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Age- and gender-specific effects on *NRAMP1* gene polymorphisms and risk of the development of active tuberculosis in Tunisian populations

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SUMMARY

Background: Studies that have assessed *NRAMP1* polymorphisms and their association with susceptibility to tuberculosis (TB) in humans have yielded conflicting results. In this study, we evaluated the association between *NRAMP1* gene polymorphisms and the risk of the development of active TB in Tunisian populations.

Methods: The distribution of 3′-UTR and D543N polymorphisms in 223 TB patients (168 patients with pulmonary TB (PTB) and 55 patients with extrapulmonary TB (EPTB)) and 150 healthy donors was determined by PCR-restriction fragment length polymorphism (RFLP) method.

Results: We found that AA and AG genotypes appeared to be associated with susceptibility to PTB (odds ratio (OR) 10.8, 95% confidence interval (CI) 1.37–230.8; p corrected for the number of genotypes (pc) = 0.018) and EPTB (OR 4.37, 95% CI 1.64–11.82; pc = 0.0024), respectively, in patients aged less than 30 years. However, wild-type GG genotype appeared to be associated with resistance against PTB in females (OR 0.1, 95% CI 0.01–0.74; pc = 0.03). The 3′-UTR del/del genotype appeared to be associated with susceptibility to PTB in patients aged less than 30 years (OR 3.75, 95% CI 1.5–9.52; pc = 0.003). In contrast, TGTG+/del might be associated with resistance against the development of active PTB (OR 0.23, 95% CI 0.08–0.65; pc = 0.003). A-del haplotype appeared to be associated with susceptibility to PTB (OR 1.79, 95% CI 1.11–2.9; pc = 0.04).

Conclusions: Collectively, our results suggest an association of *NRAMP1* 3′-UTR and D543N polymorphisms with susceptibility to mycobacterial infection in Tunisian populations in relation to age and sex.

1. Introduction

Tuberculosis (TB) is a major cause of morbidity and mortality worldwide, especially in Southeast Asian, African, and Western Pacific regions. Globally, an estimated 8.9–9.9 million new cases and 1.3 million deaths among HIV-negative individuals were reported in 2009.[1] In addition, it has been estimated that only 10% of HIV-negative individuals infected with *Mycobacterium tuberculosis* will develop clinical disease in their lifetime. A minority of individuals have a higher susceptibility to TB, most likely resulting from interplay between multiple host and environmental factors.[2–4] Some genetic variations, such as Mendelian-inherited mutations in the genes encoding interferon gamma (IFN-γ) receptor, interleukin-12, and signal transducers, are rare and are associated with severe mycobacterial infection.[5–8] We have recently reported that IFN-γ polymorphism is associated with an increased risk of the development of active TB in Tunisian patients.[9] Other genetic variations, such as polymorphism of genes encoding human leukocyte antigen (HLA) type,[10,11] vitamin D receptor (VDR),[12] dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN),[13] complement system components C2, C4, BF, and C3,[14] and the solute carrier family 11 member a1 protein (SLC11A1, formerly known as NRAMP1),[4,15,16] are known to be key factors in the development of TB.

The *NRAMP1* gene encodes an ion transporter that localizes to the lysosomal membrane during the phagocytosis of mycobacteria and other pathogens, and it might regulate ion or divalent cation transport.[17,18] It is well established that the *NRAMP1* gene product plays a pivotal role in resistance to intracellular pathogens, including bacillus Calmette–Guérin (BCG), Leishmania, Salmonella, and mycobacteria.[19] Taken together, these data suggest the importance of *NRAMP1* in the human immune response to *M. tuberculosis* infection.

NRAMP1 has been cloned and mapped to human chromosome 2q35.[20,21] Several functional polymorphisms have been described in the *NRAMP1* gene.[20,22] In addition, these polymorphisms have been associated with a number of diseases, including TB,

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autoimmune disease, meningococcal meningitis, and leishmaniasis.[23] Four polymorphisms – 3′-UTR, D543N, INT4, and 5′(GT)n – have been examined for their association with susceptibility to TB in different studies, and the data have been summarized in recent reviews, as well as in a meta-analysis of various studies.[16,24–27] In the meta-analysis, a subgroup analysis of the four separate polymorphisms indicated that three of them are associated with susceptibility to TB in Asian populations: 3′-UTR, D543N, and 5′(GT)n. No significant results were obtained when European studies were grouped. African populations showed significance in all studies, except the 3′-UTR.[4,15,25,26] Collectively, these data showed ethnic and geographic differences in human susceptibility to active TB and *NRAMP1* polymorphisms.[4–15]

Tunisia is a region with moderate TB prevalence (9.5 new cases per 100 000 population) and incidence (21 cases/100 000/year) and a predominating *M. tuberculosis* strain.[28] Because the *NRAMP1* gene is an important candidate factor for human susceptibility to active TB and there is no report investigating its impact on active TB outcomes in Tunisian populations, the present study focused on the association between *NRAMP1* polymorphisms and susceptibility to active TB in Tunisia. The association between 3′-UTR and D543N *NRAMP1* polymorphisms and the risk of developing active TB (pulmonary and extrapulmonary) was investigated in the Tunisian population grouped by sex and age.

2. Materials and methods

2.1. Studied populations

2.1.1. Patients with tuberculosis

From January 2009 to September 2010, a total of 168 patients with active pulmonary TB (PTB) (127 males and 41 females; age range 14–78 years, mean age 44 years) and 55 patients with extrapulmonary TB (EPTB) (33 males and 22 females; age range 17–84 years, mean age 42 years), from the Pneumology Unit, CHU Farhat Hached and the Health Care Service, Sousse, Tunisia were enrolled in this study. Informed written consent was obtained from all individuals prior to blood sampling, and this study was approved by the Ethics Committee of the Farhat Hached University Hospital. Demographic, clinical, and microbiological data for all adult subjects were collected on individual data collection forms and entered into a computer database. None of the patients with active TB or the controls had a history of severe pathologies, including HIV infection, cardiovascular diseases, diabetes, asthma, atopy or autoimmune diseases, and cancer. All patients had been vaccinated with BCG.

The patients had not yet started anti-tuberculosis treatment when the blood samples were taken.

Patients with active PTB were diagnosed clinically, including a chest radiograph, and the diagnosis subsequently confirmed by conventional bacteriological examination, including microscopic examination for acid-fast bacilli and culture on Lowenstein–Jensen medium, in accordance with the statements of the American Thoracic Society.[29]

The diagnosis of EPTB cases was based on conventional bacteriological examination and/or histological features (granulomatous formation). The extrapulmonary locations comprised: stomach ($n = 1$), genitourinary tract ($n = 2$), lymph nodes ($n = 36$), meninges ($n = 2$), peritoneum ($n = 3$), pleura ($n = 8$), and spleen ($n = 3$).

2.1.2. Controls

Blood samples were obtained from healthy control subjects (135 males and 15 females; age range 24–55 years, mean age 35 years) admitted to the Transfusion Unit, CHU Farhat Hached, Sousse, Tunisia. None of the control subjects had previously suffered from TB and all had negative chest X-rays and negative sputum culture results for *M. tuberculosis*.

2.2. DNA extraction

Genomic DNA was prepared from 300 μl of fresh blood peripheral blood mononuclear cells (PBMCs) using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) in accordance with the manufacturer's recommendations; this was then quantified using a NanoDrop spectrophotometer (UV-Visible NanoDrop 1000, Thermo Fisher Scientific Inc.) and standardized to 50 ng/μl. Aliquots were stored at −20 °C for further genotyping.

2.3. NRAMP1 genotyping

The following two *NRAMP1* polymorphisms were typed: (1) a nonconservative single-base substitution at codon 543 in exon 15 that changes aspartic acid to asparagine (D543N), and (2) a TGTG deletion in the 3′-untranslated region (3′-UTR) located at 55 nucleotides downstream of the last codon in exon 15 (1729 +55del4).[30,31]

A large number of single-nucleotide polymorphisms (SNPs) have already been identified, with more than 21 000 entries in the NCBI SNP database alone (http://www.ncbi.nlm.nih.gov/snp). The ability to genotype this abundant source of variation rapidly and accurately is becoming ever more important in the genetics community.[32] A variety of available technologies have the potential for use in the high-throughput genotyping laboratory.[33] Of these technologies, polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP), which is relatively simple and economical, can be applied to a large number of samples.[34]

PCR-RFLP analysis was used to type polymorphisms of the *NRAMP1* genes, as described in a previous study.[30] Briefly, the 3′ region of *NRAMP1*, including two sites of polymorphism (D543N and 3′-UTR), was amplified using sense primer 5′-GCATCTCCC-CAATTCATGGT-3′ and antisense primer 5′-AACTGTCCCACTC-TATCCTG-3′. The product size was 240 or 244 bp. PCR amplifications were performed in 25-μl reaction volumes containing 100 ng genomic DNA, 2.0 mmol/l $MgCl_2$, 200 μmol/l of each deoxynucleotide triphosphate, 10 pmol of each primer, and 1 U Taq DNA polymerase (Promega, USA). The mixture was then initially subjected to 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; the final extension was at 72 °C for 7 min; a MyCycler thermal cycler (Bio-Rad, USA) was used. To determine each polymorphism, restriction enzymes were used: *Ava*II (G↓GWCC) was used for D543N, with allele G (aspartic acid) showing at three bands of 126 bp, 79 bp, and 39 bp, and allele A (asparagine) showing at two bands of 201 bp and 39 bp (Figure 1); *Fok*I (GGATG(9/13)) was used for 3′-UTR, with allele TGTG+ showing 211 bp and 33 bp fragments, and allele del showing a 240 bp fragment (Figure 2). The digestion products were separated by electrophoresis on 4% agarose gels (Sigma, USA) containing ethidium bromide (0.5 μg/ml) (Sigma, USA) and were visualized under UV illumination using Gel Doc XR (Bio-Rad, USA).

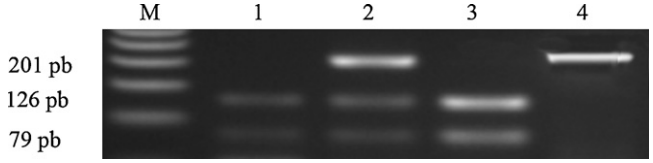

Figure 1. D543N genotyping by PCR-RFLP. GG genotype = lanes 1 and 3; AG genotype = lane 2; AA genotype = lane 4. M: 50 bp DNA size markers. Numbers on the left indicate the size of the amplicons.

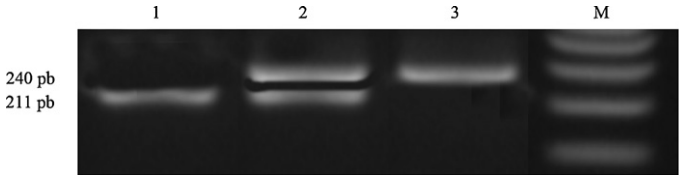

Figure 2. 3′-UTR genotyping by PCR-RFLP. TGTG+/+ genotype = lane 1; TGTG+/del genotype = lane 2; del/del genotype = lane 3. M: 50 bp DNA size markers. Numbers on the left indicate the size of the amplicons.

2.4. Statistical analysis

For genetic association analyses, each polymorphism was tested for deviations from Hardy–Weinberg equilibrium in patients and controls.[35]

The statistical analysis was performed using Epi Info 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA). The associations between the allelic/genotype frequencies and the clinical forms of TB, as well as the odds ratio (OR) for the susceptibility to infection, were obtained by Chi-square test. A *p*-value of <0.05 was considered statistically significant. The Bonferroni correction was applied for multiple tests, and $pc < 0.05$ was considered to show a statistically significant difference.

In the present study, sample sizes were calculated considering a frequency of the *NRAMP1* D543 AA genotype in the general population of 45.58%, as reported in a previous case–control study carried out in Africa in 1998.[24] Considering a 5% level of significance and a power of 80%, the minimum number of subjects needed for the study to detect an OR of 2.0 was 148 cases and 148 controls.

Haplotype frequencies were inferred by exact solution from the Hill equation using the program CubEX (http://www.oege.org/software/cubex). This program estimates the haplotype frequencies, the normalized linkage disequilibrium (LD) parameter (D′), and the LD correlation coefficient between two loci (r^2).[36]

3. Results

3.1. Hardy–Weinberg equilibrium

The genotype frequencies of *NRAMP1* D543N and 3′-UTR polymorphisms were in Hardy–Weinberg equilibrium in both the patient and control groups ($p > 0.05$).

3.2. Associations of NRAMP1 D543N with TB susceptibility

In this study, genotyping of 3′-UTR and D543N *NRAMP1* polymorphisms was investigated by two PCR-RFLPs, as reported previously.[30,31] In their study, Roy et al.[31] confirmed the results obtained by PCR-RFLP for the SNPs investigated using direct sequencing.

The frequencies of the A and G alleles were the variables investigated in four studied groups: PTB and EPTB together (Table 1), PTB alone (Table 2), EPTB alone (Table 3), and controls. Compared with healthy controls, the A allele was significantly more frequent in the TB group and seemed to be associated with an increased risk of the development of these clinical forms (PTB and EPTB; 20% vs. 12%; OR 1.85, 95% confidence interval (CI) 1.2–2.88, $p = 0.003$) (Table 1), and of PTB (21% vs. 12%; OR 1.93, 95% CI 1.22–3.06; $p = 0.002$) (Table 2).

The three genotypes AA, AG, and GG were observed in the PTB and control groups (Figure 1). Even after correcting *p*-values (*pc*) using the Bonferroni method for multiple tests, a significant association was found between the AA genotype and an increased risk of the development of PTB (OR 9.43, 95% CI 1.22–199.3; $pc = 0.03$). However, a low OR value (OR 0.55, 95% CI 0.32–0.92; $pc = 0.03$) suggested that the presence of the wild-type GG genotype might be protective against the development of active PTB.

When TB patients and healthy individuals were stratified by sex, significant differences in the distribution of genotypes was observed in females for wild-type GG genotype in the PTB group (5% vs. 33%, OR 0.1, 95% CI 0.01–0.74; $pc = 0.03$). In addition, patients aged less than 30 years with AA genotype appeared to be at increased risk of developing PTB (OR 10.8, 95% CI 1.37–230.8; $pc = 0.018$) (Table 4), and with AG genotype at increased risk of developing EPTB (OR 4.37, 95% CI 1.64–11.82; $pc = 0.0024$) (Table 5). However, patients with GG genotype appeared to be protected against the development of active EPTB (OR 0.25, 95% CI 0.09–0.65; $pc = 0.003$) (Table 5).

3.3. Associations of NRAMP1 3′-UTR with TB susceptibility

The del allele frequencies were similar in the different studied groups – PTB, EPTB, and healthy controls (Tables 2 and 3). There was no statistically significant difference in the frequency distribution of the three genotypes del/del, TGTG+/del, and TGTG+/+ in the TB patient and control groups (Tables 1–3).

Table 1
D543N and 3′-UTR allele and genotype frequencies (*n* (%)) in pulmonary and extrapulmonary tuberculosis cases and in controls

	PTB + EPTB ($n = 223$)	Controls ($n = 150$)	*p*-Value (*pc*[a])	OR (95% CI)
D543N				
Genotype				
AA	10 (5)	1 (1)	0.02[b] (NS)	7 (0.91–147.61)
AG	70 (31)	34 (23)	0.06 (NS)	1.56 (0.95–2.58)
GG	143 (64)	115 (76)	0.01 (0.03)	0.54 (0.33–0.89)
Allele				
A	90 (20)	36 (12)	0.003	1.85 (1.2–2.88)
G	356 (80)	264 (88)		
3′-UTR				
Genotype				
del/del	102 (46)	68 (46)	0.93 (NS)	1.02 (0.66–1.57)
TGTG+/del	88 (39)	62 (41)	0.71 (NS)	0.93 (0.59–1.44)
TGTG+/+	33 (15)	20 (13)	0.69 (NS)	1.13 (0.6–2.14)
Allele				
del	292 (65)	198 (66)	0.88	0.98 (0.71–1.35)
TGTG+	154 (35)	102 (34)		

PTB, pulmonary tuberculosis; EPTB, extrapulmonary tuberculosis; OR, odds ratio; CI, confidence interval; NS, not significant.
[a] *pc*: Bonferroni correction. As multiple comparisons were made, Bonferroni's correction was applied to a significant *p*-value that was multiplied by the number of genotypes (three).
[b] Fisher's exact test.

Table 2
D543N and 3′-UTR allele and genotype frequencies (*n* (%)) in pulmonary tuberculosis cases and in controls by gender

	PTB (*n* = 168)			Controls (*n* = 150)			*p*-Value[a] (*pc*)	OR[a] (95% CI)	*p*-Value[b] (*pc*)	OR[b] (95% CI)	*p*-Value[c] (*pc*)	OR[c] (95% CI)
	M+F (%)	M (%)	F (%)	M+F (%)	M (%)	F (%)						
D543N												
Genotype												
AA	10 (6)	7 (6)	3 (7)	1 (1)	1 (1)	0 (0)	0.01 (0.03)	9.43 (1.22–199.3)	0.02[d] (NS)	7.82 (0.95–171.49)	0.38[d] (NS)	ND
AG	50 (30)	14 (11)	36 (88)	34 (23)	24 (18)	10 (67)	0.15 (NS)	1.45 (0.85–2.47)	0.16 (NS)	0.61 (0.28–1.3)	0.07[d] (NS)	3.6 (0.71–18.75)
GG	108 (64)	106 (83)	2 (5)	115 (76)	110 (81)	5 (33)	0.01 (0.03)	0.55 (0.32–0.92)	0.67 (NS)	1.15 (0.58–2.28)	0.01 (0.03)[d]	0.1 (0.01–0.74)
Allele												
A	70 (21)	28 (11)	42 (51)	36 (12)	26 (10)	10 (33)	0.002	1.93 (1.22–3.06)	0.59	1.16 (0.64–2.12)	0.09	2.1 (0.81–5.53)
G	266 (79)	226 (89)	40 (49)	264 (88)	244 (90)	20 (67)						
3′-UTR												
Genotype												
del/del	80 (48)	60 (47)	20 (49)	68 (46)	63 (47)	5 (34)	0.68 (NS)	(0.69–1.75)	0.92 (NS)	1.02 (0.61–1.71)	0.3 (NS)	1.9 (0.48–7.86)
TGTG+/del	67 (40)	52 (41)	15 (36)	62 (41)	54 (40)	8 (53)	0.79 (NS)	0.94 (0.59–1.51)	0.87 (NS)	1.04 (0.62–1.76)	0.25 (NS)	0.5 (0.13–1.95)
TGTG+/+	21 (12)	15 (12)	6 (15)	20 (13)	18 (13)	2 (13)	0.82 (NS)	0.93 (0.46–1.88)	0.71 (NS)	0.87 (0.39–1.92)	0.63[d] (NS)	1.11 (0.17–9.2)
Allele												
del	227 (68)	172 (68)	55 (67)	198 (66)	180 (67)	18 (60)	0.67	1.07 (0.76–1.51)	0.79	1.05 (0.72–1.54)	0.48	1.36 (0.52–3.5)
TGTG+	109 (32)	82 (32)	27 (33)	102 (34)	90 (33)	12 (40)						

PTB, pulmonary tuberculosis; M, male; F, female; OR, odds ratio; CI, confidence interval; NS, not significant; ND, not determined; *pc*, Bonferroni correction (as multiple comparisons were made, Bonferroni's correction was applied to a significant *p*-value that was multiplied by the number of genotypes (three)).
[a] Male and female cases of pulmonary tuberculosis vs. male and female control cases.
[b] Male cases of pulmonary tuberculosis vs. male control cases.
[c] Female cases of pulmonary tuberculosis vs. female control cases.
[d] Fisher's exact test.

Table 3
D543N and 3′-UTR allele and genotype frequencies (*n* (%)) in extrapulmonary tuberculosis cases and in controls by gender

	EPTB (*n* = 55)			Controls (*n* = 150)			*p*-Value[a] (*pc*)	OR[a] (95% CI)	*p*-Value[b] (*pc*)	OR[b] (95% CI)	*p*-Value[c] (*pc*)	OR[c] (95% CI)
	M+F (%)	M (%)	F (%)	M+F (%)	M (%)	F (%)						
D543N												
Genotype												
AA	0 (0)	0 (0)	0 (0)	1 (1)	1 (1)	0 (0)	0.7[d] (NS)	0 (0–47.94)	0.8[d] (NS)	0 (0–72.71)	ND	ND
AG	20 (36)	1 (3)	19 (86)	34 (23)	24 (18)	10 (67)	0.04 (NS)	1.95 (0.95–4.01)	0.02[d] (NS)	0.14 (0.01–1.07)	0.1 (NS)	3.17 (0.5–21.81)
GG	35 (64)	32 (97)	3 (14)	115 (76)	110 (81)	5 (33)	0.06 (NS)	0.53 (0.26–1.09)	0.02 (NS)	7.27 (0.98–149.64)	0.1[d] (NS)	0.32 (0.05–2.01)
Allele												
A	20 (18)	1 (2)	19 (43)	36 (12)	26 (10)	10 (33)	0.1	1.63 (0.86–3.08)	0.02 (NS)	0.14 (0.01–1.03)	0.3	1.52 (0.52–4.47)
G	90 (82)	65 (98)	25 (57)	264 (88)	244 (90)	20 (67)						
3′-UTR												
Genotype												
del/del	22 (40)	12 (36)	10 (46)	68 (46)	63 (47)	5 (34)	0.4 (NS)	0.8 (0.41–1.58)	0.2 (NS)	0.65 (0.28–1.53	0.46 (NS)	1.67 (0.35–8.14)
TGTG+/del	21 (38)	15 (46)	6 (27)	62 (41)	54 (40)	8 (53)	0.6 (NS)	0.88 (0.44–1.73)	0.56 (NS)	1.25 (0.54–2.87)	0.1 (NS)	0.33 (0.06–1.59)
TGTG+/+	12 (22)	6 (18)	6 (27)	20 (13)	18 (13)	2 (13)	0.1 (NS)	1.81 (0.76–4.29)	0.31[d] (NS)	1.44 (0.46–4.35)	0.27[d] (NS)	2.44 (0.34–21.17)
Allele												
del	65 (59)	39 (59)	26 (59)	198 (66)	180 (67)	18 (60)	0.1	0.74 (0.46–1.2)	0.24	0.72 (0.4–1.3)	0.93	0.96 (0.34–2.75)
TGTG+	45 (41)	27 (41)	18 (41)	102 (34)	90 (33)	12 (40)						

EPTB, extrapulmonary tuberculosis; M, male; F, female; OR, odds ratio; CI, confidence interval; NS, not significant; ND, not determined; *pc*, Bonferroni correction (as multiple comparisons were made, Bonferroni's correction was applied to a significant *p*-value that was multiplied by the number of genotypes (three)).
[a] Male and female cases of extrapulmonary tuberculosis vs. male and female control cases.
[b] Male cases of extrapulmonary tuberculosis vs. male control cases.
[c] Female cases of extrapulmonary tuberculosis vs. female control cases.
[d] Fisher's exact test.

Table 4
D543N and 3′-UTR allele and genotype frequencies (*n* (%)) in pulmonary tuberculosis cases and in controls by age group

	≤29 years		*p*-Value[a] (*pc*)	OR[a] (95% CI)	30–49 years		*p*-Value[b] (*pc*)	OR[b] (95% CI)	≥50 years		*p*-Value[c] (*pc*)	OR[c] (95% CI)
	PTB (%)	Controls (%)			PTB (%)	Controls (%)			PTB (%)	Controls (%)		
D543N												
Genotype												
AA	10 (12)	1 (1)	6.10^{-3} (18.10^{-3})	10.8 (1.37–230.8)	0 (0)	0 (0)	ND	ND	0 (0)	0 (0)	ND	ND
AG	15 (18)	17 (21)	0.6 (NS)	0.82 (0.35–1.89)	25 (43)	10 (22)	0.02 (NS)	2.65 (1.02–7)	10 (40)	7 (30)	0.4 (NS)	1.52 (0.39–5.98)
GG	60 (70)	64 (78)	0.2 (NS)	0.68 (0.32–1.44)	33 (57)	35 (78)	0.02 (NS)	0.38 (0.14–0.98)	15 (60)	16 (70)	0.4 (NS)	0.66 (0.17–2.54)
3′-UTR												
Genotype												
del/del	30 (68)	20 (36)	10^{-3} (3.10^{-3})	3.75 (1.5–9.52)	20 (40)	30 (57)	0.09 (NS)	0.51 (0.22–1.2)	30 (41)	18 (43)	0.8 (NS)	0.91 (0.39–2.1)
TGTG+/del	7 (16)	25 (46)	10^{-3} (3.10^{-3})	0.23 (0.08–0.65)	20 (40)	15 (28)	0.21 (NS)	1.69 (0.69–4.18)	40 (54)	22 (52)	0.8 (NS)	1.07 (0.47–2.45)
TGTG+/+	7 (16)	10 (18)	0.7 (NS)	0.85 (0.26–2.74)	10 (20)	8 (15)	0.86 (NS)	1.09 (0.35–3.47)	4 (5)	2 (5)	0.6[d] (NS)	1.14 (0.17–9.46)

PTB, pulmonary tuberculosis; OR, odds ratio; CI, confidence interval; NS, not significant; ND, not determined; *pc*, Bonferroni correction (as multiple comparisons were made, Bonferroni's correction was applied to a significant *p*-value that was multiplied by the number of genotypes (three)).
[a] Cases of pulmonary tuberculosis vs. control cases aged ≤29 years.
[b] Cases of pulmonary tuberculosis vs. control cases aged 30–49 years.
[c] Cases of pulmonary tuberculosis vs. control cases aged ≥50 years.
[d] Fisher's exact test.

Table 5
D543N and 3′-UTR allele and genotype frequencies (*n* (%)) in extrapulmonary tuberculosis cases and in controls by age group

	≤29 years		*p*-Value[a] (*pc*)	OR[a] (95% CI)	30–49 years		*p*-Value[b] (*pc*)	OR[b] (95% CI)	≥50 years		*p*-Value[c] (*pc*)	OR[c] (95% CI)
	EPTB (%)	Controls (%)			EPTB (%)	Controls (%)			EPTB (%)	Controls (%)		
D543N												
Genotype												
AA	0 (0)	1 (1)	0.7[d] (NS)	0 (0–48.69)	0 (0)	0 (0)	ND	ND	0 (0)	0 (0)	ND	ND
AG	16 (53)	17 (21)	8.10^{-4} (24.10^{-4})	4.37 (1.64–11.82)	3 (19)	10 (22)	0.5[d] (NS)	0.81 (0.15–3.98)	1 (11)	7 (30)	0.2[d] (NS)	0.29 (0.01–3.2)
GG	14 (47)	64 (78)	10^{-3} (3.10^{-3})	0.25 (0.09–0.65)	13 (81)	35 (78)	0.5[d] (NS)	1.24 (0.25–6.76)	8 (89)	16 (70)	0.2[d] (NS)	3.5 (0.31–89.3)
3′-UTR												
Genotype												
del/del	7 (54)	20 (36)	0.2 (NS)	2.04 (0.52–8.16)	5 (28)	30 (57)	0.03 (NS)	0.29 (0.08–1.07)	10 (42)	18 (43)	0.9 (NS)	0.95 (0.31–2.96)
TGTG+/del	2 (15)	25 (45)	0.04 (NS)	0.22 (0.03–1.21)	7 (39)	15 (28)	0.4 (NS)	1.61 (0.46–5.67)	12 (50)	22 (52)	0.8 (NS)	0.91 (0.3–2.79)
TGTG+/+	4 (31)	10 (19)	0.3 (NS)	2 (0.42–9.32)	6 (33)	8 (15)	0.09[d] (NS)	2.81 (0.69–11.44)	2 (8)	2 (5)	0.4[d] (NS)	1.82 (0.17–19.84)

EPTB, extrapulmonary tuberculosis; OR, odds ratio; CI, confidence interval; NS, not significant; ND, not determined; *pc*, Bonferroni correction (as multiple comparisons were made, Bonferroni's correction was applied to a significant *p*-value that was multiplied by the number of genotypes (three)).
[a] Cases of extrapulmonary tuberculosis vs. control cases aged ≤29 years.
[b] Cases of extrapulmonary tuberculosis vs. control cases aged 30–49 years.
[c] Cases of extrapulmonary tuberculosis vs. control cases aged ≥50 years.
[d] Fisher's exact test.

Table 6
D543N and 3′-UTR haplotype and diplotype frequencies (*n* (%)) in pulmonary tuberculosis cases, extrapulmonary tuberculosis cases, and controls

	Controls (*n* = 150) (%)	PTB + EPTB (*n* = 223) (%)	*p*-Value[a] (*pc*)	OR[a] (95% CI)	PTB (*n* = 168) (%)	*p*-Value[b] (*pc*)	OR[b] (95% CI)	EPTB (*n* = 55) (%)	*p*-Value[c] (*pc*)	OR[c] (95% CI)
Diplotype										
AA/deldel	1 (1)	10 (4)	0.02[d] (NS)	7 (0.91–147.61)	10 (6)	0.01 (NS)	9.43 (1.22–199.29)	0 (0)	0.73[d] (NS)	0 (0–47.94)
AG/deldel	30 (20)	58 (26)	0.18 (NS)	1.41 (0.83–2.39)	40 (24)	0.41 (NS)	1.25 (0.71–2.21)	18 (33)	0.05 (NS)	1.95 (0.92–4.1)
AG/TGTG+/+	4 (3)	12 (5)	0.2 (NS)	2.08 (0.61–7.79)	10 (6)	0.15 (NS)	2.31 (0.65–8.95)	2 (4)	0.51[d] (NS)	1.38 (0.17–9.1)
GG/deldel	37 (25)	34 (15)	0.02 (NS)	0.55 (0.32–0.95)	30 (18)	0.13 (NS)	0.66 (0.37–1.18)	4 (7)	0.005 (0.03)	0.24 (0.07–0.75)
GG/TGTG+/del	62 (41)	88 (40)	0.71 (NS)	0.93 (0.59–1.44)	67 (40)	0.79 (NS)	0.94 (0.59–1.51)	21 (38)	0.68 (NS)	0.88 (0.44–1.73)
GG/TGTG+/+	16 (10)	21 (10)	0.69 (NS)	0.87 (0.42–1.82)	11 (6)	0.18 (NS)	0.59 (0.24–1.39)	10 (18)	0.15 (NS)	1.86 (0.72–4.74)
Haplotype										
A-del	33 (11)	76 (17)	0.02 (NS)	1.66 (1.05–2.64)	61 (18)	0.01 (0.04)	1.79 (1.11–2.9)	20 (18)	0.05 (NS)	1.8 (0.94–3.42)
G-del	165 (55)	214 (48)	0.06 (NS)	0.75 (0.56–1.02)	168 (50)	0.2 (NS)	0.82 (0.59–1.13)	47 (43)	0.02 (NS)	0.61 (0.38–0.97)
A-TGTG+	3 (1)	13 (3)	0.07 (NS)	2.97 (0.78–13.24)	10 (3)	0.07 (NS)	3.04 (0.76–14.03)	0 (0)	0.39[d] (NS)	0 (0–6.12)
G-TGTG+	99 (33)	143 (32)	0.78 (NS)	0.96 (0.69–1.33)	97 (29)	0.26 (NS)	0.82 (0.58–1.17)	43 (39)	0.25 (NS)	1.3 (0.81–2.1)

PTB, pulmonary tuberculosis; EPTB, extrapulmonary tuberculosis; OR, odds ratio; CI, confidence interval; NS, not significant; *pc*, Bonferroni correction (as multiple comparisons were made, Bonferroni's correction was applied to a significant *p*-value that was multiplied by the number of diplotypes (six) and haplotypes (four)).

[a] Cases of pulmonary and extrapulmonary tuberculosis vs. control cases.
[b] Cases of pulmonary tuberculosis vs. control cases.
[c] Cases of extrapulmonary tuberculosis vs. control cases.
[d] Fisher's exact test.

When studying the frequency distribution of the different genotypes by age group, we found that del/del genotype might be associated with an increased risk of the development of the PTB form in those under 30 years of age (OR 3.75, 95% CI 1.5–9.52; *pc* = 0.003) (Table 4). TGTG+/del appeared to be associated with increased resistance against the development of active PTB (OR 0.23, 95% CI 0.08–0.65; *pc* = 0.003) (Table 4).

3.4. NRAMP1 (D543N and 3′-UTR) haplotype and diplotype distributions

We found that the correlation (r^2) between the two *NRAMP1* polymorphisms was low and that D′ showed evidence of significant LD only in the PTB+EPTB (r^2 = 0.05; D′ = 0.614), PTB (r^2 = 0.03; D′ = 0.56), and EPTB (r^2 = 0.14; D′ = 1) groups, but not in the control group (r^2 = 0.03; D′ = 0.67).

When we analyzed the four possible haplotypes, only A-del showed significant differences between patients with PTB and control subjects (Table 6). This haplotype was over-represented in the PTB group and associated with susceptibility to the clinical form of PTB (18% vs. 11%; OR 1.79, 95% CI 1.11–2.9; *pc* = 0.04) (Table 6).

Only six of the nine possible diplotype combinations were found in patients with PTB and control individuals, and only five in the EPTB patients. The GG/deldel diplotype was significantly more frequent in control subjects in comparison to EPTB patients and appeared to be associated with a decreased risk of the development of this clinical form of TB (25% vs. 7%, OR 0.24, 95% CI 0.07–0.75; *pc* = 0.03).

4. Discussion

Polymorphisms in the *NRAMP1* gene have been found to be risk factors for the development of TB among different ethnic adult populations in a number of genetic studies.[37,38] However, no distinction was made between the different clinical forms of active TB (PTB and EPTB) in the patients enrolled in these studies, or age–sex-specific effects. Such a study design might miss or underestimate genetic control mechanisms that differ in the development of PTB or EPTB. In the present study, we focused our genetic analysis on different clinical forms of active TB in relation to the age and sex of the patients enrolled, for the first time in Tunisia. We found strong associations between the *NRAMP1* 3′-UTR and D543N polymorphisms and susceptibility to mycobacterial infection in Tunisian populations grouped by age and sex. These results could explain in part the role of *NRAMP1* in susceptibility to TB disease and provide a plausible explanation for *NRAMP1* genetic heterogeneity in TB susceptibility.

There have been significant advances in our understanding of the fundamental principles governing bacterial–host interactions. However, the clinical management of TB is very complicated because of the non-homogeneous nature of the patients.[1] Resistance to bacterial infection is a heritable trait that appears to be controlled by multiple genes. The innate immune system is of central importance to the early containment of infection. Hence, genetic variations or mutations that disrupt innate immune sensing of infectious organisms could explain the ability of the immune system to respond to infection, the diversity of clinical presentations of TB, the response to current medical treatment, and the genetic predisposition to infection in each individual patient. Several lines of evidence suggest that the *NRAMP1* gene is an important candidate factor for susceptibility to active TB. In the mouse, it has been shown that the absence of mature Nramp1 protein is the result of a G169D polymorphism, which causes increased susceptibility to several intracellular macrophage pathogens, including BCG, *Salmonella typhimurium*, and *Leishmania*

donovani.[39] In phagocytosing macrophages, Nramp1 is rapidly recruited to the membrane of late endosomal–phagosomal vesicles.[18,40] At the phagosome membrane, Nramp1 functions as a divalent cation pump,[18,41] and Nramp1-altered cation fluxes are thought to abrogate pathogen-induced blockage of phagosome maturation.[42–44] The mechanism of action of NRAMP1 in human macrophages is not known, but is thought to follow similar mechanisms.

A number of recent studies now provide evidence that *NRAMP1* polymorphisms are involved in determining autoimmune and infectious disease susceptibility, and immune responses to mycobacterial products.[45] To date, studies that have assessed *NRAMP1* polymorphisms and their association with the risk of development of active TB in humans have yielded conflicting results. The initial study reported by Bellamy et al. demonstrated that *NRAMP1* polymorphisms were significantly associated with susceptibility to TB among West Africans.[24] However, other studies involving patients living in Morocco, Japan, and Brazil, have suggested that *NRAMP1* polymorphisms are not a risk factor for TB.[25,46,47] This discrepant data may be attributed to a number of variant factors, such as the genetic background and clinical status of the TB patients.

In the present study, genotyping of the two SNPs of the *NRAMP1* gene was done correctly using previously reported PCR-RFLP protocols in TB patients divided into groups according to age and sex and compared to a control group.[30] We found that the D543N-A allele was significantly over-represented in the PTB group in comparison to the control group. This finding suggests that the D543N-A allele might be associated with an increased risk of the development of active PTB. In addition, this result appears to argue for the association of the D543N-AA genotype with susceptibility to PTB in our setting (OR 9.43; $pc = 0.03$). However, our finding is in disagreement with those of previous studies, which have reported a positive association of heterozygote D543N-AG genotype with susceptibility to PTB in Gambia[24] and Peru.[48] Moreover, in some studies with large sample sizes, no association between D543N polymorphisms and TB were found.[25,46,47] A possible explanation for the apparent inconsistency between these studies is that ethnic-specific genetic variations could greatly influence host immunity to TB, causing different TB susceptibility depending on the ethnic population studied. Another possible explanation might be the relatively small size of the studied populations.

After adjusting the frequency distribution of D543N genotypes by sex, we observed a significant increase in resistance to the development of active PTB in females in relation to wild-type GG (OR 0.1; $pc = 0.03$). Interestingly, only patients aged less than 30 years harboring AA and AG genotypes were at increased risk of developing PTB (OR 10.8; $pc = 0.018$) and EPTB (OR 4.37; $pc = 0.0024$), respectively. However, patients with GG genotype appeared to be protected against the development of active EPTB (OR 0.25; $pc = 0.003$). Hence, this finding could explain in part the heterogeneity in results reported by several studies on the associations of D543N polymorphism and the risk of developing active TB. Other studies should investigate genetic associations of D543N polymorphism in relation to the gender and age of TB patients.

The 3′-UTR del allele frequencies were similar in the different patient TB groups in comparison to healthy controls and did not show any significant statistical differences. Moreover, there were no statistically significant differences in the frequency distributions of the different genotypes between PTB patients, EPTB patients, and the control group. Our findings are in agreement with recently published data.[25,46,47] However, we found that after stratification of the different genotypes by age group, the del/del genotype was over-represented in PTB patients aged less than 30 years in comparison to the control group (68% vs. 36%). Moreover, this genotype appeared to be associated with an increased risk of the development of PTB in this age group (OR 3.75; $pc = 0.003$). In contrast, TGTG+/del genotype might be associated with increased resistance against PTB (OR 0.23; $pc = 0.003$). This finding argues for the importance of stratifying TB patients by age to better understand the possible association between this polymorphism and resistance/susceptibility to active TB.

Interestingly, in our study *NRAMP1* 3′-UTR and D543N polymorphisms appeared to be associated with an increased susceptibility or protection against the risk of developing active PTB, particularly in males aged less than 30 years. This association could be related to one or both of the following factors: (1) The polygenic aspect of susceptibility to active TB;[4] in fact, other genetic polymorphisms affecting genes encoding for cytokines or their receptors that play a pivotal role in immunity against *M. tuberculosis* could be associated with an increased risk of the development of active EPTB. (2) Associations between cytokines or receptors that play a role in the immune response in TB and the risk of development of this disease could be influenced by age and sex. In this setting, we recently reported that the variant AA genotype of +874 A/T affecting the IFN-γ gene was associated with active PTB in men (OR 2.42) aged 30–49 years.[9] However, patients aged more than 50 years had a six-fold increased risk of developing active EPTB. The A-del haplotype was significantly more frequent in the PTB group ($pc = 0.04$) in comparison to the control group. Additionally, this haplotype appeared to be a risk factor for the development of active PTB with a 1.79-fold increased risk. Finally, of the nine possible diplotypes, only six were present in PTB patients and in the control group, and only five were present in EPTB patients. The GG/deldel diplotype was much more frequent in controls ($pc = 0.03$) and might increase resistance against the development of EPTB (OR 0.24).

We have reported here the first study in the Tunisian population showing how *NRAMP1* genetic polymorphisms can affect TB susceptibility in relation to age and sex. There were a number of limitations to the current study. The first is the lack of matched age and gender distribution between the controls and study subjects. A second is that the patients were from a single geographic region, limiting the certainty that these data apply to other regions. The third limitation is the size of the population, which could also be expanded.

In summary, our data based on molecular genetics indicate that 3′-UTR and D543N polymorphisms of the *NRAMP1* gene are associated with an increased susceptibility–resistance to different clinical forms of active TB with sex- and age-specific effects in the Tunisian population. Additional studies with TB patients from diverse geographic and ethnic backgrounds will be required to further investigate the relationships underlying these interesting findings.

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