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Association of HLA-DR, -DQ, and Vitamin D Receptor Alleles and Haplotypes with Tuberculosis in the Venda of South Africa

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ABSTRACT: The vitamin D receptor (VDR) and the human leukocyte antigen (HLA) class II complex affect innate and/or adaptive immunity against *Mycobacterium tuberculosis*. HLA-DRB1, HLA-DQB1, and VDR gene (*VDR*) polymorphisms were previously associated with tuberculosis (TB) and are here investigated as candidates for TB susceptibility in the Venda population of South Africa. Genomic DNA from 95 patients with pulmonary tuberculosis (PTB) and 117 ethnically matched, healthy controls were typed for HLA-DRB1, DRB3, DRB4, DRB5, DQB1, and *VDR* polymorphisms *Fok*I, *Bsm*I, *Apa*I, and *Taq*I using polymerase chain reaction-sequence specific primers (PCR-SSP). Allele and haplotype frequencies were calculated by the estimator maximum (EM) algorithm. DRB1*1302 phenotype was significantly associated with TB occurring at a significantly higher allele frequency in cases than controls and found in haplotype with DQB1*0602/3. DQB1*0301-0304 phenotype was significantly associated with TB and found in haplotype with DRB1*1101-1121, showing significant linkage disequilibrium (LD) in both cases and controls. Only DRB1*1101-1121-DQB1*05 was significantly associated with TB based on the sequential Bonferroni *p* value. *VDR* SNP phenotypes were not associated with TB, but the haplotype F-b-A-T significantly protected from TB. In conclusion, common African HLA-DRB1 and -DQB1 variants, previously associated with protection from malaria and hepatitis B/C virus persistence, predispose the Venda to TB, whereas the proposedly active *VDR* haplotype F-b-A-T showed significant protection. *Human Immunology 67, 643–654 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: HLA-DRB1; HLA-DQB1; vitamin D receptor; VDR; tuberculosis; TB; Venda

ABBREVIATIONS

ARMS	amplification refractory mutation system
CI	confidence interval
HLA	human leukocyte antigen
LD	linkage disequilibrium
MDR	multi-drug resistant
OR	odds ratio
PCR-SSP	polymerase chain reaction-sequence specific primers
PCR-SSO	polymerase chain reaction-sequence specific oligonucleotide probe hybridization
PTB	pulmonary TB
TB	tuberculosis
VDR	vitamin D receptor

INTRODUCTION

A growing body of evidence suggests that host genetics play a role in the predisposition to tuberculosis (TB), in addition to pathogen, environmental, and socioeconomic factors [1, 2]. Studies to identify the genetic loci responsible for TB susceptibility showed that the genetic component is specified by several minor susceptibility genes, rather than a single major TB susceptibility gene [3]. Genetic factors contributing to TB susceptibility include variants of the human leukocyte antigen (HLA) class II complex [4–7] and the vitamin D receptor gene (*VDR*) [8–11].

The HLA class II molecules present peptide antigens to $CD4^+$ T cells and are essential for T cell-mediated

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Received February 11, 2005.

0198-8859/06/$–see front matter
doi:10.1016/j.humimm.2006.04.008

adaptive immunity. DQ and DR are two of five isotypes of HLA class II proteins; heterodimers composed of α and β chains, encoded by the highly polymorphic loci, HLA-DQ and -DR, respectively. The HLA class II variant, DR2 encoded by alleles DRB1*15 and DRB1*16, is associated with TB in several populations [4, 5], but not in others [6, 7]. The less common DQB1*0503 HLA class II allele associated with TB in Cambodia [7] was shown to bind a peptide from the central region of the *Mycobacterium tuberculosis* immunogenic protein, ESAT-6, with low affinity, stimulating weak effector T-cell responses [12].

The vitamin D receptor (VDR) influences both innate and adaptive immunity. In human macrophages, VDR and the active form of vitamin D, $1,25(OH)_2D_3$, mediate the antimicrobial response triggered by Toll-like receptors (TLRs), leading to the induction of the antimicrobial peptide cathelicidin and the killing of intracellular *Mycobacterium tuberculosis* [13]. The role of VDR and $1,25(OH)_2D_3$ in adaptive immunity is more controversial. *In vitro* studies suggest that $1,25(OH)_2D_3$ enhances chemotactic and phagocytic capacity of monocytes and macrophages, but reduces MHC class II expression, T-cell stimulatory capacity, and synthesis of the Th1 cytokines IL-2 and IFN-γ, inducing $CD4^+$ T lympocytes to polarize toward a Th2 phenotype [14, 15]. On the contrary, *in vivo* studies have suggested that a functional VDR is essential for Th1 cell development, though there are indications that high levels of $1,25(OH)_2D_3$-liganded VDR transcriptional activity may support the development of strong Th2 cell-mediated responses [15]. The *VDR*, located on 12q12-14, contains more than 25 known polymorphisms, although more than 100 are expected based on the observed genome-wide frequency of SNPs [16]. Mutations in the *VDR* that impair VDR function, such as that occurring in hereditary vitamin D resistant rickets (HVDRR), is associated with frequent and severe episodes of infection [15]. On the other hand, subtle polymorphisms appear to influence the quality of the immune response when sufficient levels of $1,25(OH)_2D_3$ are present [15].

The most commonly studied SNPs of the *VDR* are *Fok*I, *Bsm*I, *Apa*I, and *Taq*I. *Fok*I is a functional SNP at the 5′ end of *VDR* in exon II (alleles F/f or nucleotides C/T) producing a more active 3 amino acid-truncated VDR when encoded by the 'F' allele. Nonfunctional SNPs at the 3′ end of the gene include *Bsm*I (B/b or nucleotides T/C) and *Apa*I (A/a or nucleotides T/G) in intron VIII, and the synonymous SNP *Taq*I (T/t or nucleotides T/C) in exon IX. A 3′ UTR singlet A repeat in exon IX, proposed to influence mRNA stability, is not commonly included in TB association studies. *Bsm*I, *Apa*I, and *Taq*I likely serve as markers within an extended haplotype covering disease-causing alleles. They are located in a linkage disequilibrium (LD) block at the 3′ end of the *VDR*, including the singlet A repeat [16]. Associations of *VDR* polymorphisms with TB are inconsistent between population groups and likely due to the use of nonfunctional markers, different LD patterns and haplotype blocks in populations, small sample sizes, population stratification, and variation in environmental factors between geographically separated areas [17]. In a case-control and family-based association study in West Africa we found significant support for the association of *VDR* haplotypes with TB rather than individual alleles and proposed that haplotypes more accurately and consistently reflect associations between TB and *VDR* variants than that to any individual polymorphism [8].

Africa, being the ancestral homeland of mankind, sustained ethnically and genetically diverse populations [18]. The study of the African genome may contribute to a better understanding of the genetic basis of complex diseases such as TB. J.B.S. Haldane was the first to propose that infectious diseases are the main selective force in human evolution [19]. Genes involved in protective immunity are under greater selective pressure, showing greater variability than other genes [20, 21]. For a disease to be a selective pressure in the evolution of a population, the gene would have to have a significant impact for long periods of time, influencing morbidity and mortality before reproductive age [20, 21]. Tuberculosis was a major selective force in the evolution of Western European populations, whereas malaria served a similar role in Africa [20, 21]. The World Health Organization estimated that, in 2004, more than 80% of all TB patients lived in sub-Saharan Africa and Asia. The incidence of TB per capita in sub-Saharan Africa was almost 400 cases per 100 000 population, nearly twice that of the South-East Asia Region [22]. Before the TB epidemic became driven by the human immunodeficiency virus (HIV), the uneven geographic distribution of TB has been attributed to differential susceptibility of ethnic groups [23]. It was postulated that sub-Saharan Africa was virgin soil for *Mycobacterium tuberculosis*, brought to Africa for the first time during European colonization and trade with the East.

Despite the TB epidemic in sub-Saharan Africa, studies on the role of genetic factors in TB in populations indigenous to southern African are few [24]. The Venda is regarded to be one of the last population groups to migrate to southern Africa, and their extreme location in the Limpopo Province of South Africa, bordering Zimbabwe, ensured little admixture with population groups in South Africa. They constitute approximately 12% of the total population residing in the Limpopo Province, an area with intermediate to high malaria risk. An epidemiologic study conducted in this area (D.-L.D., unpublished data) revealed that 37% of the total number of

TABLE 1 TB cases and controls included in the analyses of HLA and *VDR* as candidate genes in TB susceptibility in the Venda of South Africa

	HLA genotyping			*VDR* genotyping			
	DRB1	DQB1	DRB3/4/5	*Fok*I	*Bsm*I	*Apa*I	*Taq*I
TB cases	92	95	95	66	85	84	84
Controls	117	117	117	86	88	82	82

Family data excluded.

Abbreviations: HLA = human leukocyte antigen; TB = tuberculosis.

TB cases registered in the Limpopo Province were of Venda descent. Knowledge of genetic factors contributing to TB susceptibility in southern Africa is essential for the development of tailored treatment strategies to reduce the burden of TB in this region.

Here, we investigate association of HLA-DRB1, DRB3, DRB4, DRB5, DQB1, and *VDR* polymorphisms *Fok*I, *Bsm*I, *Apa*I, and *Taq*I in pulmonary TB (PTB) and ethnically matched, healthy controls from the Venda using polymerase chain reaction-sequence specific primers (PCR-SSP). The more active *VDR* haplotype F-b-A-T showed significant protection, whereas TB-associated HLA variants in the Venda correspond to those in TB-naive populations.

MATERIALS AND METHODS

Cases and Controls

All participants of this study were of Venda descent. The Venda region is 2 771 km^2 located in the Limpopo Province of South Africa with an approximate population of 1 million. Hospitals and clinics in the region assisted in the identification of individuals of Venda descent who had tested positive for acid-fast bacilli in sputum. The American Thoracic Society classification system was used as a guideline to identify suitable participants. A total of 104 patients who suffered from either pulmonary or meningeal TB were recruited for this case-control study. Only patients who were shown to be HIV-negative by participating hospitals and clinics were included in the study. A control group composed of 117 ethnically matched, unrelated individuals were also included in the analyses. This group consisted primarily of other hospitalized patients and nurses, who were indicated to have no history of TB by participating hospitals and clinics. DNA was isolated from whole venous blood using the Nucleon BACC2 DNA extraction kit (Amersham Inc., Buckinghamshire, UK). Table 1 shows how many of the samples from recruited cases and controls could be utilized in the HLA and *VDR* typing experiments, respectively.

HLA-DRB1 and -DQB1 Typing

All samples were typed for HLA-DRB1 and -DQB1 using PCR-SSP, as described previously [25, 26]. The 3rd intron of HLA DRB1 was amplified in each reaction as a positive control. Amplified products were resolved using agarose (Roche, Mannheim, Germany) gel electrophoresis.

VDR SNP Typing

Allele-specific oligonucleotide primers for *Fok*I, *Apa*I, and *Taq*I were previously designed and described by Mullighan *et al.* [27], whereas *Bsm*I primers were designed based on published *VDR* sequences (GenBank accession no **J03258**). Sequences of the primers used to amplify *Bsm*I were: 5′AGCCTGAGTACTGGGAATGT 3′ ('B' allele primer) and 5′AGCCTGAGTACTGGGAATGC 3′ ('b' allele primer). The consensus primer used was 5′GGGAGGGAGTTAGGCACC 3′. Single amplification refractory mutation system (ARMS) polymerase chain reaction (PCR) was used to detect *Fok*I and *Bsm*I mutations; each in two separate reactions and double ARMS PCR was used to detect *Apa*I and *Taq*I mutations in four hybrid reactions. The 3rd intron of HLA-DRB1 was amplified in each reaction as a positive control. DNA sequencing of 6 random samples from the cases and controls confirmed the accuracy of the double ARMS PCR used for genotyping of *Apa*I and *Taq*I mutations. A selection of samples were sequenced in duplicate, using ABI Prism BigDye™ terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). Reactions were precipitated under vacuum and resuspended in dextran/formamide dye, denatured for 2 minutes at 95°C, and analyzed on a polyacrylamide gel using the ABI377 sequencer (Applied Biosystems). Analysis was performed using ABI prism sequencing analysis software (Applied Biosystems).

Statistical Analysis

Phenotype counts and frequencies were calculated, and the statistical significance verified by the Fisher exact test, using the Statistical Package for Social Sciences (SPSS software [2001]; SPSS Inc., Chicago, IL, USA).

TABLE 2 Tests of association between TB and HLA-DRB1 and -DQB1 in the Venda of South Africa

Allele	Serologic specificity	Frequency		p value	OR (95% CI)
		Cases	Controls		
DRB1					
1302	DR13b (6)	0.239	0.060	<0.001	5.05 (1.94–13.67)
DQB1					
0301-0304	DQ7 (3)	0.453	0.231	0.001	2.58 (1.37–4.88)
0604-0609	DQ6d	0.189	0.350	0.013	0.43 (0.229–0.821)

The sequential Bonferroni p value for 19 haplotypes was $p_{SB} = 0.0026$. Insignificant associations omitted. Allele frequencies shown in separate table below.
Abbreviations: TB = tuberculosis; HLA = human leukocyte antigen; OR = odds ratio; CI = confidence interval.

Association tests were done with phenotype counts. Given the multiple comparison problem presented by the data, significance of the association of phenotypes with disease was determined by the sequential Bonferroni test [28, 29]. Odds ratios (OR) and confidence intervals (CI) were calculated using Epi-Info 1.1.2 (CDC, 2001).

Allele and haplotype frequencies were calculated by the estimator maximum (EM) algorithm of Long *et al.*, 1995 [30]. Statistical significance of allele and haplotype frequencies was determined by a Monte Carlo algorithm in which the estimated allele frequencies were used to set up a 1000 samples of size N in Hardy-Weinberg and gametic equilibrium. To each Monte Carlo sample, the EM algorithm was applied to estimate allele and haplotype frequencies. Boundaries for 95% confidence intervals were determined by the 25th and 975th value of the ordered sets of allele and haplotype frequencies. A frequency that lies outside these boundaries was considered significant at the $p = 0.001$ level of significance.

Haplotypes were assigned to each subject in the cases and controls by computing the normalized probabilities of the linkage phases of each phenotype. The genotype with the maximum probability was assigned to each person. Tests for the association of haplotype with disease were performed by the Fisher exact test because cells often had expected values less than five. Given the multiple comparison problem presented by the data, significance of the association of haplotypes with disease was determined by the sequential Bonferroni test [28, 29].

RESULTS

Analysis of Association between TB and HLA-DRB1 and -DQB1 Variants

The HLA-DRB1 and -DQB1 phenotype counts for TB cases and controls were calculated independently, and the probability of association was determined using a sequential Bonferroni p value for 19 haplotypes, $p_{SB} = 0.0026$ (Table 2). The counts for DRB1*1302 and DQB1*0301-0304 were significantly higher in cases compared with controls. DQB1*0604-0609 was less frequent in cases than in controls, although this difference was not significant based on the sequential Bonferroni p value.

Allele frequencies for HLA-DRB1 and -DQB1 and their 95% confidence intervals for cases and controls are shown in Table 3. The frequency of allele DRB1*1301 was significantly lower in the cases (0.049) compared with controls (0.128), whereas DRB1*1302 was significantly higher in the cases (0.125) compared with controls (0.030).

Seventy-two haplotypes were identified in the cases and controls. Haplotypes with either statistically significant frequencies or LDs are shown in Table 4. Using an uncorrected p value of 0.05, seven haplotypes had a significant association with disease by the Fisher exact test, and two of these had an odds ratio significantly different from 1.0. The four haplotypes with the lowest Fisher two-sided p values are shown in Table 5. Three of the seven haplotypes, DRB1*0801-0805-DQB1*0301-0304 (Table 4, Cases), DRB1*1101-1121-DQB1*05 (Table 4, Controls, and Table 5), and DRB1*1302-DQB1*0602 (Table 5), had no observed haplotypes in the controls; two haplotypes, DRB1*0301-DQB1*04 (Table 4, Controls) and DRB1*1301-DQB1*05 (not shown), had no observed haplotypes in the cases. Therefore, even though there is a result at the $p < 0.05$ level of significance for two of these haplotypes in Table 5, no odds ratio could be calculated. None of these haplotypes had a frequency significantly different from 0.0 in the Monte Carlo simulation; that is, the lower boundary for the 95% confidence interval was 0.0. However, the genetic disequilibrium, D', for DRB1*1101-1121-DQB1*05 in the controls was -0.043 and significantly different from 0.0. The lower and upper boundaries of the 95% confidence interval for the Monte Carlo simulation were 0.031 and 0.034, respectively (Table 4, Controls).

Two haplotypes had nonzero values in each cell and a statistically significant odds ratio: DRB1*1302-DQB1*0603 and DRB1*1101-1121-DQB1*0301-

TABLE 3 Allele frequencies of HLA-DRB1 and -DQB1 in TB cases and controls in the Venda of South Africa

Allele	Serologic specificity	Frequency	
		Cases (95% CI)	Controls (95% CI)
DRB1			
0101-0103	DR1	0.100 (0.055–0.141)	0.103 (0.064–0.141)
0301	DR17a (3)	0.100 (0.055–0.141)	0.137 (0.093–0.181)
0304	DR17b (3)	0.044 (0.014–0.073)	0.077 (0.043–0.111)
0302	DR18 (3)	0.087 (0.046–0.128)	0.090 (0.053–0.126)
0401-0422	DR4	0.022 (0.001–0.043)	0.064 (0.033–0.096)
0701-0702	DR7	0.022 (0.001–0.043)	0.056 (0.026–0.085)
0801-0805	DR8	0.038 (0.010–0.066)	0
0901	DR9	0.005 (0.000–0.016)	0.009 (0.000–0.020)
1001	DR10	0.016 (0.000–0.035)	0.004 (0.000–0.013)
1101-1121	DR11 (5)	0.255 (0.192–0.318)	0.184 (0.134–0.233)
1201-1203	DR12 (6)	0.016 (0.000–0.035)	0.013 (0.000–0.027)
1301	DR13a (6)	0.049 (0.018–0.080)	0.128 (0.085–0.171)
1302	DR13b (6)	0.125 (0.077–0.173)	0.030 (0.008–0.052)
1301-1304	DR13c (6)	0	0
1305	DR13d (6)	0	0
1401, 1404, 1405	DR14a (6)	0	0
1402, 1403, 1409	DR14b (6)	0	0
1501-1505	DR15 (2)	0.109 (0.064–0.154)	0.073 (0.039–0.106)
1601-1606	DR16 (2)	0.016 (0.000–0.035)	0.034 (0.011–0.058)
DQB1			
02	DQ2	0.065 (0.030–0.101)	0.111 (0.071–0.151)
0301-0304	DQ7 (3)	0.223 (0.163–0.283)	0.124 (0.082–0.166)
0302	DQ8a (3)	0	0
0305	DQ8b (3)	0	0
03032	DQ9 (3)	0	0
04	DQ4	0.044 (0.014–0.073)	0.094 (0.057–0.131)
05	DQ5	0.223 (0.163–0.283)	0.235 (0.181–0.289)
0601	DQ6a	0.011 (0.000–0.026)	0
0602	DQ6b	0.158 (0.105–0.210)	0.133 (0.089–0.176)
0603	DQ6c	0.185 (0.129–0.241)	0.124 (0.082–0.166)
0604-0609	DQ6d	0.092 (0.051–0.134)	0.180 (0.130–0.229)

Abbreviations: HLA = human leukocyte antigen; TB = tuberculosis; CI = confidence interval.

0304 (Table 5). Haplotype DRB1*1302-DQB1*0603 had an odds ratio of 6.235 (95% CI 1.313–29.611). This haplotype occurred at a frequency that would be expected at genetic equilibrium in both the cases and controls; likewise its genetic disequilibrium was not different from 0.0 in either the cases or controls (data not shown). For haplotype DRB1*1101-1121-DQB1*0301-0304, the odds ratio is 2.762 (95% CI 1.386–5.503). However, in contrast with the previous observation, haplotype DRB1*1101-1121-DQB1*0301-0304 had both a haplotype frequency and genetic disequilibrium that were significantly different from the expected values at gametic equilibrium in both cases and controls (Table 4). For the cases, haplotype frequency was 0.148 (95% CI 0.015–0.106), whereas genetic disequilibrium (D') was 0.091 (95% CI −0.039–0.044). For the controls, the haplotype frequency was 0.066 (95% CI 0.000–0.051), whereas D' was 0.043 (95% CI −0.024–0.026).

Analysis of Association of *VDR* SNPs, *Fok*I, *Bsm*I, *Apa*I, and *Taq*I with TB

Table 6 summarizes the tests of association for genotypes of the *VDR* SNPs, *Fok*I, *Bsm*I, *Apa*I, and *Taq*I in the studied case and control groups. No significant differences were observed in the distribution of genotype frequencies between controls and TB cases for any of the *VDR* SNPs.

Allele frequencies and the 95% confidence interval for the four *VDR* loci for cases and controls are shown in Table 7. The distribution of allele frequencies was not significantly different between cases and controls for any of the SNPs. Thirteen of the sixteen possible 4-locus *VDR* haplotypes were assigned by the EM algorithm in the cases and controls. Haplotype frequencies or gametic disequilibria that lie outside the Monte Carlo simulation of Hardy-Weinberg and gametic equilibrium are listed in Table 8.

TABLE 4 Statistically significant frequencies and/or linkage disequilibrium measurements for the HLA-DRB1-DQB1 2-locus haplotypes in TB cases and controls

2-Locus haplotype DRB1-DQB1	Serologic specificity	Haplotype frequency (95% MCCI)	D′ (95% MCCI)
Cases			
DRB1*0901-DQB1*0601	DR9-DQ6a	0.005 (0.000–0.000)	0.005 (−0.000–0.000)
DRB1*0401-0422-DQB1*02	DR4-DQ2	0.011 (0.000–0.011)	0.009 (−0.001–0.009)
DRB1*0701-0702-DQB1*02	DR7-DQ2	0.016 (0.000–0.011)	0.015 (−0.003–0.009)
DRB1*0301-DQB1*02	DR17a-DQ2	0.027 (0.000–0.025)	0.021 (−0.010–0.017)
DRB1*1501-1505-DQB1*0603	DR15-DQ6c	0.065 (0.000–0.054)	0.045 (−0.024–0.029)
DRB1*0302-DQB1*04	DR18-DQ4	0.038 (0.000–0.016)	0.034 (−0.007–0.013)
DRB1*0101-0103-DQB1*05	DR1-DQ5	0.053 (0.000–0.055)	0.031 (−0.025–0.028)
DRB1*1001-DQB1*05	DR10-DQ5	0.016 (0.000–0.016)	0.013 (−0.008–0.011)
DRB1*0801-0805-DQB1*0301-0304	DR8-DQ7	0.027 (0.000–0.031)	0.019 (−0.013–0.019)
DRB1*1101-1121-DQB1*0301-0304	DR11-DQ7	0.148 (0.015–0.106)	0.091 (−0.039–0.044)
Controls			
DRB1*0701-0702-DQB1*02	DR7-DQ2	0.027 (0.000–0.024)	0.022 (−0.009–0.016)
DRB1*1501-1505-DQB1*0603	DR15-DQ6c	0.029 (0.000–0.029)	0.020 (−0.013–0.018)
DRB1*0301-DQB1*04	DR17a-DQ4	0.033 (0.000–0.035)	0.020 (−0.016–0.020)
DRB1*0302-DQB1*04	DR18-DQ4	0.048 (0.000–0.027)	0.040 (−0.012–0.016)
DRB1*0101-0103-DQB1*05	DR1-DQ5	0.062 (0.000–0.052)	0.031 (−0.024–0.026)
DRB1*1101-1121-DQB1*05	DR11-DQ5	0.000 (0.010–0.080)	−0.043 (−0.031–0.034)
DRB1*1101-1121-DQB1*0602	DR11-DQ6b	0.052 (0.000–0.054)	0.028 (−0.024–0.026)
DRB1*1101-1121-DQB1*0301-0304	DR11-DQ7	0.066 (0.000–0.051)	0.043 (−0.024–0.026)
DRB1*0901-DQB1*0604-0609	DR9-DQ6d	0.009 (0.000–0.009)	0.007 (−0.004–0.007)

Values are accompanied by their Monte-Carlo–simulated 95% confidence intervals (MCCI).

Abbreviations: HLA = human leukocyte antigen; TB = tuberculosis.

Four *VDR* haplotypes were associated with disease (Table 9). The only significant association, based on the sequential Bonferroni p value, was negative ($p < 0.001$) for haplotype F-b-A-T with an odds ratio of 0.211 (95% CI 0.092–0.486). The remaining haplotypes listed in Table 9 were positively associated with disease. However, they had marginal statistical significance: for F-b-A-t, $p = 0.017$, odds ratio 3.500 (95% CI 1.300–9.454); F-b-a-T, $p = 0.047$, OR 2.363 (95% CI 1.057–5.283); and haplotype f-b-A-T, $p = 0.011$, odss ratio 11.000 (95% CI 1.323–91.494), for which two cells had expected frequencies less than five. None of the four *VDR* haplotypes associated with disease (Table 9) had a frequency significantly different from 0.0 in the Monte Carlo simulation (Table 8), whereas genetic disequilibrium for F-b-A-T (D', −0.090, 95% CI 0.069−0.080) and F-b-a-T (D', 0.092, 95% CI −0.061–0.068) was significantly different from 0.0 in the case and control groups respectively (Table 8).

DISCUSSION

The aim of this study was to analyze the association of HLA-DRB1, HLA-DQB1, and *VDR* polymorphisms with TB in the Venda population of South Africa. HLA class II alleles commonly found in Africa, some previ-

TABLE 5 Associations of HLA-DQB1-DRB1 haplotypes with TB

2-Locus haplotype DRB1-DQB1	Serologic specificity	Cases		Controls		Fisher p value	OR (95% CI)
		Present	Absent	Present	Absent		
DRB1*1302-DQB1*0602	DR13b-DQ6b	7	85	0	117	0.003	N.A.
DRB1*1302-DQB1*0603	DR13b-DQ6c	9	83	2	115	0.009	6.235 (1.313–29.611)
DRB1*1101-1121-DQB1*0301-0304	DR11-DQ7	28	64	16	101	0.002	2.762 (1.386–5.503)
DRB1*1101-1121-DQB1*05	DR11-DQ5	9	83	0	117	<0.001[a]	N.A.[b]

The sequential Bonferroni p value for 72 haplotypes was $p_{SB} = 0.0007$.

Abbreviations: HLA = human leukocyte antigen; TB = tuberculosis; OR = odds ratio; CI = confidence interval.

[a] $p = 0.0002$.

[b] NA = not applicable.

TABLE 6 Tests of association between TB and genotypes for the *VDR* SNPs, *Fok*I, *Bsm*I, *Apa*I, and *Taq*I in cases and controls collected from the Venda of South Africa

Locus	Genotype	Frequency		χ^2	df	*p* value
		Cases	Controls			
*Fok*I				2.50	2	0.287
	FF	0.652	0.767			
	Ff	0.318	0.209			
	ff	0.030	0.023			
*Bsm*I				1.78	2	0.411
	bb	0.576	0.648			
	bB	0.365	0.273			
	BB	0.059	0.080			
*Apa*I				2.82	2	0.245
	aa	0.012	0.037			
	aA	0.821	0.720			
	AA	0.167	0.243			
*Taq*I				3.13	2	0.209
	tt	0.060	0.012			
	tT	0.345	0.415			
	TT	0.595	0.573			

Allele frequencies shown in separate table below. Family data omitted.
Abbreviations: TB = tuberculosis; SNP = single nucleotide polymorphism.

ously associated with malaria and hepatitis resistance, were found to be associated with TB in the Venda, alone or in haplotype (HLA-DRB1*1302, *1101-1121 and DQB1*0301-0304, *05, *0602/3). The *VDR* haplotype F-b-A-T was strongly associated with protection from TB.

DRB1*1302 and DQB1*0301-0304 (Table 2) phenotypes were significantly associated with TB. DRB1*1302 and DRB1*1301 alleles, encoding identical β: chain allotypes, except for a Val or Gly at residue 86, occurred at significantly higher frequencies in cases and controls respectively (Table 3). This study did not support the association of DR2 (DRB1*15 and DRB1*16) with TB susceptibility commonly found in other populations [4, 5]. Instead, DRB1*1302 and DRB1*1101-1121, alone or in haplotypes, (Table 5) featured in associations with TB in the Venda. HLA alleles found to be associated with TB in different populations often vary [4–7, 31–37] and may be influenced by allele frequency in a population. All the alleles found associated with TB in the Venda, alone or in haplotypes, except for DQB1*0301-0304, are more common in African-American populations than in Caucasian populations, whereas alleles for the DR2 antigen (DRB1*15 and DRB1*16) occur at a higher frequency in Caucasoid than African-American populations [38].

A similar trend was found in a survey of HLA class II disease associations in southern Africa [24]. A comparison of TB-associated Venda phenotypes with alleles, antigens, or haplotypes previously reported to be associ-

TABLE 7 Allele frequencies of the four *VDR* SNPs in TB cases and controls

Locus	Allele	Frequency	
		Cases (95% CI)	Controls (95% CI)
*Fok*I	F	0.813 (0.734–0.891)	0.821 (0.751–0.892)
	f	0.188 (0.109–0.266)	0.179 (0.108–0.250)
*Bsm*I	B	0.208 (0.127–0.290)	0.241 (0.162–0.320)
	b	0.792 (0.710–0.873)	0.759 (0.680–0.838)
*Apa*I	A	0.594 (0.496–0.692)	0.625 (0.535–0.715)
	a	0.406 (0.308–0.505)	0.375 (0.285–0.465)
*Taq*I	T	0.771 (0.687–0.855)	0.795 (0.720–0.870)
	t	0.229 (0.145–0.313)	0.205 (0.131–0.280)

Abbreviations: TB = tuberculosis; SNP = single nucleotide polymorphism; CI = confidence interval.

TABLE 8 Statistically significant frequencies and/or linkage disequilibrium measurements of the 4-locus *VDR* *Fok*I-*Bsm*I-*Apa*I-*Taq*I haplotypes in TB cases and controls

4-Locus haplotype *Fok*I-*Bsm*I-*Apa*I-*Taq*I	Haplotype frequency	
	(95% MCCI)	D′ (95% MCCI)
Cases		
F-b-A-T	0.178 (0.171–0.411)	−0.090 (−0.069–0.080)
Controls		
F-B-A-t	0.084 (<0.001–0.076)	0.056 (−0.027–0.035)
F-b-a-T	0.259 (0.099–0.286)	0.092 (−0.061–0.068)
F-b-a-t	0.010 (0.000–0.109)	−0.052 (−0.041–0.042)

Values are accompanied by their Monte-Carlo simulated 95% confidence intervals (MCCI).

TABLE 9 Associations of *VDR FokI-BsmI-ApaI-TaqI* haplotypes with TB

4-Locus haplotype *FokI-BsmI-ApaI-TaqI*	Cases		Controls		Fisher *p* value	OR (95% CI)
	Present	Absent	Present	Absent		
F-b-A-T	14	34	37	19	<0.001[a]	0.211 (0.092–0.486)
F-b-A-t	16	32	7	49	0.017	3.500 (1.300–9.454)
F-b-a-T	33	15	27	29	0.047	2.363 (1.057–5.283)
f-b-A-T	8	40	1	55	0.011	11.000 (1.323–91.494)

The sequential Bonferroni *p* value for 13 haplotypes was 0.004.

Abbreviations: OR = odds ratio; CI = confidence interval.

[a] $p = 0.0002$.

ated with TB in other populations is summarized in Table 10 (Venda-corresponding data are indicated in bold font style). Seven populations showed TB-associated alleles or antigens corresponding to those of the Venda (first entry in Table 10). The lack of some allele subtypes did not allow direct comparison of effects. Most of the HLA-DQB1 alleles found associated with TB in the Venda (alone or in haplotypes) matched with alleles that predispose to progressive TB in Cambodia when present in homozygous form (last entry in Table 10, [12]). All these alleles encode Asp at position 57 of the HLA-DQ β-chain. This variant binds the central region of the ESAT-6 immunogenic protein from *Mycobacterium tuberculosis* with fivefold less affinity and is significantly inferior to HLA-DQ β:57-Ala in stimulating effector T-cell responses when presenting the same peptide [12]. A similar mechanism may explain the HLA-DQB1 associations found in the Venda and needs to be verified.

Haplotypes DRB1*1302-DQB1*0602, DRB1*1302-DQB1*0603, and DRB1*1101-1121-DQB1*0301-0304, carrying the TB-associated alleles, as well as DRB1*1101-1121-DQB1*05, were found to be associated with TB based on odds ratio or *p* value (Table 5). Two sources of error are inherent in the association of EM-assigned haplotypes with a disease. First, the assignment of the genotype depends on a probabilistic model based on the estimated haplotype frequencies. When only two loci are considered, as for HLA-DQ and HLA-DR, and one of the loci is a homozygote, then the linkage phase of the alleles is known and the probability of the genotype is 1.0. However, with double heterozygotes, the linkage phase is unknown and is assigned as the larger of the two normalized genotype probabilities. Therefore, there can be unknown error in the assignment of genotypes.

A second source of error is the problem of multiple comparisons. Seventy-two haplotypes were identified in the cases and controls for the HLA-DRB1 and HLA-DQB1 loci. Each EM-assigned haplotype was used in a Fisher exact test, yielding a sequential Bonferroni *p* value (p_{SB}) of 0.0007 with which to begin the determination of significance. Only one haplotype, DRB1*1101-1121-DQB1*05, reached this level of significance ($p = 0.0002$, Table 5). However, the significantly different frequency of DRB1*1302 suggest that this allele is associated with disease (Table 3). Therefore, the significance of the association of haplotypes DRB1*1302-DQB1*0602 and DRB1*1302-DQB1*0603 might be more than what is suggested by the strict sequential Bonferroni threshold of $p = 0.0007$. Haplotype DRB1*1101-1121-DQB1*0301-0304, though not meeting the sequential Bonferroni threshold for significance, is of interest because of the statistically significant positive genetic disequilibria in both the cases and controls (Table 4).

The comparison of TB-associated Venda phenotypes and haplotypes with alleles, antigens, or haplotypes previously reported to be associated with TB in other populations (Table 10) showed DRB1*11-DQB1*03 to be protective from TB in a Polish population [34] in contrast to DRB1*1101-1121-DQB1*0301-0304 that predisposes the Venda. Five of the seven populations showing TB-associated alleles similar to the Venda are located in geographic areas with intermediate to high malaria risk [21], like the Venda, and similarly they were not subject to the TB epidemic that claimed more than a billion Caucasian lives in Western Europe over the previous two centuries. Alleles associated with TB in the Venda count among those common in West Africans and include alleles previously associated with protection from severe malaria (DRB1*1302-DQB1*0501) [39] and spontaneous clearance of hepatitis B virus infection (HLA-DRB1*1302) in The Gambia [40].

HLA-DRB1*1302 is also commonly associated with hepatitis B virus clearance in several Caucasian and Asian populations [41], whereas DRB1*1101-DQB1*0301 is consistently associated with hepatitis C virus clearance in Europeans [42]. These HLA class II alleles or haplotypes may have been selected during a history of exposure to these pathogens, providing a survival advantage from

TABLE 10 A comparison of TB-associated Venda phenotypes and haplotypes (indicated in bold) with alleles, antigens, or haplotypes previously reported to be associated with TB in other populations

Phenotype/allele/ antigen	Haplotype	Effect	Nation	Method	Study size and design	Reference
DRB1*1302	—	Predispose to PTB	Venda	PCR-SSP	95 TB cases	Current study
—	**DRB1*1302-DQB1*0602**	Predispose to PTB			117 controls	
—	**DRB1*1302-DQB1*0603**	Predispose to PTB				
DQB1*0301-0304	—	Predispose to PTB				
—	**DRB1*1101-1121-DQB1*0301-0304**	Predispose to PTB				
—	**DRB1*1101-1121-DQB1*05**	Predispose to PTB				
DRB1*11	—	Protect from PTB	Chinese	PCR-SSP	74 PTB cases 90 controls	[31]
DRB1*13	—	Predispose to PTB	Russian	PCR-SSP	14 families	[32]
DRB1*13	—	Protect from PTB	Polish	PCR-SSP	31 PTB cases 58 controls	[6]
DQB1*0301	—	Protect from PTB	Thai	PCR-SSO	82 PTB cases	[33]
DQB1*0502	—	Predispose to PTB			160 controls	
DQB1*0502	—	Predispose to TB	Polish	PCR-SSP	61 TB cases	[34]
—	DRB1*16-**DQB1*05**	Predispose to TB			125 controls	
—	DRB1*1601-**DQB1*05**02	Predispose to TB				
—	DRB1*04-**DQB1*03**	Predispose to TB				
—	**DRB1*11-DQB1*03**	Protect from TB				
—	DRB1*14-**DQB1*05**	Predispose to TB				
DQB1*05	—	Predispose to PTB	Polish	PCR-SSP	38 PTB cases 58 controls	[35]
DQB1*0501	—	Predispose to PTB HIV-	Mexican	PCR-SSP PCR-SSO	50 PTB cases 95 controls	[36]
DQB1*0503	—	Predispose to MDR-TB	Indian	PCR-SSO	55 MDR TB cases	[37]
DQB1*0502	—	Predispose to MDR-TB			59 drug sensitive TB	
DQB1*0503	—	Predispose to TB	Cambodian	PCR-SSO	78 & 48[a] clinical TB 49 & 39 controls	[7]
HLA-DQβ57-Asp alleles **DQB1*0301,** -0303 DQB1*04 (-0401, -0402) **DQB1*05**03 **DQB1***0601, **-0602, -0603**	—	Homozygosity predispose to progressive PTB Reduced ESAT-6 binding Reduced IFN-γ from $CD4^+$ T cells	Cambodian	PCR-SSO	436 PTB cases 107 controls	[12]

Abbreviations: TB = tuberculosis; PTB = pulmonary tuberculosis; PCR-SPP = polymerase chain reaction-sequence specific primers; PCR-SSO = polymerase chain reaction-sequence specific oligonucleotide probe hybridization; MDR = multi-drug resistant.

[a] Study done in two stages.

malaria and/or hepatitis viral infections. However, in the Venda, they may predispose individuals to *Mycobacterium tuberculosis* infection, a selective force proposed to be foreign to the genome of the African people, unlike malaria. Through a similar mechanism it was proposed that Caucasians gained an advantage over TB at the expense of increased susceptibility to autoimmune type-1 diabetes through selection of HLA-DQ β:57-Ala (HLA-DQ2 and –DQ8) and elimination of HLA-DQ β57-Asp, a variant shown to protect from autoimmune type-1 diabetes [12]. Similar to the protection afforded by a balance in the polymorphisms encoding hemoglobin S and A in high-risk malaria Africa, HLA heterozygosity may protect from both malaria and TB [20, 21].

Independent *VDR* SNPs showed no significant association with TB (Tables 6 and 7). This observation agrees with a number of case-control studies that failed to detect associations between independent *VDR* polymorphisms and TB [10, 43]. Substantial differences between races and ethnic groups in LD patterns and haplotype blocks as well as allele frequencies for the widely studied *VDR* polymorphisms have been reported [44]. Therefore, nonfunctional, disease-associated alleles may differ notably between populations if association is based on LD with a functional disease allele.

The F-b-A-T haplotype, reconstructed from case-control data and found associated with TB (Table 9) in the Venda, supports family-based global association be-

tween TB and the *Fok*I-*Bsm*I-*Apa*I-*Taq*I SNP combination reported for West Africa [8] and seen in the Venda (D.-L.D., unpublished data from 25 families). This supports the proposal that *Bsm*I, *Apa*I, and *Taq*I are markers for unidentified, functional disease loci with which they are in LD, located within the *VDR* or flanking regions. Furthermore, the significant association of F-b-A-T in the absence of individual SNP associations (Table 9) is in agreement with the literature that the influence of *VDR* polymorphisms on VDR activity is collective rather than individual [16]. For example, haplotypes of *VDR* polymorphisms located in the promoter region, coding regions for ligand- or DNA-binding domains, and the 3′UTR, respectively influencing *VDR* expression, VDR transactivation activity, and mRNA stability, may collectively reflect functional effects more accurately. This may explain why *VDR* haplotypes more significantly mark TB predisposition or protection.

However, the particular alleles comprising an associated haplotype should be interpreted with caution if not obtained through transmission disequilibrium tests of family data, due to erroneous haplotypes reconstruction from case-control data. Nonetheless, the F-b-A-T haplotype shown to protect from TB in the Venda corresponds to alleles and haplotypes encoding a more active VDR [15]. This association agrees with 'f/ 'ff' that was associated with predisposition to TB in the Gujarati Asians, UK [10] and Chinese Han [45], and 'TT' that protects from TB in female Tamil-speaking south Indians [11]. In contrast, the 'A' allele and 'FA' haplotype is transmitted more often than expected to affected offspring in West Africa [8] and the presence of the 't' allele is protective in both The Gambia [9] and Gujurati Asians in the UK [10]. Thus, particular alleles/haplotypes are not consistently associated with protection/predisposition. These inconsistencies may result from unreliable haplotypes reconstruction, population stratification, and low statistical power of studies [17]. It emphasizes the need for large family-based studies that will address these limitations.

This study showed TB-associated HLA class II variants in the Venda, common to Africa and previously associated with malaria and hepatitis resistance. *VDR* results confirmed the importance to investigate haplotypes instead of individual SNPs.

Acknowledgements

The authors thank Professor Adrian VS Hill, University of Oxford, for fruitful discussions. We are grateful to the Venda people for their participation, and thank Sr Ritha Haywood and other employees of the Department of Health and Welfare, Limpopo Province, for recruitment of participants. Mr Hans Onya, Director, Health Promotion, and Ms Stokana Mphahlele, School of Health Sciences, Faculty of Science, Health and Agriculture, University of the North, are acknowledged for facilitating sample collection. The project was financially supported by the Medical Research Council and the National Research Foundation (NRF Grant number 2053199 to L.B.) of South Africa, the Hendrik Verwoerd Research Trust, and the Walker Trust Fund, University of Johannesburg.

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