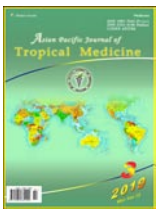

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X-linked Toll-like receptor 7 polymorphism associated with susceptibility to Chikungunya Fever

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ABSTRACT

Objective: To investigate the association between *TLR3* and *TLR7* polymorphisms with susceptibility and clinical manifestations of Chikungunya Fever.

Methods: A total of 177 individuals were studied: 73 patients with a confirmed diagnosis for Chikungunya virus and 104 non-infected individuals. Polymorphisms were determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

Results: Our analysis showed an increased CC genotype frequency of the *TLR7* rs3853839 polymorphism in male patients compared to control (29% *versus* 2%, respectively; *OR*=20.69; 95 % *CI*= 2.55-167.36; *P*<0.001). Furthermore, arthritis (acute and chronic) was frequently found in CC male patients. On the contrary, 65% of CG carriers were no-infected males (29% *versus* 65%, respectively; *OR*=0.23, 95% *CI*=0.48-3.04; *P*=0.002). Finally, we observed a higher frequency of lymphopenia in CG male patients (CG=666.86±233.77, GG=1,314.27±752.29 cells/mm3, *P*=0.047).

Conclusions: Our results suggest the *TLR7* rs3853839 polymorphism is associated with lymphopenia and increased susceptibility to Chikungunya Fever in males.

1. Introduction

Chikungunya Fever is an acute, arthritogenic, and re-emergent alphavirus-vector disease caused by Chikungunya virus (CHIKV)[1]. Suspected diagnosis includes fever (>38.3 ℃) and acute arthropathies (arthralgia/arthritis) not explained by other causes[2]. Although articular inflammation results spontaneously, 12% of cases debut with post-chikungunya chronic inflammatory rheumatism[3]. Notwithstanding, Ross River, Barmah Forest, O'nyong nyong, Sindbis, Karelia, Pogosta and Ockelbo are also considered arthritogenic viruses, according to PAHO, CHIKV is the only arthritogenic epidemic in America with 326 616 confirmed CHIKV patients since 2014; and utterly risk for endemicity[4,5]. Moreover, this infection is related to transitional positive nuclear autoantibodies and increased severity for Systemic Lupus Erythematosus[6,7]. Thus, previous conditions, vector presence and host genomic might determine susceptibility to CHIKV. Genetic variability in Toll-like Receptors have been associated with risk to infectious and inflammatory diseases[8]. Both *TLR3* and *TLR7* play a pivotal role in the innate immune response to CHIKV, inducing IFNα/β expression for replicative inhibition[9]. While the rs3775296

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polymorphism in the *TLR3* gene has been related to osteoarthritis[10], the X-linked rs3853839 polymorphism in the *TLR7* is associated with modified risk for CHIKV infection in Indian population[11]. Therefore, the aim of our study was to determine the association between *TLR3* and *TLR7* polymorphisms with clinical manifestations and laboratory testing in Chikungunya infected Venezuelan population.

2. Materials and methods

2.1. Subjects

A total of 400 patients with clinical symptoms of Chikungunya Fever were examined at the IVIC Medical Service, Caracas, Venezuela, in late 2014. Seventy-three adult (≥18 years old) patients with confirmed diagnosis for Chikungunya virus were selected. Diagnoses were accomplished by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) performed by the Virus Biology laboratory at IVIC. After patients were enrolled, a peripheral blood sample was taken from each of them, and demographic and medical information was collected. At the interview, medical information was focused on symptoms, laboratory testing data at the onset of the disease, and continuous arthropathy signs appeared during and after CHIKV infection. The data was completed with the help of Medical Services and entered into case report forms. There were no follow up of participants. Therefore, the time of resolution for those with chronic illness is unknown. Patients under 18 years old and immunocompromised for transplant, chemotherapy, and/or autoimmunity were excluded. The control included 104 healthy individuals. The peripheral blood samples were collected during 2011 and 2012 (no cases were reported in the Americas by that time). Case and control groups were residents of the capital district. Detailed written informed consent was obtained from all participants, and study approval was received from the IVIC Ethics Committee.

2.2. TLR3 and TLR7 genotype analysis

Genomic DNA was extracted from blood samples following the procedure described by Bunce[12]. The *TLR3* polymorphic variants rs3775296, rs3775290, rs3775291, and rs5743312; as the rs179008, rs3853839, and rs179010 in *TLR7* were studied by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism, using primers and restriction enzymes reported by Yang and Cheng, respectively[10,13].

2.3. Statistical analysis

Allele and genotypic frequencies were determined by direct counting. The Hardy-Weinberg equilibrium was calculated with a *Chi*-squared test. Significant differences between patients and control were estimated by Fisher's exact test using 2×2 contingency tables. Relative risk with 95% of confidence interval (95% *CI*) was calculated as odds ratios (*OR*) according to Woolf's formula[14]. *P* value <0.05 was considered statistically significant and were corrected according to Bonferroni formula[15]. Hence, for genetic analysis, all *P* values are two/three times more than the exact value according to the Fisher's exact test; depending on the number of genotypes. Moreover, package SPSS version 20.0 was used for statistical analysis.

3. Results

3.1. Subjects

Four hundred patients were screened, but only 73 (18.25%) met the eligibility criteria. Forty-one (41) were men (56.16%) and 32 were women (43.84%). The mean age was (36.25±13.89) years with a minimum of 18 and a maximum of 83. Clinical characteristics of infected individuals are shown in Table 1. A total of 104 healthy individuals were selected as control group; 51 were men (49.04%) and 53 were women (50.96%). The mean age was (50.75±11.65) years, with a minimum of 21 and a maximum of 83 years.

Table 1. Baseline clinical characteristics of infected individuals (*n*=73).

Characteristics		Patients [*n* (%)]
Coexisting conditions	Infectious disease	17(51.5)
	Metabolic	13(39.4)
	Cardiovascular	10(30.3)
	Rheumatologic	10(30.3)
Acute clinical manifestations*	Fever	58(79.5)
	Arthralgia	50(68.5)
	Cutaneous manifestations	47(64.4)
	Chills	47(64.4)
	Myalgia	43(58.9)
	Headache	4 (57.5)
Arthritis		27(36.9)
	Acute	3(4.1)†/ 5(6.8)‡
	Sub-acute	7(9.6)† / 3(4.1)‡
	Chronic	5(6.8)† / 4(5.6)‡

*None of the patients had atypical manifestations. †Documented arthritis in females. ‡Documented arthritis in males. Acute arthritis refers to symptoms lasting up to 10 days; sub-acute refers > 10 days up 90 days, and chronic arthritis for more than 3 months[5].

3.2. Allelic and genotypic frequency of the TLR3 gene in case and control

This analysis allowed us to establish allelic and genotypic frequencies of the *TLR3* polymorphisms. Hardy-Weinberg

equilibrium was found in the control group, except for the *TLR3*-rs5743312 polymorphism. This polymorphism was excluded from the association study. Table 2 shows allele frequency and genotype distribution of *TLR3* polymorphisms. Statistical differences were not observed.

Table 2. Allele frequency and genotype distribution of *TLR3* polymorphisms [*n*(%)].

SNP		Case (*n*=73)	Control (*n*=104)	*OR* (95% *CI*)	*P*-value§
rs3775296	GT	26 (35.6)	32 (30.8)	1.24 (0.66-2.35)	1.58
	TT	47 (64.4)	72 (69.2)	0.80 (0.43-1.52)	1.58
	G	26 (17.8)	32 (15.4)	1.19 (0.68-210)	1.54
	T	120 (82.2)	176 (84.6)	0.84 (0.47-1.48)	1.54
rs3775290	AA	26 (35.6)	51 (49.0)	0.57 (0.31-1.06)	0.15
	AG	38 (52.1)	45 (43.3)	1.42 (0.78-2.59)	0.48
	GG	9 (12.3)	8(7.7)	1.69 (0.62-4.6)	0.66
	A	90 (61.6)	147 (70.7)	0.67 (0.43-1.04)	1.94
	G	56 (38.4)	61 (29.3)	1.49 (0.96-2.35)	1.94
rs3775291	AA	31 (42.5)	51 (49.0)	0.77 (0.42-1.40)	0.72
	AG	36 (49.3)	42 (40.4)	1.44 (0.78-2.63)	2.73
	GG	6 (8.2)	11 (10.6)	0.76 (0.27-2.15)	1.20
	A	98 (67.1)	144 (69.2)	0.91 (0.57-1.43)	1.40
	G	48 (32.9)	64 (30.8)	1.10 (0.7-1.73)	1.40

§Fisher's exact test with *P* value correction. *P* values according to genotypes frequency comparison in case and control.

3.3. Allelic and genotypic frequency of the TLR7 gene in case and control

The *TLR7* gene is located in a sex chromosome. Therefore the Hardy-Weinberg equilibrium was not performed, and the comparisons between case and control were made separately by sex. Table 3 shows the allele frequency and genotype distribution of *TLR7* polymorphisms by sex. Moreover, when comparing genotypic frequencies, a higher frequency of the rs3853839 CC genotype was found in male patients related to healthy individuals (29.3% *versus* 2.0%, respectively; *OR*=20.69; 95% *CI*=2.55-167.36; *P*<0.001). In contrast, the rs3853839 CG genotype frequency was higher in controls than in patients (64.7% *versus* 29.3%, respectively; *OR*=0.23; 95% *CI*=0.48-3.04; *P*=0.002).

3.4. The TLR7-rs3853839 polymorphism in males with arthralgia and/or arthritis during or after CHIKF

Distribution in males with the CC (*n*=12) and CG+GG (*n*=29) were matched with those with arthralgia and/or arthritis during the acute, subacute and chronic phase for CHIKV disease; establishing a dominant inheritance pattern. Therefore, a single copy of the G allele is enough to change the risk; and being a carrier of two copies have an equal effect. Thus, CG carriers and GG carriers have the same risk, and the combination CG+GG is compared to the CC genotype[16]. The dominant pattern was established according to our association results in the present study. Then, arthralgia was frequently found in male carriers with CG+GG genotypes compared to CC carriers [22 (53.7%) CG+GG *versus* 7 (17.1%) CC]; and also during the acute phase compared to sub-acute or chronic stages of the disease [acute=13 (31.7%); subacute+chronic=9 (22.0%) for CG+GG *versus* acute=4 (9.8%); subacute+chronic=3 (7.3%) in CC males]. On the contrary, acute and chronic arthritis were frequently found in CC carriers compared to CG+GG, respectively [3 (7.3%) CC *versus* 2 (4.9%) CG+GG; 3 (7.3%) CC *versus* 1 (2.4%) CG+GG].

3.5. Blood cell count in infected males according to the rs3853839 genotypes

Distribution of neutrophils, lymphocytes, and platelets counting in

Table 3. Allele frequency and genotype distribution of *TLR7* polymorphism by sex [*n* (%)].

SNP		Female			Male		
		Case/control (*n*=32/53)	*OR* (95% *CI*)	*P*-value	Case/control (*n*=41/51)	*OR* (95% *CI*)	*P*-value
rs179008	AT	26(81.3)/49(92.5)	0.35 (0.09-1.37)	0.24	31.0 (75.6)/37 (72.5)	1.17 (0.46-3.00)	0.920
	TT	6(18.8)/4(7.5)	2.83(0.73-10.92)	0.24	10.0(24.4)/14(27.4)	0.85(0.33-2.18)	0.920
	A	26(40.6)/49(46.2)	0.79(0.42-1.49)	0.58	31.0(37.8)/37(36.3)	1.07(0.58-1.95)	1.280
	T	38(59.4)/57(53.8)	1.26(0.67-2.35)	0.58	51.0(62.2)/65(63.7)	0.94(0.51-1.71)	1.280
rs179010	CC	22(68.8)/40(75.5)	0.72(0.27-1.89)	2.46	35.0(85.4)/42(82.4)	1.25(0.40-3.85)	1.380
	CT	9(28.1)/12(22.6)	1.34(0.49-3.65)	2.37	0.0/1(1.9)	0.41(0.02-10.22)	1.650
	TT	1(3.1)/1(1.9)	1.68(0.10-27.78)	1.44	6.0(14.6)/8(15.7)	0.92(0.29-2.91)	1.680
	C	53(82.8)/92(86.8)	0.73(0.31-1.74)	1.66	70.0(85.4)/85(83.3)	1.17(0.52-2.61)	0.860
	T	11(17.2)/14(13.2)	1.36(0.58-3.22)	1.66	12.0(14.6)/17(16.7)	0.86(0.38-1.91)	0.860
rs3853839	CC	5(15.6)/3(5.7)	3.09(0.68-13.91)	0.39	12.0(29.3)/1(2.0)	20.69(2.55-167.36)	0.000**
	CG	23(71.9)/88.6(47)	0.33(0.10-1.03)	0.15	12.0(29.3)/33(64.7)	0.23(0.48-3.04)	0.002**
	GG	4(12.5)/5.7(3)	2.38(0.49-11.41)	0.72	17.0(41.4)/17(33.3)	1.42(0.66-3.32)	0.360
	C	33(51.6)/50(53)	1.06(0.57-1.98)	0.96	36.0(43.9)/35(34.3)	1.49(0.82-2.72)	0.240
	G	31(48.4)/53(50)	0.94(0.50-1.75)	0.96	56.1(46)/65.7(67)	0.67(0.37-1.21)	0.240

§Fisher's exact test with *P* value correction. *P* values according to genotypes frequency comparison in case and control. **Statistically significant *P*<0.05.

infected males according to the rs3853839 genotypes was analyzed. The total available laboratory data was 39 patients (25 males and 14 females). However, possible blood counting associations were study only in men according to our results. Thus, The mean for neutrophils, lymphocytes, and platelets counting were higher in GG carriers compared to CC and CG genotypes.

When comparing the groups studied, statistical differences were found in the lymphocyte counting among males with CG and GG genotypes [(666.86±233.77) cells/mm^3 and (1 314.20±752.29) cells/mm^3, respectively, P=0.047)]. Then, male patients with CC and CG genotypes had lymphopenia (<1 000 cells/mm^3) compared to male patients with GG genotype.

4. Discussion

Susceptibility to infections depends upon the interaction of the host genome, the environment and the microbiota[17]. Particularly, variation in polymorphisms frequencies explains clinical differences and geographical distribution in communicable diseases.

Thus, our study shows no statistical differences between the case and control group for *TLR3* polymorphisms- rs3775296, rs3775290, and rs3775291- indicating that the *TLR3* polymorphisms studied might not modify the risk of CHIKV infection.

However, the analysis showed an increased frequency of the *TLR7* rs3853839 CC genotype in male patients compared to control (29.3% *versus* 2.0%, respectively; *OR*=20.69; 95 % *CI*= 2.55-167.36; *P*<0.001). This suggests the CC genotype confers almost 21 times more risk for CHIKV infection in men, which is similar to data previously reported by Tripathi *et al*[11]. In contrast, an increased CG genotype frequency in the control group was observed when compared to patients (64.7% *vs.* 29.3%, *OR*=0.23, 95% *CI*=0.48-3.04, *P*=0.002), indicating a protective effect in CHIKF.

This data is similar to previous reports regarding viral infections[18]. Lymphopenia in viral infections is related to IFNα/β. IFNα/β induces cell death in lymphocytes and expression of chemokines as CXCL10 and CCL5. These chemokines trigger lymphocyte migration from the vascular compartment to the tissues, reducing lymphocyte count in the peripheral blood[19]. On the other hand, *TLR7* in human platelets induces translocation of P-selectin to the cell membrane mediating the formation of cellular aggregates, and consequently thrombocytopenia[20]. Moreover, the rs3853839 G allele has been associated with *TLR7* and IFNα/β overexpression in plasmacytoid dendritic cells compared to C allele carriers[21]. Likewise, *TLR7* overexpression has also been associated with IL-1β up-regulation in the inflammasome of infected cells. Then, IL-1β binds to its specific IL-1R receptor triggering signaling in uninfected cells[22]. Therefore, *TLR7* plays a pivotal role in limiting and preventing cellular infection.

Moreover, our study shows an association between lymphopenia and rs3853839 polymorphism. Lymphopenia was frequent in CG men than GG carriers. We assumed lymphopenia in CG males was associated with G dominance over the C allele, driving apoptosis by IFNα/β. Hence, there is a need for further studies to clarify this point.

Furthermore, no association between rs3853839 genotypes and arthralgia/arthritis in men was established probably due to a low number of subjects. Although 50% of the patients had suffered previous viral infections, such as dengue and hepatitis, only Chikungunya virus can be maintained in joint tissues and causes prolonged arthralgia associated with a persistent immune response[23]. In addition, infected monocytes migrate massively during the early phase of CHIKV to the synovial tissues where they contribute to the inflammatory process. The CHIKV symptoms generally resolve within 7-10 days, except for joint stiffness and pain; and 12% of patients still have chronic arthralgia three years after the onset of illness[3]. In addition, the arthralgia experienced by CHIKV patients resembles the symptoms induced by other viruses like Ross River and Barmah Forest; both only widely disseminated in Australia and absent in America[4]. Hence, those documented patients with arthralgia/arthritis were directly linked to CHIKV infection; and validated through confirmatory diagnostic.

In the present study, CC males (n=12) had 12 times more risk of chronic arthritis than CG+GG carriers (n=29), although no statistical association was found [3 (7.3%) CC *vs*. 1 (2.4%) CG+GG, *OR*=11.5, *P*=0.15]. Likewise, the *TLR7*-rs3853839 polymorphism has not been related to rheumatoid arthritis susceptibility nor ankylosing spondylitis[24,25]. On the contrary, *TLR3* polymorphisms as rs3775296 and rs3775290 are associated with osteoarthritis susceptibility in males[10]; meanwhile, the TLR9 rs5743836 is related to rheumatoid arthritis predisposition in females[26]. In this study, we did not identify other associations besides the rs3853839 polymorphism and CHIKF. Contrarily, the *TLR7* rs3853839 polymorphism is related to susceptibility for chronic disease as SLE in Asian and European populations[21,27–29]. Moreover, it appears that Chikungunya Fever can induce an SLE flare[7].

Finally, our findings provide evidence the rs3853839 polymorphism is associated with susceptibility to Chikungunya infection and with lymphopenia in men with Chikungunya Fever. Although we did not find an association among rs3853839 polymorphism and chronic arthritis, severe SLE has been documented in carriers with this polymorphism. The molecular connections among SLE, CHIKV infection and the rs3853839 polymorphism have been not described yet.

Conflict of interest statement

All authors declare that they have no potential conflict of interest.

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