Infection, Genetics and Evolution 43 (2016) 412–417

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Research paper

Analysis of TLR4 (Asp299Gly and Thr399Ile) gene polymorphisms and mRNA level in patients with dengue infection: A case-control study

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ARTICLE INFO

Article history:
Received 10 February 2016
Received in revised form 7 June 2016
Accepted 10 June 2016
Available online 11 June 2016

Keywords:
Dengue
Genetic susceptibility
Humans
Haplotypes
Polymorphism

ABSTRACT

Background: Dengue is a systemic viral infection that spreads to humans by the bite of infected *Aedes* mosquitoes. The secreted NS1 protein of dengue virus activates macrophages and human PBMCs via TLR4 and induce the release of pro-inflammatory cytokines which is responsible for the pathogenesis of disease. Mutations in TLR4 gene have been associated with the increased susceptibility to many viral, bacterial and parasitic diseases.
Objective: To study the impact of TLR4 Asp299Gly (rs4986790) and Thr399Ile (rs4986791) gene polymorphisms with susceptibility to dengue infection.
Methods: A total of 120 dengue infected (57; DHF/DSS and 63; DF) and 200 healthy controls were included in the study. TLR4 Asp299Gly and Thr399Ile gene polymorphisms was studied by PCR-RFLP. Expression of TLR4 mRNA was evaluated by rRT-PCR.
Results: Individuals with heterozygous genotype for TLR4 Asp299Gly and Thr399Ile polymorphisms had increased susceptibility to dengue infection (OR-1.70, 95% CI = 1.01–2.86 $P = 0.042$ and OR-2.17, 95% CI = 1.10–4.28, $P = 0.024$, respectively). The frequency of Gly and Ile alleles were higher in dengue patients as compared to controls (OR-1.67, 95% CI = 1.05–2.64, $P = 0.029$ and OR-2.20, 95% CI = 1.19–4.07, $P = 0.011$, respectively). Ile/Gly haplotype was associated with the risk of the disease when compared with controls (OR = 3.15, 95% CI = 1.09–9.09, $P = 0.035$). The mRNA expression was higher in DF when compared with DHF/DSS and controls ($P = 0.040$ and 0.009, respectively).
Conclusion: A higher expression of TLR4 mRNA was associated with DF. The TLR4 Asp299Gly and Thr399Ile gene polymorphisms were associated with the susceptibility of dengue infection probably by altering the immune response.

1. Introduction

Dengue is a well-known systemic viral infection caused by dengue virus serotype (1–4). It spreads in humans by the bite of infected *Aedes* mosquitoes (*Ae. aegypti* and *Ae. albopictus*). The spread of dengue virus and its pathogenesis depends on effective immune response. Alteration in immune response leads to an increase in viremia and pathogenesis of dengue infections (Clyde et al., 2006; Coffey et al., 2009). Dengue infection manifest clinically as dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). As per WHO criteria, DF is defined as an acute febrile illness associated with two or more manifestations such as headache, *retro*-orbital pain, myalgia, arthralgia, rash which lasts for 2–7 days. DHF is diagnosed when the patient presents with persistent high fever, pleural effusion, ascites, hemoconcentration (>20%), and thrombocytopenia (<100.000). DSS is defined using DHF criteria associated either hypotension or narrow pulse pressure (>20 mm Hg) in the presence of clinical signs of shock, e.g., slow capillary filling and cold clammy skin (WHO, 1997).

There are several factors associated with pathogenesis of dengue infection and its transformation from mild (DF) to severe form (DHF/DSS). The major factors associated with dengue pathogenesis are viral strain virulence, host immune status and host genetic factors (Tee et al., 2009). Innate immune response plays a crucial role in the determination of dengue pathogenesis as dengue virus targets innate immune cells like monocyte, dendritic cells and endothelial cells (Hall et al., 1991; Jessie et al., 2004; Scott et al., 1980). When dengue virus interact with monocytes/macrophage, these cells produce proinflammatory mediators such as TNF-α and nitric oxide through Toll-like receptors 4 (TLR4) activation (Braga et al., 2001). TLR4 is known to recognize bacterial LPS but it also interacts with dengue virus NS1 protein and activates

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http://dx.doi.org/10.1016/j.meegid.2016.06.027
1567-1348/

the peripheral blood mononuclear cells (PBMCs) followed by disruption of endothelial cell monolayer (Modhiran et al., 2015). In TLR4, two non-synonymous single nucleotide polymorphisms (SNPs) have been described that change the ligand-binding site of the TLR4. These two SNPs show an amino acid substitution of Gly in place of Asp at 299 position and Ile in place of Thr at 399 position (Chanock et al., 2007), and increase susceptibility to innate immune diseases (Bustamante et al., 2008). Therefore, we studied the TLR4 Asp299Gly (rs4986790) and Thr399Ile (rs4986791) gene polymorphisms in dengue infected and healthy control subjects.

2. Materials and methods

2.1. Study design and study population

The present study was conducted at Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, during 2013 outbreak. Patients coming to emergency department and general hospital of SGPGIMS were enrolled in the study. Patients were diagnosed to have dengue infection as per WHO guidelines (Pinheiro and Corber, 1997; WHO, 1997; Yeh et al., 2006). Written information consent was taken through a questionnaire from the patient or from the patient parent (in the case of minors) regarding the clinical information and their residing areas. SGPGIMS is a tertiary care hospital and patients came from different parts of the country. Therefore, according to the questionnaire, only those who belong to North Indian ethnicity were included in the present study. The control population was a group of healthy volunteers coming to the blood bank of SGPGIMS, Lucknow. They all belong to the same ethnicity, age, and gender with neither signs nor a previous history of dengue infection. Individuals with other viral symptoms were excluded from the study. The mean age of 120 dengue patients was 33.47 ± 14.66 years and the male to female ratio was 3:1. The mean age of 200 controls was 32.54 ± 9.22 years and the male to female ratio was 7:3.

2.2. Definition of cases and controls

Blood was drawn once from the patients after the onset of symptoms (2–11 days). We collected 2 ml blood in a serum separator tube. Further, it was allowed to stand at room temperature for 30 min for complete coagulation. Subsequently, tube was centrifuged at 1000 ×*g* for 10 min at 4 °C for separatoin of serum. Aliquots of serum (100 μl) were transferred to 1.5 ml centrifuge tube and stored at −80 °C for further use. Dengue infection was confirmed by clinical symptoms and signs as per WHO guidelines (Pinheiro and Corber, 1997; WHO, 1997; Yeh et al., 2006) and detection of NS1 antigen, IgM and IgG antibodies by ELISA (Bio-Rad, Marnes-La-Coquettee, France) in serum.

Table 1
Haematological and biochemical parameters in patients with dengue infection.

Variables	(mean ± SD)		*P*-value
	DHF/DSS ($N = 57$)	DF ($N = 63$)	
Haemoglobin	12.77 ± 2.7	12.13 ± 2.03	0.700
Hematocrit (%)	39.05 ± 4.59	34.26 ± 5.89	<0.001
Platelet Count (counts X 10^3 /mm^3)	64,108.7 ± 52,954.58	135,339.6 ± 36,281.99	<0.001
SGOT U/l	271.80 ± 391.57	78.21 ± 73.88	<0.001
SGPT U/l	160.98 ± 194.22	90.34 ± 93.90	0.020
Serum Bilirubin	0.64 ± 0.58	0.48 ± 0.52	0.370
Alkaline Phosphate	87.84 ± 72.31	95.71 ± 56.4	0.214

Unpaired *t*-test was applied to compare the mean of multiple variables in DHF/DSS and DF patients, and $P \leq 0.05$ was considered as significant.
SGOT, serum glutamic oxaloacetic transaminase; SGPT, Serum glutamic pyruvic transaminase.

2.3. Genotyping of TLR4 Asp299Gly and Thr399Ile SNPs

We also collected 3 ml blood from each patient by venipuncture in a vacutainer tube containing 15% EDTA solution for DNA and RNA extraction. Genomic DNA was isolated from EDTA –anti coagulated blood samples using Pure Link Genomic DNA Mini Kit (Invitrogen, Carlsbad, USA) as per manufacturer's instruction. Genotyping for TLR4 variants were carried out using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Lorenz et al., 2001). All the PCR amplification reactions were performed in 20 μl volume containing 10 μl 2× PCR master mix (Fermentas, Vilnius, Lithuania), 0.1 mM of each primer and 100 ng of extracted DNA. DNA was amplified by initial denaturation at 95 °C for 3 min, followed by 35 amplification cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 35 s followed by a final extension at 72 °C for 5 min. Amplification products were digested by *Nco*I and *Hin*fI restriction endonucleases (Fermentas, Vilnius, Lithuania) for TLR4 Asp299Gly and Thr399Ile, respectively, and separated on 3% agarose gel followed by ethidium bromide staining. To validate the genotyping, 10% of samples were re-genotyped by other laboratory personnel and genotyping results were reproducible with no discrepancy. All the experiments were performed in duplicate and repeated twice for the confirmation of PCR-RFLP results.

2.4. Extraction of RNA, cDNA synthesis and real-time reverse transcription–polymerase chain reaction (rRT-PCR)

Total RNA was extracted from blood samples using Trizol reagent as per the manufacturer's instruction (Ambion, Carlsbad, USA). cDNA was synthesized using high capacity cDNA reverse transcription kit as per the manufacturer's protocol (Applied Biosystem, Carlsbad, USA). Following cDNA synthesis, rRT-PCR was performed in ABI 7500 Real Time PCR system, using TaqMan Assays-on Demand reagents (Applied Biosystems, Carlsbad, USA), to quantify mRNA levels of TLR4. Comparisons of gene expressions were calculated after normalizing cycle thresholds against the housekeeping gene GAPDH and presented as the relative fold change by $2^{-\Delta Ct}$ comparative Ct method (Schmittgen and Livak, 2008).

Table 2
Genotypic and allele frequencies of Toll-Like Receptor-4 polymorphisms (Asp299Gly and Thr399Ile) among dengue infected cases and control subjects.

TLR4 gene polymorphisms	No. of subjects (%)		P-value	OR (95% Cl)
	Patients ($N =$ 120)	Control ($N =$ 200)		
Asp299Gly genotype				
Asp/Asp	81 (67.5)	157 (78.5)	–	Ref.
Asp/Gly	37 (30.8)	42 (21.0)	0.042	1.70 (1.01–2.86)
Gly/Gly	2 (1.7)	1 (0.5)	0.272	3.89 (0.34–43.39)
Allele				
Asp	199 (82.9)	356 (89.0)	–	Ref.
Gly	41 (17.1)	44 (11)	0.029	1.67 (1.05–2.64)
Thr399Ile genotype				
Thr/Thr	97 (80.8)	181 (90.5)	–	Ref.
Thr/Ile	21 (17.5)	18 (9.0)	0.024	2.17 (1.10–4.28)
Ile/Ile	2 (1.7)	1 (0.5)	0.285	3.73 (0.33–41.68)
Allele				
Thr	215 (89.6)	380 (95.0)	–	Ref.
Ile	25(10.4)	20 (5.0)	0.011	2.20 (1.19–4.07)

P-value was determined by χ^2 test and $P \leq 0.05$ was considered as significant. OR, odds ratio; CI, confidence interval.