Original Article

Genetic diversity of TLR2, TLR4, and VDR loci and pulmonary tuberculosis in Moroccan patients

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Abstract

Introduction: Toll-like receptors (TLRs) 2, 4, and the vitamin D receptor (VDR) are central components of the innate and adaptive immunity against *Mycobacterium tuberculosis* (*Mtb*). TLR2, TLR4, and VDR polymorphisms were previously associated with tuberculosis (TB) and were here investigated as candidates for pulmonary TB (PTB) susceptibility in a Moroccan population group.

Methodology: Genomic DNA from 343 PTB patients and 203 healthy controls were analyzed for 12 single nucleotide polymorphisms (SNPs) located in TLR2, TLR4, and VDR genes using polymerase chain reaction-based restriction fragment length polymorphism and TaqMan SNP genotyping assays.

Results: The TLR2 +597 CT genotype was associated with protection against PTB (corrected p [pc] = 0.04; odds ratio (OR) = 0.65; 95% confidence interval (CI) = 0.45 - 0.94), and the TLR4 +7263 C allele was significantly associated with PTB susceptibility (pc = 0.04; OR = 1.63; CI = 1.06 - 2.57). The VDR [f,b,a,T] haplotype was found to confer protection (pc < 0.00001; OR = 0.18; CI = 0.09 - 0.35), while the TLR2 [-16934T,+597C,+1349T] haplotype seemed to be at risk (p = 0.03; OR = 1.52; CI = 1.01 - 2.30), but statistical significance was not reached. Finally, cross-analysis between polymorphisms of the three studied genes revealed significant interaction between TLR2 +597 and TLR4 +4434 SNPs towards protection against PTB (pc = 0.036), suggesting that the functionally relevant TLR4 +4434 SNP may act synergistically with TLR2 SNPs.

Conclusions: TLR2 and TLR4 interaction and a specific VDR haplotype influence protection against PTB in Moroccans patients.

Key words: toll-like receptors; vitamin D receptor; polymorphism; tuberculosis; Morocco

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Introduction

Tuberculosis (TB) remains an important cause of death. Every year, more than eight million people develop TB, of whom three million die [1,2]. In Morocco, the incidence has been estimated to be 81 per 100,000 inhabitants, affecting mostly young adults with a consequent negative socio-economic impact [3]. Nevertheless, while the total number of subjects infected with *Mycobacterium tuberculosis* (*Mtb*) is far larger (approximately two billion), the vast majority keeps the pathogen under control and never develops the disease. Genetic variation among individuals could influence the susceptibility to developing active disease [4]. Innate immunity genes are important in modulating host susceptibility to TB because the first line of defense against *Mtb* involves the identification and uptake of the pathogen by macrophages and dendritic cells. Potentially relevant genes for susceptibility to pulmonary TB (PTB) that contribute to this immune response include toll-like receptors (TLRs) 2 and 4 and the vitamin D receptor (VDR).

The TLRs are pattern recognition receptors (PRRs) expressed on macrophages and other leukocytes. They are key players of the innate immune system, and there is substantial evidence that single nucleotide polymorphisms (SNPs) in TLR encoding genes could modulate receptors/ ligands interactions and consequently the individual susceptibility to infectious

diseases [5]. TLR2 interacts with *Mtb* cell-wall components to induce cellular activation, killing of intracellular pathogens, and apoptosis [6]. TLR polymorphisms have been associated with regulation of TLR expression and development of active TB [7-10]. TLR4 was initially identified as the mediator of lipopolysaccharide (LPS) inflammatory responses [11]. It interacts with both a heat-labile soluble mycobacterial factor and whole viable *Mtb* to trigger innate responses [12,13]. *M. tuberculosis*-induced tumor necrosis factor and nitric-oxide production can be blocked by the LPS lipid A antagonist E5531 [14], and it has been shown that TLR4-deficient mice develop a chronic lung infection resembling human disease when exposed to aerosolized *Mtb* [15]. Increased mortality and mycobacterial load in the lungs have also been observed in mice with non-functional TLR4 [16]. In TLR4, two non-synonymous mutations have been reported in the extracellular domain (*Asp229Gly* and *Thr399Ile*) and have been found to be associated with hypo-responsiveness to LPS in alveolar MPs, epithelial cells, and peripheral blood mononuclear cells [17]. A recent study from India found that the *Asp229Gly* amino-acid referred as TLR4 +4434 SNP is associated with TB [18], whereas it could not be associated with TB in previous studies from West Africa [19] and Mexico [20]. Susceptibility to other infectious diseases such as malaria [21] and brucellosis [22] was also associated with the TLR4 +4434 variation.

In this context, another interesting candidate is the vitamin D receptor (VDR) gene. Vitamin D is an immune-modulatory molecule which, via its receptor, can modulate cytokine responses by T cells [23,24], and hence represents an important link between activation of TLRs and antibacterial responses in innate immunity. Lui *et al.* [25] showed that TLR1 and TLR2 activation of human MPs up regulates the expression of the VDR and vitamin D1-hydroxylase genes, leading to the induction of cathelicidin and consequent killing of intracellular *Mtb*. The authors showed that African-Americans individuals with high susceptibility to TB have low 25-hydroxyvitamin D levels, leading to inefficient cathelicidin mRNA expression, [25] while VDR polymorphisms have been associated with active TB disease in West African population case-control and family studies [26].

In our study, we analyzed the potential role of polymorphisms within TLR2, TLR4, and VDR genes in 343 PTB cases and 203 healthy controls from Morocco.

Methodology

Population study

The present study included 343 newly diagnosed patients with PTB attending the Moulay-Youssef Hospital (Rabat, Morocco), which covers a large geographical area of the country in enrolling such patients. The diagnosis was based on sputum positive microscopy, confirmed by positive *Mtb* culture tests. Cases with absence of any previous history of TB along with positive clinical and chest radiology findings were diagnosed as a first episode of the disease. All patients were negative for HIV, hepatitis B and C, and had no antecedents of immune-mediated disorders. The mean age at diagnosis was 38 ± 16.85 years (mean ± SD) (range, 18 to 86 years) and the sex ratio was 1.4 (199 males/144 females). The control group, recruited at the blood bank center of Rabat, consisted of 203 healthy blood donors free of histories of TB or immune-related diseases. The sex ratio and the mean age of this cohort were 1.3 (115 males/88 females) and 30 ± 10.73 years (mean ± SD) (range, 18 to 57 years), respectively.

All cases and controls were from either Arab or Berber population groups (or issued from both ethnicities) and shared globally similar socio-economic conditions and lifestyle. The medical school ethics committee of Mohamed V University of Rabat approved the study and informed consent was obtained from patients and controls.

Genotyping

Genomic DNAs were isolated from peripheral blood leukocytes using a standard salting-out procedure. The genotyping of the three TLR2 SNPs (+596 C/T: rs3804099; -16934 A/T: rs4696480; +1349 T/C: rs5743699) and the five TLR4 SNPs (-6143 A/G: rs1927914; -5724 T/C: rs10759932; +4434 A/G: rs4986790; +7263 G/C: rs11536889; +8469 T/C: rs11536891) was carried out using TaqMan allelic discrimination assay (ABI PRISM 7000 Sequence Detection System, Applied Biosystems, Foster City, CA, USA). For all reactions, 50 ng of genomic DNA was pre-amplified in a total volume of 25 µL containing 7.5 µL of distilled water, 1 µL of each probe (Table 1) (100 pM each), 1 µL of each primer (Table 1) (25 pM each), and 12.5 µL of Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). In each essay, three samples with known genotypes together with three no-DNA blanks were included. After PCR amplification, sample genotypes were attributed by measuring the allele-specific fluorescence using an ABI Prism 7700

Sequence Detection System, using SDS 1.7 software for allele discrimination (Applied Biosystems Foster City, CA, USA).

Four VDR SNPs (Fok T/C [rs10735810], Apa T/G [rs7975232], Taq C/T [rs731236], and Bsm A/G [rs1544410]) were genotyped using restriction fragment length polymorphism analysis. The primers used for this procedure are provided in Table 1. PCR amplified products were digested overnight with ApaI, TaqI, FokI, and BsmI restriction endonucleases (New England BioLabs, Beverly, MA, US) and visualized in 2.5% agarose gel electrophoresis. The presence of a restriction site was assigned a lowercase letter and its absence an uppercase letter, according to convention (*i.e.*, a and A for ApaI, t and T for TaqI, f and F for FokI, b and B for BsmI).

Statistical analysis

Comparisons of genotype, allele and haplotype frequencies between patients and controls were performed using the Chi-square test with Yates's correction or Fisher's exact test wherever appropriate. P values (two tailed) were corrected (pc) using the Bonferroni method, and findings were considered statistically significant for pc equal to or less than 0.05. Odds ratio (OR) and 95% confidence interval (95% CI) were calculated to assess the relative risk conferred by a specific allele, genotype, or haplotype. Furthermore**,** a logistic regression including sex and age as covariates was also performed to estimate the effect of genotype when these potential confounding factors were taken into account. Deviation from the Hardy-Weinberg equilibrium was analyzed using the Chi-square test. TLR2, TLR4 and VDR haplotypes were constructed, and frequencies were calculated using PHASE version 2.1 software [27,28].

Table 1. Single nucleotide polymorphisms, db SNP rs, chromosomic location and oligonucleotide sequences used for genotyping

Gene (chromosome)	Mutation **(db SNP rs)**	**Primer-forward**	**Primer-reverse**	**Probe VIC**	**Probe FAM**
TLR2 4q32	+596 C/T (rs3804099)	5'CAGATCTACAGAGC TATGAGCCAAAA3'	5'CATTCCACGGAACTT GTAACATCTAC3'	5'AGATGACTTA CATTCTG3'	5'TGACTTACGTC TGAATT 3'
	-16934 A/T (rs4696480)	5'TGGTTCTGGAGTTG GGAAGTC3'	5'CTCACCATGTGATGC TTTCCAT 3'	5'TCTGGTGAGG GTCAT3'	5'ATCTGGAGAG GGTCAT3'
	+1349 T/C (rs5743699)	5'ATTTGAACTTATCCA GCACACGAAT 3'	5'TCCAGTGTCTTGGGA ATGCA3'	5'CACGTGTAAC AGGC3'	5'CACAGCGTAA CAGG3'
TLR4 9q32-q33	-6143 A/G (rs1927914)	5'AAGTGCTTGGAGGA TATTACAGTAGAACT A3'	5'GGAAAGTAGCAAGT GCAATGTAAGTTT3'	5'ACTTAGCATA CATAATAT T3'	5'ACTTAGCATG CATAATA3'
	-5724 T/C (rs10759932)	5'GTGATTACCACATT TTACAGACCAGAA3'	5'GGA AAG TAG CAAGTGCAATGTAAG TTT3'	5'TTCACCAACA CTTATT3'	5'CACCAACGCT TATT3'
	+4434 A/G (rs4986790)	5'GGCCTGTGCAATTT G ACCAT3'	5'AGTCACACTCACCA GGGAAAATG 3'	5'TCGATGGTA TTATTG3'	5'CTCGATGATA TTATTG 3'
	+7263 G/C (rs11536889)	5'GTTTCCTGTTGGGC AATG CT3'	5'CATTAATTCCAGCA CATTGTTTTCTC3'	5'CATCCACTCT TCC3'	5'AACATCCACT GTTCC3'
	+8469 T/C (rs11536891)	5'GGTGTTTCCATGTCT CATGTACTAGTG3'	5'CCTGATAGG GATACATAGGGATAT GTG3'	5'CAAATGCACA CATCT3'	5'AAATGCGCAC ATCT3'
VDR 12q12-14	Fok T/C (rs10735810)	5'AGCTGGCCCTGGCA CTGACTCTGGCTCTG3'	5'ATGGAAACACCTGC TTCTTCTCCCTC3'		
	Apa T/G (rs7975232)	5'CAGAGCATGGACAG GGAGCAA3'	5'GCAACTCCTCATGG CTGAGGTCTC3'		
	Taq C/T (rs731236)	5'CAGAGCATGGACAG GGAGCAA3'	5'GCAACTCCTCATGG CTGAGGTCTC3'		
	Bsm A/G (rs1544410)	5'CAACCAAGACTCAA GTACCGCGTCAGTGA3 '	5'AACCAGCGGAAGAG GTCAAAGGG3'		

Table 2. TLR2+597 T/C allele and genotype frequencies among patients and healthy controls

Polymorphisms TLR2 +*597*	Patients (n = 343)		Controls (n = 202)				
Allele	**n**	**f**	**n**	**f**	**p**	**pc**	**OR, 95% CI**
+597 T	369	0.55	221	0.55			0.96 (0.75-1.24)
+597 C	317	0.46	183	0.45	0.770	NS	1.04 (0.80-1.34)
Genotype	**n**	**f**	**n**	**f**	**p**	**pc**	**OR, 95% CI**
+597 TT	100	0.29	50	0.25	0.266	NS	1.25 (0.83-1.89)
+597 CT	169	0.49	121	0.60	0.016	0.048	0.65 (0.45-0.94)
+597 CC	74	0.22	31	0.15	0.075	NS	1.52 (0.93-2.47)

n: number; OR: odds ratio; f: frequency; CI: confidence interval; pc: corrected p-value; NS: non-significant

Table 3. TLR4+7263 G/C allele and genotype frequencies among patients and healthy controls

Polymorphisms TLR4 +7263	Patients (n = 342)		Controls (n = 199)				
Allele	**n**	**f**	**n**	**f**	**p**	**pc**	**OR, 95% CI**
+7263 G	596	0.87	365	0.92	-	-	0.61 (0.39-0.95)
+7263 C	88	0.13	33	0.08	0.021	0.04	1.63 (1.06-2.57)
Genotype	**n**	**f**	**n**	**f**	**p**	**pc**	**OR, 95% CI**
+7263 GG	267	0.78	167	0.84	0.100	NS	0.68 (0.42-1.10)
+7263 GC	62	0.18	31	0.15	0.448	NS	1.20 (0.73-1.98)
+7263 CC	13	0.04	1	0.005	0.040	NS	7.82 (1.06-161.44)

n: number; OR: odds ratio; f: frequency; CI: confidence interval; pc: corrected p-value; NS: non-significant

Table 4. Distribution of both the TLR2 +597 T/C and TLR4+7263 C/T genotypes by logistic regression analysis adjusted for age and sex

Polymorphisms	p	Adjusted OR	95% CI
TLR2 +597 T/C			
+597 CT *vs.* CC + TT genotypes	0.012	0.64	0.44-0.90
TLR4 +7263 C/T	**p**	**Adjusted OR**	**95% CI**
+7263 C allele	0.024	1.63	1,07-2,48
+7263 CC *vs.* GG + GC genotypes	0.048	7.86	1.02-60.65

Adjusted OR: odds ratio adjusted for age and sex; CI: confidence interval

Table 5. Frequencies of the 3-locus TLR2 -16934 A/T, +597 T/C, and +1349 T/C haplotypes among patients and healthy controls

TLR2 gene Haplotypes*	Patients (n = 343)		Controls (n = 202)				
	n	**f**	**n**	**f**	**p**	**pc**	**OR (95% CI)**
1/ A-C-T	171	0.25	116	0.29	0.170	NS	0.82 (0.62-1.10)
2/ A-C-C	40	0.06	21	0.05	0.661	NS	1.13 (0.64-2.01)
3/ A-T-T	139	0.20	65	0.16	0.088	NS	1.33 (0.95- 1.86)
4/ A-T-C	3	0.004	3	0.007	0.676	NS	0.59 (0.078-4.407)
5/ T-C-T	96	0.14	39	0.10	0.036	NS	1.52 (1.01-2.30)
6/ T-C-C	7	0.01	9	0.02	0.123	NS	0.45 (0.142-1.378)
7/ T-T-T	227	0.33	149	0.37	0.204	NS	0.85 (0.65-1.10)
8/ T-T-C	3	0.004	2	0.005	1.000	NS	0.88 (0.101-10.613)

n: number; OR: odds ratio; f: frequency; CI: confidence interval; pc: corrected p-value; NS: non-significant
*Haplotype nomenclature refers to 3 loci composed of A-16934T, T+597C and T+1349C SNPs in TLR2. Haplotype frequencies < 5% among patients and healthy controls were not considered.

Table 6. Frequencies of the 4-locus VDR FokI-BsmI-ApaI-TaqI haplotypes among patients and healthy controls

VDR gene Haplotypes *	Patients (n = 274)		Controls (n = 203)				
	n	f	n	f	p	pc	OR, 95% CI
1/ F-B-A-T	53	0.10	48	0.12	0.286	NS	0.80 (0.52-1.23)
2/ f-B-A-T	23	0.04	3	0.01	0.001	0.016	5.89 (1.75-30.78)
3/ B-A-t-F	125	0.19	105	0.26	0.276	NS	0.85 (0.62-1.15)
4/ -B-A-t	36	0.06	20	0.05	0.286	NS	1.36 (0.75-2.47)
5/ F-B-a-T	22	0.04	24	0.06	0.176	NS	0.67 (0.35-1.25)
6/ f-B-a-T	17	0.03	5	0.01	0.079	NS	2.57 (0.90-8.97)
7/ F-B-a-t	3	0.005	1	0.002	0.641	NS	2.23 (0.18-117.32)
8/ f-B-a-t	3	0.005	0	0.000	0.266	NS	ND
9/ F-b-A-T	46	0.08	24	0.06	0.146	NS	1.46 (0.85-2.51)
10/ f-b-A-T	38	0.07	30	0.07	0.787	NS	0.93 (0.55- 1.58)
11/ F-b-A-t	14	0.02	13	0.03	0.551	NS	0.79 (0.35-1.81)
12/ f-b-A-t	7	0.01	0	0.00	0.023	NS	ND
13/ F-b-a-T	141	0.26	84	0.21	0.070	NS	1.33 (0.97-1.83)
14/ f-b-a-T	13	0.03	48	0.12	<0.00001	<0.00001	0.18 (0.09-0.35)
15/ F-b-a-t	1	0.002	1	0.002	1.000	NS	0.74 (0.01-58.26)
16/ f-b-a-t	6	0.01	0	0.000	0.041	NS	ND

n: number; OR: odds ratio; f: frequency; CI: confidence interval; pc: corrected p-value; NS: non-significant

*Haplotypes nomenclature refers to 4 loci composed of FokI-BsmI-ApaI-TaqI SNPs in VDR. Haplotype frequencies < 5% among patients and healthy controls were not considered.

Results

For all the studied SNPs, the observed genotype distribution among controls and cases satisfied the expected Hardy Weinberg ratio. Logistic regression adjusted for age and sex was performed and no potential role of these confounding factors was found in all SNP results (Table 4).

TLR2 (-16934, +597, +1349) polymorphisms

Among TLR2 SNPs, the TLR2 +597 TC heterozygous genotype was found to be more prevalent in controls than in patients (60% *vs.* 49%, respectively; pc = 0.04, p = 0.01, OR = 0.65, 95% CI = 0.45-0.94 (Table 2). The observed association between the TC genotype and protection against PTB was further confirmed by a logistic regression analysis (p = 0.012, adjusted OR = 0.64, 95% CI = 0.44-0.90) (Table 4). A trend towards an increase of the CC genotype frequency in patients as compared to controls was also observed, but this difference was slightly significant (p = 0.07). Nevertheless, the allelic distribution of this variant did not show any significant difference between patients and controls (p > 0.05) (Table 2).

Concerning the TLR2 -16934 and TLR2 +1349 genetic variations, no significant difference was noted in the distribution of allele and genotype frequencies between both groups (*TLR2 -16934* :allele frequencies: *A*: 51% vs 51%, *T*: 48% vs 49% and genotype frequencies: *AA*: 26% vs 25%, *AT*: 50% vs 52%, *TT*: 23% vs 23% and *TLR2 +1349*: allele frequencies: *T*: 92% vs 91%, *C*: 08% vs 09% and genotype frequencies: *TT*: 88% vs 86%, *TC*: 08% vs 10% and *CC*: 03% vs 04% among patients and controls respectively). Nevertheless, haplotype analysis showed that the TLR2 T-C-T (-16934T, +597C, +1349T) haplotype was more prevalent in patients than in controls, but failed to reach statistical significance after correction for the number of comparisons (14% *vs.* 10% in patients and controls, respectively; p = 0.03, pc = 0.24, OR = 1.52, 95% CI = 1.01-2.30 (Table 5).

TLR4 (-6143, -5727, +4434, +7267, +8469) polymorphisms

Among the five SNPs analyzed in the TLR4 gene, the TLR4 +7267 C allele was found to be more prevalent in PTB patients than in healthy controls (13% *vs.* 8%; p = 0.04, pc = 0.02, OR = 1.63, 95% CI = 1.06-2.57) (Table 3). Analysis of the TLR4 +7267 CC genotype distribution revealed that the homozygous state of the TLR4 +7267 C allele was also more prevalent in PTB patients than in healthy controls, but with marginal statistical significance (4% *vs.* 0.5%; pc = 0.12, p = 0.04, OR = 7.82, 95% CI = 1.06-161.44) (Table 3). These results were further confirmed by a logistic regression analysis (C allele: p = 0.02, adjusted OR = 1.63, 95% CI = 1.07- 2.48; CC genotype: p = 0.048, adjusted OR = 7.86, 95% CI = 1.02-60.65) (Table 4). No associations were seen for the remaining TLR4 SNPs and no additional information was provided upon analysis of the TLR4 haplotypes distribution (Data not shown).

VDR (FokI, BsmI, ApaI, TaqI) polymorphisms

The distribution of allele and genotype frequencies was not significantly different between cases and controls for any of the VDR studied SNPs (Data not shown). However, among the 16 PHASE-generated VDR haplotypes, the VDR f-b-a-T (FokI f, BsmI b, ApaI a, TaqI T) haplotype was significantly higher in controls than in patients, and remained statistically significant after correction for the number of comparisons (12% *vs.* 3%, respectively; pc < 0.00001, p < 0.00001, OR = 0.18, 95% CI = 0.09-0.35), suggesting a protective effect (Table 6).

Gene-gene interaction analysis

In addition to single locus and haplotype analyses, a statistical cross-analysis was also performed in order to determine whether gene-gene interactions may influence susceptibility to or protection against PTB in the studied cohort. The minor allele frequencies of TLR2, VDR, and TLR4 single SNPs were ≥ 0.05, and these SNPs were therefore included in the cross-analysis. This allowed observation of three interactions between TLR2 +597 SNP and TLR4 +4434 or VDR (FokI and BsmI) SNPs (0.004 < p < 0.031) (Table 7). The combination of the TLR4 +4434AA and the TLR2 +597 CT genotypes was found to be significantly associated with protection against PTB, (p = 0.004, OR = 0.59, 95% CI = 0.41-0.86) (Table 7). Furthermore, the VDR Ff heterozygote genotype showed a trend either to associations with susceptibility to or protection against PTB respectively depending on the presence of the TLR2 +597 CC genotype (p = 0.02) or the TLR2 +597 CT genotype (p = 0.01), respectively (Table 7). A trend towards protection was also observed with the combination of the VDR Bb and the TLR2 +597 CT genotypes (p = 0.03) (Table 7). Among the observed interactions, only the interaction detected between TLR2 +597CT and TLR4 +4434AA remained statistically significant after correction for the number of comparisons (pc = 0.03), suggesting a strong synergistic effect between the two SNPs with respect to protection against PTB (Table 7). Indeed, this effect was found to be more stringent towards protection as compared with those that yielded (i) by a single TLR2 +597CT genotype effect (TLR2 +597CT/TLR4 +4434AA: pc = 0.03, p = 0.004, OR = 0.59, 95% CI = 0.41-0.86 *versus* TLR2 +597CT: pc = 0.04, p = 0.01, OR = 0.65, 95% CI = 0.45-0.94) (Table 2) or (ii) by genotypic combinations of TLR2 +597 CT and VDR Ff or VDR Bb genotypes (TLR2 +597 CT /VDR Ff : p = 0.01, OR = 0.56, 95% CI = 0.35-0.89 and TLR2 +597 CT/ VDR Bb : p = 0.03, OR = 0.65, 95% CI = 0.43-0.98 respectively) (Table 7). Of note is that the TLR4 +4434 and VDR (FokI, BsmI) SNPs showing genetic interactions did not have any statistically significant effects individually (data not shown).

Discussion

In this study, we analyzed the potential associations between TLR2, TLR4, and VDR functionally relevant polymorphisms and PTB in a Moroccan population group. Single locus association analyses allowed us to identify significant association with resistance and susceptibility to PTB at TLR2 +597 SNP (CT genotype) and TLR4 +7263 (C allele), respectively, in our population. No significant associations were observed between TLR2 (-16934, +1349), TLR4 (-6143, -5727, +4434, +8469), and VDR (FokI, BsmI, ApaI, TaqI) SNPs and PTB risk.

In terms of TLR2 polymorphism, our finding concerning the observed protective effect of the TLR2 +597 CT genotype is in contradiction with data from a previous study on Vietnamese patients. Indeed, the authors of that study showed that such genotype did not influence the susceptibility to PTB while the C allele and the CC genotype were both associated with TB, particularly with tuberculous meningitis severity and co-existence of miliary TB, suggesting that this variant could affect the dissemination of *Mtb* [29]. These conflicting results could be explained by genetic heterogeneity between the two studied samples or by potential difference in the involved *Mtb* strains. The molecular mechanism by which the TLR2 +T597 T/C change could influence the susceptibility or the resistance to PTB is still unknown. As this variant is a synonymous mutation, it could merely reflect another, yet-to-be-identified marker in close linkage disequilibrium (LD). In this context, a microsatellite region within the TLR2 intron-2 (GT repeat) affecting gene regulation was previously described with significant frequency variations across populations [7]. Interestingly, association between this microsatellite and PTB was found among Koreans on one hand [7,8], and a high level of linkage disequilibrium (LD) between the C allele of the +597 T/C SNP and the allele of the GT microsatellite has been suggested on the other [29]. Since LD patterns can vary among population groups, this may cause differences between disease associations of this SNP in different populations. Thus, it is possible that the LD between the causal mutation and TLR2 +597 T/C may be different between Moroccan and Asian individuals.

Future studies examining this in our population are warranted.

The analysis of TLR2 polymorphisms in terms of haplotypes revealed a trend towards susceptibility status of the TLR2 (-16934T, +597C, +1349T) haplotype. This latter contains mutant alleles of both TLR2 -16934 and TLR2 +597 together with the wild-type allele of TLR2 +1349. It is important to highlight that the latter has been associated with susceptibility to sepsis, Gram-positive bacteria [30], severe atopic dermatitis [31], and asthma in farmers' children [32], and that carriage of the A allele has been linked to increased production of TNF-α, IL-6 and IL-12 cytokines [33], all known to be major components of immune responses against *Mtb*. This suggests that the TLR2 -16934 T allele involved in the TLR2 (-16934T, +597C, +1349T) haplotype may result in a reduction of the production of these inflammatory cytokines, which may deregulate Th1 response and would, in turn, impact the individual's susceptibility to PTB. Moreover, as described above, the TLR2 +597 C allele variant could, within this specific haplotype, reflect a susceptibility status. Of note, the TLR2 -16934 and +1349 single SNPs did not have statistically significant individual effects. Altogether, these observations are consistent with a recent study involving Taiwanese PTB patients, reporting an association between a specific TLR2 haplotype including the TLR2 -16934 SNP and PTB susceptibility rather than this SNP alone [34]. This suggests that this variant may act through an additive effect in the presence of other genetic variations. These results together suggest that the observed effect is more likely caused by interactions between the three TLR2 SNPs, which may capture their effects or those of other functional polymorphisms within/or linked to this haplotype. They may also explain conflicting association data of TLR2 genetic diversity in populations since it is possible that statistically significant associations may be obtained only in presence of specific alleles of other SNPs within this gene. Further association studies involving large Moroccan cohorts and functional haplotype studies are needed to replicate/validate our results and to potentially precise the role of such polymorphisms and the mechanistic of their interactions in PTB susceptibility.

Concerning the TLR4 genetic diversity, we found that the TLR4 +7263 C allele is associated with susceptibility to PTB. A trend towards such association was also observed with the homozygous CC genotype and could be significant with increasing sample size for both the cases and control groups. The functional significance of this polymorphism is not known. However, it is possible that TLR4 +7263 SNP may have an effect on mRNA stability and transcription and/or translation efficiency. This might impair function of the TLR4 molecule and interfere with the host innate immunity. Interestingly, this allele was previously demonstrated to be associated with the risk of developing emphysema [35], severe periodontitis [36], and gastric atrophy in *Helicobacter pylori*-seropositive subjects [37]. However, this variant was not associated with autoimmune pancreatitis [38] or sarcoidosis-related uveitis [39]. Our study is the first, to our knowledge, to describe a significant association of this variant with PTB susceptibility. However, this variant seems to have no effect within reconstructed TLR4 haplotypes and it did not interact with any of the TLR2 SNPs. Despite the relevant functionality of the TLR4 +4434 polymorphism [17], we did not observe an association with PTB susceptibility, in agreement with previous studies examining such SNP and TB susceptibility in Gambia [19], Mexico [20], and Guinea [40]. Similarly, a lack of association was also reported in Dutch sarcoidosis patients [41]. However, TLR4 +4434 has been associated with PTB, particularly with severe forms of the disease, in an Indian population [18], and was found to increase the risk of acute TB and decline in CD4 +T cell counts in HIV-infected patients in Tanzania [42]. The considerable differences in the allele frequencies of this polymorphism among different populations might explain such discrepancy. This polymorphism was shown to be much rarer in Asian than in European and African populations; it was almost absent in population groups from China [43], Japan [44], and Vietnam [45]. However, our results indicate that this mutation occurred with a frequency of 6% observed in the healthy Moroccan population group. This is relatively high as compared with the reported frequency of 3% among European Caucasians [46-48] and lower than the frequency of 9% in a North Indian population [18] and 5%-13% in ethnic groups of Gambia [19]. Further cross-analysis showed, however, that the combination of the TLR4 +4434 AA and the TLR2 +597 CT genotypes exerted a strong protective effect against PTB. In this context, Arbour *et al.* previously showed that epithelial cells and alveolar macrophages of individuals carrying the minor TLR4 +4434 G allele exhibited a decreased response to LPS stimulation *in vitro*, both at the homozygous and the heterozygous state, and that the clinical phenotype could be reversed with the wild

type allele [17]. Because of its situation within the extracellular domain of TLR4, the impact of the TLR4 +4434 is likely caused by a decreased recognition of specific TLR4 ligands. This variant is located close to the TLR4-MD2 binding region [49], and the amino-acid replacement changes the negative charge (Asp) at position 299 in TLR4 to a neutral charge (Gly) and increases the rotational freedom of the peptide bond [50]. This conformational change in the extracellular domain may therefore modulate the interaction of ligands such as LPS with the TLR4 receptor [51], leading to impaired immune response. As the observed interaction in our study included the wild type allele at the homozygous state, the above-mentioned phenotype could be theoretically reversed, leading to an adequate TLR4 recognition of *Mtb* ligands with consequent protection. This suggests that TLR4 +4434 may contribute to resistance to PTB through a synergistic effect of TLR2 +597. As tuberculosis is a multifactorial and polygenic disorder, these results suggest cooperation between both TLRs conferring greater specificity or broader recognition capacity of microbiological components to the TLR proteins by, for example, the concomitant activation of MyD88 (myeloid differentiation primary response protein 88), and TRIF (TIR domain containing protein inducing IFN-β) adaptor proteins by TLR4 [52-54], and the recognition of large variety of *Mtb* components by TLR2 [55-57]. It is therefore tempting to speculate that cooperation of both pattern recognition receptors may play important role in innate responses to *Mtb*.

Regarding VDR genetic diversity, no significant association was found between alleles or genotypes of such locus and PTB (Table 4). Our observations fit within the accumulated discordant results provided by numerous studies in terms of susceptibility or protection [26,40,58-66]. However, our data concerning the VDR f-b-a-T haplotype's protective effect is in agreement with the literature describing associations between VDR haplotypes and TB [26,60] and the collective influence of VDR polymorphisms on the VDR activity rather than an individual SNP effect [67]. Furthermore, the b and T alleles observed in our protective haplotype are in common with those of the F-b-A-T haplotype previously found in Venda, West Africa [26]. Moreover, in a large South African study, the F-a-T haplotype tends to be associated with resistance to TB, whereas the F-A-T haplotype represent a risk factor [68]. On the other hand, Selvaraj *et al*. suggested that the FF genotype may be associated with increased VDR expression, leading to an overuse of vitamin D3 in patients carrying this genotype with consequent vitamin D3 deficiency and greater risk for spinal TB [66]. The protective f-b-a-T haplotype found in our study is also in agreement with the protective effect conferred by the TT genotype in South Indians [62] and by the A allele or the F-A combination that is transmitted more often than expected to affected offspring in West Africa [26]. In contrast, this association seems in disagreement with that of the F allele, which increases the VDR function in combination with the L allele of poly A microsatellite in exon IX (L/S) [69]. It seems also in disagreement with both f allele and ff genotype, which were previously associated with TB susceptibility in China [64] and Gujurati Asians in the UK [58], and with the presence of the t allele found to be protective in both Gambians [61] and Gujurati Asians in the UK [58]. In another study involving Guineans, no association with PTB was detected either with single VDR polymorphisms or haplotypes [40]. These inconsistencies may result from the use of non-functional SNPs (BsmI, ApaI and TaqI), which are more likely markers of truly causative polymorphisms. The association of non-functional variants depends on the LD patterns across the relevant chromosomal region, which may differ between populations [70] and contribute to heterogeneity among associations found [71]. Moreover, variations in haplotype blocs and in allelic and genotypic distribution of the four VDR SNPs between populations have been reported [70,72] and could explain this inconsistency. This may suggest that the association found between the f-b-a-T haplotype and protection from PTB in our population is likely due to linked polymorphisms within this haplotype that might be directly involved in protection from PTB. In addition, the interactions between different genes and environmental factors are known to play a role in the action of VDR [58,73]. These interactions most likely differ between populations and may also generate conflicting association data. The multiple influences of vitamin D3 on the transcription processes of several endocrine pathways [74] may also contribute to this heterogeneity. Finally, statistical cross-analysis of the TLR2 and VDR SNPs among patients and controls detected a trend towards genetic interactions between TLR2 +597 and VDR FokI or VDR BsmI SNPs, which requires confirmation by a large sample size of patient and control study. TLR2 and VDR both have important roles in the control of *Mtb* infection. TLR2/TLR1 activation of human MPs induces up-regulation of the VDR and vitamin D1-hydroxylase gene expression, leading to induction of

cathelicidin with consequent elimination of intracellular *Mtb* [25].

In conclusion, the present study highlights the importance of investigating haplotypic distribution and gene-gene interaction in association studies. In the future, an ongoing much more larger study will allow to replicate/confirm the present findings and to look for and possibly to identify the causal variant(s). Along theses lines, functional studies will possibly shed light on the underlying mechanism involving such variants and their interactions hopefully leading to new personalized therapeutic options .

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