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Contribution of Toll like receptor polymorphisms to dengue susceptibility and clinical outcome among eastern Indian patients

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ABSTRACT

Dengue infection has been one of the major public health concerns in India causing simple dengue fever (DF) to severe dengue infection. In the present study, contribution of TLR3, 7 and 8 polymorphisms towards dengue disease susceptibility and severity among Eastern Indian patients was analysed. Genomic DNA was extracted from blood of 201 dengue infected patients and 157 healthy individuals, followed by genotyping of eight polymorphisms of TLR3 (rs3775290), TLR7 (rs5741880, rs3853839, rs179008 and rs179010) and TLR8 (rs3764879, rs3764880 and rs5744080) genes by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Functional analyses of the polymorphisms were predicted. Genotypic association of polymorphisms, alone and in combination, with dengue disease susceptibility and development of WHO-defined warning signs among patients was calculated by using SPSS software. TLR7-rs179008 & TLR8-rs3764880 were implicated to be non-synonymous polymorphisms. Specific genotypes of majority of the analysed TLR polymorphisms exhibited significant positive association with disease susceptibility. CC/C and AA/A of TLR7-rs179008 ($p < 0.0001$) and TLR8-rs3764880 ($p < 0.00001$) respectively were significantly associated with development of warning signs among dengue infected patients. Particular genotypic combinations of rs3853839-rs5744080 and rs179008-rs3764880 increased the risk of dengue infectivity, whereas, presence of last combination was more prevalent among dengue patients with warning signs. Thus these polymorphic variants of TLR3, 7 and 8 might act as potential prognostic biomarkers for predicting disease severity among dengue virus infected patients.

1. Introduction

Dengue infection has been one of the major public health concerns in India and other tropical countries worldwide, which is increasingly spreading in new geographical locations (Dengue, 2009). This infection is transmitted by infected *Aedes aegypti* vector, which results in diverse clinical outcomes ranging from simple febrile illness of dengue fever (DF) to severe dengue infection. World Health Organisation (WHO) has categorized the large group of non-severe dengue patients into two subgroups - patients without and with warning signs; the latter subgroup being at higher risk of developing severe dengue. Several studies from the Americas and South-east Asian countries have proposed presence of any warning signs among non-severe dengue cases to be strongly associated with severe dengue disease outcome (Leo et al., 2013; Horstick et al., 2015; Alexander et al., 2011).

Though the reasons behind such variations in clinical outcome among dengue virus (DENV) infected individuals remain poorly understood, host immunity and genetic predisposition might play crucial role in such differential disease pathogenesis (Yacoub et al., 2013; Chen et al., 2015). Host innate immune system receptors, mainly Toll-like receptors (TLRs) are the key sensors to primarily recognise any viral genomic RNA within patient-body. In general, endosomally-localized TLR3, TLR7, TLR8 and cytoplasmic RIG like receptors recognize viral RNA genome (Bustos-Arriaga et al., 2011). Thus, genetic variations in TLR genes viz. single nucleotide polymorphisms (SNPs) might influence the innate immune responses towards pathogenic challenges and affect the dengue disease susceptibility and clinical outcome among infected individuals. Several polymorphisms of TLR genes viz. rs3775290, rs179008, rs179010, rs5741880, rs3853839, rs3764879, rs3764880 and rs5744080 have been reported to be associated with hepatitis-B (HBV), hepatitis-C (HCV), chikungunya, dengue, HIV, Cytomegalo and Crimean–Congo Hemorrhagic fever virus infectivity among French, German, Spanish, Brazilian, Chinese, Japanese, Polish, Moroccan and Turkish populations (Dutta and Tripathi, 2017; Studzińska et al., 2017; Goktas et al., 2016; Huang et al., 2015; Fakhir et al., 2017; Valverde-Villegas et al., 2017; Askar et al., 2010; Zhu et al., 2017; Wang et al.,

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2014; Alagarasu et al., 2015a). Variations in genotype of rs3764879 within TLR-8 gene have been predicted to modulate patient's immune responses during HCV infection (Wang et al., 2014). The G allele and A/G genotype of rs3764880 has been related to clearance of HCV infection and protection from progression of HIV infection (Fakhir et al., 2017; Oh et al., 2008). All these studies indicated the importance of TLR polymorphisms towards viral disease infectivity and disease pathogenesis among different ethnic human populations.

First virologically proved dengue epidemic occurred in Kolkata and Eastern Coast of India in 1963–1964 and afterwards several massive outbreaks took place in Kolkata (West Bengal, Eastern India) (Gupta et al., 2012). Also recently, dengue infection has been detected among 54–61% febrile patients from several recent outbreaks in Eastern India during the last five years (Pal et al., 2014; Mukherjee et al., 2017).

All these previous data indicated the importance of host genetics of Eastern Indian patient population towards dengue disease susceptibility. In the present study, attempts have been made to understand the contribution of TLR3, 7 and 8 polymorphisms towards dengue disease susceptibility and development of disease severity among infected Eastern Indian patients.

2. Materials and methods

2.1. Ethical approval

All procedures performed in this study involving collection of blood from human participants as well as healthy controls were in accordance with ethical standards of Clinical Research Ethical Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013) and with that of the 1964 Helsinki Declaration and its later amendments. Prior to participation in the study, written consents were obtained from patients and healthy control individuals.

2.2. Patients and healthy individuals

After obtaining institutional ethical committee approval, 5 ml of blood samples were collected from each of 425 symptomatic febrile patients (within 0–7 days of symptomatic onset), visiting Calcutta School of Tropical Medicine, Institute of Post Graduate Medical Education and Research and Medical College Hospital, Kolkata, West Bengal, India from July 2014 to October 2016. All patients exhibiting fever along with any of the following symptoms: headache, body ache, myalgia, arthralgia, rash, with or without haemorrhagic manifestation, were selected as per WHO criteria of dengue infection. Among those 425 symptomatic febrile patients, 201 (47.29%) were positive for dengue infection by anti-dengue-IgM ELISA/dengue-NS1ELISA/quantitative RT-PCR. Symptoms of dengue patients were also monitored after next 7 days (symptoms monitored during first and subsequent visit to hospital) and patients were categorised into two groups: with and without warning signs, according to WHO classification. Blood from healthy age-matched unrelated individuals of same ethnicity (n = 157), without any signs and history of other infections and without dengue infection as tested by anti-dengue-IgM ELISA/dengue-NS1 ELISA/quantitative RT-PCR, were collected from the same community, to carry out case control study, as described previously (Dutta and Tripathi, 2017).

2.3. SNP selection and genotyping

SNPs in the TLR genes, TLR3 (rs3775290), TLR7 (rs179008, rs5741880, rs179010, rs3853839) and TLR8 (rs5744080, rs3764879, rs3764880) were selected based on their minor allele frequencies (MAF) and previously reported associations with other viral infections (Dutta and Tripathi, 2017; Studzińska et al., 2017; Goktas et al., 2016; Huang et al., 2015; Fakhir et al., 2017; Valverde-Villegas et al., 2017; Askar et al., 2010; Zhu et al., 2017; Wang et al., 2014; Alagarasu et al., 2015b). SNP genotyping was carried out by using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based procedure. Briefly, genomic DNA was extracted from peripheral blood samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Seven primer pairs were designed using GenBank database sequences and Primer3 software, to amplify rs3775290 polymorphic region of TLR3; rs179008, rs5741880, rs179010, rs3853839 polymorphisms of TLR7 and rs5744080, rs3764879, rs3764880 polymorphisms of TLR8 (Table S1). PCR was carried out in 20 µl reaction volume as mentioned previously (Dutta and Tripathi, 2017). The respective PCR products were digested with TaqI (Himedia, India), Bsh1285I, ER1381, MvaI, Eco130I, TaiI, Hin1II (Fermentas, USA) and Hpy188I (NEB, UK) as mentioned in Table S1, followed by agarose gel electrophoresis. Various RFLP band patterns were confirmed by sequencing the PCR products by using ABI Prism Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) in ABI-Prism 3100 Avant Genetic Analyser (Applied Biosystems, USA).

2.4. Statistical analysis

Association of any particular genotype of respective SNPs with dengue disease susceptibility and specific dengue symptoms (WHO-defined with/without warning signs) was calculated by using SPSS software version 22. Allele and genotype frequencies were compared between different study groups using Pearson's Chi square test. For genotypic associations, *p*-values, odds ratio (OR) & minor allele frequency (MAF) were calculated. A p-value of < 0.05 was considered statistically significant. For controls, Hardy-Weinberg equilibrium was analysed for eight polymorphisms with the programme Haploview (Barrett et al., 2005). The LDlink 3.0 program of National Institute of Health (NIH) was used for Linkage disequilibrium (LD) measurements (D′) by using south Asian population data, present within 1000 genome project of Genome Wide Association Studies (GWAS), the largest public catalogue of human variation and genotype data (Machiela and Chanock, 2015). Functions of the SNPs were predicted using SNPinfo web server and effect of amino acid changes in case of non-synonymous polymorphisms was predicted by using SIFT, Polyphen2, MutationTaster and by G23D softwares.

3. Results

In this case-control study, genetic polymorphism of TLR genes was investigated among 201 dengue infected patients, collected during 2014–2016 dengue outbreaks in eastern India along with age-sex matched 157 healthy control volunteers from the same region (Table S2). The male to female ratio of dengue patients was 1:1.27, whereas that of the control group was 1:1.01. Mean age of dengue patients was 32.01 ± 14.7 years (range: 5–70 years), whereas that of control was 36.2 ± 11.8 years (range: 18–63 years). Among 201 dengue patients, 126 were positive by qRT-PCR, 144 by anti-dengue-IgM and 63 by dengue NS1 ELISA. Among PCR positive dengue patients, Dengue serotype 2 was most prevalent, followed by serotype 4 (Table S2). At the time of blood collection, major symptoms among dengue patients were myalgia (61.69%), arthralgia (41.25%), headache (32.33%), rash (26.86%), nausea (25.37%), persistent vomiting (23.88%), abdominal pain (19.90%) joint swelling (11.94%), bleeding (6.96%) and retro-orbital pain (6.46%). Among these dengue infected patients, 30.84% (62/201) exhibited WHO classified Warning signs, whereas 69.16% (139/201) were without any Warning signs. DENV serotypic distribution among patients with and without warning signs was similar (Fig. S1).

3.1. Prediction for TLR3, 7 & 8 polymorphisms

According to SNPinfo web server, two polymorphisms (rs179010 and rs3764879) might affect the transcription factor binding sites of

Table 1
(a) Genetic characterization of TLR polymorphisms and (b) predicted effect of non-synonymous TLR polymorphisms on protein structure.

(A)

Gene	SNP ref. No.	Chromosomal Location	Position	Alleles	Transcription factor binding site	Affects splicing regulation	Non-synonymous polymorphism
TLR3	rs3775290	Chr. 4	187241211	T/C	–	–	–
TLR7	rs5741880	Chr. X	12797337	G/T	–	–	–
	rs179010	Chr. X	12812806	C/T	Yes	–	–
	rs179008	Chr. X	12813580	A/C	–	–	Yes
	rs3853839	Chr. X	12817579	C/G	–	–	–
TLR8	rs3764880	Chr. X	12834747	A/G	–	ESE[†]/ESS[‡] and abolishes domain	Yes
	rs5744080	Chr. X	12834618	C/T	–	–	–
	rs3764879	Chr. X	12847725	C/G	Yes	–	–

(b)

Gene	SNP ref. No.	Amino acid change	Effect				
			SIFT prediction	Polyphen2 prediction	MutationTaster prediction	FATHMM prediction	MetaSVM prediction
TLR7	rs179008	gly11leu	Tolerated	Benign	Neutral	Tolerated	Tolerated
TLR8	rs3764880	met1val	Damaging	Benign	Polymorphism	Tolerated	Tolerated

[†] ESE = Exonic splicing enhancer.
[‡] ESS = Exonic splicing silencer.

TLR7 and 8 genes respectively (Table 1). The rs3764880 polymorphism might affect the splicing activity of TLR8 due to its location near exon-intron junction. The TLR7-rs179008 (gly11leu) and TLR8-rs3764880 (met1val) were predicted to be non-synonymous polymorphisms. The rs3764880 was also predicted to be damaging by SIFT server. Except one SNP of TLR7 (rs3853839), remaining seven polymorphisms followed Hardy-Weinberg equilibrium at $p > 0.05$. However data of rs3853839 was incorporated in this study as its genotypic distribution might arise by chance or could reflect a significant association with dengue disease symptoms. The pair-wise LD measuring D′ for the seven TLR7 and TLR8 SNPs indicated that TLR8 polymorphisms, rs3764879 and rs3764880 were in complete linkage disequilibrium, with R^2: 0.969, D′: 1.0 and *p*-value: < 0.01 (Fig. 1).

3.2. Genotypic association of the polymorphisms with dengue disease susceptibility

Systematic analysis revealed that CC genotype of TLR3-rs3775290 was significantly positively associated with dengue disease susceptibility [OR = 4.15; 95% CI 1.86–9.24; p = 0.0002] (Table 2). The distribution of CC genotype was more than three-fold among patient population compared to control group. The rs3775290 C-allele was found to be significantly associated with dengue disease susceptibility. Similarly, CC genotypes of TLR7-rs179008 and TLR7-rs3853839, were found to be significantly associated with dengue disease susceptibility [rs179008: OR = 2.89, 95% CI 1.20–6.94, p = 0.01; rs3853839: OR = 2.01, 95% CI 1.23–3.25, p = 0.004]. Compared to healthy individuals, the frequency of CC genotype of TLR7-rs179008 was more than double among dengue infected patients. The C-allele of each of TLR7-rs179008 and TLR7-rs3853839 were found to be significantly associated with the disease. The GT genotype of TLR7-rs5741880 and CT genotype of TLR7-rs179010 were significantly related with decreased risk of dengue infection.

In case of TLR8 gene, AA genotype of TLR8-rs3764880 and CC genotype of TLR8-rs5744080 demonstrated significant association with dengue disease susceptibility [rs3764880: OR = 2.58, 95% CI 1.32–5.07, p = 0.004 and rs5744080: OR = 2.49, 95% CI 1.30–3.36, p = 0.001] (Table 2). The G-allele of TLR8-rs3764880 and C-allele of TLR8-rs5744080 exhibited significant association with dengue infectivity. Frequency of TLR8-rs3764880-AG genotype markedly decreased in disease population compared to healthy individuals, indicating significant association of this genotype with lower risk of dengue infection.

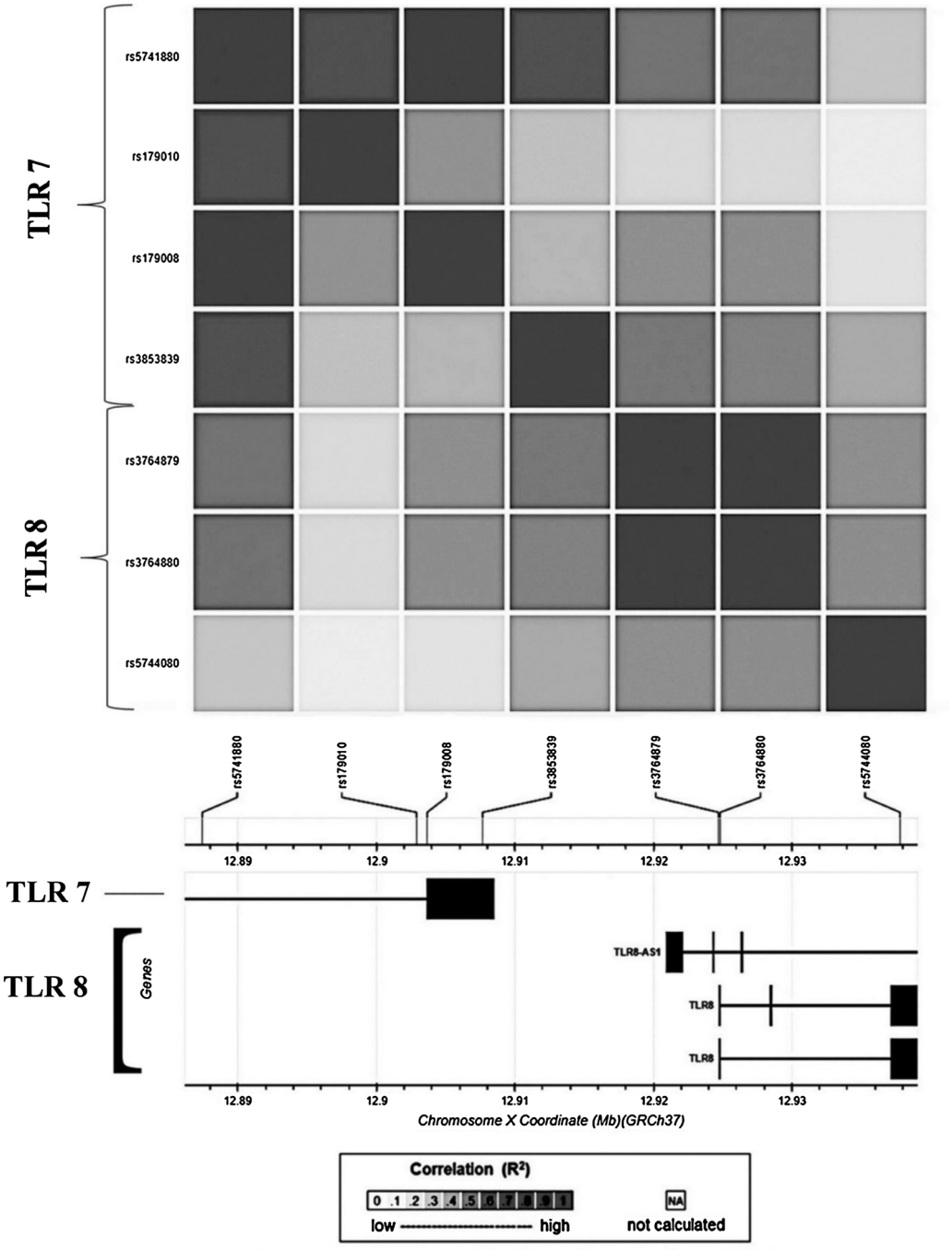
As TLR7 and 8 genes are located on X chromosome, any gender biasness of these SNPs among DENV patients needed to be analysed. The frequency of C genotype of rs179008 and rs3853839 polymorphisms of TLR7 and rs5744080 polymorphisms of TLR8 was significantly higher among DENV male patients than that of the control males (Table 2). The TLR7-rs3764880-AG and TLR8-rs179008-AC genotypes were present in significantly higher frequency among healthy female controls compared to female dengue patients. The TLR8-rs5744080-CC genotype was more prevalent among female dengue infected patients.

3.3. Genotypic association of polymorphism-combinations with disease susceptibility and severity

Various genotypes of SNPs of TLR7 and TLR8 that significantly associate with dengue infection were further analysed to find out their combined effect on enhancement of dengue disease susceptibility (Table 3a). Statistical analysis revealed that combination of CC/C-CC/C of TLR7-rs3853839-TLR8-rs5744080 [OR = 6.59, 95% CI 0.81–53.25, p = 0.042] and CC/C-AA/A of TLR7-rs179008-TLR8-rs3764880 [OR = 7.31, 95% CI 0.91–58.34, p = 0.028] were significantly higher among patient population compared to healthy control group.

3.4. Correlation between SNP-genotypes with clinical symptoms and WHO-defined warning signs

To determine the correlation between different SNP-genotypes with dengue disease symptoms, Pearson's Chi square test was performed (Table 4). The CC genotype of TLR3-rs3775290 and TT/T of TLR7-rs5741880 exhibited significant association with development of rash among infected patients compared to reference genotypes of respective polymorphisms. The CT genotype of TLR7-rs179010 and AA/A of TLR8-rs3764880 exhibited significant association with development of myalgia among dengue infected patients. Only CC/C of TLR7-rs179010 was significantly correlated with arthralgic manifestation of the patients. The TT/T and GT of TLR7-rs5741880, CC/C of both TLR7-rs179010 and TLR7-rs179008 and AA/A of TLR8-rs3764880 demonstrated significant correlation with development of headache among patients. The CC genotype of TLR3-rs3775290 and CC/C of TLR7-rs179010, TLR7-rs179008 and TLR8-rs5744080, TT/T of TLR7-rs5741880 and AA/A of TLR8-rs3764880 demonstrated significant association with persistent vomiting of dengue patients. The CC/C of



rs_number	rs5741880	rs179010	rs179008	rs3853839	rs3764879	rs3764880	rs5744080
rs5741880	1.0	0.115	0.015	0.093	0.078	0.074	0.017
rs179010	0.115	1.0	0.042	0.163	0.088	0.078	0.016
rs179008	0.015	0.042	1.0	0.031	0.049	0.051	0.009
rs3853839	0.093	0.163	0.031	1.0	0.489	0.465	0.245
rs3764879	0.078	0.088	0.049	0.489	1.0	0.969	0.308
rs3764880	0.074	0.078	0.051	0.465	0.969	1.0	0.322
rs5744080	0.017	0.016	0.009	0.245	0.308	0.322	1.0

Fig. 1. Pairwise linkage disequilibrium pattern of seven SNPs in TLR7 & TLR8 genes from South Asian BEB data from 1000 genome project.
Legends: Genomic location of these genetic polymorphisms on chromosome X. Dark colour indicated strong linkage disequilibrium between SNPs. Pairwise LD (R2) is shown for each combination of SNPs in table form.

TLR7-rs179008 and AA/A of TLR8-rs3764880 were strongly associated with development of abdominal pain among dengue infected patients. The CC genotype of TLR3-rs3775290 and CC/C of TLR7-rs179008 and AA/A of TLR8-rs3764880 exhibited significant association with haemorrhagic manifestation of dengue patients. The TT/T of TLR7-rs5741880, CC/C of TLR7-rs179008 and AA/A of TLR8-rs3764880 were significantly related with development of retro-orbital pain among the patients. Overall, the CC/C of TLR7-rs179008 and AA/A of TLR8-rs3764880 exhibited significant association with WHO-defined dengue warning signs viz. persistent vomiting, abdominal pain and

Table 2
Genotypic and allelic distribution of TLR3, 7 and 8 polymorphisms among Dengue patients and healthy controls.

SNP Ref. No.	Chromosomal Location	Genotype/ Haplotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
TLR 3 rs3775290	Chr.4		n = 157	n = 192 NA = 9				
		CC	5.09	18.22	4.15 [1.86 - 9.24]	0.0002*		
		TT	33.75	31.77	0.91 [0.58 - 1.43]	0.69		
		CT	61.14	50	ref			
		C allele	35.66	43.22	2.13 [1.58 – 2.86]	0.04*	0.35	0.43
		T allele	64.33	56.77	0.728 [0.53 - 0.98]			
		MALE	n = 78	n = 113				
		CC	6.41	21.23	3.93 [1.43-10.83]	0.005		
		TT	28.20	28.31	1 [0.59-1.90]	0.98		
		CT	65.30	50.44	ref			
		FEMALE	n = 79	n = 79				
		CC	3.9	13.92	4.09 [1.09-15.30]	0.02*		
		TT	39.24	36.70	0.89 [0.47-1.70]	0.74		
		CT	56.96	49.36	ref			
TLR 7 rs5741880	Chr. X		n = 157	n = 196 NA = 5				
		TT	12.73	13.26	1.04 [0.56 – 1.95]	0.88		
		GT	11.46	5.61	0.45 [0.21 – 1.00]	0.046*		
		GG	75.79	81.12	ref			
		G allele	81.52	83.92	1.18 [0.79 – 1.75]	0.40	0.18	0.16
		T allele	18.47	16.08	0.84 [0.57 – 1.25]			
		MALE	n = 78	n = 109				
		T	25.64	20.18	0.73 [0.36-1.46]	0.37		
		G	74.35	79.81	ref			
		FEMALE	n = 79	n = 87				
		TT	0	4.59	3.90 [0.42-35.67]	0.19		
		GT	22.27	12.64	0.49 [0.21-1.11]	0.08		
		GG	77.21	82.75	ref			
TLR 7 rs179010	Chr. X		n = 157	n = 196 NA = 5				
		CC	30.57	34.18	0.98 [0.72 – 1.32]	0.47		
		C T	17.83	9.69	1.01 [0.75 – 1.38]	0.02*		
		TT	51.5	56.12	ref			
		C allele	39.49	39.03	1.07 [0.77 – 1.47]	0.90	0.39	0.39
		T allele	60.50	60.96	0.93 [0.67 – 1.28]			
		MALE	n = 78	n = 112				
		C	48.71	44.64	0.84 [0.47-1.51]	0.73		
		T	51.28	55.35	ref			
		FEMALE	n = 79	n = 84				
		CC	12.6	20.23	1.75 [0.74-4.09]	0.19		
		TC	35.4	22.61	0.53 [0.26-1.05]	0.07		
		TT	51.8	57.14	ref			

(*continued on next page*)

Table 2 (*continued*)

SNP Ref. No.	Chromosomal Location	Genotype/ Haplotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
TLR 7 rs179008	Chr. X		n = 157	n = 193 NA = 8				
		CC	4.45	11.91	2.89 [1.20 – 6.94]	0.01*		
		AC	3.82	0	0.12 [0.01 – 1.08]	0.02*		
		AA	91.71	88.08	ref			
		C allele	6.36	11.91	1. 98 [1.15 – 3.49]	0.01*	0.06	0.12
		A allele	93.6	88.01	0.50 [0.29 – 0.86]			
		MALE	n = 78	n = 108				
		C	3.84	12.96	3.72 [1.03-13.43]	0.03*		
		A	96.15	87.03	ref			
		FEMALE	n = 79	n = 85				
		CC	5.06	10.58	2.24 [0. 65-7.52]	0.19		
		AC	7.59	0	0.13 [0.01-1.18]	0.03*		
		AA	87.34	89.41	ref			
TLR 7 rs3853839	Chr. X		n = 157	n = 175 NA = 26				
		CC	22.29	35.42	2.01 [1.23 – 3.25]	0.004*		
		GC	0	0	–	–		
		GG	77.7	64.57	ref			
		C allele	22.29	35.42	1.91 [1.35 – 2.69]	0.0002*	0.22	0.35
		G allele	77.07	64.57	0.52 [0.37 – 0.73]			
		MALE	n = 78	n = 100				
		C	19.24	37.00	2.46 [1.12-4.93]	0.009*		
		G	80.76	63.00	ref			
		FEMALE	n = 79	n = 75				
		CC	25.3	33.33	1.47 [0.73-2.96]	0.27		
		GC	0	0	–	–		
		GG	74.6	66.66	ref			
TLR8 rs3764879	Chr. X		n = 157	n = 196 NA = 5				
		CC	22.29	27.55	1.32 [0.81 – 2.16]	0.25		
		GC	9.55	8.16	0.84 [0.40 – 1.76]	0.64		
		GG	68.15	64.28	ref			
		C allele	27.07	31.63	1.24 [0.89 – 1.72]	0.18	0.27	0.31
		G allele	72.92	68.36	0.80 [0.57 – 1.11]			
		MALE	n = 78	n = 110				
		C	28.20	38.18	1.57 [0.84-3.18]	0.15		
		G	71.79	61.81	ref			
		FEMALE	n = 79	n = 86				
		CC	16.4	13.95	0.82 [0.35-1.92]	0.65		
		GC	18.9	18.60	0.97 [0.44-2.13]	0.94		
		GG	64.5	67.44	ref			

(*continued on next page*)

Table 2 (*continued*)

SNP Ref. No.	Chromosomal Location	Genotype/ Haplotype and allele distribution	Healthy Controls (%)	Dengue infected patients (%)	Odds Ratio (O.R.)	*p*-value at 95% C.I	MAF (Healthy control)	MAF (Dengue patient)
TLR8 rs3764880	Chr. X		n = 157	n = 190 NA = 11				
		AA	8.28	18.94	2.58 [1.32 – 5.07]	0.004*		
		AG	23.56	1.05	0.03 [0.008 – 0.14]	0. < 0001*		
		GG	68.15	80.00	ref			
		A allele	20.06	19.47	0.57 [0.40 – 0.81]		0.20	0.19
		G allele	79.93	80.52	1.73 [1.22 – 2.45]	0.001*		
		MALE	n = 78	n = 111				
		A	8.97	23.42	3.10 [1.21-7.57]	0.009*		
		G	91.02	76.57	ref			
		FEMALE	n = 79	n = 79				
		AA	7.59	12.65	1.76 [0.60-5.11]	0.29		
		AG	46.83	2.53	0.02 [0.006-0.12]	< 0.0001*		
		GG	45.56	84.81	ref			
TLR8 rs5744080	Chr. X		n = 157	n = 191 NA = 10				
		CC	12.73	26.70	2.49 [1.30 – 3.36]	0.001*		
		CT	14.64	13.08	0.87 [0.47 – 1.61]	0.67		
		TT	72.61	60.20	ref			
		C allele	20.06	33.24	1.98 [1.40 – 2.81]	0.0001*	0.20	0.33
		T allele	79.93	66.75	0.50 [0.35 – 0.71]			
		MALE	n = 78	n = 103				
		C	12.82	38.83	1.84 [0.96-3.50]	0.06		
		T	74.35	61.16	ref			
		FEMALE	n = 79	n = 88				
		CC	0	12.50	11.57 [1.45-91.77]	0.004*		
		CT	29.11	28.40	0.96 [0.49-1.89]	0.92		
		TT	70.88	59.09	ref			

"ref"= reference genotype, "NA"= Could not be PCR-amplified.

* $p < 0.05$ at 95% CI was considered as statistically significant.

Table 3
Association of genotype combinations of different TLR7 and TLR8 polymorphisms with (a) Dengue disease susceptibility and (b) development of WHO classified warning signs among patients.

(a)

SNP combinations	Frequencies (%)		Odds Ratio	*p* value at 95% C.I.
	Control (N = 157)	Dengue infected (N = 201)		
rs5741880-rs3853839 TT/T-CC/C	0	1 (0.49)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs179008 TT/T- CC/C	0	1 (0.49)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs3764880 TT/T-AA/A	0	1 (0.49)	0.79 [0.04 – 12.80]	0.87
rs5741880-rs5744080 TT/T-CC/C	0	1 (0.49)	0.79 [0.04 – 12.80]	0.87
rs3853839-rs179008 CC/C- CC/C	0	6 (2.98)	4.89 [0.58- 41.05]	0.10
rs3853839-rs3764880 CC/C-AA/A	4	12 (5.97)	2.42 [0.76 – 7.68]	0.11
rs3853839-rs5744080 CC/C- CC/C	0	8 (3.98)	6.59 [0.81 – 53.25]	**0.042***
rs179008-rs3764880 CC/C-AA/A	1	9 (4.47)	7.31 [0.91 – 58.34]	**0.028***
rs179008-rs5744080 CC/C-CC/C	0	4 (1.99)	3.22 [0.35 - 29.17]	0.27
rs3764880-rs5744080 AA/A-CC/C	0	5 (2.48)	4.80 [0.57 – 40.33]	0.11

(b)

SNPs Combination	Frequency (%) Symptomatic Dengue patients (n = 201)	Frequency of Symptoms (%)	
		With WHO-defined warning signs	Without warning signs
rs3853839-rs5744080 CC/C- CC/C	8(3.98)	3 (37.5)	5 (62.5)
rs179008-rs3764880 CC/C-AA/A	9 (4.47)	**9 (100)**	0

* $p < 0.05$ at 95% CI was considered as statistically significant.

Table 4
Association of TLR polymorphisms with development of different clinical symptoms among Dengue patients.

Gene	SNP Ref. No.	Genotype (female)/ Haplotype (male)	Statistical parameter	Symptoms (%)									
				Nausea	Rash	Myalgia	Arthralgia	Joint Swelling	Retro orbital pain	Headache	WHO-defined dengue warning signs		
											Persistent Vomiting	Abdominal pain	Haemorrhage
TLR3	**rs3775290**	CC	n/N (%)	28.57	45.71	68.57	45.71	14.3	14.3	40	40	31.42	14.3
			p value	0.65	**0.007**	0.52	0.67	0.31	0.08	0.34	**0.01**	0.06	**0.04**
			O.R.	0.82	3.007	1.30	1.17	1.83	3.03	1.46	2.70	2.29	3.88
		CT	n/N (%)	30.20	26.04	62.5	41.66	12.5	5.20	31.25	13.54	16.66	4.16
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		TT	n/N (%)	13.11	16.39	59.01	39.34	6.55	4.91	29.50	21.31	19.67	8.19
			p value	**0.01**	0.15	0.66	0.77	0.68	0.93	0.81	0.90	0.63	0.28
			O.R.	0.34	0.55	0.86	0.90	0.77	0.94	0.92	0.95	1.22	2.05
TLR7	**rs5741880**	TT/T	n/N (%)	12.5	75	87.5	75	0	25	62.5	50	37.5	12.5
			p value	0.34	**0.001**	0.24	0.06	–	**0.03**	**0.03**	**0.04**	0.19	0.55
			O.R.	0.37	9.23	3.30	4.33	–	5.55	4.20	3.96	2.58	1.92
		GG/G	n/N (%)	27.67	24.52	56.60	40.88	12.57	5.66	27.04	20.75	18.86	6.91
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		GT	n/N (%)	13.79	24.13	79.31	34.48	10.34	6.89	48.27	31.03	20.68	6.89
			p value	0.11	0.96	0.21	0.51	0.73	0.79	**0.02**	0.22	0.81	0.99
			O.R.	0.41	0.97	1.81	0.76	0.80	1.23	2.51	1.71	1.12	0.99
TLR7	**rs179010**	CC/C	n/N (%)	29.26	41.46	65.85	58.53	9.75	7.31	53.65	34.14	21.95	9.75
			p value	0.33	0.12	0.29	**0.01**	0.61	0.66	**0.003**	**0.03**	0.60	0.47
			O.R.	1.48	1.78	1.49	2.57	0.74	1.38	2.95	2.33	1.26	1.59
		TT/T	n/N (%)	21.81	25.45	56.36	35.45	12.72	5.45	28.18	18.18	18.18	6.36
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		CT	n/N (%)	35.55	17.77	84.44	28.88	8.88	8.88	28.88	26.66	24.44	6.66
			p value	0.07	0.30	< **0.01**	0.66	0.49	0.42	0.92	0.23	0.23	0.94
			O.R.	1.97	0.63	4.208	0.84	0.66	1.69	1.05	1.63	1.63	1.05
TLR7	**rs3853839**	CC/C	n/N (%)	27.41	27.41	53.22	43.54	8.06	4.83	30.64	25.80	16.12	4.83
			p value	0.43	0.90	0.11	0.80	0.71	0.71	0.52	0.67	0.58	0.71
			O.R.	1.32	1.04	0.59	1.08	0.81	0.77	0.80	1.16	0.79	0.81
		GG/G	n/N (%)	22.12	26.54	65.48	41.59	9.73	6.19	35.39	23.01	19.46	6.19
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
TLR7	**rs179008**	CC/C	n/N (%)	28.57	23.80	71.42	47.61	14.28	19.04	61.90	76.19	71.42	42.85
			p value	0.73	0.40	0.26	0.50	0.72	**0.01**	**0.003**	< **0.01**	< **0.01**	< **0.01**
			O.R.	0.83	1.51	1.81	1.35	1.26	4.26	4.33	14.55	14.70	25.05
		AA/A	n/N (%)	25.00	27.32	62.20	40.11	11.62	5.23	30	18.02	14.53	2.90
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
TLR8	**rs3764880**	AA/A	n/N (%)	38.88	33.33	86.11	38.88	8.33	19.44	55.55	66.66	61.11	19.44
			p value	0.051	0.23	**0.001**	0.65	0.68	**0.0003**	**0.001**	< **0.01**	< **0.01**	**0.007**
			O.R.	2.12	1.61	4.63	1.19	0.77	6.81	3.27	10.66	11.69	5.00
		GG/G	n/N (%)	23.02	23.68	57.23	39.47	12.50	3.94	27.63	15.78	11.84	4.60
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		GA	n/N (%)	50	0	50	100	0	0	0	0	0	0
			p value	0.37	NA	0.83	0.08	NA	NA	NA	NA	NA	NA
			O.R.	3.34	NA	0.74	7.64	NA	NA	NA	NA	NA	NA
TLR8	**rs5744080**	CC/C	n/N (%)	30.43	43.47	65.21	60.86	17.39	13.04	43.47	52.17	34.78	8.69
			p value	0.47	0.06	0.81	0.09	0.41	0.24	0.47	**0.01**	0.29	0.89
			O.R.	1.42	2.39	1.11	2.17	1.65	2.31	1.38	3.15	1.65	0.90
		TT/T	n/N (%)	23.47	24.34	62.60	41.73	11.30	6.08	35.65	25.21	24.34	9.56
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R.	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		TC	n/N (%)	26.41	22.640.80	58.49	28.30	11.32	5.66	22.64	9.43	5.66	1.88
			p value	0.68	0.90	0.61	0.09	0.99	0.91	0.09	**0.01**	0.03	0.07
			O.R.	1.17		0.84	0.55	1.001	0.92	0.52	0.301	0.18	0.18
TLR8	**rs3764879**	CC/C	n/N (%)	36.36	27.27	57.57	48.48	12.12	6.06	33.33	30.30	18.18	9.09
			p value	0.14	0.97	0.33	0.45	0.92	0.82	0.72	0.33	0.83	0.58
			O.R	1.82	1.01	0.67	1.33	0.94	0.83	1.15	1.52	0.89	1.47
		GG/G	n/N (%)	23.80	26.98	66.66	41.26	12.69	7.14	30.15	22.22	19.84	6.34
		(ref)	*p value*	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			O.R	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		GC	n/N (%)	21.62	27.02	48.64	35.13	8.10	5.40	40.54	27.02	24.32	8.10
			p value	0.78	0.99	**0.04**	0.50	0.44	0.71	0.23	0.54	0.55	0.70
			O.R	0.88	1.002	0.47	0.77	0.60	0.74	1.57	1.29	1.29	1.30

*$p < 0.05$ at 95% CI was considered as statistically significant.
NA = Not applicable, "ref" = reference genotype.

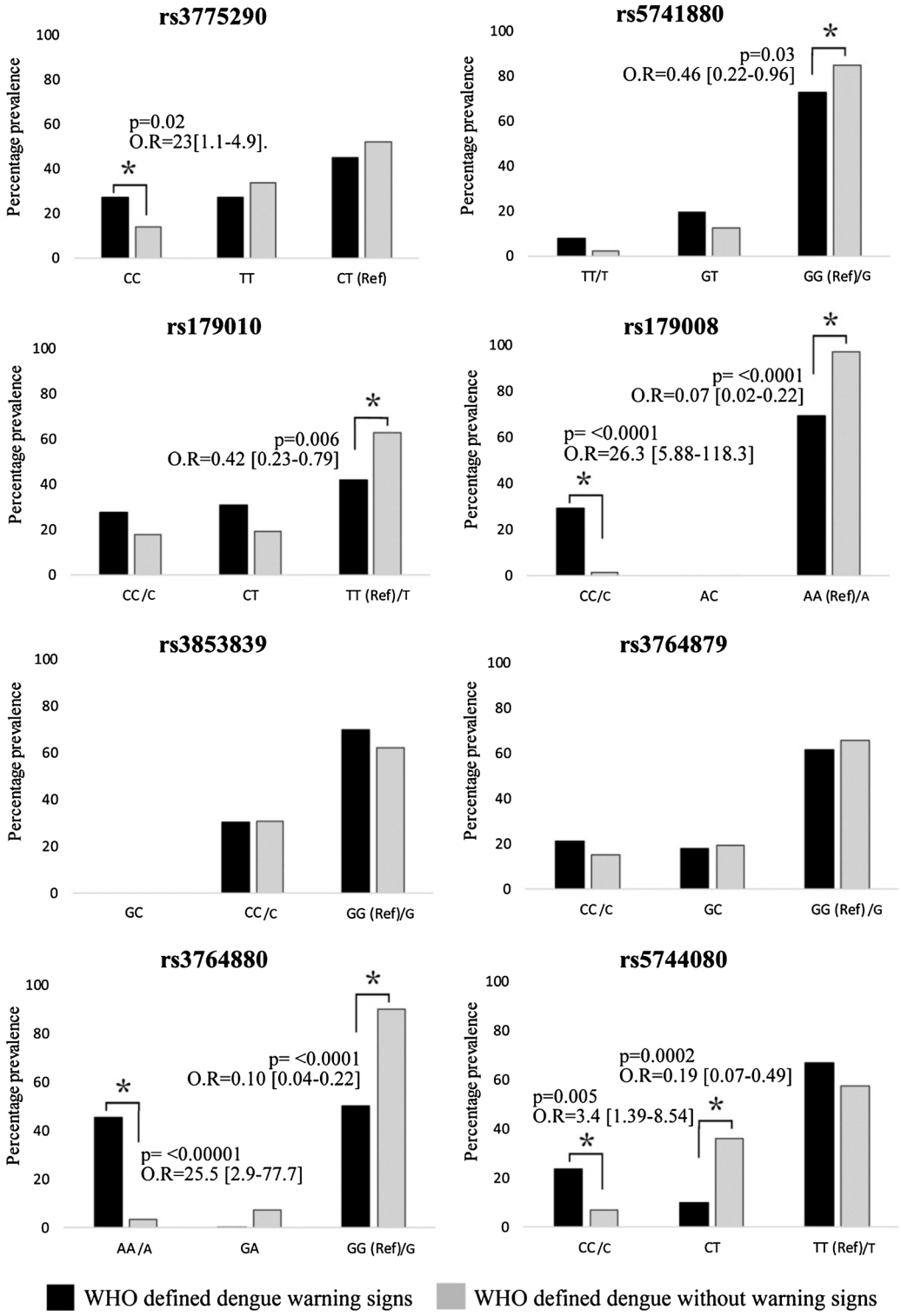


Fig. 2. Genotypic distribution pattern of the polymorphisms with/without warning signs among dengue patients.

haemorrhagic manifestation.

Association of particular genotypes with presence or absence of any WHO classified warning signs among dengue infected patients were analysed (Fig. 2). The TLR3-rs3775290-CC genotype, TLR7-rs179008-CC/C, TLR8-rs3764880-AA/A and TLR8-rs5744080-CC/C were found to be present in significantly higher frequencies among dengue patients exhibiting WHO-defined warning signs; whereas, TLR7-rs5741880-GG/G, TLR7-rs179010-TT/T, TLR7-rs179008-AA/A, TLR8-rs3764880-GG/G and TLR8-rs5744080-CT genotype, were considerably more prevalent among patients exhibiting no warning signs. Further, prevalence of specific genotypic combinations of significantly associated polymorphisms were analysed among dengue patients with/without warning signs (Table 3b). The CC/C-AA/A combination of TLR7-rs179008-TLR8-rs3764880 was predominantly present among patients with warning signs.

4. Discussion

Though TLR3, 7, 8 has long been known to be the first line of defence in innate immune recognition of dengue viral RNA genome, there has been very limited study regarding the role of TLR polymorphisms in dengue disease susceptibility. The present study clearly depicts the importance of various polymorphisms of TLR3, 7, 8 genes in determining dengue disease pathogenicity among Eastern Indian patient population.

Both TLR3 and TLR7 are important for controlling dengue virus replication and eliciting cellular antiviral response within the host (Urcuqui-Inchima et al., 2017). The CC genotype of TLR3-rs3775290 was significantly associated with dengue disease susceptibility among Eastern Indian patients, with three fold higher prevalence of this genotype among diseased population. This genotype of rs3775290 has also been previously detected to be higher among active chronic HBV patients of Turkish origin (Goktas et al., 2016). Involvement of this polymorphism has also been implicated among Tunisian chronic HCV patients and intrauterine transmissibility of HBV among Chinese patients (Huang et al., 2015; Sghaier et al., 2017).

TLR7 plays an important role in sensing viral genomic RNA which leads to the production of antiviral response (Urcuqui-Inchima et al., 2017). The CC genotypes of TLR7-rs179008 and TLR7-rs3853839 respectively of TLR7 gene demonstrated significant association with dengue infectivity among the study population. The TLR7-rs179008 polymorphism has been previously correlated with increased risk of disease progression among HCV mediated liver disease among Moroccan population and with post-bronchiolitis lung function deficiency of Finnish patients (Fakhir et al., 2017; Lauhkonen et al., 2016). Similar to the current study, the GC genotype of TLR7-rs3853839 was significantly higher among chikungunya virus infected Indian patients (Dutta and Tripathi, 2017). This TLR7 polymorphism has also been previously associated with protection against HCV persistence and susceptibility to enterovirus-71 mediated Hand foot and mouth disease among Chinese population (Yue et al., 2014).

The AA and CC genotypes of TLR8-rs3764880 and TLR8-5744080 respectively exhibited significant association among dengue infected patients. This A-allele of TLR8-rs3764880 was previously strongly associated with advanced liver disease among HCV infected Moroccan patients (Fakhir et al., 2017). Similar to this study, frequency of G-allele of rs3764880 was found to be significantly higher among pulmonary tuberculosis patients of Pakistani origin (Bukhari et al., 2015). On the contrary, this allele conferred significantly protective effect regarding progression of disease among HIV positive patients of German origin (Oh et al., 2008). The TLR8-rs5744080 polymorphism has been significantly different between osteoarthritis patients and healthy individuals of Japan (Yang et al., 2013).

Further, the present study demonstrated significantly increased risk of dengue disease susceptibility with two genotypic combinations of these polymorphisms. Contribution of such genotypic combinations of polymorphisms of JAK1 and several cytokines towards increased risk of Dengue haemorrhagic fever (DHF) development has been previously reported (Silva et al., 2010; Alagarasu et al., 2015a). Involvement of various genotypic combinations of TLR polymorphisms towards disease phenotype has also been previously demonstrated in ulcerative colitis, Crohn's disease and severe malarial anaemia (Meena et al., 2015; Munde et al., 2012).

The TLR7-rs179008-CC/C, TLR8-rs5744080-CC/C and TLR8-rs3764880-AA/A could be associated with the risk of developing WHO warning signs among dengue infected patients. Interestingly, reference genotypes of TLR7-rs5741880, TLR7-rs179010, TLR7-rs179008, TLR8-rs3764880 polymorphisms were significantly more prevalent among patients without any warning signs - thus these genotypes seemed to impart protection against development of warning signs among infected individuals. Presence of CC/C of TLR7-rs179008 and AA/A of TLR8-rs3764880 among dengue patients significantly correlated with development of WHO-classified warning signs viz. persistent vomiting, abdominal pain and haemorrhage, observed in this study. The genotypic combination CC/C of rs179008 and AA/A of rs3764880 was significantly prevalent among patients with warning signs. Contribution of such genotypic combination of various SNPs towards increased risk of DHF development has been previously demonstrated among several polymorphisms of JAK1, interleukin-10 and interferon gamma (Silva et al., 2010; Alagarasu et al., 2015b).

In addition to the role of TLR polymorphisms in imparting dengue disease severity, prevalence of any specific DENV serotype or presence of secondary dengue infection among these patients was also investigated (Table S2, Fig. S1). No significant difference in DENV serotypic distribution and prevalence of secondary dengue infection was observed between patients with and without WHO-defined warning signs.

5. Conclusion

In conclusion, the present study indicated increased risk of dengue disease susceptibility among individuals with specific genetic variants of majority of the analysed TLR3, TLR7 and TLR8 polymorphisms – indicating important role of TLR polymorphisms in dengue disease pathogenesis. Increased risk of dengue infectivity was also predicted among certain genotypic combinations of these polymorphisms. Moreover, specific genotype(s) of certain polymorphism(s), alone or in combination, were associated with development of WHO-classified warning signs, which might act as potential prognostic biomarkers for predicting disease severity among dengue infected patients.

Authors' contribution

Tripathi A: Research idea formation, manuscript writing and correction and overall supervision; Mukherjee S: Performed the experiment, data analysis and manuscript writing. All authors read and approved the final manuscript.

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Ethical approval and consent to participate

All procedures performed in this study involving collection of blood from human participants were in accordance with ethical standards at the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments. The study was approved by the Research Ethics Committee of Calcutta School of Tropical Medicine (CREC-STM/53 dated 26.09.2013). Prior to participation in the study, written consents were received from patients and healthy control individuals regarding their willingness to participate in the study, patients' rights and responsibilities, treatment information, confidentiality of the patient identity, permission to investigators to release information to the sponsors, regulatory authorities, Government agencies and ethics committee.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.08.009.

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