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***IFNG* rs1861494 Polymorphism is Associated with IBD Disease Severity and Functional Changes in both *IFNG* Methylation and Protein Secretion**

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Abstract

Background—Mucosal expression of IFN- γ plays a pivotal role in IBD pathogenesis and IBD-risk regions flank *IFNG*. The conserved *IFNG* rs1861494 T/C, introduces a new CpG methylation site, and is associated with disease severity and lack of therapeutic response in other infectious and immune mediated disorders, and is in linkage-disequilibrium with a UC disease severity region. It seems likely that CpG-altering SNPs modify methylation and gene expression. This study evaluated the association between rs1861494 and clinical, serologic and methylation patterns in IBD patients.

Methods—Peripheral T cells of UC and CD patients were genotyped for rs1861494 and analyzed for allele-specific and *IFNG* promoter methylation. Serum ANCA and IFN- γ secretion were measured by ELISA and nucleo-protein complex formation by EMSA.

Results—*IFNG* rs1861494 T allele carriage in IBD patients was associated with enhanced secretion of IFN- γ . T allele carriage was associated in UC with high levels of ANCA and faster progression to colectomy. In CD, it was associated with complicated disease involving a stricturing/penetrating phenotype. Likewise, *IFNG* rs1861494 displayed genotype specific modulation of DNA methylation and transcription factor complex formation.

Conclusions—This study reports the first association of *IFNG* rs1861494 T allele with enhanced IFN- γ secretion and known IBD clinical parameters indicative of more aggressive disease, as well as serological markers associated with treatment resistance to anti-TNF therapy in IBD patients. These data may be useful prognostically as predictors of early response to anti-TNF therapy to identify IBD patients for improved personalized therapeutics.

Keywords

Human; T Lymphocytes; methylation; interferon; Crohn's disease; ulcerative colitis; serological antibodies

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Introduction

Inflammatory bowel disease (IBD) is generally believed to be triggered by an inappropriate immune response to the commensal flora in genetically susceptible individuals (1). Diagnosis of Crohn's disease (CD) or ulcerative colitis (UC) is based on clinical, endoscopic and histological criteria. However, clinical and genetic disease heterogeneity detected between UC and CD suggests that they are likely a spectrum of mucosal inflammatory diseases. Predicting severity of disease course at time of diagnosis and response to therapy are challenges faced by clinicians and underscore the need for prognostic biomarkers. Serologic responses to commensal bacteria and auto-antigens have proven helpful to identify patients with distinct disease phenotypes and risk of complications. Of these, antibodies against the cell wall mannan of *Saccharomyces cerevisiae*, ASCA, primarily associated with CD and perinuclear anti-neutrophil cytoplasmic autoantibodies (ANCA) associated with UC (2–4) have been the focus of the most attention. Additional markers have also been identified, including antibodies to the outer-membrane porin C of *Escherichia coli* (OmpC), a *Pseudomonas fluorescens*-associated sequence (I2), and antibodies against the flagellin CBir1 (anti-CBir1) (5–7). Much effort has been expended to identify the clinical relevance of antibody response and utility as a diagnostic tool. High antibody response toward multiple microbial antigens is associated with more aggressive disease and risk for surgery in CD (8–10). In UC, high antibody response towards ANCA is associated with a more aggressive disease course and need for surgery early in the disease course (11, 12).

Although the actual causes of IBD remain unknown, genetic factors contribute to the pathogenesis of disease. Genome wide association studies (GWAS) support the presence of both common and distinct shared susceptibility loci for UC and CD. Of the 163 IBD-associated loci identified to date, 110 loci are common to both diseases (13). Many of the IBD loci are shared with other immune-mediated diseases, with a significant overlap in loci of genes associated with immune cell signaling, T-cell differentiation, and innate immune response (13). Risk regions on chromosome 12 upstream and downstream of *IFNG*, a key modulator of intestinal inflammation, are associated with CD and UC, as well as other inflammatory and autoimmune diseases such as asthma, systemic lupus erythematosus, tuberculosis and rheumatoid arthritis (13–17). A single nucleotide polymorphism (SNP) within the third intron of *IFNG*, rs1861494 T/C, has been linked to severity of disease in asthma, hepatic schistosomiasis and tuberculosis (18–20). This SNP resides in linkage disequilibrium with a region correlated with the development of severe, medically refractory UC (21). In this study, we further explored the association of rs1861494 T/C SNP with severity of disease in IBD and found a significant association of the T allele to severity in both UC and CD. Furthermore, the rs1861494 T allele functionally correlated with increased IFN- γ expression. In this context, the rs1861494 T/C polymorphism introduces a new CpG dinucleotide sequence that serves as an epigenetic target for DNA methylation resulting in altered transcription factor binding to this region that might have a functional consequence on transcription of IFN- γ expression.

Methods

Isolation of T cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers or IBD patients by separation on Ficoll-Hypaque gradients. CD3⁺ T cells (PB T) were isolated using CD3-immunomagnetic beads (Miltenyi Biotech, Auburn, CA) and were at least 95% pure.

Study Subjects

Patients with IBD were recruited through the Inflammatory Bowel Disease Center at Cedars-Sinai Medical Center. Diagnoses of Crohn's disease and ulcerative colitis were confirmed using standard clinical, radiological, endoscopic and pathological criteria. All subjects were Caucasian non-Hispanic with the average age of 41 for CD (range 15–78) and 46 for UC (range 11–77) and were genotyped for rs1861494. All genotyping was performed at the Medical Genetics Institute at Cedars-Sinai Medical Center using Infinium technology (Illumina, San Diego, CA). Control subjects were healthy individuals, free of medication and with no known personal or family history of autoimmune disease or IBD.

IFN- γ Assay

IBD T cells were stimulated with anti-CD3 antibody for 24 hours. IFN- γ was measured by an amplified ELISA. Greiner Bio-One (Longwood, FL) ELISA plates were coated overnight with 100 μ l of 5 μ g/ml monoclonal anti-IFN- γ (BD Biosciences, Woburn, MA). Samples and standards were added for 24 h followed by addition of 100 μ l of 2.5 μ g/ml polyclonal biotinylated rabbit anti-IFN- γ (BD Biosciences) for 2 h. This was followed by addition of 100 μ l of 1/1000 diluted alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h. Substrate, 0.2 mM NADP (Sigma-Aldrich, St. Louis, MO) was added for 30 min followed by addition of amplifier (3% 2-propanol, 1 mM iodonitrotetrazolium violet, 75 μ g/ml alcohol dehydrogenase, and 50 μ g/ml diaphorase; Sigma-Aldrich) for 30 min. Plates were read at 490 nm using an E max plate reader (Molecular Devices, Sunnyvale, CA).

Microbial Antibody Responses

All blood samples were taken at the time of consent and enrolment. Sera were analyzed for expression of ASCA, anti-OmpC, anti-I2 anti-CBir1 antibodies in a blinded fashion by ELISA as previously described (7, 8, 22). Antibody levels were determined and results expressed as ELISA units (EU/ml) relative to a Cedars-Sinai Laboratory standard that was derived from a pool of patient sera with well-characterized disease found to have reactivity to this antigen.

Pyrosequencing

DNA was extracted from T cells using a QIAmp DNA isolation kit (Qiagen Inc., Valencia, CA). All samples were analyzed in a blinded fashion using the EpigenDx custom pyrosequencing service (EpigenDx, Inc., Hopkinton, MA). Briefly, bisulfite treatment of 2 μ g of DNA was carried out using the EZ DNA methylation kit (Zymo Research, Orange, CA) according to manufacturer's instructions. Hot-start PCR was carried out with HotStart

Taq (Qiagen Inc.) using 100 ng of bisulfite treated DNA. PCR and pyrosequencing primers are shown in Table 1. Direct quantification of the ratio of unmethylated to methylated cytosines was determined for each site using Pyro Q-CpG software. The *IFNG* non-CpG cytosine at site -181 bp served as an internal control and revealed that bisulfite conversion of DNA was greater than 95%. Likewise, only slight variability was detected in DNA samples treated with bisulfite on different days. The naïve NK92 cell line demonstrated complete conversion following bisulfite treatment and served as a demethylation control.

EMSA

PBMC from healthy volunteers were stimulated up to 4 h with PMA/ionomycin and nuclear protein extracts were obtained. Nuclear protein extract (3–6 µg) was incubated at 25°C with 0.25 mg/ml poly (dI-dC), in 20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris pH 7.5 for 10 min. Oligonucleotides 5'-IRD700-labeled (Integrated DNA Technology, Coraville, Iowa) were then added (250 fmol) and the binding reactions incubated for an additional 30 min. The DNA-protein complexes were separated from unbound probe on a pre-run native 6% polyacrylamide gel in low ionic strength buffer (22.3 mM Tris pH 7.4, 22.3 mM Borate, 0.5 mM EDTA pH 8.0) and analyzed with Odyssey infrared imaging system (Li-Cor Biosciences). The rs1861494 oligonucleotides used were (polymorphic nucleotides are bolded, only upper strand is shown):

- 5'-TCAGTACTCCCTGTGCTTCTTCCTCA -3',
- 5'-TCAGTACTCCCCGTGCTTCTTCCTCA -3',

For methylated oligonucleotides, the following sequence was used with either methylation of one or both strands:

- 5'-TCAGTACTCCCC(Me)GTGCTTCTTCCTCA -3',

Statistical Analysis

Tests for statistical significance was performed using JMP Statistical Software (SAS Institute GmbH, Heidelberg, Germany) as follows: Test for significance between of rs1861494 SNP and ANCA, IFN-γ secretion and methylation levels was calculated by parametric Student's T test; test of association and trend using Fisher's exact test, cox proportional hazards model and Kaplan-Meier Survival Curves and Log-Rank Test.

Ethical Considerations

All studies involving human subjects were approved by the Institutional Review Board at Cedars-Sinai Medical Center.

Results

rs1861494 T allele carriage was increased among IBD patients with more aggressive disease

The *IFNG* +2109 SNP rs1861494 is located within a conserved regulatory region of the third intron of *IFNG* (Fig. 1). Disease-specific response to microbial and auto-antigens is associated with IBD (2–7). Sero-positivity to ANCA is detected in the majority of UC

patients, but only a small percentage of CD patients, and is associated with a more aggressive disease phenotype in UC (11, 12). In order to investigate whether rs1861494 was associated with disease severity in IBD, we first stratified our IBD patient population by disease phenotype and assessed the SNP allele distribution in individuals with seroreactivity to ANCA. We found, in UC populations, the proportion of T (TT and TC) versus C allele (CC) carriers was significantly higher in the ANCA positive compared to ANCA negative patients ($p < 0.015$) (Fig. 2A), and 95% of ANCA positive were T allele carriers (Fig. 2A). We also found that the rs1861494 T allele carriers were associated with a significant increase in ANCA levels ($p < 0.001$) (Fig. 2B) and an increase in the need for earlier surgical intervention for disease management ($p < 0.05$) (Fig. 2C) in UC patients.

In CD populations, no significant association between rs186194 and ANCA was detected. However, we found a significant increase of complicated disease behavior, structuring/penetrating phenotype, in CD patients carrying the T allele (Fig. 3). No significant association between rs186194 and other CD serological markers (ASCA, OmpC, CBir, and I2) was detected (data not shown). Thus, although the clinical manifestations differed, T allele carriers were associated with a more severe disease course for both UC and CD.

rs1861494 T allele carriage was associated with increased IFN- γ protein expression

Mucosal expression of IFN- γ is key not only to the development and maintenance of inflammation but, additionally, the absolute levels of IFN- γ influence the severity of disease (23). Therefore, we hypothesized that IBD T allele carriers would display a greater level of IFN- γ . Indeed, IFN- γ secretion was enhanced in IBD patients carrying the T allele compared to patients homozygous for the C allele following stimulation of peripheral T cells (Fig. 4).

Methylation of rs1861494 was allele-specific

The rs1861494 C to T SNP (+2109 bp) introduces a new potential CpG methylation site. An additional two CpG methylation sites reside 52 and 102 bp upstream at +2052 and +2007 bp.

DNA methylation is considered an epigenetic marker for expression competency, with reduced methylation usually correlated with enhanced gene expression. It is generally presumed that for most expressed genes each of the two alleles contributes equally. However, introduction of a new potential *IFNG* CpG methylation SNP might lead to allelic methylation differences, ultimately affecting IFN- γ expression levels. It has been suggested that unequal allele methylation occurs preferentially in sites adjacent to CpG SNPs (24). In order to ascertain the methylation status of rs1861494, allele/strand specific pyrosequencing methylation analysis was carried out in IBD patients heterozygous for rs1861494. Strand specific methylation levels for each of the two adjacent upstream CpG sites, as well as the rs1861494, were determined. The minor rs1861494 C allele did function as a methylation site (average methylation 60%, Fig. 5A). As expected, no significant methylation was seen for the non CpG common T allele. Furthermore, a significant decrease in the methylation of the +2052 bp CpG site was detected when comparing the minor C to the common T allele strands ($p < 0.001$) (Fig. 5B). No difference was seen for the +2007 bp CpG site (Fig. 5B). We have previously demonstrated a significant inverse correlation between the de novo

IFNG expression and the corresponding overall methylation index (MI) of the promoter *IFNG* region (-54, -186, and -295 bp) (25). Therefore, we examined the relationship between the overall MI of the three sites adjacent to rs1861494 and the MI of the *IFNG* promoter region. There was a highly significant correlation ($p<0.001$) between the MI of these two regions for both the T and C alleles (Fig. 5C), suggesting that the methylation status of the rs1861494 region paralleled that of a promoter region known to contribute to regulation of gene expression.

Allele-specific nucleo-protein binding was detected to methylated rs1861494 DNA-binding sites

To investigate how the C to T SNP influences transcription factor binding to the SNP rs1861494 region, nuclear extracts were prepared from resting or activated PB T cells. Nucleoprotein binding assays were carried out using dsDNA oligonucleotides corresponding to the common T or minor C SNP allele sequence. Activation-dependent binding of nucleoprotein complex to the T or C SNP oligonucleotide was detected (Fig. 6). Although similar complexes were seen for the common T and minor C oligonucleotides, binding to the T SNP was enhanced compared to the C SNP allele (Fig. 6A).

To investigate how methylation of the rs1861494 CpG influenced transcription factor complex formation, binding assays were set up using a non-methylated, asymmetrically methylated (one strand) C SNP or symmetrically methylated (both strands) C SNP oligonucleotide. Asymmetrical methylation of one C oligonucleotide strand displayed a pattern similar to that seen for the unmethylated oligonucleotide (Fig. 6B, C SNP vs. C/MC SNP). In contrast, symmetric methylation of both C oligonucleotide strands markedly enhanced binding and the appearance of an additional protein complex (Fig. 6B, C/MC SNP vs. MC/MC SNP). These results suggested that both the specific allele sequence and methylation levels could modulate binding of transcription factors to the rs1861494 region and likely regulate IFN- γ expression.

Discussion

Mucosal expression of IFN- γ and other pro-inflammatory cytokines is critical not only to the development and maintenance of inflammation but, additionally, the absolute amount of IFN- γ appears to modulate the severity of Crohn's disease (26). In this study, we examined the association between the *IFNG* rs1861494 T/C polymorphism in IBD. *IFNG* rs1861494 T allele carriage in IBD patients was associated with enhanced secretion of IFN- γ . In addition, *IFNG* rs1861494 T allele carriage was associated with clinical/serological parameters indicative a more severe disease course in both UC and CD patients. In UC, T allele carriage was associated with seropositivity and higher levels of the IBD associated autoantibody ANCA. To our knowledge this is the first study to report a genetic association, outside the HLA region, with ANCA levels. Among patients with medically refractory UC, T allele carriage was associated with a severe disease course that progressed more rapidly toward colectomy. In the CD patient population, T allele carriage was associated with a complicated disease behavior, characterized by stricturing/penetrating phenotype.

Our findings are consistent with previously reported studies demonstrating an involvement of *IFNG* rs1861494 in severity and treatment resistance in other infectious and immune disorders. In tuberculosis, rs1861494 T allele carriage has been associated with susceptibility as well as a more severe microscopy-positive and bacterial positive form of the disease (18). Furthermore a recent study demonstrated the presence of persistent, elevated levels of IFN- γ in T allele carriers following anti-tuberculosis treatment supporting an association with therapeutic resistance (27). These data suggests a possible mechanistic role in which elevated IFN- γ expression in T allele carriers may lead to a worse prognosis for the resolution of active disease or accelerated progression to complicated and severe disease. Likewise, in hepatic schistosomiasis, T allele carriage has been associated with poorer control of disease and higher risk of developing severe and extended hepatic fibrosis (19). In chronic myeloid leukemia, T allele carriage has been associated with poorer response to Imatinib therapy and slower progression of complete cytogenic response (28).

One of the major challenges in treating IBD is the inter-individual variability of response to therapeutic intervention. The association of rs1861494 T allele carriage with disease severity may help identify patients at risk for aggressive disease before complications occur and thereby reduce the need for surgery. In CD, half of all patients will develop stricturing or penetrating complications within the first 20 years of disease. Most of these patients will go on to require surgery with up to a 61% of CD patient requiring surgery at 10 years (29). In UC, the expression of ANCA autoantibodies is associated with a complicated disease course and the need for surgical intervention (30). In fact, a subset of UC patients expressing high levels of ANCA are more likely to develop chronic pouchitis after ileal pouch-anal anastomosis (12). Moreover, studies have suggested that ANCA reactivity may be associated with poor response to anti-TNF therapy in UC patients. ANCA seronegative UC patients have been shown to be more likely to initially achieve clinical response to anti-TNF therapy than ANCA seropositive patients (31–33). Likewise, in children ANCA reactivity has been independently associated with primary nonresponse to anti-TNF therapy (34). Similar findings have been shown in the CD population in which patients who displayed seropositivity to ANCA were more likely to fail anti-TNF therapy (35, 36). The initial lack of response to anti-TNF therapy is of clinical importance in CD and even more so in UC. It is likely that lack of primary response to anti-TNF therapy at least in part may reflect that TNF is not the primary mediator of the inflammatory process in this group of patients. Since patients who are seropositive to ANCA are more likely to fail anti-TNF therapy, the present findings of rs1861494 association with disease severity, elevated ANCA and IFN- γ protein levels, suggest that IFN- γ might be what is driving active inflammation in this subset.

IFNG is located on chromosome 12 and is highly conserved through evolution. T cell production of IFN- γ is determined primarily at the transcriptional level through cis and trans factor binding regions. DNA methylation of CpG dinucleotides provides an additional level of regulation and has been associated with transcriptional silencing. The rs1861494 T/C polymorphism is located in the third *IFNG* intronic region which has been previously reported to possess a T-bet binding site and enhancer activity (37, 38). Functionally, T allele carriage was correlated with enhanced IFN- γ secretion. The T to C substitution introduces a new CpG dinucleotide sequence that was associated with increased DNA methylation and

decreased IFN- γ expression. Electromobility shift assays indicated more intense binding of nuclear protein complex to the T compared to C allele following T cell activation. Moreover, methylation of the CpG sequence within the context of oligonucleotides complementary to the C allele, demonstrated altered nuclear complex formation and the presence of an additional complex. More intense binding of nuclear protein complex to the T and C alleles has been previously reported in nuclear extracts from the Jurkat cell line (20) and additional complex was detected binding the C allele in PHA-blasted T cells (19). This report is the first to demonstrate methylation dependent binding of nucleoprotein in the context of the rs1861494 C allele and suggests that nucleoprotein binding to this region may be regulated on two levels: one via allele specific affinity for DNA-binding proteins and a second regulated by DNA-methylation-mediated nucleoprotein binding.

We have previously reported distinct epigenetic *IFNG* DNA methylation patterns in patients with IBD compared to normal controls (25, 39). Decreased *IFNG* methylation is associated with patients requiring surgery and, in UC, is functionally correlated with enhanced IFN- γ secretion and a higher seroreactivity to what are commonly thought of as “CD-associated” antigens (34). These data, together with GWAS discovery of UC risk regions on chromosome 12 upstream and downstream of *IFNG*, suggest that *IFNG* may play a more central role in the pathogenesis of UC than was originally proposed. One of the fundamental challenges of GWAS is to define the functional consequences of these genetic variations. Surprisingly, many SNPs associated with disease are in fact CpG sites, which are statistically underrepresented in the human genome. Although CpG sites are underrepresented in most non-coding DNA, they are preserved in promoter and other regulatory genomic regions (40). Thus, in addition to differences detected in DNA methylation patterns in disease, CpG SNPs can introduce sites for allele-specific DNA methylation, the functional consequences of which may affect gene expression via epigenetic mechanisms. Recent studies have suggested that allele specific methylation occurs preferentially in sites adjacent to CpG SNPs (24). *IFNG* rs1861494 sits within a region adjacent to two upstream CpG sites. We observed C allele specific methylation at the polymorphic +2109 bp site and differential DNA methylation of the adjacent +2052 CpG site but no allele specific differences were detected for the +2007 bp site. More importantly, a significant correlation was observed between the average methylation index across all three sites for both the C and T alleles and the *IFNG* promoter methylation index. Methylation of the *IFNG* promoter region has been extensively studied and is believed to play a major role in overall regulation of *IFNG* expression. While it has been generally reported that enhanced IFN- γ expression corresponds with a decrease in *IFNG* methylation for some promoter region such as the -22 CNS, we and others have demonstrated that the reverse appears to be the case (25, 39, 41). These findings suggest that epigenetic modulation via methylation of *IFNG* expression encompasses cooperative interaction across multiple regions.

The data presented here link a genotype-dependent association of the conserved *IFNG* rs1861494 SNP with allele specific DNA methylation, transcription factor complex formation and IFN- γ protein expression associated with an altered and more aggressive course of disease progression in IBD. Moreover, the data demonstrated an association of

rs1861494 with seropositivity and higher levels of ANCA autoantibodies, a biomarker for poorer therapeutic response to anti-TNF therapy. These findings suggest that *IFNG* rs1861494 may provide new insight into stratifying IBD patients based on whether their mechanism of disease is more dependent of IFN- γ expression and less dependent on TNF. This would be of great prognostic value not only in selecting patients likely to respond to anti-TNF therapy but in potentially identifying patients likely to fail primary anti-TNF therapy and initiate evaluation of other treatment options.

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References

1. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 2007; 448:427–434. [PubMed: 17653185]
2. Duerr RH, Targan SR, Landers CJ, et al. Neutrophil cytoplasmic antibodies: a link between primary sclerosing cholangitis and ulcerative colitis. *Gastroenterology*. 1991; 100:1385–1391. [PubMed: 2013383]
3. Duerr RH, Targan SR, Landers CJ, et al. Anti-neutrophil cytoplasmic antibodies in ulcerative colitis. Comparison with other colitides/diarrheal illnesses. *Gastroenterology*. 1991; 100:1590–1596. [PubMed: 2019365]
4. Halbwachs-Mecarelli L, Nusbaum P, Noel LH, et al. Antineutrophil cytoplasmic antibodies (ANCA) directed against cathepsin G in ulcerative colitis, Crohn's disease and primary sclerosing cholangitis. *Clin Exp Immunol*. 1992; 90:79–84. [PubMed: 1327593]
5. Cohavy O, Bruckner D, Gordon LK, et al. Colonic bacteria express an ulcerative colitis pANCA-related protein epitope. *Infection and immunity*. 2000; 68:1542–1548. [PubMed: 10678972]
6. Landers CJ, Cohavy O, Misra R, et al. Selected loss of tolerance evidenced by Crohn's disease-associated immune responses to auto- and microbial antigens. *Gastroenterology*. 2002; 123:689–699. [PubMed: 12198693]
7. Targan SR, Landers CJ, Yang H, et al. Antibodies to CBir1 flagellin define a unique response that is associated independently with complicated Crohn's disease. *Gastroenterology*. 2005; 128:2020–2028. [PubMed: 15940634]
8. Mow WS, Vasilias EA, Lin YC, et al. Association of antibody responses to microbial antigens and complications of small bowel Crohn's disease. *Gastroenterology*. 2004; 126:414–424. [PubMed: 14762777]
9. Papadakis KA, Yang H, Ippoliti A, et al. Anti-flagellin (CBir1) phenotypic and genetic Crohn's disease associations. *Inflamm Bowel Dis*. 2007; 13:524–530. [PubMed: 17260364]
10. Spivak J, Landers CJ, Vasilias EA, et al. Antibodies to I2 predict clinical response to fecal diversion in Crohn's disease. *Inflamm Bowel Dis*. 2006; 12:1122–1130. [PubMed: 17119386]
11. Vecchi M, Bianchi MB, Calabresi C, et al. Long-term observation of the perinuclear anti-neutrophil cytoplasmic antibody status in ulcerative colitis patients. *Scandinavian journal of gastroenterology*. 1998; 33:170–173. [PubMed: 9517528]
12. Fleshner PR, Vasilias EA, Kam LY, et al. High level perinuclear antineutrophil cytoplasmic antibody (pANCA) in ulcerative colitis patients before colectomy predicts the development of chronic pouchitis after ileal pouch-anal anastomosis. *Gut*. 2001; 49:671–677. [PubMed: 11600470]

13. Jostins L, Ripke S, Weersma RK, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 2012; 491:119–124. [PubMed: 23128233]
14. Silverberg MS, Cho JH, Rioux JD, et al. Ulcerative colitis-risk loci on chromosomes 1p36 and 12q15 found by genome-wide association study. *Nat Genet*. 2009; 41:216–220. [PubMed: 19122664]
15. Casanova JL, Abel L. Genetic dissection of immunity to mycobacteria: the human model. *Annu Rev Immunol*. 2002; 20:581–620. [PubMed: 11861613]
16. Smart JM, Horak E, Kemp AS, et al. Polyclonal and allergen-induced cytokine responses in adults with asthma: resolution of asthma is associated with normalization of IFN-gamma responses. *The Journal of allergy and clinical immunology*. 2002; 110:450–456. [PubMed: 12209093]
17. Baccala R, Kono DH, Theofilopoulos AN. Interferons as pathogenic effectors in autoimmunity. *Immunological reviews*. 2005; 204:9–26. [PubMed: 15790347]
18. Etokebe GE, Bulat-Kardum L, Johansen MS, et al. Interferon-gamma gene (T874A and G2109A) polymorphisms are associated with microscopy-positive tuberculosis. *Scandinavian journal of immunology*. 2006; 63:136–141. [PubMed: 16476013]
19. Chevillard C, Moukoko CE, Elwali NE, et al. IFN-gamma polymorphisms (IFN-gamma +2109 and IFN-gamma +3810) are associated with severe hepatic fibrosis in human hepatic schistosomiasis (*Schistosoma mansoni*). *J Immunol*. 2003; 171:5596–5601. [PubMed: 14607968]
20. Kumar A, Ghosh B. A single nucleotide polymorphism (A → G) in intron 3 of IFN-gamma gene is associated with asthma. *Genes Immun*. 2008; 9:294–301. [PubMed: 18385742]
21. Haritunians T, Taylor KD, Targan SR, et al. Genetic predictors of medically refractory ulcerative colitis. *Inflamm Bowel Dis*. 2010; 16:1830–1840. [PubMed: 20848476]
22. Targan SR, Landers C, Vidrich A, et al. High-titer antineutrophil cytoplasmic antibodies in type-1 autoimmune hepatitis. *Gastroenterology*. 1995; 108:1159–1166. [PubMed: 7698584]
23. Blumberg RS, Saubermann LJ, Strober W. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Current opinion in immunology*. 1999; 11:648–656. [PubMed: 10631550]
24. Shoemaker R, Deng J, Wang W, et al. Allele-specific methylation is prevalent and is contributed by CpG-SNPs in the human genome. *Genome Res*. 20:883–889. [PubMed: 20418490]
25. Gonsky R, Deem RL, Targan SR. Distinct Methylation of IFNG in the Gut. *J Interferon Cytokine Res*. 2009; 29:407–414. [PubMed: 19450149]
26. Camoglio L, Te Velde AA, Tigges AJ, et al. Altered expression of interferon-gamma and interleukin-4 in inflammatory bowel disease. *Inflamm Bowel Dis*. 1998; 4:285–290. [PubMed: 9836081]
27. Peresi E, Oliveira LR, da Silva WL, et al. Cytokine Polymorphisms, Their Influence and Levels in Brazilian Patients with Pulmonary Tuberculosis during Antituberculosis Treatment. *Tuberculosis research and treatment*. 2013; 2013:285094. [PubMed: 23634300]
28. Kim DH, Kong JH, Byeun JY, et al. The IFNG (IFN-gamma) genotype predicts cytogenetic and molecular response to imatinib therapy in chronic myeloid leukemia. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2010; 16:5339–5350. [PubMed: 20959405]
29. Peyrin-Biroulet L, Loftus EV Jr, Colombel JF, et al. The natural history of adult Crohn's disease in population-based cohorts. *Am J Gastroenterol*. 2010; 105:289–297. [PubMed: 19861953]
30. Sandborn WJ, Landers CJ, Tremaine WJ, et al. Association of antineutrophil cytoplasmic antibodies with resistance to treatment of left-sided ulcerative colitis: results of a pilot study. *Mayo Clinic proceedings*. 1996; 71:431–436. [PubMed: 8628021]
31. Ferrante M, Vermeire S, Katsanos KH, et al. Predictors of early response to infliximab in patients with ulcerative colitis. *Inflamm Bowel Dis*. 2007; 13:123–128. [PubMed: 17206703]
32. Jurgens M, Laubender RP, Hartl F, et al. Disease activity, ANCA, and IL23R genotype status determine early response to infliximab in patients with ulcerative colitis. *Am J Gastroenterol*. 2010; 105:1811–1819. [PubMed: 20197757]
33. Chaparro M, Guerra I, Munoz-Linares P, et al. Systematic review: antibodies and anti-TNF-alpha levels in inflammatory bowel disease. *Alimentary pharmacology & therapeutics*. 2012

34. Dubinsky MC, Mei L, Friedman M, et al. Genome wide association (GWA) predictors of anti-TNF α therapeutic responsiveness in pediatric inflammatory bowel disease. *Inflamm Bowel Dis.* 2010; 16:1357–1366. [PubMed: 20014019]
35. Taylor KD, Plevy SE, Yang H, et al. ANCA pattern and LTA haplotype relationship to clinical responses to anti-TNF antibody treatment in Crohn's disease. *Gastroenterology.* 2001; 120:1347–1355. [PubMed: 11313304]
36. Esters N, Vermeire S, Joossens S, et al. Serological markers for prediction of response to anti-tumor necrosis factor treatment in Crohn's disease. *Am J Gastroenterol.* 2002; 97:1458–1462. [PubMed: 12094865]
37. Young HA, Ghosh P. Molecular regulation of cytokine gene expression: interferon-gamma as a model system. *Progress in nucleic acid research and molecular biology.* 1997; 56:109–127. [PubMed: 9187053]
38. Szabo SJ, Kim ST, Costa GL, et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell.* 2000; 100:655–669. [PubMed: 10761931]
39. Gonsky R, Deem RL, Landers CJ, et al. Distinct IFNG methylation in a subset of ulcerative colitis patients based on reactivity to microbial antigens. *Inflamm Bowel Dis.* 2011; 17:171–178. [PubMed: 20848535]
40. Kondrashov FA, Ogurtsov AY, Kondrashov AS. Selection in favor of nucleotides G and C diversifies evolution rates and levels of polymorphism at mammalian synonymous sites. *J Theor Biol.* 2006; 240:616–626. [PubMed: 16343547]
41. Schoenborn JR, Dorschner MO, Sekimata M, et al. Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. *Nat Immunol.* 2007; 8:732–742. [PubMed: 17546033]

Figure 1.
Schematic of *IFNG* gene showing conserved regions between human and mouse.

Figure 2.

Relationship of rs1861494 to serological markers and time to surgery. (A), rs1861494 SNP allele distribution in IBD patients as a function of sero-reactivity to ANCA; (B), rs1861494 T allele association with ANCA EU in UC patients ($n=17$ for C, $n=39$ for T); (C), Time to surgery curve of medically refractive UC patients based on rs1861494 allele genotype ($n=13$ for C, $n=153$ for T). Bars represent means $\pm$ SEM.

Figure 3.

rs1861494 T allele, compared to C allele, genotype is associated with more severe disease behavior in CD patients. Frequency of non-stricturing/penetrating vs. complicated (stricturing/penetrating) disease phenotype was determined in 44 CD patients.

Figure 4. Secretion of IFN- γ from IBD T cells stimulated with anti-CD3 antibody for 24 h ($n=28$ for C, $n=104$ for T). Bars represent means $\pm$ SEM.

Figure 5.

Allele/strand specific methylation of IBD patients heterozygous for rs1861494. (A), rs1861494; (B), CpG at +2052 and +2007; (C), Correlation of methylation of rs1861494 with methylation index of *IFNG* promoter (CpG sites at -54, -186, and -295 bp) ($n=36$). Bars represent means $\pm$ SEM.

Figure 6.

EMSA analysis of rs1861494 regulatory binding proteins. PBMC were stimulated up to 4 h with PMA/ionomycin and nuclear protein extracts were obtained. EMSAs were performed for binding activity to the (A), C or T SNP; (B) kinetics of nucleo-protein binding to the non-methylated (C SNP), asymmetrically methylated (one strand, C/MC SNP) or symmetrically methylated (both strands, MC-MC SNP) oligonucleotide. Representative of 4 experiments.

Table 1

PCR and pyrosequencing primers and conditions.

Assay CpG	Primers	PCR Conditions
-295, -186	Fwd PCR	GTGAATGAAGAGTTAATAATTTATTAGG
	Rev PCR	Biotin-TTCCTTTAAACTCCTTAAATCCTT
	Seq	GTGAATGAAGAGTTAATAATTTATTAGG
	Seq	GGTGGGTATAATGGGTTTG
-54	Fwd PCR	GGATTTAAGGAGTTTAAAGGAAA
	Rev PCR	Biotin-AAAACAATATATACTACACCTCCTCT
	Seq	TTAAAAAATTTGTGAA
	Seq	TTAAAAAATTTGTGAA
Allele-specific	Fwd PCR	TTTGGGTGATTTTGTAAAGTTAGTAAAGAGA
	Rev PCR	CCATTAAAAACAACAACCTCTCAT
	Seq	AAACTACTTCTCAATACTCC
	Seq	ACTACTTCTCAATAGTCCCT
T-specific		ACTACTTCTCAATAGTCCCT
C-specific		ACTACTTCTCAATAGTCCCT