Influence of interleukin 10 promoter polymorphisms in polymyalgia rheumatica: disease susceptibility and functional consequences

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Abstract

Objective

To investigate the functional consequences of IL10 (-592C/A *and* -1082A/G*) gene polymorphisms and their association with susceptibility to, and disease phenotype, in patients with polymyalgia rheumatica (PMR).*

Methods

A total number of 168 with PMR and 124 age-matched controls were genotyped using allele-specific primers and restriction fragment length polymorphism analysis. The levels of circulating IL10 and the production of IL10 by PBMCs after in vitro stimulation were studied by Cytometric Bead Array.

Results

No significant differences were observed in genotype or allele frequency distribution between patients and controls. The clinical characteristics and prognosis of these patients were also unrelated to the presence of these polymorphisms. No significant differences between PMR patients with low ESR (<40 mm/hr) and classic PMR (>40 mm/hr) were found. Furthermore, we did not observe any influence of circulating IL10 with the intensity of the acute phase response. In both, PMR patients and age-matched controls, no differences in circulating IL10 levels or IL10 production were observed depending on the genotypes of the IL10 gene.

Conclusion

These results do not support the impact of IL10 variants in susceptibility or clinical phenotype of PMR patients. In this aged population no functional association was found between IL10 gene variants and IL10 production.

Key words

interleukin 10, IL10 gene polymorphism, polymyalgia rheumatica

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Introduction

Polymyalgia rheumatica (PMR) is a clinical syndrome characterised by pain and stiffness in the neck, shoulder and pelvic girdles that occur only in patients older than 50 years of age (1). PMR is characterised by a marked acute phase response and an excellent response to low-dose corticosteroid therapy (1-3). Cytokines are involved in the pathogenesis of PMR and a number of studies have been conducted to investigate whether a genetic basis for disease may be found in polymorphisms of cytokine genes (4). These cytokines may also affect the gene regulation and upregulate surface expression of the endothelial cell adhesion molecules to allow subsequent migration of leukocytes into the inflamed tissues (5).

Giant cell arteritis (GCA) is a large- and medium-sized blood vessel systemic vasculitis that shares many clinical features with PMR and often overlaps in the same patient (6, 7). However, the main pathogenic mechanisms involved in the development of a full-blown vasculitis or in the predominance of musculoskeletal complaints characteristic of PMR are currently unknown (3, 8).

Interleukin (IL) 10 is an important immunoregulatory and anti-inflammatory cytokine (9). The reasoning behind the proposed involvement of IL10 gene polymorphisms in chronic inflammatory conditions or their manifestations is that it may influence the serum concentrations and *in vitro* production of IL10 (10-13). This presumption is based on the observation that individuals differ with respect to the level of cytokine production after *in vitro* culture of their cells and that these differences may be attributed partially to single nucleotide polymorphisms (SNPs). Indeed, individuals with cytokine genotypes are being called 'high', 'intermediate' or 'low' producers, which is based upon that genotype and the corresponding *in vitro* cytokine production (14, 15). Although it is likely that IL10 is involved in the pathogenesis of many diseases, a paucity of data exists on their expression and functional consequences and their role in age-restricted chronic inflammatory conditions like GCA (16, 17) and PMR (18).

The aims of this study were to investigate the genetic association of *IL10* (*-592C/A* and *-1082A/G*) polymorphisms with susceptibility to disease, clinical manifestations and prognosis in patients with PMR, and to investigate the functional consequences of IL10 SNPs on circulating levels of IL10 and *in vitro* IL10 production in patients as well as in controls.

Material and methods

Study subjects

The present study included 168 with PMR and 124 age-matched healthy controls. The main demographic, clinical and laboratory characteristics of the study population are shown in Table I. PMR was diagnosed according to the criteria proposed by Chuang *et al.* (1). In patients with PMR the possibility of GCA was excluded either by a normal temporal artery biopsy or by the absence of manifestations of GCA and cure with low-dose prednisone after a long-term follow-up. Both patients and controls were non-related Caucasians of Spanish ancestry and lived in the same geographic area of north Spain, Cantabria. All the patients and controls gave written informed consent, and the study was approved by the regional Ethics Committee. The clinical findings at diagnosis and during follow-up, the ESR and CRP values at diagnosis, as well as the initial prednisone dosage, were ascertained by reviewing the patients' medical records. For the analysis of some variables such as relapses/recurrences, duration of corticosteroid therapy, and accumulated dose of prednisone, only patients with a follow-up ≥2 years were included.

Blood collection, DNA extraction and genotyping

Genomic DNA was isolated from 5 ml of whole blood, using a DNA isolation kit (GENTRA, GENERATION DNA Purification kits; Minneapolis, MN), according to the manufacturer's instructions. The two gene polymorphisms of *IL10*, at positions *-592* (rs1800872) and *-1082* (rs1800896) from the transcription start site were amplified by polymerase chain reaction, as described by Boiardi *et al.* (16). Briefly, each PCR for IL10 was performed in a 25-μl volume

containing 1µl of DNA, 1.25 U of Taq polymerase (GoTaq Flexi DNA polymerase, Promega, Madison, WI) in PCR buffer 5×, 2 µmol/l deoxyribonucleotide triphosphates (dNTPs), 1.5 mmol/l $MgCl_2$, and 2 µmol/l of each primer. The forward primer for *–1082* position was 5´-AACACTACTAAGGCTCCTTTGGGA-3´ and the reverse primer was 5´- CAAGGAAAAGAAGTCAGGATTCCATGGA-3´. The parameters for the thermocycling were as follows: denaturation at 94ºC for 2 min followed by 35 cycles of denaturation at 94ºC for 20 s, annealling at 60ºC for 20 s and extension at 72ºC for 20 s. This was followed by final extension at 72ºC for 3 min. The PCR products were digested with 2U of *EcoNI* (New England Biolabs, Beverly, MA) restriction enzyme at 37ºC for overnight and fragments were separated by electrophoresis on a 3% agarose gel. The PCR products in *IL10A/G -1082* produce fragments of 82bp and 20bp. The forward primer for *-592* position was 5´- CTCAGTTAGCACTGGTGTAC-3´ and the reverse primer was 5´- TGTTCCTAGGTCACAGTGAC-3´. The parameters for the thermocycling were as follows: denaturation at 94ºC for 4 min, 60ºC for 2 min and 72ºC, followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 30 s and extension at 72ºC for 40 s. This was followed by final extension at 72ºC for 4 min. The PCR products were digested with 2U of *RsaI* (New England Biolabs, Beverly, MA) restriction enzyme at 37ºC for 3 hours and fragments were separated by electrophoresis on a 2% agarose gel. The PCR products with *IL10C/A -592* produce fragments of 480bp and 240bp. Two investigators who were blinded for the clinical data annotated all genotypes independently.

Table I. Demographic data and main clinical features of patients with polymyalgia rheumatica (PMR) and healthy controls.

	Controls	PMR
Number of patients	124	168
Age (mean ± SD)	74.3 ± 11.0	73.1 ± 7.7
Gender (% females)	68.3	61.3
Time to diagnosis (months)	–	3.3 ± 2.6
PMR symptoms (%)	–	100
Ischemic symptoms (%)	–	0
TAB (positive /done)	–	0 /61
Pre-treatment ESR (mm/1 hr)	–	56.5 ± 30.2
Pre-treatment CRP (mg/dl)	0.3 ± 0.2	4.7 ± 5.2

PMR: polymyalgia rheumatica; SD: standard deviation; TAB: temporal artery biopsy; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

Supplementary Table I. Hardy-Weinberg *p*-value distribution of IL10 gene in patients and controls.

SNP	Observed Heterozygosity	Expected Heterozygosity	*p*-value
Controls			
IL10 -592C/A	0.38710	0.35105	0.30624
IL10 -1082A/G	0.57258	0.49259	0.09837
PMR			
IL10 -592C/A	0.37500	0.38491	0.84001
IL10 -1082A/G	0.45833	0.48214	0.52295

PMR: polymyalgia rheumatica.

Detection of soluble cytokines in serum

The serum was isolated from 2.5 ml of blood obtained in tubes without additives from each individual (PMR=57 and HC=22) and stored at -80ºC until analysis. The quantitative determination of IL10 in serum was performed using the Cytometric Bead Array (CBA) Human Inflammation kit (BD Biosciences, San Diego, CA) as previously reported (19). The fluorescence produced by CBA beads was measured on a FACScalibur Flow Cytometer (BD Biosciences) and was analysed using Software CBA Folder Excel 98 (BD Biosciences). Detection limit was 3.3 pg/ml for IL10.

Detection of cytokines in cell culture supernatants

PBMC from heparinised blood were obtained by gradient centrifugation (Ficoll Histopaque 1077, Sigma Aldrich). PBMCs from 10 PMR patients and 10 HC were cultured in RPMI 1640 supplemented with 10% FCS and stimulated with PMA (100 ng/ml) and ionomycin (4 µg/ml) for 24 hours. Supernatants were obtained and stored at -80ºC until analysis. Release of IL10 into the culture supernatants was determined using the CBA kit as described above.

Statistical analysis

All the statistical analysis of data was carried out using SPSS 12.0 (Chicago, IL, USA). Arlequin v3 software was used to determine the Hardy-Weinberg equilibrium and haplotype analysis. The strength of the association between patients and alleles or genotypes of the *IL10* gene was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either chi-square or Fisher exact test analysis. For expression and functional studies, the statistical comparisons of data between patients and controls were performed using the Mann-Whitney U-test. Differences were considered significant when *p*-values were <0.05.

Results

Genotypic and allelic frequencies of IL10 -1082A/G *and* -592C/A *promoter polymorphisms*

The studied population was in Hardy-Weinberg equilibrium for both *-592C/A* and *-1082A/G IL10* promoter polymorphisms (Supplementary Table I). No significant differences in the genotypic or allelic frequency between patients and controls for the IL10 polymorphisms were observed (Table II). None of the haplotypes in patients differ significantly when compared to controls (Table III).

Table II. Distribution of IL10 -592C/A and -1082A/G genotype, allele frequency and allele carriage frequency in patients with polymyalgia rheumatica (PMR) and control group.

Genotype	Controls (n=124)	PMR (n=168)	*p*-value	OR (95% CI)
IL10 –592 C/A (db SNP ID rs1800872) polymorphism				
Genotype Frequency				
CC	72/124 (58.1)	93/168 (55.4)	–	Reference
CA	48/124 (38.7)	63/168 (37.5)	0.948	1.016 (0.625-1.651)
AA	04/124 (3.2)	12/168 (7.1)	0.149	2.323 (0.719-7.503)
Allele Frequency				
C	192/248 (77.4)	249/336 (74.1)	0.358	1.198 (0.815-1.760)
A	56/248 (22.6)	87/336 (25.9)		
Allele carriage frequency				
C allele carriage	120/124 (96.8)	156/168 (92.9)	0.146	0.433 (0.136-1.377)
A allele carriage	52/124 (41.9)	75/168 (44.6)	0.645	1.117 (0.699-1.784)
IL10 -1082A/G (db SNP ID rs1800896) polymorphism				
Genotype Frequency				
AA	35/124 (28.2)	62/168 (36.9)	–	Reference
AG	71/124 (57.3)	77/168 (45.8)	0.066	0.612 (0.362-1.035)
GG	18/124 (14.5)	29/168 (17.3)	0.796	0.909 (0.443-1.867)
Allele Frequency				
A	141/248 (56.9)	201/336 (59.8)	0.472	0.885 (0.635-1.234)
G	107/248 (43.1)	135/336 (40.2)		
Allele carriage frequency				
A allele carriage	106/124 (85.5)	139/168 (82.7)	0.528	0.814 (0.429-1.544)
G allele carriage	89/124 (71.8)	106/168 (63.1)	0.120	0.672 (0.407-1.110)

PMR: polymyalgia rheumatica; OR: odds ratio; CI: confidence interval.

Table III. Haplotype distribution of IL10 -592C/A and -1082A/G among polymyalgia rheumatica (PMR) and healthy control groups.

Haplotype	Controls (n=124)	PMR (n=168)	*p*-value	OR (95% CI)
IL10 -1082 A/G, -819 C/T and -592C/A				
ACC	92/248 (37.1%)	118/336 (35.1%)	0.623	0.918 (0.652-1.291)
ATA	49/248 (19.8%)	83/336 (24.7%)	0.158	1.332 (0.894-1.986)
GCC	100/248 (40.3%)	131/336 (39.0%)	0.744	0.946 (0.676-1.323)
GTA	07/248 (2.8%)	04/336 (1.2%)	0.152	0.415 (0.120-1.433)

PMR: polymyalgia rheumatica; OR: odds ratio; CI: confidence interval.

IL10 gene polymorphisms are not associated with clinical manifestations and disease prognosis in PMR patients

For PMR patients, low ESR (<40 mm/hr) and classic PMR (>40 mm/hr) subgroups were analysed. No significant differences between both subgroups were found. Furthermore, we did not observe any influence of circulating IL10 with the intensity of the acute phase response (Fig. 1).

The prognosis of PMR was addressed by analysing the presence of at least one relapse/recurrence, number of relapses, duration of corticosteroid treatment and cumulative prednisone dose. As patients had different time of follow-up, only patients with at least two years of follow-up were included. No significant association between the IL10 gene polymorphisms and any of these variables were observed.

IL10 gene polymorphisms are not associated with production of IL10 in patients with active disease and controls

Although the results are probably limited by the sample size, we did not find an association of the *IL10* (*-1082A/G, -592C/A*) gene polymorphisms studied with circulating IL10 levels (Fig. 2) or with *in vitro*-induced IL10 production (not shown).

Discussion

Cytokines are involved in the pathogenesis of most inflammatory chronic diseases and an increasing number of studies have addressed their role in the pathogenesis of PMR. During the last few years, several approaches have been taken to study the role of cytokine gene polymorphisms in patients with PMR and GCA (4). As both disorders are characterised by a strong acute phase systemic response, one approach has been to study the genes of proinflammatory cytokines that might be involved in this phenomenon. As a delicate balance between pro- and anti-inflammatory cytokines regulates the inflammatory response, another approach might be to study those genes involved in the suppression of the proinflammatory response such as IL-10 (20).

Nowadays, there is evidence that the net effect of IL10 is to suppress inflammation in a variety of diseases that are characterised by a prevalent Th1 response (20, 21). In PMR, over expression of IL10 may be a positive circumstance that may ameliorate the disease (18). With respect to possible common pathogenic mechanisms in PMR and GCA, one might speculate that patients with PMR may have an increased tissue IL10 expression compared to patients with GCA, which may protect them from overt arteritis (18). This hypothesis is also consistent with the results of Salvarani and co-workers (16) that suggest that a genetically determined down-regulation of the IL10 synthesis may be implicated in the pathogenesis of GCA and a relative impairment of IL10 synthesis could favour the development of a full-blown Th1 response, leading to the classic histological and clinical features of GCA. This issue has been addressed in the present study by analysing a large and well-defined population of PMR patients. However, our results do not support the hypothesis of Salvarani *et al*. (16). We did not find significant differences in the frequencies of the *IL10 -592C/A* and the *IL10 -1082A/G* gene polymorphisms between PMR and GCA patients (data not shown). Furthermore, the gene variants analyse did not influence the presence of polymyalgic symptoms in GCA patients (data not shown).

Although our results did not show a sig-

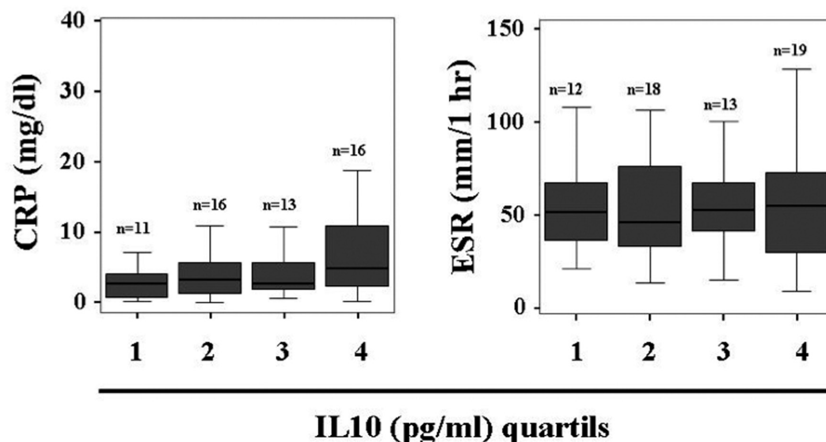

Fig. 1. The levels of circulating IL-10 do not influence the intensity of the acute phase reaction. Circulating levels of IL-10 in patients with PMR were measured by Cytometric Bead Array and grouped according to the quartile distribution (1: <25 quartile; 2: 25-50 quartile; 3: 50-75 quartile; 4: >75 quail). The figure show the levels of C-reactive protein (CRP)(Left panel) and erythrocyte sedimentation rate (ESR)(Right panel) according to the levels of circulating IL-10.

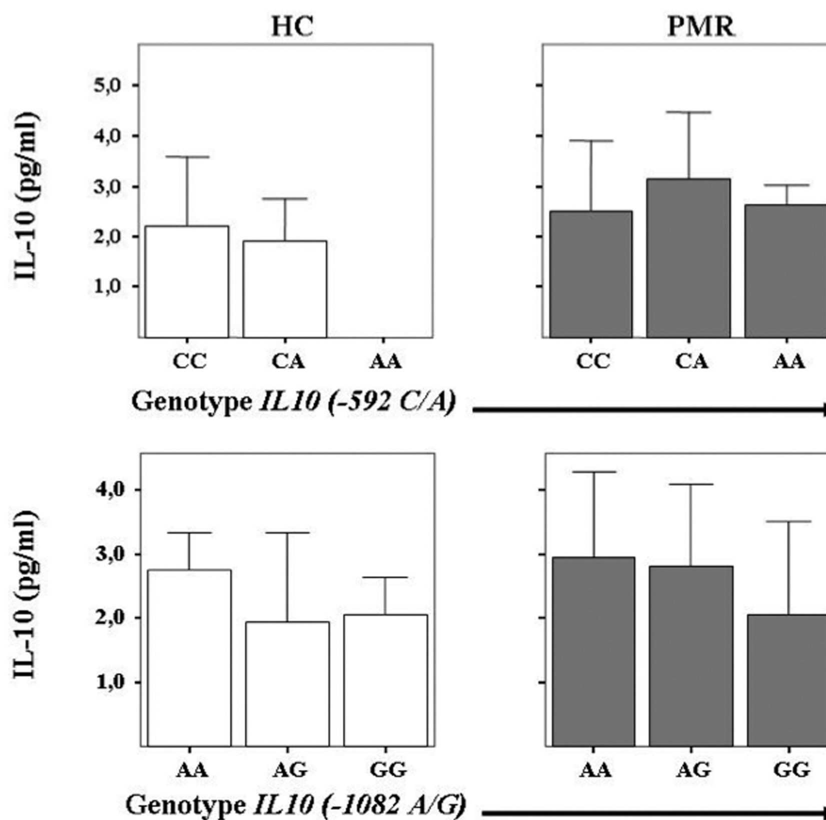

Fig. 2. Circulating levels of IL-10 according to IL10 -1082A/G and -592C/A genotypes at disease onset in patients with polymyalgia rheumatica (PMR) and healthy controls (HC). Circulating levels of IL-10 in PMR patients and HC were measured by Cytometric Bead Array.

nificant association between the IL10 gene polymorphisms studied and susceptibility and/or clinical phenotype in patients with PMR, we cannot exclude a false negative result. The main two explanations for a false negative association include population heterogeneity and statistical power (22). Although PMR is a clinical syndrome, and therefore may include a heterogeneous patient population, the first explanation is less likely due to uniformity in the diagnostic criteria between the different rheumatologist included in patient selection, and specially the close follow-up of these patients and the exclusion of those patients who changed the initial diagnosis during long-term follow-up. Despite the fact that this is the largest series of patients with PMR included in genetic studies, the major limitation of the present study is the low statistical power. Therefore, and taking into consideration the contradictory results in another close-related disorder such as GCA (16, 17), it is necessary to confirm these results in other patient populations.

The reasoning behind the proposed involvement of cytokine gene polymorphisms in susceptibility or disease severity is that they may influence *in vivo* cytokine levels (23). *In vitro* studies of human PBMC have revealed a genetic component in the large interindividual differences in the production of IL10 (23, 24). More specifically, the *A592* allele has been found to correlate with low levels of LPS-stimulated IL10 production. The same is true for the *A* allele of the *-1082A/G* polymorphism and for the *ATA* haplotype (23). To date, there are no available reports correlating the influence of IL10 gene polymorphisms and their functional consequences in PMR. In the present study, we examined for the first time the contribution of two polymorphisms (*-592C/A, -1082A/G*) in the promoter region of the IL10 gene along with their functional consequences for the susceptibility to PMR. Thus, we compared *in vivo* and *in vitro* cytokine production between patients and healthy individuals according to the IL10 gene polymorphism. As shown here, and although probably limited by the sample size, no significant association of any of these gene variants was found in patients and controls. Our results are in agreement with the work done by Warle *et al*. (20) in liver transplant patients and healthy volunteers. They reported no significant associations between *in vitro* ILl0 production and *-1082A/G* polymorphism. However, in another study (25), the *-1082A* allele was associated with low, and the *-1082G* allele with high *in vitro* IL10 production in rheumatoid arthritis patients. In any case, the poly-

morphisms studied in the present study have been associated with variability in IL10 production (10-13) and some other genetic influence, such as the microsatellites IL10.R and IL10.G (26), could contribute to this lack of association. Besides, we have only searched for the production of IL10 after *in vitro* stimulation of T cells but some other cells residing in the tissues, such as macrophages, have been also described to produce IL10 (19).

In summary, our results do not support the impact of IL10 variants in PMR patients. Due to the low statistical power, it is necessary to confirm these results in other patient populations. In this aged population no functional association was found between IL10 gene variants and IL10 production.

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