

Role of *SLC22A4*, *SLC22A5*, and *RUNX1* Genes in Rheumatoid Arthritis

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ABSTRACT. *Objective.* Excessively suppressed expression of the *SLC22A4* gene by *RUNX1* is associated with the pathogenesis of rheumatoid arthritis (RA). Two etiological polymorphisms in the *RUNX1* and *SLC22A4* genes have been defined in a Japanese population. We studied additional polymorphisms to ascertain whether any *SLC22A4/SLC22A5* haplotype is relevant for RA predisposition in a Spanish population. *Method.* We performed a case-control study comprising 416 patients with RA and 501 healthy subjects. *Results.* The etiologic *SLC22A4* mutation was rarely found in homozygosis (0.72% patients vs 0.40% controls). None of the 4 haplotypes present in the *SLC22A4/SLC22A5* region in 5q31 showed significant association with RA in our Spanish cohort. The causative *RUNX1* variant found in a Japanese cohort displayed the same genotype distribution in our population. However, no difference was observed when allele or genotype frequencies were compared between Spanish patients with RA and controls. *Conclusion.* The *SLC22A4* and *RUNX1* polymorphisms described as etiological in the Japanese study did not show a significant role in RA susceptibility in our population. The mechanism proposed by these Japanese investigators could underlie RA susceptibility irrespective of ethnicity, but the lower mutation rate present in our population hampered detection of a significant effect. Most probably the lack of mutated *SLC22A4* substrate explains the absence of *RUNX1* association with RA observed in our population. (J Rheumatol 2006;33:842–6)

Key Indexing Terms:

RHEUMATOID ARTHRITIS

SUSCEPTIBILITY

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The interplay of both genetic and environmental factors contributes to the development of rheumatoid arthritis (RA). Clinical manifestations of RA are a consequence of localization of lymphocytes in synovial tissue initiating inflammation through the production of mediators^{1,2}. Genetic analysis of human populations clearly contributes to the understanding of autoimmune diseases. RA is a common complex genetic disease and HLA-DRB1 has been clearly shown to be associated with the disease³. However, this association accounts for less than half the overall genetic susceptibility. Identifying specific genetic mechanisms involved in RA continues to present considerable challenges. There are 3 genetic methods often

used for detecting genes contributing to susceptibility or resistance to multifactorial diseases: nonparametric linkage analysis, case-control association study, and transmission disequilibrium test (TDT). Case-control association study has the highest power for detecting disease genes if there is no population stratification between patients and controls.

Human chromosomal region 5q31 has been recently related to autoimmune disorders with an inflammatory component, like RA and Crohn's disease^{4,5}. In this bowel disease, association was found with functional variants in 2 nearby genes, *SLC22A4* and *SLC22A5*, encoding the organic cation transporters OCTN1 and OCTN2, respectively. Tokuhiro, *et al*⁴ mapped RA susceptibility to the *SLC22A4* gene and also located the causative variant in this gene, slc2F2 (rs3792876). The mutant allele of this single nucleotide polymorphism (SNP) within a Runt-related transcription factor 1 (*RUNX1*) binding site further suppresses the expression of the *SLC22A4* gene. In this Japanese work, the *RUNX1* gene was associated with RA as well. The *SLC22A4* gene is expressed in hematological tissues⁶ and a 2-fold increase in its expression in cultured synovial fibroblasts was observed upon treatment with proinflammatory cytokines. Moreover, the collagen induced arthritis mouse model expresses the counterpart of the human *SLC22A4* gene in the joints, adding evidence in favor of its role in the disease⁴. *RUNX1* regulates the expression of various genes implicated in hematopoiesis and myeloid differen-

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tiation⁷, and its defective binding to target genes has been associated with 2 other autoimmune disorders: systemic lupus erythematosus and psoriasis^{8,9}. *RUNX1* also plays a role as a negative regulator of *GATA3* expression, thereby inhibiting Th2 cell differentiation¹⁰.

In summary, the exacerbated suppression of the *SLC22A4* gene by *RUNX1* has been associated with RA in a Japanese population, but these risk factors should be confirmed in different ethnic groups. For instance, none of the 3 mutations in the *NOD2/CARD15* gene was found in 483 Japanese patients with Crohn's disease¹¹, which provides evidence for the presence of genetic heterogeneity among patients of different ethnicity. Indeed, no association of the Japanese putative *SLC22A4* etiological variant has been replicated in Canadian or British cohorts with RA^{12,13}. We studied the association of both *SLC22A4* and *SLC22A5* genes with RA in a Mediterranean population to assess the impact of these 2 functionally and physically closely related genes. To our knowledge, there is only one replication study in a Caucasian population¹⁴ of the *RUNX1* gene, which was associated with RA in the Japanese report.

MATERIALS AND METHODS

Patients. Four hundred sixteen consecutively recruited patients with RA and 501 ethnically matched controls from the same Madrid area were included; all gave informed consent according to the Helsinki Declaration. Clinical data for most of the patients have been described¹⁵: female:male ratio 3:1, age at onset 54 ± 14 years, 83% of patients presented erosive disease, and 62% and 76% were positive for shared epitope (SE) and rheumatoid factor (RF), respectively. TDT was performed with 56 RA patients who belonged to the European Consortium of RA Families (ECRAF): 84% were women aged 32 ± 9 years, 78% had erosive disease, and 64% were positive for SE and 86% for RF. The Hospital Ethical Board approved the design of the study.

Genotyping. SNP of the *SLC22A4*, *SLC22A5*, and *RUNX1* genes were genotyped by TaqMan Assays on Demand under conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA), with the following identification numbers: C_3170428_10 for *slc2F2* (rs3792876), C_3170458_1_ for *slc2F11* (rs2306772), C_3170445_1_ for the exon 5 polymorphism in the same gene, T306I (rs272893), and C_1173605_1 for the intronic rs274559 in *SLC22A5* and C_2444499_1 (rs2268277) for the one in *RUNX1*. TaqMan Assay by Design was performed for promoter -207 *SLC22A5* (rs2631367).

Statistical analysis. Chi-square statistics or Fisher's exact test, when necessary, were applied in case-control analyses and associations were estimated by the odds ratios (OR) with 95% confidence intervals (CI) (Epi Info v. 6.02; CDC, Atlanta, GA, USA). Case-control groups used in this study (832:1002 alleles) had 80% power (at the 0.05 level) to detect an allelic association conferring an arbitrary 1.5-fold increase in risk, which is usually the one observed for these complex diseases, considering the observed allelic frequency of 7.5% (calculated using <http://calculators.stat.ucla.edu/powercalc/binomial/case-control>). Haplotypic frequencies were estimated using the expectation-maximization (EM) algorithm¹⁶ implemented in Arlequin v2.000 software, with number of iterations set at 5000 and initial conditions at 50, with an epsilon value of 10^{-7} .

RESULTS

Twelve SNP in absolute linkage disequilibrium, 11 of which mapped to *SLC22A4* and the other to the adjacent *SLC22A5* gene, were found to be associated with RA susceptibility⁴. We

first tested the rate of linkage disequilibrium of *SLC22A4* polymorphisms by analyzing 2, *slc2F2* (rs3792876) and *slc2F11* (rs2306772). Both SNP were in complete disequilibrium as previously shown, but allelic distribution in the Spanish population was clearly different from that present in Japan ($p < 10^{-42}$), with the minor allele frequency ranging from about 31% in the Japanese cohort to 7.5% in Spain.

A total of 416 patients with RA and 501 ethnically matched controls were then studied for association of the intronic *slc2F2* polymorphism. This SNP was originally described in a Japanese study as the etiological variant, being the mutant homozygous genotype associated with RA. The susceptible *slc2F2**T within a *RUNX1* transcription factor-binding site in the *SLC22A4* gene further diminished the expression of the gene. Table 1A shows the genotype distribution in the Spanish patients with RA and control cohorts and no significant difference was observed. Analysis by TDT of 12 informative heterozygous progenitors showed no distorted transmission of the alleles (allele C transmitted 6 times; allele T 6 times; $p = 0.61$). When we analyzed the etiologic *RUNX1* intronic polymorphism (rs2268277) tested by Tokuhira, *et al*⁴, similar genotype distribution to that found in Japan was observed in the Spanish controls. However, this SNP did not increase the risk for RA in the Spanish population in contrast to the significant association found in the Japanese report (Table 1B). Again, no preferential transmission of any allele was found by TDT analysis of 30 heterozygous progenitors (allele G and allele C were transmitted 14 and 16 times respectively; $p = 0.43$). Previous stratification for the well-established major histocompatibility complex (MHC) risk factor for RA, the SE¹⁷, did not modify the observed absence of association of either polymorphism (data not shown).

Both organic cation transporters OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*) show 88% homology and 77% identity in their sequences; the latter is physiologically important for the maintenance of plasma carnitine concentration, while the physiological substrate of the *SLC22A4* gene, ergothioneine, an intracellular antioxidant, has recently been reported¹⁸. Given this sequence similarity and taking into account the previously observed association of putative functional variants in both genes with Crohn's disease, we studied the role of these genes in RA predisposition. We tested another *SLC22A4* polymorphism, a C/T substitution in exon 5, T306I (rs272893), which displayed a minor allele frequency of 37.6% in the Spanish population. Similar results showing no association with RA were observed from the analysis of this second SNP (Table 2). Stratifying the RA cohort by SE status did not alter these results (data not shown).

Additionally, an intronic *SLC22A5* SNP (rs274559) was typed and found to be in complete linkage disequilibrium with the T306I variant. No association was therefore apparent. A final confirmation came from the lack of association with RA found for another polymorphism in the promoter of the

Table 1. Genotype distribution of *SLC22A4* slc2F2 (A) and *RUNX1* rs2268277 (B) polymorphisms in Spanish patients with RA and healthy controls.

A. <i>SLC22A4</i> slc2F2	CC (%)	CT (%)	TT (%)	TT vs (CT+CC)
Controls, n = 501	428 (85.4)	71 (14.2)	2 (0.40)	p = 0.41 OR 1.81 (95% CI 0.21–21.78)
RA, n = 416	362 (87.0)	51 (12.3)	3 (0.72)	
B. <i>RUNX1</i> rs2268277	GG (%)	GC (%)	CC (%)	(GC+CC) vs GG
Controls, n = 494	181 (36.6)	234 (47.4)	79 (16)	p = 0.76 OR 1.04 (95% CI 0.79–1.39)
RA, n = 385	145 (37.7)	189 (49.1)	51 (13.2)	

Table 2. Genotype distribution of the *SLC22A4* T3061 polymorphism in Spanish patients with RA and healthy controls.

<i>SLC22A4</i> T3061	CC (%)	CT (%)	TT (%)	(CT+TT) vs CC
Controls, n = 496	198 (39.9)	234 (47.2)	64 (12.9)	p = 0.64 OR 0.94 (95% CI 0.71–1.24)
RA, n = 381	158 (41.5)	182 (47.8)	41 (10.8)	

SLC22A5 gene, 207 nucleotides upstream of the start codon (Table 3).

All studied polymorphisms conformed to the Hardy-Weinberg equilibrium in the control population.

Finally, we ascertained whether any specific haplotype increased susceptibility to RA. The EM algorithm performed by Arlequin software allowed comparison among haplotype frequencies displayed for RA and control groups. No significant difference was observed for any slc2F2 *SLC22A4*/T306I *SLC22A4*/(-207-promoter) *SLC22A5* haplotype ($p = 0.70$; Table 4).

DISCUSSION

Case-control studies have been the traditional means to check specific hypotheses regarding candidate genes in disease susceptibility. The significant association of *SLC22A4* with RA reported in a Japanese population⁴ was not reproduced in either Canadian or British cohorts^{12,13}. A recent study in a Japanese population showed only a trend for association of the *SLC22A4* gene ($p = 0.15$, OR 1.24)¹⁹. Considering these conflicting results, we investigated the *SLC22A4* putative etiological polymorphism found by Tokuhiko. Moreover, the etiological *RUNX1* gene polymorphism had no effect on RA suscepti-

bility in a Caucasian population¹⁴, and we observed negative results in our cohorts.

The original Japanese report found no influence of the heterozygous genotype on RA susceptibility, but only with carriage of a homozygous mutant, indicative of a dose-dependent effect. Analysis of our data suggests that the same mechanism of the *SLC22A4* causative variant increasing RA risk might be effective in Spain. The mutant homozygous frequencies' ratios are very similar in our population to those present in the original Japanese study (RA patients/controls: 0.72/0.40 vs 16/8). Although we were unable to confirm a significant association, this may be due to the low minor allele frequency in Caucasian populations. Supporting this interpretation is the fact that the Canadian study¹² also found similar percentages of the homozygous mutant genotype in their RA (1%) and control (0.5%) cohorts. This effect must be taken into account when the power of a study is calculated, and would necessarily lead to a small power in Caucasian populations, as the etiological allele is very rare and only homozygosity increases RA risk. In our cohorts (416 cases, 501 controls), for the observed frequency of the risk factor (homozygous mutant genotype: $0.075 \times 0.075 = 0.0056$) and a standard significance level (0.05), a power of 80% would require a relative risk of 4.47.

Table 3. Genotype distribution of the -207 *SLC22A5* polymorphism in Spanish patients with RA and healthy controls.

-207 <i>SLC22A5</i>	CC (%)	CG (%)	GG (%)	(CG+GG) vs CC
Controls, n = 489	142 (29)	221 (45.2)	126 (25.8)	
				p = 0.65 OR 1.13 (95% CI 0.83–1.54)
RA, n = 384	102 (26.6)	201 (52.3)	81 (21.1)	

Table 4. Haplotype distribution in 362 Spanish patients with RA and 438 healthy controls.

	slc2F2 <i>SLC22A4</i>	T306I <i>SLC22A4</i>	-207 <i>SLC22A5</i>	Patients		Controls	
				Relative Frequency	Absolute Frequency	Relative Frequency	Absolute Frequency
1	C	C	C	0.526	380	0.503	440
2	C	T	G	0.274	198	0.285	250
3	C	C	G	0.131	95	0.126	110
4	T	T	G	0.065	47	0.076	67

We also tested some other polymorphisms in *SLC22A4* and *SLC22A5* showing no major association with RA in the Spanish population. None of the 4 haplotypes found in our cohorts evidenced a role in RA predisposition. A report describing the haplotype structure of chromosome 5q31 in Canada defined a 92 kb block comprising most of the *SLC22A4/SLC22A5* genes²⁰. Four major haplotypes accounted for 93% of the haplotypes, similar to our findings in our population; however, no increase in RA susceptibility could be explained by any of them. These results are concordant with the etiological role of the Japanese polymorphism. Because the Caucasian populations that have been studied seem virtually deprived of the only etiological variant described in the Japanese cohort, one would not expect any of the 4 haplotypes we found to have a role in predisposition for RA.

Some authors¹³ claim that the Japanese study showed a paradoxical result: the *SLC22A4* susceptibility allele expresses fewer transcripts than the wild-type allele despite its expression in inflammatory joints in mice with collagen-induced arthritis but not in normal mice, which argues against the etiological role of this polymorphism. However, this observation could be explained as an insufficient response to the inflammatory process in susceptible individuals. The real effect should be detected by comparison of the OCTN1 levels between patients with RA with and without the *SLC22A4* risk factor. For instance, levels of interleukin 1 receptor antagonist (IL-1Ra), a natural antiinflammatory protein, were significantly higher in patients with polyarthritis than in controls, which could seem a paradoxical finding too²¹. Partial OCTN1 response to an initial inflammatory activity would yield a whole clinical phenotype.

The *RUNX* family members include 3 mammalian *RUNX* proteins (*RUNX1*, 2, and 3). The requirement of *RUNX2*, a runt homology-domain transcription factor essential for bone formation and osteoblast differentiation, is well established^{22,23}. A recent study using the knock-in mouse model for *RUNX1* showed that the *RUNX1* gene contributes to early stages of skeletogenesis and remains active in progenitor cells of tissue that support bone formation in adults²⁴. Indeed, *RUNX1* contains *RUNX* binding sites in its promoter region, suggesting a possible cross-regulation with *RUNX2*²⁵, and most probably both transcription factors act cooperatively in the induction of skeletal development²⁶. Tokuhiro, *et al* have reported that *RUNX1* binds as an inhibitory factor to the sequence containing the etiologic variant in the *SLC22A4* gene⁴. Moreover, *RUNX1* participates in T-lymphocyte differentiation²⁷, and many proteins encoded by genes that are targets of *RUNX* transcription factors have relevant activity in autoimmunity²⁸. We did not find the described association of the putative etiological *RUNX1* polymorphism observed in a Japanese cohort. However, it should be noted that the *RUNX1* effect found in the Japanese study was restricted to individuals homozygous for *SLC22A4*, a sufficiently numerous subgroup to reveal a significant association with RA in the general Japanese population, but not in our Caucasian population. Therefore, in our population, a lack of association of *RUNX1* is to be expected, and our data indirectly support the etiological mechanism of the *RUNX1/SLC22A4* pathway proposed for the Japanese population.

Our results suggest that these genes could at best have an effect on RA predisposition only in a very reduced percentage of Caucasian patients. The low frequency of homozygosity for

SLC22A4 in Caucasian populations may explain the lack of a significant association observed in our Spanish and other Caucasian cohorts. However, when the causative variant is present, a common mechanism might be responsible for RA susceptibility irrespective of ethnic background. Although comparison of the homozygous *SLC22A4* genotype between cases and controls in the second Japanese study did not reach statistical significance, there was still an 85% probability ($p = 0.15$) that the referred homozygous genotype increased the RA risk (OR 1.24) in this independent Japanese cohort. Only a trend toward an association of the *SLC22A4* gene was found in these 2 ethnically similar cohorts, but usually associations described for the first time are stronger than any subsequent studies²⁹. Therefore, findings from the second study were not irrefutable proof against the original association. Replication of the original Japanese findings in another Asiatic cohort with high frequency of the *SLC22A4* homozygous genotype would be reassuring; however, it is most likely that the real effect will be more moderate than initially reported.

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