Experimental colitis and translation to human inflammatory bowel disease

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CHAPTER 4

DEFINITION OF POLYMORPHISMS AND HAPLOTYPES IN THE INTERLEUKIN-12B GENE: ASSOCIATION WITH IL-12 PRODUCTION BUT NOT WITH CROHN'S DISEASE

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Abstract

Interleukin-12 (IL-12) is a key cytokine for the induction of Th1 immune responses. Recently, functional polymorphisms in IL-12p40 (*IL12B*) were found to be associated with susceptibility to several autoimmune diseases. Similarly, variation in *IL12B* might be involved in susceptibility to Crohn's disease (CD), a chronic inflammatory bowel disorder associated with high IL-12 expression. We searched for additional polymorphism in *IL12B* and genotyped a large cohort of CD patients. Differential in-vitro secretors of IL-12 were tested for polymorphism. Polymorphisms were analyzed using the intrafamilial TDT test and by case control analysis. A novel polymorphism was strongly associated with differential expression of IL-12. However, no association with susceptibility to CD was seen for this and other polymorphisms. The high level of conservation is consistent with the key regulatory role of Il-12. The lack of association with *IL12B* makes it unlikely that this gene is directly involved in the susceptibility to CD.

Introduction

Interleukin-12 (IL-12) is a pro-inflammatory cytokine that induces the production of interferon-γ (IFN-γ), favors the differentiation of T helper 1 (TH1) cells and forms a link between innate resistance and adaptive immunity (1, 2). Biologically active IL-12 is a heterodimer formed by a 35-kDa light chain (known as p35 or IL-12α) and a 40-kDa heavy chain (known as p40 or IL-12β) each encoded by separate genes on different chromosomes. Given its pivotal role in Th1 differentiation, the IL-12 genes might be important candidate genes for Th1 mediated diseases. Indeed, evidence for a direct genetic association with polymorphisms in *IL12B* has been found in both type 1 diabetes and severe asthma (3, 4).

The Inflammatory Bowel Diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are chronic idiopathic inflammatory disorders of the gastrointestinal tract that are in part genetically determined. CD, but not UC, has been shown to be associated with high secretion of IL-12 and IFN- γ at lesional sites (reviewed in (5)). In addition, treatment with antibody specific for the p40 chain of IL-12 has been shown to be highly effective in a significant percentage of CD patients (G Bouma, personal communication with W.Strober, NIH, Bethesda MD USA). What drives the aberrant IL-12 responses is currently unknown, but similar to what has been suggested for asthma and type 1 diabetes, genetic variation in *IL12B* might be involved in the propensity to mount such responses. In support of this, we have previously demonstrated strong evidence for a disregulated *IL12B* as one of the main genetic determinants for colitis susceptibility in the trinitrobenzene sulfonic acid (TNBS) mouse model of colitis (6). In an other study we have explored the relation between a single nucleotide polymorphism at position 1188 in the 3'UTR and *in vitro* secretion of IL-12 (7). While the association between genotype and phenotype was notably strong, there was no 100% concordance. This may implicate that the polymorphic variant is not primarily responsible for the association with secretion, but rather part of a high secretor haplotype in which another polymorphic variant is responsible for the phenotypic effect.

Results and Discussion

In the current study we intended to build further on these previous observations. We analyzed the *IL12B* gene for additional polymorphism in a group of 12 individuals that were either high (>300 pg/ml) or low (<50pg/ml) secretors of IL-12 p70 (Fig. 1). We screened all coding exons

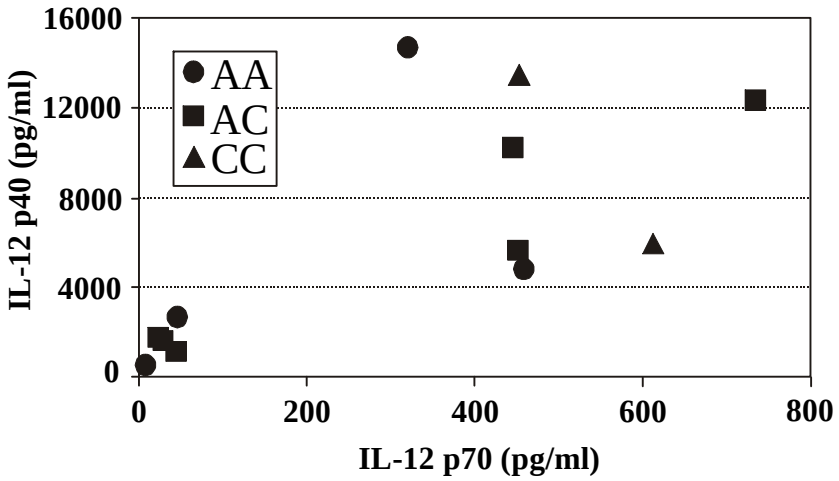

Figure 1. High and low secretors of IL-12 p70. SAC/IFNg stimulated monocytes from healthy donors were selected for high- and low expression of IL-12 p70. Genotypes refer to a *Taq*I polymorphism in the 3'UTR of *IL12B*. Data were generated in a previously published study (7). PCR analysis was done under standard conditions. Primers used generated amplicons (where necessary for contiguity overlapping each other) ranging in size between 150 and 300 bp. For Single-Strand Conformation Polymorphism (SSCP) Analysis the amplicons were denatured and loaded onto precast GeneGel Excel 12.5/24 gels (Amersham Biosciences, Sweden) and subsequently electrophoresed using a thermostatically controlled GenePhor electrophoresis system (Amersham) at 1000 V for 1.5–2.5 hr. at two different temperatures (18 °C and 5°C). Visualization of the DNA strands after electrophoresis was done with silver staining.

(i.e., exons 2 through 6; exons 1 and 7 represent non-coding exons) using SSCP. In addition, 1 kB proximal of the ATG site as well as a stretch of 1 kB located 5' upstream of the transcription initiation site were analyzed (sequences are available upon request).

Despite an extensive analysis in 12 individuals with up to 25 fold inter-individual differences in IL-12 p70 responses, no sequence variation in any of the exons was found. This is in accordance with a previous study (8) and indicates a high level of conservation among hu-

mans. However this is notably different from the situation in inbred strains of mice, where polymorphism is found that affects the structure and expression of IL-12 (6, 9).

In the 5'-UTR region, at position –822 relative to the ATG site, SSCP showed a differential banding pattern which was accounted for by a G→T single nucleotide polymorphism (SNP). More upstream Morahan et al. have previously described an *IL12B* promoter polymorphism (4). In order to design real-time PCR primers and probes for this, we sequenced the region surrounding it. It appeared that the polymorphism was not a single insertion/deletion, but rather a compound event involving a GC/TT transition combined with an AGAG microinsertion (Fig. 2).

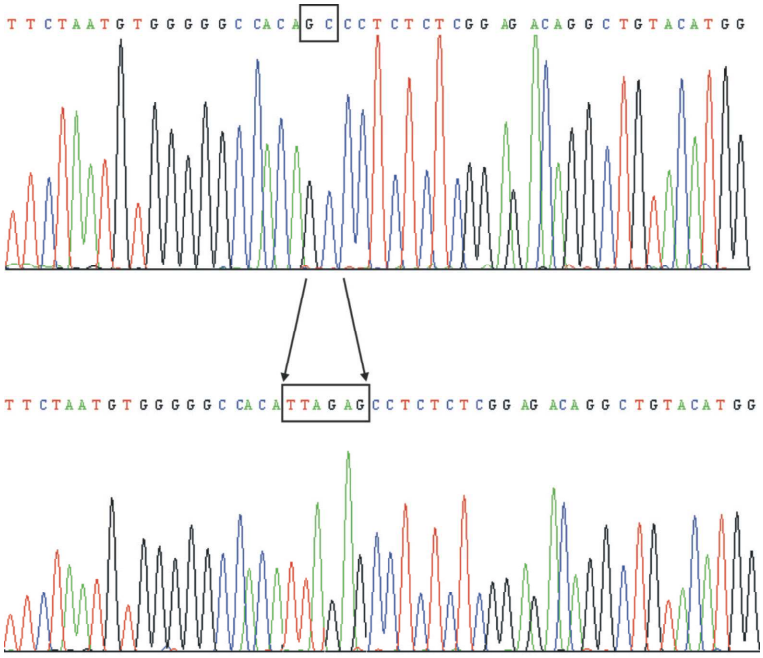

Figure 2. Peak plots showing the compound polymorphism in the upstream promoter region, sequence differences are boxed. The lower sequence shows the AGAG microinsertion together with the GC/TT transition. SNP analysis using real-time PCR primer and probe combinations for all three polymorphisms identified in *IL12B* was done on Biometra T1 or Perkin Elmer GeneAmp 9700 thermocyclers. Plate fluorescence was measured automatically with an ABI Prism 7900HT sequence detection system. For quality control individual plates are tracked during the whole process using a barcode system. Allele calling for each plate was done non-automatic to ensure data quality. Genotyping data were checked for MENDEL errors and HARDY-WEINBERG-equilibrium.

The occurrence of the three polymorphisms in *IL12B* was determined in a large sample of families. The transmission of the three polymorphisms was consistent with the presence of four common haplotypes (Table 1). Regarding the IL-12 secretor status, we found no evidence that these haplotypes were more informative to a high or low secretory type than were the individual genotypes. Interestingly, allele G of the -806 SNP was in 100% linkage disequilibrium with the A allele of the 3'UTR SNP but not with the upstream promoter polymorphism, despite the fact that it is physically more distant from the 3'UTR SNP than from the promoter polymorphism. The frequencies of the different alleles in the healthy Caucasian population are shown in Table 1. The frequency distribution of the upstream promoter polymorphism was similar to that described in the Australian population (4), and the distribution of the *Taq*I polymorphism in the 3'UTR was comparable to that described in European Caucasian populations (10).

Subsequently, we analyzed whether any of the three individual polymorphisms was associated with susceptibility to Crohn's disease or ulcerative colitis. No differences however were seen in the frequency distributions between patients and controls (Table 1). In addition, we determined whether any of the polymorphisms or haplotypes was preferentially transmitted from heterozygous parents to affected children using the transmission disequilibrium test (Table 1).

Based on the results from the case-control study and the intra-familial study, it can be concluded that a direct role for *IL12B* in susceptibility to IBD is unlikely. Although, it can not be excluded that *IL12B* variants may play a role in a subgroup of patients or in different populations.

The results suggest that the strong correlation previously found between the 3'UTR polymorphism and *in vitro* secretion is either a direct effect of this SNP, or from a more distant polymorphism that was not included in the current analysis, or from other, as yet unknown mechanism(s). In this regard it is of interest that re-analysis of our previous data revealed that similar to the results of Morahan and colleagues (4), the median expression of IL-12 p40 (but not that of p70) was decreased in individuals heterozygous for the promoter polymorphism.

In conclusion, we have confirmed a high level of conservation of the IL-12 gene. In addition, our data do not provide a direct role for this gene in the predisposition to human

Crohn's disease. Further studies to identify the factors that drive the high IL-12 response in these patients are warranted.

Table 1.Transmission of *IL12B* haplotypes and alleles from heterozygous parents to affected children and frequency distribution of three polymorphisms in *IL12B* in patients and healthy controls (HC).

Transmission of *IL12B* alleles and haplotypes

	CD transmissions		UC transmissions		IBD transmissions	
	*n** (total)	%	*n** (total)	%	*n** (total)	%
Haplotype**						
A	128 (237)	54.0	67 (118)	56.8	195 (355)	54.9
B	12 (27)	44.4	5 (12)	41.7	17 (39)	43.6
C	105 (216)	48.6	43 (98)	43.9	148 (314)	47.1
D	64 (138)	46.4	38 (78)	48.7	102 (216)	47.2
Allele						
-6416 INS	155 (294)	53	75 (145)	52	230 (439)	52
-6416 GC	139 (294)	47	70 (145)	48	209 (439)	48
-806 G	95 (186)	51	57 (114)	50	152 (300)	51
-806 T	91 (186)	49	57 (114)	50	148 (300)	49
+10841 A	95 (186)	52	54 (107)	50	152 (300)	51
+ 10841 C	91 (186)	48	53 (107)	50	148 (300)	49

Frequency distributions of three polymorphisms in *IL12B*

Genotypes	CD (*n*=305) %	UC (*n*=151) %	HC (*n*=520) %
-6416 INS/INS	26	27	23
-6416 INS/GC	49	48	54
-6416 GC/GC	24	25	24
-806 G/G	64	58	65
-806 G/T	33	37	31
-806 T/T	3	5	4
+10841 A/A	64	58	65
+10841 A/C	33	37	31
+10841 C/C	3	5	4

*n**, number of transmissions.

Haplotype**, distribution of the three common polymorphisms in *IL12B* (–6416 promoter, –806 5'-UTR, +10841 3'-UTR) is as follows: A (-6416 INS; -806 T; +10841 C), B (-6416 INS; -806 G; +10841 A), C (-6416 CG; -806 T; +10841 C), D (-6416 CG; -806 G; +10841 A).
To analyze the involvement of *IL12B* variants in the susceptibility to IBD, a total of 464 trios (i.e., families including both parents and at least one affected child) were studied. This cohort included 311 trios with Crohn's disease and 153 with ulcerative colitis which have been described in detail elsewhere (11). For case-control analysis, a total of 520 healthy Caucasian blood donors from the same geographic area were recruited.

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