IL-10 and TNF-α promoter haplotypes are associated with childhood Crohn’s disease location

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Author contributions: Levy E and Sinnett D designed research; Sanchez R performed experiments; Costea F contributed new analytic tools; Sanchez R and Sinnett D analyzed data; Sanchez R, Levy E and Sinnett D wrote the manuscript.

Supported by Crohn’s and Colitis Foundation of Canada and Valorisation Recherche Quebec

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Received: February 19, 2009 Revised: June 19, 2009 Accepted: June 26, 2009
Published online: August 14, 2009

Abstract

AIM: To determine the distribution and frequencies of the genotypes and haplotypes of the genes encoding for the glucocorticoid receptor (GR), the tumor necrosis factor (TNF)-α and the interleukin (IL)-10 in childhood Crohn’s disease (CD) and to assess the impact of the corresponding DNA variants on clinical and disease phenotypes.

METHODS: Ten variants in GR, TNF-α and IL-10 were genotyped in 113 childhood CD cases and 95 healthy subjects, both of French-Canadian origin.

RESULTS: For the GR polymorphisms (R23K and N363S) and IL-10 variants in the 5’flanking region (-1082 G > A, -819 T > C and -592 A > C), no difference was observed in allele and genotype frequencies between CD patients and controls. At the haplotype level, we found three IL-10 haplotypes previously described in Caucasians (GCC, ACC and ATA) and three novel haplotypes only present in IBD patients. When we analyzed the haplotype distribution with the anatomical location of the disease, the GCC haplotype was associated with the colonic and the ACC haplotype with the terminal ileum location, respectively. The genotyping of five polymorphisms in the promoter region of the TNF-α gene (-1031 T > C, -863 A > C, -857 T > C, -308 A > G and -238 A > G) revealed a significant overrepresentation of homozygous -1031 CC among CD patients (OR = 9.9) and an association with the colonic location. For TNF-α, eleven haplotypes were inferred, including two frequent ones, TCCGG and CACGG, which were significantly observed more frequently in controls and cases, respectively.

CONCLUSION: This is one of the first studies investigating the association between haplotype structure and disease location in a CD pediatric cohort. Our results will help to increase our understanding of the genetic determinants of childhood CD.

Key words: Haplotype; Polymorphism; Crohn’s disease; Glucocorticoid receptor; Interleukin-10; Tumor necrosis factor-α

Peer reviewer: Tamara Cacev, MSc, Division of Molecular Medicine, Rudjer Boskovic Institute, Bijenicka c. 54, Zagreb 10000, Croatia

Sanchez R, Levy E, Costea F, Sinnett D. IL-10 and TNF-α promoter haplotypes are associated with childhood Crohn’s disease location. World J Gastroenterol 2009; 15(30): 3776-3782 Available from: URL: http://www.wjgnet.com/1007-9327/15/3776.asp DOI: http://dx.doi.org/10.3748/wjg.15.3776

INTRODUCTION

Inflammatory bowel diseases (IBD) represent a complex genetic disease that is defined by at least two major disorders: Crohn’s disease (CD) and ulcerative colitis. These diseases are distinct in terms of disease extension, localization, behaviour and the occurrence of extra-intestinal manifestations[1]. The incidence of IBD is bimodal, with the first peak occurring in the second and third decades of life and the second peak between the fifth and seventh decades. However, in North America, IBD also occurs during childhood or adolescence in up to 25% of patients[2]. Recent studies suggest that genetic predisposition may play an important role. For instance, the NOD2/CARD15 gene (chromosome 16)
and the IBD5 locus (chromosome 5) are associated with susceptibility to CD in adults[3]. Although initial studies suggested that the association of CARD15 with susceptibility to CD was higher for pediatric-onset disease, recent work with children suggests that this association in pediatric cases is roughly comparable to adult-onset disease. Furthermore, the association between the IBD5 locus and the risk of CD is weaker for children than for adults[3]. Despite extensive research, the etiology of childhood IBD is still unknown, but the immune-mediated chronic intestinal inflammation results from complex interactions between genes conferring susceptibility, exogenous or endogenous triggers, and environmental factors. In this study, we investigated the association between DNA variants in genes encoding for the glucocorticoid receptor (GR), the tumor necrosis factor (TNF)-α and the interleukin (IL)-10 and childhood CD phenotypic and clinical features. We found significant associations between IL-10 and TNF-α promoter haplotypes and disease location.

**MATERIALS AND METHODS**

**Subjects**

Incident cases of CD (n = 113), ranging from 5 to 20 years old, were diagnosed in the Division of Gastroenterology of Ste-Justine Hospital, Montreal, Canada. The criteria for inclusion in this group were: (1) Complete clinical history; (2) Caucasians of French-Canadian origin residing in the Province of Quebec as judged by their names, languages and places of birth; (3) Availability of biological material. Patient characteristics are given in Table 1. The control group (n = 95) was composed of unrelated healthy French-Canadian individuals who had agreed to donate their blood on an anonymous basis and to provide the information on their geographic origin, age, and gender. The targeted population was the one served by Ste-Justine Hospital, and our requests were addressed to parents and/or children that visited the Hospital for non-gastroenterological conditions.

The French-Canadian population represents a suitable model for genetic epidemiological studies because of its relative homogeneity in terms of genetics, socio-demographics and history[3]. The Ste-Justine's Institutional Review Board approved the research protocol and informed consent was obtained from all participating individuals and/or their parents.

**Genotyping**

Genomic DNA was isolated from peripheral blood cells using standard methods (GENTRA Kit). Individuals were genotyped for TNF-α (-1031 C > T, -863 A > C, -857 T > C, -308 A > G, -238 A > G), IL-10 (-1082 G > A, -819 T > C, -592 A > C) and GR (R23K G > A and N363S A > G) polymorphisms by allele specific oligonucleotide (ASO) hybridization assay, as described in Labada et al[6]. Briefly, primers flanking the polymorphic sites were used to amplify PCR products that were dot-blotted in duplicate on nylon membranes and assayed for the absence or presence of a specific mutation by hybridization with ASO for both alleles in parallel experiments. Samples with known genotypes were interspersed among study samples (positive controls) to ensure genotyping accuracy. For each experiment, the genotypes were read manually by two independent individuals and only concordant readings were accepted. To detect potential genotyping errors, Hardy-Weinberg equilibrium was tested. The oligonucleotides and assay conditions used for ASO are given in the supplementary materials (Table 2).

**Haplotype solving and linkage disequilibrium (LD) analysis**

Haplotypes were inferred for all sites with minor allele frequency > 5% using the software PHASE, v. 2.1.1[3]. The LD statistics D and D' were both computed for each pair of single nucleotide polymorphisms (SNPs) with the software Arlequin v. 2.0[5].

**Haplotype networks**

The NETWORK 4.1.1.2 program was used to infer the likely genealogical history between the most frequent haplotypes using the Median-Joining (MJ) algorithm[3]. In an MJ network, circles represent distinct haplotypes and are scaled to reflect the frequency of these haplotypes. The branches connect the haplotypes and indicate the mutational steps between the haplotypes. The MJ network was generated only for IL-10 and TNF-α haplotypes.

**Statistical analysis**

The chi-squared test was used to examine the differences in the distribution of genotypes between cases and controls.

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Table 1 Characteristics of IBD patients

| Variables | CD (n = 94) |
|-----------|------------|
| Boys | 44 |
| Girls | 50 |
| Age of onset (yr) | 11.3 ± 3.7 |
| Location of disease | 11 |
| Terminal ileum | 11 |
| Ileocolonic | 56 |
| Colon | 8 |
| Upper digestive tract | 19 |
| H-B index | 5.0 ± 1.5 |
| ESR (mm/h) | 39.0 ± 14 |
| Albumin (g/L) | 32.0 ± 7 |
| WBC (× 10^9/L) | 9.3 ± 166 |
| Hgb (g/L) | 110.0 ± 14 |
| MCV (fL) | 76.3 ± 7.2 |
| PLT (× 10^9/L) | 468 ± 146 |
| ABS polys (%) | 6.1 ± 3 |
| ABS lymphs (%) | 2.0 ± 2.1 |
| ALT (IU/L) | 13.0 ± 8.9 |
| AST (IU/L) | 19.5 ± 7.3 |

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* Vienna classification (1998); * Always associated with other location. H-B index: Harvey-Bradshaw index; ESR: Erythrocyte sedimentation rate; WBC: White blood cells; Hgb: Haemoglobin; MCV: Mean corpuscular value; PLT: Platelet; ABS polys: Absolute value of neutrophils; ABS lymphs: Absolute value of lymphocytes; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IBD: Inflammatory bowel disease; CD: Crohn's disease.
The level of significance was calculated by Fisher’s exact test (two-sided). Odds ratios (OR) were given with 95% confidence intervals (CI). Log linear analysis was used to include potential confounding factors in the analysis (age and gender). All analyses were performed using the SPSS statistical package (version 11.0.1). A proportional test was done to compare the association of various genotypes and haplotypes with different anatomical disease locations.

RESULTS

Using PCR ASO hybridization assays, we genotyped 10 SNPs in GR, IL-10 and TNF-α genes in 113 CD children and 95 healthy subjects, all of French-Canadian origin. All SNPs tested were in Hardy-Weinberg equilibrium in the control group, although no homozygous genotypes were observed for the GR minor alleles.

For GR, we genotyped two SNPs in the exon 2, a G > A substitution, resulting in an amino acid change from arginine to lysine in the codon 23 (R23K), and an A > G substitution, resulting in an asparagine to serine change at codon 363 (N363S). The observed allele frequencies were consistent with those reported elsewhere for Caucasian populations [11-14]. The distribution of the alleles and genotypes did not differ between control and patient groups (supplementary materials, Table 3). Using the genotyping data, we were able to infer three haplotypes, including the major one GG (94%) and two minor ones, GA and AG, both at 3% (supplementary materials, Table 4). No significant differences were observed between the distribution of these haplotypes among cases and controls. No association was found between any GR genotypes and haplotypes and any clinical or phenotypic features (data not shown).

For IL-10, we genotyped three SNPs in the 5' flanking region of IL-10: -1082 G > A, -819 T > C and -592 A > G. The frequencies and the distribution of the alleles and genotypes were similar in CD patients and controls (Table 5) and similar to those reported in other Caucasian populations [15,16]. However, we found that the homozygous
The homozygous GG is significantly associated \((P = 0.008)\) with the terminal ileum location. No other significant associations were found in the colonic region. The haplotype GCC was statistically significantly associated with the ileo-colonic \((P = 0.01)\) and colonic \((P = 0.01)\) locations, whereas the haplotype ACC was significant associated with the terminal ileum \((P = 0.01)\) and upper digestive tract \((P = 0.032)\) (Figure 1). We estimated pair-wise LD and found a strong LD (\(D' = 0.93\)) between -819 T > C and -592 C > A, but a weak LD between -1082 G > A and -819 T > C (\(D' = 1.0, \ r^2 = 0.27\)) (data not shown). These results confirmed the putative independent role for the -1082 G > A variant. Altogether, these results suggest an important role for the SNP -1082 G > A in determining disease location.

For TNF-\(\alpha\) we genotyped five SNPs in the promoter region: -1031 T > C, -857 T > C, -863 A > C, -308 A > G and -238 A > G. The allelic and genotypic frequencies are shown in Table 7. The homozygous CC genotype for the SNP -1031 T > C was significantly overrepresented in CD patients \((11\%, P = 0.008)\) when compared to controls \((1%)\) suggesting an increased risk of disease \((OR = 9.9, 95\% CI: 1.1-78)\). Although not significant, we also observed differences for the SNP -863 A > C, with the allele A present in 28\% of cases and 19\% of controls. The -1031 CC homozygotes were associated \((P = 0.02)\) with the colonic region and the heterozygote CT with the terminal ileum location \((P = 0.03)\). For SNPs -308 A > G and -238 A > G, both homozygous GG genotypes were associated with the upper digestive tract \((P = 0.008 \text{ and } P = 0.05, \text{ respectively})\), whereas the heterozygous GA was associated with the terminal ileum location \((P = 0.008 \text{ and } P = 0.05)\). Using the genotyping data, we inferred eleven haplotypes (Table 8), including 3 major ones (H1, H3 and H7). These results were similar to those reported by Bennet et al.[19] in myocardial infarction (MI) patients. The haplotype H1 (TCCGG) was more frequent in the control group \((56\%)\), suggesting a protective effect \((OR = 0.69, 95\% CI: 0.49-0.99, P = 0.04)\) when compared with patients \((48\%)\), whereas the haplotype H7 (CACGG) was more frequent in CD patients \((25\%)\), suggesting an increased risk effect \((OR = 1.64, 95\% CI: 1.05-2.56, P = 0.03)\) when compared with the control group \((17\%)\). The SNPs -1031 T > C and -863 A > C might explain this effect because the other three SNPs are shared by both haplotypes. Furthermore, the haplotype H3 (TCCAG) was observed more frequently in cases with terminal

### Table 5 Allele and genotype distribution of the IL-10 polymorphisms \(n (\%)\)

| Position          | Controls \((n = 188)\) | CD \((n = 222)\) |
|-------------------|------------------------|-----------------|
| -1087 G > A1      |                        |                 |
| GG                | 19 (20)                | 24 (22)         |
| GA                | 42 (45)                | 50 (45)         |
| AA                | 33 (35)                | 37 (33)         |
| A                 | 108 (57)               | 124 (56)        |
| G                 | 80 (43)                | 98 (44)         |
| -824 T > C        |                        |                 |
| TT                | 7 (7)                  | 14 (13)         |
| TC                | 37 (39)                | 39 (35)         |
| CC                | 50 (53)                | 64 (58)         |
| T                 | 51 (27)                | 55 (25)         |
| C                 | 137 (73)               | 167 (75)        |
| -597 A > C        |                        |                 |
| AA                | 1 (1)                  | 8 (7)           |
| AC                | 37 (39)                | 40 (36)         |
| CC                | 50 (53)                | 63 (57)         |
| A                 | 51 (27)                | 56 (25)         |
| C                 | 137 (73)               | 166 (75)        |

The homozygous GG is significantly associated \((P = 0.008)\) with disease location in the colonic region.

### Table 6 IL-10 haplotypes inferred in patients and control groups \(\%\)

| Haplotype | Controls \((n = 188)\) | CD \((n = 222)\) |
|-----------|------------------------|-----------------|
| GCC       | 43                     | 44              |
| ACC       | 30                     | 30              |
| ACA       | -                      | 0.9             |
| ATC       | -                      | 0.45            |
| ATA       | 27                     | 24              |

The SNP positions within a haplotype are the following: -1082 G > A, -819 C > T, -592 C > A. \(n\): Number of chromosomes.
ileum localization ($P = 0.015$), whereas the haplotypes H6 (CCCCG) and H7 (CACCGG) were overrepresented in the colonic region ($P = 0.02$) and in the upper digestive tract ($P = 0.02$), respectively (Figure 1).

To gain knowledge about the history of each SNP found in a given haplotype and possibly about their functional impact, we built haplotype networks (Figure 2). For IL-10, the network suggests that the haplotype GCC was derived from the ancestral haplotype ACC (based on chimpanzee sequence), while the third major haplotype ATA was probably created through a recombination event involving one of the rare haplotypes (ATC or ACA) only present in CD children. We found that the homzygous -1031 CC is associated with an increased risk of developing the disease at the ileo-colonic ($P = 0.015$) and terminal ileum locations, respectively. Conversely, a SNP position within a haplotype are the following: -1031 T > C, -863 C > A, -857 C > T, -308 G > A, -238 G > A; $\alpha^1$ = 0.09, 95% CI = 0.09-0.99; $\alpha^2$ = 1.64, 95% CI = 0.05-2.56; $n$: Number of chromosomes.

| Position | Controls | CD |
|----------|----------|----|
| -1031 C > T |           |    |
| CC       | 1 (1)    | 12 (11) |
| CT       | 39 (47)  | 45 (40) |
| TT       | 43 (52)  | 55 (49) |
| C        | 41 (25)  | 69 (31) |
| T        | 125 (75) | 155 (69) |
| -863 A > C |          |    |
| AA       | 4 (5)    | 10 (10) |
| AC       | 22 (28)  | 32 (32) |
| CC       | 52 (67)  | 58 (58) |
| A        | 30 (19)  | 52 (26) |
| C        | 126 (81) | 148 (74) |
| -857 T > C |         |    |
| TT       | 1 (1)    | 1 (1) |
| CT       | 14 (18)  | 17 (17) |
| CC       | 63 (80)  | 82 (82) |
| T        | 16 (10)  | 19 (10) |
| C        | 140 (90) | 181 (90) |
| -308 A > G |        |    |
| AA       | 1 (1)    | - |
| AG       | 27 (29)  | 29 (26) |
| GG       | 66 (70)  | 83 (74) |
| A        | 29 (15)  | 29 (13) |
| G        | 159 (85) | 195 (87) |
| -238 A > G |         |    |
| AA       | 2 (2)    | - |
| AG       | 7 (8)    | 11 (10) |
| GG       | 80 (90)  | 97 (90) |
| A        | 11 (6)   | 11 (5) |
| G        | 167 (94) | 205 (95) |

The homozygous -1031 CC is associated with an increased risk of disease (OR = 9.9, 95% CI: 1.3-78, $P = 0.008$); the homozygous -1031 CC is associated (P = 0.02) with the colonic region, the heterozygous -1031 CT is associated with the terminal ileum location (P = 0.03); the homozygous -308 GG (P = 0.008) and -238 GG (P = 0.05) with the upper digestive tract.

### DISCUSSION

In the present study, we determined the allelic and genotypic distribution of SNPs and the corresponding haplotypes in the GR, TNF-$\alpha$ and IL-10 genes among CD children and healthy controls, all of French-Canadian origin.

Genes encoding for immunoregulatory molecules clearly constitute important candidate susceptibility loci for IBD, and a number of recent studies have highlighted the central roles played by IL-10 in orchestrating the immune response. With regard to the IL-10 promoter SNPs (-1082 G > A, -819 C > T, -592 C > A), we found three promoter haplotypes (GCC, ACC and ATA), previously described in Caucasians, and two haplotypes (ACA, ATC) only present in CD children. We found that children carrying the haplotypes GCC (OR = 2.0, CI = 1.08-3.69) and ACC (OR = 2.5, CI = 1.02-6.2) were at increased risk of developing the disease at the ileo-colonic and terminal ileum locations, respectively. Conversely, a protective effect was noted when carrying haplotype GCC against the terminal ileum (OR = 0.34, CI = 0.12-0.96).

![Figure 2](https://www.wjgnet.com)
and the upper digestive tract (OR = 0.4, CI = 0.17-0.91) locations, while the ACC haplotype protected against the ileo-colonic location (OR = 0.49, CI = 0.26-0.93). These observations suggest that the SNP -1082 G > A plays an important role in determining disease location. The functional impact of this SNP has been shown in other studies: the -1082 GG genotype was the most important genetic factor in the regulation of high constitutive IL-10 expression (mRNA levels) and high IL-10 serum levels (protein basal expression)\[18\]; the homozygous -1082 AA was associated with decreased IL-10 production in CD patients and controls\[17\]; and the -1082 G > A was implicated in the production of IL-10 in whole blood samples stimulated with LPS for 24 h\[16\].

TNF-α is a multifunctional cytokine involved in the promotion of inflammatory responses and plays a critical role in the pathogenesis of inflammatory, autoimmune and malignant diseases\[19\]. The functional changes in intestinal mucosa of patients with IBD lead to an increased secretion of proinflammatory cytokines in small and large bowel lamina propria. In fact, TNF-α levels are elevated in serum, mucosa and stool of patients with IBD and the infusion of monoclonal anti-TNF-α antibodies is a highly effective treatment against IBD\[20\]. Intestinal phagocytes or activated macrophages secrete ample amounts of TNF-α, which play an essential role in the pathogenesis of IBD. Variability in the capacity to produce TNF-α seems to be genetically determined\[22\]. For the TNF-α promoter SNPs (-1031 T > C, -863 A > C, -857 T > C, -308 A > G and -238 A > G), we found that children carrying at least one -1031 C allele were associated with the disease in certain locations, i.e. the colon in the case of the homozygous -1031 CC (P = 0.02), and the terminal ileum in the case of the heterozygous -1031 TC (P = 0.03). For SNPs -308 A/G and -238 A/G, both homozygous GG genotypes were associated with the upper digestive tract (P = 0.008 and P = 0.05, respectively), whereas the heterozygous GA (P = 0.008 and P = 0.05, respectively) was associated with the terminal ileum location. The promoter allele -308 A was reported to be associated with greater TNF-α transcription in vitro\[23\]. There are some studies confirming the association between the SNP -308 G > A and susceptibility to IBD\[24,25\] and others showing no association between any of the SNPs tested (-308 A > G, -857 T > C and -238 A > G) and IBD\[16,26,27\]. Furthermore, among the eleven TNF-α promoter haplotypes resolved, TCCGG was more frequent in the control group whereas CACGG was overrepresented in haplotypes resolved, TCCGG was more frequent in the control group whereas CACGG was overrepresented in IBD\[23\].

In several inflammatory diseases, variations in glucocorticoid sensitivity have been reported to be associated with SNPs in the GR gene\[13,28-30\]. In this study we found no evidence of independent involvement of GR allelic variants (R23K and N363S) and CD phenotypic and clinical parameters, despite the reported correlations of the variants N363S with glucocorticoid hypersensitivity\[19\] and R23K with decreased response to dexamethasone\[29\].

In conclusion, this is one of the first studies assessing the impact of candidate gene haplotypes and disease location in childhood CD. The fact that certain associations were observed for given haplotypes rather than at individual SNPs reflects the benefit of haplotype-based analysis. Our results highlight the importance of knowing the haplotype structure because haplotypes potentially capture more genetic diversity that single-locus genotypes and therefore serve as better hallmarks to understand complex traits. Finally, one of the limitations of studying pediatric CD is that a relative small number of patients are available for study. Because of the small sample size, this report should be considered as exploratory and further studies are required to confirm these genetic associations with childhood CD.

ACKNOWLEDGMENTS

Emile Levy and Daniel Sinnett were supported by scholarship awards from Le Fonds de la Recherche en Santé du Québec. We thank Hanni Kabbbara for his contribution with statistical analysis and Vania Yotova for her interesting suggestions.

COMMENTS

Background

Crohn’s disease (CD) is a chronic inflammatory bowel disease that affects more than half a million North Americans. The disregulated expression of the glucocorticoid receptor (GR) and the cytokines interleukin (IL)-10 and tumor necrosis factor (TNF-α) plays a crucial role in the pathogenesis of inflammatory diseases. Single nucleotide polymorphisms (SNPs) in the promoter regions of TNF-α, IL-10 and GR genes may influence the expression of these genes, thereby modulating the susceptibility to CD. This study assesses the impact of specific SNPs and haplotypes on the risk of developing childhood CD.

Research frontiers

Some variants in the promoter region of genes may affect either the expression or activity levels of proteins and therefore may be mechanistically associated with CD risk. Although several gene loci have been associated with susceptibility to CD in adults, the etiology of childhood CD is still unknown. The current study is one if the first studies assessing the impact of candidate gene’s haplotypes and disease location in childhood CD. Furthermore, it highlights the importance of haplotype structures as better hallmarks than single-locus phenotypes to understand the genetics underlying complex traits.

Innovations and breakthroughs

It is important to investigate the genetic variation in susceptibility to CD and identify markers that will facilitate identification of individuals at risk of developing this disease. Although no significant association was found between GR (R23K and N363S) polymorphisms and risk of CD, TNF-α promoter SNPs and IL-10 and TNF-α promoter haplotypes were overrepresented in children with CD and were associated with specific disease location.

Applications

This is one of the first studies investigating the association between haplotype structure and disease location in a CD pediatric cohort. The results of this study will help us to further understand the genetic determinants of childhood CD. Future studies in larger pediatric cohorts and further analysis of the biological function of the identified variants are required to understand the role of TNF-α and IL-10 polymorphisms and related haplotypes in determining the risk of CD.

Peer review

The distribution and frequencies of the genotypes and haplotypes of the genes encoding for TNF-α, IL-10 and GR and the impact of the corresponding variants on risk of CD in a childhood French-Canadian cohort was studied. The results show that polymorphisms in the TNF-α promoter region as well as specific TNF-α and IL-10 haplotypes are associated with the risk of CD, at least in this study population.
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