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Interleukin 10 Gene Polymorphisms and Susceptibility to Nephropathy in Egyptian Diabetic Patients

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Background: Type 2 diabetes mellitus (T2DM) is recognized as one of the most common causes of end-stage renal disease. In T2DM patients, a certain cytokine genotype is associated with an increased susceptibility to diabetic nephropathy.
Aim: In this study we clarify the relation between IL-10 gene polymorphisms at position (-592 A/C) and its plasma levels in T2D Egyptian patients with and without nephropathy.
Methods: 80 subjects were enrolled in this study, 30 diabetic patients without nephropathy, 30 DN patients and 20 healthy control subjects. For all subjects, kidney function tests, FBG, HbA1c, estimation of micro-albuminuria, plasma IL-10 level and IL-10 gene polymorphism were done.
Results and Conclusions: Diabetic patients with and without nephropathy exhibited significantly higher FBG, HbA1c than healthy subjects. Creatinine levels were increased in DN patients compared to both diabetic without nephropathy and healthy subjects. There is significant increase in micro-albuminuria levels in DN patients compared to diabetic without nephropathy and healthy control subjects. There is significant increase in IL-10 levels in DN patients compared to diabetic without nephropathy and healthy control subjects. The IL-10-(592) CC genotype associated with an increased risk of type 2 DM and the C allele was significantly associated with an increased risk of type 2 DM, there are no significant differences between the two T2D patient groups as regard IL-10 gene polymorphism.

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Keywords: IL-10; DM; gene polymorphisms; micro-albuminuria; Egyptian patients; kidney function; nephropathy.

1. INTRODUCTION

Interleukin 10 (IL-10) is a multifunctional regulatory cytokine involved in the inflammatory response that functions as a general inhibitor of the proliferative and cytokine response of both type 1 and type 2 helper T cells. Type 2 diabetes mellitus (T2DM) is a group of metabolic disorders characterized by high blood sugar levels, which results from defects in insulin secretion or action or both, and associated with micro-vascular and macro-vascular complications, including diabetic nephropathy [1].

Diabetic nephropathy (DN) is viewed as a state of low-grade chronic inflammation and role for interleukin 10 (IL-10) in its pathogenesis was proposed [2]. This was evidenced by the elevation in IL-10 levels in the sera of T2DM [3] and type 1 diabetes [4], patients with nephropathy, and as IL-10 levels correlated with the extent of renal damage in DN [3]. This suggests that IL-10 promoter gene variants influence DN development and progression by regulating IL-10 production [4]. The human IL-10 gene is located on chromosome 1q31-32, a locus genetically linked to susceptibility to a number of autoimmune diseases, most notably SLE [5]. A number of polymorphisms have been identified within the IL-10 locus, including 23 single nucleotide polymorphisms (SNPs) localized to the promoter alone [6]. Three single nucleotide polymorphisms (SNPs) in particular have been reported to play an important causal role in regulating IL-10 promoter activity. These SNPs situated at positions −1082, −819, and −592 are relative to the translational start site. Previous studies showed that the secretion of IL-10 can be affected by polymorphisms in its promoter region [7]. Therefore, this study was aimed to investigate the relation between the polymorphisms of the −592 region of IL-10 in T2D Egyptian patients with and without nephropathy.

2. PATIENTS AND METHODS

This study was carried out in the Medical Biochemistry & Molecular Biology and Internal Medicine Departments, Faculty of Medicine, Al-Azhar Assuit University.

2.1 Patient's Enrollment

This retrospective case-control, observational study was planned to compare patients with type 2 DM and disease–free controls. All participants were recruited from Al- Azhar Assuit University Hospital. All subjects were Egyptian citizens. An informed written consent was signed by all participants. The patient groups included 60 patients with type 2 DM under control, the patient group further categorized into 2 sub-groups; group suffering from type 2 DM without DN (30 patients) and 30 patients with DN. The ages of the patients between 40 -70 years old and the duration of DM disease was more than or equal to 5 years. Patients were excluded if they had type 1 DM, non-diabetic renal disease, urinary tract infection and liver diseases within the previous 3 months before the enrolment. Pregnant females were also excluded. The control group included 20 matched subjects to the patient group in sex, age and socio-economic status. They were selected randomly from the same population as the patients, with no known personal or family history of DM.

2.2 Methods

All participants were subjected to:

- History: Demographic data was collected from the patients in the form (age, gender, and any systemic disorder as renal or hepatic affections), past history of systemic treatment.
- Examination: All subjects underwent complete clinical examination.
- Detection of IL-10 gene polymorphism by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) according to Lin et al. [8].
- Estimation of plasma IL -10 levels by Enzyme Linked Immuno sorbent Assay (ELISA) according to Sabat et al. [9].
- Estimation of micro-albuminuria by ELISA according to Bretzel [10].
- Determination of fasting blood glucose by enzymatic method according to Trinder [11].
- Estimation of HbA1c by exchange resin method according to Abraham et al. [12].
- Estimation of blood creatinine by enzymatic method according to Bowers and Wong [13].

2.3 Sampling

- Blood sampling - Five ml of blood sample was taken under complete aseptic

condition from every participant, on (EDTA) tube. Each sample was divided into two parts, the first part was used for genetic examination and the second part centrifuged at 10.000 r.p.m for 10 minutes to separate the plasma for IL-10 estimation; plasma was separated and stored at −20°C until use.

- Urine sampling 5- 10 ml of mid-stream urine sample was collected in clean dry container. The patients were asked to refrain from heavy exercises 24 hours before the test. The patients with too much or too little muscle mass were excluded. Microbially contaminated or turbid samples were not suitable for nephelometric measurements. So, these samples have been centrifuged at 1000 r.p.m for 10 minutes.

2.4 Detection of Interleukin-10 Gene Polymorphism

All the reagents were highly purified analytical PCR-materials. All the tubes, tips and pipettes used for DNA extraction were DNAse, RNAse free tubes to avoid contamination all purchased from Gentra *(Minnapolis.* USA*).*

2.5 DNA Extraction

Genomic DNA was extracted from whole blood using the commercially available G-spin TM Total DNA Extraction Kit *(iNtron bio-technology, Seongnam-Si, Gyeonggi-do, Korea),* as described by Bubbon [14]. DNA purity and concentration were determined spectro-photometrically at 260 and 280 nm [15].

2.6 Amplification of the Interleukin-10 Promoter Gene Polymorphism

Polymerase chain reaction (PCR) for detection of IL-10 (-592 C/A; (reference SNP ID (rs) 1800872) polymorphism as described by Lin et al. [8] using the following primers:

Forward primer: 5'-GTG AGC ACT ACC TGA CTA GC-3'
Reverse primer: 5'-CCT AGG TCA CAG TGA CGT GG-3'

The PCR was carried out in a final volume of 25 µl containing 100 ng of template DNA (5 µl), 1.0 µM of each primer (1 µl) *(Biosearch Technologies, Novato, CA, USA)* and 12.5 ul of 2x *i-TaqTM* PCR Master Mix (*iNtRON Biotechnology*, *Sangdaewon-dong, Jungwon-gu, Seongnam-si, Gyeonggi-do, 462-120, Korea)* and 5.5µl deionized water.

2.7 Estimation of Plasma Levels of Interleukin-10

Plasma levels of IL-10 were measured using BMS215HS human IL-10 High Sensitivity ELISA provided by *eBioscience (eBioscience, San Diego, Calif., USA)* following manufacturer's instruction for sample collection, storage, and assay procedure [9].

2.8 Estimation of Microalbuminuria [10]

Micro-Albumin ELISA kit was purchased from *ORGENTEC Diagnostika GmbH (Mainz Germany).*

2.9 Statistical Analysis

The data were tabulated and statistically analyzed using SPSS version-20 software package.

3. RESULTS

The study was carried out on 80 patients who were classified into 3 groups:

Group I: 20 healthy subjects as control.
Group II: 30 patients of type 2 diabetes without nephropathy (DWN).
Group III: 30 patients of type 2 diabetes with nephropathy (DN) Table (1).

3.1 Genotypes and Allele Frequencies of *IL-10 (-592 C/A)*(1800872) Gene in the Studied Groups

In patients with type 2 DM (Group II, Group III), the frequencies of CC, CA, and AA genotypes were 60, 31.7 and 8.3%, respectively; and in controls, the frequencies were 25, 55 and 20%, respectively. The frequencies of C and A alleles in type 2 DM patients (Group II, Group III) were 75.8 and 24.2%; and in controls were 52.5 and 47.5%, respectively. The CC genotype associated with an increased risk of type 2 DM (OR = 4.50, 95% CI = 1.44–14.01, and P = 0.006) and the C allele was significantly associated with an increased risk of type 2 DM (OR = 2.83, 95% CI = 1.34– 5.99, and P = 0.005) (Table 2 and Figs. 1,2).

Table 1. Demographic data of studied groups

	Group I No. 20	Group II No.30	Group III No. 30	P value
Age (yrs),				
Range:	48- 60	46 - 62	47 - 64	
Mean ± SD:	54.55±4.07	54.3±4.19	53.83±4.40	0.83*
Sex,				
Male:	12 (60.0)	15 (50.0)	17 (56.7)	
Female:	8 (40.0)	15 (50.0)	13 (43.3)	0.51**

Table 2. Odds ratio and 95% confidence interval, genotype distributions and allelic frequencies of IL-10 (-592 C/A) polymorphism in type 2 DM patients (Group II, Group III) and Group I

IL-10 (-592C/A) polymorphism	Controls, No (%)	DM patients (Group II, Group III) No(%)	Odds ratio (95% confidence interval)	*P* value*
Genotype,				
CC	5 (25.0)	36(60.0)	4.50 (1.44–14.01)	0.006
CA	11 (55.0)	19 (31.7)	0.29 (0.10 – 0.85)	0.06
AA	4 (20.0)	5 (8.3)	0.63 (0.14–2.79)	0.21**
Alleles,				
C	21(52.5)	91(75.8)	2.83	0.005
A	19 (47.5)	29(24.2)	(1.34–5.99)	

** Chi-square (χ2) test*
*** Exact Fischer test*

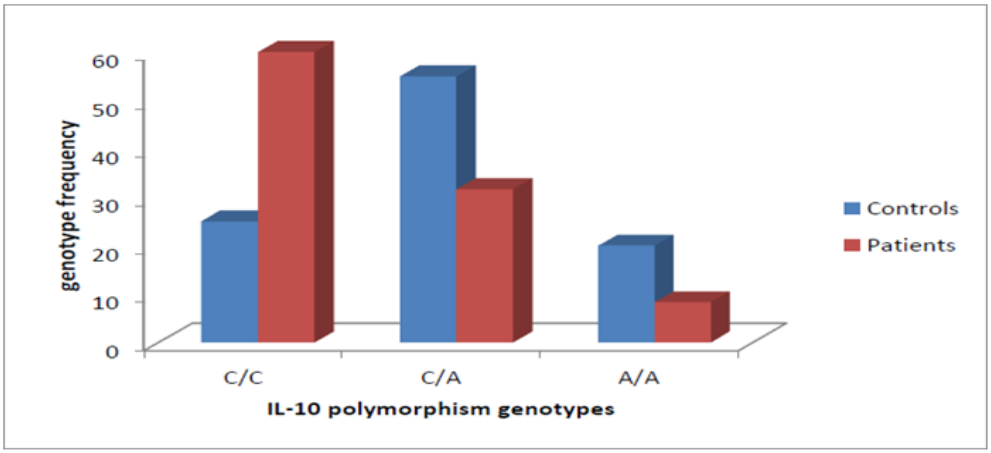

Fig. 1. Genotypes frequencies of *IL-10 (-592 C/A)* in type 2 DM patients Group II, III and controls

In Table 3 the genotype frequencies of CC, CA and AA were 63.3%, 30.0% and 6.9% in group III; and 56.7%, 33.3%, and 10.0% in group II. When they compared with controls, the CC genotype frequency was significantly higher in group III (P = 0.007; OR, 5.18; 95% CI, (1.47–18.18) and in group II (P = 0.02; OR, 3.92; 95% CI, (1.13–13.60). The frequencies of C and A alleles in group III, were 78.3% and 21.7%; and in group II were 73.3% and 26.7%, respectively. As regard C allele frequency there was statistical significant difference between group III and group II versus control.

3.2 IL-10 (-592 C/A) Polymorphism Analysis

PCR – RFLP analysis of IL-10 (-592 C/A) polymorphism revealed 412-bp band for C/C genotype, 175- and 237-bp bands for A/A genotype and 412, 237, and 175 bp bands for C/A genotype (Fig. 3).

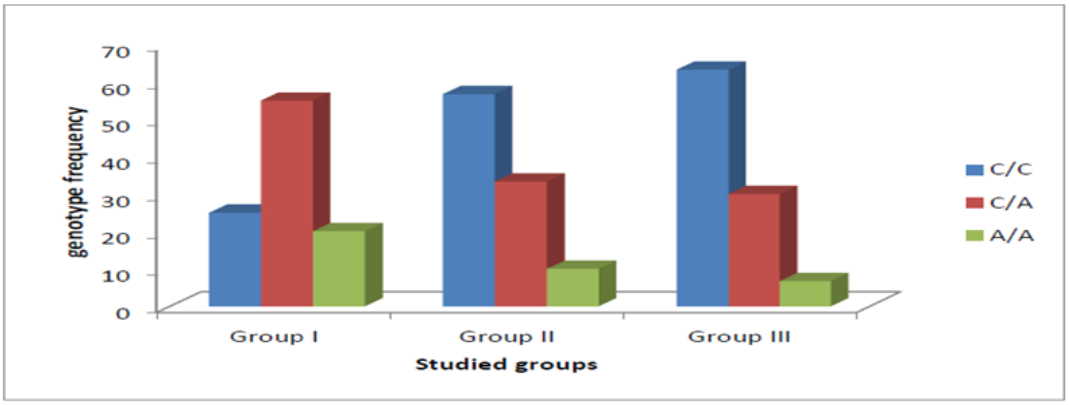

Fig. 2. Genotypes frequencies of *IL-10 (-592 C/A)* in studied groups

Table 3. Odds ratio (95% confidence interval), genotype distributions and allelic frequencies of IL-10 (-592 C/A) polymorphism in studied groups

IL-10 (-592 C/A) polymorphism	*Group I* No.%	*Group II*			*Group III*		
		No.%	OR(95%CI)	*P**	No.%	OR(95%CI)	*P**
C/C	5 (25.0)	17(56.7)	3.92 (1.13-13.60)	0.02	19(63.3)	5.18 (1.47–18.18)	0.007
C/A	11(55.0)	10(33.3)	0.409 (0.12–1.30)	0.12	9(30.0)	0.35 (0.108–1.13)	0.07
A/A	4 (20.0)	3(10.0)	0.444 (0.08–2.24)	0.41**	2(6.7)	0.28 (0.047–1.73).	0.20**
C	21(52.5)	44(73.3)	2.48	0.03**	47(78.3)	3.27	0.006**
A	19(47.5)	16(26.7)	(1.06-5.78)		13(21.7)	(1.36–7.83)	

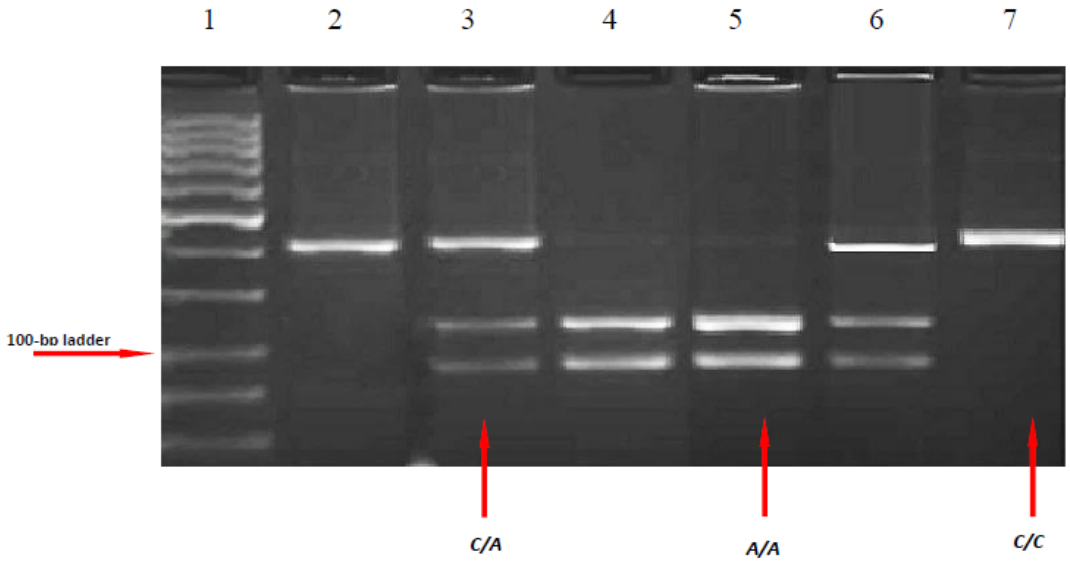

Fig. 3. Agarose gel electrophoresis showing the bands pattern obtained by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) for genotyping the *IL-10 (-592 C/A)* polymorphism. Lane 1 contains a 100-bp DNA ladder; lane 2,7 for *C/C genotype* with 412-bp band lane 3, 6 for *C/A* with 412-, 237-, and 175-bp bands; and lane 4,5 for *A/A* with 237- and 175-bp bands

3.3 Plasma Levels of IL—10 (pg/ml) in Studied Groups

In group I, IL-10 levels ranged from 0.29 to12.3 pg/ml with a mean ± SD of 6.13±3.56 pg/ml. In group II the plasma IL-10 levels ranged from 1.5 to 12.4 (pg/ml) with a mean ± SD 7.74 ± 2.4 and the IL-10 levels in group III ranged from 102 to 222 with a mean ± SD 156±33.12, patients with a statistically highly significance difference in groups III versus control (Table 4,5,6,7 and Figs. 4,5,6).

Table 4. Plasma IL-10 levels (pg/ml) in studied groups

Plasma IL-10 (pg/ml)	Group I No. 20	Group II No.30	Group III No.30
Range	0.29-12.3	1.5-12.4	102-222
Mean ± SD	6.13±3.56	7.74±2.4	156±33.12
P value *	------	0.09	<0.001

** Two tailed t-test*

Table 8 showed that: there was a highly significant difference in Blood creatinine, FBG and HbA1C (%) between the three groups.

In Table 9 and Fig. 7 there was a highly statistical significant difference in urinary micro-albumin levels (mg /l) between the studied groups.

Table 5. Plasma IL-10 levels (pg/ml) in Group III (DN) and Group II

Plasma IL-10 (pg/ml)	Group II No.30	Group III No.30	P value
Range	1.5-12.4	102-222	<0.001*
Mean ± SD	7.74±2.4	156±33.12	

** Two tailed t-test*

4. DISCUSSION

Clinical biochemical manifestations, plasma IL-10 levels and IL-10 gene polymorphisms among diabetic patients with and without nephropathy and healthy subjects were studied in this work. Patients in both diabetic with and without nephropathy groups exhibited significantly higher FBG and HbA1c levels than did healthy subjects. Plasma urea and creatinine levels were significantly elevated in DN subjects when compared to both diabetic without nephropathy and healthy subjects.

Table 6. Mean±SD of plasma IL-10 levels (pg/ml) among the different genotypes in the DN group III

Plasma IL-10 (pg/ml)	C/C	C/A	A/A	P value
Mean±SD	176.3±23.2	125.4±87.7	105±4.24	<0.029

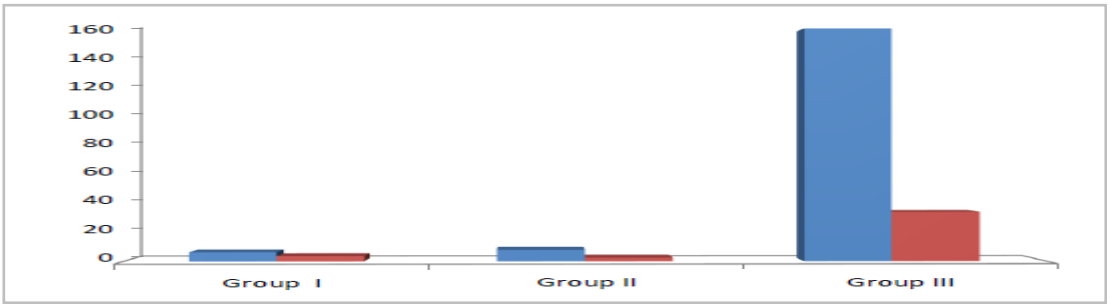

Fig. 4. Comparison between the studied groups according to the mean±SD of IL 10 levels

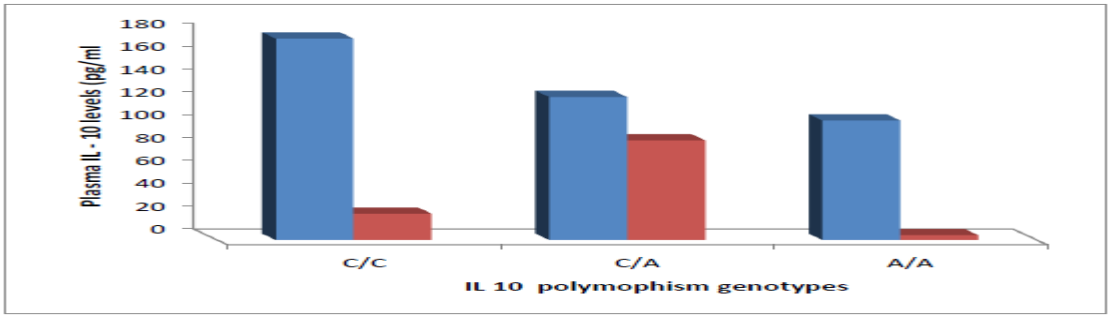

Fig. 5. Mean ±SD of plasma IL-10 levels among the different genotypes in the DN group III

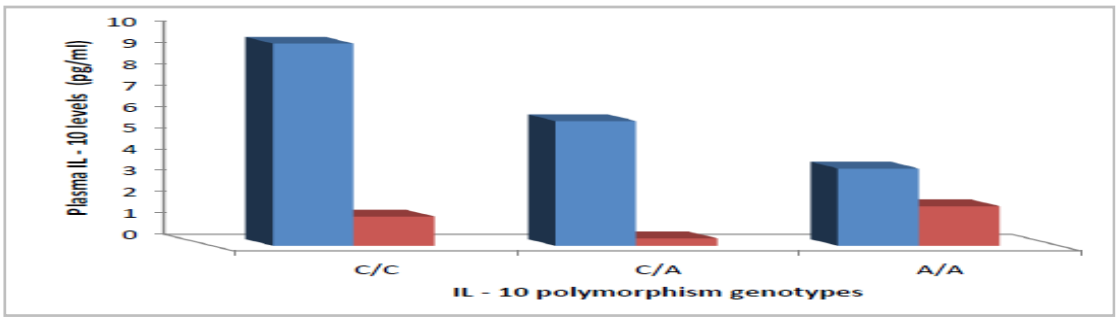

Fig. 6. Mean ±SD of plasma IL-10 levels among the different genotypes in the DWN group II

Table 7. Mean ±SD of plasma IL-10 levels (pg/ml) among the different genotypes in the group II (DWN)

Plasma IL-10 (pg/ml)	C/C	C/A	A/A	P value
Mean±SD	9.5±1.39	5.88±0.36	3.66±1.88	<0.0001

Table 8. Biochemical data of the studied groups

Laboratory data	Group I No. = 20	Group II No. = 30	Group III No. = 30	P. value
Blood creatinine (mg/dl)				
Mean±SD	1.12±0.2	1.14±0.21	4.37±1.73	<0.001
Range	0.67-1.4	0.72-1.6	1.92-8.5	
FBG (mg/dl)				
Mean±SD	88.8±8	166.4±14.3	182.9±16.6	<0.001
Range	75-106	146-205	159-215	
HbA1C (%)				
Mean±SD	4.44±0.35	8.5±0.42	8.87±0.73	<0.001
Range	4-5.1	7.8-9.3	7.8-11.1	

Table 9. Urinary micro-albumin levels (mg /l) in the studied group

Micro-albumin (mg/l)	Group I No. (20)	Group II No. (30)	Group III No. (30)	P. value
Mean±SD	19.7±6	22.6±6.2	250.5±122.4	<0.001
Range	9-29	10-42	69-514	

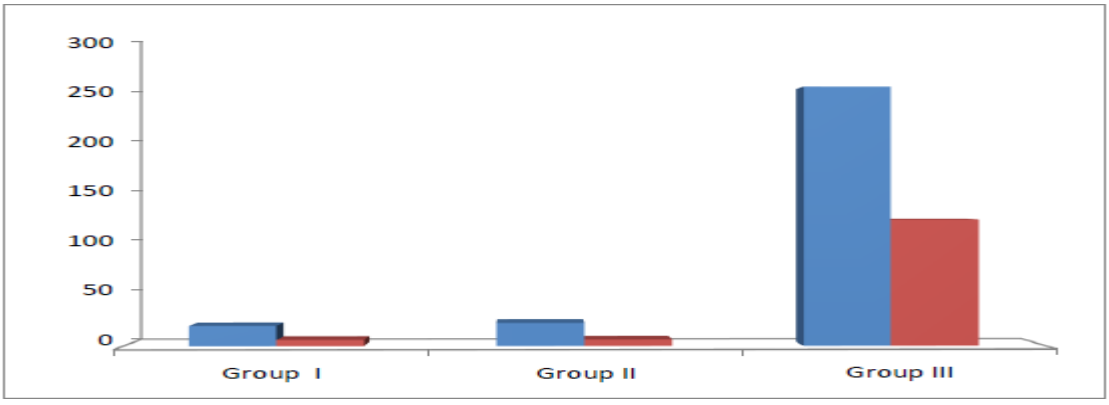

Fig. 7. Mean ±SD of urinary micro-albumin levels (mg /l) in the studied groups

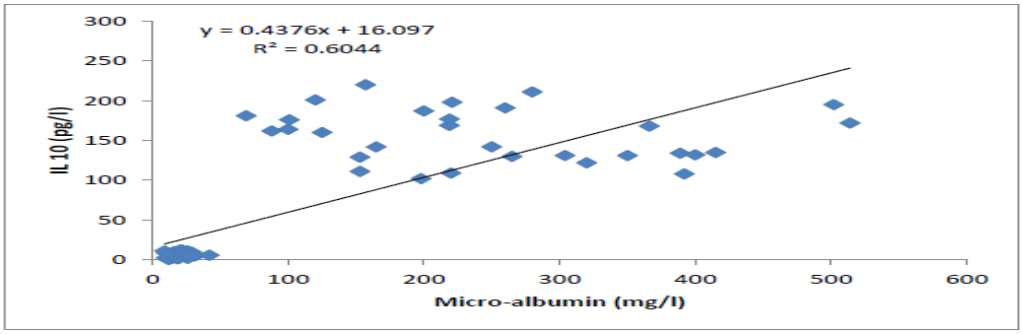

Fig. 8. Significant positive correlation between micro-albumin (mg/l) and plasma IL- 10 in the studied groups

Micro-albuminuria levels were significantly higher in DN patients compared to diabetic without nephropathy and healthy control subjects, this is consistent with the results obtained by Fraser and Phillips [16] who reported that clinical progression to DN is defined in terms of changes in urinary albumin excretion rate (UAER) and decline in GFR. Our results have revealed significant elevated concentrations of IL-10 in the plasma of DN patients compared to diabetic without nephropathy and healthy control subjects. Similar result was reported by Mysliwska et al. [4]. Wong et al., [3] reported that adiponectin, IL-10, Chemokine and IL- 8, 9 concentrations in DN were significantly higher compared to the NDN group and control subjects. Changes in IL-10 levels correlated with the extent of renal damage in DN.

Our study provides a positive correlation between IL-10 and micro-albuminuria with strong evidence that IL-10 may influence the degree of micro-albuminuria (Fig. 8). The possible mechanism of action of IL-10 may be inferred by analogy to other nephropathies. It has been found that inflammatory/TH1 and anti-inflammatory/TH2 cytokine transcripts are already weakly expressed in the normal, healthy kidney [17] and an enhancement of their expression is noticeable from the very beginning in various nephropathies [18]. The highest expression of IL-10 was found in those patients with severe proteinuria and extensive glomerular sclerosis, both in immune mediated as well as non-immune nephropathies [19]. Myśliwska et al. [4] demonstrated a role for IL-10 in the onset and progression of DN in which IL-10 was a strong independent predictor of albuminuria, and a positive correlation between IL-10 levels and albuminuria in DN patients was made. This suggests that dys-regulated IL-10 production induced by IL-10 genotype may influence DN progression [20,4], prompting the speculation as to whether IL-10 represents a potential DN genetic susceptibility locus, worthy of replication [21]. The elevated plasma IL-10 concentration in type 2 DN patients included in this study may contribute to the progression of DN indirectly because the production of IL-10 has been documented to be under the strong control of TGF-β [22] and these two cytokines mutually regulate the activity of each other's activity [23]. IL-10 exerts an immunosuppressive role by down regulating the expression of pro-inflammatory cytokines. The proposed role for IL-10 in DN pathogenesis was based on a number of observations. Increased systemic IL-10 concentrations were detected in the sera of DM patients with nephropathy [20, 3] and changes in IL-10 levels correlated with the extent of renal damage in DN [3]. Since its production is genetically controlled and evidenced by the altered IL-10 secretion mediated by specific IL-10 promoter polymorphisms [24]. Approximately 75% of the variation in IL-10 secretion capacity in humans derives from genetic factors, and these genetic differences contribute to disease susceptibility [25]. The best document of the IL-10 gene promoter polymorphisms are −592C/A, −1082G/A, and −819T/C. The −592C/A polymorphism, which lies within negative regulatory elements in the promoter, has been reported to associate with diminished IL-10 production [26].

In the present work, IL-10 -592 C/A polymorphism was chosen for analysis because of the common SNPs in the IL-10 gene promoter (-1082 G/A, -819 C/T, -592 C/A) show strong linkage disequilibrium and form two common haplotypes, designated as [ATA] and [GCC] haplotypes, the presence of these haplotypes can be fully determined by the analysis of the -592 C/A polymorphism [27]. The distribution of

IL-10 (-592) CC, CA, and AA genotypes in type 2 DM, were 60%, 31.7% and 8.3%, respectively; and in controls, the frequencies were 25%, 55% and 20%, respectively. The frequencies of C and A alleles in type 2 DM patients were 75.8% and 24.2%; and in controls were 52.5% and 47.5%, respectively. The CC genotype associated with an increased risk of type 2 DM (OR = 4.50), and the C allele was significantly associated with an increased risk of type 2 DM (OR = 2.83). Distributions of IL-10 -592 CC, AC, AA in healthy subjects are 10.0%:45.6%:44.4% in Japanese [28], 7.5%:38.8%:53. 7% in Taiwanese [29], 54.4%:39.7%:5.9% in Caucasian Italians [30] and 51.4%:41.6%:7.0% in Australians [31].

In our study the genotype frequencies of DM with nephropathy CC, CA and AA were 63.3%, 30.0% and 6.9% respectively; and 56.7%, 33.3%, and 10.0% respectively in DM without nephropathy. When they compared with controls, the CC genotype frequency was significantly higher in DM with nephropathy and DM without nephropathy. The frequencies of C and A alleles in DM with nephropathy, were 78.3% and 21.7%; and in DM without nephropathy were 73.3% and 26.7%, respectively. As regard C allele frequency there was statistical significant difference between DM with nephropathy and DM without nephropathy versus control. Our data revealed that there are no significant differences between the two T2D patient groups. Similar results were reported before in genotyping for Iranian diabetic patients by Arababadi et al. [32] who reported that T2D patients rather than its nephropathy complications show a significant correlation between their disease and the IL-10 −592 C/A polymorphism. IL-10 promoter polymorphisms -1082G/ A, -819C/T, 592 C/A have been consistently associated with Tunisian T2DM patients [33].

In Taiwanese population, DN patients showed a different genotype distribution in IL-10-(-592) but not in TNF-α (-308), IL-10- (-1082), or IL-10-(-819) compared to those in DM and healthy groups [6]. The SNPs -1082G/A and -592 A/C increased the risk for type 2 DM, and could be potential targets for screening for the early detection of the risk of type 2 DM in Chinese population [34]. Most studies reported that IL-10 gene -592 A allele was associated with the risk of T2DM [30,35,1].

However, other studies demonstrated that IL-10 gene -592 C allele was associated with the risk of T2DM [29,32].

Saxena et al. [1] found that heterogeneity of the -592C/A allele is involved in regulation of IL-10 secretion, and its level is significantly higher in diabetes patients than in controls. Among diabetes patients, the IL-10 level is slightly lower in the AA genotype than in the CC genotype. This suggests that the -592A/C polymorphism plays a major role in IL-10 transcription. Sinuani et al. [36] has suggested that the selective targeting of IL-10 expression and IL-10-related pathways may provide therapeutic approaches for many kidney diseases.

5. CONCLUSION

Plasma IL-10 levels were significantly increased in diabetic nephropathy compared to diabetic without nephropathy and control groups.

The results of the present study supported the suggestion that IL-10 -592 A/C genetic polymorphisms may contribute to increase the susceptibility and the development of diabetes.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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