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High Frequency Haplotypes in the X Chromosome Locus TLR8 are Associated with Both CD and UC in Females

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Abstract

Background—TNF- α and IL-1 have been associated with mucosal inflammation in both CD and UC. Innate immune defects have been associated with CD, specifically CARD15/NOD2. Recently Toll-like receptor 8 (TLR8) signaling has been shown to enhance generation of both cytokines. Interestingly, TLR8 is located on the X chromosome and IBD has been associated with abnormalities of the X chromosome.

Aims—To test whether TLR8 haplotypes are associated with IBD.

Methods—Subjects (735 CD, 343 UC, 245 controls) were genotyped. SNPs were chosen to tag common Caucasian haplotypes.

Results—Both a “risk (H4)” and a “protective (H1)” TLR8 haplotypes were observed associated with CD in females. Eighteen percent of CD females had H4 compared with 9% of controls ($p=0.02$). Fifty-nine percent of CD females had H1 compared with 72% of controls ($p=0.01$). H1 was also negatively associated with UC in females (59% of UC, 72% of controls $p=0.03$). Diplotype analysis of CD, UC, and all IBD in females revealed that two protective haplotypes (H1/H1) had a markedly diminished odds ratio, 0.4–0.5. The presence of a risk haplotype (H4/not H1) had a significantly increased odds ratio, 2.0–2.2. Thus, the risk for IBD was 4 to 5 times higher in females with one risk haplotype than with the protective/protective diplotype.

Conclusions—TLR8 is an X-linked IBD susceptibility gene, with both common predisposing and protecting haplotypes. These associations further emphasize the importance of genetic variation in innate immunity as determinants, not only of CD, but of UC as well.

Keywords

Toll-like receptor 8; haplotype; diplotype; Crohn's Disease; ulcerative colitis

INTRODUCTION

It has been increasingly well recognized that innate immunity is important in protection from pathogenic organisms. The innate immune system recognizes microbial components,

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known as pathogen-associated molecular patterns (PAMPs), via pathogen-sensing receptors, also known as pattern recognition receptors (PRRs). PRRs include transmembrane TLRs and cytosolic nucleotide oligomerisation domain (NOD) proteins containing leucine-rich repeats (LRRs). NOD2/CARD15 gene is one of the NOD-LRR protein families. Furthermore, mutations of CARD15/NOD2 have been strongly associated with CD in multiple Caucasian populations,¹⁻⁴ i.e. inherited defects in innate immunity contribute to CD.

Toll-like receptors (TLRs) play a central role in the initiation of innate cellular immune responses. To date, at least 10 members of the TLR family in humans and 13 members of the TLR family in mice have been identified.⁵ The activation of TLR signaling pathways is initiated from the cytoplasmic Toll/IL-1 receptor (TIR) domains. TLRs mediate specific responses in association with different combinations of four TIR domain-containing adaptors such as MyD88, TIR-associated protein/MyD88 adaptor-like (TIRAP/MyD88), TIR-domain-containing adaptor protein inducing IFN- β (TRIF), and TRIF-related adaptor molecule (TRAM).⁶ In general, signaling from all TLRs except TLR3 activates MyD88 which finally leads to NF- κ B activation through IL-1R-association kinase (IRAK)-1, IRAK-4, tumor necrosis factor-associated factor 6 (TRAF6),⁷ transforming growth factor- β -activated kinase (TAK1), and canonical I κ B kinase (IKK) complex.^{6, 8, 9} Then activated NF- κ B induces multiple proinflammatory cytokines genes such as TNF- α , IL-6, and IL-1 β . This major pathway for TLRs is called the MyD88-dependent pathway.⁶

TLR8 induces proinflammatory cytokines such as IL-1, TNF- α , IL-6, and IL-12 from PBMCs, and also induces TNF- α and IL-12 from monocyte, myeloid dendritic cells via the MyD88-dependent pathway.¹⁰ TNF- α and IL-1 play important roles in mucosal inflammation in both CD and UC.¹¹ Recently, evidence has been presented that regulatory T cells (Treg) play important roles in colonic homeostasis, but do not work well in inflammatory intestinal mucosa in IBD, even though the number of Tregs has increased in IBD.¹²⁻¹⁵ Interestingly, TLR8 is expressed on Tregs, and the ligands for TLR8 can reverse Treg suppressive activity.¹⁶

Uridine-rich or uridine/guanosine-rich single-stranded RNA, derived from viruses such as human immunodeficiency virus (HIV-1) and influenza virus, or synthetic antiviral imidazoquinoline component Resiquimod (R848), can bind to TLR8 in the endosomal membrane,¹⁷⁻¹⁹ and then TLR8 signaling consequently activates NF- κ B through the MyD88-dependent pathway. Activated NF- κ B enters the nucleus to activate the proinflammatory and immune response genes. In addition to this common pathway, TLR8 has another signaling pathway which proceeds via MyD88, IRAK4, IRAK1 and IRF-7. Then activated IRF-7 also enters the nucleus and induces IFN- α/β , which mediates both immune responses and the subsequent development of adaptive immunity to viruses.^{6, 9, 20-22}

The human TLR8 gene is located on the X chromosome, specifically Xp22,^{23, 24} and abnormalities of X chromosome number and structure have been repeatedly observed with IBD.^{25, 26} For example, the incidence of IBD among the patients with Turner's syndrome, that is individuals who lack all or part of one X chromosome, is many times higher than that seen in the general population.^{27, 28} Vermeire et al. reported that molecular analysis data for the X chromosome in patients with IBD and provided suggestive evidence for the presence and chromosomal location of an X linked susceptibility gene in IBD.²⁹ Therefore, in this study, we analyzed whether genetic variation in the TLR8 gene is associated with CD, with UC, and with all of IBD.

METHODS

Subjects

We enrolled patients with CD and UC who attended the IBD clinic at Cedars-Sinai Medical Center, Los Angeles, as well as geographically and ethnically matched controls. We analyzed 735 CD samples, 343 UC samples, and 245 control samples (table 1). They were almost equally distributed between males and females. Clinical characteristics have been previously described clinically.^{4, 30} All studies were approved by the Cedars-Sinai Human Subjects Institutional Review Board.

Selection of SNPs and genotyping

DNA was isolated from Epstein Barr virus transformed lymphoblastoid cell lines using proteinase K digestion, organic extraction, and ethanol precipitation.³¹ SNPs were selected using “Tagger”³² and data from the International HapMap Project.^{33–35} SNPs rs3764879, rs2109134, rs1548731, rs 4830806, and rs5744068 were selected to tagged common Caucasian haplotypes in Tlr8 and were genotyped using Illumina Golden Gate technology^{36, 37} following the manufacturer's protocol (Illumina, San Diego, CA).

Statistical Analysis

None of the individual markers exhibited significant deviation from the Hardy-Weinberg equilibrium in our sample set ($p=0.05$ level). The haplotype block structure of TLR8 gene was evaluated by Haploview.³⁵ Since the TLR8 gene is located on the X chromosome and males only have one copy of the X chromosome, their individual haplotypes were directly observed. Thus, the male haplotypes were assigned with 100% probability. For females, since they have two copies of the X chromosome, individual haplotypes were reconstructed using the PHASE 2.0 by assigning each haplotype with maximum probability.^{38, 39} Seventy-two percent of female haplotype assignments had probabilities of 100%, and 98% had probabilities of 80% or better. For males, the presence of the haplotype was analyzed. For females, a dominant model was assumed, i.e., carriers of the particular haplotype versus non-carriers were compared. Chi-square (χ^2) tests were used to test associations between the haplotypes and disease status and disease phenotype characteristics. Multiple testing-corrected P values were calculated by permutation tests after performing 1,000 random permutations of disease status for each tested haplotype. The empirical P value was determined as a proportion of generated samples for which the value of the chi-square statistics was greater than for the observed samples. To further evaluate the gene's effect on disease among females, a haplo-genotype, also known as a diplotype analysis, was conducted by comparing the odds ratio among patients with two protective haplotypes vs. one protective haplotype vs. other combinations vs. one risk haplotype and two copies of the risk haplotype using the mean score test.⁴⁰ The latter two categories were combined because of small numbers of homozygous individuals for the risk haplotype. All analyses were first done in female and male populations separately, and then in combined samples. Similarly, analyses were done for CD alone, UC alone, and then IBD (CD and UC combined). Statistical analysis was conducted by SAS software v9.1 (SAS Institute; Cary, NC.).

RESULTS

In the TLR8 gene, two haplotype blocks were observed, but significant differences were detected only in block 1

In the TLR8 gene, two haplotype blocks were observed (fig. 1). Significant differences were only detected in block 1; therefore all subsequent analysis was limited to block 1 data. First, haplotypes in block 1 were classified in order of frequency as haplotype 1 through haplotype

4 (H1-H4). The specific alleles comprising the haplotypes in block 1 are shown in table 2; 1 refers to the major allele, and 2 refers to the minor allele. We then analyzed the association between these haplotypes in block 1 with CD, with UC, and with all of IBD.

Haplotype 1 is a protective haplotype and Haplotype 4 is a risk haplotype for IBD

In females, the carrier frequencies of haplotype 1 were 72% in controls, 59% in CD, 59% in UC, and 59% in IBD (fig. 2A). The carrier frequencies of haplotype 1 in CD, in UC, and in IBD were significantly lower than in control females, i.e. a protective effect (empirical $p=0.01$, 0.03 , 0.01 , respectively). In males, the carrier frequencies of haplotype 1 were 46% in controls, 44% in CD, 32% in UC, and 40% in all IBD (fig. 2A). The carrier frequency of haplotype 1 in UC was significantly lower than in control males (empirical $p=0.01$), i.e. a protective effect. Taken together, these data indicate haplotype 1 is a protective haplotype.

In females, in CD and in IBD, the carrier frequencies of haplotype 4 were both 18%. In controls, the carrier frequency was 9% (fig. 2B). The carrier frequencies of haplotype 4 in CD and in IBD were significantly higher than in control females (empirical $p=0.02$, 0.02 , respectively), i.e. a risk effect. In UC females, the carrier frequency of haplotype 4 was 17%, and was also higher than in controls, but this did not attain statistical significance at this sample size. In males, the carrier frequencies of haplotype 4 were 6% in controls, 9% in CD, 11% in UC, and 9% in IBD (fig. 2B). In males, the carrier frequencies of haplotype 4 in CD, in UC, and in IBD were higher than in controls i.e. suggesting a risk effect, but they didn't attain statistical significance. Taken together, these data indicate that haplotype 4 is a risk haplotype.

Diplotype analysis indicates the risk of CD, UC, and IBD is 4 to 5 times higher with one risk haplotype than with the protective/protective haplotype combination

Since females have two X chromosomes, they have two TLR8 haplotypes. Therefore, we performed a haplogenotype analysis, also known as a diplotype analysis. The goal was to assess the comparative effects of protective and risk haplotypes. The baseline risk ratio was defined as those individuals without either a risk or protective haplotype (i.e. no H1 and no H4), and was set at an odds ratio of one.

As can be seen fig. 3, two protective haplotypes (H1/H1) had a markedly diminished odds ratio of 0.4–0.5. The presence of one protective risk haplotype (H1/not H4) had an odds ratio of 0.7–0.8. However, in the presence of a risk haplotype (H4/not H1), the odds ratio increased to 2 or above (2.0–2.2).

Thus, in females, the risk of IBD was 4 to 5 times higher in those individuals with one risk haplotype than with the protective/protective diplotype. These were all highly statistically significant by the trend test, and were true among CD, among UC, and among all IBD ($p=0.0002$, $p=0.0032$, $p=0.0002$, respectively).

Single SNP cannot explain all the haplotype results

Single SNP associations with disease were also evaluated. Two SNPs, rs2109134 and rs1548731, in haplotype block1 were found significantly associated with CD but not UC (supplement table 1, 2). Therefore, single SNPs cannot explain fully the results that we observed in the haplotype analysis.

DISCUSSION

In this study, we observed that TLR8 has two haplotype blocks, and that different haplotypes in the first block provide protection (H1) and risk (H4) respectively to IBD patients. This genetic variation in TLR8 is associated with CD, with UC, and with all IBD.

Recently, a number of papers have been published regarding TLRs; however, comparatively little is known regarding TLR8 function because TLR8 does not function in mice. The TLR8 gene shows high homology to TLR7, and both the TLR8 and the TLR7 gene are located on the X chromosome. Both TLRs are expressed within the endosomal membrane and play an important role in the innate immunity, especially for recognition of viral components. TLR8 can recognize viral component by PRRs and induces type I IFN (IFN- α/β) production that can activate target cells such as dendritic cells, T cells, B cells, and macrophages via autocrine and paracrine manners.⁹

Several reports have shown that the incidence of IBD among the patients with Turner's syndrome, that is individuals who lack all or part of one X chromosome, is many times higher than that seen in the general population.^{25, 27, 28} This may be related to the abnormal state of single X chromosome. In addition, some genome-wide linkage studies have identified putative loci on the X chromosome.^{29, 41} Furthermore, genetic associations in IBD have demonstrated defects in innate immunity, such as the now well established CARD15/NOD2 association with CD. Thus it is of interest to study the relationship of genes related to innate immunity and that are located on the X chromosome. TLR8 is such a gene. This suggested that an investigation of the TLR8 gene would be of interest in IBD.

We observed two haplotype blocks in the TLR8 gene and detected significant differences only in block 1. We then analyzed the association between these haplotypes in block 1 and CD, and UC, and all of IBD. The frequency of haplotype 1 in control in females was significantly higher than CD, UC, and IBD. In males, the frequency of haplotype 1 in UC was also significantly higher than controls. These data suggest that haplotype 1 is a protective haplotype. The protective effects of haplotype 1 were more apparent in females than in males.

In males, the frequency of haplotype 4 in both CD and UC was higher than in controls, but did not attain statistical significance. In females with CD and IBD, the frequency of haplotype 4 was significantly higher than in controls. In females with UC, the frequency of haplotype 4 was also higher than in controls, but did not attain statistical significance at this sample size. These results suggest that haplotype 4 is a risk haplotype.

Since females have two X chromosomes, they have two TLR8 haplotypes. Therefore, we examined the combined effects of the protective haplotype and the risk haplotype in females. The risk of IBD was 4 or 5 times higher with one risk haplotype than with the protective/protective haplotype combination. There were all highly statistical significances by the trend test among CD, UC, and controls.

Haplotype 1 is the most common haplotype, and is a protective haplotype. Haplotype 4 is a rare haplotype, but it is significantly associated with the risk for IBD. We have not analyzed TLR8 function in this study. TLR8 is known as an important TLR against viral infection; thus these data suggest that the ability to react against viral infection via TLR8 may contribute to the occurrence of IBD. In the past, some work has suggested a relationship between viral infection and IBD; for example one paper reported that atypical paramyxovirus infections in childhood such as mumps infection before 2 years old or measles and mumps infections in the same year of life may be risk factors for later IBD.⁴² Recent papers have concluded that measles, rubella, and mumps are not associated with IBD

43. However, measles virus, rubella virus, and mumps virus are all ssRNA virus, so TLR8 may be able to recognize them. Alternatively, some other ssRNAs which are able to bind to TLR8 may cause specific immune response, whose overall effect may well be modified by the presence of a risk haplotype or absence of a protective haplotype, and these responses may be related to occurrence of a subpopulation of IBD who have relevant TLR8 genetic variants.

TLR8 function in vivo has not been well characterized. Some experiments in vitro have showed that TLR8 agonists can induce proinflammatory cytokines via TLR8 and also inhibit the suppressive effects by Tregs. We have already reported that Tregs are correlated with mucosal inflammation in intestinal mucosa and mesenteric lymph nodes (MLN) in the patients with UC and CD,^{15, 44} and Tregs can also inhibit the proliferation of effector cells similar to those in normal intestinal mucosa. However, patients with UC and CD still have severe inflammation in their intestinal mucosa. Therefore, we have considered that some factors may abrogate Tregs function in vivo. Recently, Peng et al. showed that TLR8 agonists abrogate Tregs suppressive effects in vitro¹⁶, and thus the activation of TLR8 may be one cause of disarray of the intestinal homeostatic regulation in patients with IBD. Therefore, to delineate the relationship between TLR8 and IBD, we will need to identify natural ligands for TLR8 and to analyze TLR8 functions in vivo in relevant subgroups of patients.

In this study, we found that “protective” and “risk” TLR8 haplotypes were associated with CD and UC in females. In males, a significant association was detected with UC, but not with CD in the currently studied sample size. The odds ratio for CD and UC in females increased progressively as a function of haplotype combinations/diplotypes from protective to risk. Our data suggest that variation in the innate immunity gene TLR8 is a genetic determinant in a subpopulation of IBD, both for UC and CD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Genotyping with Illumina technology was performed at Cedars-Sinai Medical Center. Single Nucleotide Polymorphisms (SNPs) were chosen to tag common Caucasian haplotypes using information from the Innate Immunity PGA. Haplotype blocks were evaluated by Haploview 3.3. Individual haplotypes were obtained by PHASE V2.0 and ordered by frequency in the entire sample.

Figure 2.

The presence of each haplotype was analyzed as regards its frequency with CD, with UC, and with all IBD respectively. Males have one X chromosome and females have two X chromosomes, therefore we analyzed each gender individually. The data shown represent the carrier frequencies of haplotype 1 (A) or haplotype 4 (B) in CD, in UC, or in all of IBD. The Chi-Square test was used to evaluate association of IBD haplotypes. We evaluated 362 CD males, 373 CD females, 174 UC males, 169 UC females, and 133 male and 112 female controls.

Figure 3.

Since females have two X chromosomes, they have two TLR8 haplotypes. Therefore, the combined effects of the protective haplotype (H1) and the risk haplotype (H4) in females were examined using diplotype haplogenotype analysis. The data shown represent the odds ratios for CD, for UC, and for all IBD together. We evaluated 373 CD females (H1/H1; 53, H1/not H4; 142, others/others; 135, H4/not H1; 43), 174 UC females (H1/H1; 28, H1/not H4; 67, others/others; 58, H4/not H1; 21), 547 IBD females (H1/H1; 81, H1/not H4; 209, others/others; 193, H4/not H1; 64), and 112 females control (H1/H1; 30, H1/not H4; 46, others/others; 31, H4/not H1; 5).

Table 1

Distribution of the patients and healthy donors

	Total	Females	males
CD	735	373	362
UC	343	174	169
Control	245	112	133

We enrolled patients with CD, UC who attended the IBD clinic at Cedars-Sinai Medical Center, Los Angeles, as well as geographically and ethnically matched controls.

Table 2

Definition and frequency of TLR8 Haplotypes in Block 1

Haplotype	rs3764879	rs2109134	rs1548731	Frequency in total sample	
				Female	Male
H1	1	1	1	40%	41%
H2	2	1	1	31%	31%
H3	1	1	2	20%	17%
H4	1	2	2	8%	8.6%

Haplotypes in block 1 were classified in order of frequency as haplotype 1 to haplotype 4. The specific alleles consisting haplotype are shown in the table, 1 referring to the major allele, and 2 referring to minor allele.