PREVALENCE OF THE «PRO12ALA» MUTATION OF PPAR-γ2 GENE IN AN ARABIC IRAQI POPULATION

Amjad Hazim Al-Naemi, MBChB, MSc; Akram Jarjees Ahmad, MBChB, MSc

Objective: Pro12Ala is a common functional mutation of PPAR-γ2 gene whose frequency varies widely worldwide. In Iraq, there are no studies of the frequency of Pro12Ala. The aim of this study is to find out the frequency of Pro12Ala (rs1801282) in an Iraqi population and to compare it with those of other populations.

Methods: Pro12Ala was genotyped in 95 healthy unrelated Arabic native Iraqi adult subjects using PCR-RFLP. Its frequency was compared to those of other populations.

Results: Genotype frequencies were within Hardy-Weinberg Equilibrium (HWE). Allelic frequencies were 90.53% for the C (Pro) allele and 9.47% for the G (Ala12) allele. The homozygous wild type genotype (Pro12Pro) frequency was 81.05%. The heterozygous mutant genotype (Pro12Ala) was evident in 18.95% of subjects with no cases of (Ala12Ala). Allelic and genotypic frequencies were statistically different from PREV ALENCE OF THE «PRO12ALA» MUTATION OF PPAR-γ2 GENE IN AN ARABIC IRAQI POPULATION

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those of Ukrainian (p=0.007 and 0.017), Chinese (p=0.041 and 0.034), and African Americans (p=0.000 and 0.000).

Conclusions: Iraqis have relatively high prevalence of Pro12Ala mutation which differs from several populations confirming the need for understanding the genetic background of each population.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and members of the nuclear hormone receptor superfamily whose other members include receptors for thyroid hormones and steroids. They form heterodimeric molecules with the retinoid X receptor (RXR) and bind to co-repressors. Ligand binding results in conformational changes in the receptor leading to dissociation of the co-repressor complex and recruitment of co-activator proteins. This process ultimately leads to activation of gene expression mainly those genes involved in energy homeostasis and lipid metabolism.

There are three well identified types of PPARs; PPARα, PPARδ (or PPARβ) and PPARγ whose DNA-binding domains are about 80% identical. Human PPAR-γ gene is located in chromosome 3 and it spans a genomic segment of more than 150 kb. It consists of 9 exons (A1, A2, B and 1-6) from which two distinct isoforms of PPAR γ mRNA and protein -PPAR γ1 and PPAR γ2- are derived through the use of separate promoters and 5’ exons.

PPAR γ1 mRNA specie is composed of exons A1, A2 and 1-6- and is translated from P1 promoter while PPAR γ2 mRNA is a combination of exons B and 1-6 and is translated from P2 promoter. PPAR γ2 is 28 amino acids longer than PPARγ1.

While PPAR γ1 is widely expressed in adipose tissue, colon, macrophages, heart, skeletal muscles, liver, kidney, bone and other tissues, PPAR γ2 is expressed mainly in the adipose tissue. It has been demonstrated that PPAR γ2 is essential for the long-term survival, differentiation and homeostatic function of the adipocytes through transcription of many genes that are responsible for lipogenesis and insulin signaling, and that its deficiency causes substantial fat cell loss and compensatory hypertrophy.

Activation of PPAR-γ is through a variety of agonists like polyunsaturated fatty acids, prostaglandins, eicosanoids and the insulin sensitizing anti-diabetic agents thiazolidinediones (TZDs) like pioglitazone, which have been shown to improve insulin sensitivity in a variety of insulin resistant animal models and in human diabetics.

PPAR-γ has been evidently and strongly related to diseases like cancers, osteoporosis, arthritis, inflammation and hypertension, cardiovascular diseases, insulin resistance and obesity, type 2 diabetes, kidney diseases, cystic fibrosis and skin immunological disorders.

Several hereditary mutations have been identified in the ligand binding domain of PPAR-γ gene. Barroso et al. in 1999 reported two dominant negative mutations in human PPAR-γ (Pro467Leu and val290met) that are associated with severe insulin resistance, hypertension and diabetes. A rare gain of function mutation (Pro115Gln) has been identified earlier in 4 out of 121 subjects with morbid obesity.

However, and more interestingly, Yen et al. in 1997 have identified a CCA to GCA missense mutation in codon 12 of the PPAR γ2 specific exon (exon B) that encodes the amino-terminal residue of the PPAR γ2 protein. As a result «alanine» substitutes the ancestral «proline» in the mutant protein (Pro12Ala) which would consequently have a less functional activity as a transcription factor.

This single nucleotide polymorphism (SNP) of PPAR γ2 gene has been the most widely studied. This SNP has been linked to risk of development of diabetes mellitus, hypertension and different kinds of cancer in different populations worldwide. However, results have been conflicting in various populations and various ethnic groups pointing to the fact that gene-environmental interactions and/or gene-gene interactions are possibly
standing behind whether it is a «risky» or a «protective» player in the causation of different human health disorders among different populations.

As an example, many studies showed that there is a significant body of evidence that this gene mutation is associated with type 2 diabetes and that the Pro12 allele is its «risk allele».

However, others revealed no evidence of association between Pro12Ala and diabetes. Moreover, and on the contrary, other studies observed a higher Ala12 allele frequency in the diabetic patients conferring to its possible «risky» contribution in the onset of diabetes.

The minor allele frequency of Pro12Ala has been reported to extend over a wide range among different world populations. Asian people were reported to have the lowest prevalence of Ala12 allele where only 4% of Chinese people had the minor allele of Pro12 Ala. People from European descent are, on the other hand, the highest in minor allele frequency which may reach up to 21%.

In Iraq there have been no studies of genotype distribution and minor allele frequency of Pro12Ala. To best of our knowledge, this is the first study of the prevalence of Pro12Ala (rs1801282) in Iraq. We believe that understanding the genotype frequency of this common mutation would help in managing the clinically relevant associated diseases.

METHODS

This study was approved by the Human Research Ethics Committee of Ninevah Health Directorate, Mosul, Iraq on April 17th 2013 and informed consents were obtained from all participants. Thus this study was performed in accordance with the ethical standards of Declaration of Helsinki II.

Ninety-five (51 males and 44 females) apparently healthy unrelated native Arabic Iraqi adult subjects (31-70 years old) were randomly selected to participate in this study over a period of three months starting from 15th May through 15th August 2013. All these subjects were residents of urban districts of Mosul City, Ninevah Province and were generally the relatives of patients admitted in Al-Joumhour Teaching Hospital in Western Mosul, Mosul, Iraq in addition to the members of the nursing staff.

Ninevah is the second largest province in Iraq and Mosul is the biggest city in this province. Mosul citizens who are around 3 million subjects- are of different religious, national and ethnic backgrounds. This study focused on the native Arabic Iraqi people without mixing marriages with other ethnic groups till the third generation.

Subjects were excluded from participation if they have positive personal and/or family history of diabetes, liver or kidney diseases or any other major health problem like cardiovascular events in the last six months. Other exclusion criteria included current or recent participation in an interventional study or those with drug abuse or history of malignancy or terminal ill diseases.

Blood samples were obtained by non-stressful antecubital venipuncture under completely aseptic conditions. Two ml venous blood sample was transferred into a clean EDTA tube for DNA extraction and subsequent genetic analyses.

The EDTA-treated whole blood samples were refrigerated at 2-8 C° for subsequent DNA extraction within 48 hours or otherwise kept frozen at -20 C° if the extraction was to be postponed. Long storage of blood samples (more than two weeks) was avoided to increase the chance of getting higher DNA yields.

Genomic DNA was extracted using ReliaPrep™ Blood gDNA MiniPrep System (Promega Corporation, USA) following the manufacturer’s protocol. The whole molecular work was carried out in the PCR laboratory of Ibn Al-Atheer Teaching Hospital, Eastern Mosul, Iraq.

DNA samples were genotyped for the Pro12Ala mutation of PPAR-γ2 gene using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) assay as designed by Hamann et al. A 306 bp DNA sequence embracing the ambiguity site was first amplified using
PCR. The following primers (GenScript, USA) were used:

- Forward: 5’- GCCAATTCAAGCCCAGTC- 3’
- Reverse: 5’- CGTCCCCAATAGCCGTATC- 3’

Genomic DNA (2.5 μl) was added to the reaction mixture (22.5 μl) to make the final reaction volume 25 μl. The reaction mixture consisted of 12.5 μl of the 2X Master Mix (Qiagen, Germany), 0.5 μl of each primer (forward and reverse) in 10 μM concentration and 9 μl of RNase-free water. The ready to use 2X Master Mix contains HotStar Taq® Plus DNA polymerase (5 units/μl), ultrapure dNTPs and MgCl2 (pH 8.7, 20°C) in a final reaction concentration of 3 mM. The PCR was performed using PXE 0.2 Thermal Cycler (Thermo Electron Corporation, France). The cycling program is shown in Table 1. Ten μl of PCR products (306 bp) were digested with 3 units of the restriction enzyme Hgal (New Englands Biolabs, UK) in its buffer [NEBuffer 1.1 (10 mM Tris Propane- HCl, 10 mM MgCl2, 1 mM dithiothreitol, pH 7.0 at 25°C)] in a final volume of 50 μl at 37°C for 60 minutes followed by enzyme inactivation at 65°C for 20 minutes based on the manufacturer’s instructions. The C/G variation in the target sequence creates the following restriction site for Hgal:

5’- G A C G C (N) 5  …3’
3’- C T G C G (N)10 …5’

Following digestion, the DNA fragments were separated on 2% agarose gel following a standard gel electrophoresis for around 25 minutes at 125 V, stained with SYBR® Safe DNA Gel Stain, visualized on the UV transilluminator and photographed. A single band of 306 bp indicated a homozygous wild type allele (Pro/Pro), while the heterozygous mutant allele (Pro/Ala) should produce three bands; 306 bp, 220 bp and 86 bp. However, in case of homozygous mutant allele (Ala/Ala) only two bands would be produced; 220 bp and 86 bp. Figure 1 is a gel photograph of digestion products.

Frequencies of alleles and genotypes were calculated and checked if they fall within Harday-Weinberg

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Table 1. Thermal conditions to amplify Pro12Ala for subsequent RFLP.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>90 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Final extension</td>
<td>68°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

Final volume= 25 μl, cycles= 35

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Figure 1. PCR-RFLP detection of Pro12Ala on 2% agarose gel. Lane 1:100 bp DNA ladder (Promega Corporation, USA) with the prominent band is 500 bp, lanes 2, 3, 4, 5, 7, 8, 9, 11, 12, 13, 14: wildtype for codon 12 variant (Pro/Pro) showing one (306bp) band only, Lanes 6, 10 and 15 (arrowed): heterozygous for codon 12 variant (Pro/Ala) showing 306bp and 220 bp bands while the 86 bp fragment was so faint and did not appear on photographing.

Statistical analysis: Chi-square test was used to compare the allelic and genotypic frequencies with those of other populations. Statistical Package for Social Sciences (SPSS) version 19 was used for statistical analyses. p-value was significant when <0.05.

RESULTS

Figure 1 is a photograph of a gel of PCR-RFLP products of fourteen subjects. The genotypes frequencies of Pro12Ala (rs1801282) were checked whether they are normally distributed or not. For this purpose, observed frequencies were entered into software to assess Hardy-Weinberg Equilibrium (HWE). Ideally, its p-value has to be >0.05 to indicate normal distribution. Figure 2 shows the output of the used software to calculate the p-value of HWE of Pro12Ala genotypes in our studied population.

Genotypes frequencies of our subjects were within HWE ($\chi^2 = 1.04$, p = 0.308). The allelic frequencies were 90.53% (172 out of 190) for the wild type allele (Pro12) and 9.47% (18 out of 190) for the mutant allele (Ala12). The homozygous wild type genotype (Pro12Pro) was found in 81.05% (77 out of 95) of the study population. However, the heterozygous mutant genotype (Pro12Ala) was evident in 18.95% (18 out of 95) with no any case of homozygous mutant genotype (Ala12Ala) observed. Allelic and genotype frequencies of the studied population are shown in Table 2.

The allelic and genotypic frequencies revealed in our population were compared to those of other populations. Table 3 compares these frequencies using $\chi^2$ test. A simple calculator to determine whether observed genotype frequencies are consistent with Hardy-Weinberg equilibrium.

Table 3 compares these frequencies using $\chi^2$ test.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Observed #</th>
<th>Expected #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygote</td>
<td>77</td>
<td>77.9</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>18</td>
<td>16.3</td>
</tr>
<tr>
<td>Homozygote variant:</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>Var allele freq:</td>
<td>^Put your values here^</td>
<td>0.09</td>
</tr>
</tbody>
</table>

$\chi^2 = 1.04027258$
$\chi^2$ test $P$ value = 0.307722 with 1 degree of freedom
1. If P<0.05-not consistent with HWE.
2. Not accurate if <5 individuals in any genotype group.

Michael H. Court (2005-2008)

DISCUSSION

Since its identification by Yen et al. in 1997, the missense mutation “Pro12Ala” has been widely studied all over the world. A lot of light has been shed on the association of this common polymorphism with several clinical disorders like obesity, insulin resistance, diabetes mellitus, hypertension, inflammation, atherosclerosis and tumorigenesis and there have been controversies in such relationships among different populations.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>Allelic frequency n (%)</th>
<th>Genotypic Frequency n (%)</th>
<th>p- value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR-γ</td>
<td>Pro12Ala</td>
<td>C (Pro) 172 (90.53%)</td>
<td>CC (Pro/Pro) 77 (81.05%)</td>
<td>0.308</td>
</tr>
<tr>
<td></td>
<td>(rs1801282)</td>
<td>G (Ala) 18 (9.47%)</td>
<td>GC (Pro/Ala) 18 (18.95%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG (Ala/Ala) 0 (00.00%)</td>
<td></td>
</tr>
</tbody>
</table>

*Genotypic distribution using HWE calculator. p-value for $\chi^2$ has to be >0.05 for the genotype distribution to fall within HWE.

Table 2. Genotypic and allelic frequencies of Pro12Ala (rs1801282) in the study population.
<table>
<thead>
<tr>
<th>Authors/Year</th>
<th>Population/Size</th>
<th>Allelic frequency n (%)</th>
<th>Genotyping frequency n (%)</th>
<th>p-value for allele</th>
<th>p-value for genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>Iraqi (95)</td>
<td>C (Pro) 172 (90.53)</td>
<td>CC (Pro/Pro) 77 (81.05)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Frederiksen et al./2002</td>
<td>Danish Caucasian (2245)</td>
<td>G (Ala) 18 (9.47)</td>
<td>GC (Pro/Ala) 18 (18.95)</td>
<td>0.075</td>
<td>0.146</td>
</tr>
<tr>
<td>Kaydashev et al./2007</td>
<td>Ukrainian (39)</td>
<td>C (Pro) 61 (72.60)</td>
<td>CC (Pro/Pro) 1671 (74.50)</td>
<td>0.007</td>
<td>0.017</td>
</tr>
<tr>
<td>Kim et al./2007</td>
<td>Korean (129)</td>
<td>G (Ala) 17 (27.40)</td>
<td>GC (Pro/Ala) 24 (61.55)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ghoussaini et al./2005</td>
<td>French Caucasian (318)</td>
<td>C (Pro) 243 (94.19)</td>
<td>CC (Pro/Pro) 77 (81.05)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Namvaran et al./2011</td>
<td>Iranian (128)</td>
<td>G (Ala) 15 (5.81)</td>
<td>GC (Pro/Ala) 115 (89.10)</td>
<td>0.349</td>
<td>0.327</td>
</tr>
<tr>
<td>Canbay et al./2012</td>
<td>Turkish (129)</td>
<td>C (Pro) 244 (94.57)</td>
<td>CC (Pro/Pro) 77 (81.05)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tellechea et al./2009</td>
<td>Argentineans (572)</td>
<td>G (Ala) 1059 (92.57)</td>
<td>CC (Pro/Pro) 1059 (92.57)</td>
<td>0.328</td>
<td>1.042</td>
</tr>
<tr>
<td>Prasad et al./2008</td>
<td>Indian (241)</td>
<td>C (Pro) 424 (87.97)</td>
<td>CC (Pro/Pro) 186 (77.18)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gao et al./2010</td>
<td>Han Chinese of Inner Mongolia  (137)</td>
<td>G (Ala) 264 (96.40)</td>
<td>CC (Pro/Pro) 264 (96.40)</td>
<td>0.010</td>
<td>0.000</td>
</tr>
<tr>
<td>Gupta et al./2011</td>
<td>Indians (280)</td>
<td>C (Pro) 493 (88.04)</td>
<td>CC (Pro/Pro) 213 (76.00)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Badii et al./2008</td>
<td>Qatari consanguineous (450)</td>
<td>G (Ala) 847 (94.11)</td>
<td>CC (Pro/Pro) 847 (94.11)</td>
<td>0.349</td>
<td>0.316</td>
</tr>
<tr>
<td>Kao et al./2003</td>
<td>African Americans (1005)</td>
<td>C (Pro) 1964 (97.71)</td>
<td>CC (Pro/Pro) 1964 (97.71)</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 3. Comparison of genotypic and allelic frequencies of Pro12Ala (rs1801282) in Iraqi people with those of other populations.

The insulin sensitizing anti-diabetic agents TZDs have been identified in the mid1990s to be high affinity ligands for PPAR-γ. The proline substitution by alanine at codon 12 of the PPAR-γ2 protein is well known to reduce its functional capacity as a transcriptional factor for a large number of genes. This functional attenuation of the PPAR-γ2 protein is...
probably behind the Pro12Ala associations with the wide spectrum of diseases mentioned above. Also from here, possibly, emerges the variation in drug responses among diabetics treated with TZDs. This is what «pharmacogenetics» concerned with. Individualizing treatments for patients based on their genetic background will revolutionize the management regimens and plans in a successful and modern way mainly for the sufferers of chronic diseases. 

Owing to its importance, the allelic and genotypic distributions of Pro12Ala have been studied worldwide. Different ethnic groups in different populations showed different figures.

In Iraq, however, there has been no work right now to analyze the frequency of this mutation among Iraqis. To the best of our knowledge, this is the first study of this common mutation in Iraq. Iraq is a multinational and multi-ethnic country in the North-West Asia. This study has focused on a population of Native Arabic Iraqi adult healthy subjects living in urban areas of Mosul, the biggest city of Ninevah Province which is the second largest province in Iraq following the capital Baghdad.

The allelic frequencies are 90.53% and 9.47% for the C «Pro» and the G «Ala» alleles respectively. The homozygous wild-type genotype «Pro/Pro» was evident in 81.05% while the heterozygous mutant genotype «Pro/Ala» was found in 18.95% of our population. No one had the homozygous mutant genotype.

These frequencies were compared with those of other world populations. Allelic and genotypic frequencies in the studied Iraqi population were statistically different from those of Ukrainian, Chinese, Han Chinese, and African Americans populations. Compared to another Middle East population, a significant statistical difference was observed in the genotypic frequencies of Pro12Ala among Iraqi and Native Qatari subjects (p=0.030).

On the other hand, allelic and genotypic frequencies of the studied Iraqi population did not differ statistically (p>0.05) from reports of Danish Caucasians, Koreans, French Caucasians, Iranians, Turks, Argentineans from Southern European ancestries in addition to Indians.

Differences in the alleles and genotypes frequencies of the common mutation Pro12Ala across population may partially explain the variations in the prevalence rates of the common associated diseases. Among these are diabetes, hypertension and cancer diseases. Gene-environmental interactions together with gene-gene interactions may further influence the variation in diseases frequencies across different populations across the world.

CONCLUSIONS

This study revealed significant differences in the distribution of the Pro12Ala polymorphism of PPAR-γ gene (rs1801282) between an Iraqi Arabic population and those across different parts of the world mainly Caucasians (Ukraine), African Americans and Chinese. Even some Arabic populations nearby Iraq, like Qatari, have a different genotypic frequency. These differences confirm the need for understanding the genetic background of each population and lead us to recommend analyzing the association of the relatively common Pro12Ala with the common clinically-important chronic clinical diseases in Iraqis like insulin resistance, diabetes, hypertension, cardiovascular disease, cancer and others.

ACKNOWLEDGMENT

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