Vitamin D receptor and interleukin-1 receptor antagonist gene polymorphism in spinal tuberculosis

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Our earlier studies revealed that both MHC (Major Histocompatibility Complex) and non-MHC genes are associated with the susceptibility to pulmonary tuberculosis (TB). To find out whether non-MHC genes such as vitamin D receptor (VDR) and interleukin-1 receptor antagonist (IL-1RA) genes are associated with the susceptibility to spinal TB (extrapulmonary form of TB), the present study was carried out in spinal TB patients ($n = 66$) and spouses of TB patients (spinal-TB and pulmonary-TB) ($n = 80$) (family contacts). A trend towards an increased per cent genotype frequency of IL-1RA genotype variant 22 (12.1%) was seen in spinal TB patients when compared to the controls (3.8%) (spouses of the patients) ($P = 0.057$; odds ratio 3.5). No difference was observed in the frequency of VDR genotypes between the overall spinal TB patients and the family contacts. However, the VDR mutant genotype *tt* was seen at a higher frequency in female patients with TB spine (TBS) (12.8%) than female contacts (4.2%) ($P > 0.05$ not significant; odds ratio 3.5). Among the contacts, a significantly increased frequency of wild type genotype *TT* (wild homozygotes) was seen in female contacts (55.1%) than male contacts (16.1%) ($P = 0.0012$). Similarly a significant decrease in *tt* genotype was seen in female contacts (4.1%) than male contacts (25.8%) ($P = 0.012$). The present study suggests that IL-1RA genotype 22 may be associated with the susceptibility to spinal TB. Moreover, vitamin D receptor *tt* genotype may be associated with the susceptibility to spinal TB in female patients. The study reveals that multicandidate genes may be associated with the susceptibility to spinal TB.

OUR studies showed that apart from Major Histocompatibility Complex[1] (MHC), non-MHC genes such as functional mutant homozygotes (FMH) of mannose binding protein (MBP) (also known as mannose binding lectin, MBL)[2] and mutant variant genotype (*tt*) of vitamin D receptor (VDR)[3] are associated with the susceptibility to pulmonary tuberculosis (TB). In the present study, attempts have been made to find out whether VDR and interleukin-1 receptor antagonist (IL-1RA) gene variants are associated with the susceptibility to spinal TB. Vitamin D_3 (1,25 dihydroxy vitamin D_3) is an immunoregulatory hormone and activates monocytes and stimulates cell-mediated immunity[4]. Vitamin D plays a vital role on monocyte/macrophage activation through vitamin D receptor and it is known that vitamin D is one of the few mediators shown to impair the growth of *M. tuberculosis* in the macrophages[5]. The effects of vitamin D are exerted by the interaction with VDR. VDR is a nuclear hormone and a member of the super family of steroid receptors which has a wide role in the regulation of calcium homoeostasis in a variety of tissues[6].

A cluster of VDR alleles is linked to other genes in the 3′ untranslated region (3′ UTR) of VDR gene complex[7,8]. Biallelic polymorphisms have been shown in the VDR gene. The 3′ UTR allele polymorphisms are in strong linkage disequilibrium with restriction fragment length polymorphisms (RFLPs) located in intron 8 (*Bsm*I and *Apa*I) and exon 9 (*Taq*I) of the VDR region. The polymorphisms identified in the gene cluster of VDR region are (*Bsm*I site, alleles *B* and *b*; *Apa*I site, alleles *A* and *a*; *Taq*I site, alleles *T* and *t*; *B*, *A* and *T* are wild type alleles and *b*, *a* and *t*, mutant alleles). Among the three polymorphisms, *Taq*I polymorphism has been shown to be functionally more important.

The *Taq*I RFLP defines a single base change *C* to *T* in codon 352 at the 3′ end of the *VDR* gene[7]. The less common allele of *Taq*I site designated as *t* (mutant allele) has been associated with higher levels of mRNA expression[8]. The *tt* genotype of the less common allele of *Taq*I site has been shown to be associated with bone mineral density[8], resistance to primary and secondary hyperparathyroidism[9], resistance to prostatic cancer[10] and susceptibility to various infectious diseases[11].

In TB, monocytes/macrophages play a major role in the host defence against *M. tuberculosis* and secrete inflammatory cytokines at the site of lesion[12]. IL-1RA is a cytokine factor which competes for the IL-1 binding site. IL-1RA is also known as an acute phase protein with anti-inflammatory activities. The production of IL-1α and IL-1β is antagonized by the IL-1RA genes/gene products[13]. The genes for IL-1α, IL-1β, IL-1 receptor, IL-1RA are located in chromosome 2. IL-1RA gene sequence revealed 86 base pair tandem repeat sequences (minisatellite) in the intron 2 region[14]. The IL-1RA allele 2 of the repeat has been found to be associated with several autoimmune diseases[15–17]. Macrophages from heterozygous carriers of IL-1RA allele 2 have been shown to produce more IL-1RA and less IL-1α than other genotypes[18].

Numerous studies have revealed the association of variant genotypes of VDR and IL-1RA genes in many infectious and non-infectious diseases in various populations. Though VDR and IL-1RA genes play an immunoregulatory role in infectious diseases, including TB, little attention has been paid to the VDR and IL-1RA gene polymorphisms and their association with the susceptibility/resistance to pulmonary and extrapulmonary form of TB. The goal of the present study was to find out whether non-MHC genes such as VDR and IL-1RA gene variants

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are associated with the susceptibility to spinal TB (extrapulmonary-TB).

Subjects included in this study were 66 spinal TB patients and 80 family contacts (spouses of pulmonary TB [$n = 47$] and spinal tuberculosis [$n = 33$] patients). Among the spinal TB patients, 27 were males and 39 were females. The mean age with standard error was 40.9 ± 3.0 for males and 47.1 ± 2.2 for females. Among the total control subjects, 31 were males and 49 were females. The mean age with standard error was 55.3 ± 3.5 for males and 43.9 ± 2.1 for females.

Patients with clinically and radiologically active form of spinal TB involving any vertebral body from the first thoracic to the first sacral, inclusive, were studied. The pre-treatment investigations were radiograph of the chest, examination by culture of two specimens of pus from any abscess or sinus, with radiographic evidence of pulmonary TB. All these patients had received a supervised short course chemotherapy of 6 to 9 months duration and followed up for a period of 5 years after treatment. These patients were treated 15 to 20 years earlier. At the time of blood sample collection, all these cured patients were in a quiescent stage of the disease[19].

Control subjects consisted of family contacts. They were living together with the patients before, during and after treatment for a period of 10 to 15 years. All the family contacts were clinically normal at the time of blood sample collection. The patients and the contacts were not consanguineous to each other. The patients and the contacts were randomly selected and belonged to the same ethnic origin (Indo-Dravidian descent). They were Tamil-speaking south Indian population (belonging to different communities/castes) living in and around Chennai.

From the patients and control subjects, DNA was extracted from peripheral blood white cells using salting out procedure[20].

Genotyping of VDR for spinal TB patients was carried out by PCR–RFLP (polymerase chain reaction–restriction fragment length polymorphism) method. The *Taq*I site gene polymorphisms at codon 352 of the VDR gene were studied as described earlier[10]. Codon 352 in exon 9 is polymorphic, existing as either ATC or ATT, both of which code for isoleucine and the C > *T* change is associated with the loss of a *Taq*I restriction site. The resulting alleles are designated *t* (*Taq*I site present) or *T* (*Taq*I site absent) and three possible genotypes result: *TT*, *Tt* and *tt*. A 740 bp fragment of the VDR gene was amplified by PCR using the primers 5′–CAG AGC ATG GAC AGG GAG CAA–3′ and 5′–GCA ACT CCT CAT GGC TGA GGT CTC–3′ located within intron 8 and exon 9 of the VDR gene[8].

The PCR conditions were 94°C for 30 s (denaturing), 63°C for 30 s (annealing) and 72°C for 1 min (extension) (36 cycles) using 10 mM TAPS [3-Tris (hydroxymethyl) methyl aminopropane sulfonic acid], pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (Bangalore Genei, India), 0.2 mM dNTPs, 0.01 mM of each primer, 100 ng of DNA and 1 unit of *Taq* polymerase (Bangalore Genei, India) in a 25 µl reaction.

Ten microlitre of the PCR product was subjected to *Taq*I restriction enzyme digestion at 65°C for 3 h using manufacturer's buffer (Bangalore Genei, India). The *Taq*I enzyme digested product was electrophoretically (80 volts for 1 h) run on a 2% agarose gel containing 0.2 µl of ethidium bromide (10 mg/ml). Three banding patterns are observed after digestion of the 740 bp amplification fragment, depending upon genotype (a) wild homozygous (*TT*): absence of the *Taq*I polymorphism results in two fragments of 495 bp and 245 bp, (b) mutant homozygous (*tt*): presence of the *Taq*I polymorphism results in three fragments of 290 bp, 245 bp and 205 bp, and (c) heterozygote carrier (*Tt*): presence of all four fragments 495 bp, 290 bp, 245 bp and 205 bp. The 245 bp fragment is constant among all genotypes having been created by a nonpolymorphic *Taq*I site within the amplification fragment and acts as an internal control for digestion[10].

Genotyping of IL-1RA was carried out as described earlier[3,21]. The intron 2 of the IL-1RA gene contains a minisatellite consisting of 2 to 6 copies of an 86 bp tandem repeat sequence. IL-1RA gene was amplified using the following primers: 5′–CTC AGC AAC ACT CCT AT–3′, and 5′–TCC TGG TCT GCA GGT AA–3′.

The PCR cycle condition was 94°C for 20 s, 58°C for 30 s and 72°C for 30 s (35 cycles), using 10 mM Tris-HCl, pH 8.3, 25 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 mM of each primer, 100 ng of DNA and 1 unit of *Taq* polymerase (Bangalore Genei, India) in a 15 µl reaction. The size variants were typed by electrophoresis of the PCR products in an ethidium-bromide stained 2% agarose gel. Based on the size (base pair) of the PCR products different alleles were determined. Further, based on the frequencies of the alleles, they are termed as allele-1 (410 bp), 2 (240 bp), 3 (325 bp), 4 (500 bp) and 5 (595 bp). Frequencies of the VDR and IL-1RA genotypes in the spinal TB patients and controls were analysed using χ^2 analysis with Yates correction by employing the Statcalc program (Epi Info, Version-5; USD; Stone Mountain, GA). The odds ratio (approximate relative risk) and the *P*-values for significance have been represented wherever necessary.

No difference was observed in the VDR genotype frequencies of the overall spinal TB patients and contacts (Table 1). However, a trend towards an increased frequency of mutant homozygotes (*tt*) was seen in female patients (12.8%) than female contacts (4.1%) ($P > 0.05$ not significant; odds ratio 3.5). Similarly, a trend towards an increased frequency of wild type homozygotes (*TT*) was seen in female contacts (55.1%) than female patients (43.6%). An opposite picture on the genotype frequencies was seen in male patients and male contacts. An increased frequency of wild type homozygotes (*TT*) was observed in

Table 1. Per cent genotype frequencies of VDR genes in female and male patients with spinal TB and contacts

VDR genotype variants	% Genotype frequency					
	Overall contacts (n = 80)	Overall patients (n = 66)	Female contacts (n = 49)	Female patients (n = 39)	Male contacts (n = 31)	Male patients (n = 27)
TT (Wild homozygotes)	40.0	40.9	55.1*	43.6	16.1*	37.0
Tt (Heterozygote carrier)	47.5	45.5	40.8	43.6	58.1	48.2
tt (Mutant homozygotes)	12.5	13.6	4.1**,@	12.8@	25.8**	14.8

T, wild type allele; *t*, mutant allele.
@χ^2 *P* uncorrected > 0.05 (not significant; Odds ratio : 3.5); *χ^2 *P* (corrected) = 0.0012; **χ^2 *P* (corrected) = 0.012.

male patients (37.0%) than male contacts (16.1%). Similarly, an increased frequency of mutant homozygotes (*tt*) was seen in male contacts (25.8%) than male patients (14.8%). However, these increases were not significant. A significantly increased *TT* (wild type homozygotes) genotype frequency was seen in female contacts (55.1%) than male contacts (16.1%) (P = 0.0012). On the other hand, a significantly increased mutant genotype (*tt*) was seen in male contacts (25.8%) than female contacts (4.1%) (P = 0.012). However, such differences were not observed between male patients and female patients.

No difference was observed in the frequency of the variant IL-1RA genotypes between patients and control subjects (Table 2). However, a trend towards an increased percentage frequency of the variant IL-1RA genotype 22 was seen in spinal TB patients (12.1%) than patient contacts (3.8%). (P = 0.057; odds ratio 3.5).

In the present study, VDR gene variants are not associated with the susceptibility to spinal TB in the overall patient group. However, a trend towards an increased frequency of VDR mutant genotype (*tt*) was seen in female patients with spinal TB compared to female contacts. Our earlier study in pulmonary TB revealed an increased VDR mutant genotype (*tt*) in female patients with pulmonary-TB[3]. Moreover, the wild type homozygotes (*TT*) are found to be decreased in female patients than female contacts. This suggests that the mutant genotype (*tt*) of VDR may be associated with the susceptibility to both pulmonary and spinal TB in female patients. On the contrary, a trend towards an increased frequency of VDR wild type genotype (*TT*) was seen with male spinal TB patients than male contacts. Moreover, a decreased frequency of mutant genotype *tt* was seen with male patients than male contacts. This suggests that wild type genotype *TT* may be associated with the susceptibility to spinal TB in male patients and the mutant genotype *tt* may be associated with resistance in male patients. However, to substantiate this preliminary study further work is needed. The present study suggests that mutant homozygotes (*tt*) of the VDR gene may contribute to host genetic variation in the outcome of TB in female subjects and resistance in male subjects. Allelic variants of the VDR appear to be associated with differential susceptibility to several infectious and non-infectious diseases[11]. In the Gambian pulmonary TB patients, the *tt* genotype (mutant homozygotes) was found less frequently in cases of pulmonary TB suggesting that this genotype is associated with resistance to pulmonary TB[22]. Further, in a study carried out in Indian leprosy patients, Roy *et al.*[23] have found *tt* genotype to be associated with tuberculoid leprosy, whereas the opposite *TT* genotype was associated with lepromatous leprosy.

Table 2. Per cent genotype frequencies of IL-1RA alleles in spinal TB patients and contacts

IL-1 RA genotype variants	% Genotype frequency	
	Contacts (n = 80)	Spinal-TB patients (n = 66)
11	48.7	50.0
12	41.3	31.8
14	6.3	4.6
22	3.8*	12.1*
24	0.0	1.5

*χ^2 *P* (uncorrected) 0.057; Odds ratio : 3.5.

Our earlier study in pulmonary TB[3] and the present study in spinal TB suggest that *tt* genotype may be associated with the susceptibility to pulmonary TB as well as extrapulmonary forms of TB in females. Mutant alleles of the VDR gene region have been shown to be associated with increased (*Taq*I site: mutant allele *t*) and decreased (*Bsm*I site: mutant allele *b*) transcriptional activity of the VDR region[8]. It has been shown that allele *t* plays an important role in female subjects. The VDR genotype *tt* is associated with bone mineral density as well as susceptibility to TB in females[24,25]. The female sex hormones as well as calcium intake probably influence the expression of the VDRs. This may influence the vitamin D-mediated monocyte/macrophage activation through VDRs. This in turn may influence the cidal activity of the monocyte/macrophage function against *M. tuberculosis*. It has been suggested that VDR gene polymorphism may be of immunoregulatory importance for many disease processes[22,23]. However, the immune mechanism of susceptibility to pulmonary or extrapulmonary TB as far as *Taq*I polymorphism of VDR gene is concerned, has to be further explored with the other genes of the VDR region.

In the present study, increased genotype frequency of IL-1RA 22 was seen in the patients compared to control subjects. The less common allele 2 (containing just two repeats of 86 bp) has been shown to be associated with increased IL-1RA protein production as well as decreased

IL-1α production[18]. It has been suggested that the polymorphism in the IL-1RA gene may be present in the regulatory area itself or in linkage disequilibrium with another polymorphism that regulates the transcription of both IL-1RA and IL-1α genes[18]. The variable repeat sequence has been shown to contain three potential protein binding sites: an IFN-α silencer A, an IFN-β silencer *B*, and an acute phase response element[21]. Further, it has been suggested that the variable tandem repeat polymorphism in intron 2 of the human IL-1RA gene may affect the activity of enhancer sequences that affect the transcription of the IL-1RA gene and other cytokine genes[18]. Several autoimmune diseases have been shown to be associated with IL-1RA allele 2 (refs 15–17). Our earlier study did not show any association with IL-1RA allele 2 and pulmonary TB[3]. However, increased frequency of IL-1RA genotype 22 was seen in spinal TB patients. IL-1RA allele 2 has been shown to be associated with increased production of IL-1RA protein (anti-inflammatory) and decreased IL-1α production (one of the inflammatory cytokines). The association of IL-1RA genotype 22 with spinal TB found in the present study may be due to increased production of IL-1RA protein at the site of lesion in the spinal column to antagonize the inflammatory action of IL-1α as well as the IL-1β cytokines. This in turn gives protection against the detrimental effects of the inflammatory cytokines such as IL-1α and IL-1β at the site of lesion.

The present study suggests that VDR and IL-1RA genotypes may be associated with the susceptibility to spinal TB. Moreover, these genes may also play an immunoregulatory role in the mechanism of susceptibility/resistance to pulmonary as well as extrapulmonary forms of TB.

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Hospital effluent: A source of multiple drug-resistant bacteria

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The present work was carried out to study the spread of multiple drug-resistant (MDR) bacteria from hospital effluent to the municipal sewage system. The MDR bacteria population in hospital effluents ranged from 0.58 to 40% for ten hospitals studied while it was less than 0.00002 to 0.025% for 11 sewage samples from the residential areas. Further, the MDR bacteria carried simultaneous resistance for most of the commonly used antibiotics and obviously the spread of such MDR bacteria to the community is a matter of grave concern.

DEVELOPMENT of drug resistance has followed the discovery of antimicrobial agents like a faithful shadow. Drug resistance observed till 1954 was through solitary events of bacterial chromosomal gene mutations. However,

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