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Original article

Haptoglobin gene polymorphism in type 2 diabetic patients with and without nephropathy: An Egyptian study

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Abstract

Background: The development and progression of diabetic microvascular complications including nephropathy are related to the degree of glycemic control and oxidative stress and may be influenced by genetic factors. The aim of the present study was to investigate the association between haptoglobin (Hp) gene polymorphism and the occurrence of diabetic nephropathy in patients with type 2 diabetes mellitus and to find a possible link between Hp phenotypes and the inflammatory parameters; serum C-reactive protein (CRP), interleukin- 6 (IL-6), and Hp.

Methods: The study included 60 normotensive type 2 diabetic patients (>5 years duration) categorized into three equal groups (normo-, micro-, and macroalbuminuric), according to urinary albumin excretion (UAE). In addition, 20 age- and sex-matched individuals were selected to serve as a control group. Serum CRP, IL-6, and Hp concentrations were measured and Hp phenotyping was conducted using polyacrylamide gel electrophoresis.

Results: The frequency of Hp phenotype 1-1 (Hp 1-1) in diabetic patients with normoalbuminuria was 7/20 (35%) as compared with 1/20 (5%) in diabetics with macroalbuminuria ($p=0.02$). However, the frequency of Hp 2-2 was greater in diabetics with macroalbuminuria (12/20, 60%) than in those with normoalbuminuria or controls (5/20, 25%; $p=0.03$). Patients with diabetic nephropathy (micro- or macroalbuminuria) had higher levels of serum CRP, IL-6, and Hp than those without nephropathy (normoalbuminuria). Serum Hp levels in type 2 diabetics were higher in Hp phenotype 2-2 than in Hp 1-1; however, serum CRP and IL-6 levels did not differ significantly between Hp phenotype groups. Moreover, there were significant positive correlations between UAE and serum levels of CRP, IL-6, and Hp in diabetic patients.

Conclusions: Hp phenotype 2-2 is considered to be a major susceptibility gene for the development of nephropathy in type 2 diabetic patients. In addition, the significant association between inflammatory parameters and UAE indicates that inflammation may be a pathogenic mechanism of renal injury in type 2 diabetics. Moreover, serum IL-6 and Hp may be good prognostic factors for the development of nephropathy in the course of diabetes mellitus. Future research on the use of anti-inflammatory therapy may result in a new approach to the treatment and prevention of diabetic nephropathy.

Keywords: Diabetic nephropathy; Oxidative stress; Inflammation; Haptoglobin; Interleukin-6; Susceptibility genes

1. Introduction

Diabetes is a major worldwide health problem, and long-term diabetic vascular complications are the leading cause of morbidity and mortality [1]. Hyperglycemia has been shown to be a necessary but not sufficient condition for the development of these complications. Genetic differences between diabetic patients might play an important role in determining why some diabetic patients develop these complications while others do not [2].

The pathogenesis of diabetic nephropathy is still a matter of debate, although strong evidence suggests that it results from an interaction between susceptibility genes and the

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Table 1
Clinical and biochemical data of type 2 diabetic patients with and without nephropathy

Parameters	Groups			
	Control (n=20)	DM with normoalbuminuria (n=20)	DM with microalbuminuria (n=20)	DM with macroalbuminuria (n=20)
Age (years)	52.1±8.3	53.2±11.2	55.1±9.2	58.0±10.3
Sex (M/F)	10/10	7/13	8/12	9/11
Disease duration (years)	–	7.13±2.3	8.8±3.3	10.7±4.5
Fasting blood glucose (mg/dl)	86.3±7.5	190.5±6.9[a]	240.6±8.7[c]	294.2±9.5[f, h]
Postprandial blood glucose (mg/dl)	112.2±10.1	273.8±13.2[a]	314.2±12.9[c]	370.0±19.4[f, h]
$HbA1_c$ (%)	4.3±0.4	6.7±1.0[a]	8.2±1.3[c]	10.3±2.4[f, h]
Blood urea (mg/dl)	37.6±6.6	41.81±8.9	47.5±11.8	58.3±12.3[f, i]
Serum creatinine (mg/dl)	0.98±0.21	1.13±0.37	1.41±0.49[e]	1.75±0.53[g, j]
Creatinine clearance (ml/min)	104.3±9.5	98.0±13.3	80.4±15.8[e]	64.5±17.2[f, i]
Urinary albumin (mg/24 h)	14.5±4.1	19.2±5.23	147.75±50.39 [c]	684±115.73[f, h]
Serum CRP (mg/l)	1.0±0.39	2.31±1.21[a]	3.09±1.10[e]	3.59±1.31[g]
Serum IL-6 (pg/ml)	1.09±0.69	2.69±1.01[a]	3.66±1.33[e]	4.78±1.83[f, j]
Serum haptoglobin (mg/dl)	54.95±11.88	72.0±20.91[b]	95.25±30.41[d]	154.4±48.34[f, h]

Data are expressed as mean±SD [a]p< 0.001, [b]p<0. 01 normoalbuminuria vs control; [c]p<0.001, [d]p<0. 01, [e]p<0. 05 microalbuminuria vs normoalbuminuria; [f]p<0.001, [g]p<0. 01 macroalbuminuria vs normoalbuminuria; [h]p<0.001, [i]p<0. 01, [j]p<0. 05 macroalbuminuria vs microalbuminuria.

diabetic milieu. Considerable evidence has shown the importance of oxidative stress in the pathogenesis of diabetic complications, especially enhancement of atherosclerosis and diabetic microangiopathies [3].

Haptoglobin (Hp), a hepatocyte-derived serum α_2-sialoglycoprotein, is a positive acute-phase reactant and hemoglobin-binding protein that is essential in protecting against heme-driven oxidative stress [4]. Transgenic mice with targeted disruption of the Hp gene show a considerable increase in oxidative stress and oxidative tissue damage, particularly in the kidney [5]. Haptoglobin is expressed by a genetic polymorphism as three major phenotypes: Hp 1-1, Hp 2-1, and Hp 2-2 [6].

It is well established that the functional properties of Hp are type-dependent. Hp 1-1 is a better antioxidant and binds more strongly with free hemoglobin than Hp 2-2 [7,8]. The increased antioxidant function of Hp 1-1 is thought to confer protection from angiopathies; however, Hp 2-2 is believed to be a major risk factor in several oxidative stress-related disease states [9]. This has been reported for coronary [10] and peripheral [11] atherosclerotic lesions, cardiac transplant vasculopathy [12], mortality in coronary heart disease [13], restenosis after coronary angioplasty [14]or stenting [15], and cardiovascular disease in diabetic individuals [16]. Also, the Hp phenotype is an apparent risk factor for the development of gestational diabetes mellitus [17].

Recent studies demonstrated that acute-phase markers of inflammation were associated with nephropathy status, suggesting a role for inflammation in the pathogenesis of diabetic nephropathy [18,19]. Interleukin-6 (IL-6) is a multifunctional cytokine, the main function of which appears to be the induction of acute inflammatory responses. It also promotes the growth and differentiation of a wide variety of

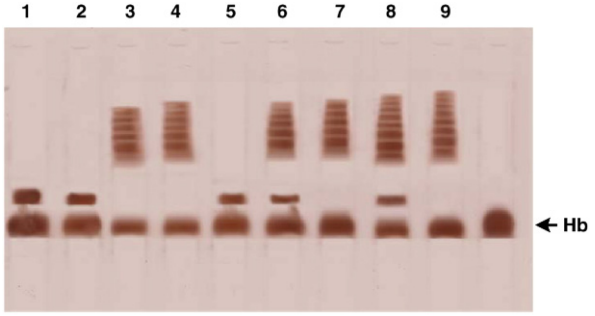

Fig. 1. Representative patterns of haptoglobin (Hp) phenotypes after polyacrylamide gel electrophoresis of hemoglobin-enriched serum. Bands correspond to Hp–Hb complexes. A band visible in each lane corresponding to free unbound hemoglobin (Hb) is indicated with an arrow. Hp phenotypes: Hp 1-1 (lanes 1, 2, and 5); Hp 2-1 (lanes 6 and 8); Hp 2-2 (lanes 3, 4, 7, and 9). Lane 1 of diabetic normoalbuminuria, lane 2 of control, lanes 3, 4, 5, and 6 of diabetic microalbuminuria, lanes 7, 8, and 9 of diabetic macroalbuminuria.

Table 2
Distribution of haptoglobin polymorphism in type 2 diabetic patients with and without nephropathy

Groups	Parameters		
	Hp 1-1	Hp 2-1	Hp 2-2
Control			
No. (%)	6/20 (30%)	9/20 (45%)	5/20 (25%)
Normoalbuminuria			
No. (%)	7/20 (35%)	8/20 (40%)	5/20 (25%)
p- value	0.74	0.75	1
Microalbuminuria			
No. (%)	2/20 (10%)	10/20 (50%)	8/20 (40%)
p-value	0.12	0.75	0.31
*p-value	0.06	0.53	0.31
Macroalbuminuria			
No. (%)	1/20 (5%)	7/20 (35%)	12/20 (60%)
p-value	0.05	0.52	0.03
*p-value	0.02	0.74	0.03
**p-value	0.50	0.34	0.21

p-value<0.05 was considered significant. p-value vs control, *p-value vs normoalbuminuria and ** p-value vs microalbuminuria.

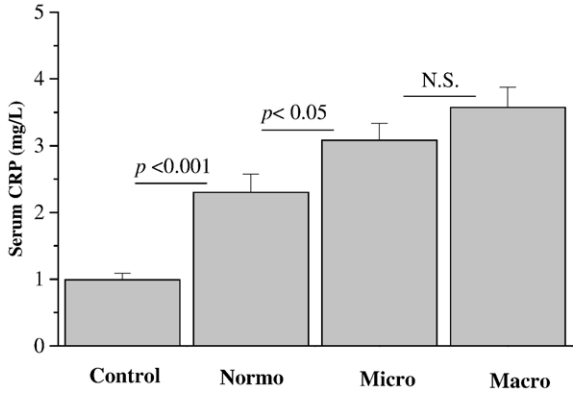

Fig. 2. Mean±S.E. of serum CRP concentrations in type 2 diabetic patients with and without diabetic nephropathy.

cells, including renal mesangial cells [20]. Furthermore, it has been reported that IL-6 mRNA expression was found in renal tissue of patients with diabetic nephropathy [21].

As the pathogenesis of microvascular complications of diabetes is considered to be associated with endothelial oxidative stress [22,23], we expected the different antioxidative capacity of Hp phenotypes to contribute to the development of diabetic nephropathy. The aim of the present study was to investigate the association between Hp gene polymorphism and the occurrence of diabetic nephropathy in patients with type 2 diabetes mellitus and to find a possible link between Hp phenotypes and the inflammatory parameters; serum C-reactive protein (CRP), interleukin-6 (IL-6), and Hp levels.

2. Subjects and methods

2.1. Subjects

This study was conducted on 80 subjects. Sixty normotensive type 2 diabetic patients were selected from those attending the Internal Medicine Diabetic Clinic of Tanta University Hospital. Twenty healthy age- and sex-matched volunteers (10 females and 10 males) with a mean age (±SD) of 52.1±8.3 years were recruited for participation as control subjects.

Patients were divided into three groups according to their level of urinary albumin excretion (UAE).

Group 1 included 20 diabetic patients, 13 females and 7 males, with normoalbuminuria (albuminuria<30 mg/24 h); their mean age (±SD) was 53.2±11.2 years, while the mean±SD of the disease duration was 7.13±2.3 years. Group 2 included 20 diabetic patients, 12 females and 8 males, with microalbuminuria (albuminuria 30–300 mg/24 h); their mean age (±SD) was 55.1±9.2 years, while the mean±SD of the disease duration was 8.8±3.3 years.

Group 3 included 20 diabetic patients, 11 females and 9 males, with macroalbuminuria (albuminuria>300 mg/24 h); their mean age (±SD) was 58.0±10.3 years, while the mean±SD of the disease duration was 10.7±4.5 years.

Patients with associated hypertension, ischemic heart disease, acute inflammation, rheumatoid arthritis, and concomitant kidney or liver disease were excluded from the study. Also, subjects with no evidence of diabetic retinopathy by fundus examination but with evidence of microalbuminuria or macroalbuminuria were excluded from this study as we considered very likely that the albuminuria was not because of diabetic renal disease.

All cases included in this study were subjected to history-taking, a complete clinical examination, and abdominal ultrasonography.

2.2. Biochemical assay

An early morning urine sample was collected from each subject for complete urine analysis and detection of microalbuminuria with a Micral test [24]. Twenty-four-hour urine volume was also collected for estimation of creatinine and quantitative assessment of albuminuria using an immunoturbidimetric assay [25].

A fasting blood sample was collected aseptically from all subjects and a 2-hour postprandial blood sample was also

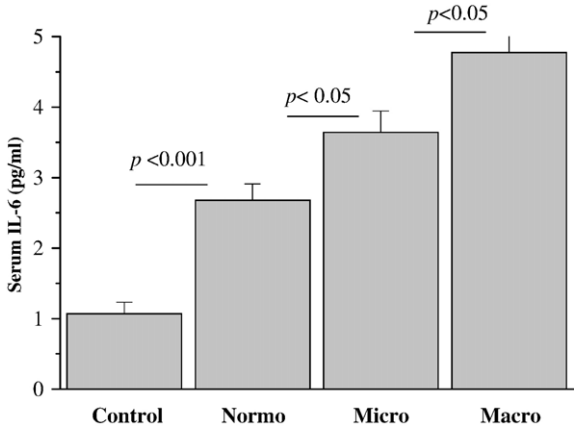

Fig. 3. Mean±S.E. of serum IL-6 concentrations in type 2 diabetic patients with and without diabetic nephropathy.

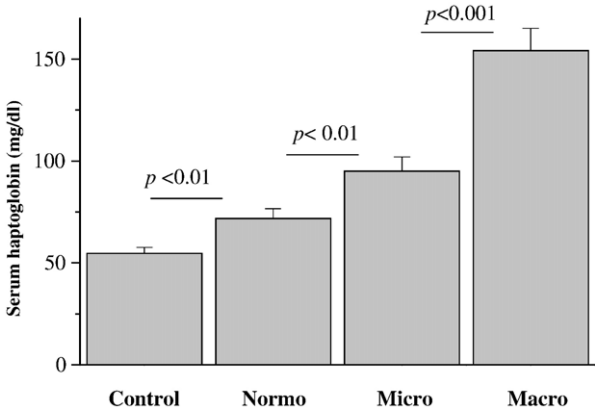

Fig. 4. Mean±S.E. of serum haptoglobin concentrations in type 2 diabetic patients with and without diabetic nephropathy.

Table 3
Relation between haptoglobin phenotypes (Hp 1-1, Hp 2-1, and Hp 2-2) and serum CRP, IL-6, and Hp levels in all type 2 diabetic patients

Hp phenotypes	Parameters		
	CRP (mg/l)	IL-6 (pg/ml)	Haptoglobin (mg/dl)
Hp 1-1 (10/60) (16%)	2.54±1.09	3.2±1.23	73.9±25.43
Hp 2-1 (25/60) (42%)	3.08±1.34	3.56 +1.73	104.4±46.5
p-value	NS	NS	<0.05
Hp 2-2 (25/60) (42%)	3.09±1.36	4.05±1.69	123.3±52.83
p-value	NS	NS	<0.001
**p*-value	NS	NS	NS

Data are expressed as mean±SD. *p*-value<0.05 was considered significant; NS: not significant; *p*-value vs Hp 1-1; **p*-value vs Hp 2-1.

collected for postprandial blood glucose estimation. An aliquot of fasting blood sample was collected on EDTA and analyzed within a few hours to estimate the percentage of glycosylated hemoglobin ($HbA1_c$) using a fast ion exchange resin separation method [26], with kits supplied by Stanbio Laboratory (Texas, USA).

The remaining fasting blood sample was allowed to clot at room temperature for about 1 h; it was then centrifuged for at least 10 min at 2000 rpm, and the serum was aspirated and divided into aliquots in small plastic tubes. One aliquot was ready for measurement of fasting blood glucose, blood urea, and serum creatinine using commercial assay kits supplied by Bicon Co. (Germany) and the creatinine clearance was calculated.

The other aliquots were stored at −20 °C for measurement of serum C-reactive protein (CRP) using a high-sensitivity latex-enhanced immunonephelometric assay (Dade Behring, Germany) [27], serum IL-6 using a commercially available double-sandwich enzyme-linked immunosorbent assay (Quantikine, R&D Systems, Minneapolis, Minn) [28], and serum Hp concentration based on the absorbance of haptoglobin–hemoglobin (Hp–Hb) complex in acid solution pH 3.7 [29].

2.3. Haptoglobin phenotyping

Haptoglobin phenotype distribution was determined using 5% polyacrylamide gel electrophoresis (PAGE), as previously described [30,31]. All chemicals for the determination of Hp phenotyping were purchased from Sigma. A 10% hemoglobin solution in water was prepared from heparinized blood after washing the blood cells three times in phosphate–buffered saline.

For each sample, Hp–Hb complex solution was prepared by adding 2 μl of 10% Hb A to 10 μl of serum and mixing for 5 min at room temperature. Then, 40 μl of sample buffer (50%, v/v, glycerol and 0.001%, w/v, bromophenol blue) was added to each sample prior to running on the gel. The Hp–Hb complex was resolved by PAGE at a constant voltage of 250 V for 4 h. After the electrophoresis was completed, the Hp–Hb complexes were visualized by immersing the gel in benzidine solution with H_2O_2 for 30 min.

Benzidine solution was prepared by dissolving 200 mg of benzidine in 250 ml of boiling water. Glacial acetic acid (1.5 ml) and H_2O_2 (600 μl) were added to the benzidine solution just prior to staining.

2.4. Statistical analysis

Results were expressed as mean±SD. Comparisons between groups were made using Student's *t*- test for continuous variables. Correlation between the two parameters was determined by Pearson's correlation coefficient (*r*). The Chi square and Fisher's exact tests were performed to compare the frequencies of Hp phenotypes between subjects with and without nephropathy. A *p* value less than 0.05 was considered statistically significant.

3. Results

The main clinical and biochemical data of the type 2 diabetic patients are shown in Table 1. The three Hp phenotype distributions (Hp 1-1, Hp 2-1, and Hp 2-2) in type 2 diabetics and controls were easily distinguished by a characteristic pattern of bands representing the Hp–Hb complex, as shown in Fig. 1.

Table 2 demonstrates that the frequency of Hp 1-1 in diabetics with normoalbuminuria was 7/20 (35%) and was significantly greater than in diabetics with macroalbuminuria (1/20, 5%; $p=0.02$).

On the other hand, 10/20 (50%) diabetics with microalbuminuria and 7/20 (35%) diabetics with macroalbuminuria had Hp 2-1. The frequency of Hp 2-2 was greater in diabetics with macroalbuminuria: 12/20 (60%) as compared to those with normoalbuminuria or controls (5/20, 25%; $p=0.03$).

Serum CRP, IL-6, and Hp levels were higher in diabetic patients with normoalbuminuria than in healthy controls ($p<0.001$, $p<0.001$, and $p<0.01$, respectively; Figs. 2–4).

Among the diabetic patients, those with diabetic nephropathy (micro- or macroalbuminuria) had higher concentrations of serum CRP, IL-6, and Hp than those without nephropathy (normoalbuminuria), as shown in Table 1 and Figs. 2–4. Moreover, serum IL-6 and Hp levels were significantly increased in diabetics with macroalbuminuria as compared to those with microalbuminuria ($p<0.05$ and $p<0.001$, respectively; Figs. 3, 4).

Table 4
Correlation between levels of urinary albumin excretion (UAE), serum CRP, IL-6, and HP concentrations in type 2 diabetic patients

Parameters	UAE (mg/24 h)	CRP (mg/l)	IL-6 (pg/ml)	Haptoglobin (mg/dl)
UAE (mg/24 h)	–	$r=0.4$*	$r=0.53$**	$r=0.71$**
CRP (mg/l)	$r=0.4$*	–	$r=0.35$*	$r=0.53$**
IL-6 (pg/ml)	$r=0.53$**	$r=0.35$*	–	$r=0.78$**
Haptoglobin (mg/dl)	$r=0.71$**	$r=0.53$**	$r=0.78$**	–

*$p<0.01$; **$p<0.001$.

Table 3 shows that there was no significant difference in serum CRP and IL-6 concentrations between Hp phenotypes (Hp 1-1, Hp 2-1, and Hp 2-2) in all diabetic patients, whereas serum Hp concentration was significantly increased in diabetics with Hp 2-1 and Hp 2-2 as compared to those with Hp 1-1 phenotype ($p<0.05$ and $p<0.001$, respectively).

In the present study, UAE levels in diabetic patients were positively correlated with serum CRP, IL-6, and Hp concentrations, as shown in Table 4. Also, serum CRP levels were positively correlated with serum IL-6 ($r=0.35$, $p<0.01$) and serum Hp ($r=0.53$, $p<0.001$). Moreover, a significant positive correlation was found between serum IL-6 and serum Hp ($r=0.78$, $p<0.001$).

4. Discussion

The chief function of haptoglobin (Hp) is to bind to hemoglobin and thereby prevent hemoglobin-induced oxidative tissue damage.

This antioxidant function of Hp is mediated in part by the ability of Hp to prevent the release of iron from hemoglobin on its binding [32]. Genetically endowed differences in antioxidant protection could contribute to differential susceptibility of diabetic patients to microvascular complications including nephropathy, which is the leading cause of end-stage renal disease [3,22,23]. In humans, there are two common alleles for Hp (1 and 2), manifesting as three major phenotypes: 1-1, 2-1, and 2-2 [6].

This study demonstrated that the frequency of Hp 1-1 was greater in diabetics with normoalbuminuria than in those with macroalbuminuria ($p=0.02$), whereas Hp 2-2 frequency in diabetics with macroalbuminuria was greater than in those with normoalbuminuria ($p=0.03$). These results are in agreement with the findings of Nakhoul et al. [33], and Levy et al. [34], who reported an association of the Hp 2 allele with an increased incidence of nephropathy in diabetic patients. Furthermore, Hp 2 homozygosity in diabetic patients was shown to increase the likelihood of retinopathy [35]. These data implicate the Hp polymorphism in the increased susceptibility of individuals to diabetic vascular complications.

With respect to renal disease, Hp phenotype 2-2 was recently demonstrated to be a possible risk factor for chronic renal failure and was over-represented in diabetic nephropathy patients [36]. Similar results regarding Hp 2-2 were found in a recent study on the role of Hp phenotype in the development of end-stage kidney disease [37]. Furthermore, Burbea et al. [38] found that in young diabetic hemodialysis patients, the Hp 2-2 phenotype was over-represented compared with older ones. These findings may be attributed to the weak antioxidant activity and Hb-binding capacity of Hp 2-2 protein compared with that of Hp 1-1 protein [7,8].

Interestingly, CD163 is a monocyte/macrophage scavenger receptor with a high affinity for Hp–Hb complexes, and the Hp phenotypes differ markedly in terms of their interaction with this receptor [39]. It was found that binding of the Hp 1-1/Hb complex to the CD163 receptor results in a weaker stimulus than binding of the Hp 2-2/Hb complex and is, therefore, a weaker activator of cytokine production, such as IL-6, and contributes less to the inflammatory process [40].

There is increased evidence of inflammatory response abnormalities in type 2 diabetes mellitus [41]; however, data about the relationship of inflammation to nephropathy in type 2 diabetes mellitus are scarce. In the present study, the inflammatory parameters serum CRP, IL- 6, and Hp levels were significantly greater in diabetics than in controls.

Furthermore, patients with diabetic nephropathy (micro- or macroalbuminuria) had higher concentrations of these parameters than those without nephropathy. Serum IL-6 and Hp levels, in particular, were significantly increased in diabetics with macroalbuminuria as compared to those with microalbuminuria, indicating that they increased significantly as nephropathy progressed.

In our study, serum Hp levels in type 2 diabetics were higher in Hp phenotype 2-2 than in Hp 1-1; however, serum CRP and IL-6 levels did not differ significantly between HP phenotype groups. Interestingly, all inflammatory parameters in diabetic patients were correlated with each other and with UAE.

Thus, in addition to metabolic, hemodynamic, and genetic factors, it is possible to hypothesize on the participation of inflammation in the pathogenesis of diabetic nephropathy.

Our results are similar to those obtained by Navarro et al. [42] and Gomes and Nogueira [43], who found that serum levels of inflammatory markers are greater in diabetic patients with increased UAE than in normoalbuminuric subjects. Furthermore, findings by the Insulin Resistance Atherosclerosis study show an association of CRP and fibrinogen levels with UAE in patients with type 2 diabetes and microalbuminuria [44].

Regarding IL-6, our study was in agreement with the studies of Shikano et al. [45] and Abdelaleim et al. [46], who found a significant increase in serum IL-6 and urinary IL-6 as diabetic nephropathy progressed. This finding was in accordance with the study of Yasuhiko et al. [47], who mentioned that the level of IL-6 in all patients with NIDDM complicated with nephropathy was significantly higher than those in controls and in NIDDM patients without nephropathy.

It is well established that angiotensin-converting enzyme (ACE) inhibitor therapy has a beneficial effect on the development and progression of diabetic nephropathy. Although intraglomerular hemodynamic effects can explain the renoprotective effect of this class of agents, some studies have also suggested a role for the antioxidant effect of these agents [48]. In particular, ACE inhibitors result in a reduction of heme-driven oxidation products accumulating in the kidney. Moreover, ACE inhibitors inhibit mesangial cell nuclear factor-kB activation and expression of proinflammatory cytokines such as IL- 6 [49].

From this study, it may be concluded that Hp phenotype 2-2 is a major susceptibility gene for the development of nephropathy in type 2 diabetic patients and this might be due to the low antioxidative potential of Hp 2 compared with Hp

1. The results of this study provide a rationale for further prospective trials of vigorous antioxidant therapy to prevent the development of diabetic nephropathy. In addition, the significant association between inflammatory parameters and UAE indicates that inflammation may be a pathogenic mechanism of renal injury in type 2 diabetics. Moreover, serum IL-6 and Hp may be good prognostic factors for the development of nephropathy in the course of diabetes mellitus. Future research on the use of anti-inflammatory therapy may result in a new approach to the treatment and prevention of diabetic nephropathy.

5. Learning points

Screening of type 2 diabetic patients for Hp phenotypes is essential, as Hp 2-2 is considered to be a major susceptibility gene for the development of nephropathy.

The use of vigorous antioxidant therapy to prevent the development of diabetic nephropathy is mandatory.

Continuous measurement of serum IL-6 and Hp could be used as good prognostic factors for the development of nephropathy in the course of diabetes mellitus.

Anti-inflammatory therapy may be helpful in the prevention and treatment of diabetic nephropathy.

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