Ethnic-Specific Genetic Associations with Pulmonary Tuberculosis

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Several susceptibility-associated genetic polymorphisms have been proposed to explain differential susceptibility to tuberculosis (TB) disease progression in different populations. Here, polymorphisms in the natural resistance–associated macrophage protein 1 (NRAMP1), vitamin D receptor, tumor necrosis factor–α, interleukin (IL)–1, and IL-10 genes were evaluated in 358 Cambodian patients with pulmonary TB and 106 tuberculin-positive control subjects. Heterozygosity for the −1082 polymorphism of the IL-10 promoter and heterozygosity for 2 linked polymorphic NRAMP1 variants, D543N and 3′ untranslated region, were associated with TB susceptibility and resistance, respectively. Other polymorphisms associated with differential susceptibility to TB were not associated with susceptibility or resistance to TB in Cambodians. The novel pattern of genetic associations with susceptibility and resistance to TB detected in Cambodia is consistent with the conclusion that unique environmental and natural selective factors have resulted in the development of ethnic-specific host genetic factors associated with TB susceptibility and resistance worldwide.

Approximately one-third of the world's population is infected with the bacteria that causes tuberculosis (TB), *Mycobacterium tuberculosis*. One in 10 of those infected are estimated to progress to active TB disease. Thus, TB is a significant cause of morbidity and mortality and causes ~2 million deaths annually [1–3]. As early as 1949, Haldane [4] proposed that the maintenance of multiple genes that confer relative susceptibilities on the host to infectious diseases would be favored by evolution. In support of this hypothesis, certain populations appear to be at risk for both increased susceptibility to infection [5] and progressive clinical disease due to mycobacteria [6–9], and several case-control studies have identified associations between TB disease and candidate genes potentially involved in the immune response to TB [10, 11].

Specifically, several polymorphic-derived deletions and point mutations of the human homologue of the murine natural resistance–associated macrophage protein 1 (NRAMP1) gene [12–16], the vitamin D receptor (VDR) gene [17, 18], the interleukin (IL)–1 gene cluster [19, 20], and the IL-10 [20] and tumor necrosis factor (TNF)–α [21] genes have been associated with susceptibility or resistance to TB in different ethnic groups, although none of these genetic associations has been shown to have any relevant functional effect on the containment of *M. tuberculosis* by the host immune system.

To investigate whether such genetic associations were independent of ethnicity or are ethnic-specific markers of TB disease patterns, we examined previously identified polymorphisms of candidate genes, including NRAMP1, VDR, IL-10, TNF-α, and IL-1, in a single, well-characterized, highly TB-burdened population from Cambodia. We report a unique pattern of association of candidate gene polymorphisms and TB susceptibility and resistance that is consistent with the hypothesis that population-specific environmental factors have resulted in the development of distinct immunogenetic factors involved in effective immune responses to *M. tuberculosis*.

Patients and Methods

Study site and subjects. The study patients were Cambodian individuals >14 years old, randomly recruited since 1995 from the Cambodian Health Committee (CHC) TB treatment program in southeastern rural Cambodia. The diagnosis of clinical pulmonary TB was made on the basis of whether acid-fast bacilli (AFB) were present in sputum, as assessed by experienced microscopists, and according to medical history and physical examination. One of us (S.T.) conducted family interviews of the household members of the patients and verified that no patients in the study were related. Patients were all tested for evidence of antibodies to human immunodeficiency virus (HIV) types 1 and 2 (Sanofi Diagnostic Pasteur), and patients found to be infected with HIV-1 or HIV-2 were

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Informed written consent was obtained from all patients and control subjects. The study was approved by the Center for Blood Research Institutional Review Board.

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not included in the study. A total of 368 patients with TB were recruited. Of these, 6 tested positive for HIV-1 infection, and 4 patients were diagnosed with extrapulmonary TB and excluded. Thus, 358 HIV-negative patients with pulmonary TB were included in the study. Their mean (±SD) age was 42.2 ± 14.1 years; 62.7% were women.

Control subjects were recruited in 1995 from tuberculin-positive unrelated patients visiting the same hospitals for minor complaints. On the basis of a detailed clinical history, control subjects did not have a history of TB or current signs or symptoms consistent with TB. For tuberculin screening, 0.1 mL (5 tuberculin units) of purified protein derivative (PPD; Tubersol; Connaught Laboratories) was injected intradermally in the forearm and was evaluated for induration 48 h later using the ballpoint method [22]. Tuberculin readings were performed by trained and experienced members of the CHC staff and under the supervision of an infectious disease specialist (A.E.G.). Subjects were classified as tuberculin positive if they had an induration >10 mm. A total of 116 non–bacille Calmette-Guérin (BCG)–vaccinated, tuberculin-positive control subjects were monitored for at least 7 years, to confirm their disease status. Ten control subjects who developed clinical TB disease over the 7-year follow-up period (confirmed by the presence of AFB in sputum) were excluded. The mean age of the remaining 106 control subjects studied was 37.5 ± 12.9 years; 56.1% were women.

Candidate gene polymorphism typing. After written consent was obtained from patients and control subjects, peripheral blood was drawn, peripheral blood mononuclear cells (PBMC) were separated according to standard techniques, and genomic DNA was prepared from PBMC by a quick isolation method [23]. In total, 19 polymorphic variants of non-HLA genes were typed in the study. Four NRAMP1 polymorphisms, including a single-nucleotide change in intron 4 (469+14G/C; denoted here as "INT4"), a nonconservative single-base substitution at codon 543 of exon 15 (D543N), a TGTG deletion in the 3′ untranslated region (1729+55del4; denoted here as "3′ UTR"), and a C→T single-base transition at 236 nt upstream of the transcription start site (−236C/T), were typed by polymerase chain reaction (PCR) restriction fragment–length polymorphism (RFLP), as described elsewhere [24]. Two polymorphisms of the VDR gene defined by the restriction endonucleases Fok1 and Taq1 were typed by PCR-RFLP. [18] Three polymorphisms of the IL-1 gene complex, including a variable number of tandem repeats of 86 bp in intron 2 of the IL-1 receptor antagonist gene and 2 single-nucleotide polymorphisms (SNPs) at positions −511 and +3953 relative to the transcriptional promoter start site of the IL-1β gene were typed by PCR–sequence-specific primers (SSP). [19] Three single-nucleotide substitutions of the IL-10 gene, located at positions −1082, −819, and −592 relative to the transcription start site, were typed by PCR-SSP. [25] Finally, 7 SNPs of the TNF-α promoter region, located at positions −1030, −862, −856, −375, −307, −243, and −237 relative to the transcription start site, were typed by PCR-SSP. Specifically, a region of 1.3 kb surrounding the TNF-α promoter region was amplified using the primers TNF-L 5′-AGT GAG AAC TTC CCA GTC TAT CTA AG-3′ and TNF-R 5′-CCG TGG GTC AGT ATG TGA GA-3′. The SNPs were then detected by dot-blotting the PCR product to a nylon membrane, hybridization with digoxenin-labeled sequence-specific oligonucleotides, and signal detection with an antidigoxigenin-antibody chemiluminescence system (Boehringer Mannheim). The oligonucleotides used were 5′-CTT TTC CTT C*A*T CTT CTC AGC-3′ and 5′-CTT TTC CTT C*G*T CTT CTC AGC-3′ for the −1030 SNP, 5′-CTT CGT TAA G*G*G GGG GTC CCC-3′ and 5′-CTT CGT TAA G*T*G GGG GTC CCC-3′ for the −862 SNP, 5′-CCC TGT CTT C*G*T TAA GGG GGG-3′ and 5′-CCC TGT CTT C*A*T TAA GGG GGG-3′ for the −856 SNP, 5′-TTC CTT CTA A*C*T TCC AGA CAG-3′ and 5′-TTC CTT CTA A*T*T TCC AGA CAG-3′ for the −375 SNP, 5′-AAC CCC GTC CCC ATG C*C*C CTC-3′ and 5′-AAC CCC GTC C*T*C ATG CCC CTC-3′ for the −307 SNP, 5′-GCT CCG ATT C*C*G AGG GGG GTC-3′ and 5′-GCT CCG ATT C*T*G AGG GGG GTC-3′ for the −243 SNP, and 5′-CTC CCT GCT C*C*G ATT CCG AGG-3′ and 5′-CTC CCT GCT C*T*G ATT CCG AGG-3′ for the −237 SNP. Italic type indicates the site of the polymorphism detected by each oligonucleotide.

Interferon (IFN)–γ assay. Whole-blood IFN-γ assay (QuantiFERON-TB; Cellestis) was performed within 12 h of blood collection. Specifically, 1-mL aliquots of heparinized whole blood were stimulated with 3 drops of the standard antigens provided in the kit, including a saline control, PPD from *M. tuberculosis,* PPD from *M. avium,* and phytohemagglutinin. After 24 h of stimulation, IFN-γ was quantified by ELISA in 50 μL of plasma collected from above the settled blood cells from each blood culture, according to the manufacturer's instructions.

Statistical analysis. Statistical analyses of the polymorphic variant frequencies of candidate genes were performed in a stepwise manner. First, overall genotype frequencies of case patients with TB and control subjects were compared using a 3×2 χ^2 test; if a significant overall difference between case patients and control subjects was detected ($P < .05$), individual genotypes were compared with the more common homozygous genotype using 2×2 χ^2 analysis. Levels of significance are reported as P values for all experiments and comparisons. P values reflect 2-tailed values and are given with 95% confidence intervals (CIs). All data analyses were performed with the aid of INSTAT software (GraphPad).

Results

The distributions of the genotype frequencies of the polymorphic variants for the NRAMP1, VDR, IL-10, TNF-α, and IL-1 genes among patients with pulmonary TB and control subjects are shown in table 1. Eleven different polymorphic variants of the human NRAMP1 gene have been identified, and at least 4 (INT4, D543N, 5′ CA repeat, and 3′ UTR) have been associated with TB disease susceptibility in patients from The Gambia [12], Japan [13], Guinea [14], and Korea [15]. By contrast, in our cohort, heterozygosity for the D543N and 3′ UTR NRAMP1 polymorphisms had a significant association with resistance to pulmonary TB disease ($P = .02$; odds ratio [OR], 0.59; 95% CI, 0.38–0.91) (table 1). We note that these 2 variants and their associations with TB resistance are not independent of each other, because the D543N polymorphic variant was always associated with the 3′ UTR polymorphic deletion ($P < .00001$). Two additional polymorphisms in the NRAMP1 gene (−236C/T and INT4) were too uncommon in our cohort (allelic frequency, <1% and <3%, respectively; data not shown) to determine any potential disease association.

Table 1. Relationship between candidate gene polymorphisms and pulmonary tuberculosis (PTB) in Cambodia.

Polymorphism, genotype	No. (%) of patients with PTB (n = 358)	No. (%) of control subjects (n = 106)	χ^2 test for overall genotypic frequency (P value)	χ^2 test for individual genotypic frequency (P value)	OR (95% CI)
NRAMP 3′ UTR					
TGTG+/+	228 (64.2)	57 (53.8)	9.41 (.009)		
TGTG+/del	115 (32.4)	49 (46.2)		5.09 (.02)	0.59 (0.38–0.91)
TGTGdel/del	12 (3.4)	0 (0)		1.82 (.17)	
NRAMP D543N					
G/G	228 (64.2)	57 (53.8)	9.41 (.009)		
G/A	115 (32.4)	49 (46.2)		5.09 (.02)	0.59 (0.38–0.91)
A/A	12 (3.4)	0 (0)		1.82 (.17)	
IL-1Ra INT-2					
A1/A1	311 (89.9)	99 (93.4)	3.27 (.19)		
A1/non-A1	26 (7.5)	3 (2.8)			
Non-A1/non-A1	9 (2.6)	4 (3.8)			
IL-1β (−511)					
A1/A1	96 (26.8)	35 (33.0)	2.28 (.32)		
A1/A2	170 (47.5)	50 (47.2)			
A2/A2	92 (25.7)	21 (19.8)			
IL-1β (+3953)					
A1/A1	324 (90.5)	98 (92.5)	0.48 (.78)		
A1/A2	28 (7.8)	7 (6.6)			
A2/A2	6 (1.7)	1 (0.9)			
IL-10 (−1082)					
A/A	86 (24.2)	39 (36.8)	6.62 (.03)		
A/G	259 (72.8)	64 (60.4)		5.97 (.01)	1.84 (1.15–2.93)
G/G	11 (3.1)	3 (2.8)		0.20 (.65)	
VDR-Taq					
T/T	325 (90.8)	96 (90.6)	0.99 (.60)		
T/t	30 (8.4)	10 (9.4)			
t/t	3 (0.8)	0 (0)			
TNF-α (−1030)					
T/T	130 (36.4)	40 (37.7)	1.97 (.37)		
T/C	192 (53.8)	51 (48.1)			
C/C	35 (9.8)	15 (14.2)			
TNF-α (−862)					
C/C	169 (47.5)	47 (44.3)	1.61 (.45)		
C/A	152 (42.7)	44 (41.5)			
A/A	35 (9.8)	15 (14.2)			
TNF-α (−856)					
C/C	324 (90.8)	98 (92.5)	0.96 (.61)		
C/T	18 (5.0)	3 (2.8)			
T/T	15 (4.2)	5 (4.7)			
TNF-α (−307)					
G/G	323 (90.2)	95 (89.6)	0.04 (.98)		
G/A	6 (1.7)	2 (1.9)			
A/A	29 (8.1)	9 (8.5)			

NOTE. Polymorphisms were typed in 358 patients with PTB and 106 tuberculin-positive control subjects, as described in Patients and Methods. Only results of polymorphisms with frequencies >3% are shown. Statistical analyses of the genotypic frequencies were performed in a stepwise manner. Overall genotype frequencies of patients with PTB and control subjects were compared using a 3×2 χ^2 test with 2 *df*. In cases where a significant overall difference between patients with PTB and control subjects was detected ($P < .05$), individual genotypes were compared with the more common wild-type homozygous genotype using 2×2 χ^2 analysis with 1 *df*. There were small differences in the total no. of individuals in whom typing was performed for each polymorphism, because limited quantities of genomic DNA were available from a few individuals. +, Presence of TGTG; CI, confidence interval; del, absence of these 4 bases; IL-1Ra, IL-1 receptor antagonist; OR, odds ratio; TNF, tumor necrosis factor; VDR, vitamin D receptor.

Several different SNPs of the human TNF-α promoter region have also been identified [26–30], and these SNPs occur in ethnic-specific patterns [31]. We note that 2 TNF-α SNPs located at −307 and −237 nt upstream of the transcription start site have been associated with TB susceptibility in India [21]. We found no significant association between the −307 SNP and TB in Cambodia, which is consistent with the results of a previous study [32]. We note that the −237, −375, and −243 SNPs were very uncommon in the Cambodian population (allelic frequency, <2%) (data not shown); thus, it was not possible to evaluate their potential disease associations. Furthermore, there were no significant associations between the −1030, −862, and −856 TNF-α SNPs and TB disease in Cambodia (table 1).

Polymorphisms of the VDR gene have been associated with TB resistance in The Gambia [17], whereas polymorphisms of the IL-1 gene complex have been associated with TB susceptibility in India [18]. We found no significant associations between the VDR Taq1 or any of the IL-1–associated SNPs and

Table 2. Agreement between whole-blood interferon (IFN)–γ assays and tuberculin testing.

Assay	PPD negative ($n = 8$)	PPD 1–9 mm ($n = 6$)	PPD 10–14 mm ($n = 26$)
Positive IFN-γ assay	0	3	25
Negative IFN-γ assay	8	3	1
Agreement, %	100	50	96.2

NOTE. Data are no. of individuals, unless indicated otherwise. One-milliliter aliquots of whole blood from 40 randomly selected healthy volunteers were stimulated with standardized concentrations of purified protein derivative (PPD) from *Mycobacterium tuberculosis* (Mtb), PPD from *M. avium,* phytohemagglutinin (mitogen), and saline control (nil). After 24 h of stimulation, IFN-γ production was quantified by ELISA, and the results were correlated with the PPD reading of the particular patient. The amount of IFN-γ produced in response to Mtb PPD in excess of the saline control (Mtb − nil) was calculated, as was the amount of IFN-γ produced in excess of the saline control by the avian PPD and mitogen-stimulated blood cultures (avian − nil and mitogen − nil, respectively). A positive result for Mtb infection was defined by the following 2 criteria: (1) (Mtb − nil)/(mitogen − nil) ≥ 0.15 and (2) [(Mtb − nil) − (avian − nil)]/(human − nil) ≥ −0.10. All other IFN-γ result profiles were considered to be negative for Mtb infection.

pulmonary TB disease in Cambodia (table 1). The VDR Fok1 SNP was very uncommon in Cambodians (allelic frequency, <2%) (data not shown); thus, disease association with this SNP could not be determined.

IL-10 SNPs were not associated with TB susceptibility in patients from The Gambia [20]. Strikingly, however, in our cohort, heterozygosity of the −1082 IL-10 SNP (A/G) was significantly associated with TB disease ($P = .01$; OR, 1.84; 95% CI, 1.15–2.93) (table 1). We note that 2 linked SNPs of the IL-10 promoter at −819 and −592 were very uncommon in Cambodia (allelic frequencies, <3%) and thus occurred too infrequently for the determination of disease associations.

Environmental mycobacterium and prior BCG vaccination are common causes of false-positive tuberculin results [33, 34]. To evaluate the specificity of positive PPD results in Cambodian control patients, we performed whole-blood IFN-γ ELISA assays in response to in vitro stimulation with PPD from *M. tuberculosis* and *M. avium* on 40 randomly selected healthy volunteers, and these results were correlated with PPD readings of the same individuals. As shown in table 2, the agreement between PPD readings and the IFN-γ assay was 100% (8/8) for individuals with negative PPD results, 50% (3/6) for individuals with PPD readings of 1–9 mm, and 96.2% (25/26) for individuals with positive tuberculin testing (PPD, >10 mm). All 40 individuals denied having received a BCG vaccination and did not have evidence of a BCG scar. Thus, consistent with data from other highly TB-burdened countries, Cambodian individuals with skin induration >10 mm in response to PPD were likely previously infected with *M. tuberculosis*.

Discussion

Case-control studies are useful to identify genetic associations with disease. In the case of TB disease, studies in diverse populations have identified a variety of associations of non–major histocompatibility complex (MHC) genes with susceptibility or

Table 3. Summary of reported association of candidate gene polymorphisms with tuberculosis (TB) in various populations.

Candidate gene, polymorphism studied	Population in which association with polymorphism has been reported		
	Susceptibility	Resistance	No association
NRAMP1			
D543N	The Gambia [12], Japan [13]	Cambodia	Guinea [14], Taiwan [16]
3′ UTR	The Gambia [12], Korea [15]	Cambodia	Taiwan [16]
INT4	The Gambia [12], Guinea [14]		Cambodia
5′ CA repeat	The Gambia [12]	Japan [13]	Guinea [14]
−236 SNP			The Gambia [12], Cambodia
VDR			
Taq1		The Gambia [17]	Gujarati Hindus [18], Cambodia
Fok1			Gujarati Hindus [18], Cambodia
TNF-α promoter			
−307 SNP	India [21]		Cambodia
−237 SNP	India [21]		Cambodia
−1030 SNP			Cambodia
−862 SNP			Cambodia
−856 SNP			Cambodia
−375 SNP			Cambodia
−243 SNP			Cambodia
IL-10 promoter			
−1082 SNP	Cambodia		The Gambia [20]
−819, −592 linked SNPs			The Gambia [20], Cambodia
IL-1 gene complex			
IL-1β −511 SNP			India [19], The Gambia [20], Cambodia
IL-1β +3953 SNP	India [19]		The Gambia [20], Cambodia
IL-Ra INT2	India [19]	The Gambia [20]	Cambodia

NOTE. Previously reported candidate gene associations with TB are shown in relation to the population where they were described. All results from Cambodia are from the present study. IL-1Ra, IL-1 receptor antagonist; SNP, single-nucleotide polymorphism; TNF, tumor necrosis factor; UTR, untranslated region; VDR, vitamin D receptor.

resistance in the past decade. Our findings of the molecular typing of 19 polymorphisms of non-MHC candidate genes produced different results in comparison with previous studies (table 3). First, in contrast to findings in West Africa [20], we found an association between heterozygosity for the −1082 SNP of the IL-10 promoter region and susceptibility to pulmonary TB in Cambodia. Second, although frequencies of various NRAMP1 polymorphisms are overrepresented among patients with TB from The Gambia [12], Japan [13], Guinea [14], and Korea [15], we found an association between heterozygosity for 2 linked polymorphic NRAMP1 variants (D543N and 3′ UTR) and resistance to pulmonary TB. Third, we found no association between previously associated genetic polymorphisms of the VDR, TNF-α, and IL-1 gene complex and pulmonary TB susceptibility in Cambodia.

An explanation for these apparently divergent findings may involve whether a polymorphism itself is functional and confers a truly altered susceptibility to TB disease or the associated allele is in linkage disequilibrium with an unknown disease susceptibility allele. In this regard, it is important to note that NRAMP1 is of limited importance in *M. tuberculosis* containment by the immune system in mice deficient in NRAMP1 [35]. However, a recent study with mice has demonstrated that the NRAMP1 gene is linked to a new locus, susceptibility to tuberculosis 1, which has a major effect on TB susceptibility [36]. Thus, polymorphisms identified to date may, in fact, serve as markers for other genes with a functional impact in population-specific genetic backgrounds.

Since 10% of the population that becomes infected with *M. tuberculosis* will develop clinical TB disease, our study design has several advantages that allow us to identify genetic factors associated with susceptibility to progressive pulmonary TB disease. Tuberculin-positive Cambodian control subjects will likely have been exposed to *M. tuberculosis,* given that they are living in a country estimated to have the highest incidence of *M. tuberculosis* infection in the world [3]. Furthermore, we have shown that common causes of false-positive tuberculin testing, such as BCG vaccination and environmental mycobacterium exposure, are unlikely in adult Cambodians with a PPD reaction >10 mm. More important, the control population was followed up for a minimum of 7 years, and patients who developed pulmonary TB disease during that time were excluded from the analysis.

Stead [37] has proposed that susceptibility to infection by *M. tuberculosis* has changed from being the norm for all humans to being an infection of certain populations as a result of the natural selection of resistance among ancestors who came in contact with the bacterium and survived illness during the preantibiotic era. Although it is unclear when Cambodia first experienced TB, it appears that it was well after Africa and the Middle East [38]. Thus, distinct environmental and natural selective factors have likely resulted in population-specific immunogenetic adaptations to clinical TB. In this regard, it is interesting to note that, although malaria has resulted in ethnic-specific adaptations of common erythrocytic variants and hemoglobinopathies associated with resistance to plasmodial parasite infection in populations from Africa, Asia, and the Mediterranean [39], other associations of candidate genes of less clear functional impact on susceptibility or resistance to malaria, including polymorphisms of the MHC [40], TNF-α [41, 42], intracellular adhesion molecule–1 [43], and nitric oxide synthase 2 genes [44], appear to be geographically heterogeneous or contradictory [45].

Recently, we demonstrated that TNF-α promoter polymorphisms occur in ethnic-specific patterns [31]. Here, we have shown that the frequencies of genetic variants of VDR, NRAMP1, IL-10, and IL-1 genes are also population-specific, thus underscoring the need for understanding the frequency of a particular polymorphism in specific populations before assigning to it specific infectious-disease associations. Because HLA-DQ genes appear to be critical for TB susceptibility in Cambodia [32], other genetic variants, including the IL-10 and NRAMP1 polymorphisms described here, may serve as markers of unidentified genetic factors that may play a critical role in host immunity to TB in Cambodia. We anticipate that the determination of ethnic-specific genetic associations with TB susceptibility may guide TB therapy and prophylaxis in an ethnic-specific manner.

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