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Complement alternative pathway genetic variation and Dengue infection in the Thai population

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Summary

Dengue disease is a mosquito-borne infection caused by Dengue virus. Infection may be asymptomatic or variably manifest as mild Dengue fever (DF) to the most severe form, Dengue haemorrhagic fever (DHF). Mechanisms that influence disease severity are not understood. Complement, an integral component of the immune system, is activated during Dengue infection and the degree of activation increases with disease severity. Activation of the complement alternative pathway is influenced by polymorphisms within activation (factor B rs12614/rs641153, C3 rs2230199) and regulatory [complement factor H (CFH) rs800292] proteins, collectively termed a complotype. Here, we tested the hypothesis that the complotype influences disease severity during secondary Dengue infection. In addition to the complotype, we also assessed two other disease-associated CFH polymorphisms (rs1061170, rs3753394) and a structural polymorphism within the CFH protein family. We did not detect any significant association between the examined polymorphisms and Dengue infection severity in the Thai population. However, the minor allele frequencies of the factor B and C3 polymorphisms were less than 10%, so our study was not sufficiently powered to detect an association at these loci. We were also unable to detect a direct interaction between CFH and Dengue NS1 using both recombinant NS1 and DV2-infected culture supernatants. We conclude that the complotype does not influence secondary Dengue infection severity in the Thai population.

Keywords: alternative complement pathway, Dengue infection, genetic polymorphism

Introduction

Dengue disease, a mosquito-borne infection, is a public health concern in many subtropical and tropical countries. The causative agent is Dengue virus (DV), a positive-sense single-stranded RNA virus and a member of the *Flaviviridae* family. This virus can be classified into four serotypes (DV 1–4) whose genomes are closely related but distinct. The genome expresses three structural proteins (Capsid, PrM and Envelop protein), and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5). DV infection may be asymptomatic or manifest as undifferentiated fever, Dengue fever (DF) or Dengue haemorrhagic fever (DHF) with plasma leakage. DHF can be classified on severity into four subtypes (DHF 1–4). The most severe disease, which has been termed Dengue shock syndrome (DSS), comprises a syndrome of plasma leakage with shock and severe bleeding [1]. Both the host immune response and the DV strain contribute to the development of DHF [2–4]. Non-neutralizing antibodies enhance infectivity in an Fc-dependent manner [5]. A number of host susceptibility genes have been identified and include genes encoding: Fc gamma receptor II (*FcγRIIA*), human leucocyte antigens (*HLA-A1*, and *HLA-A2*), the vitamin D receptor, tumour necrosis factor (TNF)-α and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) [6–8].

The complement system is an integral component of host defence and appears to influence the host response to flavivirus infection in murine models. Mice lacking complement components [C3, C1q, C4 or factor B (FB)] were more susceptible to West Nile virus (WNV) infection [9]. Activation of complement increased antibody-mediated neutralization of flavivirus, as C1q reduced the antibody-

dependent enhancement of both WNV and DV infection [10]. Complement activation occurs during the course of DV infection. An increase in C3 activation, together with reduced levels of C4 and FB, has been shown in DHF [11]. C3 levels were reduced while the levels of C3a and C5a (anaphylatoxins) were increased in DHF compared to DF [12]. C5b-9 complexes, along with high levels of DV NS1 protein, correlated with DV infection severity [13].

The molecular mechanisms through which complement could influence flavivirus infection are incompletely understood. DV NS1 can bind complement C4, a classical and lectin pathway activation protein and trigger C4 degradation [14]. WNV NS1 binds the complement regulatory protein, complement factor H (CFH), an interaction that prevented the lysis of WNV-infected cells [15]. In contrast, Japanese encephalitis virus (JEV) NS1 did not bind CFH and did not modulate the host innate immune response [16].

Recently, it has been shown that polymorphisms (collectively termed 'complotype') in complement proteins C3, FB and CFH can be associated with an up to sixfold variation in complement haemolytic activity *in vitro* [17]. C3 and FB are activation proteins of the complement alternative pathway, while CFH is the major regulator of this pathway. Therefore, the description of the complotype demonstrated that alternative pathway activation is influenced by genetic factors. These polymorphisms have been associated with susceptibility to age-related macular degeneration (AMD [18–20]), suggesting that the degree of haemolytic activation driven by these polymorphisms *in vitro* is biologically relevant. Individuals with the 'high activation' complotype have increased susceptibility to AMD [FB-32 R/R: rs12614 (C) and rs641153 (G); CFH-62 V/V: rs800292 (G); and C3-102 F/F: rs2230199 (G)], whereas risk is reduced in those with the 'low activation' complotypes [FB-32 Q/Q rs12614 (C) and rs641153 (A); CFH-62 I/I: rs800292 (A); and C3-102 S/S: rs2230199 (C)]. Consistent with these data was the finding that serum complement activation appears to be enhanced in AMD [21].

In addition, genetic variation across the *CFH–CFHR* locus (the region encoding CFH and CFH-related proteins) is linked to AMD susceptibility. This variation includes a polymorphism within CFH (CFH-402 Y/H, rs1061170, [22]) and a copy number polymorphism that results in deletion of the *CFHR1* and *CFHR3* genes (Δ*CFHR3-1*, [23]). Recently, CFH promoter (–332 C/T, rs3753394) and CFH (V62I, rs800292) polymorphisms have been associated with Dengue disease [24]. The functional consequences of these polymorphisms are not understood.

Although studies have implicated a role for complement in DV infection severity, only one study to date has explored the relationship between complement genetic variation and DV infection [24]. In this study we specifically analysed the relationship between DV infection severity and the complotype (CFH rs800292, FB rs12614/rs641153 and C3 rs2230199), as this variation has been associated with differences in complement activation. We also included the AMD-associated CFH rs1061170 variant, the CFH promoter rs3753394 variant and the Δ*CFHR3*-1 deletion polymorphism. We did not find any strong correlation between DV infection severity and these specific polymorphisms. Our data demonstrate that variation in these polymorphisms is not a major host factor for determining the phenotypic response to this infection.

Materials and methods

Patients and controls

Genomic DNA samples were extracted from patients attending either Khon-Kean or Song-Khla Hospitals, Thailand. Viral diagnosis was established using reverse transcription–polymerase chain reaction (RT–PCR) and severity classified using World Health Organization (WHO) guidelines: DF and DHF grades 1–4. Dengue immunoglobulin (Ig)G/IgM capture enzyme-linked immunosorbent assay (ELISA) was used to classify primary and secondary infection. An IgM : IgG ratio equal to or greater than 1·8 was used to define primary infection [25]. Patients with other febrile illnesses (OFI) ($n = 63$) were those presenting with fever and having undetectable Dengue viral genome and levels of anti-Dengue antibodies lower than the cut-off value for an acute Dengue infection. Healthy volunteers were recruited from Siriraj Hospital, Thailand. Written informed consent was obtained and sample collection approved by the Institutional review board.

Genotyping

Genomic DNA was amplified by PCR using high-fidelity polymerase (Certamp; Biotools, Madrid, Spain) and primers listed in Table S1. Following purification (ChargeSwitch PCR clean-up kit; Invitrogen, Carlsbad, CA, USA) amplicons were sequenced and single nucleotide polymorphism (SNP) genotype-recorded. The CFH rs800292 variant was determined using the *Taq*man SNP genotyping assay (no. 4351379; Applied Bioscience, Island, NY, USA). Δ*CFHR3-1* copy number was detected using a combination of (i) *Taq*man copy number assay probes (Applied Bioscience), (ii) genotyping of rs6677604 (a SNP within intron 12 of the *CFH* gene that has been shown to tag the Δ*CFHR3*-1 deletion) [26] and (iii) multiplex ligation-dependent probe amplification assay (MLPA; MRC Holland, the Netherlands). MLPA was performed according to the manufacturer's instructions. PCR products were mixed with GeneScan 500 ROX Size Standard (Applied Bioscience) in Hi-Di formamide (Applied Bioscience) and separated using capillary electrophoresis (3730xl DNA Analyser; Applied Biosystems, Grand Island, NY, USA).

Dengue virus infection *in vitro*

C6/36 cell lines were cultured in 10% fetal bovine serum (FBS), 10% tryptose-phosphate-broth (TPB) Leibovitz L-15 with streptomycin and penicillin at 28°C. Vero cell lines were maintained with modified Eagle's medium (MEM) supplemented with 10% FBS at 37°C in a CO_2 incubator. Dengue virus serotype 2 strain 16681 (DV2) was propagated in C6/36 cells. Infected cells were incubated further at 28°C until a cytopathic effect (CPE) could be seen. Mock was used as a negative control. To titrate the virus in the supernatant, Vero cells were plated on each well in a 96-well plate and incubated for 2 days in a CO_2 incubator. On the day of infection, the supernatant were serially diluted 10-fold with 3% MEM and added to the cells for 2 h at 37°C. The infected cells were then overlaid with 1·5% carboxymethylcellulose and incubated further for 2 days. Infected foci were observed by immunoperoxidase staining. Briefly, the infected cells were washed with PBS, fixed and permeabilized with 3·7% formaldehyde and 2% Triton-X 100, respectively. Monoclonal antibody against Dengue E protein (4G2) and rabbit anti-mouse-horseradish peroxidase (HRP) diluted in 1/1000 (Dako, Glostrup, Denmark) were added sequentially before adding 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, St Louis, MO, USA). The number of foci were counted and reported as foci-forming units per ml.

CFH and NS1 interaction assays

To determine if CFH interacted with NS1 we utilized ELISA, far Western and immunopreciptiation techniques.

For ELISA, 5 μg/ml human CFH (Quidel, San Diego, CA, USA) in carbonate buffer was coated on a 96-well MaxiSorp plate (Nunc, Roskilde, Denmark) at 4°C overnight. Either mock or DV2 supernatant was then added to wells and incubated at 37°C for 1 h. NS1 was detected by adding 5 μg/ml monoclonal mouse anti-NS1 antibody (1F11) followed by alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma-Aldrich) and substrate (0·001 mg/ml p-nitrophenylphosphate; Bio-Rad, Hercules, CA, USA). The reciprocal approach comprised coating of plates with 10 μg/ml 1F11 followed by sequential addition of supernatants and 5 μg/ml CFH. CFH was detected using goat anti-human CFH (Quidel) followed by HRP-conjugated mouse anti-goat antibody (Sigma-Aldrich) and substrate (3,3′,5,5′-tetramethylbenzidine).

For far Western blotting, mock and DV2 supernatant was separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Following membrane transfer, membranes were incubated with 5 μg/ml CFH. Following washing, either goat anti-human factor CFH (Quidel) or 1F11 was used to detect CFH and NS1, respectively. CFH (0·5 μg) was also applied to non-reducing gels, and following transfer, membrane-incubated with either mock or DV2 supernatant. Following washing, either goat anti-human factor CFH (Quidel) or 1F11 was used to detect CFH and NS1, respectively. Recombinant NS1 (rNS1; Abcam, Cambridge, UK) was also tested for interaction with CFH.

For co-immunoprecipitation, mock or DV2 supernatant was incubated with 5 μg/ml CFH for 3 h at 4°C followed by 1F11 for 1 h. Protein A (Roche, San Francisco, CA, USA) was then added and the mixture was rotated overnight. After centrifuging, beads were washed three times using 0·1% PBS-T, bound protein eluted using non-reducing loading buffer and eluate analysed by SDS-PAGE. NS1 and CFH were detected using 1F11 and goat anti-human CFH antibody, respectively (Quidel).

Statistical analyses

The analyses were performed on SPSS version 19·0 (SPSS, Inc., Chicago, IL, USA). Hardy–Weinberg equilibrium and association tests were carried out by using the χ^2 test. Odds ratio (OR) was the odds of the genotype comparing either DHF to DF or DHF with shock to DHF without shock. Logistic regression model was used to adjust OR with sex and age. Bonferroni's correction was performed by dividing α-level with the total number of polymorphisms compared ($n = 6$) to obtain an adjusted P-value of $0{\cdot}05/6 = 0{\cdot}008$.

Results

Study population

The study population demographics are depicted in Table 1. The age of patients with either Dengue infection or non-Dengue febrile infection was 2–15 years. The range for healthy controls was higher, at 19–47 years. The number of males and females within each of the three groups was comparable with the exception of the DHF4 subgroup, which was comprised of two females. We included only patients with secondary infection.

Complement gene polymorphisms did not differ in frequency between patients with severe and non-severe Dengue infection in the Thai population

The individual SNP frequencies in FB (rs12614, rs641153), C3 (rs2230199) and CFH (rs3753394, rs1061170 and rs800292) did not differ between patients with Dengue infection and either healthy controls or individuals with non-Dengue febrile illness (Table S2). We next examined whether the SNP frequencies differed among Dengue infection subgroups, defined as those with DF ($n = 121$) and those with DHF ($n = 187$). We also compared genotypes between DHF patients with ($n = 43$, defined as DHF3 and DHF4) and without shock ($n = 144$ defined as DHF1 and DHF2, Table 2). No differences were noted for any of the

Table 1. Study population demographics.

Group	*n*	Sex		Age	
		Male	Female	Median	Range
Healthy	22	9	13	21	19–47
Other febrile illnesses (OFI)	63	28	35	8	2–15
DF	121	61	60	10	3–15
DHF1	68	35	33	9·5	2–15
DHF2	76	47	29	11	5–15
DHF3	41	19	22	10	4–15
DHF4	2	0	2	7·5	5–10

Healthy and other febrile illness (OFI) subgroups were utilized as non-Dengue controls. DF: Dengue fever; DHF1–4: Dengue haemorrhagic fever 1–4.

tested polymorphisms. Notably, the minor allele frequency of functional C3 rs2230199 polymorphism was low (2%), as were the minor allele frequencies of the adjacent rs12614 and rs641153 FB polymorphisms (<10%). In contrast, the CFH rs800292 polymorphism had a minor allele frequency of 42% in this population, but nevertheless its frequency did not differ between Dengue infection subgroups. Due to the rarity of variation at C3 rs2230199, FB rs12614 and FB rs641153FB we did not detect any individual in our Dengue infection cohort with either the most [C3F/F, FB-32R/R (C/G at the adjacent rs12614/rs641153) and CFH 62V/V] or least [C3S/S, FB-32Q/Q (C/A at the adjacent rs12614/rs641153) and CFH 62I/I] active complotype described *in vitro* (Table 3) [17]. One high (defined as FB-32R/R and CFH 62V/V) and one low (defined as CFH 62I/I and C3S/S) activation complotype were present in our Dengue infection cohort, but neither was associated with infection severity (Table 3).

The CFHR3-1 deletion polymorphism is not associated with Dengue infection severity

The Δ*CFHR3-1* deletion allele frequency in our controls (healthy and non-Dengue febrile illness) and Dengue infection was 6·79 and 6·94%, respectively, and did not differ significantly between these two groups or between subgroupings of Dengue infection (Table 4). In this analysis, we noted one individual with three copies of CFHR3-1, suggesting duplication of these genes on one allele. In addition, nine individuals had heterozygous deletion of the *CFHR1* gene in the presence of two copies of the CFHR3 genes. This is most likely to indicate the presence an allele containing deletion of both the *CFHR1* and *CFHR4* genes (Δ*CFHR4-1*), a variant reported in healthy controls, but at a lower frequency than that of the Δ*CFHR3-1* deletion allele [27].

Dengue NS1 protein did not bind to the complement factor H protein

NS1 protein of WNV virus has an immune evasion activity by binding to CFH protein, resulting in decreased complement recognition of infected cells [15]. We next determined if Dengue NS1 interacts with CFH using either recombinant NS1 or NS1 from DV2-infected cell supernatants (Fig. 1). Using a combination of ELISA, far Western blotting and immunoprecipitation assays we could not detect any evidence of an interaction between CFH and Dengue NS1 (Fig. 1). As a positive control for the far Western blotting, we utilized the known interaction between CFH and the meningococcal factor H-binding protein (Fig. S1).

Discussion

In this study we assessed the relationship of selected complement gene polymorphisms and Dengue infection severity. We included variation within four nucleotides, two adjacent in FB and one in each of C3 and CFH, that have been shown to determine a sixfold variation in complement haemolytic activity *in vitro* [17]. Our data showed that the minor allele frequency in the C3 and FB polymorphisms was very low, and consequently any population effect of variation at this locus would be minimal and difficult to detect without large numbers. However, although the minor allele frequency at the CFH rs800292 polymorphism was 42% (Table 2), we did not detect any significant variation in the distribution of genotypes among the subgroups of Dengue infection. A recent study of 121 Brazilian patients with DENV-3 (genotype III Sri Lankan–Indian strain) assessed the relationship between four *CFH* polymorphisms (rs3753394, rs800292, rs3753396, rs1065489) and infection severity [24]. The minor alleles at rs3753394 and rs800292 were associated with reduced infection severity. Our analysis included these loci, but did not replicate the association. Hence, if the association is robust in the Brazilian population, it does not apply in the Thai population.

Retrospective studies of Dengue infection have shown a correlation between the disease severity and the complement activation [11,12]. Plasma CFH was lower in patients with DHF during the acute phase of the illness, returning to normal during convalescence. In contrast CFH levels did not alter during DF infection [12]. However, in this study

Table 2. Relationship between complement gene polymorphisms and Dengue infection severity.

Locus of SNP	Genotype	Phenotype†	All Dengue infection	DF	DHF	*P*-value§	OR (95% CI)	Adjusted OR (95% CI)‡	DHF without shock	DHF with shock	*P*-value¶	OR (95% CI)	Adjusted OR (95% CI)‡
CFH	TT	402 Tyr	247 (80·2)	89 (73·6)	158 (84·5)	0·01	1·96 (1·11–3·45)	2·03 (1·15–3·60)	121 (84·0)	37 (86·0)	1	1·17 (0·44–3·10)	0·99 (0·36–2·70)
rs1061170	CT	402 Tyr/His	57 (18·5)	29 (24·0)	28 (15·0)		0·56 (0·31–1·00)	0·53 (0·30–0·96)	22 (15·3)	6 (14·0)		0·90 (0·34–2·38)	1·06 (0·39–2·1)
	CC	402 His	4 (1·3)	3 (2·5)	1 (0·5)		0·21 (0·02–2·06)	0·24 (0·02–2·32)	1 (0·7)	0 (0·0)		–	–
	Frequency		0·89:0·11	0·86:0·14	0·92:0·08		2·01 (1·19–3·38)	–	0·92:0·08	0·93:0·07		1·16 (0·46–2·95)	–
	T : C		*n* = 308	*n* = 121	*n* = 187				*n* = 144	*n* = 43			
CFH	CC	–	84 (27·4)	36 (29·8)	48 (25·8)	0·1	0·82 (0·49–1·37)	0·79 (1·47–1·32)	34 (23·8)	14 (32·6)	0·22	1·55 (0·74–3·26)	1·66 (0·77–3·57)
rs3753394	CT	–	154 (50·2)	65 (53·7)	89 (47·8)		0·79 (0·50–1·25)	0·80 (0·51–1·28)	69 (48·3)	20 (46·5)		0·93 (0·47–1·85)	0·92 (0·46–1·84)
	TT	–	69 (22·5)	20 (16·5)	49 (26·3)		1·81 (1·01–3·23)	1·83 (1·02–3·28)	40 (28·0)	9 (20·9)		0·68 (0·30–1·55)	0·66 (0·29–1·50)
	Frequency		0·52:0·48	0·57:0·43	0·50:0·50		0·76 (0·55–1·05)	–	0·48:0·52	0·56:0·44		1·37 (0·85–2·23)	–
	C : T		*n* = 307	*n* = 121	*n* = 186				*n* = 143	*n* = 43			
CFH	GG	62 Val	104 (34)	33 (27·7)	71 (38·0)	0·18	1·60 (0·97–2·63)	1·61 (0·98–2·66)	59 (41·0)	12 (27·9)	0·13	0·56 (0·27–1·17)	0·57 (0·27–1·22)
rs800292	GA	62 Val/Ile	150 (49)	65 (54·6)	85 (45·5)		0·69 (0·44–1·10)	0·70 (0·44–1·11)	63 (43·8)	22 (51·2)		0·74 (0·38–1·47)	0·78 (0·39–1·56)
	AA	62 Ile	52 (17)	21 (17·6)	31 (16·6)		0·93 (0·50–1·70)	0·90 (0·49–1·66)	22 (15·3)	9 (20·9)		1·47 (0·62–3·48)	1·54 (0·64–3·71)
	Frequency		0·58:0·42	0·55:0·45	0·61:0·39		1·26 (0·91–1·75)	–	0·63:0·37	0·53:0·47		0·68 (0·42–1·11)	–
	G : A		*n* = 306	*n* = 119	*n* = 187				*n* = 144	*n* = 43			
FB	CC	–	270 (88·8)	105 (87·5)	165 (89·7)	0·72	1·24 (0·60–2·55)	1·21 (0·59–2·50)	126 (88·7)	39 (92·9)	0·58	1·65 (0·46–5·96)	1·64 (0·45–6·02)
rs12614	CT	–	33 (10·9)	15 (12·5)	18 (9·8)		0·76 (0·37–1·57)	0·78 (0·37–1·61)	15 (10·6)	3 (7·1)		0·65 (0·18–2·37)	0·67 (0·18–2·46)
	TT	–	1 (0·3)	0 (0·0)	1 (0·5)		–	–	1 (0·7)	0 (0·0)		–	–
	Frequency	–	0·94:0·06	0·94:0·06	0·95:0·05		1·16 (0·58–2·31)	–	0·94:0·06	0·96:0·04		1·72 (0·49–6·01)	–
	C : T		*n* = 304	*n* = 120	*n* = 184				*n* = 142	*n* = 42			
FB	GG	–	252 (83·2)	99 (83·2)	153 (83·2)	1	1·00 (0·54–1·85)	1·04 (0·56–1·93)	120 (84·5)	33 (78·6)	0·27	0·67 (0·28–1·60)	0·65 (0·27–1·56)
rs641153	GA	–	50 (16·5)	20 (16·8)	30 (16·3)		0·96 (0·52–1·79)	0·92 (0·50–1·73)	22 (15·5)	8 (19·0)		1·28 (0·53–3·14)	1·34 (0·54–3·31)
	AA	–	1 (0·3)	0 (0·0)	1 (0·5)		–	–	0 (0·0)	1 (2·4)		–	–
	Frequency		0·91:0·09	0·92:0·08	0·91:0·09		0·96 (0·54–1·73)	–	0·92:0·08	0·88:0·12		0·62 (0·28–1·37)	–
	G : A		*n* = 303	*n* = 119	*n* = 184				*n* = 142	*n* = 42			
C3	CC	102 Arg (C3S)	300 (98)	118 (99·2)	182(97·3)	0·1	0·31 (0·04–2·67)	0·30 (0·03–2·63)	139 (96·5)	43 (100)	0·13	–	–
rs2230199	CG	102 Arg/Gly (C3S/C3F)	2 (0·7)	1 (0·8)	1 (0·5)		0·63 (0·04–10·24)	0·55 (0·03–9·08)	1 (0·7)	0 (0·0)		–	–
	GG	102 Gly (C3F)	4 (1·3)	0 (0·0)	4 (2·1)		–	–	4 (2·8)	0 (0·0)		–	–
	Frequency		0·98:0·02	1·00:0·00	0·98:0·02		0·17 (0·02–1·36)	–	0·97:0·03	1·00:0·00		–	–
	C : G		*n* = 306	*n* = 119	*n* = 187				*n* = 144	*n* = 43			

Data are expressed as number (percentage) of patients. DF: Dengue fever; DHF: Dengue haemorrhagic fever; OR: odds ratio; SNP: single nucleotide polymorphism; CI: confidence interval; CFH: complement factor H; FB: factor B. †Number refers to amino acid number where methionine = 1. C3S and C3F refer to 'slow' and 'fast' alleles, ‡adjusted for sex and age at presentation using logistic regression, §DF *versus* DHF and ¶no shock *versus* shock.

Table 3. Relationship between complotype and Dengue infection severity.

Complotype†	Complement activation	DF ($n = 115$)	DHF ($n = 184$)	*P*-value§	OR (95% CI)	Adjusted OR (95% CI)‡	No shock ($n = 142$)	Shock ($n = 42$)	*P*-value¶	OR (95% CI)	Adjusted OR (95% CI)‡
FB32-R/FH62-V/C3F	High	0 (0·0)	0 (0·0)	–	–	–	0 (0·0)	0 (0·0)	–	–	–
FB-32R/FH-62V	High	25 (21·7)	53 (28·8)	0·22	1·46 (0·84–2·51)	1·46 (0·84–2·52)	45 (31·7)	8 (19·0)	0·12	0·51 (0·22–1·18)	0·51 (0·22–1·20)
FB32-R/C3F	High	0 (0·0)	4 (2·2)	0·16	–	–	4 (2·8)	0 (0·0)	0·56	–	–
FB32-Q/FH62-I/C3S	Low	0 (0·0)	0 (0·0)	–	–	–	0 (0·0)	0 (0·0)	–	–	–
FH62-I/C3S	Low	20 (17·4)	29 (15·8)	0·75	0·89 (0·48–1·66)	0·87 (0·46–1·63)	20 (14·1)	8 (19·0)	0·47	1·46 (0·84–2·51)	1·46 (0·84–2·52)
FB32-Q/C3S	Low	0 (0·0)	1 (0·5)	1	–	–	0 (0·0)	1 (2·4)	0·23	–	–

Data are expressed as number (percentage) of patients. DF: Dengue fever; DHF: Dengue haemorrhagic fever; OR: odds ratio; CI: confidence interval. †Number refers to amino acid number where methionine = 1, R: arginine; Q:, glutamine; V: valine; I: isoleucine. C3S and C3F refer to 'slow' and 'fast' alleles, variants are homozygous at each loci. High and low complement activity based on haemolytic activity using purified proteins *in vitro* [17], ‡adjusted for sex and age at presentation using logistic regression, §DF *versus* DHF and ¶no shock *versus* shock.

we did not find a significant correlation with severity and the examined CFH polymorphisms.

The absence of the genes encoding the CFH-related proteins, CFHR1 and CFHR3, is a frequent polymorphism (*ΔCFHR3-1*), which is thought to have developed through non-allelic homologous recombination within the *CFHR* locus [22,23]. This allele confers protection against AMD and IgA nephropathy [23,28]. The frequency of the *ΔCFHR3-1* allele in our Dengue population was approximately 7% (Table 4). The reported allele frequencies in other populations include 4·7% in European Caucasians, 16% in African populations and 2% in Asians [22]. In our analysis there was no evidence that this allele conferred protection against Dengue infection or its severity. During copy number analysis across the *CFHR* locus we noted uncommon but previously reported variation, including one individual with three copies of the *CFHR3* and *CFHR1* genes, most probably a result of duplication of theses genes on one allele [29,30]. We also detected nine individuals with deletion of the *CFHR1* gene but normal copies of the *CFHR3* genes. One explanation for these findings is the presence of a less common allele in which there is deletion of the *CFHR4* and *CFHR1* genes (*ΔCFHR4-1*). Alternatively, this could represent deletion of the *CFHR1* genes in isolation [27,31]. Our copy number analysis did not include probes for *CFHR4* gene, so this remains to be shown.

It is important to note that in our study we excluded individuals with primary infection, because in many cases infection is asymptomatic. Secondly, our sample size for severe disease (DHF3 and DHF4) was relatively small, and it remains possible that we could lack the power to detect small effects. Thirdly, the low frequency of the minor allele of the studied C3 and FB polymorphisms means that we cannot conclude what effects variation could have on infection severity in non-Thai populations, where there is greater variation at these loci.

Levels of Dengue soluble NS1 protein correlates with disease severity [32] and Dengue soluble NS1 protein can directly activate the complement cascade, a phenomenon that is enhanced by the presence of anti-Dengue antibodies [13]. Dengue NS1 has been shown to interact with Clusterin, a complement regulatory protein [33]. Recently, some studies have shown that WNV NS1, but not JEV, could bind to CFH, and thereby reduce complement activation on the virus *in vitro* [15,16]. We were unable to detect a direct interaction between CFH and Dengue NS1 protein, so we currently have no evidence to suggest that NS1 interferes with CFH-mediated regulation of complement during infection, at least in a direct manner.

In summary, our data demonstrate that the severity of Dengue infection is not influenced strongly by the CFH rs3753394 promoter polymorphism, the AMD-associated CFH rs1061170 polymorphism, the AMD-protective ΔCFHR3-1 allele and the complotype-associated CFH rs800292 polymorphism in Thai population. We also show

Table 4. Copy number variation in *CFHR3* and *CFHR1* genes and Dengue virus infection severity**.**

		CFHR3-1					*CHFR3-1*	*CFHR1*
Sample:	*n*	100% (2 copies)	50% (1 copy)	0% (0 copy)	*P*-value	*CHFR1-CFHR3* deletion *ΔCFHR3-1* allele frequency (%)	150% (3 copies)	50% (1 copy)
Controls	82	70	11	0		6·79	0	1
Dengue	297	251	34	3	1·00[†]	6·94	1	8
DF	113	95	14	0		6·42	0	4
DHF	184	156	20	3	0·74[‡]	7·26	1	4
No shock	142	118	16	3		8·03	1	4
Shock	42	38	4	0	0·47[§]	4·76	0	0

Controls include healthy controls and other febrile illness, 150%, three copies of *CFHR3-1*; 100%, two copies of *CHFR3-1*; 50%, one copy of *CHFR1-3*; 0%, no copies of the *CFHR3-1* genes *ΔCFHR3-1* allele frequency is the percentage of *ΔCFHR3-1* alleles divided by total *CFHR3-1* alleles. [†]Dengue *versus* controls; [‡]DF *versus* DHF; [§]no shock *versus* shock.

that the complotype-associated C3 rs2230199 and FB rs12614/rs641153 polymorphisms had very low minor allele frequencies in this population. Finally, we could not demonstrate a direct interaction between CFH and Dengue NS1. We conclude that these genetic factors do not influence the severity of Dengue infection in the Thai population and that Dengue NS1 does not influence complement activation during infection by a direct interaction with CFH.

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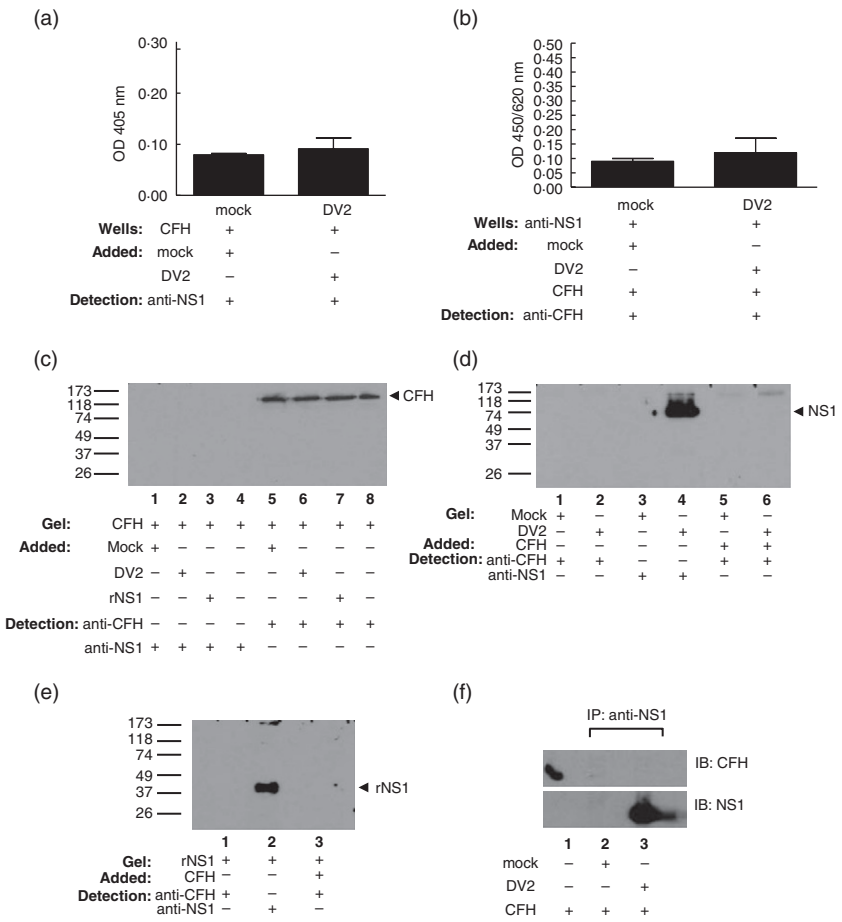

Fig. 1. Assays exploring possible interaction between complement factor H (CFH) and Dengue non-structural protein 1 (NS1). (a) and (b) enzyme-linked immunosorbemt assays (ELISA) to detect interaction between CFH and NS1. (a) Supernatants were added to plates coated with human CFH (5 μg/ml) and binding detected with anti-NS1 antibody. (b) Anti-NS1 antibody was coated on the plate and NS1 captured from Dengue virus (DV)2-infected cell supernatant. Purified CFH was then added and binding detected using an anti-CFH antibody. The experiments were performed three times independently. (c,d,e) Far Western blotting assays to detect interaction between CFH and NS1. CFH (c), supernatant (d) and recombinant NS1 (e) were immobilized using gel electrophoresis. Following addition of supernatant (c), recombinant NS1 (c) and CFH (d,e) interaction was tested by addition of the appropriate antibodies. (f) Immunoprecipitation assay to detect interaction between CFH and NS1. Immunoprecipitation using anti-NS1 antibody (lanes 2 and 3) demonstrated NS1 in the absence of CFH in DV2-infected cell supernatants only. In lane 1 purified CFH was loaded on to the gel as a positive control for CFH detection by the anti-CFH antibody.

Author contributions

M. C. P., J. M., G. R. S. and M. B. designed and supervised the research. M. C. P. and R. K. wrote the manuscript. R. K. performed the experiments. S. V., W. L., P. M. and N. T. provided the samples and cohort data.

Disclosure

The authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Far Western blot between *Neisseria* complement factor H binding protein (fHbp) and complement factor H (CFH). fHbp protein (a gift from Professor Chris Tang, University of Oxford, UK) was loaded into the gel, transferred to a membrane which was then incubated with human CFH (lane 1, 5 μg/ml; Quidel, San Diego, CA, USA) or buffer (lanes 2 and 3). The interaction between gel-immobilized fhbp and the added CFH was detected using an anti-human CFH antibody (lane 1, antibody obtained from Quidel). Gel-immobilized fHbp was visualized using an anti-fHbp (lane 2, antibody a gift from Professor Chris Tang, University of Oxford, UK). The anti-human CFH antibody did not recognize gel-immobilized fhbp in the absence of CFH (lane 3).

Table S1. Primer sequences used in custom genotyping assays.

Table S2. Relationship of complement gene polymorphisms between Dengue infection and controls.

