Interleukin-4 Polymorphism in Egyptian Patients with Type-2 Diabetic Nephropathy

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Abstract: The effects of environmental and genetic factors on the development of diabetic complications are well-documented. The roles of inflammatory processes on the development of these complications including diabetic nephropathy were established. Cytokines have great roles in the development of diabetic nephropathy. Polymorphism in the 590-region of interleukin-4 gene is associated with the regulation of expression of this gene. During these investigations, peripheral blood was collected from 100 patients with type-2 diabetes mellitus with nephropathy and 100 diabetics without nephropathy (control). DNA was extracted and a polymerase chain reaction restricted fragment length polymorphism (PCR-RFLP) technique was performed to examine polymorphisms in the -590 region of the IL-4 gene. Obtained results revealed that the frequency of allele T was higher among patients with diabetic nephropathy than among the control. In addition, most of patients with allele T had overt albuminurea, higher blood pressure, renal dysfunction and dyslipidemia than patients with allele C. In conclusion, these findings suggest that patients with allele T are more liable to develop diabetic nephropathy with most of the micro- and macro-vascular complications.

Keywords: Interleukin-4, Diabetic nephropathy, Polymorphism.

Introduction

Diabetes mellitus is one of the complex diseases that are increasing globally. Type-2 DM is the most common type (Ahluwalia *et al*, 2009). Several environmental and genetic factors had been incriminated in its pathogenesis and its complication (Kang *et al*, 2008). It has been suggested that DM is an immune-mediated disease, in which the expression of several cytokines are changed (Cruz *et al*, 2008; Yih-Hsin *et al*, 2009).

Cytokines and cytokine receptor axis (Kang *et al*, 2008) are the subject of several recent studies for their roles in the pathogenesis of DM as well as their role in the pathogenesis of its complications (Ahima, 2009; Hyum *et al*, 2009). Increase in the serum level of several cytokines such as interleukin (IL) 18, IL-6, IL-12, IL-17 and tumor necrosis factor α were documented in patients with type-2 DM and its nephropathic complications (Ahluwalia *et al*, 2009).

Interleukin 4 is secreted by T helper 2 (Th2) cells. Important roles have been identified for IL-4 in the context of the immune response (Feve *et al*, 2009). It stimulates the development of Th2 lymphocytes by acting upon the undifferentiated T cells after exposure to antigens, it induces the shift from IgM and IgG towards IgE production by plasmocytes, and it determines the secretion of the whole Th2 cytokine spectrum. In addition, it has an inhibitory effect upon interferon (IFN) secretion and the differentiation of Th2 lymphocytes (Dinarello *et al*, 2010; Enriquez *et al*, 2010).

The association of IL-4 with immunological disorders such as multiple sclerosis, systemic lupus erythematosus (SLE), nephrotic syndrome, graft rejection, asthma, and type-1 and 2 DM is well established (Colin, 2003). The key roles of IL-4 as an inhibitory cytokine of autoimmunity and inflammations raise questions concerning the impacts of this cytokine on the pathogenesis of some diseases including nephropathic type 2 DM (Elbe-Burger, 2002; Arabadadi *et al*, 2010).

In hypercholesterolemia, the accumulated low density lipoproteins (LDL) in the arterial wall would be oxidized to release oxidation products that lead to activation of inflammatory responses (Cornicelli *et al*, 2000). In mice model, severe hypercholesterolmia is associated with a switch to Th2 immune response, with increased IL-4 expression in the atherosclerotic lesions (Feve *et al*, 2009; Yuxia *et al*, 2011). IL-4 mRNA can also be detected in atherosclerotic lesions in human body. The micro-environmental IL-4 in the atherosclerotic lesions has multiple effects on atherogenesis, such as augmentation of LDL cholesterol esterification by a concentration- and time- dependent manner. In addition, IL-4 can regulate the expression of 15-lipoxygenase (15-LO), a key enzyme in LDL oxidation (Jingfang *et al*, 2010).

It had been demonstrated that the adipocyte layer in the dermis is reduced in IL-4 transgenic mice. Accordingly, local micro-environmental expression of IL-4 is suggested to be involved in the atherogenic process (Hyun *et al*, 2009).

IL-4Rα is a crucial component for binding and signal transduction of IL-4. It is reasonable that polymorphisms located in IL-4Rα, which alter the binding affinity to IL-4 or downstream signaling pathways and thus contribute to the fine tune of IL-4 responsive phenotypes, would also be linked to disease development (Dinarello *et al*, 2010; Hyun *et al*, 2009). Several studies have reported that genetic polymorphisms of IL-4 and IL-4Rα are associated with genetic predisposition to diseases, possibly through their influences on the activity of these genes or their products (Dinarello, 2011).

Although the initiation and etiology of T2DM still await identification, accumulating evidences have proved the hypothesis that DM type 2 is a state of chronic inflammation, with increased acute phase proteins and various cytokines (Ming-Yuh *et al*, 2009). Genetic studies exploring susceptible or resistant genes for T2DM could provide clues for understanding the mystery of diabetic pathogenesis and for future design of diabetic treatment (Brown, 2010).

It seems likely that the risk for diabetes-associated kidney disease is magnified by inheriting risk alleles at several susceptibility loci (Hyun *et al*, 2009). Genome-wide linkage studies have recently identified several chromosomal regions that likely contain diabetic nephropathy susceptibility genes. Previous studies had documented two polymorphisms affected gene for IL-4. One of these polymorphisms is a single nucleotide polymorphism (SNP) at region -590 in the promoter region (Mohammad, 2010).

Secretion of IL-4 can be affected by its polymorphisms in -590 region (Mohammad, 2010). This study was done aiming at studying the polymorphism of this gene at 590-region in patients with type-2 diabetic nephropathy.

2. Materials and Methods
Patients' selection:

Type 2 diabetic patients with albuminuria attending Suez Canal University hospital. 100 patients were selected up-on the following criteria as well as 100 patients as control (diabetics without nephropathy). All patients had established Diabetic Nephropathy—defined as persistent albuminuria (>300 mg/24 h or >200 μg/min or >200 mg/L) in two of three consecutive measurements on sterile urine samples—with or without renal failure.

Patients were excluded from the study if having Reno-vascular and/or uncontrolled Hypertension, congestive heart failure, chronic kidney disease, urinary tract infection, heamaturia and acute febrile illness. Also, patients with other diabetic complications other than nephropathy such as retinopathy were also excluded.

All patients underwent detailed clinical and biochemical evaluation: duration of Diabetes, overt albuminurea, renal insufficiency and hypertension.

Blood urea, serum creatinine, blood sugar fasting and post prandial, fasting lipid profile, 24-hr urinary albumin excretion (enzyme immunoassay) were measured in all patients. Serum creatinine concentration was assessed by a kinetic Jaffe method. Lipid profile was measured by a conventional laboratory technique. These laboratory investigations were done using fully-automated spectrophotometer Hitachi-912 (Roche Diagnostics, Boehringer Mannheim, Germany).

DNA extraction:

DNA was extracted using commercially available Spin-column technique kit for DNA extraction from human whole blood (QIAamp®DNA Blood Mini Kit, QIAGEN, 28159 Avenue Stanford, Valencia, CA 91355, USA). The extracted DNA samples were stored at -20°C for further use.

Determination of IL-4 genotypes:

Determination of IL-4 genotyping was done using polymerase chain reaction (PCR) using primers having the following sequences:
The sense primer:
5`-TAAACTTGGGAGAACATGGT-3`
The anti-sense:
5`-TGGGGAAAGATAGAGTAATA-3`

The PCR was carried out using a ready to use PCR buffer (Gen-Taq Master Mix, BIORON, Germany). The buffer is composed of Taq DNA Polymerase (recombinant) in reaction buffer (0.1 unit/ μl), antibodies to Taq DNA polymerase, concentration adjusted for the effective inhibition of DNA polymerase activity at 37°C, 32 mM $(NH_4)_2SO_4$, 130 mM TrisHCl, pH 8.8 at 25 ºC, 0.02% Tween-20, 5.5 mM $MgCl_2$ and dNTPs (dATP, dCTP, dGTP, dTTP): 0.4 mM of each. The PCR mixture reaction was prepared as follow: 30 μl of a ready for use master mix, 2 μl of forward primer (0.5 μmol), 2 μl of reverse primer (0.5 μmol), 3 μl of template DNA (100 – 500 ng) and 3 μl of sterile deionized water to reach a final mixture volume of 40 μl.

The PCR condition was an initial denaturation at 95ºC for 5 minutes, followed by 35 cycles of melting at 95°C for 50 seconds, annealing at 53°C for 50 seconds, and extension at 72°C for 45 seconds, with a final extension step of 5 minutes at 72°C, using thermal cycler (ThermoHybaid PX2, UK). The PCR product of IL-4 (-590 C/T) was a 195-bp fragment and was digested with AvaII (Fermentas, Germany) into 175-bp and 20-bp fragments. The digested products were run on a 2.5% agarose gel and studied after staining with ethidium bromide (Mohammad, 2010).

Statistical analysis:

Collected data was analyzed using SPSS version 13 program. For dichotomous variables,

ANOVA and regression analysis tests were used, and chi-square was used for categorical variables. A p value (two sided) of <0.05 was considered to be significant.

3. Results

The mean±standerd deviation age of this study group was 45.21±2.34 years and 46.32±2.37 years old for control group. There were statistically significant difference between both groups regarding serum triglycerides, serum total cholesterol, serum HDL-cholesterol, serum LDL-cholesterol, albuminurea level and estimated glomerular filtration rate (GFR) with a p-value of less than 0.05 (table 1).

In study patients, mean FBG, total cholesterol, triglyceride and LDL level were 230 ± 40 mg/dl, 290 ± 10 mg/dl, 350 ± 12 mg/dl and 180 ± 11 mg/dl respectively. On the other hand, in control group, mean FBG, total cholesterol, triglyceride and LDL level were 171 ± 15 mg/dl, 150 ± 6 mg/dl, 100 ± 4 mg/dl and 100 ± 9 mg/dl respectively. Mean blood urea and serum creatinine was 94.0 ± 12.8 mg/dl and 2.6 ± 0.3 mg/dl respectively among study group. Among control group, they were 71 ± 10.2 and 1.9 ± 0.2 respectively.

Regarding the distribution of the different genotypes among the study group, it was CC (n= 26), TC (n= 61) and TT (n= 13), while in the control group it was CC (n= 30), TC (n= 67) and TT (n= 3) (table 3).

In patients having genotype CC (n = 26), majority (75%) of patients developed overt albumiuria, having lesser degree of hypertension, renal dysfunction, and dyslipidemia than TC and TT genotypes (p<0.005). On the other hand, Urinary albumin excretion (UAE), SBP, DBP, TG, S.Cr. and LDL-C were significantly higher (p-value <0.05) in patients of TT type than CC and TC groups (table 2).

Odds ratio (OR) for the high risk allele (T) is 1.34 with 95% confidence interval (CI) from 0.90 to 2.00. Thus the high risk allele of IL-4 is 1.3 times in patients than in controls (p-value 0.001).

Table 1: Characteristics of Type 2 Diabetic Patient with Nephropathy and Controls:

Variable	Study	Control
Age, year	45.21±2.34	46.32±2.37
Drug therapy	Oral	Oral
Duration of DM (years)	7 ± 0.9	6 ± 0.3
Serum Triglycerides, mg/dL	350 ± 12	100 ± 4*
Serum total cholesterol, mg/dL	290 ± 10	150 ± 6*
Serum HDL- cholesterol, mg/dL	24 ± 2	40 ± 3*
Serum LDL- cholesterol, mg/dL	180 ± 11	100 ± 9*
Fasting blood sugar, mg/dL	230 ± 40	171 ± 15
Albuminurea, mg/dL	899 ± 50	25 ± 1*
Estimated GFR, mL/min	72 ± 3	120 ± 5*

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and GFR, glomerular filtration rate.
* p-value<0.05

Table 2: Patient Characteristics and Distribution of IL-4 genotypes:

Characteristics	Genotypes		
	TT (n= 13)	TC (n= 61)	CC (n= 26)
Age, Years	43.8±1.3	47.6±2.5	45.2±2.6
SBP, mmHg	178±8	162±8	146±4*
DBP, mmHg	92±6	82±4	76±6*
Urine Alb., mg/ day	1364±72	1193±38	576±30*
S. creat. mg/ dl	3.65±0.98	2.81±.45	1.97±0.31*
T. Chol, mg/ dl	233±45	229±29	185±25*
TG, mg/ dl	281±51	233±30	191±32*
LDL, mg/ dl	165±27	150±25	105±22*
Estimated GFR, ml/ min	60 ± 4	70 ± 5	78 ± 3*

*p-value <0.05

Table 3: Polymorphisms of Interleukin-4 Gene in Nephropathic Type 2 Diabetic Patients and Controls

Genetic Parameter	Study	Controls
Genotype		
CC	26 (26%)	30 (30%)
TC	61 (61%)	67 (67%)
TT	13 (13%)	3 (3%)*
Allele		
C	113 (56.5%)	127 (63.5%)
T	87 (43.5%)	73 (36.5%)*

*p-value < 0.05

Figure 1: Electrophoretic patterns of different IL-4 genotypes

4. Discussion

The main etiological cause of type 2 DM and its inflammatory complications such as nephropathy has yet to be clarified (Arababadi *et al*, 2009). It seems that immune-related factors play important roles in the etiology and pathogenesis of type 2 DM and its associated renal complications (Ikeuchi *et al*, 2009).

The crucial role of the cytokines network in orientation of immune responses is documented (Hyun *et al*, 2009). Several factors such as infectious agents, hormonal conditions, and cytokine gene polymorphisms regulate expression and secretion of cytokines (Lee *et al*, 2005). Study findings indicated a significant difference between type 2 diabetic patients with nephropathy and non-nephropathic diabetic controls regarding genotypes and alleles of the -590 region of IL-4 gene. A similar study was done by Mohammad K. on Iranian population and revealed the similar results (Mohammad, 2010).

In the present study, 74% of the patients belonged to both TC and TT genotypes. Clinical correlation revealed that most patients in this group have macro and micro vascular complications: represented by greater degree of albuminurea and

severe renal insufficiency (p<0.05). These findings indicate that the presence of the allele T of IL-4 gene is associated with greater risk of diabetic nephropathy compared with C allele.

Many studies were done to evaluate the relation of IL-4 polymorphism to the onset of DM type 2, while few only were done to show its relation to the development of its complications (Arababadi *et al*, 2009; Mohammad, 2010). Previous data revealed that there was no relation between several promotor polymorphisms including T-590C and type 2 diabetic patients without nephropathy (Ming-Yuh *et al*, 2009); therefore, based on the current and previous studies, it seems likely that the polymorphisms are associated with nephropathic complications rather than type 2 DM (Hyun *et al*, 2009).

Some studies have investigated these polymorphisms in type 1 and 2 DM without nephropathy and in non-diabetic nephropathies (Arababadi *et al*, 2009; Hyun *et al*, 2009). For example, Ikeuchi and colleagues Parry and coworkers showed that the polymorphisms in the IL-4 gene are not associated with minimal change nephrotic syndrome. Mittal and Manchanda reported that these polymorphisms are related with

susceptibility to end-stage renal disease (Mittal and Manchanda, 2007).

Another study in a Japanese population showed that IL-4 polymorphisms could influence disease susceptibility and progression in immunoglobulin A nephropathy (Masutani *et al*, 2003).

A significant relation between IL-4 polymorphisms and type 2 DM was reported by Bid and colleagues, in the north Indian population (Bid *et al*, 2008). Another study demonstrated that there were no significant differences in the IL-4 polymorphisms between patients with type 1 DM and healthy controls (Mohammad, 2010).

A probable reason for the discrepancy between results could be that populations are different in race and genetics from area to another as well as, the small number of patients studied and the probable small effect of the mutation.

Conclusion

The study explored the potential scope of one of the genetic approaches to risk assessment in development of diabetic nephropathy, with its interaction with other conventional factors. The study notified that the presence of allele T in both genotypes TC and TT is strongly associated with increased risk for developing diabetic nephropathy. These observations emphasize the need to monitor these patients to reduce the susceptibility to nephropathy and then ESRD especially in the developing countries where the transplantation is so difficult. Further, the complex interplay between genetic and environmental factors should be considered in order to evaluate the etiological role of IL-4 polymorphism in nephropathic DM.

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