Association of vitamin D receptor gene variants of BsmI, ApaI and FokI polymorphisms with susceptibility or resistance to pulmonary tuberculosis

P. Selvaraj*, G. Chandra, Sunil Mathan Kurian, A. M. Reetha and P. R. Narayanan
Tuberculosis Research Centre, Indian Council of Medical Research, Chennai 600 031, India

Vitamin D receptor (VDR) gene polymorphism was studied to find out whether the variants of this gene are associated with susceptibility or resistance to pulmonary tuberculosis (PTB) and bacteriological relapse of tuberculosis. BsmI, ApaI and FokI polymorphisms of VDR gene were studied in PTB patients (n = 120), patient contacts (spouses of the patients; n = 80), bacteriological relapse patients (n = 48) and quiescent patients (n = 48). Significant increase of Bb genotype (heterozygote carrier) of BsmI polymorphism (P = 0.028) and FF genotype (homozygotes of common allele F) of FokI polymorphism (P = 0.034) were observed in male PTB patients than male contacts. The BB genotype (homozygote of common allele B) of BsmI polymorphism and AA genotype (homozygote of common allele A) of ApaI polymorphism were increased in male contacts than male PTB patients (BB: P = 0.018; AA: P = 0.04). No significant differences were found among female patients and female contacts. In bacteriological relapse cases of PTB, a decreased frequency of AA genotype (P = 0.015) and an increased frequency of Aa genotype (P = 0.024) were observed in bacteriological relapse patients than quiescent patients of PTB. The present study suggests that Bb genotype of BsmI polymorphism and FF genotype of FokI polymorphism of VDR gene may be associated with the susceptibility to tuberculosis in males. The BB and AA genotypes may be associated with resistance to PTB in males. The genotype Aa may be associated with bacteriological relapse and AA may be associated with protection against bacteriological relapse.

VITAMIN D3 (1,25 dihydroxy vitamin D3), the active form of vitamin D regulates calcium and bone metabolism. It is an immunoregulatory hormone which plays a vital role in monocyte/macrophage activation and is shown to influence the immune response1. Vitamin D3 is one of the few mediators shown to impair the growth of Mycobacterium tuberculosis in the macrophage2. The effects of vitamin D are exerted by interaction through vitamin D receptor (VDR). Various diallelic polymorphisms have been identified in the VDR gene (BsmI site – alleles B [common allele] and b [infrequent allele]; ApaI site – alleles A [common allele] and a [infrequent allele]; TaqI site–alleles T [common allele] and t [infrequent allele]; FokI site–alleles F [common allele] and f [infrequent allele])3.4. Among these polymorphisms, BsmI and TaqI are shown to be associated with the bone mineral density6,10. The tt genotype of the infrequent allele of TaqI polymorphism of VDR gene has been shown to be associated with decreased bone mineral density, resistance to primary and secondary hyperthyroidism, and resistance to prostatic cancer5,6,7. The variant genotype aa (homozygotes of infrequent allele) of ApaI and bb (homozygote of infrequent allele) of BsmI polymorphisms of VDR gene have been shown to be associated with increased bone mineral density in females10, and the VDR haplotypes Bat and baT with the regulation of VDR expression. Moreover, there seems to be no apparent linkage disequilibrium between these polymorphisms and the VDR FokI polymorphism11. By producing a reporter gene construct under the control of a vitamin D response element, increased vitamin D-dependent transcriptional activation of VDR gene was shown with m allele (F allele of FokI polymorphism) than M allele (f allele of FokI polymorphism)12.

It has been shown that in the north Indian population, tt genotype is associated with tuberculoid leprosy and TT genotype with lepromatous leprosy13. The variant genotype tt of VDR gene has been shown to be associated with decreased risk of tuberculosis in Gambian (African) population14. Our recent studies in south Indian pulmonary tuberculosis (PTB)15 and spinal tuberculosis (extrapulmonary form of tuberculosis)16 patients revealed that variant genotype tt of VDR is associated with susceptibility to pulmonary as well as spinal tuberculosis in female
patients; whereas a trend towards an opposite picture with the \( tt \) genotype was seen in male patients and male contacts. We further studied the \( BsmI, ApaI \) and \( FokI \) polymorphisms of \( VDR \) gene to find out whether there is any association with susceptibility or resistance to PTB and bacteriological relapse of PTB.

Subjects included in this study were 120 PTB patients, and 80 patient contacts (spouses of the patients) as controls. Among the 120 PTB patients, 74 were male and 46 were female. The mean age with standard error (SE) was 40.8 ± 1.3 yrs for males and 36.2 ± 1.5 yrs for females. Among the 80 control subjects (contacts), 31 were male and 49 were female. The mean age with SE was 43.0 ± 2.3 yrs for males and 36.2 ± 1.2 yrs for females.

PTB patients (\( n = 120 \)) were selected from among subjects of an earlier chemotherapy study, which consisted of different drug regimens. Patients classified as suffering from active PTB (smear and culture positive for \( Mycobacterium tuberculosis \)) were treated at our centre. All these patients had received supervised short course chemotherapy for 6 to 8 months duration and had been followed up for five years after treatment. During follow-up, around 10% of the treated patients had a bacteriological relapse of the disease. The relapse patients were treated with other drug regimens and cured.

The relapse and quiescent patients were selected from the cured patients (\( n = 120 \)) of the previous chemotherapy study. Both the relapse and quiescent patients were treated/cured 10–15 years ago. The main criteria for relapse were clinical deterioration and persistent radiograph deterioration (chest X-ray). Moreover, two samples of sputum were examined by smear and culture monthly up to 24 months and three months thereafter. Presence of two or more cultures yielding ten or more colonies of \( M. tuberculosis \), in different months during a three-month period, of monthly follow up or a six-month period, of three-month follow-up were taken as evidences of bacteriological relapse. The relapse patients were culture-proven cases. Patients who had a bacteriological relapse (\( n = 49 \)) after 6–8 months treatment with short course chemotherapy were selected. An equal number of patients with quiescent disease (\( n = 49 \)), matched for treatment regimen and duration of follow up were also selected. The relapse and quiescent patients were age-matched. Among the 48 relapses, 39 had occurred in the first 12 months of follow-up. Care was taken to select quiescent controls who were not related to the patients with relapse. The primary treatment regimen consisted of rifampicin, isoniazid, pyrazinamide and streptomycin (or ethambutol) for the initial phase and two or three drugs in the continuation phase for 6–8 months. All the patients had bacilli sensitive to streptomycin, isoniazid and rifampicin initially. Among the relapse patients, 42 were male and 7 were female, aged 41.7 ± 3.2 yrs and 41.1 ± 11.8 yrs, respectively. Among the quiescent patients, 34 were male and 15 were female, aged 39.7 ± 3.2 yrs and 38.3 ± 5.0 yrs, respectively. The relapse patients and the quiescent patients were recruited from the same environment. They belong to the same socio-economic status and the same ethnic origin.

Control subjects consisted of spouses of the patients (family contacts; \( n = 80 \)). They were living together with the patients, before, during and after treatment. All the spouses were clinically normal at the time of blood sample collection. The patients and the contacts were not consanguineous. They were randomly selected and belonged to the same ethnic origin (Indo-Dravidian descent). The patients and the spouses were Tamil-speaking South Indian population living in and around Chennai, Tamil Nadu.

DNA was extracted from the peripheral blood white cells of patients and control subjects using salting-out procedure\(^7\). \( VDR \) gene polymorphism was studied using polymerase chain reaction (PCR) and restriction fragment length polymorphism. \( BsmI, ApaI \) and \( FokI \) polymorphisms were studied using the methods described elsewhere\(^8,9\).

For \( BsmI \) polymorphism, the following primers were used to amplify the \( VDR \) gene\(^6,10\): \( 5' – CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA – 3' \); \( 5' – AAC CAG CGG GAA GAG GTC AAG GG – 3' \). The PCR cycle conditions were: An initial denaturation step at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing 63°C for 30 s and extension 72°C for 60 s and finally 2 min extension at 72°C, using 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 mM MgCl\(_2\), 0.4 mM dNTPs, 0.01 mM of each primer (Gibco BRL, Grand Island, NY, USA), 100 ng of DNA and 1 unit of \( Taq \) polymerase (Gibco BRL, Grand Island, NY, USA) in a 25 µl reaction in a programmable thermocycler (MJ Research, Inc., Watertown, MA, USA). The amplified 825 base-pair (bp) product was subjected to \( BsmI \) restriction enzyme digestion. Five microlitres of the product was restriction digested with 3–5 units of \( BsmI \) enzyme (Gibco BRL) in a 20 µl reaction at 65°C for 3 h using manufacturer’s buffer. The \( BsmI \) enzyme-digested product was electrophoretically run on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide for 45–60 min at 80 V along with \( HaeIII \) digest as marker. Absence of \( BsmI \) restriction site (825 bp) was assigned as common allele \( B \) (wild-type allele) and presence of restriction site resulting in 650 bp and 175 bp fragments was assigned as infrequent allele \( bb \) (mutant allele). Genotypes were assigned accordingly as homozygotes for common allele (\( BB \)) and homozygotes for infrequent allele (\( bb \)). Presence of 825, 650, 175 bp was assigned as heterozygotes (\( Bb \)).

For \( ApaI \) polymorphism the following primers (Gibco BRL) were used to amplify the \( VDR \) gene\(^10\): \( 5' – CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA – 3' \); \( 5' – CAC TTC GAG CAC AAG GGG CGT TAGC – 3' \). The PCR conditions were: Initial denaturation step of 94°C for 4 min followed by 35 cycles of 94°C for 30 s
and 69 bp, designated as \((Ff)\). Wherever an expected cell value was less than 5, Fisher’s exact test was used. These analyses were carried out employing the Statcalc program (Epi Info, Version – 5; USD; Stone Mountain, GA) to find out the statistical significance; Odds ratio (OR) and 95% confidential interval (CI).

No difference in the frequencies of variant genotypes of BsmI, Apal and FokI polymorphisms of VDR gene was observed between PTB patients and contacts. However, increased trends in the frequencies of \(Bb\) genotype of BsmI polymorphism and \(FF\) genotype of FokI polymorphism were observed in total PTB patients than total controls. A trend towards a decreased frequency of \(ff\) genotype of FokI polymorphism was seen in total PTB patients than controls (Table 1).

Since tuberculosis is more prevalent in males\(^{18,19}\), the results were further analysed for males and females. In PTB, significantly increased frequency of \(Bb\) genotype \((P = 0.028; \text{OR}: 3.0; \text{CI}: 1.1–8.8)\) and \(FF\) genotype \((P = \ldots)\) was observed in total PTB patients than total contacts. However, no difference was observed in the frequencies of variant genotypes of \(Bb\), \(Bb\), and \(FF\) among male and female contacts, and patients.

**Table 1.** Genotype frequencies of BsmI, Apal and FokI polymorphisms of VDR gene in total contacts (spouses), total PTB patients, male and female contacts, and patients

<table>
<thead>
<tr>
<th>VDR BsmI Apal/FokI genotype</th>
<th>Total C(_t)</th>
<th>Total PTB C(_t)</th>
<th>Male Contacts C(_t)</th>
<th>Female Contacts C(_t)</th>
<th>Male PTB C(_t)</th>
<th>Female PTB C(_t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Bb)</td>
<td>(29)</td>
<td>(56)</td>
<td>(8)</td>
<td>(12)</td>
<td>(28.6)</td>
<td>(20.0)</td>
</tr>
<tr>
<td>(Ff)</td>
<td>(40)</td>
<td>(74)</td>
<td>(14)</td>
<td>(19)</td>
<td>(3.0)</td>
<td>(2.0)</td>
</tr>
<tr>
<td>(FF)</td>
<td>(37)</td>
<td>(70)</td>
<td>(9)</td>
<td>(13)</td>
<td>(28.6)</td>
<td>(20.0)</td>
</tr>
<tr>
<td>(Ff)</td>
<td>(30)</td>
<td>(60)</td>
<td>(6)</td>
<td>(10)</td>
<td>(0.37)</td>
<td>(0.20)</td>
</tr>
<tr>
<td>(ff)</td>
<td>(19)</td>
<td>(38)</td>
<td>(2)</td>
<td>(3)</td>
<td>(0.04)</td>
<td>(0.03)</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the subjects positive for respective genotype. \(n\), subjects studied.

\(C_t\): Contacts; PTB: Pulmonary tuberculosis; BsmI polymorphism – \(B\): Common allele, \(b\): infrequent allele, \(BB\): Homozygote of common allele, \(Bb\): Heterozygote carrier, \(bb\): Homozygote of infrequent allele, Apal polymorphism – \(A\): Common allele, \(a\): infrequent allele, \(AA\): Homozygote of common allele, \(Aa\): Heterozygote carrier, \(aa\): Homozygote of infrequent allele, FokI polymorphism – \(F\): Common allele, \(f\): Infrequent allele, \(FF\): Homozygote of common allele, \(Ff\): Heterozygote carrier, \(ff\): Homozygote of infrequent allele; *\(BB\): Male contacts vs male PTB patients; \(\chi^2\), \(P = 0.018; \text{Odds ratio (OR)}: 0.33; 95\% \text{Confidence Interval (CI)}: 0.12–0.85; *\(Bb\): Male contacts vs male PTB patients; \(\chi^2\), \(P = 0.028; \text{OR}: 3.0; \text{CI}: 1.1–8.8; *\(Aa\): Male contacts vs male PTB patients; \(\chi^2\), \(P = 0.04; \text{OR}: 0.37; \text{CI}: 0.1–1.0; *\(FF\): Male contacts vs male PTB patients; \(\chi^2\), \(P = 0.034; \text{OR}: 2.76; \text{CI}: 1.67–7.21.\)
polymorphism and a decreased frequency of towards an increased frequency of 0.12 – 0.85; whereas, increased frequency of female contacts (Table 1). An overall picture of various VDR gene polymorphisms with susceptibility or resistance to PTB is presented in Table 2.

In bacteriological relapse patients, significantly decreased frequency of AA genotype (P = 0.015; OR: 0.3; CI: 0.1–0.81) and an increased frequency of Aa genotype (P = 0.024; OR: 2.8; CI: 1.13–6.99) was observed than quiescent patients (Table 3). Moreover, trends toward a decreased frequency of BB genotype and an increased frequency of Bb genotype were also observed in bacteriological relapse patients than quiescent patients (Table 2).

The VDR gene polymorphic variants of BsmI, Apal and FokI polymorphism of the present study as well as TaqI polymorphism of our earlier study showed an association with the susceptibility or resistance to PTB in South Indian patients of Indo-Dravidian descent. However, the variant ff genotype of FokI polymorphism of VDR gene and 25-hydroxycholecalciferol deficiency have been shown to be strongly associated with PTB in Gujarati Indians living in London. Gujarati Indians belong to an ethnically different population of Indo-Aryan origin. Moreover, the production of vitamin D level may change depending on the environment as well as exposure to sunlight (ultraviolet rays). In a study carried out in the Gambian population (West Africa) PTB patients, the tt genotype of TaqI polymorphism of VDR gene was found less frequently in cases of PTB, suggesting that this genotype may be associated with resistance to PTB, whereas Apal polymorphism showed no association. This suggests that the association of variant VDR genotypes with different ethnic populations may be due to gene–environment interaction.

In the present study, male patients showed an increased frequency of heterozygous genotype BB of BsmI and FF genotype of FokI polymorphisms of VDR gene. This suggests that Bb and FF genotypes may be associ-
ated with the susceptibility to PTB in males. Whereas BB and AA genotypes are associated with resistance to PTB in male subjects. On the other hand, none of the polymorphic variants of the VDR gene studied (BsmI, Apal, FokI) showed any association with the susceptibility or resistance to PTB in female subjects. However, our earlier studies showed that the variant genotype tt of TaqI polymorphism of VDR gene is associated with the susceptibility to both pulmonary and spinal TB in female patients. The present study suggests that variant genotypes of BsmI, Apal and FokI sites of VDR gene, either alone or in combination with TaqI site of VDR gene, may be associated with susceptibility or resistance to PTB in males or females.

Differential susceptibility with variant genotypes of VDR gene in male and female subjects may be due to gene–gene interaction. It has been shown that during adulthood, rates of TB are consistently higher for men than for women. Apart from the exposure to the source of infection, one of the mechanisms suggested for this gender gap is the biological difference. Further, an X-chromosome susceptibility gene has been suggested which may contribute to the more number of males with tuberculosis observed in many populations. Moreover, besides adrenal and gonadal sex steroids, vitamin D is also included in the steroid superfamily. Since the steroid hormone receptors share substantial homologies, the sex steroid or other steroid hormones may influence VDR expression. It has been suggested that a VDR gene effect may modulate physiological responses to dietary vitamin D and calcium intake as well as the sex hormones as suggested for osteoporosis, which may play a role in the susceptibility or resistance to tuberculosis. Moreover, the effect of endogenous and/or intake of vitamin D may probably regulate the immunity to tuberculosis in a susceptible or resistant male or female host.

In the present study, while the heterozygote genotypes Aa and Bb are associated with the bacteriological relapse of PTB, the homozygotes AA and BB of the common alleles of Apal and BsmI are associated with resistance to bacteriological relapse. Our earlier study revealed the association of tt genotype of TaqI polymorphism with resistance to bacteriological relapse, irrespective of the gender of the patient. Since these genes are strongly linked, probably the haplotypes may be associated with the regulation of VDR expression. Altered VDR expression along with other host factors may play a role either in the endogenous reactivation or re-infection.

The present study suggests that Bb genotype of BsmI polymorphism is associated with susceptibility to PTB in males. In females, probably other genotypes such as tt of TaqI polymorphism may be associated with susceptibility to PTB. Heterozygote genotypes Aa and Bb may be associated with bacteriological relapse of PTB. Due to gene–environment and gene–gene interactions, the expression of VDR and the action of vitamin D through this receptor may be modulated. The polymorphic variants of VDR gene along with other gene and environmental factors may be responsible for an altered cell-mediated immunity against M. tuberculosis in a susceptible or resistant male or female host.

ACKNOWLEDGEMENTS. G.C and S.M.K. were financially supported in the form of junior and senior fellowships by the University Grants Commission, New Delhi and the Council of Scientific and Industrial Research, New Delhi respectively.

Received 18 October 2002; revised accepted 3 March 2003