Genetic Variants of MICB and PLCE1 and Associations with Non-Severe Dengue

James Whitehorn1,2,5, Tran Nguyen Bich Chau1,2, Nguyen Minh Nguyet2, Duong Thi Hue Kien2, Nguyen Than Ha Quyen2, Dinh The Trung2, Junxiong Pang3,4, Bridget Wills2,5, Nguyen Van Vinh Chau6, Jeremy Farrar2,5, Martin L. Hibberd3,4, Chiea Chuen Khor3,4,7,8, Cameron P. Simmons2,5,9

1 Department of Clinical Research, London School of Hygiene and Tropical Medicine, London, United Kingdom, 2 Oxford University Clinical Research Unit, Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam, 3 Genome Institute of Singapore, Singapore, Singapore, 4 School of Public Health, National University of Singapore, Singapore, Singapore, 5 Centre for Tropical Medicine, University of Oxford, Oxford, United Kingdom, 6 Hospital for Tropical Diseases, Ho Chi Minh City, Viet Nam, 7 Department of Paediatrics, National University of Singapore, Singapore, Singapore, 8 Department of Ophthalmology, National University of Singapore, Singapore, Singapore

Abstract

Background: A recent genome-wide association study (GWAS) identified susceptibility loci for dengue shock syndrome (DSS) at MICB rs3132468 and PLCE1 rs3740360. The aim of this study was to define the extent to which MICB (rs3132468) and PLCE1 (rs3740360) were associated with less severe clinical phenotypes of pediatric and adult dengue.

Methods: 3961 laboratory-confirmed dengue cases and 5968 controls were genotyped at MICB rs3132468 and PLCE1 rs3740360. Per-allele odds ratios (OR) with 95% confidence intervals (CI) were calculated for each patient cohort. Pooled analyses were performed for adults and paediatrics respectively using a fixed effects model.

Results: Pooled analysis of the paediatric and adult cohorts indicated a significant association between MICB rs3132468 and dengue cases without shock (OR = 1.15; 95%CI: 1.07 – 1.24; P = 0.0012). Similarly, pooled analysis of pediatric and adult cohorts indicated a significant association between dengue cases without shock and PLCE1 rs3740360 (OR = 0.75; 95%CI: 0.65 – 0.85; P = 0.018). We also note significant association between both SNPs (OR = 1.48; P = 0.0075 for MICB rs3132468 and OR = 0.75, P = 0.041 for PLCE1 rs3740360) and dengue in infants.

Discussion: This study confirms that the MICB rs3132468 and PLCE1 rs3740360 risk genotypes are not only associated with DSS, but are also associated with less severe clinical phenotypes of dengue, as well as with dengue in infants. These findings have implications for our understanding of dengue pathogenesis.

Introduction

Dengue is the most important arboviral disease of humans.[1] Dengue viruses (DENV) cause a spectrum of clinical manifestations ranging from asymptomatic infection through to life-threatening shock and haemorrhage.[1,2] The clinical outcome of an individual infection is influenced by a variety of virus and host-related factors. The host factors that influence the clinical course of an individual infection include flavivirus infection history, host genotype, sex, age, and the presence of underlying medical conditions.[3-5] The first GWAS in dengue identified susceptibility loci for dengue shock syndrome (DSS) at MHC class I polypeptide-related sequence B (MICB) (C/T, rs3132468) on chromosome 6 and phospholipase C, epsilon 1 (PLCE1) (C/A, rs3740360) on chromosome 10.[6] The MICB gene encodes an activating ligand of natural killer (NK) cells (and possibly CD8+ T cells). We have previously speculated that mutations in MICB might result in impaired induction of anti-viral effector functions in NK cells with the consequence being a greater DENV-infected cell mass in vivo [6], a recognised risk factor for severe dengue.[7]

The identification of variants of PLCE1 as being associated with severe dengue is intriguing.[6] Rare mutations of high penetrance within PLCE1 are associated with nephrotic syndrome, a condition characterised by oedema secondary to proteinuria and reduced vascular oncocytic pressure.[8] Since plasma leak, proteinuria and hypovolemia are also characteristic features of severe dengue, it’s plausible that nephrotic syndrome and severe dengue share some common underlying pathophysiological processes. Furthermore,there are data implicating PLCE1 in the homeostatic regulation of blood pressure.[9] These findings have the potential to help us define more clearly the functional basis of PLCE1 variants in severe dengue.

The SNP associations identified at MICB (rs3132468) and PLCE1 (rs3740360) by the GWAS study were in the context of
pediatric patients with DSS, leaving unanswered the question whether they are also associated with less severe clinical phenotypes of dengue. To this end, the aim of this study was to define the extent to which these alleles were associated with milder clinical phenotypes of pediatric and adult dengue. We analyzed a total of 3961 laboratory-confirmed dengue cases, independent from the initial GWAS study, and 5968 cord blood controls.[6]

Materials and Methods

Ethics statement

All participants gave written informed consent to participate in the prospective studies summarised in Table 1 (details of the inclusion and exclusion criteria are available in Supplementary Table 1). Parents or guardians provided written informed consent on behalf of the children involved in the studies. The protocols for these studies were approved by the Institutional Review Boards of each study site (Hospital for Tropical Diseases HCMC, Children’s Hospital 1 and 2 HCMC, Hung Vuong Hospital HCMC, Dong Thap Hospital, Sa Dec Hospital and Tien Giang Hospital) and by the Oxford University Tropical Research Ethics Committee. Each ethical committee approved of the consent procedure detailed above.

Enrolment and diagnosis

Blood samples for genotyping were collected in one of several prospective studies of dengue in Vietnamese patients detailed in Table 1. Dengue cases were laboratory-confirmed by one of three methods: IgM-seroconversion by ELISA assay on paired samples, detection of DENV RNA by RT-PCR, or detection of non-structural protein 1 (NS1) by ELISA (BioRad Platelia). The control samples used in this study were from blood samples collected from the umbilical cord of newborn infants enrolled into the birth cohort study detailed in Table 1. Within each cohort, dengue cases were classified in a binary fashion as being “DSS” or “not-DSS”.[6] Consistent with the original GWAS study, DSS cases were defined as laboratory-confirmed dengue cases with cardiovascular decompensation secondary to plasma leakage and requiring fluid resuscitation.[2]

Genotyping

DNA extractions were performed using a MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Germany) according to the manufacturer’s instructions. Candidate SNPs were genotyped using a TaqMan® genotyping assay to amplify and detect the specific alleles in the DNA samples per manufacturer instructions.

Statistical analysis

The data were analysed using PLINK version 1.07 and the R statistical software package version 2.12.0 (2010 The R Foundation for Statistical Computing). For each cohort study per-allele odds ratios with 95% confidence intervals were calculated to assess the relationship between SNP genotypes (rs3132468 at MICB and rs3740360 at PLCE1) and susceptibility to dengue. For the analysis we considered non-DSS cases in children separately from adults and considered infants with dengue as a distinct group. Infants were defined as less than 1 year in age, children were defined by ages between 1 and 15 years, and adults were defined by being 16 years and older. Other variables known to be associated with disease severity were not included in this analysis, consistent with the GWAS primary analysis for DSS as well as other infectious diseases.[6,10–12] Pooled odds ratios across the different sample collections were calculated using the inverse-variance, fixed effects model, as previously described.[13] This model used the weighted average of each study’s odds ratio. The weights used were the inverse of the variance of the study’s estimated odds ratio, ensuring

Table 1. Summary of the cohort studies used in the analysis.

| Group            | Number (% male) | Median age (range 5th – 95th) |
|------------------|-----------------|-------------------------------|
| Control          | 1068 (52)       | At birth                      |
| Infants          | 165 (55)        | 7 months (3 – 12 months)      |
| DC               | 59 (52)         | 6 (3 – 11)                    |
| DT               | 88 (57)         | 7 (4 – 11)                    |
| DZ               | 8 (50)          | 8 (7 – 11)                    |
| FB               | 9 (55)          | 8 (7 – 15)                    |
| Children/Young adults | 2759 (56)   | 12 years (6 – 16 years)       |
| FG               | 576 (56)        | 11 (4 – 22)                   |
| 06DX             | 220 (71)        | 13 (11 – 14)                  |
| DR               | 582 (56)        | 11 (6 – 15)                   |
| MD               | 1381 (60)       | 12 (7 – 14)                   |
| Adults           | 741 (46)        | 22 (15 – 35)                  |
| 09DX             | 159 (41)        | 23 (19 – 27)                  |
| D001             | 54 (43)         | 20 (14 – 25)                  |
| FL               | 528 (48)        | 22 (15 – 35)                  |

Figure 1. Genotyping and sample quality control process flowchart.

doi:10.1371/journal.pone.0059067.t001
that larger studies command greater weight. Forest plots demonstrating these results were created in R.

Results

Case and control cohorts

The patient cohorts that provided the laboratory-confirmed dengue cases that were genotyped in this study are summarised in Table 1. Across the 7 cohorts (4 pediatric and 3 adult) there were 297 DSS cases (161 pediatric and 136 adult), 3500 non-DSS dengue cases (2759 pediatric and 741 adult cases), and 164 cases of infants with dengue that were successfully genotyped at MICB rs3132468 and PLCE1 rs3740360. Umbilical cord blood DNA samples (n = 1068) were similarly genotyped at MICB rs3132468 and PLCE1 rs3740360 and served as population controls. Data from this was complemented with genotype data from 4900 controls studied previously.[6] In a priori analyses we considered non-DSS cases in children separately from adults. Infants with dengue (n = 164) were also treated as a distinct cohort because of the unique context of dengue in this age group. The sample quality control process is illustrated in Figure 1.

Association between MICB rs3132468 and dengue in children and adults

Pooled analysis of genotype data from the pediatric patient cohorts revealed that non-DSS dengue cases were significantly more likely to carry the MICB risk allele rs3132468 than controls (per-allele odds ratio (OR) = 1.16; 95%CI: 1.07 – 1.25). Pooled analysis of cohorts of adult non-DSS dengue cases revealed a similar pattern of effect with the MICB risk allele, but this did not reach statistical significance (OR = 1.10, \(P = 0.11\); Figure 2, and Table 2). However, a pooled analysis of all pediatric and adult patient cohorts indicated a significant association (OR = 1.15, \(P = 0.0014\); Figure 2, and Table 2) compared to controls, with no heterogeneity between children and adults (\(P_{het} = 0.19\)). In the relatively small number of adult and pediatric DSS cases (N = 297), we were able to confirm the association and effect size reported in our previously reported GWAS (OR = 1.42, \(P = 0.0014\); Figure 2 and Table 2).

Association between PLCE1 rs3740360 and dengue in children and adults

We observed significant association with non-DSS cases upon pooled analysis of all adults and children (OR = 0.92, \(P = 0.018\); Figure 3 and Table 3). Amongst DSS cases, the association at PLCE1 revealed by the previous GWAS was confirmed (OR = 0.77; \(P = 0.0094\)).

Figure 2. Forest plot illustrating the association between MICB rs3132468 and susceptibility to dengue. 06DX, DR, FG and MD were cohort studies of children, and 09DX, D001 and FL were cohort studies of adults. The oblongs represent point estimates (referring to the per-allele odds ratio, expressed on the horizontal axis), with the height of the oblongs inversely proportional to the standard error of the point estimates. Horizontal lines indicate the 95% confidence interval for each point estimate. Meta-analysis of children, adults, as well as children and adults with uncomplicated dengue are reflected by blue diamonds. Data from our previously reported GWAS on DSS is reflected by the purple diamond.6 The width of the diamonds indicates their 95% confidence intervals. Each meta-analysis is accompanied by a test of heterogeneity between the sample collections summarized by it (expressed as \(P_{het}\)).

doi:10.1371/journal.pone.0059067.g002

Uncomplicated Dengue

Infants and DSS

Figure 1. Sample quality control process in the present study.
Association between MICB rs3132468 and dengue in infants

Since each of the infant cohorts was relatively small in their own right, a meta-analysis was performed. Consistent with the findings in older children, this pooled analysis revealed a significant association between dengue in infants and MICB rs3132468 (OR = 1.48; P = 0.0075), as well as PLCE1 rs3740360 (OR = 0.75, P = 0.041) (Figures 2 and 3; Tables 2 and 3). Although the infant cohorts included 16 cases of DSS, removal of these samples did not affect the associations demonstrated.

Discussion

MICB rs3132468 and PLCE1 rs3740360 genotypes are associated with DSS in Vietnamese children.[6] Here, we have extended this finding by follow-up genotyping in a large number of Vietnamese adult and paediatric dengue cases, together with a new cohort of population control samples. The data revealed significant association between MICB rs3132468 and PLCE1 rs3740360 and clinical phenotypes of dengue less severe than DSS, albeit with smaller effect sizes than observed between these alleles and DSS.[6] In addition, we observed association between both SNP genotypes and infants with dengue at effect sizes comparable to that seen with DSS. Finally, amongst children and adults with DSS in these cohorts, we were able to confirm association of MICB rs3132468 and PLCE1 rs3740360 that was first observed in the previous GWAS.[6] Collectively, these findings provide further validation of the importance of MICB rs3132468 and PLCE1 rs3740360 risk genotypes to shaping the clinical phenotype of dengue and raises intriguing questions about their roles in disease pathogenesis.

The association of the MICB rs3132468 genotype with clinical phenotypes of dengue less severe than DSS indicates a role for this variant in susceptibility to overall clinically apparent dengue and not just severe disease. Given the role of MICB in activation of NK and CD8+ cells these findings support a central role for these cell types in shaping the outcome of DENV infection. For example, it is plausible that the MICB rs3132468 genotype is associated with an impaired NK cell response, potentially resulting in a higher 

in vivo virus titre and an increased risk of developing both symptomatic and severe dengue. Furthermore, inefficient induction of regulatory NK cells might result in dysregulated T cell responses that may also shape the clinical phenotype.[14]

The effect size between the PLCE1 rs3740360 risk genotype and milder clinical phenotypes of dengue was less pronounced than that observed between these alleles and DSS.[6] Whilst a degree of endothelial leak is likely to occur in most clinically evident DENV infections, patients with DSS experience the most severe vascular permeability.[15,16] Our current data, together with the recently demonstrated association between PLCE1 rs3740360 and DSS, may indicate a central role for PLCE1 in the context of vascular leakage.[6] Further genetic fine mapping studies will be required to pinpoint functional mutations that could mechanistically explain the association between PLCE1 and DSS. In doing so,
we expect to contribute to the wider understanding of the role of PLCE1 in health and disease, particularly in light of its association with nephrotic syndrome, regulation of blood pressure and esophageal cancer.[8,9,17] In light of the observed association with PLCE1 and nephrotic syndrome it is interesting that the degree of proteinuria has been proposed as a potential predictor in determining which dengue patients are at risk of developing more severe disease.[18]

Infants with dengue were analysed independently of other patient populations. Primary infection in the context of waning maternal antibody levels, immunological immaturity and vulnerable physiology make infants with dengue a distinct group.[19] Our data shows association between MICB rs3132468 and dengue in infants, with effect sizes in keeping with that observed in DSS patients.[6] We speculate that this reflects a prominent role for innate immunity and particularly NK cells in controlling early viral infection in infants; impaired control of viral replication could be a risk factor for clinically apparent dengue in this age group. The effect size observed at PLCE1 rs3740360 was also similar to that observed in DSS patients.[6] It has been noted that hospitalised infants with dengue represent a group with the highest risk of death, and it is thought that this is partly related to an intrinsically more permeable vascular endothelium in this age group.[4] In infants with dengue, carriage of either risk alleles thus represent an additional risk variable alongside the presence of maternally-derived non-neutralising antibodies and poor compensatory reserve.[19–20]

Our study has limitations. Misclassified control samples will be more common in this study than in the original GWAS of DSS cases because dengue without shock is a more common clinical outcome for a given cohort of children in an endemic area. Reassuringly, the fact that consistent associations were observed despite this limitation lends credence to our observations. In addition, as the functional basis of these mutations as yet to be clearly defined our conclusions are to an extent speculative. As dengue without shock includes a diverse range of clinical manifestations our ability to determine this functional basis is more limited.

We have shown that the MICB rs3132468 and PLCE1 rs3740360 genotypes are associated with clinically apparent dengue in both adults and children, which is a significant extension from the earlier GWAS on DSS cases alone. As expected, the effect sizes of these variants is small and underscores that susceptibility to symptomatic dengue is multifactorial and includes demographic risk factors (e.g. age).[21] However, we have not performed multivariate analysis in this study as the majority of risk factors for symptomatic (non-severe) dengue are not clearly defined. The challenge now is to define the functional basis for these observed genetic associations at MICB and PLCE1 and thus increase our understanding of disease pathogenesis.

Figure 3. Forest plot illustrating the association between PLCE1 rs3740360 and susceptibility to dengue. 06DX, DR, FG and MD were cohort studies of children, and 09DX, D001 and FL were cohort studies of adults. The oblongs represent point estimates (referring to the per-allele odds ratio, expressed on the horizontal axis), with the height of the oblongs inversely proportional to the standard error of the point estimates. Horizontal lines indicate the 95% confidence interval for each point estimate. Meta-analysis of children, adults, as well as children and adults with uncomplicated dengue are reflected by blue diamonds. Data from our previously reported GWAS on DSS is reflected by the purple diamond.[6] The width of the diamonds indicates their 95% confidence intervals. Each meta-analysis is accompanied by a test of heterogeneity between the sample collections summarized by it (expressed as $P_{het}$).
doi:10.1371/journal.pone.0059067.g003
Table 3. Per-collection analysis for PLCE1 rs3740360.

| Collection | Number | MAF cases | MAF controls | OR  | 95% CI | Weight | P     |
|------------|--------|-----------|--------------|-----|--------|--------|-------|
| 06DX       | 220    | 0.235     | 0.271        | 0.83| 0.59 - 1.07 | 66.09822 | 0.12  |
| OR         | 582    | 0.244     | 0.271        | 0.87| 0.73 - 1.01 | 190.7756 | 0.046 |
| FG         | 576    | 0.248     | 0.271        | 0.88| 0.74 - 1.02 | 189.726  | 0.089 |
| MD         | 1381   | 0.270     | 0.271        | 0.99| 0.90 - 1.08 | 426.8834 | 0.90  |
| All children| 2759  | 0.257     | 0.271        | 0.93| 0.86 - 1.00 | 0.054   |       |

**Adults**

| Collection | Number | MAF cases | MAF controls | OR  | 95% CI | Weight | P     |
|------------|--------|-----------|--------------|-----|--------|--------|-------|
| 09DX2      | 159    | 0.272     | 0.271        | 1.01| 0.75 - 1.27 | 56.10964 | 0.97  |
| 0001       | 54     | 0.173     | 0.271        | 0.56| 0.05 - 1.07 | 14.7929  | 0.025 |
| FL         | 528    | 0.249     | 0.271        | 0.89| 0.74 - 1.04 | 171.3221 | 0.13  |
| All adults | 741    | 0.248     | 0.271        | 0.89| 0.76 - 1.02 | 0.065   |       |
| All mild cases (Adults and children) | 3500 | 0.255     | 0.271        | 0.92| 0.85 - 0.99 | 0.018   |       |

**Collection**

| Number | MAF cases | MAF controls | OR  | 95% CI | Weight | P     |
|--------|-----------|--------------|-----|--------|--------|-------|
| All infants | 164 | 0.219     | 0.271        | 0.75| 0.48 - 1.02 | 0.041   |       |
| All children | 2759 | 0.223     | 0.271        | 0.77| 0.59 - 0.99 | 0.0094 |       |

Number: Number of cases
MAF cases: minor allele frequency in dengue cases
MAF controls: minor allele frequency in controls
OR: per-allele odds ratio.
P: P-value for association with disease (unadjusted).

Author Contributions

Critically reviewed and approved the manuscript: JW TNBC NMN DTHK NTHQ DTT JP BW NVVC JF MLH CCK CPS. Conceived and designed the experiments: JW TNBC DTT JP BW NVVC JF MLH CCK CPS. Performed the experiments: JW TNBC NMN DTHK NTHQ DTT JP CCK. Analyzed the data: JW TNBC JP MLH CCK CPS. Wrote the paper: JW TNBC CCK CPS.

Supporting Information

Table S1 Details of the cohort studies used in the analysis.

(DOCX)

References

1. Simmons CP, Farrar JJ, Nguyen v V, Wills B (2012) Dengue. N Engl J Med 366: 1423–1432.
2. WHO (2009) Dengue: guidelines for diagnosis, treatment, prevention and control - New edition. Geneva: World Health Organisation.
3. Nguyen TP, Kikuchi M, Vu TQ, Do QH, Tran TT, et al. (2008) Protective and enhancing HLA alleles, HLA-DRB1*0901 and HLA-A*24, for severe forms of dengue virus infection, dengue hemorrhagic fever and dengue shock syndrome. PLoS Negl Trop Dis 2: e304.
4. Anders KL, Nguyen NM, Chau NV, Hung NT, Thuy TT, et al. (2011) Epidemiological factors associated with dengue shock syndrome and mortality in hospitalized dengue patients in Ho Chi Minh City, Vietnam. Am J Trop Med Hyg 84: 127–134.
5. Lye DC, Lee VJ, Sun Y, Leo YS (2010) The benign nature of acute dengue infection in hospitalized older adults in Singapore. Int J Infect Dis 14: e110–413.
6. Khor CC, Chau TN, Pang J, Davila S, Long HT, et al. (2011) Genome-wide association study identifies susceptibility loci for dengue shock syndrome at MICB and PLCE1. Nat Genet.
7. Khor CC, Ramdas WD, Vithana EN, Cornes BK, Sim X, et al. (2011) Genome-wide association studies in Asians confirm the involvement of ATOH7 and TGFB3, and further identify CARD10 as a novel locus influencing optic disc area. Hum Mol Genet 20: 1064–1072.
8. Zhang J, Liu H, Chen S, Low H, Sun L, et al. (2011) Identification of two new loci at IL23R and RAB32 that influence susceptibility to leprosy. Nat Genet 43: 1247–1251.
9. Davila S, Weight VJ, Khor CC, Sim KS, Binder A, et al. (2010) Genome-wide association study identifies variants in the CFH region associated with host susceptibility to meningococcal disease. Nat Genet 42: 772–776.
10. Wongsirichat S, Malhotra D, Petersson FH, Teo YY, et al. (2010) Leprosy and the adaptation of human toll-like receptor 1. PLoS Pathog 6: e1000979.
11. Khor CC, Ramdas WD, Vithana EN, Cornes BK, Sim X, et al. (2011) Genome-wide association studies in Asians confirm the involvement of ATOH7 and TGFB3, and further identify CARD10 as a novel locus influencing optic disc area. Hum Mol Genet 20: 1064–1072.
12. Lang PA, Lang KS, Xu HC, Grusdat M, Parish IA, et al. (2012) Natural killer cell activation enhances immune pathology and promotes chronic infection by limiting CD8+ T-cell immunity. Proc Natl Acad Sci U S A 109: 1210–1215.
13. Colbert JA, Gordon A, Roxelin R, Silva S, Silva J, et al. (2007) Ultrasound measurement of gallbladder wall thickening as a diagnostic test and prognostic indicator for severe dengue in pediatric patients. Pediatr Infect Dis J 26: 850–852.
14. Trung DT, Wills B (2010) Systemic vascular leakage associated with dengue infections - the clinical perspective. Curr Top Microbiol Immunol 338: 57-66.
15. Abnet CC, Freedman ND, Hu N, Wang Z, Yu K, et al. (2010) A shared susceptibility locus in PLCE1 at 10q23 for gastric adenocarcinoma and esophageal squamous cell carcinoma. Nat Genet 42: 764–767.
16. Vasina R, Pavunandran R, Foek-Choong S, Ng JM, Suhail SM, et al. (2011) Could peak proteinuria determine whether patient with dengue fever develop...
dengue hemorrhagic/dengue shock syndrome—a prospective cohort study. BMC Infect Dis 11: 212.

19. Chau T, ANders K, Lien IB, Hung N, Hieu L, et al. (2010) Clinical and virological features of Dengue in Vietnamese infants. PLoS Negl Trop Dis 4: e657.

20. Kliks SC, Nimmanitya S, Nisalak A, Burke DS (1988) Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg 38: 411–419.

21. Egger JR, Ooi EE, Kelly DW, Woolhouse ME, Davies CR, et al. (2006) Reconstructing historical changes in the force of infection of dengue fever in Singapore: implications for surveillance and control. Bull World Health Organ 86: 187–196.