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Investigation of TNF- α and DC-SIGN promoter polymorphisms in patients with dengue fever in Lahore city of Pakistan

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ABSTRACT

Background and Objective: Dengue fever (DF) has been a major health concern globally. Pakistan is also combating this infection for the last decade. Cytokine genes play an important role in DF pathogenesis. This study aimed to analyze dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and tumor necrosis factor α (TNF- α) genes promoter polymorphisms in DF patients.

Methods: A total of 140 ($n = 140$) dDF patients were recruited for study at the Department of Human Genetics and Molecular Biology of University of Health Sciences, Lahore, Pakistan over a period of 3 years. Simple DF was noted in 105 patients (75%) while 35 (25%) showed bleeding complications. All patients were found positive for dengue non-structural protein or dengue IgM. All patients were tested for two polymorphisms in TNF- α (-238G/A, and -308G/A) and one polymorphism in DC-SIGN (-336G/A) using restriction fragment length polymorphism technique. A single nucleotide polymorphism stats program was used for statistical analysis.

Results: Susceptibility to develop dengue infection in the presence of -336G allele odds ratio (OR = 27.95, $p = <0.0001$) and GG genotype (OR = 183.77, $p = <0.0001$) was found to be significantly associated in this study. Presence of a combination of alleles -336G/-238A/-308A was noted in 59.4% of DF cases and 7.6% healthy controls, a difference with statistical significance (OR = 31.46, $p = <0.0001$). Moreover, prevalence of DF symptoms showed a trend higher in G-carriers versus non-G-carriers of DC-SIGN -336 polymorphism.

Conclusion: This work suggests a potential association of DC-SIGN -336 polymorphism with susceptibility to develop symptomatic dengue illness. However, no potential association was found between TNF- α promoter polymorphisms and dengue infection in this study.

Keywords: *Aedes aegypti*, dengue virus, genotype, polymorphisms, RFLP, TNF- α , DC-SIGN.

Received: 27 September 2022

Revised date: 22 November 2022

Accepted: 27 February 2023

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Introduction

Dengue fever (DF) is a major arthropod-borne flavivirus disease with significant morbidity and mortality in tropical and sub-tropical regions of the world. It is caused by the Dengue virus (DENV), an arbovirus with four serotypes (DENV-1 to DENV-4) and transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes. DF is usually a self-limited disease characterized by a wide range of clinical symptoms including fever, headache, myalgia, bone, and abdominal pain in combination with laboratory indications.¹ However, a more severe form of the disease called dengue hemorrhagic fever (DHF) is also recognized which can be life threatening. The global incidence of dengue infections has been calculated to be 390 million every year.²

In Pakistan, the serological confirmation of dengue infection was first reported in 1968. Since then there have

been many outbreaks of dengue infection in Pakistan, affecting thousands of people and claiming hundreds of deaths.³ All DENV strains including mixed serotypes were found to be circulating in the Pakistani population. In 2016, during the worst epidemic in Islamabad and Rawalpindi, the most commonly observed serotypes were DENV-2 and DENV-3. While DENV2 serotype was predominantly found in the 2010 and 2011 outbreaks.⁴

A significant role of genetic factors in dengue pathogenesis has been suggested by a number of observations including large heterogeneity in dengue disease outcome⁵ and epidemiological differences among different ethnic populations.⁶ There have been many genetic association studies examining the role of

different candidate loci including those of immune system receptors dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN and FcγRIIa)⁷, cytokine pathways, e.g., Tumor necrosis factor α (TNF-α)⁸, major histocompatibility complex (HLA-A and HLA-B)⁹, complement pathway (MBL2)¹⁰, and others such as Vitamin D receptor (VDR), human platelet antigen and phospholipase C epsilon-1.¹¹

The first line of defense in the innate immune system involves phagocytic cells such as dendritic cells (DCs) and macrophages which mediate early recognition and uptake of microbes through germline-encoded pattern recognition receptors (PRRs).¹² The most significant among the PRRs is the DC-SIGN molecule, which is a key player in the early interaction of a pathogen with a DC, mediating DC-T cell interaction, DC migration, and pathogen uptake.¹³

The gene for DC-SIGN is localized on the short arm of chromosome 19 and a functional single nucleotide polymorphism (SNP) in the promoter region, representing an A/G transition at position -336 (rs4804803) has been reported as a risk factor for a severe form of dengue infection, parenteral acquisition of human immunodeficiency virus, susceptibility toward tuberculosis and COVID-19.¹⁴ However, contradictory reports describing protective effect of this polymorphism against many infectious agents that use DC-SIGN receptor to infect DCs, such as human T-lymphotropic virus type 1 and tick-born encephalitis virus have also been reported in the literature.¹⁵

TNF-α, a multifunctional pro-inflammatory cytokine, which plays a critical role in several autoimmune and infectious diseases including dengue infection. It has been known to be upregulated in dengue disease leading to increased vascular permeability, inflammation, and endothelial cell apoptosis resulting in hemorrhagic manifestations.¹⁶

The TNF-α gene is localized within region III of the major histocompatibility complex on the short arm of chromosome 6. Different promoter polymorphisms of the TNF-α gene, including -238 (G/A; rs361525) and -308 (G/A; rs1800629), have been shown to influence the TNF-α transcriptional activity and levels.¹⁷ Several studies have found the TNF-α-238 and -308 polymorphisms associated with serious complications in different autoimmune and infectious diseases including dengue¹⁸, hepatitis B and C viruses, Chagas disease, malaria, leishmaniasis, and bacterial infections.¹⁹

There are only a handful of studies regarding the association of promoter polymorphisms in DC-SIGN and TNF-α with dengue disease. The results of these studies are conflicting especially for DC-SIGN, largely due to differences in allele frequencies among different ethnic groups and the definition of severe dengue infection.²⁰ However, these

studies largely focused on severe forms of dengue infection (mostly DHF) and association studies regarding host genetic factors in classical dengue infection pathogenesis are lacking. An interesting aspect would be to study the genetic modulation of classical dengue disease especially associations with clinical signs and symptoms. The present case-control study determines the genetic association of promoter polymorphisms of DC-SIGN and TNF-α with the risk of developing DF in Pakistani patients with dengue disease. The objective of the study was to investigate TNF-α and DC-SIGN promoter polymorphisms in DF patients to find out the association between these polymorphisms and the development of severe dengue disease in the local population of Pakistan.

Methods

This study included a total of 245 unrelated subjects, divided into two groups: those having DF ($n = 140$) and healthy controls ($n = 105$). Both the groups were matched for gender, age, ethnicity, and geography. The DF samples were collected during high transmissibility season of dengue disease from different tertiary care hospitals of district Lahore such as Services Hospital, Mayo Hospital, and Sir Ganga Ram Hospital, Lahore, Pakistan. Sampling was carried out during different outbreaks of DF in Lahore city from 2015 to 2018. After informed consent from the patients, 5 ml of venous blood sample was collected in ethylenediaminetetraacetic acid (EDTA)-Vacutainer. The study was approved by the Institutional Ethics Committee which follows the declaration of Helsinki, 2008.

Only clinically and serologically confirmed cases were included in the study. Diagnostic criteria of centers for disease control and prevention or dengue expert advisory group was followed. Patients with clinical symptoms, e.g., high-grade fever, body ache, pain behind the eyes, bone and joint pain along with thrombocytopenia ($\leq 100,000/\text{mm}^3$), leukopenia ($< 4,000/\text{mm}^3$) and dengue IgM positivity were included in the study and patients having any other febrile illness or co-infection were excluded. The subjects included in the healthy control group had never reported recent infection due to any other pathogen or DENV serotypes (DEV1-DENV4).

DNA was isolated from whole blood using a standard phenol-chloroform extraction procedure. DC-SIGN-336 A/G and TNF-α-238 and -308 polymorphisms were genotyped by a polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) based approach as described previously.¹⁴ The oligonucleotide sequences and optimized annealing temperatures used in PCR amplifications, PCR product sizes, and restriction enzymes used for genotyping of DC-SIGN and TNF-α polymorphisms are detailed in Table 1.

Table 1. Primer sequences, annealing temperatures, PCR product sizes, and restriction enzyme specifications.

SNP ID	Primer sequence	Annealing temperature	PCR product size	Restriction enzyme
rs4804803 (-336A/G)	Forward primer 5'ACTGGGGGTGCTACCTGGC 3' Reverse primer 5'GGATGGTCTGGGGTTGACAG 3'	58°C	152 bp	MscI
rs361525 (-238G/A)	Forward primer 5'CTGTCCAGGCTTGCCTGCTAC 3' Reverse primer 5'CTCACACTCCCCATCCTCCCCGGATC3'	64°C	376 bp	BamH1
rs1800629 (-308G/A)	Forward primer 5'AGGCAATAGGTTTGGAGGGCCAT3' Reverse primer 5'TCCTCCCTGCTCCGATTCCG3'	58°C	107 bp	NCOI

Bases in grey color are mutagenic that are necessary for restriction site creation.

Table 2. Demographic data of patients.

Demographic variables	DF patients n = 140		Controls n = 105 Frequency Percentage		Overall results n = 245	
Gender						
Male	115	81.9%	77	72.4%	192	78.4
Female	25	18.1%	28	27.6%	53	21.6
Age groups						
Under 15 years	3	2.1%	5	4.8%	8	3.3
16-30 years	37	26.4%	40	38.1%	77	31.4
31-45 years	67	47.9%	45	42.9%	112	45.7
46 years and older	33	23.6%	15	14.3%	48	19.6

Table 3. Allele frequencies and association.

Sr. #	SNP ID	Base change	Gene	Role	MAF		OR (95% CI)	p-value
					DF (%)	Controls (%)		
1	rs4804803 (-336)	A>G	DC-SIGN	Promoter	152 (72)	18 (9)	27.95 (15.80-49.43)	<0.0001
2	rs361525 (-238)	G>A	TNF- α	Promoter	30 (11)	21 (10)	1.10 (0.61-1.98)	0.86
3	rs1800629 (-308)	G>A	TNF- α	Promoter	27 (10)	16 (7)	1.32 (0.69-2.52)	0.49

Statistical analysis

Allele and genotype frequencies are represented as numbers (percentage). Allele and genotype frequencies were determined by direct counting and compared between groups using the chi-square test or Fisher's exact test as appropriate. The conformance of genotype frequencies to Hardy-Weinberg equilibrium was tested using the chi-square test. Odds ratio (OR) with a 95% confidence interval (CI) and associated p-value, adjusted for age and sex, was calculated as a measure of association/risk by the SNPstats program (online available at <http://bioinfo.iconcologia.net/SNPstats>). The combination of alleles/haplotype frequencies were also

determined and compared using the same SNPstats program. A p value less than 0.05 was considered significant in all comparisons made.

Results

In this study, a total of 245 samples (140 DF patients and 105 healthy controls) were analyzed for DC-SIGN-336, TNF- α -238, and TNF- α -308 polymorphisms. Our demographic data showed that among the enrolled patients, 81.9% were males and 18.1% were females with a mean age of 28.07 ± 14.35 years (1-67 years) while controls included 72.4% males and 27.6% females with a mean age of 22.64 ± 8.29 years (13-50 years) (Table 2). The DF group was not tested for DENV

serotypes; however, major circulating serotypes have been reported to be DENV-2 and DENV-3 in Pakistan.^{4,21}

The allelic and genotype distribution was determined to investigate the association of DC-SIGN-336 and TNF- α -238 and -308 polymorphisms with dengue infection. Analysis of allele distribution between DF and the control group showed that the frequency of the DC-SIGN-336G minor allele was significantly higher in DF patients compared to controls (OR = 27.95, p = <0.0001). However, allelic frequencies were similar among DF and control groups with no statistical difference when tested for TNF- α -238 and -308 promoter polymorphisms (Table 3).

Accordingly, genotypic distribution for DC-SIGN -336 SNP was in line with the pattern for allele frequency, as GG/AG genotypes were found to be significantly higher in DF patients than healthy controls (OR = 183.77, p = <0.0001 for GG genotype; OR = 62.22, p = <0.0001 for AG genotype, after adjusting for age and gender). Whereas for TNF- α -238 and -308 polymorphisms, the genotypic differences between DF patients and controls failed to reach any statistical significance (Table 4 shows genotypic distribution).

All the genotypic frequencies followed the Hardy-Weinberg equilibrium except for DC-SIGN-336 SNP.

In order to assess the combined effect of DC-SIGN and TNF- α polymorphisms, the possible combination of alleles or haplotype frequencies was inferred as presented in Table 5.

The frequency of the G-G-G allelic combination was found to be significantly more frequent in the DF group as

compared to the control group (OR = 31.46, p = <0.0001), implying that carrying the risk allele G for DC-SIGN-336 SNP predominantly determines the susceptibility to dengue infection in this study.

Generally, dengue patients were presented with 12 different symptoms. When the G-carriers and non-G carriers for DC-SIGN-336 SNP were compared for each clinical symptom, only back and leg pain showed a statistically significant difference (p = 0.02 for Fisher's exact test) among the two groups (Table 6).

Discussion

As with other countries in South-East Asia, dengue infection has been a major health concern in Pakistan. Although rigorous dengue control measures have decreased the risk of dengue outbreaks, cases of dengue disease are still being reported in Pakistan. The etiology of dengue infection suggests a multifactorial nature with the involvement of multiple factors including infecting DENV serotype, immunological factors, and host genetic factors.²² Multiple studies analyzing polymorphisms of different genes, such as DC-SIGN, TNF- α , Fc γ receptors, HLA, and VDR have provided published evidence that host genetic factors are significant contributors to the pathogenesis of dengue infection.⁷⁻⁹ This area has been largely overlooked from Pakistani perspective and there is only one study so far addressing the role of host genetic factors in dengue experience from Pakistan.⁷ To our

Table 4. Genotype distribution and association.

Sr. #	SNP	Genotypes	Frequency in DF (%)	Frequency in controls (%)	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
1	rs4804803 (-336 A/G)	AA	07 (6.7)	91 (86.7)	1.00	<0.0001	1.00	<0.0001
		AG	44 (41.9)	10 (9.5)	57.20 (20.41-160.34)		62.22 (20.97-184.64)	
		GG	54 (51.4)	04 (3.8)	175.50 (49.10-627.30)		183.77 (49.34-684.46)	
2	rs361525 (-238 G/A)	GG	114 (81.4)	86 (80.4)	1.00	0.07	1.00	0.05
		GA	22 (15.7)	21 (19.6)	0.79 (0.41-1.53)		1.12 (0.54-2.31)	
		AA	04 (2.9)	0 (0)	NA		NA	
3	rs1800629 (-308 G/A)	GG	113 (80.7)	91 (85.1)	1.00	0.37	1.00	0.65
		GA	27 (19.3)	16 (14.9)	1.36 (0.69-2.67)		1.20 (0.55-2.62)	
		AA	0 (0)	0 (0)	NA		NA	

Table 5. Haplotypes/allelic combinations and association.

Haplotypes			Frequency in DF (%)	Frequency in controls (%)	OR (95% CI)	p-value
DC-SIGN-336 A/G	TNF- α -238 G/A	TNF- α -308 G/A				
A	G	G	21.9	76.39	1.00	NA
G	G	G	59.4	7.6	31.46 (12.39-79.91)	<0.0001
A	G	A	5.0	5.2	3.64 (1.01-13.14)	0.05
A	A	G	1.6	8.5	0.78 (0.16-3.79)	0.76

Table 6. Association of -336 A/G polymorphism of DC-SIGN (rs4804803) and clinical features in dengue patients.

Sr. #	Clinical feature	Frequency in dengue patients (%)	Frequency in G carriers (%)	Frequency in G non-carriers (%)	p-value
1	Generalized body ache	75.2	70.47	4.76	1.000
2	Back and leg pain	70.5	68.57	1.90	0.0227
3	Headache retro-orbital pain	48.6	47.62	0.95	0.113
4	Itching	28.6	28.57	0	0.188
5	Rash	17.1	17.14	0	0.352
6	Nausea vomiting	48.6	45.71	2.86	1.000
7	Abdominal pain	51.4	48.57	2.86	0.710
8	Sore throat cough	22.9	20.95	1.90	1.000
9	Bleeding manifestations	33.3	31.43	1.90	1.000
10	Dehydration	53.3	47.61	5.71	0.118
11	Thrombocytopenia	81.9	75.24	6.67	0.346
12	Leucopenia	87.6	82.86	4.76	0.208

knowledge this is the first report investigating the association of DC-SIGN and TNF- α polymorphisms with dengue infection in Pakistan.

The present study analyzed allele and genotype frequencies of DC-SIGN-336 and TNF- α -238 and -308 polymorphisms in patients with antecedents of dengue infection, in comparison with population controls. Our results show that GG/AG genotypes and G allele of DC-SIGN-336 SNP are strongly associated with susceptibility to DF as compared to controls, suggesting a role of this polymorphism in the development of dengue disease. This finding is consistent with a study of the Taiwanese population. They analyzed 176 DF, 135 DHF, 143 other febrile illness cases, and 120 normal controls for DC-SIGN-336 SNP and found a strong association between GG/AG genotypes of rs4804803 and DF severity while AA genotype was linked with protection. This study has also shown that monocyte-derived DCs from individuals harboring the DC-SIGN-336AG genotype have higher surface DC-SIGN expression correlated to limited DENV replication but an augmented immune response with high cytokine levels including TNF- α , as compared to individuals with the AA genotype.²³ This finding provides interesting insights into dengue infection pathogenesis as severe dengue disease is attributed to altered immune response but not the viral load, which supports the role of the DC-SIGN-336 G allele in susceptibility towards dengue infection.²⁴ However, the association in this study between the G allele of DC-SIGN-336 SNP and dengue infection contrasts with the results of a study conducted on 606 dengue patients and 696 controls, where the G allele was strongly associated with controls and DHF compared to DF in a Thai cohort.²⁵ Similarly, another case-control study from Brazil suggested a population-specific effect

of DC-SIGN-336 G allele where it is associated with severe dengue susceptibility in Asians but confers protection in Brazilians. They investigated 88 severe DF patients and 335 healthy controls. They found a strong association between DC-SIGN-336G>A polymorphism and protection against severe DF.²⁶ A meta-analysis in China was conducted on nine papers and 12 studies, consisting of 1,520 severe DF cases and 1,496 clinical dengue patients. They concluded that DC-SIGN-336 SNP was strongly associated with severe DF while a few other studies found no association of DC-SIGN-336A/G SNP with dengue infection.²⁷⁻²⁹ It is well established that the relationship between an SNP and human disease is rarely forthright and complicating factors like linkage, population-specific genetic differences, epigenetic changes, and environmental factors do come into play. Therefore the discrepancy between these studies regarding protection for or no association of the G allele with DF may result from the heterogeneous distribution of the G allele among studied populations, other population-specific genetic differences, differences in clinical categories compared and/or sampling strategies, and environmental factors.

Moreover, in this study, the prevalence of the disease-associated symptoms had a trend to be higher in G-carriers as compared to G non-carriers which contrasts the results described by a study on Brazilian DF patients and may be explained by differences in G allele frequencies observed in these studies.²⁰ However both the studies revealed a putative association of DC-SIGN-336 A>G SNP with joint pain observed in dengue patients.

This study also analyzed the allele and genotype frequencies of -238 and -308 polymorphisms of the TNF- α gene in Pakistani DF patients and healthy controls. The distribution of allele and genotype frequencies of

TNF- α -238 and -308 polymorphisms failed to reach a statistically significant difference thus no association was found between these polymorphisms and dengue infection. Similarly, two studies from Mexico and India also failed to reveal any association between TNF- α -308 polymorphism and dengue disease.^{8,28} Our results are in accordance with the previous study where no association was found between TNF- α -238 and -308 polymorphisms and primary DF, DHF, and controls in ethnic Thais but a higher number of these polymorphisms were seen in secondary DHF.³⁰ Our results are in agreement with another study recently conducted in Mexico on patients of DF, DHF, and normal controls. They also did not find any difference in allele and genotype frequencies between patients and controls. They found the GA genotype in healthy controls while the GG genotype was in DF patients. They elaborated on the fact that the increased level of TNF alpha protein is independent of genetic involvement.³¹ In 2018, a meta-analysis was conducted in Malaysia to make a conclusive evidence regarding TNF alpha -308 and -238 SNPs' role in dengue severity. Eight studies comprising 640 DF patients and 1,275 controls were analyzed. They deduced that there is no association between these SNPs and dengue severity not only in Asian but also in other ethnic populations.³² Another case-control study from Brazil supports our findings for TNF alpha-238 and -308 SNPs. They also did not find any association between these SNPs and DF. In their meta-analysis, they found that the GG genotype of -308 SNP provides protection as well as risk in Asian and American population.³³ The negative associations in these studies may be attributed to low frequency of minor (A) allele observed for both TNF- α -238 and -308 polymorphisms in the studied populations including the Pakistani population. All the genotypic frequencies in the present study followed the Hardy-Weinberg equilibrium except for DC-SIGN-336 SNP. In various studies, it has been shown that many polymorphisms may not fall into the Hardy-Weinberg equilibrium for non-causal reasons, particularly if a population exhibits ethnic diversity. Probably the wide genetic admixture of the Pakistani population could have influenced the Hardy-Weinberg distribution in this study.^{17,22}

Conclusion

In conclusion, this study demonstrates the potential association of dengue infection with DC-SIGN-336 A>G polymorphism, but not with TNF- α -238 and -308 polymorphisms in a Pakistani cohort. However, genetic associations in dengue disease are influenced by population-specific genetic, epigenetic, and environmental variables which need to be analyzed in genetically distinct ethnic populations in order to completely comprehend the dengue infection pathogenesis.

Limitations of the Study

Our study has many limitations. Few of those are small sample sizes, unavailability of confirmatory tests like real-time RT-PCR, cytokines estimation to validate our molecular data, DENV type analysis, and gene sequencing to confirm our allele/genotype results.

Acknowledgement

The authors are highly thankful to the staff and faculty of the Department of Human Genetics and Molecular Biology and the institutional authorities of the University of Health Sciences Lahore Pakistan for their support during the execution and completion of the study.

List of Abbreviations

DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
TNF- α	Tumour necrosis factor α
SNP	Single nucleotide polymorphism

Conflict of interest

None to declare.

Grant support and financial disclosure

This study was completed as part of the M. Phil research, fully funded by a research grant given to the first author by the University of Health Sciences Lahore, Pakistan.

Ethical approval

The study was approved by the Institutional Ethics Review Committee of the University of Health Sciences Lahore, Pakistan on January 25, 2015.

Author's contributions

SRA, SB: Acquisition and analysis of data, drafting of manuscript, intellectual input.

SK: Concept and design of study, critical intellectual input to the manuscript

ALL AUTHORS: Approval of the final version of the manuscript to be published

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