NOD2/CARD15 and Toll-like 4 receptor gene polymorphism in Chilean patients with inflammatory bowel disease

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ABSTRACT. Crohn's disease (CD) and ulcerative colitis (UC) are multifactorial diseases with a genetic background. Genes related to the innate immune response have been observed to be involved. Polymorphisms of Toll-like receptor 4 (TLR4) and CARD15/NOD2 are thought to be involved in the pathogenesis of inflammatory bowel disease (IBD). There is no information about the frequency of these polymorphisms in South American and Chilean populations. Aim. To investigate the distribution of CARD15/NOD2 (*Arg702Trp*, *Gly908Arg* and *Leu1007fsinsC*) and TLR4 (*Asp299Gly*) polymorphisms in Chilean patients with IBD. *Methods.* DNA was obtained from 22 CD, 22 UC patients and 20 healthy individuals. Genotyping was performed by allele-specific PCR and by PCR-RFLP analysis. Clinical and demographic features were characterized. *Results*. Among the CD patients, the clinical pattern was deemed inflammatory in 14, while five had penetrating and five stricturing, variants. One patient had esophageal involvement, five perianal, seven ileal and in 16 the colon was involved. Among the UC patients, two had proctitis, two proctosigmoiditis, four left-sided colitis and 14 pancolitis. NOD2/CARD15 analysis revealed the presence of the *702Trp* allele in two CD patients (both heterozygotes), *1007fsinsC* in one CD patient (heterozygote) while *908Arg* was found in one UC patient. The *299Gly* TLR4 allele was identified in one UC and one CD patient. *Conclusion*. This genetic study shows that the alleles frequently associated with IBD (*1007fsinsC, 908Arg and 702Trp* in NOD2/CARD15 and *299Gly* TLR4) have a low incidence in Chilean, IBD patients, which is similar to European populations. It is possible that, in addition to environmental factors, other genetic polymorphisms may be involved in the pathogenesis of the disease in Chilean, IBD patients.

Keywords: inflammatory bowel disease, Crohn's disease, ulcerative colitis, genetic polymorphism, NOD2/CARD15, Toll-like receptor 4

The incidence of inflammatory bowel diseases (IBD), including ulcerative colitis (UC), Crohn's disease (CD), and indeterminate colitis (IC) seems to be increasing throughout the world [1-9] including in developing countries. There is evidence that the incidence of IBD may also be rising in Chile [10, 11]. Although the etiology of IBD is still unknown, its pathogenesis seems to be multifactorial [9, 11]. It has been proposed that in genetically predisposed individuals, IBD occurs as result of an inappropriate immunological response to commensal intestinal microflora [12, 13]. In normal subjects, to avoid an excessive and uncontrolled immune response, the gastrointestinal epithelial cells turn on molecular recognition pattern receptors. In recent years, polymorphisms affecting several genes involved in the innate immune response have been identified among patients who have developed IBD [14-16]. Some of the polymorphisms reported include NOD2/CARD15 mutations [17, 18]. This receptor belongs to a gene superfamily located on chromosome 16 and expressed mainly in monocytes, dendritic cells, intestinal epithelium and Paneth cells [12]. The NOD2/CARD15 gene encodes a cytoplasmatic receptor involved in pathogen recognition, which leads to intracellular nuclear factor κB (NFκB) mobilization [14, 16].

However, when NOD2/CARD15 is mutated, pathogen or commensal recognition may lead to altered intracellular NFκB levels. The gene polymorphisms that are most often associated with IBD are *Arg702Trp, Gly908Arg and Leu1007fsinsC*, and their relative and absolute incidence has varied according to the ethnicity of the population studied [19-23]. These polymorphism variants have not only been linked with the risk for developing CD, but also

with the disease phenotype, and especially, with aggressive disease behaviors such as early presentation, stricture development, and a predominant involvement of the ileum [24, 25].

Toll-like receptors (TLRs) also play an important role in the pathogen recognition process and in the activation of the innate immune response [14, 16]. TLR4 is expressed mainly in macrophages, dendritic cells and endothelial cells and, to a lesser extent, in the intestinal epithelium. The TLR4 recognizes gram-negative bacterial lipopolysaccharide leading to intracellular activation of NFκB and its translocation to the nucleus where it regulates gene transcription. TLR4 expression in intestinal epithelium is increased in CD patients, and the allele *299Gly* has been related with risk of developing IBD and CD, in particular [23, 24, 26].

To date there have been no studies that have specifically evaluated the frequency of NOD2/CARD15 and TLR4 gene polymorphisms in Chilean or South-American IBD patients; we undertook this study therefore, determinate the presence and prevalence of this gene mutation in Chilean IBD patients.

MATERIALS AND METHODS

Patients

All patients included in this study came from families that had been born in Chile over at least two consecutive generations. Forty-four IBD patients, (22 with CD and 22 with UC) were included and 20 healthy individuals. They were recruited from the Gastroenterology Section of the University of Chile Clinical Hospital and the Gastroenterology Section of Clínica Las Condes. All had a well-established diagnosis of CD and UC, according to current diagnostic criteria and had been followed for at least one year. The location of involved bowel segment was defined by endoscopy and imaging studies. Clinical and demographic characteristics were recorded, including, age at diagnosis, gender, family history, smoking habits (current smoking, history of past smoking, and never smoked), disease behavior, disease location, extra-intestinal manifestations, type of surgery, if any, and response to treatment.

Genetic analyses

Blood samples were collected in tubes containing heparin and total DNA was prepared from peripheral blood lymphocytes using the Chomczynski method [27]. TLR4, *Asp299Gly* (896 A/G) polymorphism was genotyped using PCR-RFLP analysis. The PCR primers employed were: (5' GATTAGCATACTTAGACTACTACCTCCATGGT 3') and (5' GATCAACTTCTGAAAAAGCATTCCCAC 3'). The underlined base in the forward primer indicates the location of the altered nucleotide used to create a NcoI (TLR4 Asp299Gly) restriction site. Following PCR amplification, the 249 bp product was digested with 1 U of NcoI restriction enzyme overnight at 37°C. After digestion, the A allele consisted of one fragment of 249bp and the G allele consisted of 223 and 26 bp fragments. The missense mutation *Arg702Trp* was genotyped by a PCR amplification of specific allele assay using two allele-specific forward primers R702WWTF: 5' ATCTGAGAAGGCCCTGCTCC 3' for the wild-type allele and R702WMUTF: 5' ATCTGAGAAGGCCCTGCTCT 3' for the *Arg702Trp* mutant allele, in combination with a common primer R702WR: 5' CCCACACTTAGCCTTGATG 3', in two separate PCR reactions. The 3'-ends of the forward primers, were able to anneal to regions that differed between the two alleles.

The missense mutation *Gly908Arg* created a restriction site for HhaI and was genotyped by a PCR-RFLP method (5' CCCAGCTCCTCCCTCTTC 3' and 5'AAGTCTGTAATGTAAAGCCAC 3'). After digestion, the wild-type *Gly908* allele resulted in an intact 380 bp fragment, whereas the RFLP profile of the *Arg908* variant was characterized by two bands of 138 bp and 242 bp. The cytosine insertion mutation was genotyped by a PCR amplification of specific allele assay using two, allele-specific forward primers L1007fsinsCWTF: 5' CAGAAGCCCTCCTGCA GGCCCT 3' for the wild-type allele and L1007fsinsCMUTF: 5' CAGAAGCCCTCCTGCAGGCCCCT 3' for the L1007fsinsC mutant allele, in combination with a common primer L1007fsinsCR: 5' TCTTCAACCACATCCCCATT 3', in two separate PCR reactions. The 3'-ends of the forward primers, were able to anneal to regions that differed between the two alleles. DNA fragments were resolved on agarose gels and visualized by ethidium bromide staining.

Ethical approval

This study was conducted after review and approval of the Ethics Committee of Hospital Clínico of the Universidad de Chile or Clínica Las Condes, as appropriate, and each patient gave written informed consent.

Statistics

All values were expressed as mean + SE. Comparisons of the frequencies of NOD2/CARD15 and TLR-4 polymorphisms were made among the CD and UC patients and healthy controls. The analyses of association with the phenotype were performed by $\chi 2$ or Fischer's exact test where appropriate p values less than 0.05 were considered statistically significant.

Results

Demographic and clinical characteristics of the CD and UC patients are shown in *tables 1, 2*. There were more women than men in both groups of patients. The demographic characteristics of the control population were similar. The prevalence of individuals with some Native American heritage was similar in CD patients (55%), UC patients (62%) and healthy controls (57%). In the CD group, the majority of patients (14 patients) had colonic involvement alone, five had terminal ileal involvement, five had perianal, two had ileo-colonic, and one, esophageal, disease. The majority (14 patients), exhibited an inflammatory phenotype. Joint involvement was present in 10 patients. Twelve patients had never smoked. Only one CD patient had a first degree relative with IBD, who had UC. Among the UC patients, the majority (14 patients) had

Table 1
Clinical characteristics of the Crohn's disease patients

Patients	
Male/Female	8/14
Age (years)	46.8 years (16-65)
Age at diagnosis (years)	41.6 years (14-65)
Disease duration before diagnosis (months)	40 months (1-216)
Familial IBD	n (%) 1(5)
Disease localization n (%)	
Upper GI	1 (5)
Ileum	5 (23)
Colon	14 (64)
Perianal disease	5 (23)
Ileocolon	2 (9)
Disease pattern (%)	
Inflammatory	14 (64)
Stricturing	5 (23)
Penetrating	5 (23)
Extra-intestinal manifestations n (%)	
Articular	10 (45)
Skin	5 (23)
Ocular	2 (18)
Oral ulceration	5 (23)
Surgery n (%)	10 (45)
Smoking habits n (%)	
Never	12 (55)
Current	5 (23)
Ex-smoker	5 (23)

Table 2
Clinical characteristics of Ulcerative Colitis patients

Patients	
Male/Female	9/13
Age (years)	37.8 years (19-67)
Age at diagnosis (years)	32 years (11-59)
Disease duration before diagnosis (months)	9 months
Familial IBD n(%)	0(0)
Disease Localization n (%)	
Proctitis	2 (9)
Rectosigmoiditis	2 (9)
Left sided colitis	4 (18)
Pancolitis	14 (64)
Extra-intestinal manifestations n (%)	
Articular	10 (45)
Skin	3 (14)
Ocular	0 (0)
Oral Ulcers	4 (18)
Surgery n(%)	3 (14)
Smoking habits n (%)	
Never	8 (36)
Current	4 (18)
Ex-smoker	10 (45)

pancolitis. Articular manifestations were reported by 10 and 10 patients were ex-smokers (defined as patients who stopped smoking more than 6 months prior to the diagnosis of IBD).

The three mutations analyzed in the gene NOD2/CARD15 were found in four patients, three with CD and one with UC (*table 3*). The *702Trp* allele was found in two patients with CD, both heterozygotes. One of these patients had experienced the onset of CD when he was 19 years-old, and the other featured ileocolonic involvement and a penetrating phenotype. The *1007fsinsC* allele was found in only one heterozygous CD patient who showed esophageal and colonic involvement with a stricturing phenotype. Finally, the mutation *Gly908Arg* was found in one patient with ulcerative pancolitis, who was heterozygous for the mutation. None of the patients studied showed more than one NOD2/CARD15 polymorphic variant. None of the control individuals showed any polymorphism.

The *299Gly* allele of TLR4 was found in two patients, one with CD with colonic and perianal disease and an inflammatory phenotype, and the other had ulcerative pancolitis *(table 4)*. None of the control individuals showed this TLR4 polymorph variant. None of patients with CD or UC showed coexistence of any of the variants analyzed for NOD2/CARD15 and the TLR4 coding genes.

DISCUSSION

This is the first study to determine the prevalence of NOD2/CARD15 and TLR4 gene mutations in Chilean patients with UC and CD. Although the number of patients in this study is small, our results confirm that, as in European populations, Chilean patients have a low frequency of the allelic variants associated with IBD.

As the ethnic background of the Chilean population is heterogeneous, as a result of the admixture of diverse racial groups to an extent that might influence our results,

Table 3
NOD2/CARD15 genotypes and alleles investigated

Mutation	**N°**	**Diagnosis**	**Disease Pattern**	**Disease location**
702Trp	1	CD	Early presentation, inflammatory	Colon
	1	CD	Penetrating	Ileo-colonic
1007insC	1	CD	Stricturing	Upper GI and colon
908Arg	1	UC		Pancolitis
Total	4			

Table 4
TLR4 alleles investigated

Mutation	N°	Diagnosis	Disease Pattern	Disease location
299Gly	1	CD	Inflammatory	Colon and perianal
	1	UC		Pancolitis
Total	2			

the racial heterogeneity of individuals involved in the present study was evaluated by analyzing the distribution of the ABO blood group. This analysis suggested that the percentage of individuals of Native American or Amerindian heritage was similar in all groups.

CD and UC are multifactorial diseases, symptomatic with variable courses and outcomes, treatment of which is primarily symptomatic. Some patients may develop complications and require surgery. Although the etiology IBD remains unsolved, its initiation may be related to an abnormal inflammatory response to enteric commensal microflora, in genetically predisposed individuals [12]. This interpretation supports the concept that genetic polymorphism may be relevant as a prognostic tool. It is also important to identify aberrant intracellular mechanisms involved in IBD pathogenesis as a means of developing new therapeutic tools. Recent studies assign roles to the pathogen recognition receptors and their mechanisms of activation as fundamental effectors in the development of IBD [16]. Among these receptors are those coded by the NOD2/CARD15 and TLR4 genes. Accordingly, polymorphisms in these genes could be risk factors for the development of IBD and could even determine disease severity and location.

CD is a heterogeneous and polygenic disease. While the three mutations of NOD2/CARD15 gene are more frequent in Caucasian patients with CD than in Caucasian control subjects, these mutations have not been observed [28], or are very rare [29], in Asian, Arabic [30], and African patients [31]. In our study, three (13.6%) of the 22 patients with CD analyzed for NOD2/CARD15 gene had one of the three variants. The relative risk of developing CD among those with these polymorphisms varies widely. In Northern European populations, the risk attributable to the NOD2/CARD15 gene diminishes when compared with others from the southern population. Even within the same country there may be differences. Arnott *et al*. reported that the risk of developing IBD, in relation to NOD2/CARD15 gene mutations in patients from Edinburgh (Scotland), was 11% compared with 27% in patients from Oxford (England) [32]. In a meta-analysis of 37 studies of variants of the NOD2/CARD15 gene in a Caucasian population, people carrying only one high risk allele had 2.4-folds (95% CI: 2.00-2.86) increased odds of CD, and the risk for those carrying two or more of the risk alleles had 17.1-fold (95% IC 10.7-27.2) compared to people without any high-risk alleles [33]. The risk of developing CD is even more variable among NOD2/CARD15 gene variants. The same analysis showed that the risk of developing CD was 4.1 for the *Leu1007fsinsC* variant (95% IC 3.2-5.2) compared with 2.2 for the *Arg702Trp* variant (95% IC 1.8-2.6) and 3.0 for the variant *Gly908Arg* (95% IC 2.4-3.7) [24]. In the present study, two patients with CD had the *Arg702Trp* variant and only one the *Leu1007fsinsC* variant. Thus, the small number of patients included in this study does not allow us to estimate the real risk of developing CD as a function of variants of the NOD2/CARD15 gene.

One recent study described that the frequency of NOD2/CARD15 variants is similar in patients and controls, suggesting that these variants do not seem to be a risk factor for developing UC [24]. Nevertheless, other studies have suggested that the NOD2/CARD15 gene can interact with the haplotype IBD5 (OCTN1/N2), thereby increasing the risk of developing UC [34]. In our analysis, only one patient with UC displayed any of the three variants (*Gly908Arg*) for the NOD2/CARD15 gene; we have not evaluated the presence of IBD5 haplotype mutations. NOD2/CARD15 variants influence the site of the intestinal segment involved in CD, being more frequent in patients with ileal involvement as compared to those with CD limited to the colon.

A meta-analysis showed that the odds ratio for terminal ileal involvement was 2.5 (95% IC 2.0-3.2) compared with 1.5 (95% IC 1.2-1.9) for colonic disease alone [24]. The association between variants of NOD2/CARD15 and terminal ileal involvement can be explained by the observation that the expression of NOD2 in the intestinal epithelium is restricted mainly to Paneth cells, which are more abundant in the ileum [12]. In our work, three patients with CD had colonic involvement, one of these had associated ileal disease. Our sample is too small to offer an explanation for the low frequency of NOD2/CARD15 variants in patients with ileal disease. Up to now, no study has evaluated the presence of NOD2/CARD15 variants in a large number of patients with CD and upper gastrointestinal involvement (*i.e.* esophageal, gastric, duodenal or jejunal). Only one of our patients had esophageal involvement, as well as colonic disease. NOD2CARD15 variants have also been associated with an increased risk of developing intestinal strictures and fistulas, which tend to have a slower evolution [35]. In this study, of three patients with one or more of the three NOD2/CARD15 variants studied, one had perforating ileal disease and the other, colonic stricturing. NOD2/CARD15 variants have also been associated with more aggressive disease, higher risk of requirement for surgical intervention and a younger age at diagnosis. On the other hand, NOD2/CARD15 variants do not seem to be associated with a higher frequency of extra-intestinal manifestations or a differential response to infliximab treatment [36]. Only one of three patients with this variant in this study, had extra-intestinal manifestations, and this individual had not received infliximab treatment; again the small number of patients included in the present study does not allow us to determine whether a relationship between NOD2/CARD15 gene mutations and the CD phenotype among Chilean patients exists.

An association between IBD and the TLR4 polymorphism has been suggested by several groups, however, its role as a genetic predictor in IBD development and evolution

seems less strong when compared to NOD2/CARD15 variants [23, 37]. The most frequently described TLR4 allele in IBD is the *299Gly*. Brand *et al*. reported that 14.2% and 14.7% of patients with CD were heterozygous for *Asp299Gly* and *Thr399Ile* variants, respectively, as compared to 37.7% of patients who presented at least one NOD2/CARD15 mutation [23]. In the present study, two patients, one with CD and one with UC, presented the *299Gly* allele. None of the control individuals presented the polymorph allele. We did not evaluate the frequency of the *Thr399Ile* polymorphism in the cases included here. The *299Gly* variant has also been associated with a stricturing phenotype [23]. However, the only CD patient that showed the *299Gly* allele, in this study, had a different clinical profile. Other research groups have not found any association between TLR4 polymorphisms and risk of developing IBD [32]. These differences might be related to the ethnic diversity of patients included in the studies and the diagnostic criteria used.

The identification of NOD2/CARD15 and TLR4 gene polymorphisms and their implications are an important contribution to our understanding of the heterogeneity of IBD. A genetic study of IBD could be a powerful tool that may help in the prediction of risk factors for developing a more aggressive version of the disease. The study of the different genetic alterations linked to IBD [38], taken together, could facilitate diagnosis and prognosis of IBD and may help in the management of individual patients with this disease.

In conclusion, TLR4 and NOD2/CARD15 gene mutations may contribute to the development and evolution of CD and UC in Chilean patients with IBD. The TLR4 polymorphism *Asp299Gly* seems to play a minor role in the population studied. It is evident however, that other genetic and environmental factors play a role in the development and evolution of IBD in this population. As the number of patients studied was small, our next goal is to perform a prospective, multicenter study to increase the power of our conclusions. By following up each individual over a longer period of time, we hope to develop better correlations between phenotype and genotype [27, 34].

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