Association of the PPARγ2 Pro12Ala and C1431T Polymorphisms with Type 2 Diabetes and Diabetic Retinopathy in a Sample of Egyptian Patients

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Abstract

Background
Peroxisome Proliferator Activated Receptor Gamma (PPAR-γ) is a pleotropic transcription factor. Given the pivotal roles of PPARγ in regulating metabolism, several studies in various ethnic populations have explored the association between variants in this gene and susceptibility to diabetes and its complications with conflicting results.

Objective
To evaluate the association of Pro12Ala and C1431T polymorphisms of PPARγ2 gene with Type 2 Diabetes (T2D), the risk of developing Diabetic Retinopathy (DR) and the severity of retinopathy, in a sample of Egyptian patients.

Methods
We recruited a total of 60 T2D patients from those attending the Ophthalmology Department, Alexandria University main Hospital - Egypt; 30 with DR (18 non-proliferative and 12 proliferative DR) and 30 patients with T2D of > 10 years' duration who showed no signs of DR. A third group of 120 non-diabetic volunteers were included as controls. Polymerase Chain Reaction followed by restriction enzyme digestion (PCR-RFLP) was used to determine the Pro12Ala and C1431T variants of PPARγ2 in patients and control subjects. Genotype and allele frequency distributions among the different groups were compared using the chi-square test.

Results
The frequency of the T allele of C1431T variant was significantly higher in T2D subjects than in controls (X²=8.5, p=0.004), whereas Pro12Ala was not significantly associated with T2D. Among diabetic subjects, no significant association was observed between these polymorphisms and DR or the severity of retinopathy.

Conclusion
Our findings support a role for the PPAR-γ2 C1431T, but not Pro12Ala, in the etiology of T2D, while they confirm lack of association between both polymorphisms and the risk to develop DR.

Keywords: Diabetes; Diabetic retinopathy; Egypt; C1431T; PPARγ2 gene; Pro12Ala

Introduction
Diabetes mellitus is a chronic metabolic disease that became a major public health problem in both developed and developing countries [1]. The prevalence of Type 2 DM (T2D), which is the most common type of diabetes, has raised markedly with the increased prevalence of obesity and sedentary life style [2]. The main cause of T2D is unclear, accumulating evidences suggest a polygenic nature of the disease marked by the interaction of environmental and genetic factors [3]. The long-term diabetic vascular complications are considered the leading cause of morbidity and mortality in diabetic patients [1]. Diabetic Retinopathy (DR) is one of the most common microvascular complications of diabetes [4], and one of the leading causes of blindness worldwide [5].

Among an array of candidate genes selected from the proposed pathogenic pathways, Peroxisome Proliferator-Activated Receptors (PPARs) have been widely studied to verify their role in diabetes and diabetic complications. PPARs are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes controlling a variety of biological functions such as cellular differentiation and metabolism [6]. Three types of PPARs have been identified: alpha, gamma, and beta [7]. Alpha (α) is expressed in liver, kidney, heart, muscle and adipose tissue. Beta (β) is expressed markedly in the brain, adipose tissue, and skin. Gamma (γ) although transcribed by the same gene, this PPAR through alternative splicing is expressed in three forms: γ1 is expressed in virtually all tissues, including heart, muscle, colon, kidney, pancreas, and spleen, γ2 is expressed mainly in adipose tissue, while γ3 is expressed in macrophages, large intestine, and white adipose tissue [7,8].

PPARγ expression in the retina has a beneficial role in the modulation of inflammation, angiogenesis and apoptosis in retinal and endothelial cells, thereby ameliorating the retinal and endothelial damage caused by high glucose induced prolonged inflammation [9]. Moreover, PPAR-γ ligands are potent inhibitors of corneal angiogenesis and neo-vascularization [10,11].

A number of genetic variants in the PPARγ2 gene have been identified. Among these variants, the most prevalent is Pro12Ala missense mutation, which was first identified in 1997 [12]. Although the initial publication [13], reported a 75% risk reduction for...
diabetes conferred by the Ala allele of this variant, the subsequent studies reported inconsistent findings [14-17]. The next most frequently occurring PPAR-y2 polymorphism is a silent C to T substitution at nucleotide 1431 in the sixth exon, (Cytosine (C) 161 → Tyrosine (T) substitution which was identified by Meirhaegue et al., [18].

Among different populations, this polymorphism has been inconsistently associated with susceptibility to T2D and diabetic complications [19-21]. These conflicting reports encouraged us to investigate the impact of these two common PPARy polymorphisms on the susceptibility to T2D and DR in a sample of Egyptian diabetic patients. Furthermore, to our knowledge, no association studies, evaluating the role of the C1431T variant in T2D, or investigating the associations of PPARy2 variants with DR, have been published.

Materials and Methods

The study was conducted on 60 unrelated T2D patients selected from those attending the outpatient clinic of the Ophthalmology Department in Alexandria University Hospital, including 30 patients suffering from T2D proved to have Diabetic Retinopathy by fundus examination (DR group) and another 30 patients with T2D of more than 10 years’ duration who showed no signs of DR (DWR group). One-hundred-twenty non-diabetic age- and sex-matched volunteers were recruited for participation as control subjects.

The study was conducted in accordance with the Declaration of Helsinki and an informed consent was obtained from all subjects after the purpose of the study was explained to them. After, medical and family histories were carefully taken; venous blood samples for the molecular analysis were collected in EDTA tubes. The molecular study was carried out in the molecular laboratory of the Human Genetics Department, Medical Research Institute, Alexandria University-Egypt.

Molecular Study

Genomic DNA was extracted from peripheral blood leukocytes by salting out technique [22].

The Pro12Ala SNP single nucleotide polymorphism in the exon B of the PPAR-y2 gene was investigated by Polymerase Chain Reaction (PCR) amplification of genomic DNA followed by Restriction-endonuclease digestion (RFLP); according to the method previously reported by Al-Shali et al., [23]. Amplification via Veriti Thermal Cycler (Applied Biosystems) was performed using the primers 5'-ACT CTG GGA GAT TCT CCT ATT GCC-3' (forward) and 5'-CTG AAT GTG AAG CCC ATT GAA -3' (reverse) (Invitrogen). The PCR reaction consisted of 30pg primers, 50ng of genomic DNA, 3mM MgCl2, 200mM each dNTPS, 1 unit Taq (Thermoscientific). The PCR program was: 94 °C for 4 min followed by 30 cycles of 30s at 94°C and 45s at 58°C and 72°C for 1 min, with a final extension step of 7 min at 72°C. The PCR products were digested with HaeIII I fast digest restriction enzyme (Thermoscientific) according to the manufacturer’s direction.

For the PPAR-y2 c.1431C_T genotyping, the same method as above was used except for an annealing temperature of 60°C and the following primers: 5'- CTG AAT GTG AAG CCC ATT GAA -3' (forward) and 5'- GTG GCC GCT GAC TCT GTG CTA G -3' (reverse). The amplified segments were digested with Pml I fast digest restriction enzyme (thermo scientific) according to the manufacturer’s instructions.

The digested PCR products were resolved by electrophoresis on 3% agarose gel stained with ethidium bromide for 20 minutes at 200V and were sized with reference to a 50-bp DNA ladder.

Statistical Analysis

Allele frequencies were estimated by direct gene counting. Chi square goodness-of-fit test was used to assess the Hardy-Weinberg equilibrium in the studied groups. Genotype and allele frequency distributions among the patients and controls were compared using chi-square test calculated to estimate the relative risk of T2D or DR.

Results

Demographic and clinical data

The T2D group included 40 females and 20 males; the male to female ratio was 1:2. Their ages ranged from 39-70 years with almost 80% of them in the age group 50-70 years with a mean and SD of 56.95±8.33. The duration of diabetes ranged from 10-46 years. Among the DR group there were 14 males and 16 females while 6 males and 24 females formed the DWR (diabetes without retinopathy) group. The age ranged from 39-70 years with a mean and SD of 57.35±8.88 among the diabetic patients with DR; while among the DWR group the age ranged from 42-70 years with a mean of 55.7±8.4 with no statistical significance.

The DR group included 18 patients suffering from Nonproliferative DR (NPDR group) and 12 patients with Proliferative DR (PDR group). In the NPDR group, there was 4 cases of severe NPDR, 4 of moderate NPDR and 10 of mild NPDR. Ophthalmologic examination revealed other ocular complications in the form of macular edema in 5 patients, vitreous hemorrhage in another 5 cases, preretinal hemorrhage, ruberosis iridis and central retinal vein occlusion each in one case.

Molecular data

The distribution of the PPAR-y2 Pro12Ala genotypes and alleles in T2D patients and controls is shown in table 1. The observed genotype frequencies followed the Hardy-Weinberg equilibrium in both groups. The Pro allele was slightly higher among diabetic patients than controls; a difference that was not statistically different (X^2=0.001, p=0.9695). Similarly, the association between Pro12Ala genotypes and T2D was considered to be not statistically significant (X^2=0.740, p=0.691).

Table 1: The allele and genotype frequencies of PPAR-y2 Pro12Ala among the T2D patients compared to control subjects.

<table>
<thead>
<tr>
<th>Gene Mutation</th>
<th>Subjects</th>
<th>Frequency (n)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2D patients</td>
<td>60</td>
<td>26 (43.33)</td>
<td>Pro/Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47 (79.17)</td>
<td>Pro/Ala</td>
</tr>
<tr>
<td></td>
<td></td>
<td>52 (86.67)</td>
<td>Ala/Ala</td>
</tr>
<tr>
<td>Non-diabetic</td>
<td>120</td>
<td>22 (36.67)</td>
<td>Pro/Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36 (53.33)</td>
<td>Pro/Ala</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21 (37.50)</td>
<td>Ala/Ala</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46 (76.67)</td>
<td>Pro/Pro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>146 (240)</td>
<td>Pro/Ala</td>
</tr>
<tr>
<td></td>
<td></td>
<td>94 (30%     )</td>
<td>Ala/Ala</td>
</tr>
</tbody>
</table>

Table 2, shows the distribution of the PPAR-y2 C1431T genotypes and alleles in T2D patients and controls. The frequency of the observed genotypes, in both patients and controls, did not deviate from the Hardy-Weinberg expectations. Among the Egyptian subjects, T2D patients showed higher frequencies of the variant...
In order to study a putative association of DR with PPAR-γ2 variants, the genotypes and alleles frequencies of each polymorphism were calculated for DR subgroup and compared to those of the DWR subgroup (Table 3). Based on chi-square tests, the distribution of neither the PPAR-γ2 Pro12Ala variant nor the PPAR-γ2 C1431T variant, showed a significant difference between the DR and DWR subgroup.

In further, we explored the impact of the PPAR-γ2 variants on the severity of retinopathy. The DR patients were classified into subjects with NPDR and those with PDR, and then the genotype and allele frequencies of the studied polymorphism were calculated separately for each group as shown in table 4. When we compared the distribution of the genotypes and alleles of both polymorphism among the two groups, no statistically significant difference was found, indicating a lack of association between the PPAR-γ2 Pro12Ala or C1431T variants and the severity of DR.

Table 2: The allele and genotype frequencies of PPAR-γ2 C1431T among the T2D patients compared to control subjects.

<table>
<thead>
<tr>
<th>Genotype/Allele</th>
<th>T2D patients (n=60)</th>
<th>Non-diabetic subjects (n=120)</th>
<th>χ²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro/Pro</td>
<td>8 (13.33)</td>
<td>42 (35.00)</td>
<td>10.4</td>
<td>0.006*</td>
</tr>
<tr>
<td>Ala/Ala</td>
<td>36 (60.00)</td>
<td>60 (50.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/T</td>
<td>16 (26.67)</td>
<td>18 (15.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>52 (43.33)</td>
<td>144 (60.00)</td>
<td>8.5</td>
<td>0.004*</td>
</tr>
<tr>
<td>T</td>
<td>68 (56.67)</td>
<td>96 (40.00)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Distribution of the genotypes and alleles of PPAR-γ2 Pro12Ala and C1431T polymorphisms among the T2D patients according the presence of retinopathy.

<table>
<thead>
<tr>
<th>Genotype/Allele</th>
<th>Pro12Ala polymorphism</th>
<th>C1431T polymorphism</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR (n=30)</td>
<td>DWR (n=30)</td>
<td>DR (n=30)</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>10 (33.34)</td>
<td>16 (53.33)</td>
</tr>
<tr>
<td>Pro/Ala</td>
<td>13 (43.33)</td>
<td>9 (30.00)</td>
</tr>
<tr>
<td>Ala/Ala</td>
<td>7 (23.33)</td>
<td>5 (16.67)</td>
</tr>
<tr>
<td>X²=2.45, p=0.294</td>
<td>X²=2.50, p=0.287</td>
<td></td>
</tr>
</tbody>
</table>
| Allele frequencies n (%) | Pro 33 (55.00) 41 (68.33) C 27 (45.00) 25 (41.67) | Ala 27 (45.00) 19 (31.67) T 33 (55.45) 35 (58.33) | X²=1.727, p=1.1887 | X²=0.34, p=0.8538

Discussion

Among several genetic variants of the PPARγ2 gene, two polymorphisms; Pro12Ala of the exon B (rs1801282) and the C1431T silent substitution (rs8586606) in the exon 6, are the most frequently occurring SNPs and have been associated with various diseases [13-21]. The association of Pro12Ala polymorphism with T2D, insulin resistance, obesity and metabolic disorders has been reported in several studies [24-26]. The C1431T polymorphism has also been studied in relation to obesity, diabetes and coronary heart disease [18,25-27]. After a meticulous review of the literature, we were able to find a single association study conducted on Egyptian individuals to explore the relation between Pro12Ala PPARγ2 variant and T2D risk [28]. However, to our knowledge, no association studies, evaluating the role of the C1431T variant in T2D, or investigating the associations of PPARγ2 variants with DR, have been published.

In the present study, we investigated the impact of Pro12Ala and C1431T PPARγ2 polymorphisms on the risk to develop T2D and DR and we came out with two important findings. The first is the significantly higher risk for diabetes among carriers of the T allele of the C1431T polymorphism. The second finding is that the frequency of the Ala12 allele of PPARγ2 in the Egyptian sample studied was very high (~38% in T2D cases and ~39% in controls) compared to that reported for Caucasians [14,16,29,30]. However, the protective effect of the "Ala" allele with T2D was not confirmed in the present work. In addition, our study failed to find any significant association of the two studied polymorphisms with the risk of DR among T2D patients.

The PPARγ2 Pro12Ala variant was not associated with either T2D or DR in the current study. In previous studies, the association between the Pro12Ala polymorphism and fasting glucose or insulin concentrations have yielded conflicting results [14,16,17,26]. Overall, combined evidences suggest that the Ala12 allele exerts a protective effect with carriers of this allele causing a decreased diabetes risk [13,15,17,24,26,31,32]. However, several reports from different ethnic origins failed to reproduce a significant association [16,19,23,33,34]. Nevertheless, some studies have even found an increased risk for T2D in subjects with the Ala12 variant [35,36]. The results of the previous Egyptian study supported an association between Pro allele of PPARγ2 gene and T2D [28]. It is noteworthy that the unexplained high frequencies of the Ala allele observed among the Egyptian control subjects, in the previous study (53%) [28] and also in the current work (~39%), exceed any frequency reported.

Regarding the relation between the PPARγ2 Pro12Ala variant and DR, few reports are available and results were also controversial. Our
findings are consistent with Petrovic et al., and Costa et al., [37,38], but contradict others who reported a protective role of the 12Ala polymorphism against proliferative DR in individuals with T2D [39,40].

In the present study, despite the increased frequency of the T allele of C1431T polymorphism and the strong association with T2D, we did not find any significant difference of this variant between the DR and DWR subgroups, indicating that it confers risk for the development T2D but not for the DR complications. The CT and TT genotypes of C1431T were significantly present in diabetic patients as a whole, but no genotype was significantly higher in patients with DR. Instead, the T allele was slightly higher in the DWR subgroup. A contradicting finding was reported by Tai et al., [25], they described a reduced risk of diabetes in carriers of the T allele of the C1431T polymorphism. In fact, only few studies [18,20,26,41] have analyzed the effects of the C1431T polymorphism, and none of them found a significant association of this variant with glucose or lipid-related variables. These inconsistencies may be attributable to the fact that this polymorphism is silent at the amino acid level and is unlikely to have any direct mechanistic link with specific phenotypes. Instead, it might be in linkage disequilibrium with an unidentified functional variation either within or flanking the PPARγ2 gene or in another gene at this locus.

We ensued to compare our results regarding the distribution of the PPARγ2 polymorphisms with the available reports from different Arab populations. In agreement with our finding, Badii et al., [42] could not confirm the potential association between this polymorphism and T2D among Qataris subjects but they reported a low frequency of the Ala allele (5.5% for diabetic and 5.9% for non diabetics) as compared to ours and to the previous reports in various Caucasians. In Tunisia, several researchers examined the association of the genetic variation of the PPARγ2 with the susceptibility to T2D and concluded that the PPARγ2 is unlikely to be responsible for T2D in the Tunisian population [43-45].

These discrepancies between studies related to these polymorphisms may be partly explained by the presence of a gene-gene or gene-environment interaction. The controversial findings may also be related to information bias caused by the studies design and the selection criteria of patients. In addition, the prevalence of these polymorphisms; which varies greatly among populations, and may be very low in some of them, may contribute to the inconsistent results by affecting the statistical power of the comparisons.

In conclusion, the present study describes a significantly higher risk for diabetes among carriers of the T allele of the C1431T polymorphism, while it reports lack of association of the Pro12Ala variants with T2D. We also found no evidence of an association between the studied variants of PPARγ2 gene with DR in the T2D patients. Thus, the PPARγ2 C1431T variant seems to confer risk for the development of T2D but not for its chronic complications. Finally, since the lack of association might come from the small sample size, further studies on larger sample sizes are required to verify the present observation.

References


Citation: Nabil KM, Desouky LMI, Abdalla EM (2016) Association of the PPARγ2 Pro12Ala and C1431T Polymorphisms with Type 2 Diabetes and Diabetic Retinopathy in a Sample of Egyptian Patients. J Ophthalmic Clin Res 3: 019.