Association of FcγRIIa Polymorphism with Clinical Outcome of Dengue Infection: First Insight from Pakistan

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Abstract. Dengue illness has been a major health concern in Pakistan during the last decade. Dengue infection can result in a spectrum of clinically distinct outcomes, ranging from asymptomatic infection to potentially life-threatening forms of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). A single-nucleotide polymorphism in FcγRIIa (rs1801274) results in altered affinity of the receptor for different subclasses of immunoglobulin G, and is a key player in determining the susceptibility to or protection from severe clinical infection of dengue. In this study, we analyzed the allelic and genotypic distribution of rs1801274 in subjects of Pakistani origin with subclinical dengue infection ($n = 40$), dengue fever (DF) ($n = 40$), and DHF/DSS ($n = 30$). We found that HH homozygotes and heterozygotes were significantly more likely to develop clinical dengue (odds ratio [OR] = 3.21, 95% confidence interval [CI] = 1.29–7.97, $P = 0.009$), either DF (OR = 2.82, 95% CI = 1.00–7.97, $P = 0.045$) or DHF/DSS (OR = 3.90, 95% CI = 1.13–13.07, $P = 0.024$) than the asymptomatic dengue infection. Results of allelic distribution comparisons and logistic regression analysis also supported the same relationship. The results suggest complex nature of interacting factors in determining the course for severe dengue illness.

INTRODUCTION

Dengue has become one of the most important arthropod-borne diseases in tropical and subtropical regions of the world. Approximately 100 million cases of dengue fever (DF) and 500,000 cases of dengue hemorrhagic fever (DHF), resulting in around 24,000 deaths, occur annually and an estimated 2.5–5 billion people are at risk of dengue virus infection.^{1,2} It is caused by any of the four dengue serotypes (DENV-1–DENV-4) that are transmitted to humans through the bite of infective female mosquitoes of genus *Aedes*. The infection with any of these DENV serotypes can result in a spectrum of clinically distinct outcomes, ranging from asymptomatic infection to potentially life-threatening forms of DHF and dengue shock syndrome (DSS).

Antibody-dependent enhancement (ADE) theory explains, at least partially, the mechanism leading to severe dengue infection. According to it, cross-reactive non-neutralizing antibodies contribute to the virus's ability to infect host macrophages via Fc receptors, leading to an increase in infected cell count, and thus paving the way for a more severe dengue infection. This observation was supported by studies from Thailand and Cuba, which indicated that a secondary dengue infection with a different serotype from the primary infection confers a greater risk of severe complications like DHF/DSS.^{3–5} Conversely, studies also highlighted the development of short-term protective immune response against all serotypes of DENV as a result of previous dengue infection, which is likely to modulate the overall incidence of DHF and/or DSS.^{6–8} Therefore, despite the wide recognition of ADE, the precise nature of the virus–antibody interaction in severe forms of dengue infection is not properly understood.⁹

A number of host genetic factors seem to be important in contributing toward variable susceptibility to severe dengue

disease. Besides ethnicity, these include genetic variants in immune system receptor genes (especially, FcγRIIa and DC-SIGN),^{10,11} major histocompatibility complex system genes,^{8,12–14} genes of complement pathway (e.g. *MBL2*),¹⁵ cytokine genes (e.g. *TNF-α*),^{16,17} and various others such as *VDR*, *PLCE1*, and *MICB*.^{18,19}

FcγRIIa (CD32), the most widely distributed member of Fcγ receptor family, is a low-affinity immunoglobulin G (IgG) receptor that can interact with IgG 1–4 subtypes by binding to their Fc fragments and is expressed on the surface of most of the hematopoietic cells. A single-point mutation A>G (rs1801274) in FcγRIIa, replacing histidine (H) at position 131 with arginine (R), results in altered affinity of the receptor for different subclasses of IgG such that the form having histidine (genotype 131HH) interacts more efficiently with IgG2 while the one with arginine (genotype 131RR) shows more affinity for binding IgG1 and IgG3 subclasses.^{20–22} Therefore, it was hypothesized that this FcγRIIa polymorphism may account for, at least partially, the variability in immune response and may shed some light on the pathogenesis of severe dengue infection. Three previous studies from Vietnam, Cuba, and Mexico also support the involvement of FcγRIIa polymorphism in dengue pathogenesis.^{10,11,18}

It is speculated that dengue virus may have been endemic in Pakistan, but due to lack of any effective surveillance and diagnostic measures, no outbreaks were reported until 1994, when introduction of DENV-1 serotype caused a major outbreak in southern Pakistan with high morbidity and mortality rates.²³ After a gap of almost 10 years, dengue outbreaks ensued again in 2005/2006 claiming many deaths,²⁴ with circulating serotypes of DENV-2 and the new DENV-3 (New Delhi genotype).^{25,26} Since 2006, the disease extended its range from southern to northern Pakistan, resulting in outbreaks that have affected thousands of people, causing hundreds of mortalities over the course of past 7 years or so. A noteworthy observation was the presence of multiple DENV serotypes in the population during the course of these outbreaks.^{27,28} The most disastrous epidemic of dengue was seen in 2011 in Punjab, with metropolitan city of Lahore (population

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of 6,319,000 in 2011) being the key affected area having over 20,000 confirmed cases and casualties exceeding 300.²⁹ Studies showed that the DENV-2 serotype was the major culprit behind this epidemic.³⁰

The province of Punjab in northern Pakistan, especially the capital city of Lahore, is a dengue-endemic region where dengue infection continues to be a major health problem. The unique epidemiological and genetic profile of this region offers an opportunity to understand the mechanism of infection and role of genetic heterogeneity in the development of severe dengue illness through the study of relevant genetic polymorphisms. The present case-control study details the comparison of genotype frequencies and allele distributions, of FcγRIIa H131R polymorphism, between asymptomatic cases and either all symptomatic cases or clinical subgroups (uncomplicated or severe), among inhabitants of Lahore, Pakistan.

MATERIALS AND METHODS

Ethics statement. The ethical approval of this study was obtained from Institutional Review Board, Postgraduate Medical Institute, Sheikh Zayed Hospital, Lahore, Pakistan. All the subjects or their legal guardians (in case of minors) gave their written informed consent to participate in the study. This study cohort consisted of 110 subjects, clinically classified as subclinical group (40 cases), DF group (40 cases), and DHF/DSS group (30 cases).

Subjects and sample collection. Samples were collected from subclinical subjects as part of a serological surveillance study conducted by Pakistan Medical Research Council Research Centre, National Health Research Complex, Sheikh Zayed Hospital, Lahore, Pakistan, during the peak of the 2011 dengue epidemic in Lahore. A cohort of healthy individuals residing in different towns of Lahore with high incidence of dengue infection was tested for subclinical or asymptomatic dengue infection through detection of either IgM or IgG dengue antibody in the sera by capture enzyme-linked immunosorbent assay. Neither febrile illness nor any other clinical indication of dengue disease had been recorded in these individuals during the course of dengue epidemic. Therefore, a seroconverted individual (positive IgM or IgG dengue antibody) who did not report fever or any other clinical symptom of dengue disease throughout the course of the epidemic period was taken as a case of subclinical or asymptomatic infection. A total of 40 such cases of subclinical dengue infection were thus enrolled in this study. A total of 70 samples were collected from subjects with the antecedent of a clinical dengue infection presenting at dengue units of different hospitals in Lahore. Of these, 40 cases represented the uncomplicated DF, while 30 cases met the criteria for severe dengue infection (DHF/DSS).

Case definitions. This study included clinical dengue cases classified as either uncomplicated or severe dengue with the intention of establishing clearly distinct groups for comparisons. The definitions of cases for severe and uncomplicated dengue infections were according to the World Health Organization (WHO) guidelines¹ and were based on medical and clinical laboratory records of each patient. According to the WHO criteria used, the cases of severe dengue (DF/DSS) included patients who presented with one or all of the following characteristics: severe plasma leakage (indicated by high or progressively rising hematocrit, hypotension, shock,

and fluid accumulation with respiratory distress), major bleeding episode(s), profound thrombocytopenia (platelet count of 50,000/mm³ or less), or severe organ impairment (liver and renal involvement, encephalopathy, cardiomyopathy, or other unusual manifestations). Cases of uncomplicated dengue were taken as subjects with confirmed febrile dengue illness but without any of the complications mentioned above. The cases of clinical dengue infection were also confirmed by dengue IgM or dengue NS1 detection from patient sera. Dengue cases with comorbidity such as cardiovascular disorders, diabetes, hepatitis B/C, or renal disease were not included in the study.

DNA extraction and genotyping. Genomic DNA was extracted from blood samples using a commercial DNA purification kit (Genomic DNA Purification Kit; Thermo Scientific, Lithuania, EU) according to manufacturer instructions. The qualitative and semiquantitative assessment of extracted DNA was performed by agarose gel electrophoresis using an in-house method and was stored at -20°C until further analysis. Genotyping for FcγRIIa H131R polymorphism (rs1801274) was performed as described by Bazilio and others.³¹ In brief, a nested polymerase chain reaction (PCR) approach was used using two sets of primers, the outer primer pair and inner primer pair. The outer primer set consisted of forward primer P63-F (5'-CAAGCCTCTGGTCAAGGTC-3') and reverse primer P63-R (5'-CAATGACCACAGCCACAATC-3'), which amplified a 1-kb segment of FcγRIIa gene that contained exon 4 and a portion of exon 5, with intronic sequence in between. Then allele-specific primers 494A and 494G (5'-ATTCTCCC[A/G]TTTGGATC-3'), along with a common P52 reverse primer (5'-GAAGAGCTGCCCATGCTG-3'), were used in a nested PCR format with subsequent agarose gel electrophoresis to determine the respective FcγRIIa rs1801274 allelic combinations for each subject. To rule out chances of any bias in results, the samples were coded and the genotypes were scored by two independent researchers.

Data analysis. χ^2 test was used to compare the FcγRIIa H131R genotypic and allelic frequencies with two-sided *P* value of < 0.05 being considered to be statistically significant. Comparisons were made between subclinical group and one or both groups of clinical infections to determine any association of FcγRIIa H131R genotypes and clinical outcome of dengue infection by means of an odds ratio (OR), as exemplified in similar studies.^{10,11} Logistic regression model was also generated to analyze the effect of FcγRIIa H131R genotypes on disease severity in the presence of other biological covariates. All the statistical analyses were performed using the SPSS version 20 software, Armonk NY.

RESULTS

Cohort summary. This study included 110 samples: 40 with subclinical dengue infection, 40 with DF, and 30 with severe form of dengue infection (DHF/DSS). There were 59 males and 31 females among the study subjects with an age range from 1 to 24 years (mean $\pm$ standard deviation [SD] = 12.26 $\pm$ 4.13 years). The cases and controls were ethnically matched as a group and all originated from Lahore District in Pakistan. The serotypes of the dengue virus were not determined, but all the samples were collected during the 2011 dengue outbreak in Lahore when the prevalent dengue serotypes circulating in Lahore were DENV-2 and DENV-3.

TABLE 1
Associations between rs1801274 genotypes and clinical outcome in dengue infection

Comparison (cases vs. controls)	Genotype (vs. baseline comparison)	Frequency in cases	Frequency in controls	OR (95% CI)	χ^2	P
All clinical vs. subclinical	HH and HR (vs. RR)	59 (84.3%)	25 (62.5%)	3.21 (1.29–7.97)	6.69	0.0096
DF vs. subclinical	HH and HR (vs. RR)	33 (82.5%)	25 (62.5%)	2.82 (1.00–7.97)	4.01	0.045
DHF/DSS vs. subclinical	HH and HR (vs. RR)	26 (86.7%)	25 (62.5%)	3.90 (1.13–13.37)	5.06	0.024
All clinical vs. subclinical	HH (vs. HR and RR)	17 (24.3%)	4 (10%)	2.88 (0.89–9.28)	3.36	0.066

CI = confidence interval; DF = dengue fever; DHF = dengue hemorrhagic fever; DSS = dengue shock syndrome; H = histidine; OR = odds ratio; R = arginine.
 χ^2 test was used to evaluate the association between genotype frequencies with clinical infection (or a subgroup, either DF or DHF/DSS) compared with subclinical infection.

Genotypic and allelic distribution of Fc γ RIIa H131R polymorphism. The H (A) allele frequency for rs1801274 was estimated to be 48% (52% for the R (G) allele) in the overall population. The distribution of Fc γ RIIa H131R polymorphism did not differ significantly from the Hardy–Weinberg equilibrium ($\chi^2 = 2.4$).

The genotypic and allelic distributions for rs1801274 in each of the clinical groups were calculated to determine the associated risk for each genetic variant as summarized in Table 1. HH homozygotes and heterozygotes were significantly more likely to develop clinical dengue than the asymptomatic dengue infection (OR = 3.21, 95% confidence interval [CI] = 1.29–7.97, $P = 0.009$). This trend was although relatively less pronounced for the DF group (OR = 2.82, 95% CI = 1.00–7.97, $P = 0.045$), but it remained the same for DHF/DSS clinical group (OR = 3.90, 95% CI = 1.13–13.07, $P = 0.024$). On the other hand, presence of RR genotype offered protection against the development of severe form of dengue infection (OR = 0.25, 95% CI = 0.07–0.87, $P = 0.024$). Results of allelic distribution comparisons also supported the same idea where frequency of H allele was also found to be associated with significantly higher rates of clinical outcome compared with subclinical outcome of dengue infection (OR = 2.08, 95% CI = 1.18–3.67, $P = 0.009$). The same relationship did not hold true exactly for DF subgroup (OR = 1.59, 95% CI = 0.84–2.99, $P = 0.14$), however, comparing asymptomatic infection to only severe dengue (DHF/DSS) also demonstrated the H allele-associated risk (OR = 3.03, 95% CI = 1.51–6.08, $P = 0.001$) (Table 2).

Logistic regression analysis. In addition to genetic factors, the clinical outcome of dengue infection can be modulated by a number of other variables including age, gender, and history of secondary infection. A logistic regression modeling approach was used to ascertain the odds of a subject presenting with subclinical outcome (in comparison with clinical infection as well as to the subgroups of DF and DHF/DSS) of dengue infection in the presence of other biological covariates (shown in Table 3). The results demonstrated that the probability of presenting clinical infection compared with subclinical infection is increased by a factor of 2.82 for a subject who is genotyped HH or HR (95% CI = 1.04–7.66, $P = 0.041$), by a factor of 2.69 when comparison is made between DF and subclinical infection (95% CI =

0.84–8.58, $P = 0.094$), and by a factor of 3.05 when comparing DHF/DSS with subclinical infection (95% CI = 0.76–12.23, $P = 0.115$). These results provide further support that in this population the H allele of rs1801274 is associated with clinical infection, even after accounting for other variables. Moreover, age was also associated with greater odds of clinical outcome of dengue infection in all models; while conversely, males seemed to have greater odds of protection against developing clinical dengue infection.

DISCUSSION

Dengue infection has been a major health problem in Pakistan for a decade or so. Although much effort has been made toward dengue health care including vector control practices, public awareness campaigns, and dedicated dengue control units in the hospitals, complete dengue control is a goal still to be achieved. Especially, effective management of severe dengue disease requires understanding of all the contributing factors involved in disease pathogenesis including viral virulence, comorbidity factors, host immune responses, and genetic factors. The dengue experience in Pakistan presents an excellent opportunity to reveal genetic immunity determinants of the severe dengue infection. The implications of such genetic association and mechanism studies can be even more substantial in the current scenario of dengue infection in Pakistan when the epidemiological conditions such as circulating serotypes and seroprevalence have stabilized.

This study reinforces the evidence for the Fc γ RIIa rs1801274 polymorphism as an inherited genetic determinant in clinical outcome of the dengue infection, this time in Pakistani population. We found that the presence of one or two histidine alleles of the Fc γ RIIa H131R polymorphism in a subject increased the odds of developing clinical dengue infection compared with subclinical infection through calculations of rs1801274 genotypic and allelic distributions by χ^2 test. The HH genotype presented with increased susceptibility toward severe form of dengue infection while homozygosity for R allele offered protection against the DHF/DSS infection. This relationship persisted even after employing logistic regression analysis to account for other biological covariates that differed between the comparison groups. These findings are consistent with the two previous studies in Vietnamese

TABLE 2
Associations between rs1801274 allele frequency and clinical outcome in dengue infection

Comparison (cases vs. controls)	Allele (vs. baseline comparison)	Frequency in cases	Frequency in controls	OR (95% CI)	χ^2	P
All clinical vs. subclinical	H allele (vs. R allele)	76 (54.3%)	29 (36.2%)	2.08 (1.18–3.67)	6.64	0.0099
DF vs. subclinical	H allele (vs. R allele)	38 (47.5%)	29 (36.2%)	1.59 (0.84–2.99)	2.08	0.14
DHF/DSS vs. subclinical	H allele (vs. R allele)	38 (63.3%)	29 (36.2%)	3.03 (1.51–6.08)	10.08	0.0014

CI = confidence interval; DF = dengue fever; DHF = dengue hemorrhagic fever; DSS = dengue shock syndrome; H = histidine; OR = odds ratio; R = arginine.
 χ^2 test was used to evaluate the association between genotype frequencies with clinical infection (or a subgroup, either DF or DHF/DSS) compared with subclinical infection.

TABLE 3
Logistic regression models of the odds of clinical compared with subclinical dengue infection

Variable	Subclinical vs. clinical			Subclinical vs. DF			Subclinical vs. DHF		
	Coefficient	OR (95% CI)	P	Coefficient	OR (95% CI)	P	Coefficient	OR (95% CI)	P
rs1801274 HH or HR	1.03	2.82 (1.04–7.66)	0.041	0.99	2.69 (0.84–8.58)	0.094	1.11	3.05 (0.76–12.23)	0.115
Age	0.69	1.99 (1.48–2.67)	< 0.0005	0.56	1.75 (1.31–2.33)	< 0.0005	1.33	3.79 (1.75–8.21)	0.001
Gender (male)	–0.64	0.52 (0.21–1.26)	0.153	–0.88	0.41 (0.14–1.18)	0.099	–0.31	0.72 (0.24–2.21)	0.57

CI = confidence interval; DF = dengue fever; DHF = dengue hemorrhagic fever; H = histidine; OR = odds ratio; R = arginine.

and Cuban populations indicating that the H allele of this polymorphism is significantly associated with susceptibility toward dengue infection and its severe form.^{11,18}

The possible mechanism behind this association of FcγRIIa H131R polymorphism with clinical outcome in dengue disease can be explained, at least partially, in the perspective of ADE theory. Previously, it has been shown that the IgG1 and IgG3 are the prime immunoglobulins produced during the course of dengue infection and FcγRIIa serves as a commonly distributed receptor for all IgG subclasses.³² FcγRIIa-H131R polymorphism alters the binding affinity of the receptor to IgG between the two possible receptor allotypes with arginine variant being causally associated with the efficient binding of IgG1 and IgG3 subclasses, but a reduced opsonization of IgG2 antibodies.^{20,33,34} Thus, it was hypothesized that the presence of H allele mediates inefficient binding of IgG1/3 from dengue immune complexes resulting in dissemination and persistence of dengue viral particles, favoring a more severe clinical course of DHF/DSS.

However, a recent study by Noecker and others¹⁰ changed the perspective of FcγRIIa-H131R SNP association with dengue infection by demonstrating that H allele is associated with protection against complicated dengue infection rather than susceptibility toward it, in a Mexican population. They highlighted a number of variables that could explain this observed disparity including structural differences between the studies in terms of case definitions of DHF/DSS infections, interacting genetic factors other than rs1801274, and other modulators of immune response such as age, viral serotype, and history of infection in the studied population. Another possible support to this contradictory theory may come from investigations of Bruhn and others,³⁵ who established a hierarchy of affinities of human Fcγ receptor toward all four subclasses of monoclonal and polyclonal IgG antibodies through a series of binding assays. They presented that, in contrast to previous indications,^{20,33} FcγRIIa H variant binds IgG1 more efficiently than FcγRIIa R variant, which implies that histidine variant is the one associated with the more effective phagolysosome formation and subsequent clearing of dengue immune complex and viral infection.^{34–36} As a whole, these findings suggest that FcγRIIa H131R polymorphism has an important role to play in severe dengue pathogenesis,

but other genetic and environmental variables complicate this relationship. Further studies exploring other genetic and epigenetic factors and affinity association of DENV/IgG complexes with FcγRIIa H/R polymorphism in view of ADE phenomenon are much required.

As per our knowledge, this is the first study enlightening the distribution of FcγRIIa H131R variant in a Pakistani population and includes subjects with subclinical dengue infection as a comparison group instead of normal healthy controls. Genetic distribution of FcγRIIa H131R polymorphism differs significantly across different populations. A comparison of genotypic and allelic distribution of R variant of FcγRIIa H131R SNP along with associated risk for developing severe dengue infection across different ethnic populations is presented in Table 4. As evident from this comparison and other studies, the RR genotype is much less prevalent in Asian population (6–10%) as compared with the rest of the studied populations where it ranges from 23% to 37%.^{37–39} This differential genotypic distribution of FcγRIIa H131R across different populations may also have implications, in addition to other variables, in different clinical outcomes in individuals with a heterotypic dengue illness under similar epidemiological conditions.

This study also determines the potential of other variables like age and sex as confounding factors in modulation of clinical severity of dengue infection. Age was found to present greater odds of clinical infection in this study. However, in a study from Latin America, age was found to offer protection against disease severity.⁴⁰

Limitations and future directions. This study demonstrates the role of an immune system polymorphism (rs1801274 of FcγRIIa) in the clinical outcome of dengue infection in a dengue-endemic metropolitan area of Lahore, Pakistan. Although, our study was slightly underpowered with respect to sample size due to financial limitations, but still it confirms the association of H allele with greater clinical severity in dengue disease as suggested by previous studies. Moreover, as part of this study, the respective serotype of dengue virus prevailing in subjects under study was not determined. Further studies, where subjects are also stratified by DENV serotypes, should be conducted to ascertain the effects of respective dengue serotypes. In addition, viral load as well

TABLE 4
Comparison of genotypic and allelic frequencies of rs1801274 and associated ORs across different studied populations

Population	RR genotype frequency (%)	R allele frequency (%)	OR with 95% CI for HH and HR vs. RR (subclinical vs. severe dengue)	P value	References
Pakistani (N = 110)	23.63	52.27	3.90 (1.13–13.37)	0.024	This study
Cuban (N = 139)	28.77	52.51	2.36 (0.79–7.03)	0.119	Garcia and others ¹¹
Mexican (N = 248)	23	49.2	0.45 (0.21–0.96)	0.026	Noecker and others ¹⁰
Vietnamese (N = 540)	8.1	27.77	1.74 (0.93–3.25)	0.075	Loke and others ¹⁸

CI = confidence interval; H = histidine; OR = odds ratio; R = arginine.

as primary and secondary infection in relation to disease severity along with genetic polymorphism studies can further strengthen the individual role of these risk factors for the development of dengue hemorrhagic fever. With respect to future directions, all such studies including this study suggest the need of analyzing as many relevant genetic variants as possible to fully ascertain the genetic determinants of severe dengue infection. A genome-wide association study approach seems appropriate in this regard; however, financial constraints and large sample size requirements prevented us from following this line of analysis in this study.

In conclusion, this study demonstrates that the role of rs1801274 of Fc γ RIIA in the clinical presentation of dengue illness is important but not straightforward. The potential effects of genetic background, prevalence of different dengue serotypes and secondary infection, and other confounding variables such as age, association with chronic diseases, and other epigenetic and environmental conditions needs to be elucidated in genetically distinct ethnic populations to enlighten the pathogenesis of severe dengue infection.

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