

VARIATIONS IN THE *NRAMP1* GENE AND SUSCEPTIBILITY TO TUBERCULOSIS IN WEST AFRICANS

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ABSTRACT

Background Genetic factors may affect the susceptibility to tuberculosis, but no specific genes governing susceptibility have been identified. In mice, natural resistance to infection with some mycobacteria is influenced by the gene for natural-resistance-associated macrophage protein 1 (*Nramp1*), but the role of the human homologue of this gene, *NRAMP1*, in tuberculosis is unknown. We typed polymorphisms in *NRAMP1* in a case-control study of tuberculosis in the Gambia, West Africa.

Methods Sequence-specific oligonucleotide hybridization and microsatellite analysis were used to type *NRAMP1* polymorphisms in 410 adults (mean age, 34.7 years) with smear-positive pulmonary tuberculosis and 417 ethnically matched, healthy controls. Patients with human immunodeficiency virus infection were excluded.

Results Four *NRAMP1* polymorphisms were each significantly associated with tuberculosis. Subjects who were heterozygous for two *NRAMP1* polymorphisms in intron 4 and the 3' untranslated region of the gene were particularly overrepresented among those with tuberculosis, as compared with those with the most common *NRAMP1* genotype (odds ratio, 4.07; 95 percent confidence interval, 1.86 to 9.12; chi-square = 14.58; $P < 0.001$).

Conclusions Genetic variation in *NRAMP1* affects susceptibility to tuberculosis in West Africans. (N Engl J Med 1998;338:640-4.)

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AMONG inbred strains of mice, natural resistance to infection with several intracellular pathogens is controlled by a single dominant gene, designated *Bcg* (also known as *Lsh/Ity*).¹⁻³ Two distinct phenotypes, *Bcg*^s and *Bcg*^r, are associated with susceptibility and resistance, respectively, to the early stage of infection with *Mycobacterium bovis* (bacille Calmette-Guérin),³ *M. avium* complex,⁴ *M. lepraemurium*,⁵ *Leishmania donovani*,^{2,6} and *Salmonella typhimurium*.¹ A candidate gene was isolated by positional cloning and designated the natural-resistance-associated macrophage protein 1 gene (*Nramp1*).⁷ *Nramp1* is expressed only in reticuloendothelial cells.⁷ A single nonconservative amino acid substitution of aspartic acid for glycine at position 169 is correlated with the susceptibility phenotype (*Bcg*^s) in 27 inbred mouse strains.^{7,8} That *Nramp1* and *Bcg* are identical has been proved by the production of an *Nramp1*

knockout mouse that is phenotypically identical to the homozygous *Nramp1*^{D169} mouse⁹ and by the restoration of the resistance phenotype in transgenic mice in which the *Nramp1*^{G169} allele was transferred onto the background of the *Nramp1*^{D169} genotype.¹⁰ These findings conclusively demonstrated that *Nramp1* has an important role in determining resistance to mycobacteria and other intracellular pathogens in mice.

In the majority of humans, an effective immune response develops after infection with *M. tuberculosis* and restricts the spread of the pathogen. Less than 10 percent of infected persons ever have clinical disease, and only a minority of such persons have an identifiable risk factor, such as diabetes, advanced age, alcohol abuse, human immunodeficiency virus (HIV) infection, or use of corticosteroids. In the remainder, a complex interaction of genetic and environmental factors probably causes the development of disease. Racial variation in the susceptibility to tuberculosis^{11,12} and studies in twins^{13,14} strongly suggest that genetic factors are important in the susceptibility to tuberculosis.

The human homologue of the *Nramp1* gene, designated *NRAMP1*, is a strong candidate gene for human tuberculosis. *NRAMP1* has been cloned and mapped to human chromosome 2q35.^{15,16} Several polymorphisms have been described in the *NRAMP1* gene, and it has been suggested that they may influence the gene's function.¹⁶⁻¹⁹ To determine whether *NRAMP1* polymorphisms are associated with susceptibility to tuberculosis, we performed a case-control study comparing the frequency of several polymorphisms in the *NRAMP1* gene in West Africans with tuberculosis and ethnically matched healthy controls.

METHODS

Patients and Controls

Patients over 16 years old with smear-positive pulmonary tuberculosis were identified at three tuberculosis-leprosy clinics in the western region of the Gambia, in and around the capital, Banjul. Patients were included only after examination of sputum specimens by experienced microscopists had confirmed the presence of

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acid-fast bacilli. In the Gambia, the majority of persons in whom tuberculosis is diagnosed are adults who present with advanced, smear-positive pulmonary disease. The patients in this study had more severe disease than that generally seen in developed countries. A total of 410 patients (more than 90 percent of those who were eligible) provided oral informed consent. Their mean (\pm SD) age was 34.7 ± 13.2 years, and 67.4 percent were male.

Sequential unrelated blood donors from the Royal Victoria Hospital, Banjul, were recruited as controls. The transfusion center serves the geographic area from which the patients were recruited. A total of 417 blood donors (95 percent of those recruited) agreed to participate. All were male, and their mean age was 30.3 ± 7.5 years. The controls were retrospectively matched with the patients according to ethnic group, as far as the numbers allowed. The study was approved by the joint ethics committee of the Gambian government and the Medical Research Council.

The following ethnic groups were represented: Mandinka (accounting for 38.5 percent of the patients with tuberculosis and 37.4 percent of the controls), Wolof (19.1 and 18.4 percent), Jola (17.9 and 16.9 percent), Fula (12.0 and 12.2 percent), Manjago (2.5 and 3.3 percent), Serrahule (2.7 and 3.8 percent), and other, less common groups such as Aku and Serere (7.3 and 8.0 percent). These ethnic groups have previously been shown to be closely related genetically.²⁰ Persons known to have come from regions outside the Gambia were not included.

Patients known to be infected with HIV were not recruited, and 95 percent of the patients who agreed to participate were screened for HIV antibodies. HIV-positive patients were excluded. All controls were HIV-negative. The combined prevalence of HIV types 1 and 2 in the Gambia is relatively low, around 1.5 percent in the general population and less than 10 percent among patients with tuberculosis (unpublished data).

NRAMP1 Genotyping

The *NRAMP1* polymorphisms typed were a (CA)_n microsatellite in the immediate 5' region of the gene,¹⁷ denoted here 5'(CA)_n; a single nucleotide change in intron 4 (469+14G/C),¹⁸ denoted here INT4; a nonconservative single-base substitution at codon 543 that changes aspartic acid to asparagine (D543N)¹⁸; and a TGTG deletion in the 3' untranslated region (1729+55del4),¹⁸ denoted here 3'UTR. DNA was extracted from whole venous blood with the use of Nucleon II kits (Scotlab).

A region of approximately 200 bp surrounding the (CA)_n microsatellite was amplified by means of the polymerase chain reaction (PCR) with the use of the primers 5'ACTCGCATTAGGCCAACGAG3' and fluorescein-labeled 5'TTCTGTGCTCCCAAGTTAGC3' (developed by John Todd and coworkers, Oxford, United Kingdom). PCR products were genotyped by electrophoresis on 6 percent polyacrylamide gels, with the use of an ABI 373 sequencer and the computer software Genescan and Genotyper (Perkin-Elmer).²¹ The PCR primers for the INT4 polymorphism were 5'CTCTGGCTGAAGGCTCTCC3' and 5'TGTGCTATCAGTTGAGCCTC3'; the primers for D543N and 3'UTR were 5'GCATCTCCCAATTCATGGT3' and 5'AAGTGTCCACTCTATCCTG3'.¹⁸ The single-base substitutions and 4-bp deletion were detected by slot blotting PCR product to a nylon membrane, hybridization with digoxigenin-labeled sequence-specific oligonucleotides, and signal detection with an antidigoxigenin-antibody chemiluminescence system (Boehringer Mannheim). The sequence-specific oligonucleotides used were 5'TTGGGGGGCCCTGGAC3' and 5'TTGGGGGGCCCTGGAC3' for INT4 variants, 5'TTGAAGAGAACCAGAA3' and 5'TTGAAGAGGACCAGAA3' for D543N, and 5'CTGGATGTGGAGGGG3' and 5'TGCTGGAGAGGGGG3' for 3'UTR.¹⁵

Statistical Analysis

Statistical analysis was performed in a stepwise manner. Overall genotype frequencies were compared initially with the use of a three-by-two chi-square test with 2 df. If there was a significant overall difference between cases and controls ($P < 0.05$), individ-

ual genotypes were compared with the use of a two-by-two chi-square test with 1 df. To allow for any potential confounding effect of ethnic group, chi-square analysis was also performed with the use of the Mantel-Haenszel test and stratification according to ethnic group. The influence of linked variation on genotypic associations was assessed by logistic regression.

RESULTS

The *NRAMP1* allelic frequencies for the INT4, D543N, and 3'UTR variants differed significantly among the ethnic groups (Table 1). This finding highlights the importance of recording accurate data on ethnic origin when carrying out genetic case-control studies. The Gambian population is well suited to genetic studies because persons can be classified precisely with respect to membership in relatively closely related ethnic groups, which is rarely possible with Western populations. Mantel-Haenszel chi-square tests were performed after stratification according to ethnic group in order to correct for this potential confounding factor (Table 2).

The four polymorphisms, 5'(CA)_n, INT4, D543N, and 3'UTR, were each significantly associated with tuberculosis ($P = 0.03$, $P = 0.009$, $P = 0.008$, and $P < 0.001$, respectively). The 5'(CA)_n microsatellite and INT4 variant were in significant linkage disequilibrium, with the 5'(CA)_n 201-bp allele strongly associated with the INT4 C allele ($P < 0.001$), and these results are therefore not independent. In addition, the D543N A allele was always associated with the 3'UTR del allele ($P < 0.001$), and these results are therefore also not independent of each other. The

TABLE 1. NRAMP1 ALLELIC FREQUENCIES AMONG 827 GAMBIAIS, ACCORDING TO ETHNIC GROUP.

ETHNIC GROUP	NO. OF SUBJECTS	ALLELIC FREQUENCY*			
		5'(CA) _n	INT4	D543N	3'UTR
		percent			
Fula	100	79	87	88	73
Manjago	24	84	94	92	83
Jola	144	84	90	91	78
Mandinka	314	87	93	96	85
Serrahule	27	83	98	96	78
Wolof	155	82	90	93	82
Other	63	84	93	94	85
Chi-square†		9.6	13.6	17.44	20.3
P value†		0.14	0.03	0.008	0.002

*The allelic frequencies shown are for the most common allele and are based on the number of persons typed. 5'(CA)_n is a microsatellite in the 5' region of the gene. INT4 is a single nucleotide transversion in intron 4. D543N is a nonconservative single-base substitution at codon 543. 3'UTR is a TGTG deletion in the 3' untranslated region.

†The chi-square and P values are for the differences among groups.

TABLE 2. RELATION BETWEEN *NRAMP1* POLYMORPHISMS AND TUBERCULOSIS IN THE GAMBIA.

POLYMORPHISM	PATIENTS WITH TUBERCULOSIS (N=410)	CONTROLS (N=417)	ODDS RATIO (95% CI)*	CHI-SQUARE TEST†		P VALUE	
				UNADJUSTED	ADJUSTED	UNADJUSTED	ADJUSTED
no. of subjects							
5'(CA) _n							
199/199	263	303	1.0				
199/other	125	99	1.45 (1.05–2.01)	6.94	6.96	0.03	0.008
Other/other	13	8	1.87 (0.71–5.02)				
INT4							
G/G	320	360	1.0				
G/C	78	48	1.83 (1.22–2.75)	9.37	7.69	0.009	0.006
C/C	3	3	1.13 (0.18–7.01)				
D543N							
G/G	337	375	1.0				
G/A	68	41	1.85 (1.20–2.85)	9.54	8.18	0.008	0.004
A/A	0	1	0.00 (0–19.35)				
3'UTR‡							
TGIG+/+	244	301	1.0				
TGIG+/del	150	100	1.85 (1.35–2.54)	16.72	8.32	<0.001	0.004
TGTGdel/del	11	16	0.85 (0.36–1.97)				

*Odds ratios are for the comparison with the more common homozygous genotype for each polymorphism. CI denotes confidence interval.

†The chi-square test with 2 df was performed for the comparison of overall genotypic frequencies. The adjusted values were obtained with the use of the Mantel–Haenszel test after stratification according to ethnic group.

‡The plus sign denotes the presence of TGIG, and *del* the absence of these four bases.

3'UTR *del*/D543N G haplotype was found in this population, whereas it is absent in Europeans and Asians¹⁸ (and unpublished data). The INT4 and 3'UTR alleles were not significantly associated with each other (chi-square=0.83, P=0.93), and both were independently and significantly associated with tuberculosis (on logistic-regression analysis, P=0.006 for INT4 and P=0.004 for 3'UTR; after stratification for the presence or absence of INT4, Mantel–Haenszel chi-square=7.73 with 1 df for 3'UTR, P=0.005; and after stratification for the presence or absence of 3'UTR, Mantel–Haenszel chi-square=7.708 with 1 df for INT4, P=0.006). There were small differences in the total number of persons in whom typing was performed for each polymorphism, since very limited quantities of DNA were available from a few persons.

Two additional polymorphisms in the *NRAMP1* gene were typed: a C-to-T single-base transition at a position 236 bp before the transcription starting site¹⁹ and a 9-bp coding deletion within exon 2.¹⁷ The 9-bp deletion was very uncommon (allelic frequency, <1 percent), and the –236C/T polymorphism was not associated with tuberculosis (chi-square=2.82, P=0.25; data not shown).

Combined analysis of the INT4 and 3'UTR polymorphisms showed a strong association with tuberculosis (chi-square=26.41 with 3 df, P<0.001) (Table 3). As compared with GG/++ homozygotes,

heterozygotes for the INT4 C allele or the 3'UTR *del* allele were overrepresented among the patients with tuberculosis (odds ratio for INT4 C, 1.79; 95 percent confidence interval, 1.07 to 3.00; odds ratio for 3'UTR, 1.85; 95 percent confidence interval, 1.30 to 2.62). This finding was consistent among all the ethnic groups studied (six of the seven ethnic groups had high numbers of INT4 heterozygotes and all seven had high numbers of 3'UTR heterozygotes among the patients with tuberculosis). Heterozygosity for both these variants was associated with the highest risk of tuberculosis (odds ratio, 4.07; 95 percent confidence interval, 1.86 to 9.12).

DISCUSSION

A mutation in *Nramp1* results in susceptibility to several mycobacterial species among inbred mouse strains.^{7–10} Our study showed that variation in the human homologue *NRAMP1* is associated with altered susceptibility to smear-positive tuberculosis. It is likely that the *NRAMP1* gene governs susceptibility to tuberculosis. Although it is possible that the association described here is due to linkage disequilibrium between variation in *NRAMP1* and another nearby susceptibility gene, this explanation is unlikely, because *NRAMP1* is a strong candidate gene for tuberculosis on the basis of studies of its murine homologue.

The study design we used does not distinguish

TABLE 3. COMBINED ANALYSIS OF *NRAMP1* INT4 AND 3'UTR VARIANTS.*

INT4/ 3'UTR GENOTYPE	PATIENTS WITH TUBER- CULOSIS		ODDS RATIO (95% CI)	CHI- SQUARE TEST	P VALUE
	no. of subjects	CONTROLS			
GG/++	191	251	1.0		
GC/++	45	33	1.79 (1.07–3.00)	5.04	0.02
GG/+del	118	84	1.85 (1.30–2.62)	12.24	<0.001
GC/+del	31	10	4.07 (1.86–9.12)	14.58	<0.001

*Odds ratios and chi-square values (with Yates' correction and 1 df) are for comparisons with the GG/++ genotype. CI denotes confidence interval. The overall comparison of combined genotypes showed a strong association with tuberculosis (chi-square=26.41 with 3 df, P<0.001).

between susceptibility to infection with *M. tuberculosis* and susceptibility to disease progression. However, most healthy Gambians in the age range we studied have been infected with *M. tuberculosis*. Since only approximately 1 in 10 persons infected with *M. tuberculosis* will ever have clinical disease,²² patients with tuberculosis have greater susceptibility to this pathogen than the general population. Human *NRAMP1* is therefore probably important in the development of overt disease, in contrast to murine *Nramp1*, which determines the ability to control bacille Calmette–Guérin in the early stages of infection. There may be differences between species in the functional consequences of *Nramp1* variation, because human and murine macrophages exhibit marked differences in their ability to inhibit the growth of virulent mycobacteria and in the mechanisms they use.²³ It is unclear whether the INT4 and 3'UTR variants affect *NRAMP1* function or whether they are in linkage disequilibrium with another functional polymorphism that has not yet been described. However, allelic variation in the 5'(CA)_n repeat may be of functional importance.²⁴

The 3'UTR variant allele associated with susceptibility to tuberculosis is very uncommon in Europeans¹⁸ but was present in about a quarter of this West African population. These findings may in part explain why American blacks have greater susceptibility to tuberculosis than whites.^{11,12} *NRAMP1* polymorphism will need to be assessed in large case-control studies of whites and Asians to determine whether the gene also governs susceptibility to tuberculosis in other racial groups.

The functions of *Nramp1* in mice and *NRAMP1* in humans remain unknown. Mice homozygous for the *Nramp1* susceptibility allele have increased susceptibility to several antigenically unrelated intracel-

lular pathogens in vivo, and macrophages from such mice have an impaired capacity to restrict the growth of mycobacteria,^{2,25,26} salmonella,²⁷ and leishmania²⁸ in vitro. Thus, studies of the *NRAMP1* susceptibility genotype identified here will be of interest in determining susceptibility to leprosy, typhoid fever, and leishmaniasis. *Nramp1* protein is localized to the late endocytic compartment of resting macrophages and, after phagocytosis, is recruited to the membrane of the phagosome.²⁹ These findings suggest that *Nramp1* may restrict the replication of intracellular pathogens by altering the phagolysosomal environment.³⁰ The related but unlinked gene *Nramp2* has been shown to encode an iron transporter.^{31,32} The yeast *Nramp1* homologue *SMF1* is important for the regulation of manganese-ion uptake, and it has been suggested that *Nramp1* may therefore function as a regulator of intraphagosomal manganese,³⁰ iron, or other divalent cations.²⁹

In the Gambia, heterozygotes for the INT4 and 3'UTR variants are at increased risk for tuberculosis, whereas among mice, only homozygotes for the *Nramp1*^{D169} variant are susceptible to intracellular pathogens. The small number of homozygotes in our study precludes a definitive analysis of their susceptibility to tuberculosis, but they do not appear to be more susceptible than heterozygotes. These findings suggest that with the human *NRAMP1* variants, the tuberculosis-susceptibility allele is dominant, whereas with the murine *Nramp1*^{D169} variant, the resistance allele is dominant. However, it is possible that incomplete linkage disequilibrium between the typed polymorphisms and an unknown mutation affecting function has obscured the true dominance pattern of the resistance and susceptibility genotypes. Alternatively, the heterozygous genotype of *NRAMP1* may lead to a divalent cation concentration in the human phagolysosome, which is more conducive to mycobacterial growth than either of the homozygous genotypes. Finally, it is also possible that *NRAMP1*-variant homozygotes who are infected with *M. tuberculosis* in childhood succumb to the disease rapidly and are therefore not overrepresented among adults with tuberculosis.

The association of *NRAMP1* variation with a major infectious disease provides support for the strategy of mapping and identifying genes for resistance to infectious disease in mice and then testing their homologues as candidate genes for susceptibility to related infections in humans. Further analysis of the mechanism of action of *NRAMP1* and its genetic variants may lead to new approaches to controlling tuberculosis, which kills more people than any other disease caused by an infectious pathogen.³³

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