Impact of Vitamin D Receptor Gene Polymorphisms on the Susceptibility to Tuberculosis among Iraqi Patients

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Abstract: Tuberculosis is a cosmopolitan disease caused by Mycobacterium tuberculosis. Even within similar environmental conditions there is obvious variation in the incidence of this disease which suggests genetic factor effects. This study aimed to assess the effect of vitamin D receptor gene polymorphisms on the susceptibility for tuberculosis among Iraqi patients. A total of 62 patients with tuberculosis and 48 apparently healthy controls were recruited for this case/control study. DNA was extracted from peripheral blood, and vitamin D receptor gene regions corresponding the FokI, BsmI and TaqI polymorphisms were amplified with specific primers using PCR. Genotyping was achieved by restriction fragment length polymorphisms. A significant association was found between homozygote mutant genotype of FokI (ff) and susceptibility to TB (ff vs. FF: OR=10.452, 95%CI=1.253-87.167, P=0.03). Allele f of this polymorphism was more prevalent among patients compared to controls (OR=3.333, 95%CI=1.635-6.797, P=0.001). On the other hand, allele t of TaqI polymorphism seems to have a protective role against TB as it had significantly higher frequency in controls compared to TB patients (OR=0.317, 95%CI=0.136-0.738, P=0.008). Therefore, the homozygote mutant genotype of FokI could be considered as a risk factor for tuberculosis, while allele t of TaqI has a protective role against the disease.

Keywords: tuberculosis, vitamin D receptor, FokI, BsmI and TaqI polymorphisms.

Introduction

Tuberculosis (TB) is one of the most important public health issues with an estimated 9.6 million new cases and 1.5 million deaths in 2014. Even with multiple virulent factor possessed by the causative bacterium, Mycobacterium tuberculosis, only 5-10% of the infected individuals develop the active disease. The effect of environmental and demographic factors such as socioeconomic status, malnutrition and gender in the susceptibility to the disease is beyond dispute, but even under similar such conditions there is a great variability in the infection rate. Such variability reflects the genetic factors which explain, at least in part, the high susceptibility or resistance to the infection in some individuals.

One candidate gene that may influence the susceptibility to TB is vitamin D receptor (VDR) gene which encodes for VDR protein. Vitamin D is a key regulator of cell mediated immunity (CMI), the effective arm of immune response against TB. Deficiency in this vitamin was found to be associated with an increased risk of TB, while vitamin D supplementation was linked with improvement in immunity against the disease. The active form of vitamin D, 1,25-dihydroxyvitamin D₃, exerts its effect by binding to VDRs which are expressed abundantly on activated macrophages, lymphocytes and dendritic cells. Thus, it is reasonable to hypothesize that polymorphisms (especially in coding regions) of VDR gene will affect the binding activity of...
this receptor with vitamin D and affect the role of this vitamin and eventually the immunity against TB. In fact, this notion was subjected for intensive studies in different populations. Usually four polymorphisms in VDR gene (FokI, BsmI, ApaI and TaqI) were the subject of these studies. In a meta-analysis included 6179 TB patients and 6585 healthy controls, Chen et al. reported significant association between the heterozygote genotype of FokI polymorphism and the increased risk of TB. In European subjects, the homozygote and heterozygote genotypes of BsmI polymorphism was significantly associated with decreased risk of TB. There are disparities among different populations regarding the frequency of different alleles, and very few studies regarding the influence of VDR gene polymorphisms on different diseases among Iraqi population. Thus, this study aimed to investigate the influence of three variants (FokI, BsmI and TaqI) in VDR gene on the susceptibility to TB among Iraqi population.

Subjects and Methods

Study Population

This case/control study recruited 62 fully informed consent TB patients (39 men and 23 women, mean age 59.18 years, range 29-83 years) who were attending Center of Chest and Respiratory Diseases/ Baghdad during the period from December 2014 to April 2015. Data including age, gender, smoking, body mass index (BMI), diabetes mellitus (DM), and first relative family history TB were obtained from direct interview or extracted from medical records.

The specific criteria for enrollment were defined as the presence of at least one of the following: clinical and radiological findings that indicate the presence of pulmonary TB, and at least one positive M. tuberculosis culture from three separate sputum examinations, or one bronchial washing specimen obtained from bronchial scopy, improvement in suspected pulmonary TB with empirical anti-TB therapy as indicated via clinical and radiological findings, positive result for Xpert test which is a modern test for molecular detection of the causative bacteria in body fluid and pathological evidence of TB as indicated from pleural or lung biopsy.

Control group consisted of 48 family unrelated, age-matched, apparently healthy blood donors attending Al-Kadhymiya Donation Center which is located at the same geographical area that the TB patients reside. As in TB patients, similar data were obtained from controls through direct interview. Exclusion criteria were defined as the presence of at least one of the following: fever greater than 38.5°C, significant weight loss according to BMI calculation, productive cough and night sweat for more than two weeks, pregnancy or nursing an infant and receiving an immuno-suppressive drug or cancer-related therapy.

Samples

Two milliliters of venous blood was collected in EDTA tubes and kept at -20°C until be used for DNA extraction.

DNA Extraction and Genotyping

DNA was extracted from blood samples using ready kit (Favor prep DNA extraction mini kit/ Favor Gene Biotechnologies/ Taiwan) according to the manufacturer's instructions. The primer sets used for amplification and fragment size are shown in table 1. Template DNA (10 µL) from each sample and primers (5 µL from each) were added to each master-mix tube (50 µL PCR master-mix (Bioneer/Korea). After mixing, the master-mix tubes were transferred to the thermocycler (Hybaid/ England) which was previously programmed. PCR conditions were similar for the three polymorphisms and involved an initial denaturation at 95°C for 3 min, 30 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, with final extension at 72°C for 5 min.

Following amplification, The PCR product was digested with restriction enzymes: FokI, ApaI and TaqI (New England Biolabs Inc./USA) that recognize specific sequences of interest. Enzyme digestion was performed in 25 µL with 10 µL PCR product, 5µl 10X NEB buffer (50mM NaCl, 10mM Tris-HCl, 10mM MgCl2, 1mM dithiothreitol, pH 7.9), and 1µl (10U) of the restriction enzyme. The reaction was adjusted to 25µl using sterile deionized H2O. The solution was mixed by flicking, followed by spinning in microcentrifuge at 5000 rpm for 30 s. The FokI and ApaI enzymes were incubated at 37°C for 5 min; TaqI enzyme was incubated...
at 65°C for 5 min. Digested fragments were separated on a 2% of agarose gel and visualized in the gel stained with ethidium bromide.

Table 2: PCR-RFLP pattern of FokI, BsmI, and TaqI polymorphisms of Vitamin D receptor gene

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Primers</th>
<th>PCR_RFLP products (bp)</th>
</tr>
</thead>
</table>
| FokI (T>C) | F:5’-AGCTGGCCCTGGCAGTCCCTCTGC-TCT-3’  
                               R:5’-ATGGGAAACACCCTTGTCTTCTTTCTTCTC-3’ | TT (FF): 196, 69  
                               TC (Ff): 265, 196, 69  
                               CC (ff): 265 |
| BsmI(A>G) | F:5’-CAACCAAGACTACAAGGTCCCGGTCTAGTGAC-3’  
                               R: 5’-AACCAGCGGGAAGAGGTCAAGGG-3’ | GG(BB): 650, 175  
                               GA(Bb):825, 650, 175  
                               AA(bb): 825 |
| TaqI (C>T) | F:5’-CAGAGCATGGACAGGGAGCAA-3’  
                               R:5’-CACTTCGAGGACAGGGCGTTAGC-3’ | TT(TT): 490  
                               TC(Tt): 490,290,200  
                               CC(tt): 290,200 |

Statistical Analysis

The distribution of the genotypes was compared with that expected from Hardy-Weinberg equilibrium (HWE) by the chi square ($\chi^2$) test. Frequencies of genotypes and alleles were compared between TB patients and controls using logistic regression test with adjustment for all the confounding factors. Odds ratios (OR) were calculated together with their 95% confidence intervals (95%CI). Differences in gender, family history, smoking, and DM were calculated by Fisher's exact test/Chi square test as appropriate, while students unpaired t test was used to compare the means and standard deviation of age and BMI. A two-tailed $P$ value of less than 0.05 was considered statistically significant. Statistical tests were performed using the software SPSS 16.0 (SPSS Inc., Chicago, Illinois).

Results

Demographic and clinical characteristics of the study population are shown in table (1). Of the studied risk factors, only BMI and smoking seemed to have significant effect on the susceptibility to TB.

Table (1): Demographic and clinical characteristics of the study population

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases N=62</th>
<th>Control N=48</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age in years (SD)</td>
<td>62.12 (8.82)</td>
<td>59.3 (7.91)</td>
<td>0.271</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39 (62.91%)</td>
<td>30 (62.5%)</td>
<td>0.561</td>
</tr>
<tr>
<td>Female</td>
<td>23 (37.09%)</td>
<td>18 (37.5%)</td>
<td></td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>57 (91.94%)</td>
<td>47 (97.92%)</td>
<td>0.174</td>
</tr>
<tr>
<td>Yes</td>
<td>5 (8.06%)</td>
<td>1 (2.08%)</td>
<td></td>
</tr>
<tr>
<td>Mean BMI (SD)</td>
<td>20.12 (4.53)</td>
<td>24.37 (4.92)</td>
<td>0.032</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>41 (66.13%)</td>
<td>43 (89.58%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Smoker (ex/current)</td>
<td>21 (33.87%)</td>
<td>5 (10.42%)</td>
<td></td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-diabetic</td>
<td>51 (82.26%)</td>
<td>41 (85.42%)</td>
<td>0.43</td>
</tr>
<tr>
<td>Diabetic</td>
<td>11 (17.74%)</td>
<td>7 (14.58%)</td>
<td></td>
</tr>
</tbody>
</table>

BMI: body mass index, N: number, SD: standard deviation
Allele frequencies in the study population were in accordance with HWE for the three SNPs.

FokI polymorphism had three genotypes in TB patients and controls. These were FF, Ff and ff (figure 1). In patients these genotypes respectively represented 50%, 35.48% and 14.52% compared to 75%, 22.92% and 2.08% respectively in controls with significant difference of ff vs FF (OR=10.452, 95%CI=1.253-87.167, P=0.03) as shown in table 2.

At allelic level, the frequency of f allele was higher in TB patients (32.26%) than controls (13.54%) with significant difference (OR=3.33, 95%CI=1.635-6.797, P=0.001).

Figure 1: FokI genotype patterns in TB patients after genotyping using RFLP-PCR visualized under U. V light after staining with ethidium bromide. M: DNA marker. Lanes 1,4,5,8 and 9 homozygote wild type (FF), lanes 2 and 6 heterozygote genotypes (Ff). Lanes 3 and 7 homozygote mutant genotypes (ff).

Similarly, the SNP BsmI appeared in three genotypes in patients and controls which were BB, Bb and bb (Figure 2). However, statistical analysis revealed no significant effect of this SNP on the susceptibility to TB neither as genotypes or alleles.

Figure 2: BsmI genotype patterns in TB patients after genotyping using RFLP-PCR visualized under U. V light after staining with ethidium bromide. M: DNA marker. Lanes 1,2,5,6 and 7 homozygote wild type (BB), lanes 4 heterozygote genotypes (Bb). Lanes 3 and 8 homozygote mutant genotypes (bb).

Although the homozygote tt and heterozygote Tt genotypes of the SNP taqI appeared to have protective role against TB, this effect was insignificant (OR=0.389, 95%CI=0.137-1.102, P=0.076 and OR=0.153, 95%CI=0.016-1.426, P=0.099) as shown in figure 3 and table 2. However, this protective effect was significant at allelic level as the frequency of t allele in controls was higher than that in TB patients (19.79% and 7.26% respectively) (OR=0.317, 95%CI=0.136-0.738, P=0.008).
**Figure 3:** TaqI genotype patterns in TB patients after genotyping using RFLP-PCR visualized under U. V light after staining with ethidium bromide. M: DNA marker. Lanes 1,2,3,5,8 and 9 homozygote wild type (TT), lanes 6 and 10 heterozygote genotypes (Tt). Lanes 4 and 7 homozygote mutant genotypes (tt).

**Table 2:** Genotypes and allele frequencies of VDR gene polymorphisms in TB patients and controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases N=62</th>
<th>Control N=48</th>
<th>P-value</th>
<th>OR(95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FokI Genotypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FF</td>
<td>31 (50%)</td>
<td>36 (75%)</td>
<td>0.025</td>
<td>1.0</td>
</tr>
<tr>
<td>Ff</td>
<td>22 (35.48%)</td>
<td>11 (22.92%)</td>
<td>0.057</td>
<td>2.232 (0.974-5.536)</td>
</tr>
<tr>
<td>Ff</td>
<td>9 (14.52%)</td>
<td>1 (2.08%)</td>
<td>0.030</td>
<td>10.452 (1.253-87.167)</td>
</tr>
<tr>
<td><strong>Alleles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>84 (67.74%)</td>
<td>83 (86.46%)</td>
<td>0.001</td>
<td>1.0</td>
</tr>
<tr>
<td>f</td>
<td>40 (32.26%)</td>
<td>13 (13.54%)</td>
<td></td>
<td>3.333 (1.635-6.797)</td>
</tr>
<tr>
<td><strong>BsmI Genotype</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>51 (82.29%)</td>
<td>35 (72.92%)</td>
<td>0.492</td>
<td>1.0</td>
</tr>
<tr>
<td>Bb</td>
<td>8 (12.9%)</td>
<td>10 (20.83%)</td>
<td>0.251</td>
<td>0.549 (0.197-1.529)</td>
</tr>
<tr>
<td>bb</td>
<td>3 (4.84%)</td>
<td>3 (6.25%)</td>
<td>0.656</td>
<td>0.686 (0.131-3.599)</td>
</tr>
<tr>
<td><strong>Alleles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>110 (88.71%)</td>
<td>80 (83.33%)</td>
<td>0.322</td>
<td>1.0</td>
</tr>
<tr>
<td>b</td>
<td>14 (11.29%)</td>
<td>16 (16.67%)</td>
<td></td>
<td>0.636 (0.294-1.378)</td>
</tr>
<tr>
<td><strong>TaqI Genotypes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>54 (87.1%)</td>
<td>33 (68.75%)</td>
<td>0.066</td>
<td>1.0</td>
</tr>
<tr>
<td>Tt</td>
<td>7 (11.29%)</td>
<td>11 (22.92%)</td>
<td>0.076</td>
<td>0.389 (0.137-1.102)</td>
</tr>
<tr>
<td>Tt</td>
<td>1 (1.61%)</td>
<td>4 (8.33%)</td>
<td>0.099</td>
<td>0.153 (0.016-1.426)</td>
</tr>
<tr>
<td><strong>Alleles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>115 (92.74%)</td>
<td>77 (80.21%)</td>
<td>0.008</td>
<td>1.0</td>
</tr>
<tr>
<td>t</td>
<td>9 (7.26%)</td>
<td>19 (19.79%)</td>
<td></td>
<td>0.317 (0.136-0.738)</td>
</tr>
</tbody>
</table>

**Discussion**

This study aimed to investigate the effect of certain SNPs in VDR gene on the susceptibility to TB among a sample of Iraqi patients. The study revealed a significant role of FokIff genotype as a risk factor for TB (OR=10.452, 95%CI=1.253-87.167, P=0.03). These results agree with that of Roth et al. who reported a strong positive association of ff genotype with TB among Asian population. Similar results were obtained by Gao et al. in a meta-analysis study in Asian subjects. However, a null result was recorded in the same study among African subjects. Same result was previously obtained by Babb et al. who studied this polymorphism in 249 newly diagnosed African patients with TB and 352 healthy controls.
Two major explanations for the effect of SNPs in VDR gene on the susceptibility or resistance to TB and may be on other diseases. The first explanation is that these SNPs may involve dramatic changes in the structure of VDR protein and/or in the VDR gene activation. Such changes could affect the cellular function of the active form of vitamin D which is role in TB resistance is well-documented. The other explanation implies linkage disequilibrium (LD) between these SNPs with the other unidentified genes which influence the outcome of TB.

FokI polymorphism most probably affects the structure of VDR protein more than its activity in LD. This polymorphism results in three amino acids elongation in VDR length. Biologically, this protein was found to be less active than the short one. Thus, the binding of vitamin D with its receptor is interrupted with some decrease in the activity of vitamin D as an immune-modulator.

Interestingly, the current study demonstrated a protective role of allele t of the TaqI variant against the disease (OR=0.317, 95%CI=0.136-0.738, P= 0.008). Worldwide, the association of TaqI tt genotype with TB is even more controversial than FokI ff. The current results are inconsistency with that of Wilbur et al. who linked this genotype with TB resistance. However, a case-control study in India documented the role of this genotype with increased susceptibility to the disease especially in women.

This polymorphism is located at the 3’end of the VDR gene neighboring the 3’UTR region, which do not result in changes in the predicted amino acid sequence of the VDR. It is involved in regulating the stability of VDR mRNA and the rate of transcription. Selvaraj et al. suggested that mRNA encoded from TaqI t allele would be more stable than that from T allele. Furthermore, T allele has been shown to cause decrease in the production of tissue inhibitor of metalloproteinase 1 which is a natural inhibitor of the MMP-9. More importantly, LD was recorded frequently between this polymorphism and the other SNPs in VDR gene.

Collectively, these candidate allele t of this SNP as protective allele against TB.

These inconsistencies between different studies regarding the effect of the SNPs on the susceptibility to TB may be attributed to two possible factors. Firstly, the potential influence of environmental factors. Dietary intake and exposure to sunlight for example are well defined factors associated with vitamin D levels, and this may, at least partially explain the null association among African subjects compare to European subjects because the former expose for more sunlight and are expected to have higher levels of active vitamin D which restrains the effect of VDR gene polymorphism.

The second factor referred to the diversity of genetic background in different populations. Of course, vitamin D status and susceptibility to TB are not restricted to VDR gene only; rather there is large number of loci which could interfere with these conditions and these loci have different genetic sequence in different populations.

The SNP BsmI appeared to have insignificant association with TB neither at genotypes nor at allelic levels. The only plausible explanation for significant effect of TaqI but not BsmI is that TaqI polymorphism but not BsmI may be in linkage disequilibrium with another marker that may be the true causative factor influencing the Vitamin D levels.

Collectively, these data strongly suggest the role of FokI ff genotype as a risk factor for TB and allele t of the SNP TaqI as a protective factor against the disease.

References