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Association of vitamin D receptor gene polymorphisms with clinical outcomes of dengue virus infection

K. Alagarasu [*,1], T. Honap [1], A.P. Mulay, R.V. Bachal, P.S. Shah, D. Cecilia

Dengue Group, National Institute of Virology, 20-A Ambedkar Road, Pune 411001, Maharashtra, India

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ABSTRACT

Vitamin D is known to affect pathogenesis of dengue through modulation of immune responses. Vitamin D exerts its effects through vitamin D receptor (VDR). The functioning of VDR is affected by the gene polymorphisms in the coding (rs2228570) and 3′untranslated region (UTR) (rs1544410, rs7975232 and rs731236). In the present study, *VDR* gene polymorphisms were investigated in 112 dengue infected patients (83 dengue fever (DF) and 29 dengue hemorrhagic fever cases (DHF)) and 105 apparently healthy controls (HCs) using polymerase chain reaction based restriction fragment length polymorphisms methods. HCs had no documented evidence of symptomatic dengue. Results revealed significantly lower frequency of 'C' allele of rs7975232 in all dengue patients (DEN) as compared to HCs [(P corrected (Pc) = 0.014, Odds ratio (OR) 0.51]. The frequency of C/C genotype of rs7975232 was significantly lower in DEN and DF cases compared to HCs (DEN vs. HCs: Pc = 0.0184, OR 0.24; DF cases vs. HCs: Pc = 0.028, OR 0.21). The frequency of T allele of rs2228570 in a dominant mode was significantly higher in DHF cases as compared to DF cases ($P = 0.034$ OR 2.58). A significantly lower frequency of the haplotype G-C-T (Pc = 0.0135) and higher frequency of the haplotype G-A-T (Pc = 0.000085) was observed in DEN and DF cases as compared to HCs. The results suggest that the 3′UTR haplotypes of VDR gene are differentially associated with risk of symptomatic dengue requiring hospitalization. The 'T' allele of rs2228570 polymorphism in a dominant mode of inheritance is associated with DHF.

1. Introduction

Dengue virus (DENV) infection in humans leads to a wide spectrum of outcomes resulting in diverse clinical manifestations ranging from asymptomatic to a mild form of the disease, dengue fever (DF) and severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1]. This heterogeneity in the outcome of infection is influenced by the virus, host and environmental factors. Epidemiological studies have shown that age, presence of other chronic diseases, secondary infection with different serotype, sequentiality of serotypes in primary and secondary infections, time interval between primary and secondary infections, nutritional status, income and genetic ancestry were risk factors for development of DHF [2]. The observation that African ancestry was associated with protection against DHF and DSS in Cuba and Brazil indicates a major role for host genetic variations in determining the clinical outcome of DENV infection [3,4]. The association of variants of human leukocyte antigen class I and class II genes, *tumor necrosis factor* (*TNF*)-α, *CD209*, *FcGRIIA*, *vitamin D receptor* (*VDR*), *transporters associated with antigen presentation* (*TAP*) and *JAK1* genes with disease severity in dengue have been reported [5].

Vitamin D receptor (VDR) gains more importance due its immunomodulatory nature. VDR mediates the functions of vitamin D, which is known to be a regulator of gene expression. VDR mediated immune functions include upregulation of expression of cathelicidin and antimicrobial peptides in monocytes and macrophages. Vitamin D also downregulates the expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ in immune cells [6,7]. It has been shown that vitamin D is known to suppress the replication of DENV [7]. A recent study has reported higher levels of 25-hydroxy vitamin D_3 in DENV infected patients compared to apparently healthy controls [8]. Vitamin D has been shown to upregulate the expression of DENV entry receptors, dendritic cell specific intercellular adhesion molecule-3 grabbing non integrin (DC-SIGN) and FcGRIIA [9–11].

VDR is coded by the *VDR* gene which is located on chromosome 12. Functionality of VDR is influenced by single nucleotide polymorphisms (SNPs) in the gene. The most commonly studied polymorphisms in the *VDR* gene include a start codon polymorphism, which is defined by the presence or absence of *FokI* restriction enzyme site and three polymorphisms in the 3′ untranslated region

* Corresponding author.
E-mail address: alagarasu@gmail.com (K. Alagarasu).
[1] These authors contributed equally to the work.

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Table 1
Primer sequences, annealing temperatures, restriction enzymes used, and restriction digestion pattern for genotyping of VDR gene polymorphisms.

SNP	Primer sequences	Annealing temperature for PCR	PCR product (bp)	Restriction enzyme	Restriction fragment lengths (bp)
rs2228570	5′-agctggccctggcactgactcttgctct-3′ and 5′-atggaaacaccttgcttcttctccctc-3′	64 °C	265	*Fok*I	CC: 265 CT: 265, 196 & 69 TT: 196 & 69
rs1544410	5′-caaccaagactacaagtaccgcgtcagtga-3′ and 5′-aaccagcgggaagaggtcaaggg-3′	65 °C	825	*Bsm*I	AA: 825 AG: 825, 650 & 175 GG: 650 & 175
rs7975232	5′-cagagcatggacagggagcaag-3′ and 5′-gcaactcctcatggctgaggtctca-3′	63 °C	740	*Apa*I	AA: 740 AC: 740, 520 & 220 CC: 520 & 220
rs731236	5′-cagagcatggacagggagcaag-3′ and 5′-gcaactcctcatggctgaggtctca-3′	63 °C	740	*Taq*I	TT: 495 & 245 TC: 495, 290, 245 & 205 CC: 290, 245 & 205

(UTR) of VDR gene. The 3′UTR polymorphisms are defined by the presence or absence of restriction sites for the enzymes namely *Bsm*I, *Apa*I and *Taq*I [12–14]. These genetic variations in the *VDR* gene have been shown to be associated with susceptibility or resistance to infectious diseases [15,16]. The minor allele of *Taq*1 polymorphism has been shown to be associated with susceptibility to respiratory syncytial virus disease in South African children [17]. The 3′UTR haplotype of *VDR* gene has been shown to be associated with protection against HIV-1 infection [18]. *VDR* gene variants are also known to influence immune functions. The 3′UTR haplotype of the *VDR* gene, B-A-t has been shown to be associated with increased production of interleukin (IL)-12p40 and IFN-γ and decreased production of the anti-inflammatory cytokine, IL-10 [19]. Since the *VDR* gene is known to influence immune responses to DENV, it is a potential candidate gene for association studies in dengue. A study carried out in Vietnamese children has reported the association of the minor allele of rs731236 polymorphism with resistance to DHF [20]. In the present study, we investigated whether coding and 3′UTR gene variants are associated with DF or DHF in a group of DENV infected patients from Pune, Western India.

2. Study subjects and methods

2.1. Study subjects

Dengue cases (DEN) include 112 subjects (68 males and 44 females) (mean age ± standard deviation (SD) 31.8 ± 12.7). All these subjects had a history of hospitalization for dengue during 2007-2010, which had been laboratory confirmed by dengue specific IgM ELISA and/or reverse transcriptase polymerase chain reaction. Apparently healthy controls (HCs) consisted of 106 subjects (69 males and 37 females) (mean age ± SD 32.6 ± 13.4). Apparently HCs had no known history of hospitalization for dengue like illness and no documented evidence of having symptomatic dengue. Among the dengue cases, 85 cases had DF (mean age ± SD 32.0 ± 12.3). Based on at least two of the DHF defining criteria of the World Health Organization, 29 cases had DHF (mean age ± SD 31.3 ± 14.0) [21]. Since most of the samples were collected retrospectively, it was not possible to investigate the infecting serotypes and immune status as a part of this study. The study was approved by the institutional ethics committee, and a written informed consent was obtained from the study participants before blood collection. All the participants were living in and around Pune, Maharashtra, Western India and were not related to each other. Both cases and controls belonged to Marathi speaking population from Western India.

2.2. Genotyping of VDR gene polymorphisms

DNA was isolated from the white blood cells using salting out procedure. Polymorphisms in the *VDR* gene were studied using PCR followed by restriction fragment length method as described earlier [22]. The primer sequences, annealing temperatures for PCR, PCR product size, restriction enzymes used for genotyping and restriction digestion pattern for assigning the genotypes are provided in Table 1.

2.3. Statistical analysis

Allele and genotype frequencies were calculated by direct counting. Genotype frequency distributions were tested for their confirmation to Hardy–Weinberg equilibrium using the Chi square test. Allele and genotype frequencies were compared between different study groups using the Chi square test or Fisher's exact test as appropriate. For allele frequencies, *P* values with Yate's correction and Odds ratio (OR) with 95% confidence limits (CI) were calculated using Statcalc program, Epi info version 6.0.4, CDC, Atlanta, GA, July 1996). For genotypic associations, P values with OR adjusted for gender and age were calculated by logistic regression using the SNPstats program [23]. A *P* value less than 0.05 was considered significant. For genotypic associations, the P values were further multiplied by number of SNPs studied to derive Pc values. Pairwise linkage disequilibrium (LD) between the *VDR* gene polymorphisms was computed, and LD plots were constructed Haploview software version 4.2 [24]. Haplotype frequencies were inferred and compared using SNPstats program and Haploview software version 4.2. For haplotypic associations, the *P* values were subjected to the Bonferroni correction in which the *P* value was multiplied by the number of haplotypes, and P corrected (Pc) value of less than 0.05 was considered significant. Function prediction analysis of *VDR* gene polymorphisms were carried out using FastSNP web server [25].

3. Results

3.1. Allele frequencies of VDR gene polymorphisms in dengue patients

When the allele frequencies of VDR gene polymorphisms were compared between DEN and HCs, a significantly lower frequency of 'C' allele of rs7975232 polymorphism was observed in DEN as compared to HCs ($P = 0.0026$, Pc = 0.0104, OR with 95% CI 0.54 (0.36–0.82)).

When the DEN cases were grouped into DF and DHF cases and compared with HCs, the frequency of 'C' allele of rs7975232 polymorphism was significantly lower in DF cases than HCs ($P = 0.0022$,

Table 2
Percent allele frequencies of VDR gene polymorphisms in healthy controls and dengue patient groups.

SNP	Alleles	Percent minor allele frequency *n* (%)[a]	Controls vs. cases		DF vs. DHF	
			Odds ratio	*p*-value	Odds ratio	*p*-value
rs2228570		T	–	–	–	–
Healthy controls		61 (29.0)	–	–	–	–
DEN	C>T	58 (25.8)	0.85 (0.55–1.33)	0.52	–	–
DF		38 (22.8)	0.73 (0.44–1.19)	0.21	–	–
DHF		20 (34.5)	1.29 (0.65–2.48)	0.52	1.77 (0.87–3.55)	0.11
rs1544410		A	99 (47.1)	–	–	–
Healthy controls		99 (47.1)	–	–	–	–
DEN	G>A	11 (491.5)	1.1 (0.74–1.63)	0.68	–	–
DF		80 (48.1)	1.04 (0.68–1.6)		–	–
				0.92		–
DHF		31 (53.4)	1.29 (0.69–2.41)	0.48	1.23 (0.65–2.35)	0.59
rs7975232		C				
Healthy controls		105.(50.0)	–	–	–	–
DEN	A>C	79.(35.2)	0.54 (0.36–0.82) [b]	0.0026[b]	–	–
DF		56.(33.7)	0.51 (0.33–0.79)[c]	0.0022[c]	–	–
DHF		23.(39.6)	0.66 (0.35–1.23)	0.21	1.29 (0.66–2.49)	0.511
rs731236		C				
Healthy controls		66 (31.4)	–	–	–	–
DEN	T>C	71 (31.6)	1.01 (0.66–1.55)	0.965	–	–
DF		51 (30.7	0.97 (0.61–1.54)	0.972	–	–
DHF		20 (34.4)	1.15 (0.59–2.2)	0.77	1.19 (0.59–2.33)	0.719

[a] Number in the parentheses represent percent allele frequencies.
[b] Pc value = 0.0104.
[c] Pc value = 0.0088.

Pc = 0.0088, OR with 95% CI 0.51 (0.33–0.79). Allele frequencies were not different between DHF cases and HCs for all the *VDR* gene polymorphisms (Table 2).

3.2. Genotype frequencies of VDR gene polymorphisms in dengue patients

The frequency distribution of genotypes of all the polymorphisms confirmed to Hardy-Weinberg equilibrium in HCs ($P > 0.05$). Significantly lower frequency of C/C genotype of rs7975232 polymorphism was observed in DEN and DF cases compared to HCs (DEN cases vs. HCs: $P = 0.0046$, Pc = 0.0184 OR 0.24 95% CI 0.10–0.59; DF cases vs. HCs: $P = 0.0052$, Pc = 0.028, OR 0.21 95% CI 0.08–0.58) (Table 3).

When DF and DHF cases were compared, the frequency of 'T' allele of rs2228570 polymorphism in a dominant mode (C/T + T/T genotypes) was significantly higher in DHF cases ($P = 0.034$, Pc = 0.136, OR 2.58 95% CI 1.06–6.30) (Table 3).

3.3. Linkage disequilibrium and haplotype frequencies of VDR gene polymorphisms in dengue patient groups

Pairwise LD was computed and LD plots were constructed using the Haploview software version 4.2. LD analysis revealed a strong LD ($r^2 > 0.8$) between rs1544410 and rs7975232 and a weak LD between rs1544410 and rs731236 or between rs7975232 and rs731236 in HCs. In patients, weak to moderate LD was observed between rs1544410, rs7975232 and rs731236. No LD was observed between rs2228570 and other polymorphisms in both HCs and DEN group (Fig.1).

Since LD patterns of 3′UTR polymorphisms (rs1544410, rs7975232 and rs731236) differed between HCs and DEN, 3 locus haplotype involving 3′UTR polymorphisms were compared between the study groups. A significantly lower frequency of the haplotype G-C-T (Pc = 0.0135) and higher frequency of the haplotype G-A-T (Pc = 0.000085) was observed in DEN and DF cases as compared to HCs. The frequency of G-C-T was lower and the frequency of G-A-T was higher in DHF cases compared to healthy controls, though statistically not significant ($P = 0.087$ & 0.133 respectively). When the DF and DHF cases were compared, the frequency of G-A-T was lower in DHF cases than in DF cases, though not significant ($P = 0.08$) (Table 4).

Analysis of four locus haplotype frequencies in the study groups revealed a higher frequency of C-G-A-T (Pc = 0.00064) and lower frequency of C-G-C-T in DEN and DF cases compared to HCs ($P = 0.0135$, Pc = 0.104). The frequencies of haplotypes having 'T' allele i.e. (T-A-A-C, T-G-C-T and T-G-A-T) were higher in DHF cases compared to DF cases, though statistically not significant ($P > 0.05$) (Table 4).

3.4. Function prediction analysis of VDR gene polymorphisms using FastSNP

The functional role of *VDR* gene polymorphisms were predicted using FastSNP which prioritizes SNPs according to 12 phenotypic risks and putative functional effects, such as changes to the transcriptional level, pre-mRNA splicing, protein structure, etc. Briefly, rs228570, which was shown to be associated with DHF, was predicted to affect splicing regulation and the presence of T allele results in the loss of an exon splicing enhancer (ESE). The rs7975232 polymorphism which was shown to be associated with symptomatic dengue was predicted to function as an intronic enhancer and the presence of C allele results in loss of the binding site for the transcription factor AML-1a.

4. Discussion

Epidemiological evidences indicate that host genetic factors play a role in determining the outcome of DENV infection. Polymorphisms in the immunomodulatory genes are known to affect

Table 3
Percent genotype frequencies of VDR gene polymorphisms in healthy controls and dengue patient groups.

SNP	Percent frequency of genotypes *n* (%)[a]			Controls vs. cases		DF vs. DHF	
				[b]Odds ratio	*p*-value	[b]Odds Ratio	*p*-value
rs2228570	C/C	C/T	T/T	–	–	–	–
Healthy controls	54 (51.4)	41 (39.0)	10 (9.5)	–	–	–	–
DEN	60 (53.6)	46 (41.1)	6 (5.4)	0.52 (0.17–1.56)	0.46	–	–
DF	49 (59.0)	30(36.1)	4 (4.8)	0.39 (0.11–1.36)	0.3	–	–
DHF	11 (37.9)	16 (55.2)	2 (6.9)	1.04 (0.20–5.57)	0.37	[c]2.58 (1.06–6.30)	[d]0.034
rs1544410	G/G	G/A	A/A	–	–	–	–
Healthy controls	33 (31.4)	45 (42.9)	27 (25.7)	1.16 (0.57–2.36)	0.84	–	–
DEN	35 (31.2)	43 (38.4)	34 (30.4)	1.08 (0.51–2.32)	0.8	–	–
DF	28 (33.7)	30 (36.1)	25 (30.1)	1.70 (0.55–5.25)	0.64	1.63 (0.51–5.16)	0.49
DHF	7 (24.1)	13 (44.8)	9 (31.0)	–	–	–	–
rs7975232	A/A	A/C	C/C	0.24 (0.10–0.59)	[e]0.0046	–	–
Healthy controls	26 (24.8)	53 (50.5)	26 (24.8)	[f]0.21 (0.08–0.58)	[f]0.0052	–	–
DEN	43 (38.4)	59 (52.7)	10 (8.9)	0.32 (0.08–1.33)	0.17	1.31 (0.27–6.46)	0.79
DF	34 (41.0)	42 (50.6)	7 (8.4)	–	–	–	–
DHF	9 (31.0)	17 (59.0)	3 (10.0)	1.42 (0.47–4.26)	0.62	–	–
rs731236	T/T	T/C	C/C	1.44 (0.45–4.59)	0.45	–	–
Healthy controls	45 (42.9)	54 (51.4)	6 (5.7)	1.43 (0.25–8.18)	0.85	1.00 (0.18–5.57)	0.51
DEN	51 (45.5)	51 (45.5)	10 (8.9)	–	–	–	–
DF	40 (48.2)	35 (42.2)	8 (9.6)	–	–	–	–
DHF	11 (37.9)	16 (55.2)	2 (6.9)	–	–	–	–

[a] Number in the parentheses represents percent genotypic frequency.
[b] Odds ratio was based on co-dominant logistic regression models adjusted for age and gender & is with respect to frequency of homozygous genotype of rare allele within cases and controls.
[c] Odds ratio was based on dominant logistic regression model.
[d] Pc value = 0.136.
[e] Pc value = 0.0184.
[f] Pc value = 0.028.

immune functions and are associated with disease outcomes. Vitamin D receptor (VDR) is an immunomodulator known to affect both innate and adaptive immune responses. Functioning of VDR is affected by polymorphisms in the coding region and 3′UTR of the gene. In the present study, the start codon polymorphism, rs2228570 and 3'UTR polymorphisms (rs1544410, rs7975232 and rs731236) were investigated among patients having a documented history of hospitalization for dengue and HCs who had no known history of hospitalization for dengue and no documented evidence of symptomatic dengue. Results revealed a significantly higher frequency of G-A-T and C-G-A-T haplotypes in DEN and DF cases suggesting their association with increased risk of symptomatic dengue requiring hospitalization. The frequencies of C allele, C/C genotype, G-C-T and C-G-C-T haplotypes were observed at a significantly lower frequency in DEN and DF cases suggesting their association with reduced risk of symptomatic dengue. Significantly higher frequency of C/T + T/T genotypes of rs2228570 polymorphism was observed in DHF cases suggesting an association of 'T' allele with DHF in a dominant mode. This was further strengthened by the observation of increased frequency of four locus haplotypes

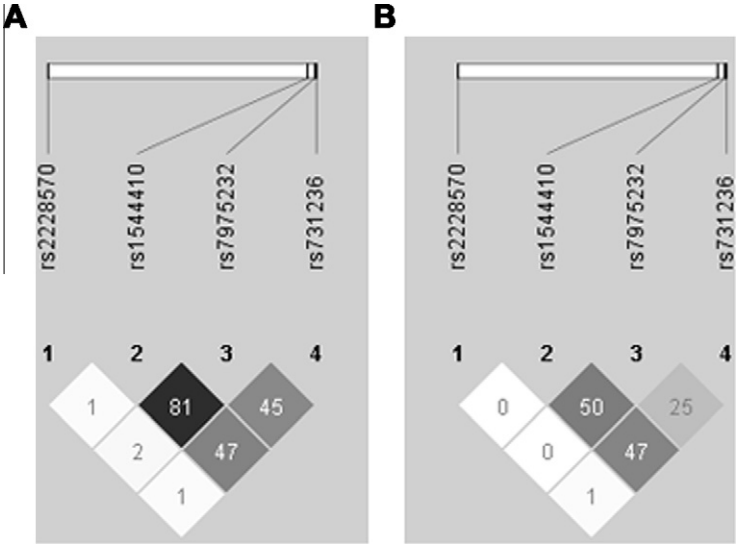

Fig. 1. Pattern of linkage disequilibrium of VDR gene polymorphisms in the healthy controls (A) and dengue patients (B). Graphical representation of VDR gene with the locations of polymorphisms in coding region (rs2228750), and 3′ untranslated region (rs1544410, rs7975232, and rs731236). Numbers in the boxes represent the correlation coefficient value of LD (r^2) value multiplied by 100. In dark boxes without any numbers, the r^2 value is 100. The intensity of the dark color of the boxes represents strength of linkage disequilibrium (r^2) with dark boxes having high LD and white boxes having low LD.

Table 4
Percent frequencies of three and four locus haplotypes of VDR gene polymorphisms in healthy controls and dengue patient groups.

Haplotype	% Haplotype frequency				Controls vs. DEN		Controls vs. DF		Controls vs. DHF		DF vs. DHF	
	HC ($n = 105$)	DEN ($n = 112$)	DF ($n = 83$)	DHF ($n = 29$)	OR with 95% CI	p-value	OR with 95% CI	p-value	OR with 95% CI	p-value	OR with 95% CI	p-value
G-C-T	49	34.8	33.7	37.9	0.55 (0.37–0.83)	0.0027[1]	0.53 (0.34–0.84)	0.0032[2]	0.63 (0.33–1.2)	0.133	1.2 (0.45–3.12)	0.566
A-A-C	31	31.7	30.7	34.5	1.04 (0.68–1.59)	0.87	0.99 (0.62–1.57)	0.95	1.17 (0.6–2.25)	0.6	1.22 (0.44–3.24)	0.59
A-A-T	15.2	17.4	17.5	17.2	1.17 (0.68–2.03)	0.53	1.18 (0.65–2.12)	0.55	1.16 (0.47–2.63)	0.7	0.95 (0.24–3.14)	0.967
G-A-T	3.4	15.6	18.1	8.7	5.37 (2.27–14.63)	0.0000173[3]	6.4 (2.64–17.7)	0.0000024[4]	2.74 (0.65–10.43)	0.08	0.52 (0.09–2.08)	0.08
C-G-C-T	36.8	25.8	25.9	26.9	0.6 (0.39–0.92)	0.0135[5]	0.6 (0.38–0.96)	0.0236[6]	0.64 (0.32–1.27)	0.14	1.12 (0.37–3.15)	0.93
C-A-A-C	19.8	21.2	22	18.6	1.06 (0.65–1.74)	0.72	1.14 (0.67–1.94)	0.55	0.96 (0.41–2.1)	0.86	0.94 (0.27–2.88)	0.74
C-A-A-T	10.8	13.6	14.5	10.2	1.66 (0.68–2.36)	0.37	1.37 (0.71–2.66)	0.29	0.99 (0.16–4.12)	0.98	0.68 (0.11–2.83)	0.57
T-A-A-C	11.2	10.5	7.9	15.9	0.93 (0.48–1.0)	0.82	0.71 (0.33–1.5)	0.38	1.42 (0.55–3.42)	0.35	2.26 (0.51–9.11)	0.2
T-G-C-T	12.2	8.9	8.7	11	0.69 (0.35–1.34)	0.27	0.6 (0.27–1.26)	0.17	0.82 (0.14–3.3)	0.86	1.74 (0.34–7.49)	0.42
C-G-A-T	2.9	13.3	14.8	8.5	5.26 (2.09–15.74)	0.000087[7]	5.75 (2.21–17.56)	0.000028[8]	2.74 (0.65–10.43)	0.082	0.44 (0.05–2.13)	0.17
T-A-A-T	4.4	3.8	3	7	0.93(0.32–2.72)	0.75	0.69 (0.18–2.36)	0.492	1.34 (0.3–4.75)	0.56	1.45 (0.02–28.6)	0.33
T-G-A-T	0.5	2.3	3.3	–	4.77 (0.53–226.75)	0.11	6.49 (0.71–308.4)	0.051	–	0.58	–	0.31

The order of SNP for three locus haplotypes is **rs15444410 - rs7975232 - rs731236**.
The order of SNP for four locus haplotypes is **rs2228570 - rs15444410 - rs7975232 - rs731236**.
[1] Pc value = 0.0135.
[2] Pc value = 0.015.
[3] Pc value = 0.000085.
[4] Pc value = 0.00001.
[5] Pc value = 0.104.
[6] Pc value = 0.184.
[7] Pc value = 0.00064.
[8] Pc value = 0.00016.

containing 'T' allele of rs2228570 in DHF cases. This is the first study to investigate all the four polymorphisms in dengue patients. The 'C' allele of rs731236 polymorphism has been shown to be associated with increased risk of DHF in Vietnamese children [19]. The lack of replication of the association of 'C' allele of rs731236 with DHF in the present study could be due to genetic heterogeneity between the two populations studied. Moreover, both the studies differed with regard to clinical presentation of cases. The Vietnamese study included only children with DHF grades III and IV while in the present study, adults with both DF and DHF were investigated. Thus, age specific differences and disease severity might also explain the failure to replicate the association reported in the Vietnam study.

Function prediction analysis using FastSNP web server revealed that rs7975232 is an intronic polymorphism which is known to affect the binding of a transcription factor AML-1a. The presence of 'C' allele abolishes the transcription factor binding site. Functional studies carried out earlier have suggested that polymorphisms in the 3′UTR region are known to collectively affect the functioning of VDR rather than individually. The haplotype b-a-T (G-C-T) has shown to be associated with 15% lower levels of VDR mRNA expression and 30% faster decay of VDR mRNA compared to B-A-t (A-A-C) haplotype in MG-63, a human bone derived osteosarcoma cell line [26]. In contrast to this report, a study which investigated differential expression of *VDR* gene transcripts in primary cells of human trabecular bone samples, reported that the b-a-T (G-C-T) haplotype is associated with over expression of *VDR* gene compared to b-A-T (G-A-T) and B-A-t (A-A-C) haplotypes [27]. It has been shown that Vitamin D enhances the expression of DENV entry receptors and suppresses the production of IFN-γ which is known to exert antiviral effects [6,7,9,10]. It is possible that b-a-T (G-C-T) haplotype might be associated with altered VDR expression. Defective VDR signaling might allow for decreased expression of DENV entry receptors and increased production of IFN-γ conferring the protection against the development of symptomatic dengue.

The present study revealed the association of 'T' allele of rs2228570 polymorphism in dominant mode with DHF. The 'T' allele codes for the long length VDR which is the least active form of VDR compared to the shorter form of VDR associated with 'C' allele. FastSNP prediction revealed that the presence of 'T' allele abolishes an exon splicing enhancer (ESE) motif and conferred a low to medium risk for the presence of allele. Levels of TNF-α and IL-10 have been shown to be associated with disease severity in dengue [28,29]. Since vitamin D is known to suppress TNF-α and enhance IL-10 levels [6,7], it is possible that defective VDR signaling might lead to high levels of TNF- α and low levels of IL-10 contributing to DHF. The delicate balance between the suppressor and enhancing effects of vitamin D exerted through VDR and other immune mediators might determine the development of severe dengue. The 'T' allele together with other polymorphisms in the promoter region and the 3'UTR might determine the availability of functional VDR and influence susceptibility to severe dengue.

In the present study, cases with a history of hospitalization for laboratory confirmed dengue were compared with apparently HCs who have no documented evidence of symptomatic dengue. There is a possibility that some of these HCs might have developed milder symptoms of dengue which may not have been recognized by the subjects. This will not change the major conclusions since the associations reported in the study pertain to symptomatic dengue requiring hospitalization. Moreover, including previously uninfected subjects with the potential to develop DF requiring hospitalization or DHF might decrease the power to detect differences between the groups. Epidemiological evidences suggest that severe dengue is more prevalent in secondary infections compared to primary infections. Moreover, even in secondary infections, severe dengue is known to occur with specific sequences of infection such

as DENV-1 in primary infection followed by secondary infection with DENV-2 or DENV-3 [30]. In the present study, the samples were collected retrospectively, it was not possible to identify the infecting serotypes and immune status of the cases due to the unavailability of acute sera. Whether this will affect the study results is not clear. Earlier studies have shown that all the serotypes of DENV were circulating in Pune [31] and have also reported that 70% of the dengue cases were secondary infections [8,32]. A recent study had shown that vitamin D levels are higher in DEN cases, and when DEN cases were categorized based on immune status, higher levels of vitamin D was associated with secondary DHF (8). It is possible that elevated levels of vitamin D in the presence of defective VDR signaling might alter VDR mediated immune functions in secondary infections and need further investigations.

Based on the frequency of minor allele of rs7975232, the present study has a power of more than 0.8 to detect association between dengue cases requiring hospitalization and apparently healthy controls. The sample size might be limited in its power to detect the minor associations based on the frequencies of other polymorphisms. Moreover, the number of DHF cases were also lesser in the present study. This could limit the power of study to detect minor associations between DF and DHF cases. Hence, further studies with large number of cases and clinically and laboratory documented asymptomatic controls (Asymptomatic controls who were positive for either IgM or an IgG titre of >1280) are needed in different populations to confirm the association of *VDR* gene variants with dengue. Experimental validation of the functionality of *VDR* gene variants predicted by FastSNP and the associated downstream signaling pathways also need to be assessed in the immune cells such as monocytes and dendritic cells in which DENV replicates. Since vitamin D is known to inhibit the replication of DENV (7), effect of VDR gene variants on DENV replication in immune cells need to be investigated and such studies might identify the VDR mediated immunoregulatory pathways that target DENV. This might be helpful in designing therapeutic strategies and may have implications for better clinical management of dengue cases. To summarize, the present study suggests that G-A-T and C-G-A-T haplotypes are associated with increased risk of symptomatic dengue and DF while C allele and C/C genotype of rs7975232, G-C-T and C-G-C-T haplotypes are associated with reduced risk of developing symptomatic dengue requiring hospitalization. The 'T' allele of rs2228570 polymorphism in a dominant mode of inheritance is associated with DHF.

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