

# THE ROLE OF NRAMP1/*SLC11A1* GENE VARIANT D543N (1730G/A) IN THE GENETIC SUSCEPTIBILITY TO DEVELOP RHEUMATOID ARTHRITIS IN THE MEXICAN MESTIZO POPULATION

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## ABSTRACT

**Background:** Rheumatoid arthritis is a chronic inflammatory disease whose cause has not been fully elucidated. However, genetic factors seem to have an important role in its pathogenesis. **Objective:** We analyzed the possible association between rheumatoid arthritis and variants of the *SLC11A1* gene, which encodes for NRAMP1, a protein involved in the activation of phagocytes and synthesis of proinflammatory cytokines. **Methods:** In a case-control study in a Mexican Mestizo population, blood samples from 188 patients with rheumatoid arthritis and 133 healthy individuals were obtained to determine the frequency of *SLC11A1* gene variants INT4 (469+14G/C or *rs3731865*), D543N (1730G/A or *rs17235409*) and 3'UTR (1729+55del4 or *rs17235416*) by polymerase chain reaction and restriction fragment length polymorphism. **Results:** We found similar frequencies of INT4 and 3'UTR polymorphisms in patients and controls ( $p = 0.18$  and  $0.89$ , respectively). In contrast, a significantly lower frequency of the D543N polymorphism was observed in patients with rheumatoid arthritis compared to controls ( $p$  corrected =  $0.016$ ; OR:  $0.48$ ; 95% CI:  $0.28-0.80$ ). **Conclusion:** Our data suggest that the D543N variant of *SLC11A1* gene has a protective effect in the development of rheumatoid arthritis, an interesting finding that has not been previously reported in any population. (REV INVES CLIN. 2017;69:5-10)

**Key words:** Rheumatoid arthritis. Phagocyte. *SLC11A1* gene variant.

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## INTRODUCTION

Rheumatoid arthritis (RA) is an immune-mediated disease characterized by chronic inflammation of diarthrodial joints with synovial hyperplasia, progressive destruction of cartilage, and juxta-articular bone resorption, which may seriously affect the quality of life and survival of these patients<sup>1</sup>. Although the cause of RA has not been fully elucidated, different studies have indicated that genetic factors may account for approximately 50-65% of the risk in the development of RA<sup>1,2</sup>.

Natural resistance-associated macrophage proteins (NRAMP) 1 and 2 are transmembrane molecules classified as solute carriers that mediate the uptake of divalent cations such as ferrous iron (Fe<sup>2+</sup>) and manganese (Mn<sup>2+</sup>)<sup>3,4</sup>. In humans, NRAMP1 is encoded by the *SLC11A1* gene, which is 14 kb in length with 15 exons and is located on chromosome 2q35<sup>3,4</sup>. NRAMP1 is expressed by monocytes, macrophages, and polymorphonuclear neutrophils, and has an important role in the control of replication of intracellular parasites by altering the intravacuolar environment of the microbe-containing phagolysosome<sup>4,5</sup>. In addition, NRAMP1 seems to be involved in the priming and activation of phagocytes, participating in the induction of synthesis of proinflammatory cytokines (tumor necrosis factor- $\alpha$ , interleukin-1) and nitric oxide as well as in the expression of class II major histocompatibility complex (MHC) molecules<sup>5-8</sup>. Therefore, it is very feasible that NRAMP1 may have a relevant role in the pathogenesis of inflammatory and autoimmune diseases. Accordingly, different studies have analyzed the possible association between variants of the *SLC11A1* gene and autoimmunity<sup>9</sup>.

At least 17 genetic variants of *SLC11A1* gene have been described and 10 of these have been characterized in depth<sup>9-11</sup>. Nine of these variants are single nucleotide polymorphisms (SNP) and the other one corresponds to a microsatellite GTn-repeat polymorphism of the promoter region<sup>9-11</sup>. Among these variants, it has been described that the INT4 SNP (469+14G/C or *rs3731865*) is located at exon 4a, generates a termination codon into exon 5, and affects the expression and function of NRAMP1. Likewise, the D543N SNP (1730G/A or *rs17235409*) generates a missense mutation in codon 543 (aspartic acid to asparagine in exon 15) that could affect

the function of the protein. Moreover, the 3'UTR (1729+55del4 or *rs17235416*) variant corresponds to a 4 bp insertion/deletion immediately 3' of the stop codon; however, the possible effect of this variant on the expression and function of NRAMP1 has not been defined yet.

Different studies have analyzed the possible association between genetic variants of *SLC11A1* and infectious or autoimmune inflammatory diseases. Thus, a significant association has been detected between several *SLC11A1* polymorphisms (mainly the GTn promoter alleles 2 and 3 or *rs534448891*) and infectious diseases, mostly tuberculosis<sup>9,10,12,13</sup>. Moreover, a significant positive association between the 469+14G/C (*rs3731865*) SNP and autoimmune diseases, including RA, has been detected<sup>9</sup>. In contrast, the 237C/T SNP (*rs7573065*) seems to exert a protective effect in the development of inflammatory bowel disease<sup>9,14</sup>. However, most of these studies have been performed in Asian and Caucasian populations, and the possible association between *SLC11A1* gene variants and RA has not been previously analyzed in the Mexican Mestizo population, which comprises almost one hundred million individuals. Therefore, we decided to assess the frequency of the INT4 SNP (469+14G/C or *rs3731865*), D543N SNP (1730G/A or *rs17235409*) and 3'UTR (1729+55del4 or *rs17235416*) *SLC11A1* gene variants in patients with RA and healthy controls. This study indicates that there is an interesting and significant negative association between the D543N SNP and RA.

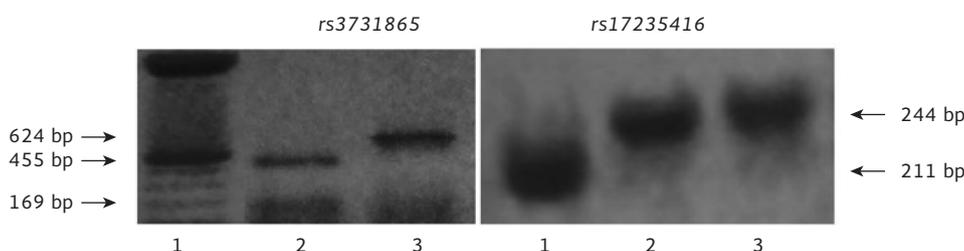
## MATERIAL AND METHODS

### Subjects

The state of San Luis Potosí, in central Mexico, has an extension of 61,000 km<sup>2</sup> with approximately 2.5 million inhabitants, mostly Mestizo population. However, in the western region of the state a significant fraction of the population corresponds to native Amerindians, which represents approximately 12% of the inhabitants of the state. The city of San Luis Potosí is located in this region and is mainly inhabited by Mexican Mestizos. We studied 321 unrelated Mexican Mestizo individuals from the city of San Luis Potosí; of them, 188 had RA, according to the classification criteria of the American College of Rheumatology<sup>15</sup>.

Figure 1. Identification of the *SLC11A1* gene variants INT4 (*rs3731865*), D543N (*rs17235409*) and 3'UTR (*rs17235416*) by polymerase chain reaction plus restriction fragment length polymorphism. In the upper panel, the primers, restriction enzymes and length of amplicon fragments are indicated. In the lower panel two examples of genotype identification are shown, for INT4 (lane 1, ladder; lane 2, allele C; lane 3, allele G) and 3'UTR variants (lane 1, allele ND; lanes 2 and 3, allele D).

Polymorphisms	Primers (5' to 3')	Restriction enzyme	Restriction fragment size (bp)
<i>rs3731865</i> (INT4)	TCTCTGGCTGAAGGCTCTCC TGTGCTATCAGTTGAGCCTC	Apa I	(G) 624 (C) 455, 169
<i>rs17235409</i> (D543N)	GCATCTCCCCAATTCATGGT AACTGTCCCACTCTATCCTG	Ava II	(G) 126, 79, 39 (A) 201, 39
<i>rs17235416</i> (3'UTR)	GCATCTCCCCAATTCATGGT AACTGTCCCACTCTATCCTG	Fok I	(D) 244 (ND) 225, 19



Patients were recruited at the Regional Center of Rheumatology and Osteoporosis in the Hospital Central "Dr. Ignacio Morones Prieto", San Luis Potosí; 20 were male and 168 were female, with an age range of 17–60 years (arithmetic mean: 47.4 years). Disease duration ranged between one and 37 years and almost all of the patients were receiving disease-modifying anti-rheumatic drugs. Most patients had shown a moderate or good response to therapy at the time of study. Patients with HIV infection, diabetes mellitus, or another inflammatory/autoimmune disease were not included in the study. As controls, 133 healthy individuals (25 male, 108 female; age range 20–50 years, with an arithmetic mean of 44.2 years) were included. All study subjects signed an informed consent; the study was approved by the institutional Bioethics Committee. Peripheral blood samples were obtained from all participants.

### Blood samples and genomic DNA isolation

Genomic DNA was extracted from peripheral blood samples by using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA), and the DNA was dissolved in sterile distilled water. Quantity and purity were determined with an Optizen Pop spectrophotometer (Mecasys Co, Daejeon, Korea).

### Detection of *SLC11A1* gene polymorphisms

The *SLC11A1* variants INT4 SNP (469+14G/C or *rs3731865*), D543N SNP (1730G/A or *rs17235409*) and 3'UTR (1729+55del4 or *rs17235416*) were determined by polymerase chain reaction (PCR) plus restriction fragment length polymorphism (RFLP). The PCRs were performed in a total volume of 50 µl of a solution containing 100 ng genomic DNA, 5 µl free Mg<sup>2+</sup> 10X buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), 200 µM dNTPs, 2 mM MgCl<sub>2</sub> and 1U of recombinant Taq DNA polymerase (Invitrogen). Thermal cycling was performed on a TC-412 device (Techne, Cambridge, UK). Cycling conditions for the variants D543N (*rs17235409*) and 3'UTR (*rs17235416*) were: 94°C for 10 minutes, 35 cycles of 94°C for 30 seconds, 68°C for 30 seconds, and 72°C for 30 seconds, with a final five-minute extension at 72°C. In the case of INT4 (*rs3731865*) cycling conditions were 94°C for 10 minutes, 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final five-minute extension at 72°C. Primers employed for each PCR are shown in figure 1. Amplicons were digested at 37°C for 24 hours with the proper restriction enzyme (Amersham Pharmacia Biotech, Piscataway, NJ) (Table 1) in a 20 µl reaction mix containing 1X restriction enzyme buffer and bovine

Table 1. Distribution of *SLC11A1* polymorphisms in patients with rheumatoid arthritis and controls

Polymorphism	Patients n (%)	Controls n (%)	p value	OR (95% CI)
<i>rs3731865</i>				
G/G	125 (66.5)	72 (59.1)	0.186	1.37 (0.86-2.2)
G/C and C/C	63 (33.5)	50 (40.9)		
<i>rs17235409</i>				
G/G	139 (76.4)	67 (60.9)	0.016*	0.48 (0.28-0.80)
G/A and A/A	63 (23.6)	43 (39.1)		
<i>rs17235416</i>				
ND/ND	67 (48.5)	50 (50.0)	0.895	0.94 (0.56-1.57)
ND/D and D/D	71 (51.5)	50 (50.0)		

\*Corrected p value (uncorrected = 0.0056).

CI: confidence interval; D: deletion; ND: non-deletion; OR: odds ratio.

serum albumin (BSA). Digested products were run on 2% agarose gels, which were stained with ethidium bromide and visualized using a UV transilluminator.

## Statistical analysis

Frequencies of genotypes were determined by direct counting, and genotype distribution and Hardy-Weinberg equilibrium were tested by using the Arlequin software version 3.11 (University of Bern, Switzerland). The statistical significance of genotype frequency differences between patients and controls was determined by Fisher's exact test using 2 × 2 contingency tables and GraphPad software (San Diego, CA). Corrected p values were obtained by multiplying raw values by the total number of variables analyzed and were considered significant when they were < 0.05.

## RESULTS

The *SLC11A1* variants INT4 (*rs3731865*), D543N (*rs17235409*), and 3'UTR (*rs17235416*) were determined by PCR-RFLP by using the Apa I, Ava II and Fok I restriction enzymes, respectively (Fig. 1). In all cases, the alleles were in Hardy-Weinberg equilibrium.

As shown in table 1, the frequency of the INT4 (*rs3731865*) SNP was not significantly different (p = 0.18, OR: 1.37; 95% CI: 0.86-2.20) in patients with RA (33.5%) and healthy controls (40.9%). Likewise, when the genotype distribution of the 3'UTR (*rs17235416*) variant was analyzed, we found a very similar frequency in patients with RA (51.4%) and

healthy controls (50.0%) (p = 0.89; OR: 0.94; 95% CI: 0.56-1.57). In contrast, we observed a significantly lower frequency of the D543N (*rs17235409*) SNP in patients with RA (23.6%) compared to healthy controls (39.1%) (p uncorrected = 0.0056, p corrected = 0.016; OR: 0.48; 95% CI: 0.28-0.80).

## DISCUSSION

Although different factors have been involved in the etiology and pathogenesis of RA, it is evident that the cause and mechanisms of tissue damage in this disorder remain as very relevant points to be fully elucidated<sup>1</sup>. Genetic factors have been found to significantly contribute to the risk for RA<sup>1,2</sup>. Thus, some MHC alleles, as well as different variants of immune genes, show a significant association with this condition<sup>2</sup>. Accordingly, different studies have addressed the possible role of MHC and other gene alleles in the susceptibility and severity of RA in Mexicans<sup>16,17</sup>.

The *SLC11A1* gene encodes for a transmembrane protein (NRAMP1) that is mainly expressed by professional phagocytes (monocytes, macrophages, neutrophils)<sup>5</sup>. NRAMP1 mediates the uptake of divalent cations in phagolysosomes and is involved in the activation and effector mechanisms of phagocytes, including their bactericidal activity and the synthesis of proinflammatory cytokines and nitric oxide<sup>5-7</sup>. Thus, it is very feasible that *SLC11A1*/NRAMP1 may be involved in the pathogenesis of chronic inflammatory conditions, including RA<sup>8</sup>. Accordingly, in the last 10 years, several studies on the association of *SLC11A1* variants and RA in different populations

have been published<sup>10,18-21</sup>. In addition, a possible association between a *SLC11A1* promoter polymorphism and RA severity<sup>22</sup>, or risk for idiopathic arthritis or juvenile rheumatoid arthritis, has been reported<sup>23,24</sup>. However, in a recent meta-analysis performed by Archer, et al.<sup>9</sup>, only a single significant association was detected between *SLC11A1* variants and RA, namely the INT4 SNP (469+14G/C or *rs3731865*), with an OR: 1.60 (95% CI: 1.20-2.13). Based on these data, we decided to analyze the possible association between three variants of *SLC11A1* gene and RA in the Mexican Mestizo population.

In contrast with previous reports<sup>9</sup>, we observed a similar frequency of the INT4 (*rs3731865*) SNP in RA patients and controls. Likewise, no significant association was detected between the 3'UTR (*rs17235416*) variant of *SLC11A1* gene and RA. However, we observed a significantly lower frequency of the D543N (*rs17235409*) SNP in RA patients compared to controls ( $p$  corrected = 0.016; OR: 0.48; 95% CI: 0.28-0.80). Since this SNP generates a missense mutation in codon 543, which results in the substitution of negatively charged aspartic acid by uncharged asparagine, it would be expected that this variant affects the function of the protein. However, this point has not been fully addressed and no data are available regarding the precise effect of this amino acid substitution on the levels of expression or the function of NRAMP1. In any case, this is the first report of a negative association between the D543N (*rs17235409*) SNP and RA. In this regard, a study by Singal, et al. suggested a possible protective effect of D543N variant in the development of RA (relative risk = 0.06; uncorrected  $p$  = 0.014); however, the value of  $p$  corrected in this study was not significant<sup>21</sup>. Moreover, it has been reported that the D543N SNP apparently confers protection against *Mycobacterium tuberculosis* reactivation in Cambodian patients infected with HIV ( $p$  = 0.04; OR: 0.21)<sup>25</sup>, an association that was not observed in Indian patients<sup>23</sup>. All these data suggest that the D543N *SLC11A1* variant has a complex role in the susceptibility to inflammatory and infectious disease, with a relevant interaction with the genetic background of individuals studied<sup>26</sup>.

It has been proposed that the high mortality by *M. tuberculosis* infection observed over the past three centuries has exerted an important selective force, increasing the frequency of allelic gene variants

(including those of the *SLC11A1* gene) that enhance the resistance to this pathogen<sup>27</sup>. Since these allelic variants are associated with an increased capability to generate proinflammatory mediators by immune cells, it has been hypothesized that the selective force generated by *M. tuberculosis* in humans has resulted in an increased susceptibility to chronic inflammatory conditions, including RA<sup>27</sup>. In this regard, our previous report on the lack of association between the D543N SNP and tuberculosis in Mexican Mestizos<sup>28</sup> is of interest.

In summary, we have identified a variant (D543N or 1730G/A, *rs17235409*) of an immune gene (*SLC11A1*/NRAMP1), which has an important role in the activation and effector functions of professional phagocytes and that apparently confers protection in the development of RA in the Mexican Mestizo population. We consider that it would be of interest to confirm this finding in a larger study, as well as to explore the possible effect of this SNP on the expression and function of NRAMP1 and its impact on the pathogenesis of RA.

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