Research Article

Study of vitamin D receptor polymorphisms (FokI, TaqI, ApaI) in acute lower respiratory infections among hospitalised Indian children

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Abstract

Background: Acute Lower Respiratory Infections (ALRI) is the most common cause of global child mortality, with the incidence almost 10 times higher in developing countries in under-5 children. Many in vitro studies have shown the role of vitamin D in promoting and regulating immune response via binding to vitamin D receptor (VDR). In this study, we aimed to find the association between vitamin D receptor polymorphism and ALRI. **Methods:** Three vitamin D receptor (VDR) polymorphisms (FokI, TaqI, ApaI) were studied by the method of PCR-RFLP in 78 hospitalised cases of acute lower respiratory infections (ALRI) and were compared with 75 age and sex matched apparently healthy children below five years of age. Serum vitamin D levels were also measured. **Results:** TaqI *tt* genotype and independent *t* allele were found to confer a significant protection against ALRI. FokI and ApaI genotypes had no significant association with the risk of ALRI in our study population. Vitamin D levels were found to be significantly low in children suffering from ALRI. No statistically significant difference in vitamin D levels was found among the three genotypes in FokI, TaqI and ApaI polymorphisms. **Conclusions:** The present study demonstrates that presence of *tt* genotype confers a significant protection against ALRI of bacterial origin.

Key words: ALRI, ApaI, FokI, TaqI, Vitamin D, VDR polymorphism

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INTRODUCTION

Acute Lower Respiratory Infections (ALRI) is the most common cause of global child mortality, accounting for approximately 2 million under-five deaths every year[1]. It is clinically classified into two groups: Bronchiolitis and Pneumonia, but both types are analysed together because there is a major clinical overlap between the two conditions. The incidence is almost 10 times higher in developing countries (20-30%) in under-5 children, the main reasons being high prevalence of malnutrition, low birth weight and indoor air pollution.[2] Further, bacterial pneumonia of streptococcal origin is the leading cause in developing countries.[3-5]

There is a high possibility of poor immune response predisposing infants and toddlers to this debilitating and fatal condition. Since the past few decades, immunomodulatory role of vitamin D metabolism is being investigated. Vitamin D plays an important role in modulating the innate immune response against infections.[6] Certain in vitro studies have even shown the role of vitamin D for promoting and regulating immune response via binding to the vitamin D receptor (VDR).[7-9] It has been observed that rachitic children are more prone to pneumonia than their normal counterparts.[10,11] Four hospital-based case–control studies from Ethiopia (Muhe *et al*. 1997)[10], India (Rehman 1994;[12] Wayse *et al*. 2004)[13] and Afghanistan (S.Manaseki-Holland *et al*. 2010)[14] suggest that vitamin D deficiency may substantially increase the risk of severe pneumonia among children younger than five. In tuberculosis, role of

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T helper cell mediated immune response has been found. Subclinical vitamin D deficiency has been associated with increased risk of tuberculosis in adults, which is modulated by polymorphisms in the vitamin D receptor.[15] Janssen *et al*. reported a significant association between genetic susceptibility to viral bronchiolitis and several single nucleotide polymorphisms (SNPs) of genes related to innate immune function, including the VDR.[16] Roth *et al*. demonstrated that children with VDR SNP FokI (ff) genotype were at a higher risk of acquiring ALRI, predominantly viral bronchiolitis.[17] These findings support the concept of vitamin D possessing important pleiotropic actions apart from calcium homeostasis and bone metabolism. The VDR belongs to the steroid receptor superfamily present on chromosome 12q13.11 and is widely expressed in many immune cell types, including lymphocytes and macrophages showing an extensive polymorphism pattern.[18] These polymorphisms are distributed throughout the complete VDR gene region: the promoter region exons 1a–1f, the coding exons 2–9, introns and the 30-UTR. Five polymorphisms of the VDR gene (Cdx-2, FokI, TaqI, BsmI and ApaI) have been most commonly studied.[19,20] The FokI (rs2225870) polymorphism is a T/Ctransition polymorphism (ATG to ACG) at the first of two potential translation initiation sites in exon 2 characterized by the presence of either two ATG start codons separated by six nucleotides in the long *f*-VDR or only one start codon due to a T-to-C substitution in the most 5' ATG codon, resulting in a 3-aa shorter *F*-VDR protein (424 aa instead of 427 aa).[21-23] VDR FokI polymorphism affects immune cell behaviour, with a more active immune system for the short *F*-VDR, thus possibly playing a role in immune-mediated diseases.[24] The TaqI (rs731236) polymorphism is a T/C nucleotide substitution(ATT to ATC) leading to a synonymous change atcodon 352 (isoleucine) in exon 9, meaning that it is present in the coding sequence (i.e., exon 9) but that it is not changing the amino acid sequence of the encoded protein. Taq1 polymorphism has been associated with increased VDR mRNA stability and/or protein translation efficiency.[25] The TaqI SNP has been shown to be associated with many metabolic and immune-mediated diseases. The t allele is associated with asthma/atopy, tuberculosis, chronic hepatitis B infection, lepromatous leprosy and type I diabetes in Eastern Europeans and South Indians.[26-28] The T allele, on the other hand, is associated with Crohn's disease, tuberculoid leprosy, and few cases of type 1 diabetes.[29-31] The functional consequences of this polymorphism are not fully understood. ApaIrestriction site polymorphism is located in intron 8 and is not affecting any splicing site and/or transcription factor binding site.[27,32] However, they may affect gene expression through regulation of mRNA stability. Due to paucity of study of VDR Polymorphisms in ALRI and considering the above facts, the genotype distribution of 3 SNPs (FokI, TaqI, ApaI) in VDR gene were analysed in this study among children below 5 years of age admitted to hospital with ALRI of bacterial origin, and age and sex matched apparently healthy children without any history of ALRI. Our study was an endeavour to find the association, if any, between VDRgene polymorphism and ALRI. Hence, this study can be useful for more insights on the genetic basis of ALRI.

MATERIALS AND METHODS

The present study is a hospital based case control study conducted in the Department of Biochemistry in collaboration with Department of Paediatrics, Vardhman Mahavir Medical College and Safdarjang Hospital, New Delhi, India during the period of January 2011 to December 2011. A predesigned proforma was used to obtain information about the child. A detailed history was taken and examination was done to look for signs of ALRI. The study was conducted after approval from Institutional Ethics Committee and written informed consent was taken from guardians of participants. 78 children below 5 years of age admitted in paediatric ward with ALRI were selected as case groups. 75 apparently healthy age and sex matched children without any history of ALRI were taken as controls. ALRI is clinically diagnosed and classified into two groups: Bronchiolitis (rhinorrhoea, coryza, cough and/or fever of <2 weeks duration; wheezes and/or crackles on auscultation of the lung fields; and increased respiratory effort) or Pneumonia (temperature > 38°C, respiratory distress and consolidation or pleural effusion on a chest radiograph) supported by other investigation parameters followed in the ward. Cases were diagnosed as per definition of severe ALRI as given by WHO (1995)[33] i.e. presence of lower chest indrawing with respiratory rate ≥ 60/minute in infants <2 months of age,≥ 50/minute in infants 3-12 months of age, ≥ 40/minute in children 13-60 months of age. Children who had taken Calcium and Vitamin D supplements and ALRI cases due to pulmonary aspiration, oropharyngeal abnormality (cleft lip/palate) or symptomatic congenital heart diseases that could predispose or complicate ALRI were excluded from the study. Healthy controls were selected from the hospital immunization clinic. Those having past history of ALRI and those on vitamin D supplements were excluded. Under strict aseptic conditions without using tourniquet 1ml of venous blood was collected in EDTA vacutainers and plain vacutainers and processed immediately. Serum was then stored at -80^{0}C which was then used later for serum vitamin D estimation by ELISA (DLD ELISA kit,

Hamburg, Germany). DNA isolation was done manually by a chloroform-ethanol method described by Daly *et al*.[34] DNA concentration and purity was checked by spectrophotometer. DNA sequences containing 3 previously described VDR SNPs identified as TaqI (rs731236) and FokI (rs 2225870) and ApaI (rs7975232) were amplified by PCR. The presence of a restriction sitewas designated by a lowercase letter and its absence by an uppercaseletter: "t" and "T" were used for the TaqI site, "f " and"F" were used for the FokI site and "a" and "A" for ApaI site . For FokI, a 265 bp fragment was amplified using the following primers : Forward 5'-AGC TGG CCC TGG CAC TGA CTC TGG CTC T-3' and Reverse 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3'(Harris *et al*.).[35] Common primers were used for amplifying a 740 bp fragment for TaqI/ ApaI: Forward 5' -CAG AGC ATG GAC AGG GAG CAA-3' and Reverse 5' -GCA ACT CCT CAT GGC TGA GGT CTC-3'(Riggs *et al*.)[36] The following PCR conditions for Fok1 was used: Initial denaturation at 94°C for 6 min., 35 cycles of following three temperatures, denaturation at 94°C for 45 sec., Annealing at 60°C for 45 sec., Extension at 72°C for 45 sec., Final extension at 72°C for 7 min., 4°C for temporary storage.The annealing temperature used for TaqI/ApaI was 66°C. Rest of the cycling conditions are same as FokI. The PCR products obtained were then subjected to digestion by corresponding restriction endonuclease from NEB (New England Biolabs, MA, USA). Digestion by FokI at 37°C for 2 hours produced bands at 265 bp position if homozygous alleles present for Wild type, at 265, 196, 69 bp positions if heterozygous alleles present for Wild and Polymorphic type and at 196 and 69 bp positions if homozygous alleles present for Polymorphic type. Digestion by TaqI at 65°C for 5 hours produced bands at 495 and 245 bp positions if homozygous alleles present for Wild type, 495, 290, 245 and 205 bp positions if heterozygous alleles present for Wild and Polymorphic type, at 290, 245 and 205 bp positions if homozygous alleles present for Polymorphic type. Digestion by ApaI at 25°C for 3 hours produced bands at740 bp position if homozygous alleles present for Wild type, at 740, 530and 210 bp positions if heterozygous alleles present for Wild and Polymorphic type, at 530 and 210 bp positions if homozygous alleles present for Polymorphic type.

Statistical Analysis

Data was statistically analysed using Graph Pad Prism (5.0 version) software program (http://www.graphpad.com). Mean and standard deviation for all parameters were calculated. Conformity towards Hardy-Weinberg equilibrium was calculated by the chi-square test. Association between groups for genotypes and alleles were determined by contingency table analysis by calculating Odd's ratio. Logistic regression analysis was performed to obtain odds ratios and 95% confidence intervals for the association between VDR genotypes and ALRI. Serum vitamin D levels in different genotypes in FokI, TaqI and ApaI were analysed using analysis of variance (ANOVA). Tukey's multiple comparison test was done to compare the intergenotypic variations in vitamin D levels.

RESULTS

In the present study, both the sexes were affected by ALRI, of which majority (64%) were males. Among 78 cases, only two patients had bronchiolitis and rest were diagnosed for pneumonia. The study and control groups were matched for age and sex, which was further verified by statistical evaluation(Table 1). History of exclusive breastfeeding for 4 months and exposure to sunlight in earlier months was significantly higher in controls than cases.

Table 1: Data distribution among Cases and Control

	CASES(n= 78)	CONTROLS(n=75)	p-value
Age in months (Mean± S.E.M.) (Min. - Max.)	8.785 ± 1.156 (0.5 - 42)	12.76 ± 1.934 (0.03 - 60)	0.0772
Male sex n (%)	50 (64%)	44 (59%)	0.51
Exclusive breastfeeding n (%)	25 (32%)	37 (49%)	0.03*
Exposed to Sunlight n (%)	62 (89%)	70 (93%)	0.017 *

*p value is significant

The mean values of vitamin D was 32.43 ± 2.801 (mean ± S.E.M.) in cases and 79.83 ± 6.781 in controls. There was a significant higher level of vitamin D in controls ($p < 0.0001$). (Table 2)

Table 2: Comparison of vitamin D levels between cases and controls

	CASES (n = 78)	CONTROLS (n=75)	p- value
Vit. D (nmol/l) (Mean ± S.E.M.) (Min. - Max.)	32.43 ± 2.801 (3 - 93.75)	79.83 ± 6.781 (11.5 - 290)	< 0.0001***

Genotypic distribution of VDR gene as given in table 3 showed that both the groups did not deviate from Hardy – Weinberg Equilibrium (p-value > 0.05) (Table3).

Table 3: VDR Genotype distribution and chi square values among cases and controls

VDR Polymorphism	Cases n = 78 n (%)	χ^2	p - value	Controls n= 75 n(%)	χ^2	p – value
FokI						
FF	49 (63%)			38 (50%)		
Ff	24 (31%)	0.739	0.389	34 (46%)	1.898	0.168
Ff	5 (6%)			3 (4%)		
TaqI						
TT	31 (40%)			22 (29%)		
Tt	41 (52%)	2.297	0.129	38 (50%)	0.037	0.847
Tt	6 (8%)			15 (21%)		
ApaI						
AA	25 (32%)			17 (22%)		
Aa	34 (44%)	1.180	0.277	39 (52%)	0.124	0.724
Aa	19 (24%)			19 (26%)		

Further analysis of genotypes was done by Odd's ratio to assess the risk factors associated with them as shown in table 4.

Table 4: Analysis of the association between vitamin D receptor (VDR) genotypes and the risk of acute lower respiratory tract infection

VDR Polymorphism	Cases n = 78 n (%)	Controls n = 75 n (%)	Odd's ratio (95% Confidence Interval)	p – value
FokI				
FF	49 (63%)	38 (50%)	1 (ref)	
Ff	24 (31%)	34 (46%)	0.547 (0.279-1.073)	0.091
Ff	5 (6%)	3 (4%)	1.293 (0.290-5.753)	1
F	122 (78%)	110 (73%)	1 (ref)	
F	34(22%)	40 (27%)	0.766 (0.453-1.295)	0.351
TaqI				
TT	31 (40%)	22 (29%)	1 (ref)	
Tt	41 (52%)	38 (50%)	0.766 (0.379-1.546)	0.481
Tt	6 (8%)	15 (21%)	0.284 (0.095-0.847)	0.037*
T	103 (66%)	82 (55%)	1 (ref)	
T	53 (34%)	68 (45%)	0.62 (0.391-0.985)	0.047*
ApaI				
AA	25 (32%)	17 (22%)	1 (ref)	
Aa	34 (44%)	39 (52%)	0.593 (0.275-1.279)	0.245
Aa	19 (24%)	19 (26%)	0.68 (0.28-1.649)	0.5
A	84 (54%)	73 (49%)	1 (ref)	
A	72 (46%)	77 (51%)	0.813 (0.519-1.273)	0.423

*p value is significant

In cases, 31(40%) had *TT*, 41(52%) had *Tt*, and 6(8%) had *tt* genotypes while in controls, 22(29%) had *TT*, 38(50%) had *Tt*, and 15(21%) had *tt* genotypes. There was a significant protective factor associated with *tt* genotype (Odd's ratio = 0.284; p value = 0.04) in controls. 66% of cases and 55% of controls had *T* allele while 34% of cases and 45% of controls had *t* allele. There was found to be a significant protective association of ALRI with *t* allele (Odd's ratio = 0.62; p value = 0.047). There was no significant difference in the FokI genotype frequency (*F* allele 78% : 73%) and (*f* allele 22% : 27%) between the cases and controls and no

significant risk associated withALRI. Also, no significant risk was associated with ApaI genotypes and their independent alleles.

Table 5,6 and 7 show the comparison of vitamin D levels in different genotypes FokI, TaqI and ApaI.

Table. 5: Comparison of serum vitamin D levels in different genotypes of FokI by ANOVA

	GENOTYPES			
Vitamin D levels (ng/ml) Mean ± S.D.	**FF**	**Ff**	**ff**	**P-value**
	54.99±54.22	58.89±46.85	39.06±19.29	0.57

Table. 6: Comparison of serum vitamin D levels in different genotypes of TaqI by ANOVA

	GENOTYPES			
Vitamin D levels (ng/ml) Mean ± S.D.	**TT**	**Tt**	**tt**	**P-value**
	61.46 ± 57.66	51.26 ± 47.31	57.7 ± 37.11	0.51

Table 7: Comparison of serum vitamin D levels in different genotypes of ApaI by ANOVA

	GENOTYPES			
Vitamin D levels (ng/ml) Mean ± S.D.	**AA**	**Aa**	**aa**	**P-value**
	61.51 ± 63.55	49.16 ± 37.19	61.99 ± 55.29	0.31

The vitamin D levels were not significantly different among the three genotypes ($P > .05$) in each of the FokI, TaqI and ApaI polymorphism. Intercomparison of the three genotypes i.e. between FF and Ff, FF and ff and Ff and ff showed no statistically significant difference. No statistically significant difference was found among intergenotypic comparisons in TaqI and ApaI polymorphisms also.

Thus, the SNP in the coding region of exon 9 i.e. Taq I displayed a significant protection associated with ALRI. The minor allele of TaqI (*t*) (OR-0.62, p-value-0.047) and the genotype (*tt*) (OR- 0.284, p-value-0.037) was found to be a significant protective factor associated with occurrence of ALRI, whereas SNP in the coding region of exon 2 (FokI) and non coding region of intron 8 (ApaI) were not significant risk factors as deduced by their odd's ratio and their p-values.

DISCUSSION

VDR mediated regulation of various genes is responsible for the immunomodulatory role of vitamin D in respiratory epithelial cells. VDR polymorphism may influence the function of immune cells.TaqI polymorphism has previously been linked to susceptibility to infectious diseases, including tuberculosis.[37] The *TT* genotype has previously been associated with varied levels of VDR expression in peripheral blood mononuclear cells that are higher than those for the *tt* genotype.[38] Controversies have clouded the definitive association of Taq polymorphism and diseases recently. Initial studies showed tt genotype had some protection against tuberculosis.[39,40] Few years later, the same genotype was seen to be causative in a different set of women population.[41] Presence of T allele contributes to high occurrence of tuberculosis in Gujarati-Asians in West London when considered in combination with vitamin D deficiency.[15] Further studies showed confusing results.[42,43] According to our study, (TaqI) *tt* genotype conferred significant protection against ALRI among young Indian children whereas TT genotype conferred no significant risk. Subclinical vitamin D deficiency was a significant independent risk factor for severe ALRI in children in India aged 5 years;[13] in the present study, we also found significant lower levels of vitamin D among children suffering from ALRI. Vitamin D fights bacterial infections by producing proteins called cathelicidin and defensins. Liu et.al demonstrated that stimulation of macrophage-bound Toll-like receptor 2/1 complex by *M tuberculosis*-derived antigens upregulates the expression of both VDR and CYP27M, an enzyme that converts 25-hydroxyvitamin D (25-OHD) to its active 1,25-dihy-droxyvitamin D form. Intracellular 1,25-$(OH)_2D$ generated though action of CYP27b 1 then interacts with the VDR and leads to induction of the antimicrobial peptide, cathelicidin and killing of intracellular *M. Tuberculosis.*[44] Although this antimicrobial mechanism of vitamin D has been demonstrated only in macrophages infected with *M. tuberculosis,* it is also well known that cathelicidin has broad-spectrum activity against a wide variety of other pathogens, including gram-negative and gram-positive bacteria. Additional studies also suggest that vitamin D may cause upregulation of the oxidative burst in activated macrophages,[45] thus augmenting another multipurpose mechanism of bacterial killing. Studies of VDR polymorphisms in humans support the hypothesis that

variability in vitamin D status and host genes encoding vitamin D-responsive elements affect the immune response to bacterial pathogens other than *M. tuberculosis*[46,47] When infections are considered, TaqI polymorphism so far has been associated in chronic infections like tuberculosis. However, in our study, this protective association of *t* allele in acute lung infection in under-5 children is a new observation. Further research need to be performed to explain the mechanism of immunomodulation by VDR with respect to TaqI polymorphic variant. Regarding the FokI allele, when diseases of immune etiology are considered, FokI allele variants are generally linked to in autoimmune disorders like grave's disease, rheumatoid arthritis.[48-50] Studies conducted by Roth *et al*.in Canada showed strong association of *ff* genotype with the risk of ALRI.[17] But, we did not find any association of FokI allele with ALRI. Thus SNP in the coding region of exon 9 (Taq I) displayed a significant protection associated with ALRI whereas SNP in the coding region of exon 2 (FokI) and non coding region of intron 8 (ApaI) were not a significant risk as per present study. But, the implications of TaqI polymorphisms for immune function are still not known. Non functional polymorphisms like TaqI, ApaI are nevertheless still useful in association studies because they can be used as markers. When association is found with a marker allele, the association is then believed to be caused by a truly functional allele which is linked to the marker allele and which is located elsewhere but usually nearby in the same gene. This type of linkage between marker allele and functional allele depends on the extent and strength of linkage disequilibrium in that area of the chromosome. Accordingly, differences in linkage disequilibrium between the marker allele and the truly functional allele can lead to varying associations. Environmental factors and genetics interactionsalso play a role in the action of this steroid hormone receptor transcription factor. For example, dietary Calcium intake is known to differ substantially between countries and populations while circulating serum Vitamin D levels, which are determined by several metabolizing enzymes as well as different levels of sunlight exposure also differ between populations. Different alleles are sometimes reported to be the risk allele, then the potential confounding effects which arise from the pleiotropy of vitamin D can influence the associations observed.[51]

CONCLUSION

In our study of ALRI patients and controls in North Indian population, tt polymorphism conferred a protective association. Vitamin D levels were found to be lower in children suffering from ALRI. The findings in this study should be considered as elementary, as the sample size was small, and false-positive associations are relatively common in case-control studies of genetic association. Confirmation of these results in a larger sample, as well as further elucidation of the effect of interplay between circulating vitamin D metabolites and the VDR on the host response to ALRI should be done so that firm conclusions can be drawn about the clinical or public health importance of these findings. Elaboration of the role of VDR polymorphism (TaqI) in immunity is also recommended..

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