Original article:

Association of interleukin-6 polymorphism (-634C/G) in the promoter region with diabetic nephropathy in type 2 diabetes mellitus

Rakshanda Talib[1], Muhammad Kashif[2], Romeeza Tahir[3], Ahmed Nadeem[4], Saba Khaliq[5], Bushra Mubarik[6], Afia Abbas[7], Shah Jahan[8], Nadeem Afzal[9]

Abstract:

Background*:** Long term microvascular complications of DM includes: nephropathy, retinopathy, and neuropathy. Increased level of IL-6 and its polymorphism have been associated with diabetic nephropathy.The present study was designed to determine serum level of IL-6 and C/G polymorphism at − 634 position in the promoter region of IL-6 gene in patients with type 2 diabetes mellitus (T2DM) with and without nephropathy. ***Method: This comparative study was comprised of 164 subjects, divided into two groups of 82 subjects in each. The subjects who had diabetes for more than 5 years without nephropathy were labeled as group-I and the subjects who had diabetes for more than 5 years with nephropathy were labeled as group-II. IL-6 polymorphism was detected by RFLP and its level was determined by ELISA technique. Data was analyzed using SPSS 20.0. ***Results:*** In group-I, 14 (17.1%) patients had CC, 47 (57.3%) had CG and 21 (25.6%) had GG polymorphism. In group-II, 9 (11%) patients had CC, 69 (84.1%) had CG and 4 (4.9%) had GG polymorphism. Pearson's correlation test determined significant association of IL-6 polymorphism (CC,CG,GG) in both the groups ($p < 0.001$). In patients of CC polymorphism, median levels of IL-6 was high in group-I (4.2) as compared to group-II (3.2),whereas in patients of CG polymorphism, median levels of IL-6 was high in group-II (3.8) as compared to group I (3.7) while in GG polymorphism, median levels of IL-6 was same in both the groups. On comparison of IL-6 polymorphism and level of IL-6 there was no statistically significant difference between the two groups (p1=0.304, p2=0.425 respectively). ***Conclusions:*** Statistically significant difference in the frequencies of allele (CC,CG,GG) between two groups is pointing towards the protective role of GG allele as it was predominant in T2DM patients without nephropathy.

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Introduction

Diabetes mellitus (DM) is a state of prolonged hyperglycemia, which may be due to a fault in insulin secretion, insulin action or combination of these conditions. The chronicity of DM can cause destruction of many organs such as kidneys, eyes and heart.[1]

In 2012, worldwide 347 million people were affected with DM and it is projected to be more than 366 million by the year 2030.[2,3] In 2013, the International Diabetes Federation (IDF) estimated 381.8 million people with diabetes and suggested a projected

1. Rakshanda Talib
2. Muhammad Kashif
3. Romeeza Tahir
4. Ahmed Nadeem
5. Saba Khaliq
6. Bushra Mubarik
7. Afia Abbas
8. Shah Jahan
9. Nadeem Afzal

Department of Immunology, University of Health Sciences, Lahore, Pakistan.

Correspondence to: Dr. Nadeem Afzal Professor & Head Department of Immunology, University of Health Science Lahore, Pakistan, email: immunology@uhs.edu.pk

increase to 591.9 million by 2035.[4]

Among all the continents, Asia has rapidly increasing number of DM patients i.e. Pakistan, India, China, and Japan.The prevalence of DM in Asia will nearly double in the coming two decades according to World Health Organization (WHO).[2] In Pakistan 6.9 million people were affected with diabetes in 2012 and this number will grow to 11.5 million by 2025.[4]

Immune-mediated diabetes (Type 1 DM) comprises only 5–10% of diabetes and also termed as IDDM while Type 2 DM (T2DM), also called NIDDM, comprises for 90–95% of DM.[1] The family history of diabetes is associated with T2DM. Involvement of genetic factors in T2DM is suggested by its increased prevalence rate in monozygotic twins as compared to dizygotic twins.[5]

DM is a chronic disorder and T2DM (90%~95%) is the most common form worldwide and it may affect every organ of the body.[3] Long term microvascular complications of DM are nephropathy, retinopathy, and neuropathy whereas macrovascular complications are ischemic heart disease (IHD), stroke and peripheral vascular diseases.[1]

Diabetic nephropathy (DN) is a common manifestation of microvascular complications of T2DM which is associated with morbidity and mortality, leading to lethal renal disease.[6]DN is defined by the presence of excess amount of extracellular matrix. The pathogenesis of DN comprises three steps, 1) glomerular hypertrophy and hyperfiltration, 2) inflammation of glomeruli and tubulo-interstitial regions, 3) cell reduction by apoptosis and accumulation of ECM. In addition to renal cells, macrophages also play a vital role in the pathogenesis of DN. The tissue reshaping in DN resembles with the wound healing after tissue injury. Pro-inflammatory macrophages, such as M1 macrophages, exasperate kidney cell injury, but anti-inflammatory M2 macrophages are also present, which stimulate epithelial and vascular repair.[7]

Inflammatory cytokines such as IL-1, IL-6, IL-18 and TNF are critically involved in the pathogenesis of DN.[8] Interleukin-6 (IL-6) is a multifunctional cytokine secreted from different cells, such as monocytes, lymphocytes, fibroblasts, endothelial cells, and mesangial cells. The role of IL-6 in T2DM is complicated and contentious, however, many researches have confirmed that IL-6 activates other inflammatory cytokines along with insulin resistance in peripheral tissues and apoptosis in pancreatic islets.[9,10,11]Therefore, IL-6 can be considered as an independent risk factor and pathological factor for impairment of insulin action and development of DM.[12]

There are several polymorphisms in the IL-6 gene. Many studies investigated association of IL-6 gene polymorphism with different diseases such as T1DM, T2DM, insulin resistance and other features of metabolic syndrome. These studies have focused on the three common SNPs of IL6 promoter region i.e. IL6 -174G>C, IL6 -634C>G, and IL6 -597A>G.[13]

IL-6 gene transcription and serum levels of IL-6 can be affected by SNP in the promoter region-634 C/G. Many epidemiological studies have been conducted in the diverse group of populations to determine association of IL-6 gene−634 C/G polymorphism with the risk of T2DM.[14]

IL-6 gene −634 G allele had been associated with the elevated risk of T2DM. SNPs in this locus can be considered as the candidate biomarkers for screening and diagnosis of T2DM.[14] IL-6 levels and its polymorphisms are related with the progression of DN. In another study, IL-6 -634C/G polymorphism has been suggested as a genetic factor for the progression of DN.[15]The effects of IL-6 gene may vary with ethnicity.[13]

The present study was designed to determine serum levels of IL-6 and C/G polymorphism at the position of − 634 in the promoter region of IL-6 gene in patients of T2DM with and without nephropathy.

Materials and methods

This comparative cross sectional study was conducted in the Department of Immunology University of Health Sciences (UHS) Lahore. The samples were collected from the Diabetes Center and Department of Nephrology, Sheikh Zayed Hospital Lahore. One hundred sixty four subjects were recruited for this study and they were grouped in to two of 82 subjects in each group strictly following the inclusion and exclusion criteria (Table 1).

Group I comprised of 82 patients of T2DM without nephropathy.

Group II comprised of 82 patients of T2DM with nephropathy (DN).

Table 1 : Selection criteria for the study groups

Inclusion criteria	Exclusion criteria
Diagnosed T2DM patients with and without nephropathy. Both male and female. Patients with ≥ 5 years duration of diabetes and not above 20 years	Patients of viral infection e.g. HBV, HCV Autoimmune disorders e.g. rheumatoid arthritis Malignancy Pregnancy Other vascular disorders

DN was diagnosed according to the presence of microalbuminuria i.e. 30 to 300 mg albumin/24 hours or albumin to creatinine ratio [ACR] of 30 to 300 mg/g or macro-albuminuria of >300 mg albumin/24 hours or ACR >300 mg/g.

After a written informed consent, all the relevant details such as age, sex and duration of disease were collected and recorded in a structured questionnaire. Five (5) ml of venous blood from each subject was drawn in a sterile 5 ml syringe and poured into two vacutainers: One vacutainer contained ethylene diamine tetra acetic acid (for DNA extraction) and the other gel vacutainer (for the estimation of serum IL-6). Soon after the sample was collected, it was transported in an ice box to the Department of Immunology, UHS, Lahore.The samples taken in the gel vacutainers were immediately centrifuged for the isolation of serum. Serum was separated into an aliquot that was marked with identification number and stored at -80°C for the estimation of IL-6 by ELISA technique.One EDTA vacutainer of each patient was stored at -20°C for DNA extraction. Following parameters were performed:

1. Serum IL-6 by ELISA
2. IL-6 polymorphism (634 C/G) by PCR and restriction fragment length polymorphism (RFLP) (Figures 2, 3).

Serum IL-6 levels were determined using commercially available ELISA kits (BOSTER,Germany). The standard IL6 curve is given below.

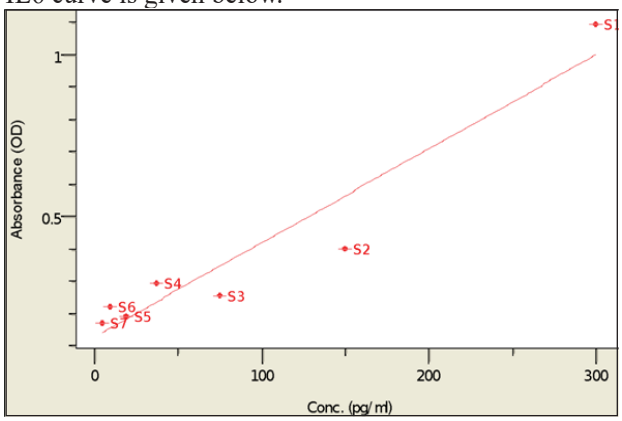

Figure 1: Standard Curve of IL-6

X-axis=concentration
Y-axis=absorbance

For PCR amplification, primers (forward and reverse) were used as described by Kitamura *et al.,2002* (Table 1). Primers were synthesized from 1st BASE (UK). Primers sequences are given in Table 2.

Table 2: Sequences of Primers for the Amplification of Loci

IL-6 polymorphism	Sequence
Forward Primer	**5′-GAGACGCCTTGAAGTAACTG-3′**
Reverse Primer	**`5′-AACCAAAGATGTTCTGAACTGA-3′**

The data was entered and analyzed using SPSS 20.0. Mean ±S.D was given for quantitative variables like age and weight. Median (Q1-Q3) was given for height, BMI and duration of disease. Frequencies and percentages were given for qualitative variables like gender and IL-6 polymorphism. Kolmogorov-Smirnov and Shapiro-Wilk test was applied to determine the distribution of data. Student *t*-test was applied where the data was normally distributed and Mann-Whitney test was applied where the data was not normally distributed. Chi-square test was applied to observe association between IL-6 polymorphism allele and study groups. The confidence interval was created at 95% as a convention. A *p*-value of ≤ 0.05 was considered as statistically significant.

Ethical clearance: The study was approved by the Ethical Review Committee and Advance Studies and Research Board of UHS and Sheikh Zayed Hospital Lahore.

Results

Detailed demographic features of the patients and their comparison in both the groups are shown in Table: 3. The median (Q1-Q3) of duration of disease of the subjects was high in group-II 10 (7-16) as compared to group-I 10 (6-14). On comparison the difference between the two groups was not statistically significant (p= 0.362).

Considering the serum levels of HbA1c, it was high in the group II (8.5±0.88) as compared to group I (8.3±0.94) with astatisticallysignificant difference between the two groups (p=0.05).

Similarly, the mean±SD of IL-6 levels was higher in group II 326.98±104.17 as compared to group I (299±81.51) but the difference was insignificant on comparison.

Table.3 Number, percentage, mean ±SD, and median (Q1-Q3) of study variables and their comparison between two groups

Variables		Group I	Group II	p-value
Gender	Male n (%)	33 (40.2%)	29 (35.4%)	0.59
	Female n (%)	49 (59.8)	53 (64.6%)	
Age (Mean±SD) years		56±11.13	54±9.76	0.12
Height [Median (Q1-Q3)] (Cm)		167(158-176)	158(152-170)	0.002*
Weight (Mean±SD) (Kg)		72±11.94	71±15.14	0.76
BMI [Median (Q1-Q3)] (kg/m^2)		25(23-28)	27(24-31)	0.012*
Duration of disease (Years) [Median (Q1-Q3)]		10(6-14)	10(7-16)	0.36
HbA1c (Mean±SD) (mmol/mol)		8.3±0.94	8.5±0.88	0.05*

Group-I=T2DM without nephropathy, Group-II=T2DM with nephropathy, n=number, %=percentage, *p≤0.05=statistically significant

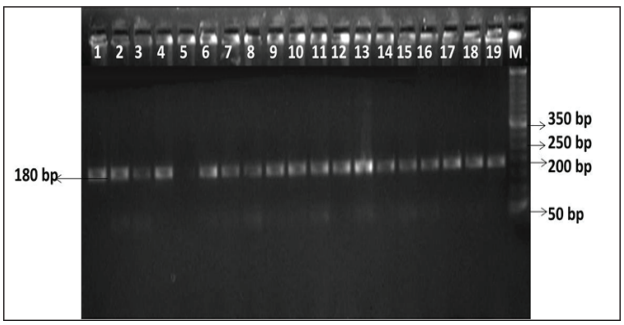

Figure 3: Gel electrophoresis showing ladder and amplified PCR product of IL-6 polymorphism (634 C/G) of different samples
M=Ladder, 1-19=Different samples,

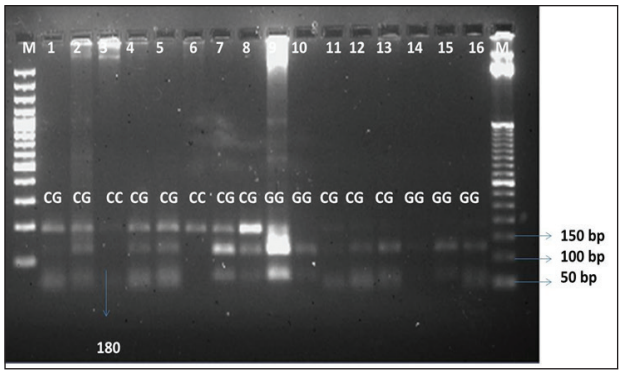

Figure 4: Gel electrophoresis showing ladder, RFLP of IL-6 gene (634 C/G) polymorphism of different samples.

In group-I, 14 (17.1%) patients had CC, 47 (57.3%) had CG and 21 (25.6%) had GG polymorphism. In group-II 9 (11%) patients had CC, 69 (84.1%) had CG and 4 (4.9%) had GG polymorphism. By applying Pearson's correlation test significant association of IL-6 polymorphism (CC,CG, GG) was determined between the two groups (p <0.001) (Table 4).

Table 4: Number, percentage and comparison of IL-6 gene (634 C/G) polymorphism between the two groups

IL-6 polymorphism	Group I n(%)	Group II n(%)	p-value
CC	14 (17.1)	9 (11.0)	<0.001*
CG	47 (57.3)	69 (84.1)	
GG	21 (25.6)	4 (4.9)	

Group-I=T2DM without nephropathy, Group-II=T2DM with nephropathy, n=Number, %=Percentage, *p≤0.05 statistically significant

Regarding median level of IL-6, in patients of CC polymorphism it was high in group-I (4.2) as compared to group-II (3.2), whereas in patients of CG polymorphism it was high in group-II (3.8) as compared to group I (3.7) while in GG polymorphism it was same in both the groups. On comparison the difference between IL-6 polymorphism and IL-6 level between two groups was not statistically significant (p1=0.304, p2=0.425 respectively).

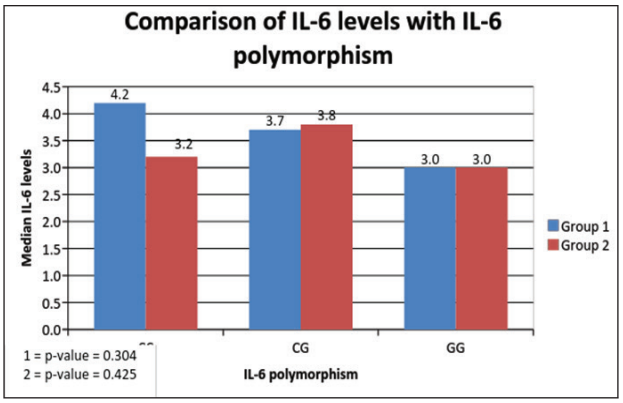

Figure 5:Median level, different polymorphisms of IL-6 gene (634C/G) and their comparison between two groups
p-1=0.304 for group 1
p-2=0.425 for group 2
p≤0.05=statistically significant

Discussion

While comparing the findings related to median duration of disease, it was higher in group-I as compared to group-II. This finding is in agreement with the studies of Tang et al. (2013), Kitamura et al. (2002) and Saxena et al. (2011). Kitamura et al (2002) documented duration of disease of subjects in group-I (normo-albuminuria) as 15.8 ± 3.8 years, of group-II (micro-albuminuria) as 16.2 ± 4.1 years and of group-III (macro-albuminuria) as 16.3 ± 4.6 years. Tang et al (2014) reported an insignificant association between duration of DM and development of DN. The current study and other studies suggest that duration of diabetes may not be the major factor that contributes towards nephropathy.[6,13,15]

The levels of HbA1c have been noted to vary significantly between both the study groups (*p*=0.05). This finding is in agreement with the study of Muammer et al (2014) who suggested mean± SD of HbA1c of subjects in group-I (DN) as 8.02± 2.39, of group–II (without DN) as 6.68 ± 1.39 and of group III (healthy controls) as 5.13 – 0.13 (p= 0.001). Further, it has been suggested that by improving the glycemic control progression of DN can be reduced.[16]

In the current study, BMI of subjects in group-II was higher as compared to group-I, with a statistically significant difference between the two groups (p=0.012). This finding is not in agreement with the study of Kitamura et al. (2002).[15] Kitamura et al (2002) reported mean ±SD of BMI of T2DM patients of group-I (normo-albuminuria) as 23.2 ± 3.3, of group-II (micro-albuminuria) as 23.1 ± 3.1 and of group-III (macro-albuminuria) as 23.4 ± 3.1. On comparison, the difference among the groups was not statistically significant (p=0.821). This non-agreement could be due to large sample size of Kitamura et al (2002) who included 454 patients while the current study had 164 patients. It could be due to differences in the genetic make-up of the studied population as Kitamura et al. (2002) studied Japanese population whereas current study was performed on Pakistani population.[15]

Considering the levels of IL-6, it was higher in group II (326.98±104.17) as compared to group I (299±81.51) but the difference was not statistically significant (p=1.153). This finding is in line with Shelbaya et al. (2012)[17] who conducted their study on patients with type 1 diabetic nephropathy. They grouped them as Group A with urine albumin excretion rate of < 20 µg/min, Group B with albumin excretion rate from 20-200 µg/min and Group C with albumin excretion rate of >200 µg/min. They suggested high levels of IL-6 in Group C 3.62± 0.36 as compared to Group A and B (2.06± 0.42) and (2.5± 0.44) respectively; these findings are similar to the findings of the current study.

In the current study, the frequency of IL-6 polymorphism of CC and GG was high in group-I 14 (17.1%) and 21 (25.6%) as compared to group-II 9 (11%) and 4 (4.9%) respectively, whereas frequency of CG was high in group-II 69 (84.1%) as compared to group-I 47(57.3%). On comparison the frequency of IL-6 polymorphism was statistically significant (p=0.001). In this way, in the present study GG allele indicated a protective role for diabetic nephropathy. These findings are in agreement with Kitamura et al (2002)[15] who studied patients of T2DM of ≥ 10 years and grouped them as normo-albuminuria, microalbuminuria and macro-albuminuria. They reported high frequency of IL-6 polymorphism of CC in normo-albuminuria 118 (72.8%) as compared to microalbuminuria 92 (66.7%) and macro-albuminuria 94 (61%), frequency of CG and GG was high in macro-albuminuria 44 (28.6%), 16 (10.4%) as compared to microalbuminuria 39 (28.3%), 7 (5.1%) and normo-albuminuria 39 (24.1%) 5 (3.1%). Both of these studies documented have high frequency of CG in the group with complications.

In the current study, the frequency of IL-6 polymorphism of CC and GG was high in group-I 14 (17.1%) and 21 (25,6%) as compared to group-II 9 (11%) and 4 (4.9%) respectively, whereas frequency of CG was high in group-II 69 (84.1%)

as comparedto group-I 47(57.3%). On comparison the frequency of IL-6 polymorphism was statistically significant (p=0.001). These findings are in agreement with Rubeaan et al. who studied IL-6 -634 C/G polymorphism. They reportedhigh frequency of CC 64 (71.9) in the control group as compared to hemodialysis patients 43(53.8). Frequency of CG 26 (32.5) and GG 11(13.8) was high in hemodialysis patients as compared to control group CG 18 (20.2), GG 7 (7.9) respectively. In both the studies, frequency of CG was higher in the groups with complications.[18]

Conclusion

Statistically significant difference in the frequencies of allele (CC,CG,GG) between two groups is pointing towards the protective role of GG allele as it was predominant in T2DM patients without nephropathy.

Future thoughts/recommendations

Studies on a larger sample size should be conducted. Other alleles of IL-6 polymorphism in the promoter region such as 174C/G should be performed to determine possible association of IL-6 polymorphism with DN.

Conflict of interest

The authors declare (s) that there is no conflict of interest regarding the publication of this paper

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Authors' contribution:

Data gathering and idea owner of this study: Talib R, Kashif M, Tahir R, Afzal N Study design: Afzal N Data gathering: Talib R, Kashif M, Abbas A, Jahan S, Afzal N

Writing and submitting manuscript: Talib R, Kashif M, Nadeem A, Afzal N

Editing and approval of final draft: Talib R, Kashif M, Tahir R, Jahan S, Afzal N

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