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Association of VDR Gene Polymorphism and Haplotype with Tuberculosis Susceptibility, Treatment Outcome, Bacillary Load and Extrapulmonary Site

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Abstract

Background: A case-control study was conducted to study three *VDR* gene polymorphism, *FokI*, *BsmI* and *TaqI*, with Tuberculosis (TB)-susceptibility, treatment outcome, bacillary load and site of extrapulmonary TB.

Methods: Three hundred and fifty seven TB patients and 392 healthy unrelated controls with no clinical symptoms or family history of TB belonging to same ethnicity were enrolled.

Results: This study reported a significant association of 'ff' genotype of *VDR FokI* gene with susceptibility to TB. These results were highly significant in extrapulmonary (EP) cases, pleural effusion patients carrying polymorphic variant 'f' were at significantly higher risk of developing the disease where as presence of 'b' allele of *BsmI* variant contributed to protection against disease. *BsmI* polymorphism analysis reported a protective role of 'bb' genotype against TB. Treatment outcome analysis revealed 'Bb' genotype conferring risk in patients with unsuccessful treatment when compared to those with successful treatment. To the best of our knowledge no other study has reported a similar association. Low bacilli load was reported to be significantly higher in 'ff' carriers when compared to control group whereas 'bb' genotype was reported to confer protection against the same. Data in this study showed a positive association of smoking status and TB risk. Forty nine percent of smoker males belong to < 35 age-group. The frequency of TBf and TBF haplotype was reported to be significantly higher in case group when compared to control group. Haplotype TbF was found to be more frequent in control group than cases.

Conclusion: A significant association of *VDR* gene polymorphisms and haplotypes with disease susceptibility, treatment outcome, bacillary load and site of extrapulmonary TB along with smoking status and TB susceptibility was found.

Keywords: VDR; Gene polymorphism; Haplotype; Tuberculosis susceptibility; Bacillary load; Extrapulmonary TB

Introduction

India accounts for an estimated one quarter (24%) of all the global tuberculosis (TB) incident cases alone [1]. It is estimated that annually around 2,40,000 Indians die due to TB [1]. Many investigations have confirmed that host genetic factors are involved in the tuberculosis disease, and these include adoption studies [2], twin studies [3], genome wide linkage [4] and population-based case–control association studies [5-7]. Recently, a number of genes have been studied in various case-control studies with reference to tuberculosis, among which are *NRAMP1* (natural resistance associated macrophage protein one), *HLA-DQB1* (major histocompatibility complex, class II, DQ beta -1) and *VDR* (vitamin D receptor). The *VDR* gene is of particular interest as vitamin D modulates monocyte-macrophage activity in the body and plays a role in human innate immunity to infectious agents like *Mycobacterium tuberculosis.* Vitamin D exerts its actions through vitamin D receptor (VDR), a nuclear hormone receptor. Liver metabolises vitamin D into 25-hydroxyvitamin D3 (calcidiol/25(OH) 2D3), which is further converted to 1, 25(OH) 2D3 by kidneys (active form of vitamin D). A meta-analysis recently showed that vitamin D deficiency might play a role as risk factor during the development of TB [8].

The *VDR* gene is located on the long arm of chromosome 12 (12q12-14) and is composed of 10 exons, the first of which is not transcribed [9]. Polymorphisms in the *VDR* gene, which may influence VDR activity and subsequent downstream vitamin D-mediated effects, are therefore studied as potential candidates of risk markers for various clinical outcomes [8]. Three of these *VDR* polymorphisms can be distinguished by digestion with restriction enzymes. Two of them do not change the translated protein (*Bsm I* and *Taq I*). The polymorphisms recognized by BsmI (BB, Bb and bb) are located in intron 8 of the *VDR* gene and the one recognized by Taq I (TT, Tt and tt) is located in exon 9, that leads to a silent codon change, with ATT and ATC, both coding for isoleucine and has been associated with increased VDR mRNA stability. *FokI VDR* polymorphism recognized by Fok I restriction enzyme (FF, Ff and ff), results from a C→T transition that creates an alternative initiation codon (ATG), and three codons from the downstream start site. The VDR encoded by the f allele from the Fok I restriction enzyme recognition is increased in length by three amino acids, and the transcription of this allele is 1-7 times less efficient than the F allele and can alter the amount of VDR produced [10].

There is strong evidence to suggest that *VDR* gene variants may be related to the development of TB [8-12]. However, these polymorphisms were seemed to play different roles in different populations. Gao et al. [8] evaluated *VDR* gene polymorphism in different ethnic population studies published prior to April 2009 with regard to TB followed by a recent meta-analysis conducted by Cao et al. [11] in Chinese Han population. A significant association was observed for Asian subjects carrying different *VDR* gene variants [8].

Vegans and other vegetarians who limit their intake of animal products may be at greater risk of vitamin D deficiency than non-vegetarians, because foods providing the highest amount of vitamin D per gram naturally are all from animal sources [12]. Asians from the Indian subcontinent having vegetarian diet were reported to have vitamin D deficiency, known to affect immunological competence which might result in increased mycobacterial reactivation [13]. To check for the same we included the nutrition status of our subjects and analysed them with respect to *VDR* genotype. Recently Sharma et al. [14] reported an association of *VDR* polymorphism with

bacillary load. Validation of this preliminary report in a different population is attempted in this study.

To the best of our knowledge, no studies have reported the influence of *VDR* genes on outcome to TB treatment in North Indian population. We therefore, conducted a well characterised case-control study and a number of other demographic and clinical factors to reproduce the reports of the role of *VDR* polymorphism with TB in our study population as well as association of the same with treatment outcome, bacillary load and site of extrapulmonary TB.

Methods

Study population

Three hundred and fifty seven TB patients were enrolled for this longitudinal study after taking a written consent from each subject, from DOTS centre Rampur Bushahr, District Shimla of Himachal Pradesh (H.P.). These tuberculosis patients were classified into five groups according to Revised National Tuberculosis Control Programme (RNTCP) standard classification: 1) Extrapulmonary (EP) 2) Pulmonary Negative (PN; sputum smear negative pulmonary patients) 3) Pulmonary Positive (PP; sputum smear positive pulmonary patients) 4) Pulmonary All (PTB, Pulmonary Positive + Pulmonary Negative) and 5) TB All (TB, Pulmonary All + Extrapulmonary). Following WHO protocol the sputum smear status of subjects was established after screening of slides for *Tubercle bacilli* and radiological (X-ray) investigations [15]. Fifty patients (14%) out of 357 cases were relapse case belonging to pulmonary positive, negative as well as extrapulmonary category. According to WHO guidelines for disease classification of extrapulmonary TB on the basis of disease severity the patients could be divided into two categories: Severe (meningitis, miliary, pericarditis *etc.*) and Less-severe (lymph node, pleural effusion, abdominal *etc.*) [16]. EP cases here included 25 abdominal, 1 breast, 16 lymph node, 27 pleural effusion, 1 spine and 1 urinary tract case. For this study the study population was stratified on the basis of sputum bacillary load, EP site and treatment outcome. For analysis of treatment outcome and *VDR* gene polymorphism, the outcome was stratified into percentages of successful and unsuccessful outcomes, among all patients who underwent standard Directly Observed Treatment, Short-course (DOTS) therapy. For this part only pulmonary positive patients (PP) were included as their follow-up sputum smear reports were available thus, making assess of disease outcome convenient and unbiased. Integrated Counselling and Testing Centres (ICTCs) for HIV counselling and testing services was started in March 2009 in our hospital. None of the study patients taken after the date of start of this facility was found to be HIV positive.

Three hundred and eighty healthy unrelated controls with no clinical symptoms or family history of TB belonging to same ethnicity were taken. A strict exclusion criterion was applied for preventing biasing of the population by selecting only those patients who were settled in this area from minimum of three generations. Marriage within the local community is a common trend observed in this population. The study was approved by Postgraduate Institute of Medical Education & Research (PGIMER), Chandigarh and Indira Gandhi Medical College (IGMC), Shimla (H.P.).

Genotyping

One millilitre peripheral blood was taken and genomic DNA was then isolated by phenol-chloroform method [17] for the purpose of genotyping. Primers were used as in Wilkinson et al. [18]. PCR conditions for *VDR TaqI* polymorphism included initial 95 ˚C for 4 minutes followed by 35 cycles at 95 ˚C for 30 seconds, annealing at 60 ˚C for 30 seconds and extension at 72 ˚C for 90 seconds and a final extension at 72 ˚C for 7 minutes. For *FokI* polymorphism conditions included initial 96˚ C for 1 minutes followed by 30 cycles at 94 ˚C for 45 seconds, annealing at 61.5 ˚C for 45 seconds and extension at 72 ˚C for 60 seconds and a final extension at 72 ˚C for 5 minutes. *Bsm*I product was amplified by initial 96 ˚C for 1 minutes followed by 30 cycles at 94 ˚C for 60 seconds, annealing at 60 ˚C for 60 seconds and extension at 72 ˚C for 60 seconds and a final extension at 72 ˚C for 1 minutes.

The PCR product was digested with excess of restriction enzyme 6-8 hrs (TaqI and BsmI at 65 ˚C and FokI at 37 ˚C). The digested product was run on 2% agarose gel pre stained with ethidium bromide and visualised using a UV transilluminator. The presence of a restriction site was assigned a lowercase letter and its absence an uppercase letter, according to convention (*i.e.,* b and B for *BsmI*, f and F for *FokI*, and t and T for *TaqI*) (Figure 1, 2 and 3, respectively).

Statistical analysis

SPSS ver. 17 and Epi Info (TM) 3.4.3 were used for statistical analysis. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. The overall genotypic frequency was compared between the groups using 3x2 χ^2 test and the allelic frequency was compared by 2x2 χ^2 test. All data were considered significant when $p < 0.05$. Whenever an expected cell value was less than 5, Fisher's exact test was used. Construction of the *VDR* haplotypes

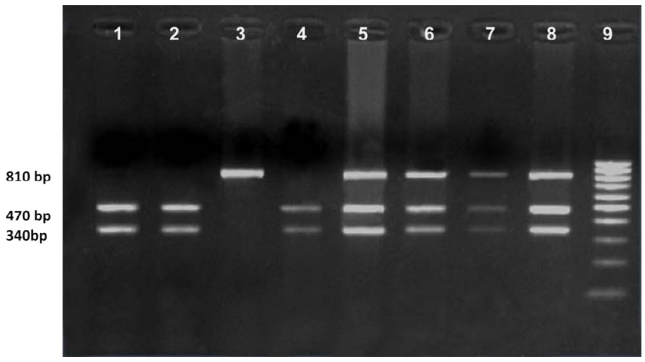

Figure 1: Genotyping of vitamin D receptor (*VDR*) BsmI polymorphism was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Lane 1, 2 and 4: polymorphic variant, Lane 3: wild, Lane 5-8: heterozygote and, Lane 9: 100bp marker.

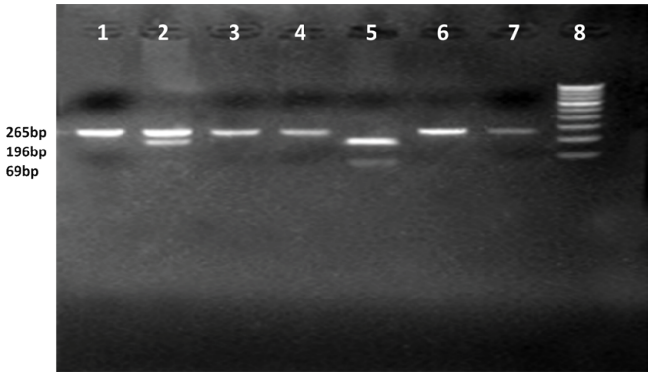

Figure 2: Genotyping of vitamin D receptor (*VDR*) FokI polymorphism was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Lane 1,3,4,6 and 7: wild type, Lane 2: heterozygote, Lane 5: polymorphic variant and Lane 8: 100bp marker.

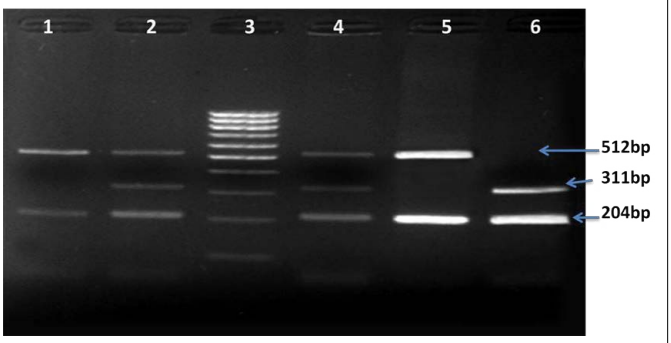

Figure 3: Genotyping of vitamin D receptor (*VDR*) TaqI polymorphism was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Lane 1 and 5: wild, Lane 2 and 4: heterozygote, Lane 6: mutant and Lane 3: 100bp marker.

was done with the help of Plink ver. 1.07-dos [19], pairwise linkage disequilibrium estimation and D' calculation was performed using the Haploview ver. 4.2 [20].

Results

Demographic characteristics of case and controls

A total of 737 subjects, including 357 tuberculosis patients (37% females) and 380 healthy controls (46% females) belonging to same ethnicity were analysed for *VDR* gene polymorphism and its role in TB susceptibility. All the three polymorphisms studied were found in Hardy Weinberg Equilibrium. The details of the baseline characteristics of the studied population are given in Table 1. The mean age of TB patients and controls was 33.14 ± 15.42 and 36.33 ± 14.87, respectively. 61.60% of the cases and 50% of the controls belonged to age-group < 35 years. Ever-smoker's were reported to be at higher risk of developing TB ($p < 0.001$, OR 2.61 95% CI 1.90-3.59) as compared to controls. Majority of cases and controls lived in semi-pucca houses (63.30% cases and 70% controls). Dietary regime analysis, as a possible indicator of vitamin D deficiency in vegetarians as compared to non-vegetarians, showed a majority of study subjects following non-vegetarian diet-style (75% cases and 69% controls) ($p = 0.11$, OR 0.77 95% CI 0.55-1.08). 12% of the cases were exposed to the disease whereas the exclusion criteria for controls were no exposure to TB and thus none of the healthy controls was exposed to disease. Indian education system has been divided into primary (up to 14 years of age), secondary (14-18 years of age) followed by higher education including college and professional courses. Stratification on basis of educational status, a risk factor for disease as well as an indicator of socio-economic status, majority of case pursued higher education (41%) followed by primary (29%) whereas in controls majority received primary (37.80%) followed by secondary education (30.60%) (Table 1).

VDR genotype in cases and controls

Genotypic distribution of *VDR BsmI* polymorphism was first analysed using 3x2 $\chi 2$ test (Table 2) reporting an overall genotypic difference between the case groups and controls. A closer look at the same showed a significantly higher prevalence of 'bb' genotype in the control group as compared to the case groups; EP($p < 0.01$, OR 0.33, 95% CI 0.14-0.79), PN ($p = 0.01$, OR 0.33, 95% CI 0.12-0.86), PP ($p < 0.001$, OR 0.42 95% CI 0.24-0.71) and PTB ($p < 0.001$, OR 0.40 95% CI 0.24-0.65) and TB ($p < 0.001$ OR 0.38 95% CI 0.24-0.60) groups. The allele frequency distribution showed a significantly higher frequency of 'b' allele in the control group as compared to all case groups (Table 2).

Characteristic	Cases (%)	Controls (%)	*p-value*
Sample No.	357	380	
Mean Age ± SD	33.14 ± 15.42	36.33 ± 14.87	
< 35	220(61.60)	191(50)	
35-59	106(29.70)	158(42)	
≥ 60	31(8.70)	31(8)	
Male	225(63)	206(54)	
Female	132(37)	174(46)	
Smoking			
Non-smoker	180(50.40)	276(73)	
Ever-smoker	177(49.60)	104(27)	$p < 0.001$
Types of house			
Kachcha	73(20.50)	59(15.50)	
Semi-pucca	226(63.30)	266(70)	
Pucca	58(16.20)	55(14.50)	
Exposure status			
Yes	43(12)	0(0)	
No	314(88)	380(100)	
Dietary- regime			
Non-vegetarian	268(75)	264(69)	
Vegetarian	89(25)	116(31)	$p = 0.11$
Educational status			
Primary	105(29)	142(37.80)	
Secondary	56(16)	117(30.60)	
Higher	147(41)	70(17.80)	
Illiterate	49(14)	51(13.80)	

Table 1: Baseline characteristics of all tuberculosis cases and controls.

Genotypic distribution of *VDR FokI* polymorphism showed a significant difference between case groups and controls (Table 3). Closer look at the same showed a significantly higher prevalence of 'ff' genotype in the case groups; EP ($p <0.001$, OR 5.86 95% CI 2.07-16.50), PP ($p = 0.02$, OR 2.36 95% CI 1.07-5.26), PTB ($p = 0.02$, OR 2.25 95% CI 1.05-4.85) and TB ($p < 0.01$, OR 2.72 95% CI 1.33-5.64). In case of EP patients, presence of a polymorphic variant ('f ') was observed to be significantly associated with disease ($p < 0.001$, OR 2.08 95% CI 1.40-3.09) (Table 3).

For *TaqI* variant of *VDR* gene no overall significant association between the allele and genotype distribution of case groups and control group was observed (Table 4).

VDR genotype and disease outcome

As per WHO definition of cases, successful cases included cured and patients who completed their treatment (174) and unsuccessful included failures and patients who died during treatment (22) [21]. Thirty two patients were excluded as they were either transfer-out or defaulters (the patient who did not take the medication for two consecutive months).

Tendency was reported for carriers of *VDR BsmI* heterozygote (Bb) and disease outcome ($p = 0.05$, OR 3.38 95% CI 0.91-18.66) (Table 5). No significant association was reported between overall risk of unsuccessful treatment and genotype and allele distribution for any other VDR variant studied (Table 5).

VDR genotype and bacillary load

	Genotype				Allele			
Subjects (*n*)	**BB** (%)	**bb** (%)	**bb** (%)	***p-value***	**B** (%)	**b** (%)	***p-value***	**OR (95% CI)**
Controls (380)	98(26)	171(45)	111(29)	Ref.	367(48)	393(52)	0	1
Extrapulmonary (EP)(71)	24(34)	38(53)	9(13)	0.01	86(61)	56(39)	<0.01	0.61(0.42-0.89)
Pulmonary Negative (PN)(58)	19(33)	32(55)	7(12)	0.02	70(60)	46(40)	<0.02	0.61(0.40-0.93)
Pulmonary Positive All (PP) (228)	68(30)	128(56)	32(14)	<0.001	264(58)	192(42)	<0.01	0.68(0.53-0.86)
Pulmonary All (PTB) (286)	87(30)	160(56)	39(14)	<0.001	334(58)	238(42)	<0.001	0.67(0.53-0.83)
TB All (TB) (357)	111(31)	198(56)	48(13)	<0.001	420(59)	294(41)	<0.001	0.65(0.53-0.81)

Table 2: Frequency distribution of the *BsmI* polymorphism of *VDR* in study population (OR=odds ratio, CI=confidence interval).

	Genotype				Allele			
Subjects (*n*)	**FF**(%)	**Ff**(%)	**ff**(%)	***p-value***	**F**(%)	**f**(%)	***p-value***	**OR (95% CI)**
Controls (392)	220(58)	147(39)	13(3)		587(77)	173(23)		
Extrapulmonary (EP)(71)	26(36.6)	36(50.7)	9(12.7)	<0.001	88(62)	54(38)	<0.001	2.08(1.40-3.09)
Pulmonary Negative (PN)(58)	37(64)	17(29)	4(7)	0.22	91(80)	25(20)	0.77	0.93(0.56-1.53)
Pulmonary Positive All (PP) (228)	136(60)	73(32)	19(8)	0.02	345(76)	111(24)	0.53	1.09(0.82-1.45)
Pulmonary All (PTB) (286)	173(60)	90(32)	23(8)	0.01	436(76)	136(24)	0.66	1.06(0.81-1.38)
TB All (TB) (357)	199(56)	126(35)	32(9)	<0.01	524(73)	190(27)	0.09	1.23(0.96-1.57)

Table 3: Frequency distribution of the *FokI* polymorphism of *VDR* in study population (OR=odds ratio, CI=confidence interval).

	Genotype				Allele			
Subjects (*n*)	**TT**(%)	**Tt**(%)	**tt**(%)	***p-value***	**T**(%)	**t**(%)	***p-value***	**OR (95% CI)**
Controls (392)	201(54)	156(42)	23(4)		576(74)	208(26)		
Extrapulmonary (EP)(71)	42(60)	25(35)	4(5)	0.62	109(77)	33(23)	0.41	0.84(0.54-1.30)
Pulmonary Negative (PN)(58)	34(59)	18(31)	6(10)	0.23	86(74)	30(26)	0.87	0.96(0.60-1.54)
Pulmonary Positive All (PP) (228)	117(51)	87(38)	24(11)	0.13	321(70)	135(30)	0.25	1.16(0.89-1.52)
Pulmonary All (PTB) (286)	151(53)	105(37)	30(10)	0.09	407(71)	165(29)	0.36	1.12(0.87-1.44)
TB All (TB) (357)	193(54)	130(36)	34(10)	0.14	516(72)	198(28)	0.62	1.06(0.84-1.34)

Table 4: Frequency distribution of the *TaqI* polymorphism of *VDR* in study population (OR=odds ratio, CI=confidence interval).

Polymorphism, genotype	**Unsuccessful* (%)**	**Successful ** (%)**	***p-value***	**OR (95% CI)**
VDR FokI				
FF	15(68)	108(62)		
Ff	4(18)	52(30)	0.31	0.55(0.13-1.86)
ff	3(14)	14(8)	0.46	1.54(0.25-6.50)
VDR BsmI				
BB	3(14)	56(32)		
Bb	17(77)	94(54)	0.05	3.38(0.91-18.66)
bb	2(9)	24(14)	0.64	1.56(0.12-14.42)
VDR TaqI				
TT	9(18.40)	94(54)		
Tt	10(20.40)	64(37)	0.31	1.63(0.57-4.67)
tt	30(61.20)	16(9)	0.39	1.96(0.31-8.98)

Table 5: Association between treatment outcome and *VDR* gene polymorphism (OR=odds ratio, CI=confidence interval, * Successful cases included cured and patients who completed their treatment, ** Unsuccessful cases included failures and patients who died during treatment).

Microscopically diagnosed sputum smear positive patients were further subcategorized into two clinical groups according to their bacilli load 1+ as low bacillary load and 2+ and 3+ as high bacillary load (Table 6).

Of the 228 pulmonary patients included for this analysis 147(64.50%) were high bacillary load carriers (2+ and 3+) and 81(35.50%) were low bacillary load carriers (1+). Each of these categories was separately compared with healthy controls for each genotype. Variant genotype (ff) of *VDR FokI* variant was seen to be associated with risk of having low bacillary load as compared to controls (*p <0.01,* OR 3.68 95%CI 1.35-9.67) (Table 6). However, the 'bb' variant of *VDR BsmI* gene was observed to be significantly higher in control group when compared to low bacilli load carriers (*p < 0.001,* OR 0.23 95% CI 0.09-0.55) (Table 6). Tendency was also observed for *VDR Taq* I mutant genotype 'tt' and low bacillary load on comparison with the control group (*p = 0.05*, OR 2.24 CI 0.91-5.42). No such association was observed for high bacillary load carriers and control group (Table 6).

VDR genotype and extrapulmonary site

EP site and *VDR* polymorphism analysis consisted of 71 patients. 68 patients belonging to abdominal, lymph node and pleural effusion

EP TB were selected (Table 7). With respect to pleural effusion site, presence of polymorphic variant 'f' for *VDR FokI* gene was reported to be highly associated with risk of developing the disease ($p < 0.001$, OR 3.39 95% CI 1.87-6.15). However, polymorphic variant for *VDR BsmI* gene (b) was observed to confer protection against Pleural TB ($p < 0.01$, OR 0.47 95% CI 0.25-0.87). No association was reported for any other EP site and *VDR* gene polymorphism (Table 7).

Linkage disequilibrium analysis and haplotype analysis

Pair-wise linkage disequilibrium (D') was analysed between all three studied polymorphisms. *TaqI* was not linked to either *BsmI* or *FokI* (D' = 0.005 and D' = 0.03). No linkage disequilibrium (LD) was observed between *BsmI* and *FokI* either (D' = 0.04). On haplotype analysis, the frequency of TBf and TBF haplotype was reported to be significantly higher in case group when compared to control group ($p = 0.03$ and $p = 0.03$, respectively). Haplotype TbF was found to be more frequent in control group than cases ($p < 0.001$) (Table 8).

Discussion

A non-silent *VDR* gene polymorphism is the *FokI* polymorphism, found in exon 2, which is at a translation initiation start site and is

Genotype	High Load *	Controls	*p-value*	OR (95% CI)	Low Load**	Controls	*p-value*	OR (95% CI)
VDR FokI								
FF	90(61)	220(58)			46(57)	220(58)		
Ff	48(33)	147(39)	0.28	0.80(0.52-1.22)	25(31)	147(39)	0.44	0.81(0.46-1.42)
ff	9(6)	13(3)	0.24	1.69(0.64-4.42)	10(12)	13(3)	<0.01	3.68(1.35-9.67)
VDR BsmI								
BB	37(25)	98(26)			31(38)	98(26)		
Bb	86(59)	171(45)	0.22	1.33(0.82-2.16)	42(52)	171(45)	0.35	0.78(0.44-1.36)
bb	24(16)	111(29)	0.06	0.57(0.31-1.06)	8(10)	111(29)	<0.001	0.23(0.09-0.55)
VDR TaqI								
TT	78(53)	201(53)			39(48)	201(53)		
Tt	55(37)	156(41)	0.64	0.91(0.59-1.39)	32(40)	156(41)	0.83	1.06(0.61-1.82)
tt	14(10)	23(6)	0.21	1.57(0.72-3.38)	10(12)	23(6)	0.05	2.24(0.91-5.42)

Table 6: Association between bacilli load and *VDR* gene polymorphism (OR=odds ratio, CI=confidence interval, * High load constitutes 2+ and 3+ bacilli load patients, ** Low load constitutes 1+bacilli load patients).

Genotype	Abdominal (25)	Controls	*p-value*	OR (95% CI)	Lymph Node (16)	Controls	*p-value*	OR (95% CI)	Pleural effusion (27)	Controls	*p-value*	OR (95% CI)
VDR FokI												
FF	10(40)	220(58)			9(56)	220(58)			6(22)	220(58)		
Ff	13(52)	147(39)	0.12	1.95(0.78-4.93)	6(38)	147(39)	1.00	1.00(0.29-3.22)	15(56)	147(39)	<0.01	3.74(1.33-12.01)
ff	2(8)	13(3)	0.16	3.38(0.33-18.4)	1(6)	13(3)	0.45	1.88(0.04-15.5)	6(22)	13(3)	<0.001	16.92(3.83-71.5)
VDR BsmI												
BB	8(32)	98(26)			5(31)	98(26)			11(41)	98(26)		
Bb	14(56)	171(45)	0.99	1.00(0.38-2.72)	8(50)	171(45)	1.00	0.92(0.26-3.67)	14(52)	171(45)	0.45	0.73(0.30-1.80)
bb	3(12)	111(29)	0.09	0.33(0.06-1.43)	3(19)	111(29)	0.48	0.53(0.08-2.81)	2(7)	111(29)	<0.01	0.16(0.02-0.77)
VDR TaqI												
TT	15(60)	201(53)			10(63)	201(53)			15(55)	201(53)		
Tt	9(36)	156(41)	0.55	0.77(0.30-1.94)	4(25)	156(41)	0.26	0.52(0.12-1.83)	11(41)	156(41)	0.89	0.94(0.39-2.25)
tt	1(4)	23(6)	1.00	0.58(0.01-4.15)	2(12)	23(6)	0.37	1.75(0.18-8.95)	1(4)	23(6)	1.00	0.58(0.01-4.15)

Table 7: Association of extrapulmonary site and *VDR* gene polymorphism (OR = odds ratio, CI = confidence interval).

Haplotype	rs731236(T/t)	rs1544410(B/b)	rs2228570(F/f)	Cases	Controls	*p-Value*
tbf	t	b	f	0.03	0.03	0.63
Tbf	T	b	f	0.09	0.10	0.54
tBf	t	B	f	0.05	0.04	0.26
TBf	T	B	f	0.10	0.07	0.03
tbF	t	b	F	0.09	0.10	0.37
TbF	T	b	F	0.20	0.29	< 0.001
tBF	t	B	F	0.10	0.09	0.57
TBF	T	B	F	0.34	0.28	0.03

Table 8: Three locus haplotypes for *VDR TaqI, BsmI* and *FokI* gene polymorphism for tuberculosis cases and controls.

predicted to change the structure of the coded protein. Although the difference between the two proteins is only three amino acids, it has been suggested that the more commonly observed shorter VDR protein is functionally more active [22]. This study reported a significant association of 'ff' genotype and 'f' allele with susceptibility to TB, as reported by Sharma et al. [14]. The role of 'ff' genotype has been found to confer a significantly increased risk to TB among Asians but no evident association was observed among African [8]. These results were highly significant in extrapulmonary cases ('f' allele frequency 0.38 versus 0.23 in controls). On further stratification of EP cases based on site of disease, pleural effusion patients carrying of polymorphic variant 'f' were at significantly higher risk of developing the disease where as presence of 'b' allele of *BsmI* variant may contribute to protection against disease. Results of this study were in concordance to those observed in a Gujarati Indian population [18] living in London having several forms of extrapulmonary tuberculosis as well as blacks with extrapulmonary TB, studied by Motsinger-Reif et al. [23] and spinal TB study in Chinese Han population [24].

Two of the most widely studied *VDR* polymorphisms are located in 3′ untranslated region - *Taq*I and *Bsm*I, which have been shown to be related to VDR activity or expression [25]. For *BsmI* polymorphism we found a significantly higher prevalence of 'bb' genotype in the control group when compared to the case groups inferring the protective role of this genotype against TB. These results are consistent with those concluded by a meta-analysis reporting a significant decreased risk for 'bb' carriers against TB [8,9]. Ates et al. [26] reported an overrepresentation of 'B' allele in case-group (48% compared to 30% in controls) similar to our results (59% in cases and 48% in controls).

With regard to *TaqI* polymorphism, no association was reported in this study similar to those reported in East Indian [10], Tanzanian [27], South African [28] and a recent analysis done on Chinese Han population [12]. However, a South Indian study reported an association of 'tt' genotype with PTB in females [29]. This difference could be contributed to Indo-Dravidian origin of the later compared to Indo-Aryan population in this study.

WHO's strategy and framework for effective TB control includes evaluation of treatment outcome of TB patients undergoing standard therapy [30]. Treatment success is defined as sum of patients cured and those who completed their treatment [30]. Analysis of *VDR* gene polymorphism and treatment outcome revealed a higher prevalence of 'Bb' genotype in patients with unsuccessful treatment when compared to those with successful treatment. To the best of our knowledge no other study has reported a similar association between treatment outcome and *VDR* gene polymorphism.

Low bacilli load was reported to be significantly higher in 'ff' carriers when compared to control group whereas 'bb' genotype was reported to confer protection against the same. A recent study on Indian population, however, reported an association of 'tt' genotype with high bacillary load not found in this study population [14]. The later might be contributed to difference in ethnicity between two populations as well as difference in study design.

Demographic analysis of studied population revealed that 61.60% of the cases belonged to < 35 age-group which includes not only the socio-economically more important generation but also woman of reproductive age. Data in this study showed a positive association of smoking status and TB risk. 90% of the ever-smoker cases were males as smoking has been widespread among Indian males since many decades but to our surprise 49% of these males belonged < 35 age-group. A study by Gajalaxami et al. [31] underlined that mortality from TB is 4 times as greater among smokers than non-smokers. It has been suggested that the link between *VDR* polymorphisms and disease susceptibility might be modulated by vitamin D status. Stratchan et al. [13] reported that vitamin D deficiency, associated with a vegetarian diet, is known to affect immunological competence and increased mycobacterial reactivation among Asians from the Indian subcontinent. To validate this aspect in our population, we included the nutrition status of cases and controls. But to our surprise 75% of the cases were non-vegetarian compared to 69% controls. Therefore, we might conclude that vegetarian diet is not one of the risk factors for TB susceptibility in our population.

In conclusion, this study reported *VDR* gene variant to play an important role in TB susceptibility as well as treatment outcome and bacillary load. The study population belongs to a small town in Himachal Pradesh with subjects living in that area from a minimum of three generations and marriage within the community is a common trend providing an unbiased population for the study. Therefore, we believe that similar studies in different populations within India as well as outside India will definitely prove beneficial with regard to better understanding of immunogenetics of TB. The era of personalised medicine and genetic markers have the potential to be used for early targeting of a high burden population or population at higher risk to TB. We strongly recommend that the variant homozygous genotype of *FokI* variant of *VDR* ('ff') should be further explored with reference to extrapulmonary tuberculosis in different ethnic groups.

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