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Association of *IL10* promoter polymorphisms with idiopathic achalasia

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Idiopathic achalasia is an esophageal motor disorder of unknown etiology. A wealth of evidence supports the concept that achalasia is an immune-mediated disease. According to this evidence, achalasia has been significantly associated with specific alleles of the human leukocyte antigen class II, *PTPN22* and *IL23R*. Several studies have demonstrated association of the *IL10* gene with different inflammatory disorders. Our aim was to evaluate the role of functional *IL10* promoter polymorphisms in susceptibility to idiopathic achalasia. A case–control study was performed with the -1082, -819, and -592 *IL10* promoter polymorphisms in 282 patients and 529 controls and in an independent replication set of 75 patients and 575 controls. The GCC haplotype of the *IL10* promoter was reported to be associated with a lower risk of achalasia in the discovery sample (odds ratio [OR] = 0.79, 95% confidence interval [CI] = 0.64–0.98, p = 0.029). This association was validated in a replication set (OR = 0.69, 95% CI = 0.48–1.00, p = 0.04). In the combined analysis no heterogeneity was observed between the 2 sample sets and the GCC haplotype was significantly associated with the disease (OR_{MH} = 0.76, 95% CI = 0.63–0.91, p = 0.003). Our results provide the first evidence for an association between *IL10* promoter polymorphisms and idiopathic achalasia, suggesting that the interleukin-10 cytokine may contribute to the pathogenesis of this disease.

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1. Introduction

Idiopathic achalasia is an esophageal motor disorder characterized by incomplete relaxation of the lower esophageal sphincter (LES) following deglutition and absence of coordinated peristalsis. Histologic examination reveals a significant decrease in the number of myenteric neurons, especially inhibitory nitric oxide–releasing neurons, in the distal esophagus and at the level of the LES [1]. This degeneration of the inhibitory innervation seems to be responsible for the reported abnormal esophageal function; however, the initiating cause remains largely unknown.

The presence of an inflammatory infiltrate within the LES [2,3], the occurrence of circulating autoantibodies against the myenteric plexus [4–6], and the increased prevalence of certain human leukocyte antigen (HLA) class II antigens [7–9] support the existence of an immunoinflammatory mechanism implicated in the pathogenesis of achalasia. It has been postulated that an environmental insult, such as a viral infection, leads to a chronic inflammatory process, resulting in damage to the myenteric plexus in genetically predisposed subjects [10].

In recent years, some studies have explored the genetic background underlying the risk to develop achalasia. The role of polymorphisms in the *ALADIN* gene, involved in the triple-A syndrome (characterized by achalasia, alacrima, and adrenal abnormalities), and *NOS* gene polymorphisms was studied with negative results [11–13]. A functional polymorphism in the lymphoid tyrosine phosphatase N22 gene (*PTPN22*) has been described as a susceptibility factor for women with achalasia [14] and Paladini et al. demonstrated an association between achalasia and the human vasoactive intestinal peptide receptor 1 gene (*VIPR1*) in patients with late disease onset [15]. In a recent study, we described that the Arg381Gln *IL23R* variant confers predisposition to achalasia [16]. Interestingly, *PTPN22* and *IL23R* genes are associated with several autoimmune disorders [17,18].

IL10 gene polymorphisms were also associated with different immunopathological conditions, such as systemic lupus erythematosus [19,20], type 1 diabetes [21], ulcerative colitis [22], and asthma [23]. Interleukin-10 (IL-10) is an important immunosuppressor cytokine involved in many aspects of immune responses.

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Levels of IL-10 are critical in immune regulation, controlling the balance between inflammatory and humoral responses, mainly through the inhibition of the proinflammatory mediator's production. IL-10 is also a potent inhibitor of antigen presentation [23,24].

Analysis of the differences in IL-10 secretion between twins and nonrelated individuals suggests that 75% of the differences in IL-10 production are genetically determined [25]. The human *IL10* gene is located on chromosome 1 (1q31–q32); its promoter region spans 5 kb and it is very polymorphic [26,27]. The 3 best characterized single nucleotide polymorphisms (SNPs) are at positions -1082 A/G, -819 T/C, and -592 A/C and the high linkage disequilibrium among them determines only 3 haplotypes (GCC, ACC, and ATA). These variants have been involved in the transcriptional rate of IL-10 and therefore in the production level of the cytokine. Different *in vitro* studies indicated that the GCC haplotype is associated with a high IL-10 production [28–31]. Furthermore, carriers of the GCC haplotype had higher mucosal IL-10 mRNA levels than carriers of other haplotypes in biopsies of patients with chronic *Helicobacter pylori* infection. This result demonstrated the functional relevance of these SNPs *in vivo* [32].

Based on the previous data, the aim of this study was to assess whether the IL-10 -1082 A/G, -819 T/C, and -592 A/C variants are associated with development of idiopathic achalasia in the Spanish population.

2. Subjects and methods

2.1. Subjects

The study group consisted of 282 nonrelated idiopathic achalasia patients and 529 healthy subjects consecutively recruited at the Hospital Clínico, San Carlos, Madrid. The gender distribution of achalasia patients was 44% female and 56% male, ranging in age from 14 to 83 years (mean 44, median 44). For replication purposes, an independent Spanish sample recruited in 2 centers in Barcelona (Hospital Vall d'Hebron and Centro Médico Teknon) was also studied. This replication group consists of 75 achalasia patients (55% female and 45% male; ranging in age from 21 to 79 years, mean 49, median 53) and 575 healthy controls. Both control groups included mainly blood donors and ethnically matched hospital employees. Individuals with immune-related diseases were excluded. All participants were of white ethnicity and provided their written informed consent to participate in the study, which was conducted in accordance with the ethics committee of the respective hospitals.

2.2. Clinical diagnosis

The diagnosis of achalasia was established on the basis of clinical, radiographic, endoscopic, and manometric criteria. Esophageal body motility and LES pressures were measured using an 8-lumen polyvinyl catheter (4.5-mm diameter) and low-compliance pneumohydraulic capillary infusion system. The LES pressures were measured by both rapid and station pull-through techniques. Contractions in the esophageal body were measured with the 4 proximal openings positioned 3, 8, 13, and 18 cm above the LES. Fifteen wet swallows (with 5 mL water) were given, separated by 30-second intervals. LES relaxation was considered normal if LES pressure dropped greater than 90% from mean resting LES to gastric baseline pressure. None of the patients suffered from diagnosed autoimmune diseases or cancer and the possibility of secondary achalasia was excluded by careful anamnesis considering radiologic and endoscopic studies. The median follow-up was 22.7 months (range 2–144 months) and the age at disease onset ranged from 14 to 82 years (mean 45, median 45). Table 1 illustrates the main clinical characteristics of the patient collection.

Table 1
Clinical features in achalasia patients studied

	Achalasia patients
Mean evolution (months)	22.7
Dysphagia (%)	100
Chest pain (%)	31.5
Regurgitation (%)	78.5
Weight loss >5 kg (%)	56.2

2.3. Genetic and antibodies analysis

Genomic DNA was extracted from EDTA-anticoagulated peripheral blood leukocytes by a salting-out procedure with a success rate of 97%.

SNPs -1082 A/G (rs1800896) and -592 A/C (rs1800872) were genotyped in patients and controls (included in the same plate) by TaqMan-MGB methodology. Primers and probes were obtained from Applied Biosystems using the TaqMan Pre-Designed Assays-on-demand kits (assay ID for *IL10*-1082: C_1747360_10; assay ID for *IL10*-592: C_1747363_10). Amplifications were carried out in a 7900HT fast real-time polymerase chain reaction system, under the conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA). In some control subjects, a third promoter polymorphism, -819 C/T (rs1800871), was also genotyped. This third SNP was demonstrated to be in complete linkage disequilibrium with the -592 A/C and therefore it could be always inferred in the rest of the samples. Quality control was assessed by regenotyping 10% of the samples. Genotyping call-rate success was over 99%.

HLA DQA1 and *DQB1* typing was carried out by polymerase chain reaction amplification and hybridization with allele-specific oligonucleotides according to the recommendations of the 11th International Histocompatibility Workshop.

Autoantibodies against myenteric plexus (AAM) were determined by indirect double immunofluorescence following the method of Ruiz de León et al. [33], based on the technique described by Storch et al. [5].

2.4. Statistical analysis

Genotype frequencies for each *IL10* gene polymorphism among control subjects were tested for Hardy–Weinberg equilibrium by a χ^2 test with 1 degree of freedom. Differences in allele, genotype, and haplotype frequencies were evaluated by the χ^2 test and associations were measured by the odds ratio (OR) with 95% confidence interval (CI). For each marker, the most common homozygous genotype was used as the reference category. p values under 0.05 were considered significant. Bonferroni correction was applied according to the number of analyzed variables. The combined analysis of the 2 data sets was performed using the Mantel–Haenszel (MH) statistical test. Statistical analyses used Epidat (Xunta de Galicia and Pan America Health Organization) version 3.1.

3. Results

The genotype frequencies of the studied polymorphism did not deviate significantly from Hardy–Weinberg expectations in controls ($p > 0.05$).

Genotype, allele, and haplotypes frequencies of the *IL10* promoter polymorphisms in patients and controls of the discovery set are shown in Table 2. The frequency of the GCC haplotype was significantly lower in patients than in controls compared with the ATA/ACC haplotypes (OR = 0.79, 95% CI = 0.64–0.98, $p = 0.029$).

Associations involving the *PTPN22*, *VIPR1*, and *IL23R* genes have been reported in achalasia patients stratified by gender and/or age at disease onset [14–16]. According to this, we decided to analyze the distribution of the *IL10* promoter haplotypes stratified by those characteristics. No statistically significant differences in haplotype frequencies were observed in any case (Table 3).

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