Association Between -592 C/A Polymorphism of *Interleukin–10* Gene and Diabetic Nephropathy in Type 2 Diabetes Mellitus Patients

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ABSTRACT

Objective: To establish the association between -592 C/A polymorphism of *interleukin (IL)–10* gene and Diabetic Nephropathy (DNP) in Type 2 Diabetes Mellitus (T2DM) patients.

Study Design: Comparative observational study.

Place and Duration of the Study: Department of Nephrology (in collaboration with Biochemistry and Advanced Research Centre for Biomedical Sciences (ARCB)), Mayo Hospital and King Edward Medical University, Lahore, Pakistan, from January to December 2021.

Methodology: The study included 282 patients with T2DM for ≥5 years, and aged ≥40 years. Patients with and without DNP were placed in Group A and B (n = 141 in each group), respectively. After drawing samples, deoxyribonucleic acid (DNA) was first extracted from whole blood, then amplified *via* specific primers, and finally genotyped through the polymerase chain reaction and restriction fragment length polymorphism (PCR – RFLP) method. Median and interquartile range (IQR) were used to describe the skewed data and frequencies were used for categorical data. Mann-Whitney U test was used for comparison of data groups. Chi-square test was used to establish an association.

Results: The median age of patients was 50.0 (12) years in Group A and 54.0 (11) years in Group B. Male-to-female distribution was 39 (27.7%) / 102 (72.3%) in Group A and 49 (34.8%) / 92 (65.2%) in Group B. The -592 C/A polymorphism of *IL–10* genes was found more frequently in Group A 72 (51.0%) than Group B 63 (44.7%) without statistical significance (p = 0.283).

Conclusion: The current study showed that the presence of single nucleotide C/A polymorphism at the -592 position of *IL–10* gene in T2DM patients does not influence (increase / decrease) their susceptibility to develop DNP.

Key Words: *Type 2 Diabetes Mellitus, Diabetic Nephropathy, Interleukin-10, -592 C/A, Single nucleotide polymorphism*.

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INTRODUCTION

Diabetes Mellitus (DM) is an endocrine metabolic disorder characterised by hyperglycaemia due to the impaired production of insulin (Type 1) or tissue resistance to insulin (Type 2).[1] The global prevalence of DM is rising and 578 million people are expected to be affected by 2030 which will demand very huge expenditure of 825 billion dollars in diabetic care.[2] T2DM constitutes 85-95% of all DM cases.[3] It has multiple micro- and macro-vascular complications, and of these, Diabetic Nephropathy (DNP) is frequently encountered. Nephropathy, secondary to DM, is the chief cause of End-Stage Renal Disease (ESRD) worldwide and it is associated with high mortality.[4]

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The precise aetiology of DNP is not well understood and many factors contribute in its development such as elevated blood pressure, high glomerular filtration rate (GFR), poor glycaemic control, obesity, hyperuricaemia, and ethnicity.[5] The pathogenesis of DNP is complex involving multiple overlapping mechanisms which include the formation of advanced glycation end-products (AGE), production of reactive oxygen species, activation of various pathways such as protein kinase C, mitogen-activated protein kinases, transforming growth factor (TGF) beta – 1, and renin-angiotensin aldosterone system (RAAS).[6] The immune system and inflammatory reactions play a crucial role in the development of DNP through the production of various cytokines, particularly interleukins. The members of the interleukin family which promote inflammation, such as IL – 1, IL – 6, and IL – 18, induce glomerular basement membrane thickening, mesangial proliferation, and endothelial apoptosis. The IL–10 is a key regulator of many immune responses and it has established anti-inflammatory protective role in DNP.[7]

Genetics play a vital role in DNP, as genetic variability influences the susceptibility of T2DM patients to DNP. It alters the expression and behaviour of various factors involved in the

pathogenesis of DNP such as IL–10, IL-4, tumour necrotic factor (TNF) – alpha, and TGF beta –1.[8] The gene that encodes for IL-10 is bi-allelic with one allele located on each of the paired chromosomes 1, q31 - q32 and exhibit five exons. The promoter region of *IL-10* gene can have several single nucleotide polymorphisms (SNPs) influencing expression as well as function of *IL–10* gene. Transcriptional start site at 5' flanking region of *IL-10* gene is highly polymorphic and its three known SNPs are: C/A (C to A substitution) at position - 592 (rs1800872), C/T (C to T substitution) at position -819 (rs1800871), and G/A (G to A substitution) at position-1082 (rs1800896).[9] The wild genotype of *IL–10* gene with respect to SNP at -592 position is CC. A mono-allelic polymorphism gives rise to the CA genotype which is much more common than the rare genotype AA produced by bi-allelic SNP. The advancement in genetics has made it possible to counter many disease processes by inducing desired changes in target genes thus altering their expression. The *IL–10* gene has now become the focus of research in nephrology and its three SNPs are being investigated for their possible association with DNP. The results, so far, are mixed and inconclusive. Some previous studies showed the association of DNP with -1082 G/A and -819 T/Cpolymorphisms of *IL–10* gene,[10,11] while other studies reported no correlations between any of the three aforementioned polymorphisms of *IL–10* genes and DNP.[12-14] A group of researchers from Mexico studied the combined effect of three SNPs of *IL–10* gene and found that the ATC (-1082A/-819T/-592C) and GTA (-1082G/-819T/-592A) haplotypes are associated with increased risk for DNP.[15]

Till date, no research has been conducted in Pakistan to determine the contribution of the known SNPs of *IL–10* gene in causing DNP and the relevant data available from other countries are also limited. So, this study was conducted to establish the association between -592 C/A polymorphism of *IL–10* gene and DNP in T2DM patients.

METHODOLOGY

It was a comparative observational study, conducted at the Nephrology Department (in collaboration with the Biochemistry Department, Diabetic Clinic, and Advanced Research Centre for Biomedical Sciences (ARCB)), Mayo Hospital, Lahore attached with King Edward Medical University, Lahore, Pakistan, after obtaining the approval from the Institutional Review Board (IRB No: 127/RC/KEMU, Dated: 10/02/2020). Patients with T2DM, aged 40 years or above and having DM for more than five years, were included in the study. The sampling was done using non-probability convenience method and 282 patients were selected. Patients with ESRD, chronic kidney disease (CKD), previous history of proteinuria, haematuria, T2DM for less than five years' duration, uncontrolled T2DM (HbA1C >08 %), urinary tract infections, and history of any cancer were excluded from the study. DNP was defined as uACR ≥0.03 mg/mg. The patients with DNP were placed in the Group A while those without DNP were put in the Group B (n = 141 each).

Five millilitres of venous blood was drawn from each patient and the samples were then transferred to ethylenediamine tetraacetic acid (EDTA) vials which were stored in refrigerator at 2-8°C. DNA was extracted from leucocyte fraction of stored whole blood samples using a Genejet genomic DNA extraction kit. The concentration of DNA extracts was checked using a spectrophotometre at wavelengths of 260 and 280 nm. The 260/280 ratio was used to assess the purity of DNA.

Commercially Synthesised Forward: 5'-GTGAGCACTACCTGAC-TAGC-3' and Reverse: 5'-CCTAGGTCACAGTGACGTGG-3' primer sets were used to detect C/A polymorphism at -592 position of *IL–10* gene. For verification purposes, primer sequences were matched with model sequences provided on Ensemble Genome Browser (https://www.ensembl.org/index.html). Important properties of primers such as length, concentration, GC content, melting point, self-complementarity, and mismatch chances were checked with online Oligo Calc software (http://biotools.nubic.northwestern.edu/OligoCalc.html). The conditions for polymerase chain reaction (PCR) were optimised using thermocycler T100 (BioRad, USA) to attain desired products. The reaction mixture (15µl) for DNA amplification was prepared using genomic DNA extract (02 µl), forward primer (01 µl), reserve primer (01 µl), nuclease free water (03 µl), and 8 µl of Dream Taq PCR master mix 2X (Taq polymerase, DNA staining dye, PCr buffer, dNTPs, and Mg^{2+}). The specificity of the amplified product was confirmed through the electrophoresis on 2% agarose gel. Finally, the amplified products were digested with the RsaI restriction enzyme and separated on 2% agarose gel. After digestion, the gene with the A allele produced two fragments of 176 and 236 base pairs while the gene with the C allele remained undigested yielding a single fragment of 412 base pairs. The collected data including demographic, clinical, and genomic parameters were analysed using SPSS version 26.0. Categorical variables such as gender and polymorphism were expressed as frequencies (percentages) while continuous variables such as age and duration of DM were presented as median and interquartile range (IQR). The distribution of continuous variables was assessed using Shapiro-Wilk's test which was found non-normal, so Mann-Whitney U test was used to compare them between groups A and B. The frequency of -592 C/A polymorphism of *IL–10* gene along with its three resultant genotypes (CC, CA, and AA) and the two constituent alleles (C and A) was also determined. Chi-Square test was performed to explore the association between DNP and polymorphism of *IL–10* gene. The confidence interval (CI) was taken as 95% and a p-value of ≤0.05 was considered statistically significant.

RESULTS

The median (IQR) age of patients was 50 (12) years and 54 (11) years in Group A and B, respectively (p = 0.024). The male-to-female ratio was 39 (27.7%) / 102 (72.3%) in Group A and 49 (34.8%) / 92 (65.2%) in Group B (p = 0.199). The comparison between medians of serum urea, creatinine, and uACR is shown in Table I.

Table I: Comparison of demographical and biochemical parameters between DNP (Group A) and non-DNP (Group B) patients.

Study variables	Study groups	Median (IQR) / frequency (%)	p-value
Age (years)	Group A	50 (12)	0.132
	Group B	54 (11)	
Gender (male / female) (n (%))	Group A	39 (27.7%) / 102 (72.3%)	0.199
	Group B	49 (34.8%) / 92 (65.2%)	
Serum urea (mg/dl)	Group A	30 (6)	0.074
	Group B	29 (5)	
Serum creatinine (mg/dl)	Group A	1.00 ± 0.13	<0.001*
	Group B	0.94 ± 0.13	
Spot uACR (mg/mg)	Group A	0.28 (0.36)	<0.001*
	Group B	0.02 (0.00)	

Mann-Whitney U test was used for non-normally distributed continuous variables. Chi-square test was used for categorical variables.
** Statistically significant p-value.*

Table II: Distribution of *IL–10* gene -592 C/A polymorphism, genotypes, and alleles in DNP (Group A) and non-DNP (Group B) patients.

IL–10 Gene -592 Polymorphism		Group A n (%)	Group B n (%)	p-value
Genotypes	C/C	69 (49.0%)	78 (55.3%)	0.468
	C/A	70 (49.6%)	60 (42.5%)	
	A/A	2 (1.4%)	3 (2.2%)	
Alleles	C	208 (73.7%)	216 (76.5%)	0.435
	A	74 (26.2%)	66 (23.4%)	
C/A	Yes	72 (51.0%)	63 (44.7%)	0.283
	No	69 (49.0%)	78 (55.3%)	

Chi-square test was performed to compare the distribution of observed genotypes, alleles, and -592 C/A polymorphism of IL–10 gene between the two study groups.

Three genotypes related to *IL–10* gene -592 polymorphism were observed in the study population in the following order of frequency: CC 147 (52.1%) > CA 130 (46.1%) >AA 5 (1.8%). The C allele was found much more abundant than the A allele in the overall population (424 (75.2%) *vs*. (140 (24.8%)). There was no statistically significant difference in the distribution of genotypes and alleles between the two study groups (p = 0.468, p = 0.435). Although, the frequency of C/A polymorphism at -592 position of *IL–10* gene was found slightly higher in Group A 72 (51.0%) as compared to Group B 63 (44.7%), yet its statistically significant association with DNP was not established (p = 0.283, Table II).

DISCUSSION

Genetics have documented a fundamental role in many known disease processes at various levels from determining the individual's susceptibility to the disease outcome. Deleterious mutations in specific sets of genes result in the production of structurally and functionally altered proteins which initiate or contribute to disease pathogenesis. Environmental factors play an additional role in the onset and progression of the disease process by modifying the behaviour of involved gene polymorphisms.[16] DNP has an established genetic basis with multiple genes and pathways found to be involved in its development and progression. Around 51 susceptibility genes with their 66 polymorphic variants have been identified which include: *ACACB* (rs2268388 and rs5186) related to pyruvate metabolism; *ADIPOQ* (-11391 G/A) and *SLC2A1* (rs841853) from adipocytokine signaling pathway; *APOC1* (rs4420638) involved in lipid metabolism, *NOS3* (-786 T/C) from oxidative stress pathway, *ACE I/D* and *AGTR1* (A1166C) component of RAAS, *TGF beta* - 1 (T869C) and *ELMO1* (rs741301 and rs10951509) from inflammatory pathways, *VEGFA* (-1499 C/T) and *EPO* (rs1617640) related to angiogenesis; *CCR5* (-59029 A/G); *IL–1 beta* (-511 C/T), *IL-6* (634 C/G), and *IL–10* (-1082 G/A) cytokines and *SIRT1* (rs4746720) from epigenetic pathway.[17]

The two major genetic mechanisms which have documented a role in DNP pathogenesis are DNA methylation that promotes the activation of immune cells and histone post-translational modifications that enhance the inflammatory genes expression.[18] As the strong genetic background of DNP has been disclosed, gene therapy has become the focus of treatment which can provide curative results. One of the important therapeutic targets is *IL–10* gene, as it has a key pathogenic role in DNP which can be modified because of its highly polymorphic expression. Among the three identified SNPs of *IL–10* gene, -592 C/A is the most neglected one with its definitive role in DNP still to be established.

The current study showed that C/A SNP at -592 position of *IL–10* gene did not affect the risk of developing DNP in T2DM patients and the results were also supported by a previous study.[19] In past, two separate researchers explored the contribution of *IL–10* gene polymorphism -592 in the pathogenesis of DNP and found that the studied SNP was associated with an increased risk of developing DM rather than DNP.[12,20] Thus, it was suggested that C/A polymorphism at -592 position of *IL–10* gene had neither causative nor protective role in relation to DNP. This could be due to several possible reasons.

The activation of immune response in DNP appears to be caused by a persistent state of hyperglycaemia rather than

antigenic stimulation. The AGE then induce an inflammatory immune response that leads to the proliferation of cortical fibroblasts, collagen production, and injury to the proximal tubular epithelial cells, all contributing to the development of DNP.[21] All DNP patients initially have normal renal functions that decline slowly over period of time as disease progresses and renal impairment may interfere with the activity of the immune system. The studied SNP of *IL–10* gene may behave differently under variable circumstances such as the degree of glycaemic state and status of renal function. As in the current study, all selected patients had better glycaemic control (HbA1C <08%) and preserved renal functions (serum creatinine <1.3 mg/dl), this might have affected the behaviour of -592 C/A SNP. Moreover, the isolated role of this *IL–10* gene polymorphism could be insufficient in determining the vulnerability of T2DM patients to DNP and might have required additional contribution of other related susceptibility gene polymorphisms. The ethnic factor may also play a role suggesting that *IL–10* gene polymorphism -592 C/A had no impact on susceptibility to DNP in the local diabetic population. Finally, studies have reported an association of *IL–10* gene polymorphism -592 C/A with other complications of T2DM most particularly retinopathy,[22] progression of non-diabetic renal diseases such as lupus nephritis,[23] susceptibility to infections such as hepatitis B,[24] and predisposition to certain malignancies.[25] Thus, *IL–10* gene polymorphism -592 C/A might be more specifically linked to pathologies other than DNP.

In the current study, the -592 C/A and C/C genotypes of *IL–10* gene were found in almost the same proportion among T2DM patients. A previous study reported that C/C was the dominant genotype in the diabetic group and the C allele had an association with the increased risk of T2DM.[19] The contrasting results suggest high occurrence of *IL–10* gene -592 C/A genotype in local diabetic population and its possible association with the risk of T2DM that needs to be evaluated with further research. In the current study, DNP patients had higher serum creatinine, uACR, and albuminuria compared to patients without DNP but no correlation between any of these laboratory parameters and SNP of *IL–10* gene was established.

The study was limited due to financial constraints, time constraints, small sample size, single targeted SNP (-592 C/A) of *IL–10* gene, and no measurement of IL–10 blood levels. Moreover, the corelation of various factors such as renal function status and degree of glycaemic control with *IL–10* gene polymorphism was not evaluated. Therefore, further studies with larger sample sizes, broader investigation of *IL-10* gene polymorphisms, and consideration of other possible associated susceptibility factors are suggested.

CONCLUSION

The current study showed that the presence of single nucleotide C/A polymorphism at -592 position of *IL–10* gene in T2DM patients does not influence (increase / decrease) their susceptibility to develop DNP.

ETHICAL APPROVAL:

The study was approved by the Institutional Review Board of the King Edward Medical University, Lahore, Pakistan, (Approval No. 127/RC/KEMU, Dated: 10.02.2020). This research was conducted by the ethical standards of the institute and in line with the 1964 Helsinki Declaration and its later amendments.

PATIENTS' CONSENT:

All participants signed a general research consent form, approved by the IRB.

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

MA: Critical revision of the manuscript for the important intellectual content and final approval of the manuscript.
UAB: Acquisition and analysis of data.
IE: Conception of the work.
MR: Analysis and interpretation of data.
MSP: Drafting of the work.
All authors approved the final version of the manuscript to be published.

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