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# Association between *SLC11A1* (formerly *NRAMP1*) and the risk of sarcoidosis in Poland

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Sarcoidosis (SA) is a systemic granulomatous disorder of unknown etiology characterized by T helper 1-type inflammatory responses at sites of disease with signs of B cell hyperactivity. Like rheumatoid arthritis and diabetes, an infectious etiology has frequently been postulated but no single infectious trigger definitively identified. Polymorphic alleles at *SLC11A1* have previously been associated with susceptibility to both the putative infectious agents and to these autoimmune disorders. We therefore investigated its candidacy as a genetic determinant of SA in Poland in an association-based study comparing 86 SA patients with 85 tuberculosis (TB) patients and 93 control subjects. The functional promoter (GT)<sub>n</sub> polymorphism and four of 10 other single nucleotide or insertion/deletion polymorphisms genotyped across *SLC11A1* were informative in our sample. Consistent with previous autoimmune disease studies, allele 3 at the functional (GT)<sub>n</sub> promoter region repeat polymorphism was significantly associated with SA when compared with healthy controls (odds ratio 1.68; 95% CI: 1.01–2.81; *P* = 0.04) or with TB patients (odds ratio 1.69; 95% CI: 1.042–0.78; *P* = 0.03).

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## Introduction

Sarcoidosis (SA) is a multisystem granulomatous disorder of unknown etiology. Diagnosis is firmly established when clinicoradiological findings are supported by histological evidence of noncaseating epithelioid cell granulomas found on tissue biopsy. Despite extensive research into the etiology of SA, no identifiable agent has been demonstrated to account for the granulomata. Chemicals, allergy, infectious antigens, autoimmunity and genetic factors

have all been explored as potential causes.<sup>1</sup> It is likely that genetically predisposed hosts are exposed to antigens that trigger an exaggerated cellular immune response with the production of antibodies, including self-antibodies, leading to granuloma formation.<sup>1–6</sup> A high frequency of endocrine autoimmunity in patients with SA has been reported, but the mechanism of this relationship is not known.<sup>7–9</sup>

The inflammatory response in SA is characterized by large numbers of activated macrophages that secrete proinflammatory cytokines/chemokines including tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and RANTES (regulation of activation, normal T-cell expression and secretion), and accumulation of CD4<sup>+</sup> cells with a Th1-type interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-2 (IL-2) cytokine profile.<sup>1</sup> These features are shared in common with many inflammatory

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and autoimmune disorders and, like inflammatory bowel disease, rheumatoid arthritis and diabetes, an infectious etiology has frequently been postulated.<sup>1</sup> Although no single infectious trigger has been definitively identified, indirect evidence<sup>10–12</sup> implicates *Mycobacterium tuberculosis* and other mycobacteria as possible agents involved in triggering disease. Mycobacteria have also been implicated in triggering<sup>13–16</sup> or protecting individuals from<sup>15–17</sup> autoimmune disease. Polymorphic alleles at *SLC11A1* have previously been associated with susceptibility to both these putative infectious agents and to these autoimmune disorders (reviewed in Blackwell *et al*<sup>18</sup>). *SLC11A1* encodes the natural resistance associated macrophage protein 1 that regulates divalent cation homeostasis in macrophages and has many pleiotropic effects on macrophage activation.<sup>18</sup> It is also known to be involved in determining Th1 versus Th2 bias in immune response to infection or specific antigens. In humans, no functional variants across the coding region of the gene have been identified, but a functional (GT)<sub>n</sub> repeat polymorphism has been identified<sup>19</sup> and characterized.<sup>20–22</sup> The most common allele in all populations studied, designated allele 3 and characterized by T(GT)<sub>5</sub>AC(GT)<sub>5</sub>AC(GT)<sub>9</sub> repeats, drives high levels of reporter gene expression and is associated with rheumatoid arthritis<sup>23</sup> and juvenile rheumatoid arthritis.<sup>24</sup> The second most common allele, designated allele 2 and characterized by T(GT)<sub>5</sub>AC(GT)<sub>5</sub>AC(GT)<sub>10</sub> repeats, drives low expression and is associated with tuberculosis (TB) in multiple populations.<sup>25–28</sup> By these criteria, we predict that allele 3 would also be associated with susceptibility to SA and allele 2 with TB. To test this hypothesis we carried out a population-based study in Poland comparing *SLC11A1* genotypes in SA patients with TB patients and control subjects. This is the first time that polymorphisms at *SLC11A1* have been analyzed in patients with SA and TB in the same European Caucasoid ethnic group.

## Materials and methods

### Patients and controls

**SA patients** In total, 86 outpatients (mean age 39.5 ± 10 years; range 21–70 years; 34 women, 52 men) with documented pulmonary SA were studied. Diagnosis of SA was based on histological, clinical and radiological evidence.<sup>1</sup> Chest radiographs were staged as stage I (bilateral hilar lymphadenopathy; 29 patients) or stage II (bilateral hilar lymphadenopathy and diffuse pulmonary infiltrations; 57 patients). A negative PPD skin-test response was an additional criterion for inclusion in the study group. Microbiological and cytological examination of the sputum samples revealed no acid-fast bacilli, fungi or atypical cells.

**TB patients** In total, 85 unrelated patients (mean age 48.5 ± 15 years; range 20–80 years; 25 women, 60 men)

with newly detected active pulmonary TB at the Pulmonology Hospital in Sopot and Wejherowo, Poland, were studied. A diagnosis of TB was established using standard clinical, radiographic and bacteriological criteria. The patients studied were at a similar clinical stage and with similarly localized disease on the initial chest radiographs (ie infiltrates with cavitation in the one or two lung zones). The diagnosis of TB was confirmed in all patients by demonstration of acid-fast bacilli in sputum smears and by positive sputum culture of the *M. tuberculosis* strains. A positive PPD skin-test response was an additional criterion included in the studied group. Patients were also classified according to their response to chemotherapy. All TB patients included in this study responded to the first line treatment (rifampin, isoniazid, ethambutol, pyrazinamide) and were therefore classified as drug responders.

**Controls** The sample studied consisted of 93 unrelated healthy blood donor volunteers (mean age 38.1 ± 13 years; range 20–80 years; 39 women, 54 men) originating from the region of Gdansk in northern Poland. They had an unremarkable physical examination, normal blood and serum analysis as well as normal CXRs and no acid-fast bacilli in sputum. All of them were negative for the PPD skin test. The controls were of the same socioeconomic status and ethnic background as the patients. The Polish population is a relatively homogenous ethnic group. Patients and controls included people living in the Gdańsk area and in the neighboring districts. As a result of postwar movements, they come from east, north and central Poland; therefore, they may be considered as a homogeneous Caucasian group. None of the TB or SA patients or controls had a familial history of TB, SA or autoimmune disease. All patients and controls were vaccinated with BCG (bacillus Calmette-Guerin). In Poland, BCG vaccinations have been mandatory at birth and again at 7, 12 and 18 years of age since 1955.

Ethical approval for the study was obtained from the Independent Bioethics Committee for Scientific Researches, Medical University of Gdańsk, Poland, and informed consent was obtained for collection of 10 ml blood by venepuncture for preparation of DNA. Samples were anonymised for genetic analysis. A total of 86 SA patients, 85 TB patients and 93 control subjects were available for genotyping of *SLC11A1* polymorphisms. All individuals included in this study were HIV negative.

### Genotyping

The functional promoter (GT)<sub>n</sub> repeat polymorphism and 10 single nucleotide (SNPs) (–237C/T; –86G/A; 274C/T; 469+14G/C; 823C/T; 1465–85A/G; D543N G/A; 1801+417C/T<sup>29–31</sup>) or insertion/deletion (IN/DEL) (3'UTR UTR TGTG<sup>29</sup>; 3'UTR CAAA<sup>32</sup>) polymorphisms were genotyped across *SLC11A1*. The (GT)<sub>n</sub> repeat in the promoter region of *SLC11A1*<sup>19</sup> was PCR amplified using NED- and

FAM-labelled forward primers and PCR products were analyzed by electrophoresis on 6% polyacrylamide using a capillary ABI3100 Genetic Analyser (Applied Biosystems, Warrington, UK) as previously described.<sup>29</sup> The two IN/DELS<sup>29,32</sup> were also typed using the ABI3100 as described.<sup>31</sup> The (GT)<sub>n</sub> repeat polymorphism and IN/DELS were analyzed using ABI computer software GeneScan™ and Genotyper® v 3.7. For the SNPs, SNaPshot assays using the ABI PRISM® SNaPshot™ Multiplex System. SNP flanking primers were designed using Primer3 software (<http://frodo.wi.mit.edu/>) with extension primers designed according to the manufacturer's recommendations (Web Table 1). Following DNA amplification, PCR products were pooled and purified by the addition of 2U exonuclease (New England Biolabs, Hitchin, UK) and 5U Shrimp Alkaline Phosphatase (SAP; USB, Cleveland, OH, USA) per 15 µl of sample and incubated for 1 h at 37°C followed by 15 min at 72°C in order to remove unincorporated primers and dNTPs. After purification, 2 µl of PCR product was mixed with 0.5 µl of deionized water, 2.5 µl of SNaPshot ready Reaction Mix and 0.5 µl of pooled SNaPshot extension primers (with each primer at 0.4 µM final concentration) and cycled according to the manufacturer's recommendations. SNaPshot products were then purified by the addition of 1U SAP and incubated for 1 h at 37°C followed by 15 min at 72°C in order to remove unincorporated [F]ddNTPs. Following purification, 0.5 µl SNaPshot product was mixed with 9 µl Formamide Ultra (Bioproducts Ltd, UK) and 0.5 µl LIZ-120 Size Standard (ABI) then denatured at 95°C for 5 min. Fragments were run on the ABI 3100 Genetic Analyser and analyzed using GeneMapper® Software v 3.0 (Applied Biosystems).

### Statistical analysis

Tests for deviation from Hardy–Weinberg equilibrium (HWE), allelic association between markers and disease,

and linkage disequilibrium (LD) between markers (reported as the *r*<sup>2</sup> statistic which ranges from 0 to 1 and takes allele frequencies into account), were performed within STATA v 8.0 (<http://www.stata.com/>) using the GenAssoc package (available from <http://www-gene.cimr.cam.ac.uk/clayton/software/stata/>). Power calculations were performed in excel using a script prepared in-house at CIMR by Dr Heather Cordell. Genotype and allele frequencies were compared using the  $\chi^2$  statistic and odds ratios (OR) and 95% confidence intervals (CI) calculated using unconditional logistic regression analysis under a multiplicative model (ie two alleles contribute twice the effect of one allele). A likelihood ratio test was used to determine dominance effects by comparing one degree of freedom (df; allele-wise) and 2 df (genotype-wise) tests.

### Results

Allele frequencies for markers in the healthy control group are shown in Table 1. Tests for deviation from HWE confirmed that all markers were in HWE in this control group. The variant alleles at –237C/T, –86G/A, 823C/T, D543N, 3'TGTG and 1801+417C/T were too low in frequency (<0.1) to be used to detect allelic association with disease, and were excluded from further analysis. Variant alleles for all other markers were  $\geq 0.2$ . Power calculations demonstrated >80% power to detect a significant (*P*<0.05) effect at an odds ratio  $\geq 2$  for a marker with variant allele frequency  $\geq 0.2$ .

Table 2 shows results of allelic association tests comparing SA patients, TB patients and controls for the five informative markers at *SLC11A1*. Allele 3 at the functional (GT)<sub>n</sub> repeat polymorphism was associated with higher risk of SA when compared to both control (OR = 1.68; *P* = 0.04) and TB patient (OR = 1.69; *P* = 0.03) groups. No dominance effects were observed for either comparison, consistent

**Table 1** Allele frequencies and results of tests for deviation from HWE (global  $\kappa$ , Z-value and *P*-value) for *SLC11A1* polymorphic markers in the healthy Polish control sample. NCBI reference SNP IDs are given where known. Position (bp) in the July 2003 human reference sequence based on NCBI Build 34 is given for all polymorphisms

Marker name	NCBI reference SNP ID (rs)	Position (bp)	Common allele (%)	Variant allele (%)	Global $\kappa$	Z-value	P-value		
(GT) <sub>n</sub>		219 449 178–219 449 218	(GT) <sub>n</sub> a3	74.73	(GT) <sub>n</sub> a2	25.27	0.003	0.030	0.9761
–237C/T	rs7573065	219 449 247	C	94.26	T	5.74	0.243	1.913	0.0557
–86G/A		219 449 397	G	100.0	A	0.00	—	—	—
274C/T	rs2276631	219 451 553	C	74.16	T	25.84	0.069	0.659	0.5101
469+14G/C	rs3731865	219 452 543	G	80.11	C	19.89	0.113	1.075	0.2824
823C/T		219 455 169	C	96.51	T	3.49	–0.035	–0.234	0.8149
1465–85G/A	rs2279015	219 461 810	G	68.28	A	31.72	0.189	1.840	0.0658
D543N G/A		219 462 272	G	99.42	A	0.58	–0.006	–0.054	0.9573
3' UTR TGTG/del		219 462 353	INS	98.40	DEL	1.59	–0.016	–0.156	0.8764
3' UTR CAAA/del		219 462 566	DEL	65.48	INS	34.52	0.064	0.589	0.5558
1801+417[CT]		219 462 715	T	100.0	C	0.00	—	—	—

a2 – allele 2 of *SLC11A1* (GT)<sub>n</sub> promoter polymorphism.

a3 – allele 3 of *SLC11A1*(GT)<sub>n</sub> promoter polymorphism.

**Table 2** Results of allele-wise (1 df) tests for association between polymorphic variants at *SLC11A1* and SA (SA) or tuberculosis (TB) in the Polish patient groups compared to controls, and to each other

Polymorphisms	SA (n = 86) versus controls (n = 93)		TB (n = 85) versus controls (n = 93)		TB (n = 86) versus SA (n = 85)	
	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P	Odds ratio (95% CI)	P
(GT) <sub>n</sub> a3/a2	1.68 (1.01–2.81)	0.04	0.92 (0.59–1.44)	0.71	1.69 (1.04–2.75)	0.03
274C/T	1.09 (0.68–1.78)	0.71	0.93 (0.59–1.46)	0.75	1.16 (0.73–1.86)	0.53
469+14G/C	1.63 (0.93–2.86)	0.08	1.02 (0.64–1.64)	0.92	1.49 (0.88–2.51)	0.14
1465–85G/A	1.28 (0.84–1.97)	0.25	1.08 (0.71–1.62)	0.73	1.17 (0.76–1.80)	0.47
3' UTR CAAA del/ins	1.06 (0.70–1.62)	0.77	0.90 (0.59–1.38)	0.63	1.17 (0.77–1.77)	0.46

with a higher OR (3.47; 95% CI 1.04–11.60;  $P=0.04$ ) for disease in SA patients homozygous for allele 3 at the (GT)<sub>n</sub> repeat compared to the TB patient group. No allelic associations between TB and *SLC11A1* were observed in this population compared to the control group, and no other informative polymorphisms across *SLC11A1* were associated with SA compared to either the control or TB patient groups, despite moderate LD between the (GT)<sub>n</sub> and 274C/T ( $r^2=0.68$ ) and between (GT)<sub>n</sub> and 469+14G/C ( $r^2=0.58$ ). There was little or no LD ( $r^2=0.24-0.39$ ) between any of the informative 5' *SLC11A1* polymorphic markers ((GT)<sub>n</sub>; 274C/T; 469+14G/C) and any of the informative 3' markers (1465–85A/G; 3' UTR CAAA), although the latter were in strong LD ( $r^2=0.92$ ) with each other.

## Discussion

Previous studies of inflammatory and autoimmune diseases (reviewed in Blackwell *et al*<sup>18</sup>) and the clinical and immune response phenotypes associated with SA (reviewed<sup>1</sup>) provided *a priori* evidence that allele 3 at the (GT)<sub>n</sub> the functional repeat polymorphism in the promoter region of *SLC11A1* would be associated with SA. Data presented here provide evidence to support this prediction, both when SA patients were compared to healthy controls and to TB patients, in this Polish population. This is consistent with previous etiological and epidemiological studies suggesting genetic predisposition to disease<sup>1</sup> and, in particular, the hypothesis that mycobacteria might provide one of the specific environmental agents that triggers the inflammatory response in genetically susceptible individuals.<sup>12</sup> In this context, we might have expected an 'equal and opposite' association between polymorphism at *SLC11A1* and susceptibility to TB in this population, especially since the low expressing allele 2<sup>20</sup> had previously been shown to be associated with both susceptibility to TB<sup>25–27,33</sup> in multiple populations, and to high anti-inflammatory IL-10 responses<sup>33</sup> in the Gambia. In the event, we observed no direct association between polymorphisms at *SLC11A1* and TB in Poland, suggesting that the two diseases are under separate genetic control in this

population. This does not, however, preclude the possibility that interaction with (environmental) mycobacteria/mycobacterial products could provide the trigger for heightened macrophage activation/proinflammatory responses associated with *SLC11A1*-regulation of susceptibility to SA in this population. Failure to observe an association between the (GT)<sub>n</sub> promoter polymorphism at *SLC11A1* and pulmonary TB is consistent with previous reports showing population-to-population variation in demonstrable positive<sup>25,27,28,33</sup> versus no detectable<sup>34</sup> association, the latter even in the presence of association with the 469+14G/C polymorphism in intron 4 of the gene. Hence, other etiological *SLC11A1* variants are likely to contribute functionally to disease association with TB. There are a number of other studies that report linkage<sup>35,36</sup> or association<sup>37–39</sup> with polymorphisms across *SLC11A1* where the promoter (GT)<sub>n</sub> polymorphism has not been examined, and other reported failures<sup>40,41</sup> to detect linkage or association between *SLC11A1* and TB. This is an expected outcome for diseases where complex inheritance of susceptibility leads to different genes influencing disease in different populations, but some caution in interpreting these data are necessary as samples sizes employed frequently have low power to detect allelic association even when present. Since the sample employed in our study is also small, further work is required both to confirm the positive association with SA and the negative finding for TB in this population.

In previous functional studies, allele 3 at the (GT)<sub>n</sub> promoter polymorphism was shown to drive high levels of reporter gene activity, even in the absence of exogenous stimuli. By implication, this allele would therefore be associated with high expression of *SLC11A1* protein, high activation status of macrophages, and concomitant enhancement of proinflammatory responses including TNF- $\alpha$ , interleukin-1 $\beta$  and chemokine responses. All these are features of immunopathology associated with SA.<sup>1</sup> In reporter gene studies the enhanced activity of allele 3 is reversed in the presence of the variant T allele at –237C/T polymorphism.<sup>21</sup> This variant was at low frequency (<0.06) in this Polish population. Hence, it was neither possible nor necessary to stratify by the –237C/T polymorphism to detect the allele 3 effect. Nor did we observe

any other allelic associations with SA across *SLC11A1*, suggesting that the (GT)<sub>n</sub> repeat is the functional variant associated with disease in this population. This is consistent with a previous study of *SLC11A1* polymorphisms and SA in an African-American population, where four polymorphisms were studied but only the (GT)<sub>n</sub> repeat showed significant association with disease.<sup>42</sup> In this case the authors pooled the rarer alleles at the (GT)<sub>n</sub> with allele 2, and reported that this combination of alleles was significantly protective for SA compared with the more common allele 3. Another study of SA in a Japanese population examined only a single polymorphism (1703G/A) at the 3' end of the gene, and failed to demonstrate an association.<sup>43</sup> Similarly, we and others<sup>42</sup> found no evidence for association with markers in the 3' end of the gene in. There was, in any case, no LD between polymorphic markers at 5' and 3' ends of *SLC11A1*, so there was no prior expectation that we should find allelic association with markers in the 3' region of *SLC11A1* in our population.

In summary and consistent with previous autoimmune disease studies, our study demonstrates that allele 3 at the functional (GT)<sub>n</sub> promoter region repeat polymorphism of *SLC11A1* is associated with susceptibility to SA in a European Caucasoid population. This provides further support for the notion of a possible infectious trigger for SA, and adds to the cumulative evidence that exposure to infectious agents has shaped the gene-by-environment interactions that now contributes to susceptibility to autoimmune-related diseases in man.

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