Differential distribution in vitamin D receptor gene variants and expression profile in Northeast Brazil influences upon active pulmonary tuberculosis

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Abstract

Tuberculosis is an infectious disease with variable outcomes. This variability is due to host immune capacity in containing the infection process initiated by the *Mycobacterium tuberculosis* (MTB). Vitamin D is able to modulate a very specific immune response against MTB infection, and its action relies on vitamin D receptor (VDR) binding. Altered VDR forms may compromise vitamin D pathway and proper immune response after MTB infection. Herein we assessed the relationship of five potentially functional polymorphisms from *VDR*: rs2228570 FokI, rs11568820 Cdx-2, rs2248098, rs1540339 and rs4760648, with tuberculosis susceptibility. The SNP rs4760648 T/T was associated with differential susceptibility to tuberculosis (OR = 2.50, 95%CI = 1.20–5.36, p = 0.01). The SNP rs1540339 presented association to both T allele (OR = 0.55, 95%CI = 0.35–0.88, p = 0.01) and the T/T genotype (OR = 0.404, 95%CI = 0.20 – 0.78, $p = 0.005$). The FokI T allele was identified as associated to diminished susceptibility (OR = 0.67, 95% CI = 0.45–0.99, p = 0.04) to active TB, as well as T/T genotype (OR = 0.15, 95%CI = 0.04–0.45, $p = 9.58 \times 10^{-5}$). We also performed the expression analyses and observed a down-regulation of *VDR* in patients (-10.717 FC, $p = 8.42e^{-12}$), and according to the presence of associated FokI SNP, we observed that the C/T and T/T genotypes presence increases VDR expression (+ 1.25 and + 2.35 FC, p = 0.425 and p = 0.506, respectively). This study shows that vitamin D receptor variants can influence upon pulmonary tuberculosis susceptibility and VDR mRNA levels are decreased in those patients.

Keywords Tuberculosis · Vitamin D · Polymorphisms · *M. tuberculosis* · VDR · Gene expression

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Introduction

Tuberculosis (TB) burdens the role of one of the most ancient, compromising and deadly infectious disease worldwide, caused by the *Mycobacterium tuberculosis,* displays quite variable clinical outcomes [9]. Recent data from the World Heathy Organization shows that about 10 million new cases and eventually 1.5 million deaths by the disease were notified in 2018 [16]. In Brazil, TB it is considered the 4th cause of death by infectious disease [9] It is estimated that one-third of the World's population is infected with *M. tuberculosis*, but only one out ten individuals developed active TB, leading to a complex interplay enclosing environmental and genetic factors, key modulators in determining individual's differential susceptibility [8].

In view of the roles of genetic and environmental factors in host response, immunomodulatory biomolecules such as

vitamin D (VD), a liposoluble secosteroid hormone, have been highlighted in studies related to TB [4]. VD in its active form 1,25α-dihydroxyvitamin D3 (VD_3) or cholecalciferol acts modulating the immune responses [13]. The VD_3 acts as an important modulator in host resistance to infection mechanism by *M. tuberculosis* in a specific manner. By binding to VDR, VD_3 is able to induce, by an independent of interferon gamma pathway, the phagosome-lysosome complex formation [4].

Vitamin D exerts its function through the binding to Vitamin D Receptor (VDR), and many of its biological activities are mediated by high or low affinity of this hormone with its cellular receptor. *VDR* is located on chromosome 12 (12q13.11) and polymorphisms within the gene lead to variations in transcript length and activity, influencing upon VD pathway [6].

Several studies have reported association between polymorphisms of the VDR gene and the immune response to various diseases. However, the role of the *VDR* polymorphisms in the susceptibility or resistance to TB remains controversial, what is justifiable once inclusion and exclusion criteria may bias the results. What, by now, only suggests that the *VDR* variations could contribute to individuals' differential immune response against *M. tuberculosis* [7].

In view of the genetic aspects involved in the development of the disease, and the peculiarities of the Brazilian Northeast population, since several factors influence individual's susceptibility, we assessed differential distribution from *VDR* most functional SNPs, covering by linkage disequilibrium (LD) most of gene region, and also evaluated its influence in *VDR* expression in a Northeast Brazilian population.

Materials and methods

Study population and design

We performed an analytical and prospective study divided in an association study and an expression assay. Study participants were recruited from the metropolitan area of Recife, in Northeast of Brazil, from 2013 to 2015 in hospitals and polyclinics of public health. For the association study we investigated two groups: patients (n = 138) with active TB, diagnosis confirmed by clinical and/or radiological evidence and Nested PCR positive for *M. tuberculosis*; and a control group (n = 195). The control group was composed by individuals presenting respiratory symptoms enclosing asthmatics, that apparently, for living in a TB endemic region, display greater chances to be exposed to bacillus, but were tested negative at tuberculin skin test and HIV test. In relative gene expression assay, we performed two major analyses. First, *VDR* gene expression was analyzed in 20 TB randomly selected patients in comparation to 20 symptomatic respiratory individuals also randomly selected (control group) to evaluate this gene expression in presence of the disease. In a second analysis, we evaluated in the same individuals *VDR* gene expression according to presence of FokI polymorphism, which is known to modulate protein size and interaction with VD [5]. The post hoc Power analysis was performed in the G*Power 3.1.9.4 software and the results are included at Table 1.

Genetic association study

Genomic DNA was isolated (25 ng/μL) from peripheral blood samples using the *Salting Out* protocol, adapted from Miller et al., (1988). VDR polymorphisms were selected using HapMap database and the SNPbrowser software 4.0 (Applied Biosystems®). The SNP selection was performed according to Minimum Allele Frequency (MAF) > 10%, functions upon protein activation and/or Tagging position throughout the gene coverage area. The selected SNPs were as follows: rs2228570 FokI [C → T], rs11568820 Cdx2 [G → A], rs2248098 [A → G], rs1540339 [C → T] and rs4760648 [C → T]. Genotyping was performed using TaqMan® fluorogenic probes for each polymorphism, C__12060045_20, C___2880808_10, C___3290630_1_, C___8716064_1_ and C___3290657_10, respectively.

The reactions were performed on ABI 7500 real-time PCR platform (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions. Allelic discrimination followed as recommended by the manufacturer and analyzed using the SDS software (Applied Biosystems®).

Relative Gene Expression Assay

RNA was isolated from peripheral blood using Trizol®, following manufacturer's instructions. For measure the concentration of RNA samples and evaluated its integrity, a spectrophotometer assay using Nanodrop 2000 (Thermo Scientific USA) and gel electrophoresis were performed, respectively. cDNA synthesis was performed using *RT M-MLV* commercial kit (Invitrogen USA) following the standard protocol with an RNA input of 500 ng for each sample.

The *VDR* and the endogenous reference gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene expression were performed using specific TaqMan Gene Expression assays (Hs00172113_m1 and Hs03929097_m1, respectively). All the assays were performed on ABI 7500 platform (Applied Biosystems, Forter City, CA, USA). The ΔCq method, according to Livak et al. (2001), were used to calculate the relative quantity (Rq) values based on technical triplicates.

Table 1 VDR assessed SNPs distribution in TB patients and controls

SNP ID	TB patients	Controls	OR (95%CI)	*p*-value
rs11568820 (G > A)	n = 123	n = 161		
Allele				
G	147 (0.60)	195 (0.60)	Ref.	
A	99 (0.40)	127 (0.40)	1.03 (0.72–1.47)	0.863
Codominant				
GG	41 (0.33)	58 (0.36)	Ref.	
GA	65 (0.53)	79 (0.49)	1.16 (0.67–2.02)	0.6
AA	17 (0.14)	24 (0.15)	1.00 (0.44–2.22)	1
rs2228570 (C > T)	n = 138	N = 191		
Allele$^{\delta}$				
C	221 (0.84)	279 (0.7)	Ref.	
T	55 (0.16)	103 (0.3)	0.67 (0.45–0.99)	0.04*
Codominant$^{\delta}$				
CC	88 (0.64)	110 (0.58)	Ref.	
CT	45 (0.32)	59 (0.31)	0.95 (0.57–1.58)	0.903
TT	5 (0.04)	22 (0.11)	0.15 (0.04–0.45)	9.59×10^{-5}*
rs4760648 (C > T)	n = 96	n = 109		
Allele				
C	89 (0.46)	117 (0.54)	Ref.	
T	103 (0.54)	101 (0.46)	1.34 (0.89–2.02)	0.16
Recessive$^{\delta}$				
CC–CT	67 (0.64)	93 (0.85)	Ref.	
TT	29 (0.36)	16 (0.15)	2.50 (1.20–5.35)	0.0107*
Codominant$^{\delta}$				
CC	22 (0.23)	24 (0.22)	Ref.	
CT	45 (0.47)	69 (0.63)	0.71 (0.33–1.50)	0.3779
TT	29 (0.30)	16 (0.15)	1.96 (0.79–5.00)	0.1406
rs2248098 (A > G)	n = 96	n = 102		
Allele				
A	90 (0.47)	100 (0.49)	Ref.	
G	102 (0.53)	104 (0.51)	1.08 (0.72–1.65)	0.6882
Codominant				
AA	16 (0.17)	23 (0.225)	Ref.	
AG	58 (0.60)	54 (0.53)	1.53 (0.69–3.47)	0.2694
GG	22 (0.23)	25 (0.245)	1.26 (0.49–3.26)	0.6652
rs1540339 (C > T)	n = 96	n = 95		
Allele$^{\delta}$				
C	145 (0.755)	120 (0.63)	Ref.	
T	47 (0.245)	70 (0.37)	0.55 (0.35–0.88)	0.0106*
Codominant$^{\delta}$				
CC	58 (0.60)	37 (0.39)	Ref.	
CT	29 (0.30)	46 (0.48)	0.40 (0.20–0.78)	0.0053*
TT	09 (0.10)	12 (0.13)	0.48 (0.16–1.38)	0.1478

The inheritance genetic models presented are the ones statistically associated to diseases' susceptibility

$p < 0.05$: statistically significant.

$^{\delta}$Power > 0.8

Statistical analyses

For the association study allelic and genotypic frequencies were calculated by direct counting, and Hardy–Weinberg equilibrium was assessed by Chi-squared ($\chi 2$) test using the SNPstats software. Fisher's Exact Test was performed to assess whether genetic variables were associated with tuberculosis susceptibility. The analyses were performed using R software version 3.1.3. (https://www.r-project.org/).

To verify the type of sample's distribution of expression analyses, we performed the Shapiro–Wilk test, applying Student's T-test for analysis of variance. Were considered as statistically significant $p < 0.05$ in a 95% confidence interval (CI).

Results and discussion

VDR genotypic profile

All SNPs tested were in Hardy–Weinberg equilibrium, except for rs11568820 (Cdx-2) and rs2248098. The SNP rs4760648 T/T was associated with differential susceptibility to tuberculosis in the recessive model (OR = 2.50, 95%CI = 1.20–5.36, $p = 0.0107$, Table 1). The rs4760648, located at chromosome 12: 47886282 (GRCh38.p12), is a Tag SNP for six other variants of the *VDR* gene. Among the SNPs marked by rs4760648, the rs2853564 is correlated with a greater susceptibility to develop asthma, mainly with hyper reactivity of the bronchi and bronchioles [15]. This leads us to suggest that this ancestral variant may be related with differential susceptibility to pulmonary diseases, correlating with a decreased of immune response capacity of macrophages and pneumocytes in the infection establishment.

Both the T allele and T/T genotype from Tag SNP rs1540339 are associated with a lower susceptibility to active TB development (OR = 0.55, 95%CI = 0.35 – 0.88, $p = 0.01$ and OR = 0.404, 95%CI = 0.20–0.78, $p = 0.005$, respectively). Tag SNP rs1540339 tags three variants within *VDR*, among them, the rs2239181, associated in a study that evaluated the adaptive immune response of children vaccinated against measles in the population of Germany. In the study, the presence of this variant of the *VDR* was related to an increased in the activity and levels of the interferon gamma (IFN-γ) in these individuals [10]. Based on this IFN-γ association, we may suggest that in our population may occur a similar action, since it is one of the earliest cytokines of the cellular immune response that arise to contain the process of infection by *M. tuberculosis* by activation of phagolysosome formation [2]. Therefore, this particular SNP might be related to lower predisposition, since these individuals have a factor associated with increased activity of this cytokine [3].

The FokI (rs2228570) T allele was identified as associated to diminished susceptibility (OR = 0.67, 95% CI = 0.45–0.99, $p = 0.04$). The T/T genotype from this particular SNP, was associated to diminished susceptibility in a codominant model (OR = 0.15, 95%CI = 0.04–0.45, $p = 9.58 \times 10^{-5}$), as showed at Table 1. The ancestral *f* allele (C) for FokI encodes a full length VDR protein, whilst *F* allele (T) results in a VDR final protein three aminoacid (aa) longer, with 427 aa. In silico analysis indicated that this longer isoform interacts more efficiently with Transcription Factor II B (TFIIB), which may increase the transcriptional machinery activation [5]. Since that the presence of SNP variant *F* may increase the complex VTD-VDR interaction, this would potentiate the vitamin D dependent immune response, making difficult the bacillus infection establishment and increasing infection resistance. In fact, in the research by [11], the authors indicated that not only FokI was associated to decreased susceptibility in TB patients but also to householders counter partners [11].

Huang et al. showed in a meta-analysis study (4894 cases and controls 5319) with an OR of 1.34 (CI = 1.091–1.645, p-value = 0.005) significant associations for a higher risk to develop active TB, our results do not corroborate this data since the polymorphism FokI, both for the T allele and genotype T/T confers a minor susceptibility in our study group [12]. In relation to the FokI polymorphism, our studies reveal that the presence of the T allele and the T/T genotype are implicated a less susceptibility against the disease in our population, indicating a biological action unlike the one described in other studies in South Africa [1], Chinese populations [7] and Latin America [14].

No statistically significant association was observed for rs2248098 and rs11568820 Tag SNPs.

VDR expression

We evaluated the expression in patients with active TB compared to control group and observed overall *VDR* expression presented down-regulated in patients (− 10.717 FC, $p = 8.42e^{-12}$, Fig. 1a). When the expression of *VDR* was evaluated according to the presence of associated FokI SNP, our analyses indicated that the C/T and T/T genotypes increases VDR expression (+ 1.25 and + 2.35 FC), when compared to C/C genotype, but was not statistically significant (p = 0.425 and p = 0.506, respectively) (Fig. 1b). The lack of statistical significance may be due to our limited number of patients involved in this study. However, in the study conducted by Panda et al., the authors identified VDR mRNA levels were increased in Fok I T allele carriers as well as cathelicidin levels [11].

Over the years several studies in different populations were performed to understand the role of *VDR* polymorphisms and the susceptibility to infectious diseases.

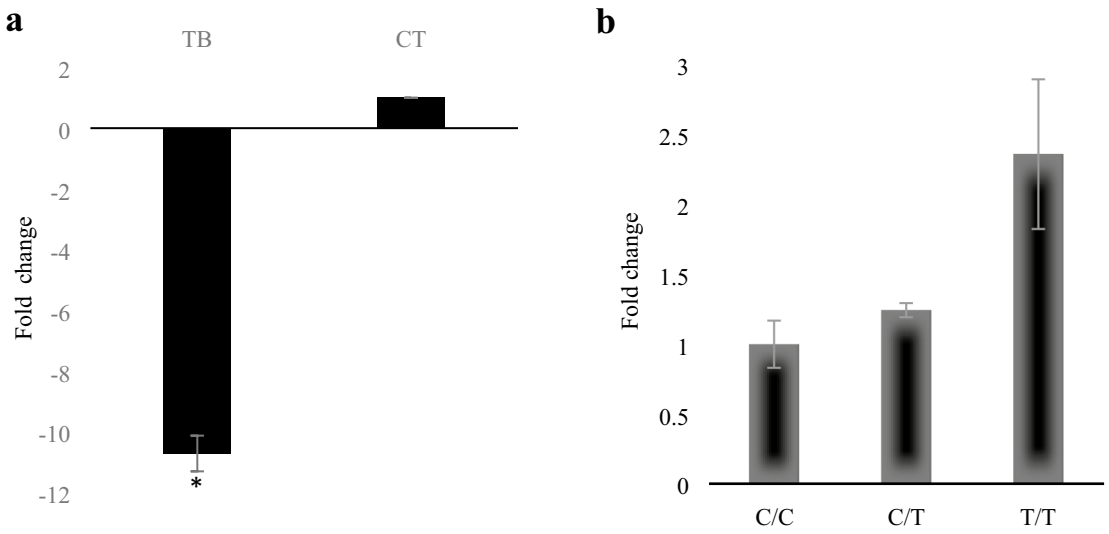

Fig. 1 **a** VDR gene expression in patients with active tuberculosis (TB) *vs.* symptomatic respiratory controls (CT), **b**. VDR gene expression in patients with active tuberculosis according to FokI (rs2228570) genotypes. The results were normalized using *GAPDH* as endogenous reference gene. Target gene expression in control group were set at 1. *p-value < 0.05

Unfortunately, the results have been conflicting so far, mainly due to the fact that the vitamin D acts according cell type and stimuli. Therefore, in our population, not only the SNPs assessed but mRNA levels indicated an influence upon active TB.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed herein involving human participants were in accordance with the ethical standards of the 1964 Helsinki declaration, its later amendments or comparable ethical standards and was approved by the national research committee PLATAFORMA BRASIL under the reference number 30667014.5.0000.5208.

Informed consent All subjects enrolled in the study signed a written informed consent term.

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