RESEARCH COMMUNICATIONS

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Association of vitamin D receptor gene variants of *Bsm*I, *Apa*I and *Fok*I 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. *Bsm*I, *Apa*I and *Fok*I 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 *Bsm*I polymorphism ($P = 0.028$) and *FF* genotype (homozygotes of common allele *F*) of *Fok*I polymorphism ($P = 0.034$) were observed in male PTB patients than male contacts. The *BB* genotype (homozygote of common allele *B*) of *Bsm*I polymorphism and *AA* genotype (homozygote of common allele *A*) of *Apa*I 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 *Bsm*I polymorphism and *FF* genotype of *Fok*I 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 D_3 (1,25 dihydroxy vitamin D_3), 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 response[1]. Vitamin D_3 is one of the few mediators shown to impair the growth of *Mycobacterium tuberculosis* in the macrophage[2]. The effects of vitamin D are exerted by interaction through vitamin D receptor (VDR). Various diallelic polymorphisms have been identified in the *VDR* gene (*Bsm*I site – alleles *B* [common allele] and *b* [infrequent allele]; *Apa*I site – alleles *A* [common allele] and *a* [infrequent allele]; *Taq*I site–alleles *T* [common allele] and *t* [infrequent allele]; *Fok*I site–alleles *F* [common allele] and *f* [infrequent allele])[3–9]. Among these polymorphisms, *Bsm*I and *Taq*I are shown to be associated with the bone mineral density[6,10]. The *tt* genotype of the infrequent allele of *Taq*I 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 cancer[3,6,7]. The variant genotype *aa* (homozygotes of infrequent allele) of *Apa*I and *bb* (homozygote of infrequent allele) of *Bsm*I polymorphisms of *VDR* gene have been shown to be associated with increased bone mineral density in females[10], 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 *Fok*I polymorphism[11]. 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* allelele of *Fok*I polymorphism) than *M* allele (*f* allele of *Fok*I polymorphism)[12].

It has been shown that in the north Indian population, *tt* genotype is associated with tuberculoid leprosy and *TT* genotype with lepromatous leprosy[13]. The variant genotype *tt* of *VDR* gene has been shown to be associated with decreased risk of tuberculosis in Gambian (African) population[14]. 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

*For correspondence. (e-mail: psraj21@hotmail.com)

patients; whereas a trend towards an opposite picture with the *tt* genotype was seen in male patients and male contacts. We further studied the *Bsm*I, *Apa*I and *Fok*I 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[17]. *VDR* gene polymorphism was studied using polymerase chain reaction (PCR) and restriction fragment length polymorphism. *Bsm*I, *Apa*I and *Fok*I polymorphisms were studied using the methods described elsewhere[8,10].

For *Bsm*I 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 MgCl₂, 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 *Bsm*I restriction-enzyme digestion. Five microlitres of the product was restriction digested with 3–5 units of *Bsm*I enzyme (Gibco BRL) in a 20 µl reaction at 65°C for 3 h using manufacturer's buffer. The *Bsm*I 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 *Hae*III digest as marker. Absence of *Bsm*I 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 *b* (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 *Apa*I 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

(denaturation), 65°C for 30 s (annealing) and 72°C for 60 s (extension) and a final extension step at 72°C for 2 min using 10 mM TAPS [3-Tris(hydroxymethyl) methyl aminopropane sulphonic acid], (pH 8.7), 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% gelatin (Genei, Bangalore, India), 0.6 mM dNTPs, 0.01 mM of each primer, 100 ng of DNA and 1 unit of *Taq* polymerase (Gibco BRL) in a 25 µl reaction and run in a programmable thermocycler (MJ Research Inc.). The amplified 2000-bp PCR product was subjected to *Apa*I restriction enzyme (Gibco BRL) digestion. Five microlitres of the PCR product was restriction digested with 5 units of *Apa*I restriction enzyme in a 20 µl reaction using manufacturer's buffer at 30°C for 3 h. The *Apa*I enzyme-digested product was electrophoretically (80 V for 1 h) run on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide along with *Hae*III digest as marker. Absence of *Apa*I restriction site (2000 bp) was assigned as common allele *A* (wild-type allele) and presence of restriction site resulting in 1700 bp and 300 bp fragments was assigned as infrequent allele *a* (mutant allele). Genotypes were assigned accordingly as homozygotes for common allele (*AA*) and homozygotes for infrequent allele (*aa*). Presence of 2000, 1700 and 300 bp fragments was assigned as heterozygotes (*Aa*).

For *Fok*I polymorphism, the following primers were used to amplify a 265 bp product from the region flanking exon 2 of *VDR* gene[8]: 5′ – AGC TGG CCC TGG CAC TGA CTC TGC TCT – 3′; 5′ – ATG GAA ACA CCT TGC TTC TTC TCC CTC – 3′. Next, 100 ng of DNA was amplified by PCR using 10 mM Tris HCl, 50 mM KCl, 1 mM MgCl$_2$, 0.4 mM dNTPs and 1 unit *Taq* DNA polymerase in a 25 µl reaction mix. The PCR condition involved an initial denaturation of 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 1 min. A final extension step at 72°C for 2 min was also given. The PCR product was verified using a 1% agarose gel containing ethidium bromide. The PCR products were digested with *Fok*I restriction enzyme at 37°C for 3 h followed by electrophoresis in a 2% agarose gel containing *Hae*III digest was used as the molecular weight marker. The *FF* genotype (homozygote of common allele) lacks a *Fok*I site and shows only one band of 265 bp. The *ff* genotype (homozygote of infrequent allele) generates two fragments of 196 and 69 bp. The heterozygote displays three fragments of 265, 196 and 69 bp, designated as *Ff*.

The frequencies of the genotypes in the patient groups and controls were analysed using χ^2 with Yates correction (χ^2_y). 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 *Bsm*I, *Apa*I and *Fok*I polymorphisms of *VDR* gene was observed between PTB patients and contacts. However, increased trends in the frequencies of *Bb* genotype of *Bsm*I polymorphism and *FF* genotype of *Fok*I polymorphism were observed in total PTB patients than total controls. A trend towards a decreased frequency of *ff* genotype of *Fok*I 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 ($P = 0.028$; OR: 3.0; CI: 1.1–8.8) and *FF* genotype ($P =$

Table 1. Genotype frequencies of *Bsm*I, *Apa*I and *Fok*I polymorphisms of *VDR* gene in total contacts (spouses), total PTB patients, male and female contacts, and patients

VDR *Bsm*I/ *Apa*I/*Fok*I genotype	Total Ct $n = 80$	Total PTB $n = 120$	Male		Female	
			Contacts $n = 31$	PTB $n = 74$	Contacts $n = 49$	PTB $n = 46$
*Bsm*I						
BB	40.0	35.0	58.1*	31.5*	28.6	44.4
	(32)	(42)	(18)	(23)	(14)	(20)
Bb	36.3	46.7	25.8@	52.1@	42.9	37.8
	(29)	(56)	(8)	(38)	(21)	(17)
bb	23.8	18.3	16.1	17.8	28.6	20.0
	(19)	(22)	(5)	(13)	(14)	(9)
*Apa*I						
AA	37.5	33.3	51.6£	28.8£	28.6	42.2
	(30)	(40)	(16)	(21)	(14)	(19)
Aa	46.3	47.5	38.7	53.4	51.0	40.0
	(37)	(57)	(12)	(39)	(25)	(18)
aa	16.3	19.2	9.7	19.2	20.4	20.0
	(13)	(23)	(3)	(14)	(10)	(9)
*Fok*I						
FF	53.8	65.0	38.7$	63.5$	61.2	67.4
	(43)	(78)	(12)	(47)	(30)	(31)
Ff	36.2	30.0	48.4	32.4	30.6	26.1
	(29)	(36)	(15)	(24)	(15)	(12)
ff	10.0	5.0	12.9	4.1	8.2	6.5
	(8)	(6)	(4)	(3)	(4)	(3)

Numbers in parentheses represent the subjects positive for respective genotype. *n*, subjects studied.
Ct: Contacts; PTB: Pulmonary tuberculosis; *Bsm*I polymorphism – *B*: Common allele, *b*: infrequent allele, *BB*: Homozygote of common allele, *Bb*: Heterozygote carrier, *bb*: Homozygote of infrequent allele, *Apa*I polymorphism – *A*: Common allele, *a*: Infrequent allele, *AA*: Homozygote of common allele, *Aa*: Heterozygote carrier, *aa*: Homozygote of infrequent allele, *Fok*I 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; c^2_y $P = 0.018$; Odds ratio (OR): 0.33; 95% Confidence Interval (CI): 0.12–0.85; @*Bb*: Male contacts vs male PTB patients; c^2_y $P = 0.028$; OR: 3.0; CI: 1.12–8.81; £*AA*: Male contacts vs male PTB patients; c^2_y $P = 0.04$; OR: 0.37; CI: 0.1–1.0; $*FF*: Male contacts vs male PTB patients; c^2_y $P = 0.034$; OR: 2.76; CI: 1.07–7.21.

Table 2. Association of VDR genotypes with susceptibility or resistance to PTB

VDR gene polymorphism	Susceptibility		Resistance	
	Male	Female	Male	Female
BsmI	Bb (Heterozygotes)	–	BB (Wild-homozygotes)	–
ApaI	–	–	AA (Wild-homozygotes)	–
TaqI	TT* (Wild-homozygotes)	tt* (Mutant-homozygotes)	–	TT* (Wild-homozygotes)
FokI	FF (Wild-homozygotes)	–	–	–

*Refs 15 and 16.

Table 3. Genotype frequency of BsmI, ApaI and FokI polymorphisms of VDR gene in quiescent and relapse patients of PTB

VDR-BsmI, ApaI and FokI genotypes		Genotype frequency (%)	
		PTB patients	
		Quiescent n = 48	Relapse n = 48
BsmI	BB	41.7 (20)	22.9 (11)
	Bb	43.7 (21)	62.5 (30)
	bb	14.6 (7)	14.6 (7)
ApaI	AA	43.7* (21)	18.7* (9)
	Aa	41.7@ (20)	66.7@ (32)
	aa	14.6 (7)	14.6 (7)
FokI	FF	68.8 (33)	60.4 (29)
	Ff	25.0 (12)	39.6 (19)
	ff	6.2 (3)	0 (0)

Failed PCR: One quiescent patient and one relapse patient out of 49 patients in each group; n, subjects studied; Numbers in parentheses represent the subjects positive for respective genotype; *AA, Quiescent patients vs relapse patients; c^2_y P = 0.015; OR: 0.3; CI: 0.1–0.81; @Aa, Quiescent patients vs relapse patients; c^2_y P = 0.024; OR: 2.8; CI: 1.13–6.99.

0.034; OR: 2.76; CI: 1.07–7.21) was observed in male patients than male contacts. Whereas, increased frequency of BB and AA genotypes was observed in male contacts than male patients (BB: P = 0.018; OR: 0.33; CI: 0.12–0.85; AA: P = 0.04; OR: 0.37; CI: 0.1–1.0). A trend towards an increased frequency of aa genotype of ApaI polymorphism and a decreased frequency of ff genotype

of FokI polymorphism was observed in male PTB patients than male contacts. However, no increase or decrease in the BsmI, ApaI and FokI polymorphic variants of VDR gene was observed in female patients and 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, ApaI and FokI polymorphism of the present study as well as TaqI polymorphism of our earlier study[15] 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[9]. 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 ApaI polymorphism showed no association[14]. 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 (*Bsm*I, *Apa*I, *Fok*I) showed any association with the susceptibility or resistance to PTB in female subjects. However, our earlier studies showed that the variant genotype *tt* of *Taq*I polymorphism of *VDR* gene is associated with the susceptibility to both pulmonary and spinal TB in female patients[15,16]. The present study suggests that variant genotypes of *Bsm*I, *Apa*I and *Fok*I sites of *VDR* gene, either alone or in combination with *Taq*I 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[18–20]. Apart from the exposure to the source of infection, one of the mechanisms suggested for this gender gap is the biological difference[20]. Further, an X-chromosome susceptibility gene has been suggested which may contribute to the more number of males with tuberculosis observed in many populations[21]. 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[22], 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 *Apa*I and *Bsm*I are associated with resistance to bacteriological relapse. Our earlier study[15] revealed the association of *tt* genotype of *Taq*I 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 *Bsm*I polymorphism is associated with susceptibility to PTB in males. In females, probably other genotypes such as *tt* of

*Taq*I polymorphism[15] 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.

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