The association between diabetic nephropathy and polymorphisms in *PPARγ Pro12Ala* and *CCR5δ 32* genes in type 2 diabetes

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Background
Diabetic nephropathy is one of the debilitating complications of type 2 diabetes and the leading cause for end-stage renal disease requiring renal replacement therapy. Currently identified risk factors do not fully explain the susceptibility of some patients to diabetic nephropathy. Peroxisome proliferator-activated receptor γ *(PPARγ) Pro12Ala* gene polymorphisms modulate insulin sensitivity and oxidative stress in diabetic patients, and conflicting data exist on its association with kidney disease in diabetes. Several polymorphisms in another immune modulator set of genes, the C–C chemokine receptor 5 (CCR5) genes, were associated with diabetic nephropathy. However, CCR5δ 32 gene polymorphisms were not studied in patients with diabetic nephropathy. The aim of this study was to assess the association between polymorphisms in both *PPARγ Pro12Ala* and *CCR5δ 32* genes and the presence of diabetic nephropathy in Egyptian patients with type 2 diabetes.

Methods
We included 51 patients having type 2 diabetes for at least 5 years. They were all normotensive patients selected from the outpatient clinic with no other clinically identifiable risk factor for kidney disease. Genotype detection for *PPARγ Pro12Ala* and *CCR5δ 32* gene polymorphisms was carried out using the PCR technique. Clinical data, HbA1c levels, lipid profile, and fasting and postprandial blood sugar levels were recorded. Serum creatinine levels and the urinary albumin/creatinine ratio were measured to stratify the participants according to the presence or absence of diabetic nephropathy.

Results
Age, sex, BMI, HbA1c, and duration of diabetes were not significantly different among patients with and those without diabetic nephropathy. Diabetic nephropathy patients had a significantly higher urinary albumin/creatinine ratio and lower estimated glomerular filtration rate levels ($P<0.0001$). Homozygotes for the *PPARγ Pro12Ala Pro–Pro* allele constituted 82% of our total study population and 86.4% of patients with diabetic nephropathy; the remaining were *Pro–Ala* heterozygotes, and we had no *Ala–Ala* homozygotes. The odds ratio for diabetic nephropathy in *Pro–Pro* homozygotes was 3.5 ($P=0.075$, 95% confidence interval, 0.8–15). The Pro allele was present in 75% of patients with nephropathy and 50% of those without nephropathy. The *Pro* allele was significantly associated with diabetic nephropathy compared with the *Ala* allele (odds ratio = 3.5, $P=0.012$, 95% confidence interval, 1.3–15). With regard to the *CCR5δ 32* insertion/deletion genotype, 24 patients were homozygous for the insertion polymorphism, two were homozygous for the deletion polymorphism, and the remaining 25 were insertion/deletion heterozygotes. There was no significant difference between nephropathic and non-nephropathic patients as regards the *CCR5δ 32* genotype ($P=0.3$) or the frequency of allele distribution ($P=0.6$).

Conclusion
The Pro allele of *PPARγ Pro12Ala* was associated with diabetic nephropathy. Polymorphisms in the *CCR5δ 32* gene did not show an association with diabetic nephropathy.

Keywords:
diabetic nephropathy, polymorphisms in *CCR5δ 32, PPARγ Pro12Ala*

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Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. By the year 2030, diabetes mellitus is expected to affect almost 5% of the world's population: an estimated 366 million people. Diabetic nephropathy eventually develops in 30% of patients with both type I and type II diabetes, and once present, will progresses in many of these diabetic patients to end-stage renal disease [1,2].

Glomerulosclerosis is a major morphological change associated with diabetic nephropathy. Mesangial cell proliferation and extracellular matrix expansion are common features of glomerulosclerosis, and the infiltration of the glomeruli and interstitium by immunocompetent cells is a common finding in both rodent models of diabetes and biopsy specimens collected from diabetic patients [3].

Peroxisome proliferator-activated receptor γ (PPARγ) is an important transcription factor for lipid and glucose metabolism.

PPARγ is also expressed in the renal glomerular tissue and in vascular walls, thus participating through various complex mechanisms in glomerular and vascular sclerosis and the development and progression of nephropathy.

Activation of PPARγ may directly attenuate diabetic glomerular disease possibly by inhibiting mesangial growth, which occurs early in the process of diabetic nephropathy, or by inhibiting PAI-1 expression [4,5].

Another important factor for the development and progression of diabetic nephropathy is the prolonged uncontrolled state of hyperglycemia that increases the secretion of cytokines, such as tumor necrosis factor-α and interleukin-1β, that stimulate the expression of a chemokine, known as regulated upon activation, normal T-cell expressed and secreted (RANTES), by human mesangial cells. Signals mediated through the interaction between RANTES and its major receptor, C–C chemokine receptor 5 (CCR5), may promote monocyte/macrophage infiltration, differentiation, and activation of the glomeruli, possibly playing a key role in the development of diabetic nephropathy [6,7].

There is evidence that susceptibility to diabetic nephropathy has a significant genetic component [8]. Studies have reported that polymorphisms in PPARγ are associated with increased risk for development of diabetes, but whether it is associated with increased risk for development of diabetic nephropathy is a matter of debate, and results did not uniformly confirm this association. This is particularly true for the *PPARγ Pro12Ala* polymorphisms for which conflicting results were obtained in different populations [9,10]. Although several *CCR5* gene polymorphisms were extensively studied in diabetic nephropathy, one specific gene polymorphism responsible for modulation of the immune responses to inflammation, the *CCR5δ 32* insertion/deletion polymorphism, was not previously explored in patients with diabetic nephropathy [11].

The aim of this study was to determine the association between polymorphisms in two of the genes suspected to be associated with the development of diabetic nephropathy – that is *PPARγ Pro12Ala* and *CCR5δ 32* – in Egyptian patients with type 2 diabetes.

Patients and methods

A total of 51 patients were recruited from the outpatient clinic of the Kasr Al-Ainy Hospital to participate in the study (between January 2011 and May 2011). The patients were divided into two groups according to the presence or absence of diabetic nephropathy. The first group (non-nephropathic) included 14 patients with no evidence of diabetic nephropathy, defined as the absence of overt or micro albuminuria and estimated glomerular filtration rate (eGFR) levels greater than 60 ml/min. The second group included 37 patients with diabetic nephropathy, which is defined as the presence of persistent proteinuria and/or reduction of eGFR levels below 60 ml/min.

All patients included had type 2 diabetes for at least 5 years. Patients with other causes of kidney disease were excluded, namely patients with hypertension, urinary tract obstructive stones, urinary tract anomalies, pyelonephritis, those who were on regular nonsteroidal anti-inflammatory drugs, as well as those with current or history of acute kidney injury or glomerulonephritis.

All participants were subjected to history taking and clinical examination. BMI was calculated in all participants as follows: BMI = mass (kg)/height (m^2). Blood samples (7 ml) were collected after fasting for 12–14 h early in the morning. They were aliquoted into plain tubes for serum, EDTA tubes for HbA1c determination and genotypic study, and heparin tubes for determination of plasma glucose levels. Aliquots for genotypic studies were frozen at −20°C until DNA extraction. Immediate determination of creatinine levels, lipid profile (total cholesterol, high-density lipoproteins, and triglycerides), fasting plasma glucose levels, and HbA1c levels was performed through routine biochemical assays on the Cobas Integra autoanalyzer (Roche Diagnostics, Indianapolis, Indiana, USA). Low-density lipoprotein levels were calculated according to the method described by Friedewald *et al.* [12].

A random sample of urine was collected from all participants for determination of the urinary microalbumin/creatinine ratio. eGFR was calculated according to the Modification of Diet in Renal Disease formula [13].

Genotype detection

Genomic DNA was extracted from blood leukocytes using the salting out technique [14] using reagents supplied by Sigma (Sigma Chemical Co., St Louis, Missouri, USA). The *CCR5δ 32* polymorphism was assessed using the PCR technique and by amplification of a fragment surrounding the deletion with the forward primer 5′-TCACT TGGGTGGTGGCTGTGTTTGCGTCTC-3′ and the reverse primer 5′-AGTAGCAGATGACCATGACAAGC

AGCGGCAG-3′ [14]. The PCR reaction was carried out in a total volume of 25 µl, containing 2.5 µl of genomic DNA, 12.5 µl of master mix containing 10 µmol/l of Tris-HCl (pH 8.4), 1.5 mmol/l of $MgCl_2$, 0.2 mmol/l of dNTPs, 40 pmol/l of each primer, 0.25 U of Taq DNA polymerase, and 9 µl of sterilized distilled H_2O. PCR was performed with an initial denaturation step at 94°C for 15 s, annealing and extension at 70°C for 15 s (30 cycles), and a final extension step at 70°C for 5 min in a Hybaid thermal cycler supplied by Promega Corporation (Madison, Wisconsin, USA). The fragment length was determined on a *2%* agarose gel using Tris–borate–EDTA as the buffer, and the gel was stained with ethidium bromide. A 193 bp PCR product corresponded to the wild type allele and a 161 bp product represented the deletion.

An assay based on PCR-restriction fragment length polymorphism was used to determine the genotype of the *PPARγ2 Pro12Ala* gene [15]. The following primers were used: forward, 5′-GCCAATTCAAGCCCAGTC-3′ and reverse, 5′-CGTCCCCAATAGCCGTATC-3′. PCR was performed with an initial denaturation step at 94°C for 5 min, 35 cycles of annealing at 55°C for 1 min and extension at 72°C for 2 min, and a final extension step at 70°C for 5 min. The PCR products were further analyzed using the standard restriction fragment length polymorphism technique. An aliquot of 12 µl of PCR products was digested with 5 U of *Hga*I fast digest restriction enzyme at 37°C for 1 h. The digest was analyzed on a *2%* agarose gel using Tris–borate–EDTA as the buffer, and the gel was stained with ethidium bromide. One fragment of 306 bp indicated the absence of the *Hga*I restriction site (wild genotype), two fragments of 220 and 86 bp indicated homozygotic presence of the restriction site, and three fragments of 306, 220, and 86 bp indicated heterozygosity for the restriction site. The *Hga*I digestion enzyme, primers, master mix, and Taq DNA polymerase were obtained from Fermentas Inc. (Burlington, Ontario, Canada).

Statistical analysis

Baseline characteristics of the study population have been summarized as mean ± SD, interquartile range, frequencies, and percentages. One-way analysis of variance and Student's *t*-test were used for the comparison of means among the study participants. Pearson's χ^2-test was used to compare the distributions of genotype and allele frequencies between the different groups; a *P*-value of less than 0.05 was considered significant. All statistical analyses were carried out using the SPSS 14 software (SPSS Inc., Chicago, Illinois, USA).

Results

Table 1 shows the baseline characteristics of the study population.

Analysis of the distribution of the *PPARγ Pro12Ala* gene polymorphism showed a trend toward an increased occurrence of *Pro–Pro* homozygotes (32 of 37, 86%) among diabetic nephropathy patients compared with *Pro–Ala* heterozygotes (odds ratio, 3.5; $P = 0.075$; 95% confidence interval, 0.8–15). There were no Ala–Ala homozygotes among our study participants. Table 2 shows the characteristics of the participants according to the *PPARγ Pro12A1a* gene polymorphism. The allelic distribution of *PPARγ2 Pro12Ala* showed a significant preponderance of the wild type allele (Pro allele) among patients with nephropathy compared with those without nephropathy. The odds ratio for carrying the wild type allele, Pro allele, was 3.5 among patients with nephropathy ($P = 0.012$, 95% confidence interval, 1.3–15; Fig. 1).

As regards the *CCR5δ 32* gene, there was no statistical difference in genotypic distribution between the two groups of patients. This is despite the fact the only two patients homozygous for the 161 bp deletion had proteinuria and were classified in the diabetic nephropathy group ($P = 0.318$). Similarly, allelic frequency showed no statistical difference among the patient groups ($P = 0.6$). Table 3 shows the demographic characteristics of patients with each of the *CCR5δ 32* polymorphisms. Figure 2 shows the relative frequency of the *CCR5δ 32* allele distribution.

Discussion

This study showed that polymorphisms in the *PPARγ Pro12Ala* gene were associated with the occurrence of diabetic nephropathy, unlike the polymorphisms in the *CCR5δ 32* gene.

We found a significant association between *PPARγ Pro12Ala* allele polymorphisms and the presence of diabetic nephropathy. The wild type allele, Pro allele, was associated with higher odds of occurrence of the disease compared with the Ala allele. Moreover, there was a trend for genotypes carrying the wild type allele to be associated with diabetic nephropathy. The explanation for this association is that Ala polymorphisms of this gene are associated with improved insulin sensitivity and lower oxidative stress. Our study confirms this genetic association among Egyptian patients,

Table 1 Baseline characteristics of the study participants

	NN	DN	*P*-value
Number	14	37	
Age (years)	54 ± 11.1	58.6 ± 14.4	0.42
Duration of diabetes (years)	17 ± 3	19 ± 1.7	0.14
BMI (kg/m^2)	30.3 ± 1.4	30.8 ± 4.3	0.9
FPG (mg/dl)	205.6 ± 50.5	177.5 ± 49.6	0.04*
HbA1c (%)	7.7 ± 3.1	9.1 ± 1.8	0.35
Creatinine (mg/dl)	0.7 ± 0.2	2.6 ± 2	<0.001**
Cholesterol (mg/dl)	195 ± 18.9	197 ± 28.3	0.72
Triglycerides (mg/dl)	120 ± 39.4	143.5 ± 83.7	0.26
HDL (mg/dl)	46 ± 7.2	43.6 ± 9.7	0.478
LDL (mg/dl)	126.9 ± 14.1	118.4 ± 17.8	0.124
Microalbumin/creatinine ratio (mg/g)	13.3 ± 8.4	590.2 ± 791.9	<0.001**
eGFR (ml/min)	123 ± 27.4	29.4 ± 27.5	<0.001**

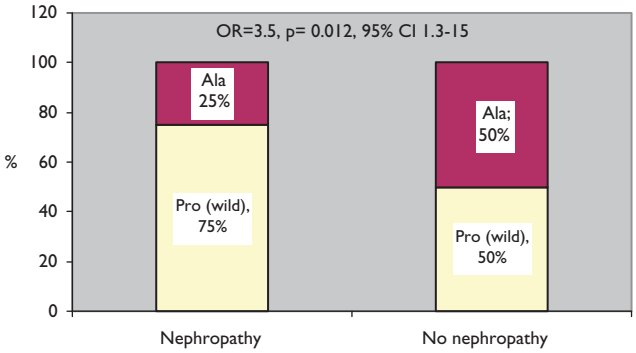
DN, diabetic nephropathy; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HDL, high-density lipoproteins; LDL, low-density lipoproteins; NN, no nephropathy.
*$P<0.05$.
**$P<0.01$.

Table 2 Demographic data and biochemical markers of the patients distributed according to *PPARγ2 Pro12Ala* gene polymorphisms

	PPARγ2 Pro12Ala gene polymorphism		
	Pro–Pro (wild type)	Pro–Ala	*P*-value
Number	41	10	
Age (years)	57.3 ± 12.5	45 ± 16.9	0.07
Duration of diabetes (years)	20 (10–20)	16 (15–20)	0.7
BMI (kg/m^2)	31 ± 4.4	30.6 ± 0.2	0.9
FPG (mg/dl)	171 (122–286)	169 (80–226)	0.53
HbA1c (%)	7.8 (6.8–9.3)	8.1 (6.3–10.3)	0.95
Creatinine (mg/dl)	1.3 (0.8–4.2)	2 (0.6–6.9)	0.81
Cholesterol (mg/dl)	190 ± 32.9	244.5 ± 81.3	0.5
Triglycerides (mg/dl)	155.8 ± 10	113 ± 61	0.86
HDL (mg/dl)	41.2 ± 9.2	48.4 ± 2.2	0.31
LDL (mg/dl)	118.8 ± 23.7	173 ± 66.5	0.2
Albumin/creatinine ratio (mg/g)	118.5 (24.9–673)	22.6 (7.7–2000)	0.65
eGFR (ml/min)	174 (140–217)	187 (95.7–248.9)	1.0

eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HDL, high-density lipoproteins; LDL, low-density lipoproteins.

Figure 1



Distribution of *PPARγ Pro12Ala* alleles. PPARγ, peroxisome proliferator-activated receptor γ.

and this has been previously demonstrated in German, Brazilian, and Chinese populations [10,16–18]. It is noteworthy that a Danish study [9] showed a contradictory association with type 1 diabetes. This may be because of the different pathophysiologies of type 1 diabetes, in which insulin deficiency is the main issue, unlike type 2 diabetes, in which insulin resistance, the metabolic syndrome, and macrovascular complications place the PPARγ pathway in a more central position. Kidney disease in type 2 diabetes has both macrovascular and microvascular components, unlike that in type 1 diabetes [19,20]. It is also important to bear in mind the ethnic variations between the studied populations when comparing associations of gene polymorphisms with the disease [5,10].

The CCR5 gene produces CCR5, which is a receptor for various proinflammatory cytokines. The deletion variant of this gene is associated with decreased inflammation-driven activity in several disorders. Pathogenesis of diabetic nephropathy is possibly associated with CCR5-driven inflammation [6,21], and this led us to hypothesize that *CCR5δ 32* deletion may be associated with a favorable renal outcome; however, our results did not demonstrate this effect. To our knowledge, this is the first study to explore this association. It is possible that this gene polymorphism has no association with diabetic nephropathy. It is also possible that patients carrying the deletion had a survival advantage over noncarriers, and thus patients with diabetic nephropathy carrying the gene became more prevalent in the studied population. Indeed it was previously shown that hemodialysis patients carrying the deletion allele have a survival advantage [22]. It is also important to bear in mind that the protective effect of the gene may be subtle and overshadowed by effects of various other genes and risk factors; hence, the size of the study population may not have been large enough to demonstrate this association.

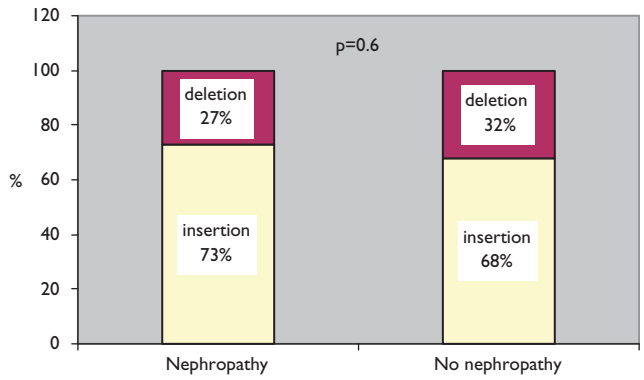
This study was capable of confirming the association of *PPARγ Pro12Ala* polymorphisms with diabetic nephropathy in Egyptian patients with type2 diabetes. To our knowledge, this is one of the first studies exploring the

Table 3 Characteristics of patients distributed according to *CCR5δ 32* gene polymorphisms

	CCR5δ 32 gene polymorphism			
	193 bp insertion (wild type)	*193, 161 bp*	*161 bp* deletion	*P*-value
Number	24	25	2	
Age (years)	61 (47.7–68.5)	58 (45.5–62)	47 (42–52)	0.267
Duration of diabetes (years)	20 (13.7–20)	16.5 (8–21.2)		0.56
BMI (kg/m^2)	31.9 ± 5.5	30.1 ± 2.4	29.6 ± 1.9	0.5
FPG (mg/dl)	174 (138–235.8)	171.5 (124.8–223.8)	280.5 (214.5–346.5)	0.29
HbA1c (%)	7.8 (7.1–10.2)	7.8 (6.3–9.4)	11.4 (9.1–13.7)	0.25
Creatinine (mg/dl)	2 (0.9–5)	1.2 (0.7–2.6)	0.9 (0.6–1.3)	0.41
Cholesterol (mg/dl)	202 ± 15	206 ± 49	155 ± 67.9	0.32
Triglycerides (mg/dl)	182 ± 58	124 ± 46.5	143 ± 13.4	0.64
HDL (mg/dl)	42.9 ± 8.3	43.3 ± 8	36 ± 10.9	0.61
LDL (mg/dl)	124 ± 12.7	137.8 ± 39.8	90.2 ± 48.4	0.23
Microalbumin/creatinine ratio (mg/g)	167.5 (12.3–652.5)	62.8 (16–842)	241 (52.2–429.8)	0.40
eGFR (ml/min)	23.1 (10.9–72.8)	46.1 (21–119.4)	89 (61.6–116.5)	0.24

eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; HDL, high-density lipoproteins; LDL, low-density lipoproteins.

Figure 2



Frequency of the *CCR5δ 32* allele distribution among the study participants. CCR5, C–C chemokine receptor 5.

association between *CCR5δ 32* polymorphisms and diabetic nephropathy. Limitations of this study are its cross-sectional nature and the relatively small number of patients included. Nevertheless, we have tried to overcome these limitations by trying to avoid the interaction of confounding factors by including patients with relatively young age, comparable duration of diabetes, and glycemic control, as well as excluding patients with hypertension.

Conclusion

PPARγ Pro12Ala Pro allele was associated with diabetic nephropathy. Polymorphisms in the *CCR5δ 32* gene did not show an association with diabetic nephropathy.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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