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Genome-wide association study identifies susceptibility loci for Dengue shock syndrome at *MICB* and *PLCE1*

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Abstract

Hypovolemic shock (Dengue shock syndrome (DSS)), is the commonest life-threatening complication of dengue. We conducted a genome-wide association study of 2,008 pediatric cases treated for DSS and 2,018 controls from Vietnam. Replication of the most significantly associated markers was carried out in an independent Vietnamese follow-up sample of 1,737 cases and 2,934

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Accession codes: *MICB* (MHC class I polypeptide-related sequence B) mRNA, NM_005931; *PLCE1* (phospholipase C, epsilon 1) mRNA, NM_001165979, NM_016341.

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controls. Polymorphisms within two genes showed genome-wide significant association with DSS ($P_{\text{meta}} = 4.41 \times 10^{-11}$, per-allele odds ratio (OR) = 1.34 for *MICB* rs3132468 located within the broad MHC region and $P_{\text{meta}} = 3.08 \times 10^{-10}$, per-allele OR = 0.80 for *PLCE1* rs3765524). Our data implicates *MICB* as an important determinant in early immune control of dengue virus infection and *PLCE1* a factor in vascular endothelial dysfunction and circulatory hypovolemia.

Introduction

Dengue is an acute systemic viral infection caused by one of four serotypes of dengue (DEN) virus and is globally the commonest mosquito-borne infection after malaria¹. The burden of dengue is growing, with an estimated 100 million infections now occurring annually and with 2.5 billion people living in areas at risk of transmission. A wide spectrum of disease manifestations is seen, ranging from subclinical infection to severe and fatal disease. Severe dengue in children is characterised by an increase in vascular permeability that leads to life-threatening hypovolemic shock (dengue shock syndrome-DSS). This is often accompanied by thrombocytopenia and haemostatic dysfunction, which may result in severe bleeding. Children are at greatest risk of developing DSS but with careful supportive care the case fatality rate is less than 1%². In southern Vietnam, serological studies have estimated the population based exposure to dengue virus infection to reach 85% by the end of childhood (15 years old)³, while the incidence of DSS is estimated to occur at less than 1% of exposed individuals² (see “The use of population controls” in the Methods section). A host genetic basis to susceptibility to severe dengue has been alluded to in epidemiological studies, and various candidate gene studies of modest sample sizes have been performed⁴⁻⁸.

To estimate the genetic contribution underlying severe dengue, we genotyped 2,118 DNA samples from Vietnamese children with established or incipient DSS and 2,089 cord blood controls in a genome-wide association study (GWAS). After exclusion of samples for discrepancies between clinical and genetically inferred gender, relatedness or for per-sample call rates of less than 95 percent (Supplementary Figure 1), there were 2,008 DSS cases and 2,018 controls available for analysis. The clinical and virological characteristics of the case population are described in Supplementary Table 1. A total of 657,366 SNPs were initially included within the Illumina 660W Beadchip used for genome-wide genotyping. After various stringent QC exclusions (Supplementary Figure 2), a total of 481,342 SNPs were retained for downstream association analysis.

Upon conducting the routine GWAS statistical tests (see Statistical findings in the Methods section), detailed examination of the overall scan results revealed strong evidence of disease association at two distinct loci; (Figure 1) *MICB* on Chromosome 6 and *PLCE1* on Chromosome 10, both represented by SNPs which were close to the formal threshold for genome-wide significance ($P = 5.38 \times 10^{-8}$ for *MICB* rs3132468 and $P = 5.84 \times 10^{-8}$ for *PLCE1* rs3740360) (Table 1). Together with the SNPs at *MICB* and *PLCE1*, a total of 85 SNPs exceeded $P < 10^{-4}$ on single SNP analysis (Supplementary Table 2). We were able to design assays for 72 out of these 85 SNPs using the Sequenom Mass-Array platform. The remaining 13 SNPs in the broad MHC region were refractory to assay design, thus necessitating ABI Taqman assays to be designed for the sentinel SNP at *MICB* (rs3132468) and rs3134899 (also within *MICB*; GWAS $P = 1.03 \times 10^{-4}$, OR = 1.31). We then genotyped these 74 SNPs (72 non-MHC SNPs and two SNPs within *MICB*) in a replication sample of 1,824 DSS cases and 3,019 controls. We applied the same GWAS QC filters for the replication set: five SNPs had poor genotyping clusters and were excluded from analysis (Supplementary Table 2), and 132 samples (87 cases and 85 controls) had per-sample call-rates of less than 95 percent; these were excluded from further analysis. This left 69 SNPs to

be analyzed in 1,737 cases and 2,934 controls for the replication stage. In keeping with the GWAS observations, the strongest evidence of association was observed with SNPs at *MICB* (rs3132468, $P_{\text{repl}} = 9.32 \times 10^{-5}$ and rs3134899, $P_{\text{repl}} = 0.0082$) and *PLCE1* (3 SNPs with P_{repl} ranging from 5.23×10^{-4} to 1.6×10^{-4} , Table 1). Using inverse-variance weights, data from both the GWAS and replication cohorts ($N = 3,745$ DSS cases and $N = 4,952$ controls) were combined in formal meta-analysis, and this revealed strong evidence of association with rs3132468 at *MICB* ($P = 4.41 \times 10^{-11}$; per-allele odds ratio (OR) = 1.34, [1.23 - 1.46]) and 7 SNPs at *PLCE1* (4.18×10^{-9} P 3.08×10^{-10} ; 0.75 OR 0.87, Table 1). To aid in refining the original signal of association, we performed imputation analysis at regions flanking both loci (Chr. 6: 30 - 32 Mb, and Chr. 10: 95.5 - 96.5 Mb). This did not reveal signals of association over and above that of the directly genotyped SNPs. The associations observed at *MICB* and *PLCE1* were not specific to any Dengue virus serotype on subgroup analysis of viral serotype, nor were they associated with the degree of thrombocytopenia or the degree of clinical shock (data not shown).

Found within the broad Major Histo-Compatibility (MHC) locus, *MICB* lies just outside both the type I and type II HLA regions, ~140,000 base-pairs centromeric to the nearest Class I gene (*HLA-B*) and more than 1 million base-pairs away from the nearest Class II gene (*HLA-DR*). Apart from the peak signal at rs3132468 which was observed directly within *MICB*, twelve other SNPs in this region also showed association signals exceeding $P < 10^{-4}$ on single-SNP analysis. We thus performed conditional analysis to assess the independence of the association observed at *MICB* rs3132468 from that of the nearby genes. Although the most significant SNP from the GWAS (rs3132468) could account for the majority of the association signal across the locus, we observed residual signals of association ($0.0003 < P < 0.05$) with SNPs near the vicinity of *HLA-B* and *HLA-C* as well as other neighboring genes (Supplementary Figure 3). These residual associations indicate that definitive identification of *MICB* as a gene associated with DSS could be complicated by its location within the broad MHC region, which is known for its extensive linkage disequilibrium (LD) spanning multiple genes (Supplementary Figure 4). This precludes definitive identification of the causative gene without extensive further fine-mapping and re-sequencing. With regards to *PLCE1* on Chromosome 10, association analysis conditioning for the lead SNP (rs3743060, directly genotyped) did not reveal any secondary signals of association (Supplementary Figure 5), which suggests that the lead SNP -or any of its close correlates in complete LD with it and confined within their distinct genomic region (Supplementary Figure 6)- best explains the association signal at the locus. We did not observe any evidence of epistasis between SNPs at *MICB* and *PLCE1* ($P = 0.11$).

MICB appears to be a promising candidate based on the present strength of the statistical associations observed in the Chromosome 6 hit region. *MICB* encodes for MHC class I polypeptide-related sequence B, an inducible activating ligand for the NKG2D type II receptor on natural killer (NK) and CD8⁺ T cells⁹⁻¹⁰. Ligation of NKG2D by *MICB* stimulates anti-viral effector functions in NK cells including cytokine expression and the cytolytic response¹¹. We have previously reported that *MICB*, together with other genes associated with NK cell activation, are highly expressed in the leukocytes of acute dengue patients¹². We therefore propose the association between the *MICB* rs3132468 genotype and susceptibility to severe dengue might reflect altered or dysfunctional NK and/or CD8⁺ T cell activation early in infection that results in a higher viral burden *in vivo*, a recognized factor in clinical outcome¹³⁻¹⁴. The recent finding that a SNP near the closely related *MICA* gene (rs2596542) is associated with Hepatitis C virus induced hepatocellular carcinoma is suggestive of a pivotal role for MIC proteins in the pathogenesis of these *Flaviviridae* infections¹⁵.

Mutations within *PLCE1* are associated with nephrotic syndrome¹⁶. Nephrotic syndrome is a kidney disorder in which dysfunction of the glomeruli basement membrane results in proteinuria and hypoproteinemia that when severe leads to reduced vascular oncotic pressure and edema. These elements of nephrotic syndrome have striking similarities with severe dengue and suggest an important role for *PLCE1* in maintaining normal vascular endothelial cell barrier function. In summary, our study identifies genetic variants in *MICB* and *PLCE1* as being associated with severe dengue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix

Methods

Patient enrolment and diagnosis

Blood samples for genotyping were collected from patients enrolled into one of two research studies of children with dengue. In both studies, children were eligible if they were 15 years of age and had clinical signs, symptoms and hematological findings that led to a clinical diagnosis of incipient or established DSS as defined by WHO criteria (WHO, see URLs). All patients were resuscitated with bolus intravenous fluid therapy (15ml/kg in the first hour). Summary laboratory and clinical findings were recorded into case record forms during the inpatient period until the patient was either discharged from hospital, died, or was transferred to another hospital. Blood samples for research and diagnostic tests were collected at the time of enrolment and again prior to patient discharge from Hospital. The first study enrolled patients in the pediatric intensive care unit of the Hospital for Tropical Diseases between 2001-2009. The second study enrolled patients in high dependency rooms or the intensive care departments of Children's Hospital No. 1, Children's Hospital No. 2, Tien Giang Provincial Hospital, Dong Thap Provincial Hospital and Sa Dec Hospital between 2008-2010. The parent or guardian of each participant gave written informed consent to participate. The Scientific and Ethical Committees of each study site approved the study protocols, as did the Oxford University Tropical Research Ethical Committee.

The GWAS was performed on DNA samples (n=1039) from patients enrolled between 2001-2009 at the Hospital for Tropical Diseases and from patients (n=969) enrolled at the other five participating hospitals during 2008 only. The replication study was performed in patients (n=1737) enrolled between 2009-2010 at Children's Hospital No. 1, Children's Hospital No. 2, Tien Giang Provincial Hospital, Dong Thap Provincial Hospital and Sa Dec Hospital. All patients represented in the GWAS and replication phases had laboratory evidence of dengue as shown by RT-PCR detection of viral RNA in plasma collected at the time of enrolment and/or by serological detection of DEN-virus reactive IgM or IgG in single or paired plasma specimens.

Cord blood DNA samples

Blood from the cord of newborn infants was collected in one of two prospective studies. The first study was conducted at Hung Vuong Hospital, Ho Chi Minh City, between 2004-2006. The second study was conducted at Hung Vuong Hospital, Ho Chi Minh City and Dong

Thap Hospital, Dong Thap Province, between 2009-2010. All participants gave written informed consent to participate. The Scientific and Ethical Committees of each study site approved the study protocols, as did the Oxford University Tropical Research Ethical Committee. DNA was extracted from cord blood using Nucleon BACC Genomic DNA Extraction Kits (GE Healthcare, USA).

The use of population controls

The number of potentially misclassified cord blood controls in the GWAS and replication stages was estimated to be 11 (out of 2,018) in the GWAS stage, and 15 (out of 2,394) in the replication stage, based on the following three assumptions that- a) all individuals in a given birth cohort will experience two sequential infections by different serotypes during their lifetime,¹⁷ b) that only up to 25% of these infections are clinically apparent,¹⁸⁻²³ c) 2% of clinically apparent secondary infections develop DSS. These assumptions estimate a lifetime population risk of DSS to be 0.5%. This is consistent with estimates of the prevalence of DSS cases expected over the first 15 years in a given birth cohort under the assumption that the incidence of DSS is constant (DSS incidence in Southern Viet Nam in 2009 was 26.59/100,000; based on statistics obtained from the Dengue Control program, Ministry of Health Viet Nam, 2010). Under this assumption we would expect 0.4% of a birth cohort to experience DSS before the age of 15yrs.

Genotyping

Cases and controls were randomized on plates and were genotyped with the Illumina Human 660W Quad BeadChips following manufacturer instructions. This successful use of this chip has been previously documented²⁴. For the replication stage, 72 of the selected SNPs which were not on the broad MHC region were genotyped with the Sequenom MassArray (Sequenom, see URLs) primer extension iPLEX system. *MICB* rs3132468 and rs3134899 were genotyped using the ABI Taqman platform (Applied Biosystems, see URLs).

Statistical analysis

Stringent QC filters were applied to remove poorly performing SNPs and samples using tools implemented in PLINK version 1.7²⁵. The QC criteria were as follows: SNPs that had > 5% of missing genotypes, gross departure from Hardy-Weinberg equilibrium (test for HWE showing $P < 10^{-7}$) or were of minor allele frequency below 1% were excluded from downstream analysis. For sample QC, samples with an overall genotyping call rate of < 95% were excluded from analysis. The remaining samples were then subjected to biological relationship verification by using the principle of variability in allele sharing according to the degree of relationship. Identity-by-state (IBS) information was derived using PLINK.²⁵ For those pairs of individuals who showed evidence of cryptic relatedness (possibly either due to duplicated or biologically related samples), we removed the sample with the lower call rate before performing principal component (PC) analysis. PC analysis was undertaken to account for spurious associations resulting from ancestral differences of individual SNPs²⁶, and PC plots were performed using the R statistical program package (R-project, see URLs).

For both the GWAS and replication stages, analysis of association with DSS was carried out using a 1 degree of freedom (d.f.) score-based test. This test models for a trend-per-copy of the minor allele on disease risk and has been extensively described²⁷⁻²⁸. It has the best statistical power to detect association for complex traits across a wide range of alternative hypotheses, with the exception of those involving rare recessive variants²⁹. The threshold for significant independent replication was set at $P < 0.05$ in the combined replication data sets³⁰.

Meta-analysis was conducted using inverse variance weights for each cohort, which calculates an overall Z-statistic, corresponding *P*-value and accompanying odds ratios for each SNP analyzed³⁰⁻³¹. Genotyping clusters were directly visualized for the 85 SNPs exceeding $P < 10^{-4}$ and confirmed to be of good quality before inclusion for statistical analysis. Supplementary Figure 7 shows the cluster plots for *MICB* rs3132468 and *PLCE1* rs3740360, the two SNPs with showing $P < 10^{-7}$ in the GWAS stage. Analysis of linkage disequilibrium was performed using Haploview³².

Statistical findings after routine GWAS analysis

Analysis of genetic ancestry using principal components (PC) revealed no significant population substructure between the DSS cases and controls (Supplementary Figure 8). As the individual PCs were non-significant (Bonferroni corrected $P > 0.05$, Supplementary Table 3) when tested as continuous covariates using logistic regression, we did not adjust for them in subsequent association analysis.

Single SNP analysis was performed using logistic regression assuming additive genetic effects relating genotype dosage (scoring for 0, 1, or 2 copies of the minor allele) to DSS. A quantile-quantile plot of the single SNP analysis showed a clear excess of extreme *P*-values compared to the null distribution (Supplementary Figure 9). As this excess was observed against a background of minimal genome-wide inflation of test statistics ($\lambda_{gc} = 1.024$), it excludes the possibility of substantial population substructure and differential genotyping call rate between cases and controls as a reason for the excess. Instead this suggests that at least some of these extreme *P*-values ($P < 10^{-4}$) may represent true genetic associations with DSS;

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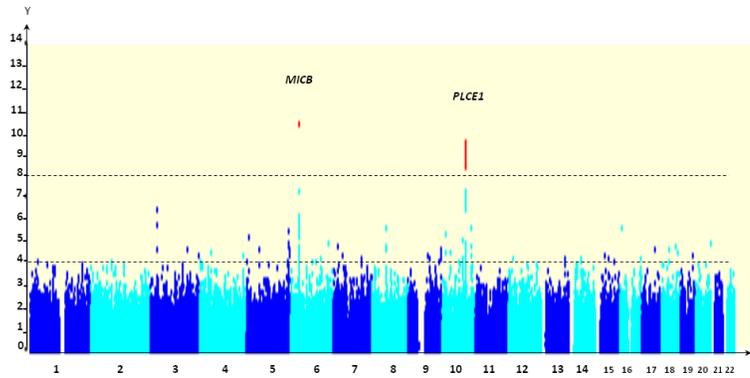


Figure 1.

Manhattan plot showing directly genotyped SNPs plotted according to chromosomal location (X-axis, with $-\text{Log}_{10}P$ -values (Y-axis) derived from the trend test. The lower horizontal dotted line indicates the threshold for bringing SNPs forward to the replication stage ($P < 10^{-4}$). SNPs surpassing $P < 10^{-8}$ (upper horizontal dotted line) on combined analysis of both GWAS and replication data are reflected by red dots, and gene names are given for these loci. SNPs in *MICB* and *PLCE1* have significant associations.

Table 1
Association analysis between Dengue shock syndrome and SNP genotypes at *MICB* and *PLCE1*.

Gene/Marker (Alleles)	Chromosome (Position)	Stage	MAF Cases	MAF Controls	OR	P	OR _{meta} (95% CI)	P _{meta}
<i>MICB</i> /rs132468 (C/T)	6 31583465	GWAS Replication	0.176 0.163	0.132 0.134	1.41 1.27	5.39×10^{-8} 9.32×10^{-5}	1.34 (1.23 - 1.46)	4.41×10^{-11}
<i>MICB</i> /rs134899 (G/A)	6 31581265	GWAS Replication	0.130 0.114	0.102 0.096	1.31 1.20	1.09×10^{-4} 0.0082	1.26 (1.14 - 1.38)	4.08×10^{-6}
<i>PLCE1</i> /rs37655 24 (T/C)	10 96048288	GWAS Replication	0.249 0.265	0.300 0.302	0.77 0.83	2.68×10^{-7} 1.60×10^{-4}	0.80 (0.75 - 0.86)	3.08×10^{-10}
<i>PLCE1</i> /rs22742 23 (G/A)	10 96056331	GWAS Replication	0.250 0.267	0.303 0.300	0.77 0.85	1.19×10^{-7} 5.23×10^{-4}	0.81 (0.75 - 0.86)	6.89×10^{-10}
<i>PLCE1</i> /rs37403 60 (C/A)	10 96015481	GWAS Replication	0.219 0.242	0.271 0.273	0.75 0.85	5.84×10^{-8} 0.0012	0.80 (0.75 - 0.86)	1.15×10^{-9}
<i>PLCE1</i> /rs12263 737 (A/G)	10 96034903	GWAS Replication	0.250 0.266	0.301 0.300	0.77 0.84	3.73×10^{-7} 3.95×10^{-4}	0.81 (0.75 - 0.87)	1.22×10^{-9}
<i>PLCE1</i> /rs11187 842 (T/C)	10 96042501	GWAS Replication	0.219 0.240	0.269 0.271	0.76 0.85	1.19×10^{-7} 0.0011	0.80 (0.75 - 0.86)	1.78×10^{-9}
<i>PLCE1</i> /rs75372 4 (T/G)	10 96041407	GWAS Replication	0.219 0.242	0.269 0.272	0.76 0.85	1.28×10^{-7} 0.0012	0.81 (0.75 - 0.86)	2.27×10^{-9}
<i>PLCE1</i> /rs37812 64 (G/A)	10 96060365	GWAS Replication	0.229 0.250	0.278 0.280	0.77 0.85	3.43×10^{-7} 0.0011	0.81 (0.76 - 0.87)	4.18×10^{-9}

MAF cases: Minor allele frequency in DSS cases

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MAF controls: Minor allele frequency in the controls

OR: Odds of DSS per-copy of the minor allele

P : P -value using the 1 degree of freedom score-test.

OR_{meta}: Odds ratio for the combined GWAS and replication cohorts.

P_{meta} : P -value for the combined GWAS and replication cohorts.

95% CI: 95% confidence interval for the OR.

P_{het} : Heterogeneity P -value.

GWAS: Sample size of 2,008 DSS cases and 2,018 cord blood controls.

Replication: Sample size of 1,737 DSS cases and 2,934 cord blood controls.