P-ISSN 2586-9000
E-ISSN 2586-9027
Homepage : https://tci-thaijo.org/index.php/SciTechAsia
Science & Technology Asia
Vol.25 No.4 October - December 2020
Page: [125-134]
Original research article

Genertic Polymorphism of Dengue Susceptibility Genes among Bhutanese Population

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Received 28 May 2019; Received in revised form 15 July 2019
Accepted 24 July 2019; Available online 24 December 2020

ABSTRACT

The objective of this study is to investigate the distribution of genetic frequency of dengue susceptibility genes among Bhutanese population. Three hundred and fifty dried blood spot samples were collected from a Bhutanese population residing in a Dengue endemic area. Single nucleotide polymorphisms at TNFα -308G/A, IL10 -592C/A, IL10 -1082A/G, TGFβ1 -509C/T and CD209 -336A/G genes were investigated using PCR-RFLP, whereas, IFNγ +874A/T was genotyped using the ARMS-PCR technique. The genotype frequencies of TNFα -308 were 97.96% and 2.04% for GG and GA genotypes, respectively. The frequencies of CC, CA, and AA genotypes of IL10 -592 were 31.1%, 45.5% & 23.4%, respectively, and AA, GA and GG genotypes of IL10 -1082 were 82.7%, 15% &2.3%, respectively. CC; 12%%, CT; 86.8% and TT; 1.17% were genotype frequencies of TGFβ1 -509. The frequencies of AA, AT, and TT genotypes of IFNγ +874 were 27.9%, 96.3% & 2.87%, respectively. 68.6% were AA genotypes, 30% were AG genotypes, and 1.4% were GG genotypes for SNP at CD209 -336. This first study gives the pattern of frequency distribution of TNFα, IL10, and TGFβ1, IFNγ and DC-SIGN genes among Bhutanese population. The genetic polymorphisms of these genes will provide the possible clinical outcome of Dengue infection in Bhutanese; further studies to evaluate association between severity of dengue infection and dengue susceptible gene will be conducted.

Keywords: Dengue; TNFα; IL10; TGFβ1; INFγ; DC-SIGN; Bhutanese population

1. Introduction

Severity of dengue virus infection is influenced by various factors including the host factors. The host immune response has been highlighted as a genetic biomarker for disease with the production of several cytokines [1]. Genetic polymorphisms may trigger gene transcription, protein

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expression and immunological reactions, which results in the individual's response towards certain pathological conditions.

The initial target of dengue virus infection is dendritic cells. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), encoded by CD209 is an important C-type lectin receptor for internalization of dengue virus in dendritic cells [2].

Cells after infection with dengue virus produces protective chemokines such as tumor necrosis factor-alpha (TNFα), Interferon-gamma (IFNγ), transforming growth factor β-1 (TGFβ1), and interleukin-10 (IL10) which attracts and activates leukocytes at the site of infection [3]. TNFα is a pro-inflammatory cytokine, and it plays a vital role in early removal of dengue virus. However, higher levels of TNFα induce endothelial apoptosis leading to hemorrhage development [4]. IFNγ is another pro-inflammatory cytokine which eliminates dengue virus by up-regulating the expression of human leukocyte antigen. The plasma level of IFNγ is higher in severe dengue than in dengue fever [5]. TGFβ1, a pleotropic cytokine which has several immunomodulatory effects on numerous cells and tissues. The increased plasma level of TGFβ1 is associated with severe dengue infection [6].

IL10 is an anti-inflammatory cytokine which inhibits the production of pro-inflammatory cytokines and reduces the function of macrophages and activation of Th1 cells. The plasma level of IL10 increases during severe dengue infection, enhancing its potential role in disease pathogenesis [7].

The six SNPs including TNFα (-308G/A), IL10 (-592C/A & -1082A/G) TGFβ1 (-509C/T), IFNγ (+874A/T) and DC-SIGN (CD209 -336A/G) were targeted in our study due to the high association with the clinical outcome of dengue infection [8-11]. However, these polymorphisms have never been investigated among Bhutanese population. Therefore, in this study, we investigated the genetic frequency of SNP of these genes among the Bhutanese in Dengue endemic areas.

2. Research Methodology

2.1 Study population

This study was approved by Research Ethical Board of Health (REBH), Ministry of Health of Bhutan (REBH/Approval/2019/006). The samples for this study were collected *via* Royal Center for Disease Control (RCDC), Ministry of Health, Bhutan. RCDC collects samples from dengue-endemic areas (dengue surveillance site) as part of their routine diagnostic and surveillance system from individuals visiting four hospitals of Dengue surveillance sites of Bhutan. The Dengue surveillance sites of Bhutan include Samtse, Phuentsholing, Gelephu and Samdrupjongkhar as shown in Fig. 1.

2.2 DNA extraction

DNA was extracted from dried blood spots by using QIAampDNA Mini Kit (Qiagen CA, USA). DNA was stored at –20ºC until further used.

2.3 SNP analysis

SNP at TNFα -308G/A, IL10 -592C/A, IL10 -1082A/G, TGFβ1 -509C/T and CD209 -336A/G were investigated using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as narrated in earlier studies with a slight modification in annealing temperature [12-15]. The primers and restriction enzymes are listed in Table 1. SNP at IFNγ +874A/T was genotyped by amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method using the primer and condition previously described [16].

2.4 Statistical analysis

The polymorphism data were presented as number and percentage. Allelic frequencies were calculated from genotype frequencies using the Hardy–Weinberg

equation. A test for deviation from the Hardy–Weinberg was performed by using the chi square test; the significance level was $\alpha = 0.05$.

3. Result

3.1 Demographic data of sample population

Genetic polymorphism of dengue susceptibility gene among Bhutanese population was examined in 350 individuals. Out of 350 individuals, 198 (56.6%) were female, and 152 (43.4%) were male. All the individuals enrolled in the study were above eighteen years of age and the mean age ± standard deviation is 45.42 ±14.29 as shown in Table 2.

3.2 Genotype and allelic distribution of TNF-α-308G/A

Genotype and allelic frequencies are given in Table 3. The genotype frequency of GG and GA were found 336 (97.96%) and 7 (2.04%), respectively, whereas no AA genotype was observed. The allele frequencies of G and A were 679 (98.98%) and 7 (1.02%), respectively.

3.3 Genotype and allelic distribution of IL-10-592C/A & -1082A/G

SNP at IL-10-592C/A & -1082A/G are shown in Table 3. The genotype frequency of CC, CA and AA genotype of IL-10-592 was observed at 109 (31.14%), 159 (45.43%) and 82 (23.43%), respectively. The allele frequency of C was 377 (53.86%), and A was 323 (46.14%). The genotype distributions of IL-10-1082 were AA (287, 82.71%), AG (52, 14.99%) and GG (8, 2.03%), respectively. The allele frequencies of A and G were 626 (90.20%) and 68 (9.80%), respectively.

3.4 Genotype and allelic distribution of TGF-β-1-509C/T

The SNP mutation of TGF-β-1 at position -509 was investigated, as shown in Table 3. The distribution of three genotypes: CC, CT and TT of TGF-β-1, were 42 (12.03%), 303 (86.82%) and 4 (1.15%), respectively. The allele frequencies of C and T were 387 (55.44%) and 311 (44.56%), respectively. The genetic distribution of TGF-β-1 gene was significantly different from the Hardy- Weinberg law ($p < 0.00001$).

3.5 Genotype and allelic distribution IFN-γ+874A/T

The genotypes of IFN-γ+874 were reported as AA, AT and TT, respectively. Among 348 samples, the frequencies of each genotype were 97 (27.88%), 241 (69.25%) and 10 (2.87%) for AA, AT and TT, respectively. The allele frequencies of A and T were 435 (62.50%) and 261 (37.50%), respectively, as shown in Table 3. The observed genetic frequencies of IFN-γ +874 gene were not compatible with Hardy-Weinberg equilibrium predictions ($p< 0.00001$).

3.6 Genotype and allelic distribution CD209-336A/G

The results of CD209 genotypes at position -336 were AA (240, 68.57%), AG (105, 30.00%) and GG (5, 1.43%). An allele frequency of A was 585 (83.57%), while the G allele was found as 115 (16.43%).

4. Discussion

This is the first study determining the distribution of the genetic pattern of dengue susceptibility gene among the Bhutanese population. The genetic distribution of TNFα-308, IL10-592 & -1082, and CD209-336 were compatible with Hardy-Weinberg law. Thus, these gene variations among Bhutanese population remain in equilibrium from one generation to the next. However, the disequilibrium of Hardy-Weinberg was observed in two genes; TGFβ1 and IFNγ. The deviation could be population-specific since we collected the samples randomly from an ethnically diverse population. The accuracy of our technical procedure was confirmed with blinded quality assurance

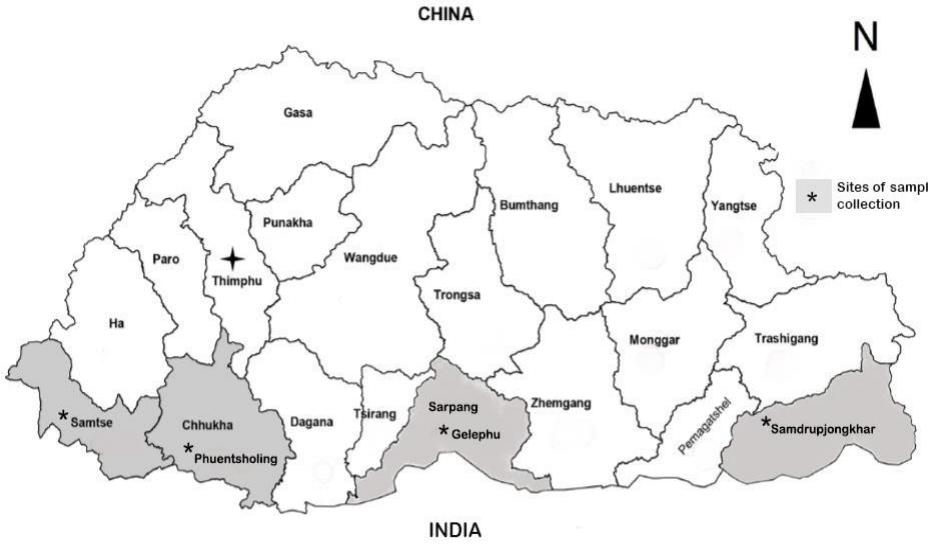

Fig. 1. Map of Bhutan showing the location of sample collection areas. (Adapted from http://www.ezilon.com/maps/asia/bhutan-maps.html).

Table 1. Primers sequences, restriction enzymes and the restriction digestion patterns for genotyping of TNFα-308, IL10-592, IL10-1082, TGFβ1-509, IFNγ+874 and CD209-336.

Gene	Primers (5'-3')	PCR product size (bp)	Restriction Enzyme	Genotype: restriction product size (bp)	Ref
TNFα -308	F-AGGCAATAGGTTTTGAGGGCCAT R-TCCTCCCTGCTCCGATTCCG	107	*Nco*I	GG:87 GA: 87&107 AA: 107	[13]
IL10 -592	F-GACTCCAGCCACAGAAGCTTA R-ATATCCTCAAAGTTCCCAAGC	437	*Rsa*I	CC: 302 CA:302 & 240 AA: 240	[12]
IL10 -1082	F-TCTGAAGAAGTCCTGATGTC R-CTCTTACCTATCCCTACTTCC	192	*Mnl*I	AA:127 AG**:** 127&93 GG:93	[12]
TGFβ1 -509	F-GTCCCTCTGGGCCCAGTTTC R-GAGGGGGCAACAGGACACCTTA	178	*Afl*II	CC:178 TC: 178&159 TT: 159	[14]
IFNγ +874	F-TTCTTACAACACAAAATCAAATCT F-'TTCTTACAACACAAAATCAAATCA R-TCAACAAAGCTGATACTCCA	264	NA*	NA*	[16]
CD209 -336	F-GGATGGTCTGGGGTTGACAG R-ACTGGGGGTGCTACCTGGC	150	*Msc*I	AA**:**150 GA**:**150 &131 GG**:**131	[15]

*Not applicable

Table 2. Demographic details and numbers of samples collected from each site. Gender was presented as number (percentage), age was presented as mean ± standard deviation.

Surveillance sites	Number of samples	Gender		Age (years)
		Male	Female	
Samtse and Phuntsholing	122	54 (44.3%)	68 (55.7%)	43.74±13.55
Gelephu	113	49 (43.4%)	64 (56.6%)	44.55±14.04
Samdrupjongkhar	115	49 (42.6%)	66 (57.5%)	47.95±14.88
Total	350	152 (43.4%)	198 (56.6%)	45.42±14.29

Table 3. The genotypes and allele distribution of TNFα-308, IL10-592, IL10-1082, TGFβ1-509, IFNγ+874 and CD209-336 among Bhutanese population.

Gene			Frequency		
			Number	%	*P* value
TNFα -308	Genotype	GG	336	97.96	0.9849
(N=343)		GA	7	2.04	
		AA	0	0	
	Allele	G	679	98.98	
		A	7	1.02	
IL10 -592	Genotype	CC	109	31.14	0.5232
(N=350)		CA	159	45.43	
		AA	82	23.43	
	Allele	C	377	53.86	
		A	323	46.14	
IL10 -1082	Genotype	AA	287	82.71	0.2550
(N=347)		AG	52	14.99	
		GG	8	2.30	
	Allele	A	626	90.20	
		G	68	9.80	
TGFβ1 -509	Genotype	CC	42	12.03	**<0.00001***
(N=349)		CT	303	86.82	
		TT	4	1.15	
	Allele	C	387	55.44	
		T	311	44.56	
IFNγ +874	Genotype	AA	97	27.88	**<0.00001***
(N=348)		AT	241	69.25	
		TT	10	2.87	
	Allele	A	435	62.50	
		T	261	37.50	
CD209 -336	Genotype	AA	240	68.57	0.4055
(N=350)		AG	105	30.00	
		GG	5	1.43	
	Allele	A	585	83.57	
		G	115	16.43	

* The statistical significance of genotypes compared with the Hardy–Weinberg law (Chi-square test)

The GG genotype of TNFα-308 gene is predominant among the Bhutanese population. Similarly, it is also predominant in other Asian populations such as Indian [17], Japanese [18] and Han Chinese [19]. The high expressing TNFα-308A was found at a frequency of 1.02% among the Bhutanese population. The same was reported at 6% among the Indian population [17]. A previous study from South Central America reported that A allele increases the risk of developing severe dengue [20, 21]. A similar study from the Thai population, which found 11.8% of A allele, reported the association of A allele and dengue infection with significant bleeding [22].

The C allele of IL10 -592 is associated with higher plasma concentration of IL10 [23]. Among Bhutanese population, C allele was found at a frequency of 53.86% which is similar to north Indian [24] and Srilankan populations [25]; however, C allele is more frequent among Africans (66.3%) [26] and Caucasians (74.2%) [27].

The genotype of IL10-1082; AA, GA and GG affects the plasma levels of IL10, which is associated with lower, intermediate and higher secretion of IL10, respectively [28]. The frequency of GG genotype associated with higher plasma levels of IL10 among Bhutanese population has closer affinity to North Indian (2.30% *vs.* 5.69%) [24] and Srilankan (2.30% *vs.* 8.1%) populations [25]. However, the GG genotype was distributed more frequently among the European population (24%-34.4%) [26, 29].

Transcription of the IFNγ gene is influenced by SNP at the position of +874A/T and secretion of high, intermediate

and low plasma levels of IFNγ were associated with TT, AT and AA genotypes, respectively [30]. Studies from different populations have reported that the AT genotype confers protection to dengue fever and severe dengue; in contrast, AA and TT genotype increases risk for dengue infection [8, 31]. Frequency of T Allele, which is associated with higher production of IFNγ, is similar to the Indian population (37.50% *vs.* 37%) [32] but it differs from native Canadians (3%) [33] and Egyptians (69.9%) [34].

The TT homozygous of TGFβ1-509C/T has a higher level of TGFβ1 plasma concentration than the TC and CC genotypes [35]. A study of the Taiwanese population reported that the CC genotype is associated with a high risk of severe dengue infection [9]. The frequency of TT genotype in this study was in closer range to the Indian population (5%) [36]. However, Asian countries like China, Korea, Iran and Thailand have reported higher frequencies of TT genotypes (22%, 21.9%, 29.7% and 48.2% respectively) [14, 37-39].

For SNP at CD209-336A/G, the frequency of G allele, related to higher expression of DC-SIGN is similar to western Indians (15.9%) [40]. Furthermore, the frequency of G allele detected among the Bhutanese population is slightly higher than the frequency reported from other Asian populations, such as those of eastern China (7.3%) [41], Northern Thailand (9.5%) [42] and Taiwan (3.5%) [43]. Case-control studies from Thai and Taiwanese populations have reported that G allele of CD209 increases risk of severe dengue infection [10, 15]. In contrast to this, studies from Mexican and Brazilian populations have reported protective association against symptomatic dengue [44, 45].

Frequency distribution of most of the genes in our study was in closer affinity to the Indian population. This could be due to similarities in ethnicities as dengue-endemic areas share an immediate border with India. However, it would be too early to come to this conclusion as the exact ethnic composition of dengue-endemic areas of Bhutan remains undefined. On the other hand, based on this comparison, we could speculate that the Bhutanese population residing in dengue-endemic areas might have the same genetic profile with the Indian population. Therefore, the similarity in the findings between the two countries can be partly attributed to similar host genetic response towards disease outcome.

The variation of genetic frequencies with other studied populations could be due to reasons such as ethnic differences among the population and a combination of various ethnicities in the same geographical location. The non-uniformity in the sample size and study design might have also influenced the result of different studies.

The limitation of this study is lack of plasma cytokines level, due to the short half-life of these substances and the lack of facilities in Bhutan. Moreover, the inclusion of samples from dengue naïve population of Bhutan (higher mountains) would provide some crucial information.

5. Conclusion

In conclusion, this study demonstrates the overview of the frequency distribution of TNFα, IL10, TGFβ1, IFNγ and DC-SIGN genes polymorphism among the Bhutanese population. Results from this study will provide baseline data for further studies to assess the association between a polymorphism in host genetic and clinical outcome of dengue infection and further examination of the role of dengue susceptible gene as potential biomarkers.

Acknowledgement

The study was supported by Chulabhorn International College of Medicines (CICM), and Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma Thammasat University

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