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Consice report

IL23R gene variants in relation to IL17A levels and clinical phenotype in patients with ankylosing spondylitis

Johannes C. Nossent[1,2], Sylvia Sagen-Johnsen[3] and Gunnstein Bakland[4]

Abstract

Background. IL23 receptor (IL23R) binding by IL23 is required for the maturation of $CD4^+$ cells into Th17 cells and subsequent generation of IL17A and TNF. As IL23R variations contribute to AS susceptibility, we investigated the effect of *IL23R* variants on cytokine levels and disease measures in an AS cohort.

Methods. This was a cross-sectional study of AS patients ($n = 334$, 90% $B27^+$, age 45 years). *IL23R* genotyping for three non-synonymous single-nucleotide polymorphisms (rs11209026, protective allele A; rs10489629, protective allele A; and rs11209032, risk allele A) was done by Taqman RT-PCR. IL23, IL17A, TNF and IL6 concentrations were determined by sandwich ELISA. Genotypic associations were analysed with non-parametric methods.

Results. Twenty-two AS patients (6.6%) carried the protective rs11209026 A allele, whereas 206 (61.7%) carried the rs11209032A risk allele ($P = 0.03$). Two patients homozygous for rs11209026A had late onset, no co-morbidity and undetectable cytokine levels. *IL23R* genotypes and five common haplotypes were unrelated with age at onset, BASFI or co-morbidity (all $P > 0.2$). There was no overall difference in the concentration of IL17A (184 *vs* 233 pg/ml, $P > 0.2$) or IL23 (276 *vs* 262 pg/ml, $P > 0.4$) between AS patients and controls, but a global haplotype association ($P = 0.01$) was observed for IL23 concentrations.

Conclusion. Homozygosity for rs11209026A is rare in AS patients, but may ameliorate the clinical presentation. IL17A and IL23 levels are similar in controls and AS patients. *IL23R* variants influence IL23 levels but not IL17A levels in AS patients, suggesting that IL23R impacts more on cell types other than Th17 cells.

Key words: ankylosing spondylitis, IL23R, haplotype, phenotype

CONSICE REPORT

Key messages

- *IL23R* variants do not influence IL17A levels in AS patients.
- The full protective *IL23R* haplotype (AAG) is associated with significantly reduced levels of IL23, TNF and IL6 in AS patients.
- The major *IL23R* risk variant (rs11209032 A allele) has no discernible impact on co-morbidity, BASFI scores or ESR levels in AS patients.

[1]*Department Rheumatology, School of Medicine, University of Western Australia,* [2]*Sir Charles Gairdner Hospital, Perth, WA, Australia,* [3]*Department Plant Science, University in AAS and* [4]*Department Rheumatology, University Hospital North Norway, Tromsø, Norway*

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Correspondence: Johannes C. Nossent, School of Medicine, University of Western Australia, M503, Stirling Highway, Crawley, WA 6011, Australia.
E-mail: johannes.nossent@uwa.edu.au

Introduction

Over the last decade, it has become clear that non-MHC genes are involved in the pathogenesis of AS [1, 2]. IL23 is produced by antigen-processing cells recognizing danger signals, and the binding of IL23 to the IL23 receptor (IL23R) on naive $CD4^+$ T cells then

induces differentiation into IL17A-producing helper T (Th17) cells [3, 4]. The relevance of IL17A and TNF-α in the pathophysiology of AS has been highlighted by the beneficial effect of blocking these two cytokines through inhibiting antibodies [5, 6]. Genome-wide association studies (GWASs) have demonstrated that variations of the IL23R alter the susceptibility for AS, presumably by altering the efficiency of IL23 signalling for $CD4^+$ cell maturation and subsequent IL17A production [1, 7, 8]. The association between the IL23R and AS is, however, complex, because the most strongly associated IL23 risk variant is rs11209032 [A allele, odds ratio (OR) = 1.3], whereas the rarer rs11209026 (A allele, OR = 0.69) and rs10489629 (A allele, OR = 0.83) variants are considered protective [1, 7]. Although the IL23/Th17 axis is an important pathway for disease development, it is also likely to influence the diverse clinical phenotype of AS. Two studies were unable to detect an effect of IL23R variants on radiographic disease severity in AS [9, 10]. As there are otherwise few data available on serological and clinical associations, we investigated whether IL23R variants impact on cytokine profiles and clinical phenotype in AS patients.

Methods

Patients

The study cohort consists of 334 patients who attended a research clinic visit and provided informed consent for usage of clinical, radiological and biological data. All patients fulfilled the modified New York criteria for AS and were TNF inhibitor naive at the time of study. Demographics, spinal function, BASFI, HLA B27 status by PCR and standard laboratory tests were recorded. The protocol for patient consent, primary data collection and biobanking of samples was approved by the regional ethics committee (REK Nord).

IL23R genotyping

DNA was isolated using the Puregene blood core kit A (Qiagen) from peripheral blood mononuclear cells in stored whole blood samples (−20°C). Samples were quantified using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Genotyping was performed using commercial Taqman (Applied Biosystems, Foster City, CA, USA)-based RT-PCR allelic discrimination assays for three non-synonymous single-nucleotide polymorphisms (SNPs) in the *IL23R* gene region [rs11209026 (A is rare but protective allele, OR = 0.69), rs10489629 (A is protective allele, OR = 0.83) and rs11209032 (A is risk allele, OR = 1.3] [2, 11].

Serum cytokine measurement

Serum concentrations of IL17A, IL6, IL23 and TNF were determined in stored serum aliquots (−20°C) by a quantitative sandwich immunoassay technique (Human TNF-alpha DuoSet, R&D Systems, Minneapolis, MN, USA). The manufacturer's recommendations were followed throughout and results for duplicate runs in each lot averaged. Only sera collected within 1 week from the date for clinical and biochemical data were included in the association analyses ($n = 260$). Control sera were obtained from a group of patients ($n = 72$) with chronic back pain who had normal radiographs of the sacroiliac joints and normal ESR levels.

Statistics

Genotype and haplotype estimation was done through the SNPStats software program [12], applying logistic regression analysis for genotypic (codominant model) and global haplotype (frequency threshold 5%) associations with clinical features and cytokine levels. The Northern European cohort (CEU) of the 1000 genomes project (http://www.internationalgenome.org/data) was used as a control group for SNP frequencies. Values given are means (S.D.) unless otherwise indicated. As most data were skewed, statistical analyses performed with SPSS (IBM, version 23.0) used non-parametric techniques (χ^2 test, Mann–Whitney *U*-test and Spearman's rank correlation coefficient (Rs)]. Two-sided *P*-values corresponding to α levels ≤ 5% were considered statistically significant.

Results

The characteristics of AS patients and controls differed as expected (Supplementary Table S1, available at *Rheumatology Advances in Practice* online). The overall levels of IL23, IL17A, TNF or IL6 were not significantly different in AS patients and controls and were not influenced by HLA B27 status or gender (Supplementary Fig. S1A–C, available at *Rheumatology Advances in Practice* online; all $P > 0.2$). In AS patients, there was a strong correlation between IL23 levels and IL17A (Rs = 0.59, $P < 0.001$), TNF (Rs = 0.57, $P < 0.001$) and IL6 (Rs = 0.59, $P < 0.001$) levels, with similar correlation coefficients observed in controls. No correlation was seen for any of the cytokines with BASFI, spinal function tests (Schober, finger floor and occiput wall distance; all $P > 0.2$) or with ESR, CRP, WBC or haemoglobin levels (all $P > 0.3$).

Genotype frequencies for rs11209026, rs10489629 and rs11209032 in AS patients and the CEU cohort in the 1000 genes project (Table 1) demonstrate a very low frequency of the protective rs11209026 A allele, which was not significantly different (4 *vs* 5%, $P > 0.3$). Although the rs11209026 AA genotype associated with absence of B27 and co-morbid conditions and low levels of BASFI, ESR, CRP and inflammatory cytokines, this failed to reach statistical significance owing to low numbers. The protective rs10489629 A allele was slightly more frequent in AS patients than in the CEU cohort (56 *vs* 52%, $P = 0.07$) and although A allele carriers were more likely to be female (OR = 2.11, $P = 0.04$), no other consistent effect was seen on co-morbidity or markers of inflammation. AS patients more frequently carried the rs11209032 risk allele A (38 *vs* 30%, $P < 0.05$), which

Table 1 *IL23R* single nucleotide polymorphism frequency and association with selected disease features in AS patients

IL23R variant	Frequency, %		Age at AS onset, mean (s.d.), years	Female, OR	B27+, OR	IBD, OR	Psoriasis, OR	Uveitis, OR	BASFI score	ESR, mm	TNF, pg/μl	IL6, pg/μl	IL17, pg/μl	IL23, pg/μl
	This cohort	1000 genes CEU cohort												
rs11209026														
A/A ($n = 2$)	1	1	25.5 (9.1)	0	0	0	0	0	0.50	7	1	1	1	1
A/G ($n = 20$)	6	8	23.1 (7.1)	1.57	1.79	0	1.22	0.67	3.71	21	85	18	141	20
G/G ($n = 312$)	93	91	23.1 (7.7)	1	1	1	1	1	2.48	19	148	92	173	259
rs10489629														
A/A (CC) ($n = 65$)	20	26	22.8 (7)	2.81*	2.29	1.36	0.56	0.88	2.84	16	165	94	188	307
A/G (CT) ($n = 165$)	49	44	23.4 (7.9)	1.87*	1.88	0.94	1.38	0.96	2.30	22*	113	76	165	187
G/G (TT) ($n = 104$)	31	30	22 (7.8)	1	1	1	1	1	2.73	14	183	104	153	295
rs11209032														
A/A ($n = 49$)	15	11	22.1 (8.2)	0.66	0.62	1.05	1.18	1.17	2.64	18	202	97	233	330
A/G ($n = 157$)	47	38	22.8 (8.2)	0.87	0.53	0.47	1.40	1.09	2.59	19	72*	58	124	144
G/G ($n = 128$)	38	51	23.8 (6.9)	1	1	1	1	1	2.44	19	218	116	201	329

Allelic and genotypic associations were analysed by dominant and codominant models, respectively. Figures indicate frequency, means for continuous variables and odds ratios (OR) for dichotomous variables.
*$P \leq 0.05$ by χ^2 or Mann–Whitney *U*-test.

had borderline associations with male gender, B27 positivity and lower TNF and IL6 concentrations ($0.05 < P < 0.1$).

The three SNPs were in strong linkage disequilibrium ($P = 0.001$) and formed five common haplotypes (Supplementary Table S2, available at *Rheumatology Advances in Practice* online). None of these haplotypes was associated with B27 status, co-morbidity development or ESR levels (all $P > 0.2$; data not shown). There was, however, a significant global association between *IL23R* haplotype and IL23 levels ($P = 0.01$), and the fully protective haplotype (AAG) had a further impact on IL6 and TNF levels but not on IL17A (Fig. 1).

Discussion

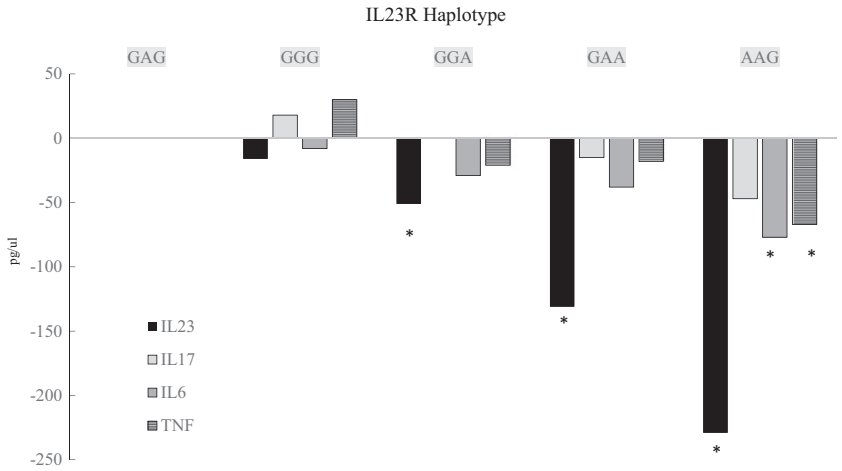
The results of this study indicate a global haplotype impact of IL23R variants on IL23 levels in AS patients with reduced IL17A levels observed only with the fully protective haplotype. Furthermore, the data provide additional evidence that serum cytokine levels do not accurately reflect clinical disease activity or severity in AS.

The contribution of IL23R to AS susceptibility has been established in multiple GWAS reports which, together with mechanistic studies, have paved the way for the successful targeting of the IL23/IL17 pathway in AS patients [6, 13]. However, AS is a diverse condition regarding clinical presentation, treatment response and disease progression. This variety remains largely unexplained, but is thought to reflect the complex genetic background of AS, indicating a significant need for translational study of these genetic traits [1, 14]. Our study cohort was predominantly of Caucasian descent, which is the patient group where IL23R variants most strongly link with AS [14]. Also, as most patients had longer-standing disease this allowed for adequate observation time to determine disease severity and co-morbidity, and all patients were TNF inhibitor naive at study to ensure that measurements of cytokine levels were not confounded.

We found no significant difference in cytokine levels between AS patients and controls and no impact of B27 status or gender on cytokine levels, which, furthermore, did not correlate with functional status or levels of acute phase reactants. Although some studies have described isolated increases in levels of IL23, IL17A, TNF or IL6 in AS patients, this remains debated because others have failed to find consistent differences, and no distinctive cytokine signature has yet emerged in AS patients [15]. It is not clear whether this is attributable to methodological differences between studies regarding ethnic background, diagnostic selection bias (e.g. AS *vs* SpA) or incompatible cytokine assays or possibly reflects the fact that serum cytokine levels in SpA are not representative of the inflammatory process at AS specific target tissues (entheses), which are difficult to examine *in vivo* [15].

The *IL23R* rs11209026 A allele leads to an arginine (R) to glutamine (Q) substitution within the cytoplasmic

Fig. 1 Effect of *IL23R* haplotypes (rs11209026, rs10489629 or rs11209032) on cytokine concentrations in patients with AS. Bars indicate mean change in cytokine concentrations compared with most frequent haplotype (GAG). *$P < 0.05$ *vs* reference.



domain of the IL23R protein at position 381 (R381Q), and this selectively reduces IL23-induced STAT3 phosphorylation in Th17 effector cells and production of IL17A [16]. Our data (although statistically hampered by small numbers of A carriers) support this R381Q effect on IL17A levels and extend the observation towards a protective clinical phenotype with less severe clinical disease and low inflammatory markers, especially in homozygous (AA) patients. An earlier study [17] described less sacroiliitis in AS patients carrying this protective A allele.

Although the *IL23R* rs10489629 A allele has been associated with reduced disease susceptibility for AS and IBD, our data show that this variant has little impact on disease phenotype. However, the increased frequency of the protective rs10489629 A allele in female and HLA B27$^-$ AS patients could help to explain the divergent disease development long observed in female and B27$^-$ AS patents in some studies [18].

The rs11209032 A allele is the major AS risk factor (GWAS studies) and also carries an increased risk for IBD and uveitis, indicating a pervasive effect across multiple immune-mediated conditions [19]. Although the mechanism through which this variant influences AS risk is unknown, a recent study found this SNP to influence Th1 cell numbers and homozygosity for the risk A allele, leading to higher numbers of IFN-γ-secreting (Th1) cells while not affecting IL-17A$^+$ CD4$^+$ T-cell numbers, suggesting a cell-specific effect of this SNP [8]. Although we found an enrichment of this risk A allele in AS patients, this was not associated with a significant effect on B27 frequency and had no discernible effect on comorbidity, BASFI scores or ESR levels. Surprisingly, the risk allele was associated with lower levels of inflammatory cytokines, although only significantly so for lower TNF levels in heterozygous (AG) patients.

Although the study of single SNPs can reveal authoritative associations, we included both risk and protective alleles for AS in our study and, despite the inevitable loss of power, we performed haplotype analysis to determine whether there were potential effects from tandem mutations that could have been missed in single SNP analyses. Although we failed to find strong associations between the various haplotypes and clinical disease measures, we detected a remarkable effect of haplotype on cytokine levels. The haplotype combining all three protective alleles (AAG) was associated with significant reductions in the levels of IL23, IL6 and TNF and representative of a significant global haplotype association with IL23 levels (Fig. 1). Remarkably, no such association was seen for IL17A across the *IL23R* haplotypes. The predominant sources of IL23 in humans are dendritic cells (DCs) and macrophages, where IL23R is embedded in the cell membrane. Given the lack of association between individual SNP or global haplotypes with IL17A levels, this suggests that in AS patients *IL23R* variants have a larger effect on the IL23R-dependent activation of DCs and macrophages than on Th17 cell activation and IL17A production, as recently shown for activated DCs [20]. Also, homozygosity for the rs11209032 risk A allele has been associated with more IFN-γ-secreting (Th1) cells [8], and together, this indicates a need for further study of the potential impact of these immune cells on pro-inflammatory cytokine production in AS.

The limitations of the present study should be recognized and included the selection of three *IL23R* SNPs with recognized disease impact, whereas other *IL23R* SNPs were not evaluated. As the fully protective AAG haplotype is rare, this reduces the accuracy of the imputation compared with more common haplotypes. The homogeneous North European make-up of the

cohort limits the generalizability of the data, and the fact that appropriately timed cytokine levels were available for 78% of the cohort may have limited the power of this study.

In conclusion, *IL23R* variants known to impact on disease susceptibility do not influence clinical disease measures of activity or severity in AS patients, have no effect on IL17A levels but impact on IL23 levels, suggesting that they are more relevant for DCs than for Th17 activation in AS.

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Supplementary data

Supplementary data are available at *Rheumatology Advances in Practice* online.

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