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Title: A novel vitamin D receptor polymorphism associated with leprosy

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Highlights

- A new polymorphism, A61894G, in VDR gene was associated to leprosy
- The transversion (G>A) conferred susceptibility for disease in PB group
- T61968C and A61894G polymorphisms interaction are crucial in M. leprae infection
- TCAA genotype and negative Mitsuda presented 28.33-fold chance to develop the leprosy

Abbreviations:

PB, paucibacillary leprosy; MB, multibacillary leprosy; RFLP, Restriction Fragment Length Polymorphism; VDR, Vitamin D receptor; SNPs, Single Nucleotide Polymorphisms; OR, Odds Ratio; CI, Confidence Interval; WHO, World Health Organization; HWE, Hardy-Weinberg equilibrium; LD, Linkage disequilibrium; Th1, T helper 1; Th2, T helper 2.

Keywords: Leprosy, SNP, Vitamin D receptor, Intron 8, Mitsuda test.

Letter to the Editor:

Leprosy is a chronic infectious disease that primarily affects peripheral nerves, and the skin, but can also compromise functions of the eyes and other organs. *Mycobacterium leprae* (*M.*

leprae) is the etiological agent and an obligate intracellular parasite [1]. Leprosy remains a serious public health problem in developing countries, such as India, Brazil and Indonesia, and it is endemic in Africa, Asia and Latin America [2].

Clinically, leprosy is a spectral disease presenting the tuberculoid pole with cell-mediated response and the lepromatous form, often with Th2 response. There is also a transition group between both forms, denominated as borderline. The World Health Organization (WHO) guidelines classify leprosy patients into two categories: Paucibacillary (PB), which includes tuberculoid leprosy and some borderline tuberculoid leprosy; and Multibacillary (MB), which includes a borderline group (borderline tuberculoid, mid borderline, borderline lepromatous) and lepromatous leprosy [3].

Several aspects related to host, pathogen and environment contribute to maintain epidemiological rates. Individual genetic susceptibility is a determining factor, and various genes and their polymorphisms have been linked to or associated with susceptibility to leprosy including the Vitamin D receptor (VDR) [4]. This receptor has several single nucleotide polymorphisms (SNPs) located in restriction sites near the 3'UTR region. The *Taq*I (rs731236, also known as T61968C) is a synonymous variation (located at +352 position of exon 9) that does not alter the amino acid sequence, but affects the stability of mRNA, altering protein expression [5]. Therefore, it is reasonable to hypothesize that VDR polymorphisms may play a critical role in leprosy.

A previous study reported interaction between the VDR T61968C polymorphism and leprosy susceptibility in a Brazilian population [6]. Here, we tested for differences in genetic variations between 132 patients with a diagnosis of leprosy (63 PB and 69 MB) and 138 household contacts (HC) to identify susceptibility and make predictions regarding development

of leprosy. Genotyping analysis of T61968C (NCBI: #AY342401) polymorphism was performed through PCR-RFLP, and we have described, for the first time, the A61894G (NCBI: #AY427834) polymorphism (also in the 3'UTR region), evaluated through LIS-SSCP. Genotypes were also compared using a Mitsuda test for each patient, and the polymorphisms were confirmed by sequencing. Statistical analyses were performed using R (2017, R Foundation for Statistical Computing, Vienna, Austria) and BioEstat 5.0 software. Significant differences for allele frequency, association of the VDR genotypes with age and Mitsuda and Hardy-Weinberg equilibrium (HWE) values were tested using Chi-square. Leprosy outcome was predicted through odds ratio (OR). $P < 0.05$ was considered significant.

In our cohort study, samples were randomly selected. The disease affected both sexes, however it is more incident in males, as demonstrated previously [7]. Moreover, genetic polymorphisms can vary depending on the population [8]. Our study was performed in a miscegenated population, and our results may be a molecular signature in a high endemic country.

Our analysis revealed that A61894G in PB patients is under selection ($P = 0.004$), and the mutant allele may still be incorporated in this group (Table 1). There was a significant different for A61894G polymorphism genotypes between HC and PB groups ($P = 0.047$). In (GG+AG)x(AA) analysis comparing HC and PB, the G allele was significantly more frequent in the HC group ($P = 0.037$). Age, Mitsuda value and gender were not correlated to genetic variations.

For OR analysis, the Mitsuda values were transformed into qualitative data (Table 2) as previously described [9]. Combined tests of T61968C and Mitsuda values among patients and HC showed that patients with a 'TT' genotype and negative Mitsuda response were 5.23 times

more chance (OR = 5.23; CI95%:2.25–12.16; P = 0.0002) to develop leprosy. This profile was also verified for the T allele in (TT+TC) analysis (OR = 2.25; CI95%:1.32–3.85; P = 0.004). Polymorphisms in VDR, an immune response gene, may influence intracellular growth of mycobacterium. Variations in T61968C and A61894G may alter mRNA stability leading to an imbalance of Th1 to Th2 cytokines, which predicts clinical evolution of leprosy in patients. Considering the importance of Mitsuda values to predict immunological responses, we suggest that the chance of leprosy occurrence is highly related to this test, and patients with negative Mitsuda responses have a higher risk of developing leprosy, which is also previously confirmed [9].

Considering A61894G SNP, we found that the AA genotype associated with negative Mitsuda values was responsible higher chances of leprosy occurrence (OR = 5.23; CI95%:1.88–14.56; P = 0.002). Allelic frequency analysis of (AA+AG) also demonstrated the important role of the A allele in leprosy occurrence (OR = 2.37; CI95%:1.30–4.31; P = 0.007). Higher stability of VDR mRNA has been associated more often with the C allele for T61968C SNP, conducting patients to cellular immune response, decreasing the risk for leprosy [10]. So, the T allele affects VDR function and alters immunological responses against the bacillus. The same behavior was verified for the AA genotype for the novel polymorphism A61894G.

The interaction between both VDR polymorphisms and the Mitsuda response was further investigated in combined genotypic analysis (data not shown). Odds Ratio (OR) analysis revealed a 5.14 higher chance to develop leprosy for TTAG genotype associated with negative Mitsuda values (OR = 5.14; CI95%:1.44–18.36, P = 0.02) compared to HC. Similarly, patients with both TTAA genotype and negative Mitsuda response had 5.60 higher chance for developing the disease (OR = 5.60; CI95%:1.24–25.17, P = 0.044). Finally, patients with the TCAA

genotype were 28.33 times more likely to contract leprosy (OR = 28.33; CI95%:2.39–336.02, P = 0.007). The combined genotype clearly and strongly affects leprosy occurrence, and the new A61894G polymorphism provides molecular evidence that genetic interactions are crucial in *M. leprae* infection. Probably, the interaction of T61968C and A61894G polymorphisms disrupt elements in UTRs and their combination alters VDR regulatory function.

In summary, our study is the first to describe the A61894G polymorphism, which is in linkage disequilibrium with T61968C, and its association with leprosy occurrence. According to our analysis, both polymorphisms are closely linked to disease development. We suggest that the combined genotype TCAA favors leprosy susceptibility.

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Competing interests:

The authors declare that they have no conflict of interest

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Table 1 - Genotypic and allelic frequencies for VDR gene polymorphisms and clinical parameters of leprosy patients (paucibacillary and multibacillary) and houlsehold contacts.

SNP	**Healthy (N = 138)**				**Leprosy (N = 132)**							
					PB (N = 63)				**MB (N = 69)**			
	N (%)	**'§Age**	**Mitsuda****	**Gender F/M (N %)**	**N (%)**	**'§Age**	**Mitsuda****	**Gender F/M (N %)**	**N (%)**	**'§Age**	**Mitsuda****	**Gender F/M (N %)**
T61968C												
TT	57 (41.30)	30.44 (17.81)	8.06 (3.21)	27 (19.57)/ 29 (21.01)	24 (18.18)	48.88 (14.21)	6.25 (3.60)	17 (12.88)/ 7 (5.30)	23 (17.43)	46.87 (18.97)	0.70 (1.94)	4 (3.03)/ 18 (13.64)
TC	56 (40.58)	26.77 (18.51)	6.80 (3.86)	37 (26.81)/ 20 (14.49)	33 (25)	43.84 (13.91)	8.38 (3.84)	17 (12.88)/ 16 (12.12)	34 (25.75)	46.18 (15.46)	0.21 (0.74)	10 (7.58)/ 25 (18.94)
CC	25 (18.12)	29.84 (17.93)	8.88 (4.45)	8 (5.80)/ 17 (12.32)	6 (4.54)	43.33 (17.34)	10 (5.69)	2 (1.51)/ 4 (3.03)	12 (9.10)	43.08 (14.39)	0.33 (1.15)	0 (0)/ 12 (9.09)
$P\chi^2(1)$	Healthy x Leprosy = 0.29				Healthy x PB = 0.17				Healthy x MB = 0.51		PB x MB = 0.41	
Alleles T/C	170/106 (61.59/38.41)				81/45 (64.29/35.71)				80/58 (57.97/42.03)			
P_{HWE}	0.095				0.26				0.92			
TT+TC	113 (81.88)	28.60 (18.16)	7.43 (3.53)	64 (46.38)/ 49 (35.50)	57 (43.18)	46.36 (14.06)	7.31 (3.72)	34 (25.76)/23 (17.42)	57 (43.18)	46.52 (17.21)	0.45 (1.34)	14 (10.61)/ 43 (32.58)
CC	25 (18.12)	29.84 (17.93)	8.88 (4.45)	8 (5.80)/17 (12.32)	6 (4.54)	43.33 (17.34)	10 (5.69)	2 (1.51)/ 4 (3.03)	12 (9.10)	43.08 (14.39)	0.33 (1.15)	0 (0)/ 12 (9.09)
$P\chi^2(2)$	Healthy x Leprosy = 0.31				Healthy x PB = 0.12				Healthy x MB = 0.90		PB x MB = 0.19	
CC+TC	81 (58.7)	28.30 (18.22)	7.84 (4.15)	45 (32.61)/ 37 (26.81)	39 (29.54)	43.56 (15.62)	9.19 (4.76)	19 (14.39)/20 (15.15)	46 (34.85)	44.63 (14.92)	0.27 (0.94)	10 (7.58)/ 37 (28.03)
TT	57 (41.3)	30.44 (17.81)	8.06 (3.21)	27 (19.57)/ 29 (21.01)	24 (18.18)	48.88 (14.21)	6.25 (3.60)	17 (12.88)/7 (5.30)	23 (17.43)	46.87 (18.97)	0.70 (1.94)	4 (3.03)/ 18 (13.64)
$P\chi^2(3)$	Healthy x Leprosy = 0.34				Healthy x PB = 0.67				Healthy x MB = 0.27		PB x MB = 0.57	

SNP	Healthy (N = 138)				Leprosy (N = 132)							
					PB (N = 63)				MB (N = 69)			
	N (%)	'§Age	Mitsuda**	Gender F/M (N %)	N (%)	'§Age	Mitsuda**	Gender F/M (N %)	N (%)	'§Age	Mitsuda**	Gender F/M (N %)
A61894G												
GG	40 (28.99)	30.18 (16.71)	8.89 (4.20)	26 (18.84)/ 14 (10.15)	20 (15.15)	47.45 (15.82)	8.25 (3.26)	10 (7.58)/10 (7.58)	21 (15.90)	44.71 (17.43)	0.14 (0.65)	5 (3.79)/16 (12.12)
AG	67 (48.55)	27.57 (19.11)	6.66 (3.30)	39 (28.26)/ 28 (20.29)	20 (15.15)	47.16 (11.09)	9.68 (3.71)	12 (9.09)/8 (6.06)	28 (21.23)	48 (16.52)	0.59 (1.80)	7 (5.30)/21 (15.90)
AA	31 (22.46)	29.63 (17.86)	8.37 (3.79)	16 (11.59)/ 15 (10.87)	23 (17.42)	43.09 (15.53)	5.61 (4.18)	14 (10.60)/9 (6.82)	20 (15.15)	44.20 (15.53)	0.40 (1.10)	3 (2.28)/17 (12.88)
$P\chi^2$ (1)	Healthy x Leprosy = 0.08				Healthy x PB = 0.04*				Healthy x MB = 0.48		PB x MB = 0.55	
Alleles G/A	147/129 (53.26/46.74)				66/60 (52.38/47.62)				68/70 (49.27/50.73)			
P_{HWE}	0.7705				0.0039*				0.1179			
GG+AG	107 (77.54)	28.87 (17.91)	7.77 (3.75)	65 (47.1)/ 42 (30.44)	40 (30.3)	47.30 (13.45)	8.96 (3.48)	22 (16.67)/ 18 (13.64)	49 (37.13)	46.35 (16.97)	0.36 (1.22)	12 (9.09)/ 37 (28.02)
AA	31 (22.46)	29.63 (17.86)	8.37 (3.79)	16 (11.59)/ 15 (10.87)	23 (17.42)	43.09 (15.53)	5.61 (4.18)	14 (10.60)/ 9 (6.82)	20 (15.15)	44.20 (15.53)	0.40 (1.10)	3 (2.28)/ 17 (12.88)
$P\chi^2$ (4)	Healthy x Leprosy = 0.06				Healthy x PB = 0.04*				Healthy x MB = 0.30			
AA+AG	98 (71.01)	28.6 (18.49)	7.51 (3.54)	55 (39.85)/ 43 (31.16)	43 (32.57)	45.12 (13.31)	7.64 (3.94)	26 (19.69)/ 17 (12.88)	48 (36.38)	46.1 (16.02)	0.49 (1.45)	10 (7.58)/ 38 (28.78)
GG	40 (28.99)	30.18 (16.71)	8.89 (4.20)	26 (18.84)/ 14 (10.15)	20 (15.15)	47.45 (15.82)	8.25 (3.26)	10 (7.58)/ 10 (7.58)	21 (15.90)	44.71 (17.43)	0.14 (0.65)	5 (3.79)/ 16 (12.12)
$P\chi^2$(5)	Healthy x Leprosy = 0.81				Healthy x PB = 0.69				Healthy x MB = 0.83		PB x MB = 0.87	

§ Mean (±SD)
*Significant data
**The Mitsuda test values do not follow a normal distribution in the three groups
PB Paucibacillary, MB Multibacillary, $P\chi^2$(1) Genotype, $P\chi^2$(2) TT+TC, $P\chi^2$ (3) CC+TC, $P\chi^2$(4)GG+AG, $P\chi^2$(5) AA+AG

Table 2 - Chance of leprosy occurrence (odds ratio) by association of VDR gene polymorphisms and Mitsuda test results in patients and household contacts.

		Mitsuda test (No. of patients[§])			
Genotypes	**Group**	**Positive**	**Negative**	**Odds ratio**	**P**
T61968C					
TT	Healthy	38	19	5.23 (2.2499-12.1609)	0.0002*
	Leprosy	13	34		
TC	Healthy	23	33	1.10 (0.5326-2.2681)	0.9437
	Leprosy	26	41		
CC	Healthy	15	10	3.90 (1.0571-14.3879)	0.0751
	Leprosy	5	13		
TT+TC	Healthy	61	52	2.25 (1.3208-3.8531)	0.0042*
	Leprosy	39	75		
CC+TC	Healthy	38	43	1.54 (0.8274-2.8641)	0.2274
	Leprosy	31	54		
All genotypes	Healthy	76	62	2.45 (1.4968-4.0156)	0.0005*
	Leprosy	44	88		
A61894G					
GG	Healthy	28	12	3.29 (1.3149-8.2523)	0.0183*
	Leprosy	17	24		
AG	Healthy	31	36	1.43 (0.6736-3.0577)	0.4554
	Leprosy	18	30		
AA	Healthy	18	13	5.23 (1.8785-14.5654)	0.0025*
	Leprosy	9	34		
GG+AG	Healthy	59	48	1.90 (1.0714-3.3568)	0.0391*
	Leprosy	35	54		
AA+AG	Healthy	49	49	2.37 (1.3019-4.3159)	0.0069*
	Leprosy	27	64		
All genotypes	Healthy	77	61	2.52 (1.5408-4.1366)	0.0003*
	Leprosy	44	88		

*Significant data with 95% CI (confidence interval)

[§]Mitsuda Test results: positive (≥7 mm), negative (<7 mm)