Resequencing of the *IL12B* gene in Psoriasis patients with the rs6887695 / rs3212227 risk genotypes.

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Abstract.

Background and aims: Recent genomic surveys have identified *IL12B* as susceptibility locus for psoriasis (Ps). Our aim was to replicate the association between *IL12B* SNPs and Ps. In addition, we sequenced the *IL12B* gene in several patients to identify new variants that could explain the disease-risk.

Results: A total of 304 Ps-patients and 422 healthy controls (all Caucasian Spanish) were genotyped for three *IL12B* polymorphisms. SNP rs6887695 (GG genotype) was significantly associated with Ps ($p=0.002$; OR=1.60, 95%CI=1.19-2.16). This genotype was also more frequent among patients with severe psoriasis ($p=0.03$). Sequencing of 30 patients with the risk genotype identified several *IL12B* reported SNPs. Allele and genotype frequencies for two putative functional variants (rs3213120 and rs3213119) did not differ between patients and controls.

Conclusions: Our study confirmed rs6887695 as a risk factor for Ps. No other *IL12B* variants that could explain this association were found in our patients.

1. Introduction

Genome wide association studies (GWAs) identified significant association between *IL12B* single nucleotide polymorphisms (SNPs) and Psoriasis (Ps) [1,2]. *IL12B* is on chromosome 5q31 and encodes the IL12 p40 subunit that heterodimerizes with the IL12 p35 subunit (*IL12A* gene) to form IL12, or with the IL23 p19 subunit (*IL23A* gene) to form IL23. In addition, IL12 p40 exists as a monomer and as a homodimer (IL12 p80). IL12b is important for the generation of T-helper 1 (Th1) cells, and this would explain its role on Ps and other inflammatory-mediated processes [3-5]. Moreover, the role of the interleukin-12/23 p40 cytokines in the pathophysiology of Ps was also supported by the efficacy of an interleukin-12/23 monoclonal antibody therapy [6].

Most of the case-control studies analyzed one *IL12B* SNP in the 3' untranslated (UTR) region (rs3212227) and two 3' extragenic SNPs (rs2082412 and rs6887695) [7-11]. The functional role of these variants was unclear. An effect of rs3212227 on the production of IL12 subunits by cultured cells has been reported, but others failed to replicate this association [12-14]. It is thus possible that these SNPs linked to Ps were surrogate markers for other functional *IL12B* variants. Our aim was to replicate the association between Ps and these *IL12B* SNPs, and to determine whether some functional *IL12B* sequence variant could explain the association.

2. Patients and Methods

2.1. Patients and Controls. A total of 304 patients with Ps (mean age 47 ± 15 years; 54% men) were recruited by Dermatologists from Hospital Universitario Central Asturias (HUCA) in the period January 2007-Oct 2010. The main characteristics of most of these patients had been previously reported, and are summarised in **Table 1** [15]. Ps was diagnosed based on clinical findings. The disease was defined as severe or non-severe according to the Psoriasis Area and Severity Index (PASI): patients with a PASI score ≥ 10 were classified as severe, and those with a PASI < 10 as non-severe. Patients who had at least one first degree relative also Ps-affected were classified as familial cases. The existence of arthritis was assessed by a rheumatologist. The control group comprised 422 non-related healthy individuals (mean age 47 ± 13 years; 55% men), recruited through the Blood Bank and the Dermatology Department of HUCA. All the patients and controls were Caucasians from the

region of Asturias (Northern Spain, total population 1 million), and gave their written informed consent to participate in the study, approved by the Ethical Committee of HUCA.

2.2. IL12b SNPs genotyping. SNPs rs6887695 and rs3212227 were genotyped through a Real-time taqman assay (assays id C_1994992_10 and C_2084293_10; www.appliedbiosystems.com). SNPs rs2082412, rs3213120, and rs3213119 were genotyped through Polymerase Chain Reaction amplification followed by restriction enzyme digestion (PCR-RFLP) (**supplementary table 1**). To confirm the accuracy of these methods, we sequenced and confirmed the genotypes in several individuals.

2.3. IL12b sequencing. We sequenced the seven coding and 3'UTR exons, as well as 1,557 bp of the promoter region (5' upstream of exon 1) in 30 patients who were rs6887695 GG + rs3212227 AA. Briefly, genomic DNA was PCR-amplified with primer-pairs (**supplementary table 2**), and the fragments were purified and sequenced with BigDye chemistry in an automated ABI3130xl equipment (Applied Biosystems). To define the nucleotide changes, sequences were compared with the reference *IL12B* sequence in the Ensembl database (accession number ENSG00000113302; www.ensembl.org).

2.4. Statistical analysis. The χ^2 test was used to compare genotype and allele frequencies between the groups, and to determinate the deviation from the Hardy-Weinberg equilibrium. Odds ratios (OR) and their 95% confidence intervals (CI). The Student's *t* test was used to compare the quantitative data between the groups. We performed multivariate logistic regression analysis to adjust for risk factors. A *p* < 0.01 was considered as statistically significant. Frequencies of the haplotypes defined by pair of loci were calculated with the Cubic exact solutions for the estimation of pairwise haplotype frequencies (Cubex; www.oege.org/software/cubex).

3. Results and Discussion.

The genotype frequencies for the three rs3212227, rs6887695, and rs2082412 SNPs did not deviate from the Hardy-Weinberg equilibrium in both, patients and controls. We found a significant association between Ps and the rs6887695 GG genotype (*p*=0.002; OR=1.60, 95%CI=1.19-2.16) (**Table 2**). Furthermore, this genotype was also significantly more frequent among patients with severe Ps (57% vs 46%; *p*=0.04) (**supplementary table 3**). SNPs rs3212227 and rs2082412 were in complete linkage disequilibrium (LD). The rs3212227 AA genotype (linked to Ps-risk in some studies) was more frequent among the patients (0.72 vs. 0.69). However, this was a non significant difference,

probably due to a limited sample size. Assuming the observed genotype frequencies, a total of 1,330 patients and controls should be necessary to reach a power of 80 at a $p=0.05$. Rs6887695 and rs3212227 were in incomplete LD. We found a significantly higher frequency of rs6887695 GG + rs3212227 AA genotypes in the Ps (0.46 vs. 0.32; $p<0.0001$; OR=1.82, 95% CI=1.34-2.47) (**supplementary table 4**).

The three SNPs were either extragenic (rs6887695 and rs2082412) or in the 3'UTR of *IL12B* (rs3212227). Rs3212227 was previously related with differences in IL-12p40 by human cultured leukocytes [12,13]. The putative functional effect on this SNP on *IL12B* expression could be explained by differential binding to microRNAs (miRNAs). These are small (approximately 20 nucleotides long) RNAs that bind to complementary sequences in the 3'UTRs of mRNAs. Through this process miRNAs control mRNA stability and transcription. An online search (www.microrna.org) indicated that this SNP was not in a binding sequence for known miRNAs. The lack of a direct effect of rs3212227 on IL-12 expression was in agreement with a recent report that found no difference in IL-12p40/p70 levels between the genotypes in patients with acute malaria [14]. It is thus possible that the SNPs linked to Ps-risk were surrogate markers in LD with the causal variant, rather than being directly implicated in Ps-risk.

To determine whether some *IL12B* variant transmitted with the risk genotypes could explain the association with Ps, we sequenced the seven coding and 3'UTR exons and 1,557 bp of the promoter region in 30 patients who were rs6887695 GG + rs3212227 AA. We found six previously reported SNPs: three intronic, two in the coding sequence of exons 7 and 8, and two in the 3' UTR (**supplementary figure 1**). Rs3213120 was in the first nucleotide of exon 8, and could thus affect pre-mRNA splicing. SNP rs3213119 was a missense change (p.298 V>P) in exon 7. The two variants could thus have a functional effect on *IL12B* expression and/or function. The two SNPs were in complete LD, and allele and genotype frequencies did not differ between patients and controls (**supplementary table 5**).

Our work has some limitations. First, some associations could be missed because an insufficient number of patients and controls were studied. This could explain the lack of association between rs3212227 and Ps, because >1,300 patients and controls should be necessary to confirm the reported association in our population. Second, the resequencing of *IL12B* was performed on 30 patients and we could thus not exclude that some rare variants (frequency <0.02) were not identified.

In conclusion, SNP rs6887695 was strongly linked to Ps-risk in the Spanish population. This polymorphism was also associated with disease severity. No *IL12B* gene variant that could explain this association was identified in our patients.

Conflicts of interests.

The authors declare no conflict of interest related with this work, including honoraria in connection with the production of this manuscript

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Table 1. Characteristics of the 304 psoriatic patients.

Gender (male/female)	164 (54%) / 140
Mean age (years $\pm$ SD)*	47 $\pm$ 15
Early-onset psoriasis	210 (69%)
Late-onset psoriasis	94 (31%)
PASI<10	188 (62%)
PASI>10	116 (38%)
Cw6 +	120 (39%)
Cw6 -	184 (61%)
Familial Ps	143 (47%)

* SD, standard deviation; PASI, Psoriasis Area and Severity Index

Table 2. Genotype frequencies of the *IL-12B* SNP rs3212227 and rs6887695 in Ps patients (n=304) and controls (n=422).

rs3212227	AA	AC	CC	MAF
Patients	219 (0.72)	76 (0.25)	9 (0.03)	0.155
Controls	291 (0.69)	113 (0.27)	18 (0.04)	0.177
rs6887695 *	GG	GC	CC	
Patients	155 (0.51)	133 (0.44)	16 (0.05)	0.271
Controls	166 (0.39)	186 (0.44)	70 (0.17)	0.386

* Patients vs controls: P=0.002; OR=1.60, 95%CI=1.19-2.16 (GG vs GC+CC).