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ORIGINAL ARTICLE

Association of Vitamin D Receptor Gene (*FokI*) (rs2228750) Polymorphism with Susceptibility to Tuberculosis

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ABSTRACT

Background: *Genetic susceptibility has been suggested as an important explanation for individual risk for tuberculosis. The association between vitamin D receptor (VDR) FokI gene polymorphisms and the risk of tuberculosis have been studied in several populations; but results were inconsistent.* ***Objectives:*** *This study aimed to determine the influence of vitamin D status and frequency of association of VDR FokI (rs2228570) polymorphism on susceptibility to pulmonary tuberculosis.* ***Methodology:*** *A case-control study was conducted between 40 pulmonary tuberculosis patients and 40 control subjects. Serum vitamin D level and VDR FokI gene (rs2228570) polymorphism were tested.* ***Results:*** *Tuberculosis patients had significant lower vitamin D level compared to controls (P = 0.002). Patients showed significantly higher frequencies of VDR FokI (rs2228750) Ff, ff genotypes and f allele than controls (P = 0.023, 0.014, <0.001 respectively) with significant lower vitamin D level in patients with ff compared to patients with FF or Ff genotypes (P = 0.017). Excess smoking, vitamin D insufficiency and VDR FokI (rs2228750) (Ff+ff) genotypes were found predictors for susceptibility to tuberculosis infection.* ***Conclusion:*** *vitamin D deficiency plays a role as a risk factor for tuberculosis and VDR FokI (rs2228750) polymorphism may be partially responsible for host susceptibility to human tuberculosis in Egyptians.*

INTRODUCTION

Tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis* (MTB) bacteria, affects mainly the lungs among other parts of the body [1]. Pulmonary TB leads to several long-term lung complications e.g. lung fibrosis, bronchiectasis, airway stenosis and chronic obstructive pulmonary disease (COPD) [2].

According to World Health Organization (WHO) 2016 statistics, TB was responsible of 10.4 million infections and 1.7 million deaths worldwide[3]. In Egypt, TB incidence rate was 26 per 100,000 individuals at 2014 and 15 per 100,000 individuals at 2018[4].

Vitamin D has been connected to various medical conditions[5]. Newborns with subclinical vitamin D deficiency have been reported to be in an increased risk of having acute lower respiratory infection [6]. The active form of vitamin D ($1,25(OH)_2D_3$) has an important immunoregulatory role by moving into the nucleus through binding to the vitamin D receptor (VDR) complex[7].

Vitamin D can improve the clinical outcomes of TB via its anti-inflammatory and anti-bacterial properties. It stimulates macrophage-mediated killing of MTB and modulates the anti- and pro-inflammatory T-helper responses to TB[8]. The inter-individual differences in the susceptibility to MTB infection can be attributed to the host genetic variability[9]. Polymorphisms in VDR *FokI* gene −located on chromosome 12q12-q14− can modify the expression of VDR and affect the plasma levels of vitamin D in TB patients[10]. The association between *FokI* polymorphism of VDR and TB susceptibility has been investigated in several populations; however, the results were inconsistent and conflicting [11]. Thus we aimed in this study to determine the influence of vitamin D status and the frequency of association of VDR *FokI* (rs2228570) gene polymorphism on susceptibility to pulmonary TB in a group of Egyptian patients.

METHODOLOGY

Study participants:

A case-control study was performed on 40 adult patients with newly-diagnosed pulmonary TB with smear-positive ZN stained sputum sample ± positive sputum culture on Löwenstein Jenseen (LJ) media, recruited from Abbasia Chest Hospital, Cairo –a national-wide tertiary-care chest referral center− between October 1, 2018 and October 30, 2019. Controls were 40 healthy age and sex-matched non-related subjects to TB patients with no history of previous tuberculosis. Subjects on anti-tuberculous therapy, had any other respiratory tract infection or on vitamin D supplementation were excluded. Informed

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consent was taken from all participants before sample collection. The study was approved from the ethical committee of the Faculty of Medicine, Benha University and Abbasia Chest Hospital, Ministry of Health, Cairo.

All enrolled subjects underwent a full history taking, thorough clinical examination, laboratory investigation (sputum ZN-stained smear ± culture on LJ media) and radiological investigations to confirm TB diagnosis.

Sampling:

Six mL of venous blood were drawn; 3 mL were transferred into EDTA containing tubes and stored at -20°C for subsequent DNA extraction, and 3 mL were added to plan tubes for further serum separation from clotted blood by centrifugation and stored at −20 °C for subsequent determination of 25(OH)D concentration.

Serum 25(OH) D assay:

Serum 25(OH) D concentration was determined by ELISA (Cat.# 201-12-1982, SunBio Red, China). Calibrators and controls provided with kits were run in duplicate. For the purpose of this study, vitamin D status according to serum 25(OH)D concentration was defined as vitamin D deficiency <20 ng/ml, insufficiency 20−30 ng/ml, sufficient 30−100 ng/ml, excess >100 ng/ml and vitamin D intoxication >150 ng/ml [12].

VDR FokI gene genotyping:

All subjects were genotyped for VDR *FokI* gene (rs2228750) SNP by PCR-RFLP technique. Genomic DNA was extracted from whole blood with Gene Purelink Whole Blood Genomic DNA Purification Mini Kit QIAamp® (Cat.# K182001, QIAGEN, Germany) according to the manufacture' protocol. Extracted DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Germany) against nuclease free water as blank. The A260/A280 ratio between 1.8 and 2 was accepted as pure DNA. DNA concentration was measured and any sample with concentration less than10 ng/µl was excluded. Enzymatic amplification of a 265 bp target from the extracted DNA was performed using VDR *FokI* gene (rs2228750) F>f polymorphism primers (Cat.# FD2144, Thermo Scientific, USA) [F: 5'−AGC TGG CCC TGG CAC TGA CTC TGG CTCT−3', R: 3'− ATG GAA ACA CCT TGC TTC TTC TCC CTC−5']. The PCR reaction mixture contained 12.5 µL of 2x EasyTaq PCR Super Mix master mix, 0.5 µL of each primer, 1 µL of genomic DNA (10 ng), and 9 µL nuclease free water. PCR was conducted on Veriti Applied Biosystem 9902 (Life Technologies, Singapore) under the following thermal conditions: initial denaturation at 94 °C for 5 min, 30 cycles were run at 94 °C for 45 seconds (denaturing) followed by 68 °C for 45 seconds (annealing) then 72 °C for 30 seconds (extension) with a final extension step at 72 °C for 15 min. FokI (BSeG1) restriction enzyme was used to digest the amplified target for 5 h at 37 °C then 20 min at 65 °C. Digested samples were run on 2% agarose gel and visualized on the gel documentation system (Figure 1).

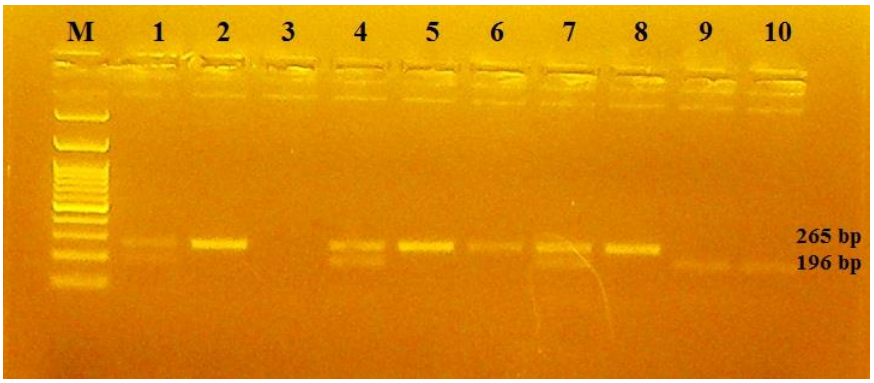

Fig. 1. Genotyping of the *FoKI* (rs 2228750) F>f Polymorphism. M: 100 bp marker. Lanes 1, 4, 7 Heterozygous (F/f) (265 bp, 196 bp). Lanes 9, 10 Homozygous (f/f) (196 bp). Lanes 2, 5, 6, 8 Homozygous (F/F) (265 bp).

Statistical analysis:

The collected data were coded and analyzed using SPSS v.20.0. Numerical data were represented as mean ±SD. Student (T) and one way analysis of variance (ANOVA) tests were used to compare means of two or more groups respectively. Categorical data were represented as frequency and percentage. Chi-Square (X^2) or Fisher's exact (FE) tests were used to examine the relationship between two qualitative variables whenever appropriate. Shapiro test was done to test the normality of data distribution. All data of the controls passed the Hardy-Weinberg equilibrium (P >0.05). The X^2 test or FET were used to determine allelic and genotypic frequencies. Odds ratio and 95% confidence interval (CI) were calculated. Logistic regression analysis was used for prediction of risk factors of TB development. P was considered significant if <0.05 at 95% CI.

RESULTS

Participant characteristics:

A total of 40 cases with smear-positive and sputum culture on LJ media-positive pulmonary TB and 40 controls with mean age 36.6±9.9 years for cases vs. 39.8±12.1 years for controls were recruited to the study. 72.5% of cases and 60% of controls were males. Eighty percent of cases and 20% of controls were smokers (P < 0.001). Family history of TB did not differ between cases and controls (P 0.644). The mean disease duration in studied patients was 0.8±0.6 years (Table 1). Cough was the presenting symptom in all cases, followed by expectoration (95%), chest pain (65%), and shortness of breath (55%), wheezes (40%), and hemoptysis (10%).

Vitamin D status:

The mean serum 25(OH) D concentration was significantly lower in TB patients (17.7±5.1 ng/ml) than controls (24.4±7.5 ng/ml) (P = 0.002). Fifty percent and 10% of TB cases had profound vitamin D insufficiency and deficiency respectively while 40% of controls had insufficient vitamin D (P = 0.048) (Table 1).

Table 1: Characteristics of studied subjects

	TB n=40	**Control n=40**	***P***
Age (years)	36.6±9.9	39.8±12.1	0.195
Sex			
Male	29 (72.5%)	24 (60%)	0.237
Female	11 (27.5%)	16 (40%)	
Smoking	32 (80%)	8 (20%)	**<0.001**
Family history of TB	2 (5%)	3 (7.5%)	0.644
Duration of TB (years)	0.8±0.6	–	–
Vitamin D (ng/ml)	17.7±5.1	24.4±7.5	**0.002**
Sufficient	16 (40%)	24 (60%)	**0.048**
Insufficient	20 (50%)	16 (40%)	
Deficient	4 (10%)	0 (0%)	

Data are represented as mean±SD or number (%)

VDR FokI gene genotyping:

VDR *FokI* (rs2228750) gene polymorphism was in HWE in the control group. Compared to control, *FokI* (rs2228750) Ff and ff genotypes and f allele were significantly increased in TB patients (P = 0.023, 0.014 and <0.001 respectively), with increased risk to develop TB (OR 2.208, 4.318 and 4.585 respectively). Distributions of VDR *FokI* (rs2228750) gene polymorphism are shown in table 2.

Table 2: Genotype and allele analysis of FokI (rs2228750) VDR gene polymorphism

	TB n=40	**Control n=40**	***P***	**OR (95% CI)**
Genotypes				
FF	20 (50%)	33 (83%)	–	reference
Ff	13 (33%)	6 (15%)	**0.023**	2.208 (1.118−4.361)
ff	7 (18%)	1 (3%)	**0.014**	4.318 (1.347−13.844)
Alleles				
F	53 (66.3%)	72 (90%)	**<0.001**	reference
f	27 (33.7%)	8 (10%)		4.585 (1.930−10.890)

OR, odds ratio; CI, confidence interval.

Vitamin D level was significantly lower in studied TB patients with ff genotypes compared to patients with FF or Ff genotypes (P = 0.017). As regards vitamin D categories in the studied TB patients, vitamin D deficiency was significantly associated with ff genotype. While, vitamin D insufficiency was significantly associated with Ff genotype (P = 0.045) (Table 3).

Logistic regression analysis was conducted for prediction of susceptibility to TB infection, using age,

gender, family history, smoking, vitamin D level and *VDR FokI* (rs2228750) (Ff + ff) genotypes as covariates. It was found that excess smoking, lower vitamin D level and VDR *FokI* (rs2228750) (Ff+ff) genotypes were considered as predictors for susceptibility to TB infection in both uni- and multivariable analyses (Table 4).

Table3: Associations between VDR gene FokI polymorphism and clinical feature of pulmonary TB.

	FF n= 20	**Ff n= 13**	**ff n= 7**	***P***
Smoking	16 (80%)	10 (76.9%)	6 (85.7%)	0.896
Family history of TB	2 (10%)	0 (0%)	0 (0%)	0.665
Vitamin D	19.5±5.9	18.2±5.7	12.1±4.3	**0.017**
Sufficient	10 (50%)	5 (38.5%)	1 (14.2%)	**0.045**
Insufficient	10 (50%)	7 (53.8%)	3 (42.9%)	
Deficient	0 (0%)	1 (7.7%)	3 (42.9%)	

Data are represented as mean±SD or number (%)

Table 4: Regression analysis for prediction of susceptibility to TB infection

	Univariable		**Multivariable**	
	P	**OR (95% CI)**	***P***	**OR (95% CI)**
Age	0.191	0.983 (0.958−1.009)		
Gender	0.237	0.703 (0.391−1.262)		
Smoking	**<0.001**	5.383 (2.878−10.069)	**<0.001**	4.292 (2.209−8.339)
Family history of TB	0.644	0.763 (0.242−2.404)		
Vitamin D level	**0.004**	0.944 (0.907−0.982)	**0.041**	0.560 (0.117−0.905)
VDR FokI (rs2228750) (Ff+ff)	**0.002**	2.607 (1.409−4.822)	**0.015**	1.906 (1.336−3.879)

OR, odds ratio; CI, confidence interval.

DISCUSSION

Pulmonary tuberculosis is a worldwide major health problem and the most infectious disease causing high mortality in humans. [13] Maintenance of bone density is the classic role of vitamin D; however the most important "non-classic" role is modulating both the innate and adaptive immune systems. [14]. The VDR gene seems to be an important factor that shapes the host susceptibility to TB due to the potential roles of VDR in the immune response to TB. [15] We studied 40 patients with pulmonary TB with mean age 36.6±9.9 years and a male to female ratio 2.6:1, along with 40 age and sex-matched apparently healthy control subjects. Several studies have reported a male predominance to MTB infection. [16] This might be attributed to the fact that men are more active and have more frequent social contacts, risk of infection, and vulnerable to stress which may modify their immune response and progression from infection to disease. Our studied cases were within middle age group. The same was reported by various studies conducted on Egyptian TB patients which reported that the highest prevalence of TB was among individuals between 15−45 years, and the lowest prevalence was in the extremes of age (<15 years and >60 years). [17] Moreover, in another study conducted at El-Minia governorate between 1997 and 2010; it was found that the highest incidence of TB occurred in the age group 15-29 years and the lowest incidence occurred in the extremes of age. [18] This could be referred to the increased prevalence of smoking behavior and alcohol abuse in this active middle age male group in our society. Moreover, poverty, malnutrition, physical, mental, and occupational stress, and greater exposure to infection are other contributing factors among this age group. In our study, smoking was a significant risk factor among TB patients (P < 0.001). This was also the case in previous studies conducted on TB patients in Egypt[19,20], Brazil[21], Japan[22], UK, China, India and USA.[23] In our study, serum level of 25(OH)D was significantly lower in TB patients than control group (P = 0.002) and TB patients showed a significant insufficient and deficient levels of 25(OH)D compared to control group (P = 0.048). The association between vitamin D and tuberculosis has been extensively investigated. Studies on populations from different origins; as Gujarat India [24], Indian, Pakistani, Somali, Afghan, Sri Lankan and African residents in London [25], African immigrants in Australia [26], Kenya [27], West Africa [28] and China [29] found a significantly lower mean of 25(OH)D concentrations higher prevalence of vitamin D deficiency in TB patients than healthy

controls. Although, 25(OH) D levels did not differ between TB patients and healthy controls in Indonesia. [30] In Egypt, plasma levels of 25(OH)D were significantly higher in controls than in TB patients with 72.5% of patients had vitamin D insufficiency. [31]

Our studied sample of individuals was selected randomly from population in Cairo Governorate in Egypt. Applying Hardy Weinberg equation, revealed that VDR FokI (rs2228750) genotypes in control as well as in TB patients groups were in Hardy Weinberg equilibrium. Taking FF genotype and F allele as references; *VDR FokI* gene (rs2228750) Ff and ff, genotypes and f allele showed significantly higher frequencies in TB patients when compared to control groups (P = 0.023, 0.014, <0.001 respectively), with increased risk to develop TB (OR 2.208, 4.318, 4.585 respectively). In South Koreans, the prevalence of the VDR *FokI* Ff genotype showed a 1.4-fold increase in TB patients compared with normal healthy groups without significant association between the genotype groups, TB patient and normal control. [32] In Chinese population, *FokI* site showed higher mutation frequency in TB patients who had relatively significant higher frequencies of ff genotype and f allele.[29]

While, in another study VDR *FokI* polymorphism didn't show significant association with TB susceptibility. [16] Additionally, a previous Egyptian study conducted in Sohag University showed that the VDR *FokI* Ff genotype was prevalent in TB patients and FF genotype in controls without association between VDR *FokI* genotypes and 25(OH) vitamin D levels in TB patients. [31] A possible explanation for this observed heterogeneity is the potential effect of relevant environmental factors on different populations such as sunlight exposures and diet that can affect serum vitamin D concentrations. Another reason is the different of genetic background of various populations.[33,34]

We found that TB patients' with ff genotype had significantly lower vitamin D level compared with patients with FF or Ff genotypes (P = 0.016), vitamin D deficiency was significantly associated with ff genotype and vitamin D insufficiency was significantly associated with Ff genotype (P = 0.045). Likewise, only VDR *FokI* ff genotype showed significant association with 25(OH) vitamin D concentration.[10] On the contrary, some studies did not show any link of serum vitamin D level or vitamin D state with different VDR *FokI* genotypes.[29,35,36] Logistic regression analysis revealed that excess smoking, lower level of vitamin D and VDR *FokI* (rs2228750) (Ff+ff) genotypes were predictors of MTB infection susceptibility. This was reported previously, where VDR *FokI* ff genotype was found to be associated with susceptibility to spinal TB [37] and VDR gene SNPs [−336 (rs4804803) and −139 (rs2287886)] were associated with susceptibility to pulmonary TB in a West African population. [38]

CONCLUSION

Our findings support the hypothesis that vitamin D deficiency plays a role as a risk factor for TB. Although, VDR polymorphism may be of immunoregulatory importance for many disease processes, *FokI* (rs2228750) polymorphism in the VDR gene may be partially responsible for host susceptibility to human tuberculosis in Egyptians.

Conflict of Interest

The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

Authors' Contributions

OE; work idea and design, laboratory experiments, analysis, and interpretation; drafting and revising manuscript and final approval of the version to be published. SA; work idea and design and revising manuscript and final approval of the version to be published. NS; data acquisition, laboratory experiments and analysis; drafting manuscript and final approval of the version to be published. SF; work idea and design, analysis, and interpretation of results; revising manuscript and final approval of the version to be published.

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