

Allele *2 of the HS1,2A enhancer of the Ig regulatory region associates with rheumatoid arthritis

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ABSTRACT

Objective: To investigate the role of the HS1,2 enhancer polymorphisms as a new candidate marker for rheumatoid arthritis (RA) and to define the possible association with autoantibody positivity and clinical outcome.

Methods: Genomic DNA was obtained from two cohorts of patients with RA (100 with early RA (ERA) and 114 with longstanding RA (LSRA)) and from 248 gender-matched controls from the same geographical area. Clinical and immunological characteristics were recorded for all the patients.

Results: The percentage of the 2/2 genotype was higher in patients with ERA (27.0%), and in patients with LSRA (34.2%), than in controls (14.9%) (ERA: OR = 2.11 (95% CI 1.20 to 3.70) vs controls; LSRA: OR = 2.96 (95% CI 1.76 to 5.00) vs controls). A lower representation of allele *3 was present in patients with ERA (2.0%) than in controls (6.0%; OR = 0.32 (95% CI 0.11 to 0.91)). No significant associations were found between polymorphisms and autoantibodies positivity.

Conclusion: The HS1,2A allele *2 associates with early and longstanding RA.

Studies of twins clearly show a genetic contribution to disease susceptibility in rheumatoid arthritis (RA).¹ The most important genetic risk factor is in the polymorphic HLA region. The HLA-DRB1 alleles, encoding for shared epitope, confer a higher risk for development of RA.² The association exists for RA that is characterised by the presence of anti-cyclic citrullinated peptide (anti-CCP) antibodies.³ Other genetic associations have been found in recent years (eg, PTPN22).⁴

Recently, a polymorphism of the enhancer HS1,2A of the Ig heavy 3' regulatory region (IgH 3'RR-1) at the 3' of the constant alpha (C-alpha) genes has been associated with IgA nephritis, coeliac disease, systemic sclerosis and psoriasis.⁵⁻⁸ In these diseases, the variation of allelic frequencies involves allele *2, which has one consensus site for the NF-κB transcription factor missing in the second more frequent allele *1.⁹ The IgH 3' regulatory region is active in the transcription of the heavy constant genes for class switch recombination and in the Ig transcription.¹⁰

The major aim of this study was to investigate a possible association of HS1,2A polymorphism with RA, the presence of autoantibodies and finally, to look for a possible relationship with response to treatment.

PATIENTS AND METHODS

Patients

A cohort of 100 patients with early RA (ERA, disease duration <12 months), at the Division of

Rheumatology of the Catholic University (Rome, Italy) was studied. Patients fulfilled the American College of Rheumatology criteria for RA.¹¹ The disease status for each patient was assessed. Detailed assessment at study entry (baseline), at 6 months and at 1 year included the disease activity score (DAS), complete tender and swollen joint count, acute-phase reactants (erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)), as well as visual analogue scale (0–100 mm) for global disease activity. All patients' serum samples were tested for anti-CCP, IgM rheumatoid factor (RF) and IgA RF autoantibodies, following ELISA standard techniques. Treatment was based on methotrexate (MTX) for 3 months (up to 20–25 mg/week) and etanercept after 3 months if the DAS was not sufficiently lowered with MTX only, to reach clinical remission (DAS44 <1.6).

A second, completely independent cohort of 114 patients with RA with a long disease duration (more than 1-year's disease duration, longstanding RA (LSRA)) was included in the study and the same measures for disease activity were collected. All these patients with LSRA were receiving MTX in combination with the tumour necrosis factor α blocker because of severe, progressive disease.

Collection of DNA samples

Genomic DNA was isolated from peripheral venous blood of patients and controls with a FlexiGene DNA kit (Quiagen, Valencia, California, USA), according to the instruction given by manufacturer. The control sample comprised 248 unrelated, healthy controls of both sexes. Each control was asked to supply name, birthplace, language and ethnicity for three generations in order to exclude recent admixture. The patients and control subjects were white subjects of Italian origin, from the same geographical area and gave their informed consent to participate in the study.

HS1,2A genotyping

Alleles of the HS1,2A enhancer were determined through two PCRs. The first was on genomic DNA, selective for the IgH 3'RR at the 3' of C-alpha 1 gene and amplified a fragment of 5404 bp, the second was a nested PCR to amplify the polymorphic HS1,2A fragment varying from 465 to 287 bp (fig 1). The first PCR was performed with primers SA2.5 forward 5'-GGATCCCTG TTCCTGATCACTG-3' and A2R reverse 5'-GCCCTTCTGCCAACCTG-3'; PCRs were carried out in 50 µl reaction volume containing



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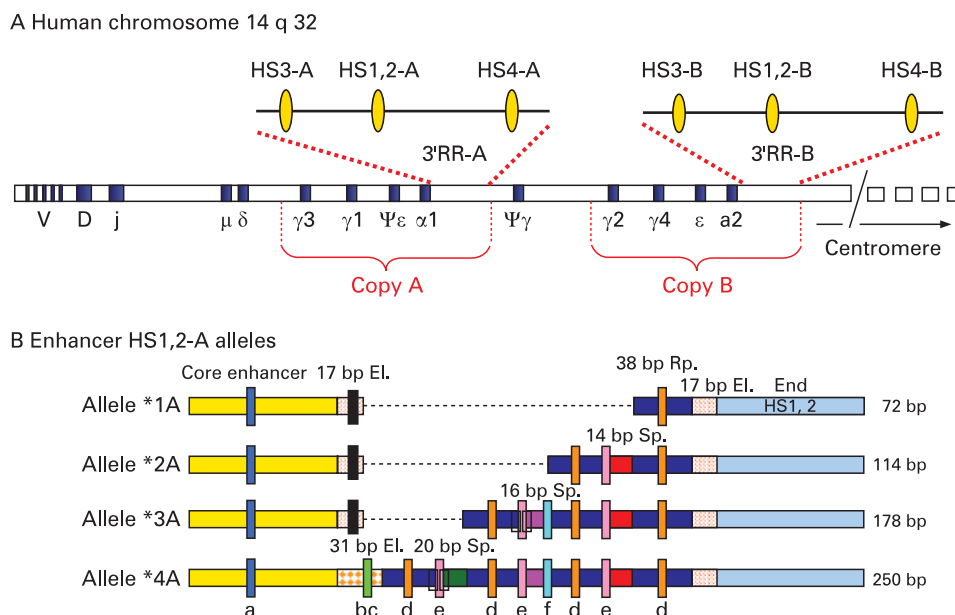


Figure 1 (A) Map of the Ig heavy chain cluster showing the duplication of four constant regions along with the two 3' regulatory regions (copy A and copy B). The three enhancers of both 3'RRs are conserved also in mice, separated by intervening sequences highly conserved for the quality of repeats units but not for number. (B) Only the enhancer HS1,2 was found to be polymorphic. The alleles of the A and B regions are identical. On the right side of the alleles is indicated the length of the polymorphic region of each allele. The polymorphism is based on the duplicated element of 38 bp minisatellite (blue strips) from one to four times separated by spacers (Sp) of 20 bp (green), 16 bp (violet) and 14 bp (red). The polymorphic region of allele *4 has the substitution of the 17 bp element 5' element (EI) (red spots) with a 31 bp EI (orange diamonds) harbouring the consensus sequence "c". The letters listed at the bottom of the figure correspond respectively to "in silico" predicted transcription factors. The site "a" within the core not polymorphic of the enhancer HS1,2 has the consensus for CEBP, CMYB; "b" presents the consensus for SP1; "c" has the site for AP4, E47, MYOD and μ E5; "d" presents the consensus for IK2, MZF1 and NF- κ B (P50); "e" has a site corresponding to NF- κ B; "f" has the consensus for CMYB site. EMSA experiments confirmed within alleles *1 and *2 different number of consensus for SP1 and NF- κ B (unpublished data).

2 μ l extracted DNA (20 ng). The nested second PCR was performed using the primers P3 forward 5'-GATTCATTCTGGGCAGACTTG-3' and D3 reverse 5'-GTCCTGGTCCCAAAGATGG-3' and with 1/25 of the volume of the first PCR, minimising the carry-over of the genomic DNA; control reactions were performed with 2 and 5 ng total genomic DNA, giving no visible amplification on gel agarose electrophoresis. Reaction, conditions and cycles were the same as described previously.⁹ Negative and positive controls, without a DNA template or with a heterozygote control DNA sample were always included. PCR products were analysed on 3% agarose gel stained with cyber green.

Statistical analysis

Data were analysed using SPSS, version 13.0 and Prism software (Graph-Pad, San Diego, California, USA). Categorical and quantitative variables were respectively described as numbers (%) and mean (SD). Sample size calculations were based on the following assumptions: power 80%, confidence 95%, estimated difference to test between 2/2 genotype frequency of cases and controls equals 15%, ratio between cases and controls 1:2. A minimum of 97 cases and 194 controls was derived from these calculations. A Mann-Whitney test was used to compare continuous variables. Categorical variables were analysed using χ^2 test or Fisher test, depending on sample size restrictions, and the odd ratios (ORs) with 95% CI were calculated. A value of $p < 0.05$ was considered significant. The Hardy-Weinberg equilibrium was calculated for all the genotyped groups.

RESULTS

Study participants' characteristics

The study cohorts consisted of 100 patients with ERA, enrolled before any treatment with disease-modifying antirheumatic drugs had been initiated and 114 with LSRA. Table 1 shows the main demographic, clinical and serological features of the patients with RA.

Distribution of HS1,2A genotypes in patients with RA and in controls

The genotype distribution of the HS1,2A polymorphism was significantly different between patients with ERA and controls ($\chi^2_{v=6} = 13.20$, $p = 0.004$) and the percentage of the 2/2 genotype was higher in patients with ERA (27.0%) than in the control group (14.9%; $p = 0.008$) (table 2). The frequency of the allele *2 was increased in patients with ERA (53.0%) as compared with the control group (40.0%; OR = 1.73 (95% CI 1.24 to 2.40); $p = 0.001$), whereas a lower representation of the allele *3 (2%) in patients with ERA was seen when compared with the controls (6%; OR = 0.32 (95% CI 0.11 to 0.91); $p = 0.03$). The alleles *1 and 4 had comparable frequency in patients with ERA (allele *1: 36.5% and allele *4: 8.5%) and controls (allele *1: 43.9% and allele *4: 10.5%).

In LSRA, 34.2% of the patients carried the 2/2 genotype ($p < 0.001$ vs controls; table 2) and the frequency of the allele *2 was 62.7% (OR = 2.58 (95% CI 1.86 to 3.56); $p < 0.001$). A lower representation of allele *3 and allele *4 was present in LSRA versus controls (allele *3: 13%, OR = 0.21 (95% CI 0.06 to 0.69); $p = 0.03$); allele *4: 1.3%, OR = 0.12 (95% CI 0.04 to 0.37);

Table 1 Demographic, clinical and laboratory characteristics of patients with early rheumatoid arthritis at baseline, and of patients with longstanding rheumatoid arthritis at the moment of the analysis

Characteristics	Patients with ERA (n = 100)	LSRA (n = 114)
Female, n (%)	75 (75.0)	97 (85.1)
Age (years), mean (SD)	54.9 (13.8)	57.1 (13.6)
Disease duration (years), mean (SD)	0.6 (0.3)	13.7 (10.0)
Tender joint count, mean (SD)	15.5 (10.1)	8.3 (8.9)
Swollen joint count, mean (SD)	11.4 (8.7)	5.3 (6.2)
Anti-CCP (U/ml), mean (SD) {median}	48.6 (63.4) {13.9}	49.6 (91.3) {17.0}
Anti-CCP ≥5 U/ml, n (%)	65 (65.0)	78 (68.4)
IgM RF (U/ml), mean (SD) {median}	61.6 (98.6) {27.1}	46.5 (78.8) {13.4}
IgM RF ≥20 U/ml, n (%)	54 (54.0)	47 (41.2)
IgA RF (U/ml), mean (SD) {median}	75.6 (149.9) {14.8}	52.0 (122.2) {10.2}
IgA RF ≥20 U/ml, n (%)	43 (43.0)	33 (28.9)
CRP (mg/l), mean (SD)	24.1 (30.6)	6.9 (11.9)
ESR (mm/1st h), mean (SD)	40.6 (24.6)	23.7 (19.5)
DAS44, mean (SD)	3.9 (1.4)	2.7 (1.4)
HAQ, mean (SD)	1.24 (0.78)	1.05 (0.80)

anti-CCP, anti-cyclic citrullinated protein; CRP, C-reactive protein; DAS, disease activity score; ERA, early rheumatoid arthritis; ESR, erythrocyte sedimentation rate; HAQ, Health Assessment Questionnaire; LSRA, longstanding rheumatoid arthritis; RF, rheumatoid factor.

$p < 0.001$). In all groups the observed genotypes were in agreement with those expected under the Hardy-Weinberg equilibrium.

Genotype distribution for phenotypes of patients with RA and relation with the autoantibody pattern

No significant association between the allele *2 of HS1,2A and the presence of autoantibodies (anti-CCP, IgM RF, IgA RF) in RA was seen; also, no difference was seen between patients with ERA and those with LSRA (data not shown).

The data suggest a strict relationship between allele *2 and RA, not with autoimmune phenotypes.

Genotype distribution in patients with ERA and relation with clinical and laboratory variables

Carriers of HS1,2A allele *2 were 5.2 years younger than patients without this allele (48.1 years vs 53.3 years), although the difference did not reach significance ($p = 0.14$). There were no differences in gender distribution, tender and swollen joint count and inflammatory parameters (ESR and CRP) at baseline

between HS1,2A allele *2 positive and negative patients with ERA (data not shown).

HS1,2A polymorphism and response of patients with ERA after 12 months' treatment

Forty-seven (48%) of 98 patients with ERA followed up for 12 months were in clinical remission (DAS44 <1.6). Two patients were lost at follow-up. The frequency of 2/2 genotype was higher in the subgroup of patients not in clinical remission (30%; OR = 2.4 (95% CI 1.2 to 4.8) vs controls) than in the cohort of patients in clinical remission (25%, OR = 2.0 (95% CI 0.9 to 4.1) vs controls).

DISCUSSION

RA is characterised by polyclonal B-cell activation and production of autoantibodies even before the appearance of arthritis, suggesting lack of tolerance as primary event conditioning autoreactivity.¹² These data suggest an important role for B lymphocytes in the pathogenesis of RA as contributors of autoimmunity, as antigen-presenting cells and T-cell stimulators, and of inflammation as cytokine producers.¹³

The central human enhancer HS1,2A of IgH 3'RR located in chromosome 14,q32 was polymorphic in humans and the involvement of allele *2 in the genetic predisposition of immune-related diseases has already been noted.⁸

We found that allele *2 of the HS1,2A enhancer is significantly more common in patients with ERA than in healthy controls, and more common in patients with RA with an aggressive disease not responding to treatment with common disease-modifying antirheumatic drugs (LSRA). Allele *2 is not associated directly with the presence of autoantibodies. The trend suggesting that HS1,2A allele *2 might also play a role in limiting the occurrence of disease remission in patients with RA under adopted treatment needs confirmation in a larger cohort with a longer follow-up.

There are no data reported with wide genome scans on the association of chromosome 14 (region q32) with RA disease; this may be because all studies focused mainly on the analysis of translated and transcribed genes and not on cis-acting regulatory regions.^{14, 15} It might be interesting to develop a study for multiple association analysis of the risk factors so far studied in different patient populations with different techniques, including wide genome screening if any, in order to obtain conclusive information.

Table 2 Distribution of genotypes of the HS1,2A polymorphism in patients with early rheumatoid arthritis (ERA), patients with longstanding rheumatoid arthritis (LSRA) and controls

HS1,2A polymorphism genotype n (%)	Controls (n = 248) n (%)	Patients with ERA (n = 100) n (%)	OR (95% CI)	p Value*	Patients with LSRA (n = 114) n (%)	OR (95% CI)	p Value*
1/1	52 (21.0)	11 (11.0)	0.47 (0.23 to 0.94)	0.03	10 (8.8)	0.36 (0.18 to 0.74)	0.004
2/2	37 (14.9)	27 (27.0)	2.11 (1.20 to 3.70)	0.008	39 (34.2)	2.96 (1.76 to 5.00)	<0.001
3/3	2 (0.8)	–	0.49 (0.02 to 10.32)	1.00	–	0.43 (0.02 to 9.05)	1.00
4/4	4 (1.6)	5 (5.0)	3.21 (0.84 to 12.22)	0.13	–	0.24 (0.01 to 4.45)	0.31
1/2	84 (33.9)	46 (46.0)	1.66 (1.04 to 267)	0.03	59 (51.8)	2.09 (1.33 to 3.29)	0.001
1/3	12 (4.8)	3 (3.0)	0.61 (0.17 to 2.20)	0.57	–	0.08 (0.00 to 1.49)	0.02
1/4	18 (7.3)	2 (2.0)	0.26 (0.06 to 1.15)	0.07	–	0.05 (0.00 to 0.91)	0.001
2/3	13 (5.2)	1 (1.0)	0.18 (0.02 to 1.42)	0.08	3 (2.6)	0.90 (0.14 to 1.75)	0.40
2/4	25 (10.1)	5 (5.0)	0.47 (0.17 to 1.26)	0.14	3 (2.6)	0.24 (0.07 to 0.82)	0.014
3/4	1 (0.4)	–	0.83 (0.03 to 20.34)	1.00	–	0.72 (0.03 to 17.84)	1.00

*p Values are calculated from χ^2 tests performed in patients (ERA and LSRA) versus controls.

In “silicio” predictive analysis shows in the allele *2 of the HS1,2A enhancer polymorphism the presence of a consensus binding site for NF- κ B, absent in allele *1.⁹ Preliminary data suggest that allele *2 of the HS1,2A polymorphism establishes a higher affinity with NF- κ B and its link with the transcription (unpublished data). If this were the case the association with RA could be more fully interpreted.

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