Infection with *Mycobacterium tuberculosis* Beijing Genotype Strains Is Associated with Polymorphisms in *SLC11A1/NRAMP1* in Indonesian Patients with Tuberculosis

Reinout van Crevel,† Ida Parwati,† Edhyana Saharatmadja,‡ Sangkot Marzuki,§ Tom H. M. Ottenhoff,¶ Mihai G. Netea,† Andre van der Ven,† Ronald H. Nelwan,§ Jos W. van der Meer,† Bachtli Alisjahbana,|| and Esther van de Vosse†

†Department of Internal Medicine and Nijmegen Institute for Infection, Inflammation, and Immunity (N4I), Radboud University Nijmegen Medical Center, Nijmegen, and ‡Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands; §Health Research Unit, Medical Faculty, Padjadjaran University/Hasan Sadikin Hospital, Bandung, and ¶Eijkman Institute for Molecular Biology, and ||Medical Faculty, University of Indonesia, Jakarta, Indonesia

Differences in host immune genes may predispose to tuberculosis caused by particular *Mycobacterium tuberculosis* genotypes. We examined this hypothesis in Indonesia by sputum isolates recovered from 336 patients with pulmonary tuberculosis and typing the patients’ *SLC11A1* gene (formerly known as “NRAMP1”), which is involved in susceptibility to tuberculosis. The *M. tuberculosis* Beijing genotype, which comprised 29.8% of all isolates, was strongly associated with 2 polymorphisms in *SLC11A1*: the D543N G allele (odds ratio [OR], 2.15; *P* = .005) and the 3′ untranslated region (3′UTR) insertion/insertion genotype (OR, 2.5; *P* < .001). This finding supports the hypothesis of coevolution of *M. tuberculosis* and the human immune system.

Different evolutionary lineages of *Mycobacterium tuberculosis* are strongly associated with specific geographical regions, suggesting that they have adapted to particular human populations. Indeed, particular *M. tuberculosis* lineages (also known as genotype families) have shown preferential spread in certain patient populations [1, 2]. This finding may be caused by differences in environmental exposure, but it might also be the result of coevolution of host and pathogen. In this case, differences in host immune genes would confer increased susceptibility—or resistance—of certain human populations to particular *M. tuberculosis* genotype families.

So far, only one study has tried to show a direct association between the genetic characteristics of patients with tuberculosis and their (own) mycobacterial isolates. In this study from Vietnam, it was shown that a particular variation in Toll-like receptor 2, a relevant receptor for *M. tuberculosis*, was more common among patients infected with strains belonging to the evolutionarily successful *M. tuberculosis* Beijing genotype than among patients infected with other genotype strains [3]. This study suggests that the outcome of exposure to *M. tuberculosis* depends on both human and bacterial genotypes. We examined this hypothesis in Indonesia, which has the third highest global burden of tuberculosis. We previously demonstrated that 33% of patients with tuberculosis in a large cohort in Indonesia were infected with strains belonging to the *M. tuberculosis* Beijing genotype [4], which globally is one of the most predominant *M. tuberculosis* families [5]. In this same population, we have now examined whether infection with the Beijing genotype *M. tuberculosis* was associated with specific polymorphisms of *SLC11A1*, which was formerly known as “NRAMP1” (natural resistance–associated macrophage protein 1), a gene that was reported to be associated with susceptibility to tuberculosis, especially among Asian subjects [6].

**Methods.** From January 2001 through December 2006, consecutively seen patients who were >16 years of age and had microscopically proven pulmonary tuberculosis were included in 2 outpatient clinics and 2 hospitals in Jakarta and Bandung (West Java, Indonesia), as part of a case-control study examining host susceptibility to tuberculosis [7]. Diagnosis of tuberculosis was based on clinical presentation, chest radiograph examination, microscopic detection of acid-fast bacilli in Ziehl-Neelsen–stained sputum smears, and culture of *M. tuberculosis* on 3% Ogawa medium. All patients were tested for human immunodeficiency virus (HIV) infection, and 13 HIV-sero-
positive patients (1.8%) were not included in further analysis. The study was approved by the ethics committee of the Faculty of Medicine, University of Indonesia, Jakarta, and the Faculty of Medicine, Padjadjaran University, Hasan Sadikin Hospital, Bandung, Indonesia.

Spoligotyping was performed on M. tuberculosis sputum cultures obtained from 769 patients with tuberculosis. Mycobacterial DNA was extracted by bringing 2 loops of bacterial mass from a M. tuberculosis culture in saline and subsequently heating it at 95°C for 5 min. Spoligotyping was performed using a commercial kit (Isogen Bioscience). The presence or absence of 43 spacers in the DR region of isolates of M. tuberculosis was detected as described elsewhere [4]. M. tuberculosis Beijing genotype was defined as a spoligopattern showing hybridization to at least 3 of the 9 spacers 1–34. Spoligotyping was done at the Hasan Sadikin Hospital, Bandung, Indonesia. For quality control, spoligotyping of 10% of the isolates and of all isolates lacking hybridization was repeated at Gelre Hospital, Apeldoorn, the Netherlands.

Typing of the SCL11A1 polymorphism was performed for 342 patients in this cohort, but 6 HIV-infected patients were excluded from further analysis. Genomic DNA was isolated from ethylenediaminetetraacetic acid blood. Two single-nucleotide polymorphisms (SNPs) in the gene NRAMP1: D543N (1703G→A in exon 15, leading to an aspartate to asparagine substitution at codon 543 [SNP identification {SNPid}, rs17235409]) and INT4 (469+14G→C in intron 4 [SNPid, rs3731865]), as well as a TGTG insertion/deletion (ins/del) polymorphism in the 3' untranslated region (3'UTR) (1729+55 ins/del4 [SNPid, rs17235416]), denoted as "3'UTR," were analyzed as described elsewhere [8]. The 5' (GT) promoter polymorphism of SLC11A1 was examined in 60 individuals, but minor allele frequencies were too low (1%–3%) to analyze in the full cohort. The Hardy-Weinberg equilibrium of each polymorphism was checked using the program HWE. The program Conting was used to calculate the χ² value and the associated values for a contingency table. All statistical analyses were 2-sided, and P<.05 was considered to be statistically significant. Associations of SLC11A1 polymorphisms with infection due to the M. tuberculosis Beijing strain (as opposed to other, or "non-Beijing," strains) were expressed as odds ratios (ORs) and 95% confidence intervals (CIs).

Results. Of the 336 patients (median age, 30 years; range, 16–75 years) included in the study, 193 (57.4%) were male and 17 (5.1%) had a history of receiving previous tuberculosis treatment. One hundred (29.8%) were infected with M. tuberculosis Beijing genotype strains. The remaining 236 patients were infected with genotypes T1 (11.1%), Haarlem (9.3%), LAM (6.3%), EAI (4.2%), or U/H3 (3.6%), remaining genotypes (4.7%), or "orphan strains" (31.0%), the latter of which had no type shared with the international spoligotype database SpolDB4. These results were representative for the total cohort of 740 patients with pulmonary tuberculosis, which was previously described elsewhere [4]. Patients infected with M. tuberculosis Beijing strains and those infected with M. tuberculosis strains of other ("non-Beijing") genotypes were not significantly different in terms of age, sex, or history of previous tuberculosis treatment. All 336 patients were HIV seronegative.

The distribution of alleles and genotypes of 3 SCL11A1 polymorphisms among patients infected with M. tuberculosis Beijing genotype strains and other genotype strains is shown in Table 1. Each polymorphism was in Hardy-Weinberg equilibrium in the total group of patients as well as in 363 control subjects analyzed in a previous study [8]. The variant allele of INT4 was present in <5% of subjects and therefore was not further analyzed. As demonstrated in the present study, significant associations were found between infection with M. tuberculosis Beijing genotype strains and the 2 remaining SLC11A1 polymorphisms. The G allele and the GG genotype of the D543N polymorphism were significantly associated with infection with M. tuberculosis Beijing genotype strains (P = .01 and P = .005, respectively). The association between M. tuberculosis Beijing genotype and the GG genotype of D543N showed an OR of 2.15 (95% CI, 1.25–3.70). Similarly, the insertion allele and the insertion/insertion genotype of the 3'UTR polymorphism were significantly associated with infection by M. tuberculosis Beijing genotype strains (P = .01 and P < .001, respectively).

Table 1. SCL11A1 Polymorphisms According to Mycobacterium tuberculosis Genotype Strain

<table>
<thead>
<tr>
<th>Polymorphism, allele or genotype</th>
<th>Frequency in patients infected with M. tuberculosis, no. (%)</th>
<th>Beijing genotype strain</th>
<th>Other genotype strains</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>D543N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>172 (87.8)</td>
<td>360 (79.3)</td>
<td>.010</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>24 (12.2)</td>
<td>94 (20.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>76 (77.6)</td>
<td>140 (61.7)</td>
<td>.005a</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>20 (20.4)</td>
<td>80 (35.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>2 (2.0)</td>
<td>7 (3.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INT4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>193 (96.5)</td>
<td>457 (98.9)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>3 (1.5)</td>
<td>5 (1.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3'UTR</td>
<td>ins</td>
<td>104 (88.1)</td>
<td>227 (77.7)</td>
<td>.016</td>
</tr>
<tr>
<td>del</td>
<td>14 (11.9)</td>
<td>65 (22.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ins/ins</td>
<td>48 (78.0)</td>
<td>87 (59.6)</td>
<td>.0013b</td>
<td></td>
</tr>
<tr>
<td>ins/del</td>
<td>12 (20.3)</td>
<td>53 (36.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>del/del</td>
<td>1 (1.7)</td>
<td>6 (4.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. 3'UTR, 3' untranslated region; del, deletion; ins, insertion; NA, not available.

a GA and AA genotypes combined for analysis.
b Ins/del and del/del genotypes combined for analysis.
The association between Beijing genotype strains and the insertion/insertion genotype of 3′UTR showed an OR of 2.40 (95% CI, 1.19–4.83).

Discussion. We report a strong association between host and bacterial genotypes among patients with tuberculosis in Indonesia. Patients carrying the most common genotypes of 2 different polymorphisms of SLC11A1 had a much higher chance of having tuberculosis caused by M. tuberculosis Beijing genotype strains than did patients carrying other genotypes of these SLC11A1 polymorphisms. The human gene SLC11A1, formerly known as “NRAMP1,” is one of the most important genes reported to be associated with susceptibility to tuberculosis [6], but this is the first study to show an association between SLC11A1/NRAMP1 polymorphisms and one of the most prevalent and most successful M. tuberculosis lineages, the Beijing genotype family.

The SLC11A1/NRAMP1 gene has been extensively examined to determine its association with tuberculosis [6]. It encodes a transmembrane protein that is exclusively expressed in macrophages and polymorphonuclear leukocytes. After phagocytosis, the SCL11A1 protein is rapidly recruited to the phagolysosomal membrane, where it mediates transport of iron and other cations. In addition, SLC11A1/NRAMP1 has pleiotropic effects on macrophage activation. Microbial pathogens, including M. tuberculosis, have homologues of SLC11A1. Iron is essential for biological functions, both for host immune defense and mycobacterial growth, and iron transporters of the human host and the mycobacterium compete intracellularly for iron [9]. SLC11A1/NRAMP1 may also play a role in susceptibility to other intracellular pathogens, including Salmonella organisms.

We found that polymorphisms of SLC11A1/NRAMP1 were associated with tuberculosis caused by strains belonging to one particular lineage of M. tuberculosis, the Beijing genotype family. This genotype family is one of the most widespread evolutionary lineages of M. tuberculosis [5]. All M. tuberculosis lineages have expanded in the modern era, but the Beijing family has had by far the largest population increase [10]. This finding suggests that Beijing strains may have a selective advantage over other M. tuberculosis strains. Indeed, Beijing strains have shown increased in vitro outgrowth in human monocytes and macrophages [11] and enhanced virulence and distinctive histopathologic findings in animal models [12]. In human patients, Beijing genotype strains were associated with treatment failure and relapse [13] and with a higher chance of progression to active tuberculosis [14].

The association between the Beijing genotype and polymorphisms of SLC11A1/NRAMP1 suggests that these polymorphisms may increase susceptibility to infection or active tuberculosis caused by this particular genotype of M. tuberculosis. Little is known about the functionality of the various polymorphisms of SLC11A1/NRAMP1. However, it is very interesting that Beijing genotype strains were associated with the most common (and, therefore, evolutionarily the most successful) alleles of SLC11A1/NRAMP1. These selected genotypes might lead to differences in transcription or translation of the SLC11A1 gene or result in different variants of the SLC11A1 protein that is involved in the bactericidal properties of macrophages. One might hypothesize that the Beijing genotype strains are better equipped to resist these selected variants of the SLC11A1 gene or protein than are other M. tuberculosis strains. In vitro studies comparing the outgrowth of different M. tuberculosis strains in human macrophages with different SLC11A1/NRAMP1 genotypes might help to further explore this hypothesis. In addition to a direct effect from SLC11A1/NRAMP1, the genetic association that we have found might also indicate linkage to other immune genes in the vicinity of SLC11A1/NRAMP1, such as the interleukin-8 receptors IL-8RA and IL-8RB. However, SLC11A1/NRAMP1 seems to be a more likely candidate, because, so far, no study has reported a significant role for the IL-8R in promoting susceptibility to tuberculosis.

Globally, M. tuberculosis shows strong geographical differences. We have previously typed M. tuberculosis isolates from 897 patients originating from 2 different islands in Indonesia, Java, and Timor [4]. Interestingly, a difference was found in the population structure of M. tuberculosis. The Beijing genotype family was found in 33.0% of patients in Java versus 14.3% of patients in Timor. Inversely, in Timor, the EAI and LAM genotype families were predominant, whereas strains belonging to these genotypes were uncommon in Java. One could speculate about the explanation for these geographic differences. First, it may be the result of a “founder effect,” with an increased chance of finding a particular M. tuberculosis genotype family closer to where it originated. Second, the predominance of certain genotype families might be explained by an easier transmission or “escape” from bacille Calmette-Guérin vaccination. However, our previous study did not support the latter hypothesis [4]. Finally, as suggested by our current study, particular mycobacterial lineages may have adapted to specific properties of the immune system in particular human populations (ie, “genetic coevolution”). Similar to the geographical phylogenetic differences (“phylogeography”) noted for M. tuberculosis, the host immune genes also show geographical differences; we ourselves have shown a unique global distribution of 2 functional polymorphisms of Toll-like receptor 4 (TLR4), one of the key pattern recognition receptors for a variety of pathogens, including mycobacteria [15]. Similar to TLR4, SLC11A1/NRAMP1 has a strong geographical variation [6], which might account for (part of) the geographical variation of M. tuberculosis. Other host genes might be involved as well, and the Indonesian archipelago provides an excellent setting in
which to further test the concept of human-mycobacterial “co-evolution,” because both \textit{M. tuberculosis} and the human host inhabiting the many islands most likely show substantial genetic variation.

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**References**