

SUSCEPTIBILITY TO DENGUE HEMORRHAGIC FEVER IN VIETNAM: EVIDENCE OF AN ASSOCIATION WITH VARIATION IN THE VITAMIN D RECEPTOR AND FC γ RECEPTOR IIA GENES

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Abstract. Dengue is an increasingly important cause of morbidity and mortality in the tropics, with more than a billion people at risk each year. Immunologic enhancement is thought to contribute to disease pathogenesis. Only a very small proportion of infected individuals develop life-threatening dengue hemorrhagic fever (DHF). In a large case-control study with 400 DHF patients and 300 matched controls, we have assessed five polymorphic non-HLA host genetic factors that might influence susceptibility to DHF. The less frequent t allele of a variant at position 352 of the vitamin D receptor (VDR) gene was associated with resistance to severe dengue ($P = 0.03$). Homozygotes for the arginine variant at position 131 of the Fc γ RIIA gene, who have less capacity to opsonize IgG2 antibodies, may also be protected from DHF (one-tailed $P = 0.03$). No associations were found with polymorphisms in the mannose binding lectin, interleukin-1 (IL-4), and IL-1 receptor antagonist genes. Further studies to confirm these associations are warranted.

INTRODUCTION

Dengue fever has become one of the most important arthropod-borne diseases. Any of the four serotypes of dengue virus can result in dengue fever (DF), an acute viral infection characterized by fever, rash, headache, muscle and joint pain, and nausea. Occasionally, DF progresses to dengue hemorrhagic fever (DHF), a potentially life-threatening illness associated with vascular leakage, hemorrhage, and shock.¹ More than 1 billion people are at risk of dengue infection and every year, there are approximately 100 million cases of DF and approximately 500,000 cases of DHF.²

The pathogenesis of DHF is poorly understood. The antibody-dependent enhancement theory of DHF pathogenesis draws support from epidemiologic studies, which show that of the presence of circulating dengue-specific IgG antibodies constitutes the largest risk factor for DHF and, *in vitro*, dengue-specific IgG can enhance viral entry and infection of cells.^{3–5} Recently, cross-reactive T cells were proposed to play a pathogenic role by causing tissue damage and secreting permeability enhancing cytokines.⁶ Pathogenesis has also been linked to viral virulence factors; in particular the Southeast Asian dengue viruses may be specifically associated with DHF.⁷

Host genetic factors may also be relevant and predispose some individuals to DHF. It is known that only a small proportion of antibody-positive individuals who experience a dengue infection actually develop DHF.⁸ In addition, there may be racial differences in susceptibility to DHF.⁹ Our group and others have shown that polymorphisms at the major histocompatibility complex (MHC) class I loci are associated with an altered risk of DHF.^{10–12} Studies in other infectious diseases have found associations with polymorphic non-MHC genes, which are linked with immune responses.¹³ In this study, we investigated whether susceptibility to DHF is associated with polymorphisms within five non-HLA candidate genes.

The vitamin D receptor (VDR) mediates the immunoregulatory effects of 1,25-dihydroxyvitamin D₃ (1,25D₃), which

include activating monocytes, stimulating cellular immune responses, and suppressing immunoglobulin production and lymphocyte proliferation.¹³ Recently, the tt genotype of a single nucleotide polymorphism (SNP) at position 352 of the VDR gene has been associated with tuberculoid leprosy, enhanced clearance of hepatitis B infection and resistance to pulmonary tuberculosis.^{14,15} Expression of VDR may affect susceptibility to DHF since activated B and T lymphocytes express VDR, and 1,25D₃ affects monocytes, the main sites of dengue virus infection and replication.¹⁶

The Fc γ receptor II (Fc γ RII) is a widely distributed receptor for all subclasses of IgG, and is able to mediate antibody-dependent enhancement *in vitro* by binding to virus-IgG complexes.^{17,18} An arginine (R) to histidine (H) substitution at position 131 of the Fc γ RIIA gene has been associated with meningococcal disease and recurrent respiratory tract infections.^{19,20} This polymorphism changes the IgG binding affinity of the receptor, with reduced opsonization of IgG2 antibodies causally associated with the arginine variant.²¹ Therefore, it seemed reasonable to investigate whether homozygosity for the arginine variant might be associated with a reduced risk of DHF caused by antibody-dependent enhancement.

Dengue shock syndrome (DHF grades III and IV) is associated with marked changes in vascular permeability potentially due to inflammatory mediators and complement activation.⁶ Interleukin-4 (IL-4) is primarily produced by Th2 subset of CD4+ T cells. It regulates B cell growth and IgG class switching, as well as suppresses Th1-type responses.^{22,23} It is a good candidate gene for DHF since it affects both antibody responses and inflammatory responses during disease. An SNP identified within the IL-4 promoter has been reportedly associated with increased levels gene transcription *in vitro*.^{24–26} IL-1RA is involved in the regulation of IL-1-mediated inflammatory responses by competitive binding to IL-1 receptors.²⁷ A two-repeat allele (IL-1RA2) of an 86-basepair variable number tandem repeat in the IL-1RA gene is associated with increased serum levels of IL-1RA, and has also been associated with a number of autoimmune diseases, including

systemic lupus erythematosus, psoriasis, and Graves disease.^{28,29} Intrinsic differences in levels of IL-1RA due to genetic polymorphisms could affect regulation of inflammatory responses, and thus DHF pathogenesis.

Mannose-binding lectin (MBL) mediates carbohydrate-dependent activation of the classical complement pathway.^{30,31} Several mutations in the MBL gene, including a G → A substitution at codon 54 (MBP54) have been associated with marked reduction in serum MBL levels and MBL-mediated complement activation.^{32,33} The MBL mutations have been associated with susceptibility to hepatitis B infection and recurrent childhood infections.^{34,35} Thus, MBL is a candidate gene for DHF since complement activation may contribute to DHF pathogenesis, and dengue virus has glycosylated envelope and non-structural (NS1) proteins that may be opsonized by MBL.^{36,37}

We have assessed these five candidate genes in the largest genetic susceptibility study of dengue yet reported. We conducted this initial study as a classical case-control study with age-, sex-, and ethnically matched healthy children as the control group and the most severe form of DHF as the cases.

MATERIALS AND METHODS

This study was carried out at Dong Nai Paediatric Centre, a provincial pediatric hospital 40 km north of Ho Chi Minh City in Vietnam. The DHF grade III and Grade IV patients were classified according to criteria of the World Health Organization.¹ Controls were selected from children admitted to the minor surgical unit in the same hospital and were age, sex, and ethnically matched.

DNA was extracted from 3–5 mL of blood from each individual using Nucleon II DNA extraction kits (Anachem Ltd Luton LU2OEB UK). The polymerase chain reaction (PCR) was carried out to amplify the genetic region of interest using the following oligonucleotide primers: 5'-CAG AGC ATG GAC AGG GAG CAA G-3' and 5'-GGT GGC GGC AGC GGA TGT A-3' for VDR; 5'-CAA GCC TCT GGT CAA GGT C-3' and 5'-GAA GAG CTG CCC ATG CTG-3' for FcγRII; 5'-ACT AGG CCT CAC CTG ATA CG-3' and 5'-GTT GTA ATG CAG TCC TCC TG-3' for IL-4; 5'-CTC AGC AAC ACT CCT AT-3' and 5'-TCC TGG TCT GCA GGT AA-3' for IL-1RA; and 5'-GCA CCC AGA TTG TAG GAC AGA G-3' and 5'-CAG GCA GTT TCC TCT GGA AGG-3' for maltose-binding protein.

The SNPs were typed by dot-blotting PCR products onto a nylon membrane, hybridization with 3' digoxigenin-labeled sequence-specific oligonucleotides, and visualization of the probes using an anti-digoxigenin chemiluminescence system (Boehringer Mannheim, Indianapolis, IN). Oligonucleotide probes used were 5'-GCG CTG ATT GAG GCC ATC-3' for VDR T and 5'-GCG CTG ATC GAG GCC ATC-3' for VDR t; 5'-ATT CTC CCG TTT GGA TC-3' for FcγRII R and 5'-ATT CTC CCA TTT GGA TC-3' for FcγRII H; 5'-GAA CAT TGT CCC CCA GTG-3' for IL-4 C and 5'-GAA CAT TGT TCC CCA GTG-3' for IL-4 T, and 5'-CGT GAT GGC ACC AAG GGA-3' for MBL G and 5'-CGT GAT GAC ACC AAG GGA-3' for MBL A. The IL-1RA repeat polymorphisms were visualized by electrophoresis of the PCR products on a standard 2% agarose gel. Results were analyzed for any differences in genotype or allele frequencies

TABLE 1

Vitamin D receptor (VDR) polymorphism genotype frequencies in a dengue hemorrhagic fever (DHF) case-control study*

VDR genotype	DHF cases (n = 327)		DHF controls (n = 247)	
	Number	Frequency (%)	Number	Frequency (%)
TT	316	96.6	231	93.5
Tt	11	3.4	15	6.1
tt	0	0	1	0.4

* Genotype frequencies for DHF cases vs. controls: 3 × 2 chi-square analysis, degrees of freedom = 2, P = 0.154.

between the cases and controls in a stepwise fashion. The StatCalc statistical analysis package (EpiInfo, Centers for Disease Control and Prevention, Atlanta, GA) was used to carry out the standard contingency table chi-square test and the chi-square test for trends.

Informed consent was obtained from the cases and controls recruited into the study or from their parents or guardians. The study was approved by the Ethical and Scientific Committee of the Centre for Tropical Diseases in Ho Chi Minh City and the Ethical Committee of the Dong Nai Paediatric Centre in the Dong Nai province of Vietnam.

RESULTS

Three hundred fifteen DHF grade III patients, 37 grade IV patients, and 251 healthy controls were recruited into the study. All patients and controls originated from Dong Nai Province in Vietnam, and cases and controls were ethnically matched as a group. It was not possible as part of this study to determine the serotype of the dengue virus. All four serotypes continuously circulate in southern Vietnam. Recruitment of subjects took place in 1994–1996 when the dominant serotypes circulating in southern Vietnam were dengue II and dengue III.

Genotype frequencies for the VDR polymorphism did not differ between DHF cases and controls (3 × 2 chi-square analysis, degrees of freedom [df] = 2, P = 0.154; Table 1). However, allele frequency analysis showed that there was an association between VDR polymorphism and DHF disease severity (chi-square test for trend analysis, df = 1, P = 0.033; Table 2). This result suggests that the t allele may be protective against severe DHF.

Genotype frequencies (3 × 2 chi-square analysis, df = 2, P = 0.178; Table 3) and allele frequencies (2 × 2 chi-square analysis, df = 1, P = 0.107; Table 3) for the FcγRII polymorphism were not significantly different between DHF patients and controls. However, based on the hypothesis that

TABLE 2

Vitamin D receptor (VDR) polymorphism allele frequencies in a dengue hemorrhagic fever (DHF) case-control study*

VDR allele	DHF Grade IV cases (n = 70)		DHF Grade III cases (n = 584)		Controls (n = 494)	
	Number	Frequency (%)	Number	Frequency (%)	Number	Frequency (%)
T	70	100	573	98.1	477	96.6
t	0	0	11	1.9	17	3.4

* DHF cases (Grades III and IV) vs. controls: 2 × 2 chi-square test, degrees of freedom (df) = 1, P = 0.056. chi-square test for trend analysis, df = 1, P = 0.0326.

TABLE 3

FCγRII polymorphism genotype frequencies in dengue hemorrhagic fever (DHF) cases and controls*

CD32 genotype	DHF cases (n = 302)		DHF controls (n = 238)	
	Number	Frequency (%)	Number	Frequency (%)
RR	19	6.3	25	10.5
RH	118	39.1	94	39.5
HH	165	54.6	119	50.0

* Comparison of RR vs. 'RH + HH' genotypes: 2 × 2 chi-square analysis, $P = 0.036$ (1-tailed test).

Genotype frequencies for DHF cases vs. controls: 3 × 2 chi-square analysis, degrees of freedom (df) = 2, $P = 0.178$.

Allele frequencies for DHF cases vs controls: 2 × 2 chi-square analysis, df = 1, $P = 0.107$.

homozygosity for the lower-affinity binding arginine variant may be protective against antibody-dependent enhancement-associated immunopathogenesis, a 2 × 2 chi-square analysis comparing R/R individuals who possess only the lower-affinity binding allele with individuals who possess copies of the higher-affinity binding allele (R/H and H/H) found a possible protective effect (2 × 2 chi-square analysis, one-tailed $P = 0.036$, odds ratio = 0.57, 95% confidence interval = 0.29–1.11; Table 3). The observed frequency of the HH genotype in this Vietnamese population, 54.6% in DHF cases, and 50% in controls, is similar to those found in Japanese (61%) and Chinese (50%) populations and higher than those found in whites.³⁸

There were no significant differences in MBL genotypes (3 × 2 chi-square analysis, df = 2, $P = 0.187$; Table 4) or allele frequencies (2 × 2 chi-square test, df = 1, $P = 0.072$) between the cases and controls. However, the relatively low frequency of the variant allele in this population limits the statistical power of this analysis.

No differences between cases and controls were observed for the IL-4 promoter polymorphism, either at the genotypic level (3 × 2 chi-square analysis, df = 2, $P = 0.688$; Table 5) or the allelic level (2 × 2 chi-square analysis, df = 1, $P = 0.624$). Interestingly, the T allele, which is infrequently found (0.02) in whites, is more common (0.45) in African populations, and is the dominant allele in the Vietnamese population (0.77).³⁹

There was no difference in genotype frequencies of the IL-1RA repeat polymorphisms between DHF cases and controls (6 × 2 chi-square analysis, df = 5, $P = 0.833$; Table 6). Allele frequencies were also not significantly different between the two groups (4 × 2 chi-square analysis, df = 3, $P = 0.94$). The frequency of IL-1RA2 (7%) was intermediate between that observed in southern Indians (25%) and European whites (24%), and that observed in West Africans (2.5%).⁴⁰

TABLE 4

MBP54 polymorphism genotype frequencies in dengue hemorrhagic fever (DHF) cases and controls*

MBP54 genotype	DHF cases (n = 340)		DHF controls (n = 264)	
	Number	Frequency (%)	Number	Frequency (%)
GG	241	70.9	202	76.5
GA	88	25.9	58	22.0
AA	11	3.2	4	1.5

* Genotype frequencies for DHF cases vs. controls: 3 × 2 chi-square analysis, degrees of freedom (df) = 2, $P = 0.187$.

Allele frequencies for DHF cases vs. controls: 2 × 2 chi-square test, df = 1, $P = 0.072$.

TABLE 5

Interleukin-4 (IL-4) promoter polymorphism genotype frequencies in dengue hemorrhagic fever (DHF) cases and controls

IL-4 promoter genotype	DHF cases* (n = 147)		DHF controls* (n = 147)	
	Number	Frequency (%)	Number	Frequency (%)
CC	12	8.2	8	5.4
CT	46	31.3	49	33.3
TT	89	60.5	90	61.2

* Only a subset of the cases and controls were studied for this polymorphism.

Genotype frequencies: 3 × 2 chi-square analysis, degrees of freedom (df) = 2, $P = 0.688$.

Allele frequencies: 2 × 2 chi-square analysis, df = 1, $P = 0.624$.

DISCUSSION

In this study of genetic susceptibility to DHF, variation in two of the five genes assessed showed evidence of association with altered risk of severe dengue. The IL-4 and the IL-1RA gene variants were not associated with altered risk, but there is no compelling evidence that the polymorphisms assessed in these genes are of functional significance. In contrast, heterozygotes and particularly homozygotes for MBL variants clearly have reduced serum levels of this lectin, indicating that MBL deficiency does not appear to affect the risk of severe dengue significantly.⁴¹

The less frequent t allele of a dimorphism at position 352 of the VDR gene was associated with dengue disease severity. This t allele was initially associated with susceptibility to osteoporosis, and some evidence of higher expression of receptor alleles of this type has been presented.⁴² However, subsequent studies of bone density association and some limited studies of the functionality of this variant have provided conflicting results.⁴³ This variant has now been studied in several infectious diseases. In case-control studies of Gambians, homozygotes for the t allele were at reduced risk of tuberculosis and of persistent hepatitis B virus infection.¹⁵ Further evidence of a tuberculosis association was obtained in a study in the United Kingdom,⁴⁴ and the tt genotype was associated with tuberculoid rather than lepromatous leprosy in Indians.¹⁴ These associations led to the suggestion that the tt genotype may be associated with a relatively stronger TH1-type cellular immune response than the TT genotype; interestingly, dihydroxyvitamin D has recently been found to alter IL-12 expression and dendritic cell maturation.^{14,45,46} By extension it might be inferred that the association of the t allele with resistance to severe dengue might reflect a protective role for enhanced cellular immunity in this disease. However, further work will be required both to confirm this association and to explore possible mechanisms.

TABLE 6

Interleukin-1 repeat allele (IL-1RA) polymorphism genotype frequencies in dengue hemorrhagic fever (DHF) cases and controls*

IL-1RA genotype	DHF cases (n = 280)		DHF controls (n = 229)	
	Number	Frequency (%)	Number	Frequency (%)
11	232	82.9	195	85.2
12	42	15.0	28	12.2
13	2	0.7	2	0.9
14	3	1.1	2	0.9
22	0	0	1	0.4
44	1	0.4	1	0.4

* Genotype frequencies: 6 × 2 chi-square analysis, degrees of freedom (df) = 5, $P = 0.833$.

Allele frequencies: 4 × 2 chi-square analysis, df = 3, $P = 0.94$.

There is also evidence of a protective effect of homozygosity for the arginine variant at amino acid position 131 of Fc γ R2 against DHF. Studies have reported a role for this variant in several infectious disease, and there is preliminary evidence that homozygotes for the arginine variant are more susceptible than homozygotes for the histidine variant to infections with encapsulated bacteria.^{20,47,48} This is consistent with the lower opsonic capacity of this variant for IgG2 and the evidence that IgG2 is protective against disease caused by many encapsulated bacteria.¹⁷ Therefore, the association of the genotype encoding homozygosity for the arginine variant with resistance to DHF may relate to a pathogenic role for disease-enhancing IgG2 antibodies in this disease.

The frequency of the RR polymorphism varies significantly. It is much less common in Asian populations (RR polymorphism = 6–10%) than in white and African populations from Europe, India, and North and South America (RR polymorphism = 23–37%).^{38,47,49} The variation in the frequency of this polymorphism between Asian (Japanese, Chinese, and Vietnamese) and South American populations (Brazil) is of particular interest, given the apparent protective effect we have demonstrated in this study and the increased incidence of severe dengue hemorrhagic disease in Asia compared with South America. Further assessments in comparative studies of the geographic variation in this gene might be important.

The availability of an increasing number of defined polymorphisms throughout the human genome will greatly increase the potential power of genetic susceptibility studies in dengue and should provide further insights into possible mechanisms of pathogenesis and protection. This large study demonstrates potentially important associations with two host genes and susceptibility to the severe form of DHF and finds no link with three other candidate genes. We used healthy children, rather than children with milder forms of dengue, as controls because we were primarily interested in what factors led healthy children to develop severe dengue, and wanted to avoid errors associated with assessment of the clinical state; the World Health Organization classification of dengue has been developed on clinical criteria and may not accurately reflect the underlying pathogenesis. It is possible that a component of susceptibility to severe dengue involves an increase in the risk of acquiring a primary dengue infection, and further studies in a separate cohort are underway to compare DHF with DF to identify factors associated solely with the severity of disease. We believe the current study is a first step in piecing together the human factors that may underlie DHF.

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