ORIGINAL ARTICLE

Association of *NRAMP 1* Gene Polymorphism with Susceptibility to Tuberculosis in Taiwanese Aboriginals

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Background/Purpose: The human homologue of mice natural-resistance-associated macrophage protein 1 (*Nramp 1*) gene, *NRAMP 1*, has been reported to play a role in susceptibility to tuberculosis in humans. The aboriginal population in Taiwan has a five-fold higher prevalence of tuberculosis than people of Han ethnicity. Whether genetic factors such as *NRAMP 1* polymorphism play a role in the prevalence of tuberculosis in Taiwanese aboriginals should be clarified.

Methods: *NRAMP 1* polymorphism was studied using a case-control design of patients with tuberculosis, including subjects of Han (Hans) and aboriginal ethnicity in Hualien, eastern Taiwan. The polymorphisms of *NRAMP 1* at loci INT4, D543N, 77-385C/T, 3-UTR (CAAA) deletion and 5-(CA)n microsatellite markers were assessed by polymerase chain reaction on tissue DNA isolated from 105 aborigines and 110 Hans with tuberculosis. Comparable numbers of ethnically-matched controls were studied simultaneously.

Results: Two *NRAMP 1* polymorphisms, INT4 and 5-(CA)n, were significantly associated with susceptibility to tuberculosis in aboriginals ($p = 0.0070$ and $p = 0.0031$, respectively). However, no association was detected at the five loci of *NRAMP 1* polymorphisms among Hans ($p > 0.08$).

Conclusion: Genetic variation in *NRAMP 1* may affect susceptibility to and increase risk for tuberculosis in Taiwanese aboriginals. Although environmental factors play an important role in tuberculosis infection, genetic factors such as *NRAMP 1* polymorphism may also contribute to the high prevalence of tuberculosis in Taiwanese aboriginals. [*J Formos Med Assoc* 2006;105(5):363–369]

Key Words: Hans, *NRAMP 1* gene, polymorphism, Taiwanese aboriginals, tuberculosis

Tuberculosis, an important infectious disease, has recently re-emerged as a major public health threat worldwide.[1] Given that only 10% of individuals infected with *Mycobacterium tuberculosis* develop the clinical disease,[2,3] host factors such as genetics may play a role in determining the outcome of *M. tuberculosis* infection.[2,4,5] In mice, the natural resistance to infection with some mycobacteria is influenced by the gene for natural-resistance-associated macrophage protein 1 (*Nramp 1*).[6] The human homologue of the *Nramp 1* gene, designated *NRAMP 1*, has been cloned and mapped to chromosome 2q35.[4] Although still controversial, several studies have indicated that the *NRAMP 1* gene is associated with susceptibility to tuberculosis in different ethnic groups.[3,7–12]

Tuberculosis remains a major infectious disease in Taiwan, especially in the aboriginal populations. According to data from the Center for Disease Control in Taiwan, the aborigines have a five-

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fold higher prevalence of tuberculosis than people of Han ethnicity (Hans).[13] Environmental factors such as poor hygiene, income, and social behavior such as alcoholism have been claimed to be responsible for the prevalence of tuberculosis in aboriginal populations. Since genetic factors have been implicated in the high occurrence of gout and alcohol tolerance in the aboriginal populations in Taiwan,[14] the significance of *NRAMP 1* gene polymorphism to the high susceptibility of Taiwanese aboriginals to tuberculosis should be clarified.

We compared the distribution of five *NRAMP 1* gene polymorphisms in a population-based design study of tuberculosis among individuals of Han and aboriginal ethnicity in Hualien, eastern Taiwan.

Methods

Samples and controls

Formalin-fixed, paraffin-embedded tissue blocks from 215 patients with clinically suspected tuberculosis treated during the period 1990–2004 were obtained from the archives of the Surgical Pathology Laboratory of National Cheng Kung University Hospital in Tainan and the Buddhist Tzu Chi General Hospital in Hualien. The patients and control subjects from Hualien were mainly of Minnan ethnicity, and the Taiwanese aboriginals were mainly from the Atayal and Ami tribes. Of the patients, 110 were of Han and 105 were of aboriginal ethnicity. The diagnosis of tuberculosis was confirmed histopathologically by the identification of caseating granulomatous inflammation and the demonstration of acid-fast bacilli by two experienced pathologists. Controls were local volunteer blood donors who had no evidence of pulmonary tuberculosis at the time of participation, and who were matched to cases based on ethnic or geographic origin. Of the control subjects, 92 were of Han and 95 were of aboriginal ethnicity. All patients and control subjects gave informed consent for participation. This study was approved by the institutional ethics review board of the National Health Research Institutes.

DNA extraction

Tissue DNA was extracted from each block by standard proteinase K digestion followed by phenol-chloroform extraction, and then precipitated with isopropanol and ammonium acetate.[15] To prevent carry-over tissue from contaminating subsequent samples, the microtome blade was cleaned with octane and 100% ethanol after sectioning each sample. DNA from controls was prepared from peripheral blood by using the QIAamp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommended protocol.

NRAMP 1 genotyping

Single nucleotide polymorphisms

The INT4, D543N and 77-385C/T single nucleotide polymorphisms (SNPs) of the *NRAMP 1* gene were genotyped using the approach provided by the Assay-by-Design Service (Applied Biosystems, Foster City, CA, USA). The method was used because its reported accuracy (> 99%) and concordance (between 99.7% and 99.8%) in SNP genotyping are extremely high.[16,17] Two primers and two probes for the amplification fragments and alternative alleles were labeled with different fluorescent markers, and amplified together with one common antisense primer, and the different fluorescent colors indicating the presence of the alternative alleles. The primers and probe sequences are listed in Table 1.[3,4,18] Polymerase chain reaction (PCR) for Assay-by-Design (Applied Biosystems) was performed in a volume of 20 μL containing 50 ng of genomic DNA, 2X TaqMan Universal PCR Master Mix, No AmpErase UNG solution and 1X assay mixture. The PCR program was: 2 minutes at 50° C and 10 minutes at 95° C, followed by 40 cycles of 95° C for 15 seconds and 60° C for 1 minute. Amplification and fluorescence detection were performed using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems).

Microsatellite and 3-UTR polymorphisms typing

The 5-(CA)n microsatellite in the promoter region of the *NRAMP 1* gene was amplified using the FAM-labeled forward primer 5′-ACTCGCATTAG-

GCCAACGAG-3′ and reverse primer 5′-TTCTGT-GCCTCCCAAGTTAGC-3′. Three alleles of 202 bp, 200 bp and 198 bp were observed in the studied populations. The 3-UTR polymorphism of the *NRAMP 1* gene was amplified with the TET-labeled forward primer 5′-CTTTAACACAGTGTCTGGCAC-3′ and reverse primer 5′-TCAAGCTCCAGTTT-GGAGCTT-3′ that yields a product of 157 bp and 161 bp.[19] The PCR cycle conditions were: 10 minutes at 95° C, followed by 30 cycles of 94° C for 30–90 seconds, 55–60° C for 15–90 seconds, 72° C for 30–60 seconds, and a final extension at 72° C for 10 minutes, using 0.2 μM of each primer, 50 ng of genomic DNA, 200 μM dNTPs and 0.5 U *Taq*Gold DNA polymerase (Applied Biosystems) in a 10 μL reaction mixture. Amplification was performed in a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems). Fragment size analysis was carried out using the ABI 310 Genetic Analyzer (Applied Biosystems).

Table 1. Primer and probe sequences used in Assay-by-Design Service single nucleotide polymorphism genotyping

Name	Primers and probes	References
D543N	F: 5'-CCA CCA CCA CTT CCT GTA TGG-3' R: 5'-AGC CAG AGG TCT CCC CTT T-3' VIC-5'-TCC TTG AAG AG**G** ACC AG-3' FAM-5'-CCT TGA AGA G**A**A CCA G-3'	3, 4
77-385C/T	F: 5'-ACG TGT GTG TGT GTG TGT GT-3' R: 5'-CCT CCC AAG TTA GCT CTG ATT TCA G-3' VIC-5'-CCC TTG C**G**T ATT CAT-3' FAM-5'-CCC TTG C**A**T ATT CAT-3'	4, 18
INT4	F: 5'-CTG CCA TCT CTA CTA CCC TAA GGT-3' R: 5'-GGA AGC TGA AAA TGG CTG TTT GG-3' VIC-5'-TCC AGG **C**CC CCC AAG-3' FAM-5'-CCA GG**G** CCC CCA AG-3'	3, 4

F = forward primer; R = reverse primer; VIC and FAM indicate the different fluorescent labels in the probes. The nucleotide substitution is indicated in bold and underlined.

Statistical analysis

All markers were analyzed for Hardy-Weinberg equilibrium in the two control groups. Differences in genotype and allele distributions between patients and controls were analyzed using Fisher's exact test and chi-square test using SAS version 8 software (SAS Institute Inc, Cary, NC, USA). Statistical significance was indicated by $p < 0.05$, and the significance level was further adjusted by the Bonferroni correction to $p < 0.01$ (i.e. five loci).

Results

The five previously published *NRAMP 1* polymorphisms – INT4, 5-(CA)n, 3-UTR, 77-385C/T and D543N – were investigated in 215 patients with tuberculosis (105 aboriginals and 110 Hans). Ethnically-matched Han (92) and aboriginal controls (95) from the Hualien area were used as controls. The demographic data of tuberculosis patients and control subjects are shown in Table 2.

As summarized in Table 3, the analyses of five genotypes of the *NRAMP 1* polymorphism revealed that INT4 ($p = 0.007$) and 5-(CA)n ($p = 0.0031$) were present in significantly different percentages of aboriginal tuberculosis patients and controls. Furthermore, 5-(CA)n polymorphism also showed significant differences ($p = 0.0041$) in allele distributions between tuberculosis patients and controls (Table 3). Regarding the 5-(CA)n microsatellite, the homozygous genotype for the 198/198 variant was seen in 81.2% of patients and 95.5% of controls, whereas the corresponding figures for the heterozygous genotype of 198/200 were 14.9% and 1.1%, respectively. In addition, the frequencies of INT4 homozygous G/G were

Table 2. Demographic data for 215 tuberculosis (TB) patients and 187 control subjects among aboriginals and Hans in Taiwan

Demographic variable	Aboriginals		Hans	
	Control (n = 95)	TB (n = 105)	Control (n = 92)	TB (n = 110)
Age at diagnosis (yr), mean ± SD	45.9 ± 13.7	50.1 ± 22.9	48.3 ± 13.1	35.1 ± 22.2
Ratio of women to men	1.40	0.95	1.33	0.81

Table 3. Frequencies of different *NRAMP 1* alleles between Taiwanese aboriginal patients with tuberculosis (TB) and ethnically-matched control subjects

NRAMP 1 locus	Aboriginal ethnicity		*p*
	TB patients	Control subjects	
3UTR-(CAAA)n, insertion/deletion			
Polymorphism, *n* (%) of subjects	85	95	
157/157	50 (58.8)	63 (66.3)	0.4674
157/161	29 (34.1)	24 (25.3)	
161/161	6 (7.1)	8 (8.4)	
Allelic frequency			
157	129 (0.76)	150 (0.79)	0.5283
161	41 (0.24)	40 (0.21)	
Microsatellite 5-(CA)n			
Polymorphism, *n* (%) of subjects	101	88	
198/198	82 (81.2)	84 (95.5)	
198/200	15 (14.9)	1 (1.1)	0.0031*
198/202	4 (3.9)	3 (3.4)	
Allelic frequency			
198	183 (0.91)	172 (0.98)	
200	15 (0.07)	1 (0.005)	0.0041*
202	4 (0.02)	3 (0.017)	
D543N missense mutation			
Polymorphism, *n* (%) of subjects	88	90	
AAC(Asn) G/G	62 (70.5)	63 (70.0)	1.0000
GAC(Asp) G/A	21 (23.9)	22 (24.4)	
A/A	5 (5.7)	5 (5.6)	
Allelic frequency			
G	145 (0.82)	158 (0.82)	0.8902
A	31 (0.18)	32 (0.18)	
77-385C/T			
Polymorphism, *n* (%) of subjects	88	93	
C/C	85 (96.6)	87 (93.5)	0.4982
C/T	3 (3.4)	6 (6.5)	
T/T	0 (0)	0 (0)	
Allelic frequency			
C	173 (0.98)	180 (0.97)	0.5038
T	3 (0.02)	6 (0.03)	
INT4			
Polymorphism, *n* (%) of subjects	105	92	
G/G	92 (87.6)	91 (97.8)	0.0070*
G/C	13 (12.4)	2 (2.2)	
C/C	0 (0)	0 (0)	
Allelic frequency			
G	197 (0.94)	184 (0.99)	0.0308
C	13 (0.06)	2 (0.01)	

**Significant differences in distribution between patients and control subjects after Bonferroni correction.*

87.6% and 97.8%, and of heterozygous G/C were 12.4% and 2.2% among patients and controls, respectively; no individuals carried the homozygous C/C genotype. Statistical analyses of the variants of 3-UTR, D543N and 77-385C/T loci did not reveal any significant differences between patients and controls. In the Han population, however, the frequencies at all five polymorphic loci did not differ significantly between patients and controls (Table 4). All of the control subjects fitted Hardy-Weinberg equilibrium criteria.

Discussion

This study demonstrated a positive association of *NRAMP 1* gene polymorphism with susceptibility to tuberculosis in Taiwanese aboriginals but not in Taiwanese Hans. These data may help to explain the high prevalence of tuberculosis among Taiwanese aboriginals. Genetic linkage has been demonstrated to play a role in ethnic susceptibility to other diseases such as gout and alcoholism in Taiwanese aboriginals.[14]

Variants of the *NRAMP 1* gene have been associated with susceptibility to clinical tuberculosis.[1,10,20] However, conflicting data have been reported from different population groups. Bellamy et al reported that *NRAMP 1* polymorphisms were convincingly associated with susceptibility to tuberculosis in West Africans.[3] However, subsequent studies from Korea, Japan and Denmark revealed no association of *NRAMP 1* polymorphism with the development of tuberculosis.[10,21,22] This study found that *NRAMP 1* polymorphism was not associated with susceptibility to the development of tuberculosis in the ethnic Han population of Taiwan, who migrated from mainland China decades or centuries ago.[23] Liaw et al also reported no significant differences in *NRAMP 1* gene polymorphism between tuberculosis patients and controls in Taiwan.[11] However, their study did not stratify cases into aboriginal and Han ethnicity. Therefore, the association of *NRAMP 1* gene polymorphisms with susceptibility to tuberculosis may depend on ethnicity. Although no significant association of *NRAMP 1* polymorphism with susceptibility to tuberculosis was found in Taiwanese Hans in this study, a recent study from China reported that variants of *NRAMP 1* were significantly associated with the severity of, rather than the susceptibility to, pulmonary tuberculosis in the Han Chinese population.[20] No data on the clinical severity of our cases with the *NRAMP 1* gene polymorphism were available to examine this correlation in the present study.

The protein encoded by the *NRAMP 1* gene plays an important role in the phagolysosomal function of pulmonary macrophages as well as antigen presentation.[8,24] The NRAMP 1 protein becomes activated and fused with lysosomes to digest the engulfed mycobacteria. In a mouse model, mutation or knockout of the *NRAMP 1* gene makes hosts susceptible to tuberculosis infection.[6,25,26] In a preliminary study, we demonstrated a weak or absent expression of *NRAMP 1* gene in the macrophages or Langerhans giant cells in the granulomatous lesions of aboriginal compared with Han subjects (data not shown). If such an observation can be demonstrated in future experiments, it may provide an explanation for the increased susceptibility to tuberculosis infection in patients with *NRAMP 1* gene polymorphism.

The genetic susceptibility of Taiwanese aboriginals to tuberculosis may imply the need for intensive public health measures to control tuberculosis infection in these populations, in addition to improvements in environmental and socioeconomic conditions.

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Table 4. Frequencies of different *NRAMP 1* alleles between Hans with tuberculosis (TB) and ethnically-matched control subjects

NRAMP 1 locus	Han ethnicity		*p*
	TB patients	Control subjects	
3UTR-(CAAA)n, insertion/deletion			
Polymorphism, *n* (%) of subjects	85	79	
157/157	44 (51.7)	46 (58.2)	0.4313
157/161	38 (44.7)	28 (35.4)	
161/161	3 (3.5)	5 (6.3)	
Allelic frequency			
157	126 (0.74)	120 (0.76)	0.8289
161	44 (0.26)	38 (0.24)	
Microsatellite 5-(CA)n			
Polymorphism, *n* (%) of subjects	110	78	
198/198	82 (74.6)	60 (76.9)	0.9100
198/200	22 (20.0)	16 (20.5)	
198/202	4 (3.6)	2 (2.6)	
200/200	2 (1.8)	0 (0)	
Allelic frequency			
198	190 (0.86)	138 (0.88)	0.8147
200	26 (0.12)	16 (0.1)	
202	4 (0.02)	2 (0.01)	
D543N missense mutation			
Polymorphism, *n* (%) of subjects	83	86	
AAC(Asn) G/G	56 (67.5)	64 (74.5)	0.5518
GAC(Asp) G/A	25 (30.1)	19 (22.1)	
A/A	2 (2.4)	3 (3.5)	
Allelic frequency			
G	137 (0.84)	147 (0.85)	0.5529
A	29 (0.16)	25 (0.15)	
77-385C/T			
Polymorphism, *n* (%) of subjects	83	85	
C/C	75 (90.4)	68 (80.0)	0.0820
C/T	8 (9.6)	17 (20.0)	
T/T	0 (0)	0 (0)	
Allelic frequency			
C	158 (0.95)	153 (0.9)	0.0952
T	8 (0.05)	17 (0.1)	
INT4			
Polymorphism, *n* (%) of subjects	108	92	
G/G	85 (78.7)	73 (79.3)	0.2626
G/C	20 (18.5)	19 (20.7)	
C/C	3 (2.8)	0 (0)	
Allelic frequency			
G	190 (0.88)	165 (0.9)	0.6361
C	26 (0.12)	19 (0.1)	

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