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genetic risk factors for *clostridium difficile* infection in Ulcerative Colitis

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Abstract

Introduction—Patients with inflammatory bowel disease (IBD) are at higher risk for *Clostridium difficile* infection (CDI). Disruption of gut microbiome and interaction with the intestinal immune system are essential mechanisms for pathogenesis of both CDI and IBD. Whether genetic polymorphisms associated with susceptibility to IBD are also associated with risk of CDI is unknown.

Methods—We used a prospective registry of patients from a tertiary referral hospital. Medical record review was performed to identify all ulcerative colitis (UC) patients within the registry with a history of CDI. All patients were genotyped on the Immunochip. We examined the association between the 163 risk loci for IBD and risk of CDI using a dominant genetic model. Model performance was examined using receiver operating characteristics curves.

Results—The study included 319 patients of whom 29 developed CDI (9%). Female gender and pancolitis were associated with increased risk while use of anti-TNF was protective against CDI. Six genetic polymorphisms including those at TNFRSF14 (Odds ratio (OR) 6.0, p-value 0.01) were associated with increased risk while 2 loci were inversely associated. On multivariate analysis, none of the clinical parameters retained significance after adjusting for genetics. Presence of at least one high risk locus was associated with an increase in risk for CDI (20% vs.

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1%) ($p=6 \times 10^{-9}$). Compared to 11% for a clinical model, the genetic loci explained 28% of the variance in CDI risk and had a greater AUROC.

Conclusion—Host genetics may influence susceptibility to CDI in patients with ulcerative colitis.

Keywords

Clostridium difficile; ulcerative colitis; tumor necrosis factor; colectomy; genetics

INTRODUCTION

Clostridium difficile (*C difficile*), a gram negative anaerobe first described as the causative organism for antibiotic associated pseudomembranous colitis, is an important cause of nosocomial morbidity and mortality^{1–9}. The past two decades have seen a rising incidence of *C difficile* infection (CDI) with the emergence of hypervirulent strains, regional epidemics, and increasing failure rates of commonly used first-line therapies^{7, 10–12}. Host, pathogen, and environmental factors all have important contributions towards the pathogenesis of CDI^{13–15}. The development of symptomatic CDI is favored strongly through the disruption of the normal host microbial flora^{8, 15–18}. *C difficile* exerts its effect primarily through the production of enterotoxins that disrupt the epithelial barrier leading to secretory diarrhea^{8, 15}. However, whether infection leads to asymptomatic colonization or symptomatic disease also depends on host immune responses¹³.

The role of CDI in patients with inflammatory bowel diseases (IBD) is an area of growing interest^{19–26}. Extrinsic factors related to CDI in IBD patients include immunosuppression²⁷ or corticosteroid use²⁸, while traditional risk factors including antibiotics or healthcare contact are less common in those with IBD²⁷. In particular, patients with ulcerative colitis (UC) are more susceptible to CDI, have greater morbidity, and need for surgery. Furthermore, active colonic disease or more extensive colitis also appears to favor development of CDI^{29, 30}. A total of 163 distinct loci that mediate susceptibility to Crohn's disease or UC have been identified encompassing a spectrum of innate and adaptive immune responses, maintenance of epithelial barrier function, pathogen sensing, and modulation of activation thresholds for adaptive immunity^{31, 32}. Several of these loci also have an important role in host-microbial interaction and are involved in susceptibility to infections. Given the key role of host microflora in determining risk of both CDI and IBD independently, and the interplay between the microbiome and the host immune system in both diseases, it is plausible that there is a shared genetic predisposition for both diseases. As well, IBD patients having a greater complement of such polymorphisms may be at a higher risk for CDI. However, the association between IBD risk loci and risk of CDI in patients with UC has not been examined previously.

The aims of our study were to use a well characterized and genotyped cohort of patients with UC to (1) identify clinical risk factors for CDI; (2) examine if any of the IBD genetic risk loci were associated with CDI; and (3) to compare the performance of predictive models using clinical and genetic risk factors in determining risk of CDI.

METHODS

Study Cohort

Patients included in the study were from the Prospective Registry in IBD Study at Massachusetts General Hospital (PRISM). This is a prospective cohort initiated in 2005 with ongoing recruitment that enrolls patients from within the physician practices at the Crohn's and Colitis center at Massachusetts General Hospital (MGH). Enrollment criteria include

age > 18 years, a diagnosis of Crohn's disease, UC, or indeterminate colitis and provision of informed consent. An initial questionnaire detailed demographic (age, gender, race) and disease-related information. For all patients, disease extent was classified as limited or pancolitis depending on extension beyond the splenic flexure according to the Montreal classification. Smoking history was classified as ever or never use. Information on medications used to treat UC was obtained at study enrollment and subsequently updated through end of follow-up (for controls) or at the time of CDI (for cases). These also included medications that were taken prior to enrollment within PRISM.

Careful medical record review was performed to identify those patients who had > 1 episode of CDI during the course of their disease. This was performed by free text search within the entire electronic medical record (EMR) comprising outpatient notes, laboratory tests, and hospitalizations (dating back to 1996) for the terms "*C difficile*" or "*clostridium difficile*" or "*c diff*" and other relevant variations. Diagnosis of CDI was then confirmed through review of the EMR and formed our case population. For patients who had their diagnosis made at our facility, a diagnosis of CDI required confirmation with a stool enzyme linked immunosorbent assay (ELISA) against toxins A and B. Patients who had their diagnosis prior to receiving care at our center were identified based on medical record notation of a history of CDI with positive stool toxin. A board certified gastroenterologist (A.N.A) confirmed all cases. The medical records were reviewed for all patients that were included in our study and case ascertainment was performed blinded to genetic status. These patients represented our entire UC cohort who had been genotyped on the Immunochip. UC patients who did not have a history of CDI formed our controls. All patients gave their written informed consent to participate in PRISM after approval of the project by the Massachusetts General Hospital Institutional Review Board.

Genotyping

All patients enrolling in PRISM provided blood samples. Genomic DNA was extracted from the buffy coat and genotyping performed at the Broad Institute (Cambridge, MA) on the Immunochip. This is an Illumina genotyping chip containing 196,524 polymorphisms designed to perform deep replication of autoimmune and inflammatory disease loci, and fine mapping of established genome wide significant loci³³. For the purpose of this study, we included the 163 distinct single nucleotide polymorphisms (SNP) that confer susceptibility to either UC or Crohn's disease³¹. We restricted our analysis to these SNPs in our exploratory analysis because of their known association with innate or adaptive immune responses, disruption of epithelial barrier, or host-microbial interaction, which are key processes in the pathogenesis of CDI. Also, given the association between CDI and underlying UC, it is mechanistically plausible that the strength of association between the two (compared to non-IBD patients) is mediated through risk loci that confer susceptibility to IBD. All 163 SNPs met the Hardy-Weinberg equilibrium threshold of $p > 0.001$ and genotyping call rate of > 99%. All patients included in this study also had a genotyping success rate > 80%. Genotype calling was made using optiCall³⁴. For our primary analysis, we assumed a dominant model based on the presence of rare allele. As the exact genetic variants represented by several of the IBD-related SNPs remain incompletely defined, the distinction between the functional effects of heterozygosity compared to homozygosity for the risk alleles remains challenging. Owing to the limited number of cases, and relatively rare occurrence of the minor alleles for some of the SNPs, we selected a dominant model for our primary analysis. However, we also examined an allelic model with most significant SNPs meeting $p < 0.05$ on both models.

Statistical Analysis

Continuous variables were summarized as medians with inter-quartile ranges and compared using non-parametric methods. Categorical variables were expressed as proportions and compared using the chi-square test with the Fisher's test used when number of patients in a cell was five or less. Genetic analysis was performed using PLINK v1.07³⁵. All SNPs meeting a p-value ≤ 0.05 were included in our multivariate analysis. As this was an exploratory hypothesis-generating study and restricted to a case-only population (i.e. those with UC), we did not perform correction for multiple testing. Both clinical variables and genetic risk loci significant on univariate analysis were entered into a combined multivariate logistic regression model. To reduce the impact of the small number of cases, the multivariate model was run using bootstrapping with 1000 replications. The performance of the model in predicting CDI was assessed using the area under the receiver operating characteristics curve (AUROC) and the proportion of variation examined was determined using the pseudo-R² of the logistic regression model. We also performed a time to event analysis examining whether the presence of the risk alleles was associated with earlier time to CDI in the course of UC. Statistical analysis was performed using Stata 11.0 (Stata Corp, College Station, TX).

RESULTS

Study Cohort

Our study included 319 patients with UC (median age 43 years) with a median age at diagnosis of 27 years (interquartile range (IQR) 20 – 38 years). All patients were of Caucasian ethnicity. Just over half the cohort were women (53%). A similar proportion had pancolitis (52%) and 40% had ever required biologic anti-TNF therapy. A total of 29 patients developed CDI (9%). The median time between UC diagnosis and CDI was 2 years (IQR 1 – 7 years) while the mean duration of disease in the no-CDI group was 11 years (IQR 7 – 21).

Table 1 compares the characteristics of patients with and without CDI. Patients who developed CDI were younger (mean age 35 vs. 45 years, $p = 0.0002$) and more likely to have pancolitis (69% vs. 51%, $p = 0.06$). A smaller proportion of CDI patients had ever been on anti-TNF therapy (24% vs. 42%, $p = 0.06$) but there was no difference in the use of immunomodulators or 5-aminosalicylates. Consistent with prior literature, CDI patients had a greater likelihood of requiring colectomy (31% vs. 14%, $p = 0.02$).

Genetic Associations: Univariate Analysis

Supplemental Table 1 presents the allelic frequencies for all the 163 immuno-chip risk SNPs. On univariate analysis, 6 genetic loci were positively associated with CDI while 2 loci were inversely associated. Figure 1 illustrates the frequency of occurrence of the rare allele (assuming dominance) for CDI cases vs. non-CDI controls. The strongest associations were seen at rs864745 (CREB5) (Odds ratio (OR) 10.93, 95% CI 1.46 – 81.66, $p = 0.01$) and rs6667605 (TNFRSF14) (OR 6.00, 95% CI 1.40 – 25.80, $p = 0.01$) (Table 2). Two loci were less common in CDI cases - rs4409764 (NKX2-3) and rs11229555 (CNTF, LPXN). For seven of the loci, the risk allele for CDI was the same as the Immuno-chip IBD risk allele. Among the 8 loci, 6 were risk alleles for both Crohn's disease and UC; TNFRSF14 was a risk allele for UC alone, while CREB5 is associated only with Crohn's disease.

Multivariate Analysis

Multivariate logistic regression was performed to identify predictors of CDI. In a model consisting of clinical variables alone, four parameters were independently associated with CDI. Female gender (OR 3.07, 95% CI 1.26 – 7.52) and pancolitis (OR 2.52, 95% CI

1.03 – 6.17) increased risk of CDI while age > 55 years (OR 0.21, 95% CI 0.05 – 0.90) and use of biologic anti-TNF (OR 0.29, 95% CI 0.12 – 0.76) reduced risk of CDI. Among the genetic predictors, 7 of the 8 polymorphisms identified on univariate analysis were independently significant on the multivariate analysis as well with one locus (rs17085007) achieving borderline significance ($p = 0.056$). The effect sizes for each individual locus remained relatively unchanged. Presence of at least one high risk locus was associated with an increase in risk for CDI (20% vs. 1%) ($p = 6 \times 10^{-9}$). There was also a linear increase in the risk of CDI with increasing number of risk loci (or absence of protective loci). Over half the patients with 7 (9 out of 14 patients) or 8 (1 out of 2 patients) at-risk polymorphisms developed CDI compared to none of those with 3 or fewer loci ($p = 1 \times 10^{-9}$). Each 1 additional locus increased risk of CDI three-fold (OR 3.57, 95% CI 2.32 – 5.51). On a stepwise multivariate model combining clinical and genetic risk factors, none of the clinical risk factors were retained while 7 of the genetic risk loci achieved independent statistical significance. We also performed a time to event analysis examining whether increasing complement of genetic risk loci was associated with CDI earlier in the course of UC. As visualized in Figure 2, increasing number of risk loci was also associated with earlier occurrence of CDI (log-rank p -value < 0.0001). All the risk alleles also demonstrated statistical significance at $p < 0.05$ in an allelic model (data not shown). For example, one of our strongest associations was for the rs6667605 allele. The frequency of CDI among those with CC, CT, and TT genotypes was 2.2%, 12.1% and 11.7% respectively confirming similar effects of the heterozygous and homozygous states. The allelic analysis also demonstrated another potentially important locus in CDI pathogenesis at the NF- κ B 1 gene (rs3774959) (OR 2.0, 95% CI 1.16 – 3.45).

The model comprising only clinical variables (age, sex, pancolitis, anti-TNF use) had a fair performance in predicting CDI with an AUROC of 0.74 (pseudo-R² 0.11). However, a model incorporating the independently significant genetic risk alleles had a superior AUROC (0.87, $p < 0.0001$ compared to the clinical model) and explained a greater proportion of the variance in CDI risk (pseudo-R² 0.28). The only clinical parameter that retained significance in a multivariate model also incorporating genetics was increasing age, with borderline significance for female sex ($p = 0.08$) and anti-TNF use ($p = 0.13$). The ROC of this combined model was 0.89 ($p = 0.008$ compared to genetics only model; pseudo-R² 0.35).

To examine the possibility whether these loci were specifically associated with CDI or were merely markers of more aggressive disease (pancolitis, need for anti-TNF use or colectomy), we analyzed the association between the genetic risk score and the above outcomes. The genetic risk loci identified were not predictive of the presence of pancolitis ($p = 0.29$), anti-TNF therapy ($p = 0.10$), or colectomy (0.21) in the entire cohort.

DISCUSSION

Clostridium difficile infection is a growing concern in patients with underlying IBD. The mechanisms for an increased risk of CDI in patients with IBD, in particular the role of host genetic susceptibility, have not been well defined. In this exploratory analysis using a large UC cohort genotyped on the Immunochip, we demonstrate that 6 IBD-risk loci were associated with increased risk of CDI while 2 risk loci were protective. Genetic risk alleles explained a larger portion of the variance in CDI risk than clinical factors suggesting that host immunity may play an important role in susceptibility to CDI.

The co-occurrence of CDI in patients with IBD is associated with increased need for hospitalizations, surgery, and escalation of therapy for underlying IBD^{26, 36}. Indeed, IBD patients with CDI may have up to four-fold increase in mortality²⁶. Extrinsic risk factors

including antibiotic use or healthcare contact appear less common in IBD patients²⁷. In contrast, intrinsic risk factors including extent and severity of colitis, and need for aggressive therapy increase risk of CDI, but may be determined by host genetics^{27–30}. Another mechanism through which host genetics may predispose to CDI risk is through regulation of host humoral immune response¹⁵. Individuals with a greater antibody response to CDI toxins are more likely to be asymptomatic carriers while those with lower antibody levels are more likely to develop diarrhea after initial colonization^{13, 37} and have greater rates of recurrence^{37, 38}. As well, the innate immune response has also been hypothesized to play a role. *C. difficile* mediates its effect primarily through the actions of two enterotoxins that catalyze glucosylation of Rho proteins leading to cytoskeletal disruption⁸. The resultant inflammatory response is mediated through several innate immunity pathways including those involving NF- κ B and MAP kinase signaling^{39, 40}. Consequently, variants in pathways involved in mediating this innate immune response could influence risk of CDI. In our study, we found 7 IBD-related risk loci to achieve statistical significance at $p < 0.05$ on multivariate analysis with one locus achieving borderline significance in its association with CDI. Indeed, several of these risk loci have been previously described as having important role in host-microbial interactions and mediating susceptibility to various infections, thus demonstrating biological plausibility.

One of the strongest associations we identified was a UC-specific locus associated with the tumor necrosis factor receptor (TNFRSF14), also known as the herpes virus entry mediator (*Hvem*)^{41, 42}. *Hvem* is expressed both in colonic epithelial cells and CD45 antigen carrying leukocytes and plays an important role in maintaining the colonic barrier against pathogenic bacteria⁴². In animal models of colitis, *Hvem* enhances NF- κ B mediated Stat3 activation. *Hvem*^{-/-} mice demonstrate impaired immune responses in the colon, greater bacterial burden following intestinal *Citrobacter rodentium* infection, and increased mortality⁴².

Another interesting finding in our study regarding the potential role of TNF-mediated pathways in CDI pathogenesis is that patients on anti-TNF biologic therapy demonstrate a lower risk of CDI, an effect that was not seen with aminosalicylates or immunomodulators. Previous studies have demonstrated an increase in CDI risk with both corticosteroids as well as immunomodulators^{27, 28}. Since most of the prior studies were done before to widespread anti-TNF use in UC, it is difficult to compare our findings with previous ones. However, one reason for lower risk with anti-TNF therapy may be that such biologics, despite being a marker for severity of disease, may be associated with greater rates of endoscopic healing than immunomodulator or steroid therapies, thereby potentially reducing the disruption of the microbiome and lowering risk of CDI. This association merits further exploration in other cohorts.

Some of the other loci associated with CDI in our study also have plausible biological mechanisms. The endoplasmic reticulum aminopeptidases (ERAP1 and 2) are involved in the regulation of innate immune responses⁴³. ERAP1 forms a complex with the tumor necrosis factor receptor (TNFR1) and is involved in cytokine receptor shedding. ERAP-1 may also play an important role in the immune response to *Toxoplasma gondii* infection as well as that to the human immunodeficiency virus (HIV), lymphocytic choriomeningitis virus, human papilloma virus, and cytomegalovirus (CMV)⁴³. Another gene proximate to the same locus is the leucyl/cystinyl aminopeptidase (LNPEP) gene within the vasopressin pathway which is associated with increased mortality in septic shock⁴⁴. One of the protective loci we identified in our study was at rs10896794; a gene associated with this locus is leupaxin (LPXN) which may play a role in B-cell receptor signaling and B cell function⁴⁵. Variants at the NKX2-3 locus were inversely associated with risk of CDI. This locus on 10q24.2 is associated with susceptibility to Crohn's disease in the Wellcome Trust Case Control Consortium genome-wide analysis and in subsequent replication studies⁴⁶,

while nominal associations have been demonstrated with UC. This gene is important for development of small intestinal epithelium and lymphoid tissue; mice deficient in this transcription factor demonstrate reduced proliferation of the intestinal epithelium and delayed formation of intestinal villi⁴⁷. In the allelic model, a mutation on chr4 at the NF- κ B1 locus was also associated with increased risk for CDI. Several studies have demonstrated that the effect of *C. difficile* toxin, in particular toxin A, is mediated through the NF- κ B signaling pathway^{39, 48}.

There are a few implications to our findings. To our knowledge, ours is the first study examining the association between host genetics and risk of CDI in a cohort of patients with IBD. Our findings suggest that host genetic variants involved in regulation of innate or adaptive immunity, or epithelial barrier function may confer increased risk or protection against CDI; indeed such genetic variants may explain a greater proportion of the variance in disease risk than clinical parameters. However, replication in larger cohorts is essential to better define the set of risk loci and magnitude of such associations. Further functional studies and knockout mouse models of the identified polymorphisms are essential to further tease out the functional consequences of the relevant loci. If genetic risks can be confirmed in other cohorts, then it is plausible that individuals at a higher genetic risk for CDI be considered for prophylaxis in the setting of specific extrinsic risk factors such as antibiotic use or hospitalization. Furthermore, we restricted this exploratory analysis to the 163 risk loci associated with IBD³¹. Whether other genetic risk loci are associated with CDI requires further exploration as are studies that examine risk alleles associated with CDI risk in the non-IBD population.

There are several limitations to our study. First, our sample size was relatively small for a genetic association study, limiting our ability to define weaker associations. However, CDI is not an outcome that is captured and validated in most cohorts with detailed genotyping; thus our cohort and analysis is novel. Furthermore, to our knowledge, no published studies have examined the association between host genetics and risk of CDI even in a non-IBD population. Second, our controls included patients who had no history of CDI on medical record review, but did not all undergo testing to rule out colonization. Diagnosis of CDI during the entire study period was made using a toxin EIA which has been demonstrated to have suboptimal sensitivity in diagnosis of CDI compared to two-step algorithms including an initial glutamate dehydrogenase (GDH) screen followed by confirmatory polymerase chain reaction (PCR) testing⁴⁹. While this may have theoretically resulted in some cases of CDI being missed and classified as 'non-cases', both of these misclassifications would bias towards the null suggesting that our effect sizes are a conservative estimate. Third, we did not have information on episodic extrinsic risk factors including antibiotic use, healthcare contact prior to CDI, acid suppressive medication or corticosteroid use. Whether such extrinsic risk factors demonstrate an interaction with host genetics in defining CDI risk merits exploration in future studies. Fourth, given the limited number of cases, we were limited in the degrees of freedom for our multivariate model. We attempted to minimize the effect of a lower event: variable ratio by using stepwise regression methods to develop the most parsimonious model, and by examining the effect of the genetic variants using a cumulative genetic risk score modeled as a continuous variable, thereby increasing statistical power. As demonstrated in Table 2, we also observed only minor variations in the strength of association on univariate and multivariate modeling, supporting the robustness of our final adjusted models. Finally, we also did not correct for multiple testing in our analysis as our study was a hypothesis-generating exploratory analysis in the absence of prior published data determining SNP selection. Larger cohorts with a greater number of CDI patients are required to replicate our associations, identify other potential risk loci, and define strength of association.

In conclusion, we demonstrate that specific host genetic risk alleles may be associated with CDI in patients with UC, particularly those involving the TNF or NF- κ B signaling pathways. However, our study included a limited number of cases and is an exploratory analysis. Further validation studies in larger cohorts including those with CD, and without IBD are required, and may help further define pathogenesis of CDI as well as identify high risk subgroups that may benefit from prophylactic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Distribution of risk alleles in *clostridium difficile* cases and controls in a cohort of patients with ulcerative colitis
 UC – ulcerative colitis; CDI – Clostridium difficile infection

Figure 2. Association between genetic risk burden and risk of *Clostridium difficile* infection in patients with ulcerative colitis

The number of patients in the risk categories 0–2 risk loci, 3–4 risk loci, 5–6 risk loci, and 7–8 risk loci 39, 138, 122, and 16. A total of 173 patients were followed-up for 10 years or longer after UC diagnosis.

Table 1

Characteristics of study cohort

Characteristic	<i>C difficile</i> infection (n = 29) %	No <i>C difficile</i> infection (n = 286) %	p-value
Mean age (in years) (SD)	35 (15)	45 (14)	0.0002
Mean age at diagnosis (in years) (SD)	30 (13)	30 (13)	0.97
Female	69	52	0.07
Pancolitis	69	51	0.06
Ever smoker	35	40	0.57
Medications ever use			
Aminosalicylates	100	92	0.11
Immunomodulator	75	66	0.32
Anti-TNF biologics	24	42	0.06
Colectomy	31	14	0.02

SD – standard deviation; *C difficile* – *clostridium difficile*; anti-TNF – monoclonal antibodies to tumor necrosis factor α (infliximab, adalimumab, certolizumab pegol)

Table 2
Univariate analysis of risk alleles associated with *Clostridium difficile* infection in ulcerative colitis patients

CHR	dbSNP ID	Key Genes	Risk allele	Univariate OR	P-value	Adjusted OR [†]	Immunochip risk allele	Disease association
1	rs6667605	TNFRSF14, MMEL1, PLCH2	T	6.00	0.01602	5.67	T	UC
5	rs1363907	ERAP2, ERAP1, LNPEP	A	2.96	0.03235	3.67	G	IBD
5	rs10051722		C	4.18	0.004729	4.74	C	IBD
7	rs864745	CREB5, JAZF1	G	10.93	0.0198	9.39	C	CD
10	rs4409764*	NKX2-3	G	0.42	0.02918	0.20	G	IBD
11	rs11229555*	CNTF, LPXN	T	0.32	0.01672	0.30	T	IBD
13	rs17085007		G	2.56	0.01803	2.36	T	IBD
13	rs941823		T	2.55	0.01736	2.76	T	IBD

UC – ulcerative colitis; CD – Crohn's disease; IBD – inflammatory bowel disease; OR – odds ratio; Immunochip

[†] Mutually adjusted for the other SNPs presented in the table