IBD versus control, and within IBD for major versus minor allelic variants). The two groups of individuals with IBD did not otherwise differ based upon age, gender, clinical disease activity or concurrent medications, suggesting that the difference in serum DCR3 concentration was due to the observed genotypic variation at 20q13.

The 21q22 signal resides in a small region of LD that harbors no genes, but the nearest gene is *PSMG1* (proteasome assembly chaperone 1). We observed a modest increase in the colonic expression of *PSMG1* in IBD cases compared to controls (Supplementary Fig. 2b). However, the expression did not vary with either the degree of mucosal inflammation or the carriage of the alleles at the 21q22 locus.

In the case-control analysis of CD alone, we also compared single-marker allele frequencies using χ^2 statistics for all markers. Nine markers were above the threshold for Bonferroni correction. As shown in Supplementary Table 1 online, all of these loci have been previously reported in GWA studies[4].

We similarly compared single-marker allele frequencies in the case-control analysis of UC alone using χ^2 statistics for all markers. Seventeen markers were above the threshold for significance after correction (Supplementary Table 2 online). However, whereas the genomic inflation factor (GIF) for IBD or CD alone was close to 1, the GIF for UC was 1.3. To address this issue, we used principle components analysis to control for cryptic population structure as implemented in Eigenstrat and as we have previously described[19]. As a consequence, four markers remained robustly genome-wide significant, all of which resided in the major histocompatibility complex (MHC) on chromosome 6q21. This reinforces previously

suggested MHC associations based on linkage studies[19] and is the first GWA study to associate UC with specific MHC alleles.

We have identified previously unreported susceptibility loci for pediatric-onset IBD at 20q13 and 21q22. On the basis of our data showing differences in serum DCR3 concentration between individuals with IBD with and without the identified SNPs, and its known biologic function, we feel that *TNFRSF6B* is the most plausible candidate within the 20q13 locus. Consistent with this, DCR3 is upregulated in inflamed intestinal epithelia and is secreted by T lymphocytes isolated from individuals with IBD[20,21]. DCR3 has multiple complex roles within the innate and adaptive immune system, which may result in a net pro- or anti-inflammatory effect based upon the precise context[22,23]. Recent studies have implicated both innate (involving *NOD2* in bacterial pattern recognition and *ATG16L1* in autophagy) and adaptive (involving *IL23R* in Th17 lymphocyte differentiation) pathways in susceptibility to IBD. Results of the current study add to our understanding of this complex pathogenesis by identifying a candidate gene, *TNFRSF6B*, involved in both antigen-presenting cell differentiation and lymphocyte function[24,25].

METHODS

Study participants

The IBD discovery cohort consisted of 1,011 individuals, including 647 with CD and 317 with UC (Supplementary Table 3 online), with the remainder having indeterminate colitis. The IBD replication cohorts consisted of (i) 1,749 cases from the WTCCC and (ii) 173 cases that were recruited and genotyped at CHOP after genotyping analysis of the discovery set was completed. The mean age at diagnosis for the discovery cohort was 11.1 years (range 1–18 years). All individuals were diagnosed under the age of 19 years and fulfilled standard IBD diagnostic criteria[26] and phenotype characterization based on the Montreal classification[26]. Out of 1,011 subjects with IBD, 55% were males. Only subjects of European ancestry were used in the analysis; the program STRUCTURE[27] was used to determine greater than 95% European ancestry based on AIMS markers. Among individuals with CD, 17% had disease confined to ileum, 29% had disease confined to the colon and the remaining 54% had ileocolonic disease. Among individuals with UC subjects, 89% had pancolonic involvement, and only 11% had disease confined to left side of the colon. Mucosal intestinal biopsies were obtained for a subset of subjects during a routine diagnostic endoscopic procedure. The research ethics boards of the respective hospitals and other participating centers approved the study, and written informed consent was obtained from all subjects.

The control group used in the discovery phase included 4,250 children, 52.9% males and 47.1% females, with self-reported European descent and mean age 9.5 years who did not have IBD (neither CD nor UC). The replication control groups consisted of (i) 10,643 WTCCC subjects (that is, the WTCCC cohort except subjects with IBD, rheumatoid arthritis or type 1 diabetes) and (ii) an independent set of 3,481 children self-reported as of European descent. All controls were recruited by CHOP clinicians and nursing staff within the CHOP Health Care Network, including four primary care clinics and several group practices and outpatient practices that included well child visits. The Research Ethics Board of CHOP approved the study, and written informed consent was obtained from all subjects.

Self-reported European ancestry proved to be accurate, as the resulting genomic inflation factor for the IBD run was less than 1.1.

Genotyping

We carried out high-throughput genome-wide SNP genotyping using Illumina Infinium II HumanHap550 BeadChip technology at the Center for Applied Genomics at CHOP, as previously described[28]. For the discovery cohort, a total of 1,011 IBD cases (including 647 CD and 317 UC, with the remainder being indeterminate colitis) of European ancestry and 4,250 controls with matching ancestry were included in the final analyses. We rejected 7,215 SNPs with call rates <95%, 22,255 SNPs with MAF <1% and 3,381 SNPs with Hardy-Weinberg equilibrium $P < 10^{-5}$. The threshold for significance was 1×10^{-7}. The only cohort that showed substantial population stratification was the UC collection (genomic inflation factor = 1.3); the results for this subset were adjusted using EIGENSTRAT. For the replication study, an independent cohort of 173 IBD cases of European ancestry and 3,481 ancestry-matched controls were analyzed. We rejected a total of 10,381 SNPs were rejected with call rates <95%, 7,141 SNPs with MAF <1% and 53 SNPs with Hardy-Weinberg equilibrium $P < 10^{-5}$. In addition, imputed allele frequencies for the WTCCC CD cohort were compared with those in all other WTCCC subjects (except those with autoimmune disease), including the 1958 UK birth cohort, yielding a total control set of 10,643 subjects. Combined *P* values were obtained using Fisher's method.

Gene expression analyses

We examined gene expression in individual colonic biopsy specimens from subjects with pediatric-onset CD and UC and in healthy controls. Biotinylated cRNA was hybridized to the Affymetrix GeneChip HG-U133 Plus 2.0 arrays, containing probes for approximately 22,634 genes, at the CCHMC Digestive Health Center Microarray Core. The images were captured using Affymetrix GeneChip Scanner 3000. We normalized data to allow for array-to-array comparisons, and we detected differences between groups in GeneSpring with significance at the 0.05 level relative to samples from healthy controls. Serum DCR3 concentration was determined by ELISA (R&D Systems) (Supplementary Methods online).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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AUTHOR CONTRIBUTIONS

S.K., R.N.B. and H.H. designed the study and H.H. supervised the genotyping data analysis and interpretation. L.A.D. designed and supervised the microarray and ELISA experiments, data analysis and interpretation. J.P.B., P.M.A.S.

and S.F.A.G. conducted the statistical analyses. S.K., R.N.B., S.L.G., S.C., G.T., V.A. and L.A.D recruited the individuals with IBD and performed all phenotyping. C.E.K. and E.C.F. directed the genotyping in a team consisting of J.T.G., J.L.S., R.M.S. and F.G.O. J.P.B., M.I., K.A., J.T.G., A.W.E. and E.S. provided bioinformatics support. The remaining authors coordinated the studies. S.K., R.N.B., S.L.G., L.A.D., S.F.A.G. and H.H. wrote the manuscript.

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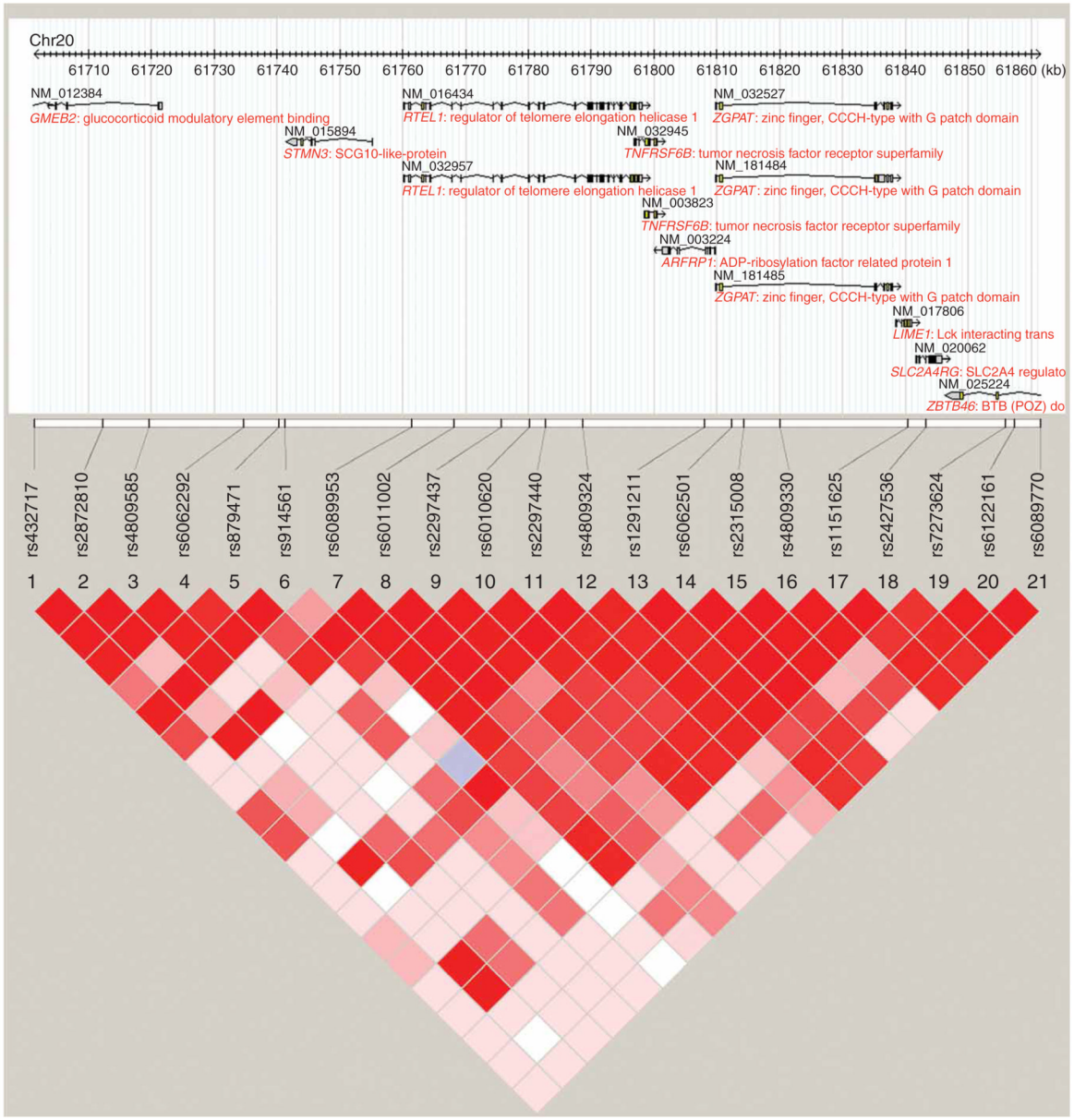

Figure 1.

Linkage disequilibrium (D) between SNPs at the 20q13 locus in the control cohort together with the corresponding Haploview gene track. The association signal resides in a region of LD that harbors several genes, including *TNFRSF6B*.

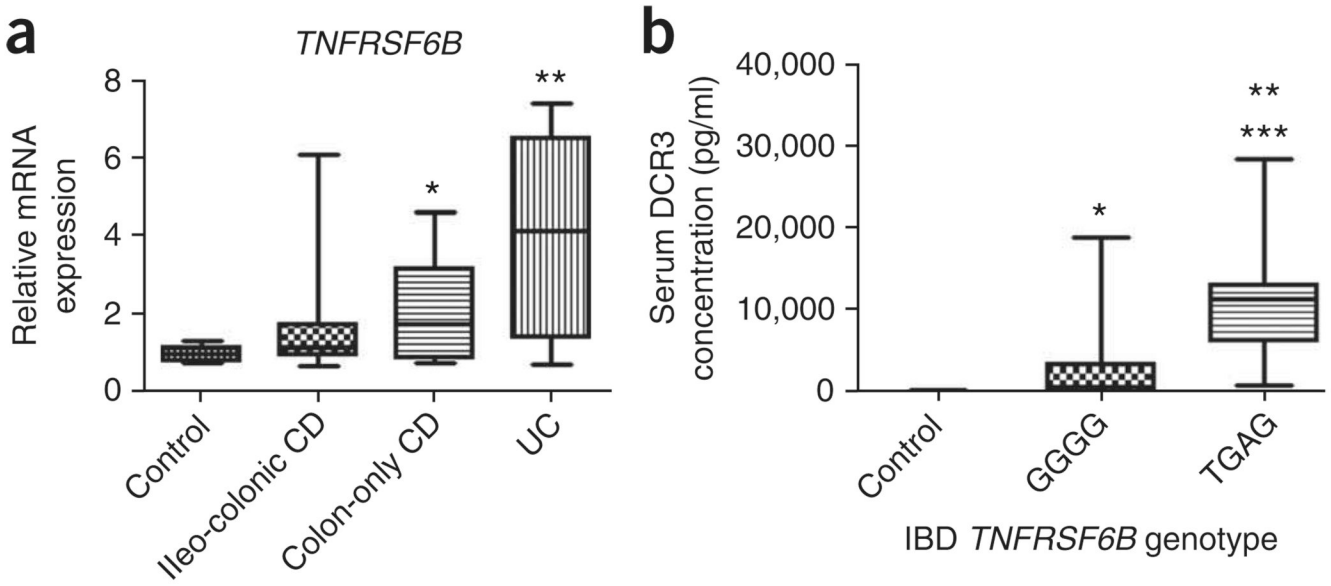

Figure 2.

Colonic *TNFRSF6B* expression and serum DCR3 concentration. (**a**) Colon biopsies were obtained from healthy controls ($n = 11$, Crohn's disease histological index of severity (CDHIS): 0) and affected segments from individuals with Crohn's disease with ileo-colonic ($n = 18$, mean ± s.e.m. CDHIS: 4.1 ± 0.7) or colon-only ($n = 14$, mean ± s.e.m CDHIS: 4.9 ± 1) location and individuals with UC ($n = 10$, mean ± s.e.m. CDHIS: 7.2 ± 0.6, $P < 0.05$ vs. CD groups). We prepared RNA and determined the global pattern of gene expression using the Affymetrix GeneChip Human Genome HG-U133 Plus 2.0 array. Data were normalized to an internal control reference sample, and then to the median value of the healthy control samples, to allow for comparison of mRNA expression in the IBD colon samples relative to the healthy control samples. Results for *TNFRSF6B* mRNA expression relative to controls are shown. (**b**) We determined the serum concentration of the *TNFRSF6B* gene product, DCR3, by ELISA in healthy controls ($n = 10$), individuals with IBD with the *TNFRSF6B* GGGG genotype ($n = 17$, carrying the major allelic variants: G at rs2315008 and G at rs4809330) and individuals with IBD with the *TNFRSF6B* TGAG genotype ($n = 11$, carrying the minor allelic variants: T at rs2315008 and A at rs4809330). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control, $^{***}P < 0.05$ vs. *TNFRSF6B* GGGG genotype.

Table 1

IBD case-control association study results for GWA significant markers

Chr.	SNP	Position (B36)	Minor allele	MAF, affected	MAF, control	*P* value	Bonferonni *P*	OR	95% CI	Relevant gene
1	rs11209026	67478546	A	0.024	0.061	7.47×10^{-11}	3.84×10^{-5}	0.385	0.29–0.52	*IL23R*
16	rs5743289	49314275	T	0.232	0.172	3.77×10^{-10}	0.00019	1.455	1.29–1.64	*NOD2*
1	rs11465804	67475114	G	0.030	0.065	1.46×10^{-9}	0.00075	0.442	0.34–0.58	*IL23R*
6	rs477515	32677669	T	0.248	0.313	1.02×10^{-8}	0.0052	0.724	0.65–0.81	*HLA-DRB1*
6	rs2516049	32678378	G	0.248	0.313	1.06×10^{-8}	0.0054	0.724	0.65–0.81	*HLA-DRB1*
6	rs9271568	32698441	A	0.238	0.301	2.95×10^{-8}	0.015	0.724	0.65–0.81	*HLA-DQA1*
9	rs6478109	116608587	A	0.251	0.314	3.20×10^{-8}	0.016	0.733	0.66–0.82	*TNFSF15*
21	**rs2836878**	**39387404**	**A**	**0.214**	**0.273**	$\mathbf{6.01 \times 10^{-8}}$	**0.031**	**0.725**	**0.65–0.81**	***PSMG1***
20	**rs2315008**	**61814400**	**T**	**0.250**	**0.311**	$\mathbf{6.30 \times 10^{-8}}$	**0.032**	**0.737**	**0.66–0.82**	***TNFRSF6B***
20	**rs4809330**	**61820030**	**A**	**0.249**	**0.310**	$\mathbf{6.95 \times 10^{-8}}$	**0.036**	**0.738**	**0.66–0.82**	***TNFRSF6B***
9	rs6478108	116598524	C	0.262	0.324	8.36×10^{-8}	0.043	0.743	0.67–0.83	*TNFSF15*
16	rs2076756	49314382	G	0.317	0.258	9.65×10^{-8}	0.050	1.332	1.20–1.48	*NOD2*

Newly identified signals are indicated in boldface. Minor allele frequencies (MAF), *P* values and odds ratios (OR) are shown. The ORs shown are for the minor alleles (as observed in the controls). Combined *P* values are also shown, together with the most relevant gene in which the markers reside or which they are nearest to. *P* values are two sided and uncorrected in each instance.

Table 2

Key signals in CD and UC separately

Chr.	SNP	Minor allele	MAF (control)	MAF (CD)	*P* value (CD)	OR (CD)	95% CI (CD)	MAF (UC)	P value (UC)	OR (UC)	95% CI (UC)
20	rs2315008	T	0.311	0.252	1.84×10^{-5}	0.747	0.65–0.85	0.238	0.00013	0.694	0.57–0.84
20	rs4809330	A	0.309	0.252	2.71×10^{-5}	0.752	0.66–0.86	0.235	8.58×10^{-5}	0.686	0.57–0.83
21	rs2836878	A	0.272	0.224	0.00026	0.772	0.67–0.89	0.194	1.71×10^{-5}	0.643	0.53–0.79

Minor allele frequencies (MAF), *P* values and odds ratios (OR) are shown. The ORs shown are for the minor alleles (as observed in the controls). *P* values are two sided and uncorrected in each instance.

Table 3

Replication of the newly identified signals in an independent replication cohort of pediatric-onset IBD and in the WTCCC CD cohort

			Independent replication cohort				WTCC CD				Combined
Chr.	**SNP**	**Minor allele**	**MAF, affected**	**MAF, control**	**OR (95% CI)**	***P* value**	**MAF, affected**	**MAF, control**	**OR (95% CI)**	***P* value**	***P* value**
20	rs2315008	T	0.248	0.311	0.734 (0.568–0.948)	0.01749	0.293	0.33	0.842 (0.753–0.939)	8.03×10^{-6}	8.85×10^{-15}
20	rs4809330	A	0.253	0.309	0.756 (0.587–0.973)	0.02943	0.293	0.33	0.842 (0.753–0.939)	7.90×10^{-6}	1.62×10^{-14}
21	rs2836878	A	0.214	0.277	0.71 (0.544–0.927)	0.01131	0.257	0.276	0.9 (0.81–1.02)	6.60×10^{-3}	4.48×10^{-12}

Minor allele frequencies, *P* values and odds ratios (OR) are shown. The ORs shown are for the minor alleles (as observed in the controls). Combined *P* values are also shown. *P* values are two-sided and uncorrected in each instance.