Immunobiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Short Communication

Polymorphisms in RNA sensing toll like receptor genes and its association with clinical outcomes of dengue virus infection

K. Alagarasu*, R.V. Bachal, R.S. Memane, P.S. Shah, D. Cecilia

Dengue Group, National Institute of Virology (ICMR), 20A, Dr. Ambedkar Road, Pune 411001, Maharashtra, India

ARTICLE INFO

Article history:
Received 23 August 2014
Accepted 26 September 2014
Available online xxx

Keywords:
Toll like receptor
Dengue
Gene polymorphisms
Dengue fever
Dengue hemorrhagic fever

ABSTRACT

Functional polymorphisms in RNA recognizing toll like receptors (TLR) 3, 7, 8 and toll-interleukin-1 receptor domain containing adapter protein adapter (TIRAP) coding genes were investigated in 120 dengue cases [87 dengue fever (DF) cases and 33 dengue hemorrhagic fever (DHF) cases] and 109 healthy controls (HC) to identify their association with clinical outcomes of dengue virus infection. Results revealed significantly lower frequency of *TLR3* rs3775291 T allele [DHF vs. DF $P = 0.015$ odds ratio (OR) with 95% confidence interval (CI) 0.390 (0.160–0.880); DHF vs. HC $P = 0.018$ OR with 95% CI 0.410 (0.170–0.900)] and 'T' allele carriers [DHF vs. DF $P = 0.008$ OR with 95% CI 0.288 (0.115–0.722); DHF vs. HC $P = 0.040$ OR with 95% CI 0.393 (0.162–0.956)] and higher frequency of *TIRAP* rs8177374 'C/T' genotype [DHF vs. HC $P = 0.020$ OR with 95% CI 2.643 (1.167–5.986)] in DHF. Higher frequency of *TLR8* rs3764879–rs3764880 haplotype C-A was observed in male DF cases compared to male HC [$P = 0.025$ OR with 95% CI 2.185 (1.101–4.336)]. The results suggest that *TLR3* and *TIRAP* gene variants influence the risk for DHF.

Introduction

Dengue fever (DF) is a major public health problem of concern in tropical and sub tropical countries and is spreading to newer areas where the disease has not been reported previously. Dengue virus (DENV), is an RNA virus, has four serotypes, and is transmitted by the bite of the infected mosquito vector, *Aedes aegypti*. Infection with the virus results in diverse clinical outcomes ranging from asymptomatic infections or mild DF to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Guzman et al., 2010). The reason behind the variable clinical outcomes in different individuals is not completely understood and factors involving host, virus and environment have been investigated to understand the pathogenesis of DF and its complications (Yacoub et al., 2013).

Innate immune responses play an important role in restriction of viral infections and reduction of the disease severity. Recognition of pathogens by the innate immune components is the first step in the initiation of immune responses. The RNA genomes of the viruses are recognized by toll like receptors (TLR) that are expressed in the intracellular compartments. TLR3 is known to recognize double stranded RNA while TLR7 and TLR8 recognize single stranded RNA. Recognition of viral RNA by TLRs leads to the expression of antiviral responses (Jesper, 2013). TLR3 and TLR8 have been shown to be upregulated in DENV infected HepG2 cells (Conceição et al., 2010). Activation of TLR3 is known to block the replication of DENV2 through production of IFN-β (Liang et al., 2011). Decreased DENV replication and increased anti viral humoral response have been observed with the use of combined TLR3/7/8 agonists in macaques (Sariol et al., 2011).

Functioning of TLRs is affected by single nucleotide polymorphisms in the genes coding for TLRs. Several functional polymorphisms have been reported in various TLR genes. A leucine to phenylalanine polymorphism in the 412th codon (rs3775291) of *TLR3* gene is known to affect the functioning of TLR3 and has been shown to be associated with reduced risk of severe tick borne encephalitis (TBE) virus infection, human immunodeficiency virus (HIV)-1 and herpes simplex virus (HSV)-2 infections and geographic atrophy (Sironi et al., 2012; Svensson et al., 2012; Kindberg et al., 2011; Barkhash et al., 2013; Zhou et al., 2011). Functional polymorphisms in the *TLR7/8* (*TLR7* rs179008; *TLR8* rs3764879 & rs3764880) genes have been shown to be associated with susceptibility to auto immune and infectious diseases (Pierik et al., 2006; Engin et al., 2010; Wang et al., 2011, 2014). Apart from SNPs in the TLR genes, polymorphisms in the genes coding for adapter proteins that are involved in the downstream signaling pathways of TLRs also affect susceptibility to infectious diseases. The Mal or Toll-interleukin-1 receptor domain containing adapter protein (TIRAP), is an essential component of TLR2 and TLR4 signaling. A serine to leucine SNP (rs8177374) in the coding region of *TIRAP* gene is known to affect functioning of mal protein and has been shown to be associated with various bacterial diseases (Khor et al., 2007; Selvaraj et al., 2010). Since DENV is an RNA virus, it is plausible

* Corresponding author. Tel.: +91 020 26006256.
E-mail address: alagarasu@gmail.com (K. Alagarasu).

http://dx.doi.org/10.1016/j.imbio.2014.09.020
0171-2985/

that functional variations in the RNA recognizing TLRs might affect clinical outcome after infection. In the present study we investigated whether functional SNPs in the *TLR3/7/8* and *TIRAP* genes are associated with DF or DHF.

Study subjects and methods

Study subjects

One hundred and twenty subjects [mean age ± standard deviation (SD) 31.3 ± 13.0], who, had a history of hospitalization for dengue during 2007–2010 and were laboratory confirmed by dengue specific IgM capture ELISA were included in the study. Among the 120 dengue cases, 73 were males and 47 were females. Based on at least two of the DHF defining criteria of the World Health Organization (1999) (WHO, 1999) 33 cases had DHF [mean age ± SD 30.5 ± 14.7] and 87 cases had DF [mean age ± SD 31.6 ± 12.3]. One hundred and nine apparently healthy controls (HCs) were also included [mean age ± SD 29 ± 8.0]. Healthy controls included 67 males and 42 females. The study was approved by the institutional ethics committee, and a written informed consent was obtained from the participants before blood collection. All the participants were from a Marathi speaking population living in and around Pune, Maharashtra, Western India and were not related to each other. DENV is endemic in Pune and all the serotypes are in circulation.

Genotyping of TLR3/7/8 and TIRAP polymorphisms

Genotyping of *TLR3* (rs3775291), *TLR8* (rs3764879 & rs3764880) and *TIRAP* (rs8177374) gene SNPs were investigated using commercially available Taqman genotyping assays (Applied Biosystems) using the protocol as described by the manufacturer. *TLR7* (rs179008) was genotyped using a PCR-RFLP based procedure as described earlier (Pierik et al., 2006).

Statistical analysis

Genotype frequency distributions were tested for their confirmation to Hardy–Weinberg equilibrium using the Chi square test. Allele and genotype frequencies were compared between different study groups using the Chi square test or Fisher's exact test. For genotypic associations, *P* values and odds ratio (OR) adjusted for gender and age were calculated by logistic regression analysis. The significant *P* values were further multiplied by the number of SNPs studied to derive *Pc* values. For *TLR3* and *TIRAP* combined genotypes, the *P* values were further corrected for multiple comparisons using Bonferroni correction by multiplying the *P* value with number of combined genotypes and expressed as *Pc* values. Since *TLR7* and *TLR8* genes were located in the X chromosome, analysis was done separately for males (one X chromosome) and females (two X chromosomes). For *TLR8*, in males, due to the presence of haploid X chromosome, haplotypes were directly inferred from the genotyping results. However, in females, the haplotype frequencies were inferred and compared between the study groups using SNPstats web server (Sole et al., 2006). All statistical analyses were performed using the SPSS software version 18. A *P* value of less than 0.05 was considered statistically significant. Functions of the SNPs were predicted using SNPinfo web server (Xu and Taylor, 2009).

Results

Properties of single nucleotide polymorphisms as predicted by SNPinfo web server

Among the five SNPs studied, 4 are non synonymous polymorphisms. Results from SNPinfo web server revealed that *TLR7* rs179008 and *TLR8* rs3764880 are predicted to affect splicing of exons while *TLR8* rs3764879 is known to affect the binding of transcription factors. *TLR3* rs3775291 is predicted to have a possibly damaging effect on the protein structure. Though, *TIRAP* rs8177334 is a non synonymous polymorphism, the SNP is predicted to have a benign effect on the protein structure (Table 1).

TLR3 and TIRAP gene polymorphisms

The genotype frequency distributions of all SNPs confirmed to Hardy–Weinberg equilibrium (HWE) in healthy controls and dengue patient groups ($P > 0.05$) except for *TIRAP* rs8177374 in DEN group ($P < 0.05$). However, when the patients were categorized into DF and DHF, *TIRAP* rs8177374 genotype frequencies in both the groups confirmed to HWE ($P > 0.05$).

Frequency of *TLR3* rs3775291 C/T genotype was significantly lower in DHF as compared to DF and HC [DHF vs. DF OR with 95% CI 0.287 (0.110–0.753); DHF vs. HC OR with 95% CI 0.390 (0.154–0.989)]. The frequency of rs3775291 T allele was significantly lower in DHF compared to DF and HC [DHF vs. DF OR with 95% CI 0.390 (0.160–0.880); DHF vs. HC OR with 95% CI 0.410 (0.170–0.900)]. The carrier frequency of rs3775291 T allele (C/T + T/T) was significantly lower in DHF as compared to DF and HC [DHF vs. DF OR with 95% CI 0.288 (0.115–0.722); DHF vs. HC OR with 95% CI 0.393 (0.162–0.956)] (Table 2).

Significantly higher frequency of *TIRAP* rs8177374 'C/T' genotype [DHF vs. HC OR with 95% CI 2.643 (1.167–5.986)] and T allele carriers [DHF vs. HC OR with 95% CI 2.486 (1.099–5.628)] were observed in DHF cases compared to HC (Table 2). Analysis of combination of *TLR3* and *TIRAP* gene variants revealed higher frequency of C/C–C/T genotype [DHF vs. HC OR with 95% CI 3.54 (1.30–9.44); DHF vs. DF OR with 95% CI 3.95 (1.36–11.35)] and lower frequency of C/T–C/C genotype [DHF vs. HC OR with 95% CI 0.330 (0.08–1.05)] in DHF compared to HC (Table 2).

TLR7 and TLR 8 gene polymorphisms

TLR7 rs179008 polymorphism was observed at a very lower frequency in the study population. *TLR8* rs3764879 and rs3764880 were in complete linkage disequilibrium as observed by the presence of only two haplotypes. In males, higher frequency of C-A haplotype was observed in DF cases compared to healthy controls [DF vs. HC OR with 95% CI 2.185 (1.101–4.336)] while the frequency of G-G haplotype was higher in DHF cases as compared to DF cases [DHF vs. DF OR with 95% CI 2.644 (0.964–7.249)] (Table 3).

Discussion

In vitro studies have shown that activation of TLR3/7/8 is an important component of the innate immune response against DENV. Functioning of TLRs are affected by SNPs in the TLR genes and have been shown to be associated with HIV-1 and HSV-2 infections, viral encephalitis, and mortality in Crimean Congo hemorrhagic fever (CCHF) (Sironi et al., 2012; Svensson et al., 2012; Kindberg et al., 2011; Barkhash et al., 2013). In the present study, functional variations in the *TLR3/7/8* genes were investigated in a group of patients hospitalized for DF, DHF and healthy individuals. The results suggest that the T allele of *TLR3* rs3775291 is associated with decreased risk of DHF in a dominant mode. The 'T' allele codes for phenylalanine and has been previously shown to be associated with protection against HIV-1 and HSV-2 infections, severe TBEV disease and geographic atrophy, a type of age related macular degeneration (Sironi et al., 2012; Svensson et al., 2012; Kindberg et al., 2011; Barkhash et al., 2013; Zhou et al., 2011). Although, there are contradicting results with regard to the effect of rs3775291 on the expression of TLR3, reverse electrophoretic mobility shift assay

Please cite this article in press as: Alagarasu, K., et al., Polymorphisms in RNA sensing toll like receptor genes and its association with clinical outcomes of dengue virus infection. Immunobiology (2014), http://dx.doi.org/10.1016/j.imbio.2014.09.020