Association between PON1-rs662 Gene Polymorphism and Diabetic Retinopathy in Population of the Qom, Iran

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Abstract

**Background:** Diabetic retinopathy is the most severe diabetic microvascular complication that causes changes in the vessel wall. One of the genes involved in this disease is PON1, which encodes paraoxonase1 protein in liver and kidney. It might regulate inflammatory and microvascular responses to the disease. The rs662 T>C is one of the single nucleotide polymorphisms of this gene that changes glutamine to arginine at position 192.

**Methods:** In this study, 300 samples were collected, including 100 healthy and 100 diabetics without retinopathy, and 100 diabetics retinopathies were studied and their age range was from 30 to 80 years. Then 2.5 ml of blood was collected from all relevant individuals in tubes containing EDTA-Na2. This polymorphism was examined by tetra-ARMS PCR.

**Results:** Results showed that there is no significant correlation between genotypes and alleles related to PON1 and Diabetes (CC genotype: p=0.609; C allele: p=0.228). On the other hand, an association was observed between PON1 and diabetic retinopathy (CT+CC genotype: p<0.001; CT allele: p<0.001). Considering that the Polyphen database examined the changes caused by replacing the amino acid arginine instead of glutamine at position 129 on the protein, it does not consider these changes dangerous and has introduced this polymorphism as benign.

**Conclusion:** Based on the findings of this study, the rs662 locus could be considered as one of the molecular markers in future research.

**Keywords:** Diabetic angiopathies, Diabetic retinopathy, Polymerase chain reaction, Polymorphism, PON1


Introduction

Diabetes mellitus is the most common noncommunicable disease worldwide. It is among the leading causes of death in all socioeconomic circumstances. 1. Diabetic Retinopathy (DR) is known as a major complication of diabetes mellitus, which is a leading cause of visual loss. DR is characterized by vascular abnormalities in the retina. 2. Clinically, DR includes two stages: Non-Proliferative Diabetic Retinopathy (NPDR) and Proliferative Diabetic Retinopathy (PDR). NPDR is the early stage of DR. In this stage, retinal pathologies such as hemorrhages and microaneurysms are detectable. However, neovascularization occurs in PDR. 3. A meta-analysis study showed that the incidence of diabetic retinopathy ranges from 22 to 127% 4. In addition, the prevalence of DR in Iranian diabetic patients is 30%. 5. The pathophysiology of this disease is complex and has not been fully understood. Oxidative stress is implicated in hyperglycemia-induced abnormalities in the retina. 6. In recent years, it is generally believed that genetic factors are involved in the occurrence, prevention, and treatment of DR.

Today, various polymorphisms in the promoter or coding regions have been documented in the paraoxonase 1 (PON1) gene. 7. PON1, a calcium-dependent enzyme known as a serum esterase/lactonase which is synthesized in liver, 8. PON1, a polymorphic protein

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Received: 4 May 2023
Accepted: 20 Jul 2023

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Vol. 15, No. 4, October–December 2023

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prevents low-density lipoprotein oxidation in diabetes\textsuperscript{9}. PON1 is an HDL-associated protein that hydrolyzes oxidized LDL-cholesterol and exerts potential atheroprotective effects \textsuperscript{10}. However, few studies reported a relationship between PON1 polymorphisms with DR. Hampe \textit{et al} demonstrated that the PON1 R allele is associated with susceptibility to DR \textsuperscript{11}. Another study showed that genotype L/L was significantly associated with DR \textsuperscript{12}. Therefore, one of the polymorphisms that may play a role in DR is rs662(Q192R), and this study aimed to investigate the relationship between rs662-PON1 gene polymorphism and DR in the Qom’s population.

Materials and Methods

Samples

In the case-control study, intravenous blood samples were collected from diabetic patients with retinopathy (n=100), without retinopathy (n=100), and healthy subjects (n=100). The inclusion criteria were age from 30 to 80 years old, and the diagnosis of retinopathy by an optometrist. All blood samples were collected from Qom hospitals in Iran from 2018 to 2022 years. This study was approved by the Ethics Committees of the Mazandaran University of Medical Science (#1R.UMZ.REC.1399.035) and all subjects signed an informed consent form before entering the study.

DNA extraction and PCR-RFLP

Genomic DNA was extracted from leukocytes of the blood samples by a standard salting out method. Extracted DNA stored at -20°C after determining the relevant concentrations and analysis on gel electrophoresis. PON1-rs662 gene polymorphisms were examined by tetra-ARMS PCR. All primers used in this research were designed by a primer design program, Oligo7 (Table 1). Each PCR reaction was performed in a final volume of 20 μl, including 100 ng of genomic DNA, 3.5 μl of 10× solution buffer, 1.5 μl of a 10 μM of four mixed dNTPs, 1.5 μl of 50 mM of MgCl\textsubscript{2}, 0.25 μl of 5μl Taq DNA polymerase (Cinnagene, Co., Iran) and appropriate concentrations of each primer. 1.4 μl, of IF and IR primers and 1.2 μl, of the OR and the OF primers were added to the tubes at a concentration of two picomoles (pmol), and after adding 1 μl, of template DNA, finally 3.4 μl, of Taq DNA polymerase enzyme was added. After a short vortex and then spinning the samples, the PCR steps of the desired fragments were performed using the thermal cycler: my cycler (BioRad). The amplified PCR products were checked using a primer map in the \textit{tetra}-ARMS, which is shown in figure 1.

Table 1. PCR primer sequences of PON1-rs662 locus

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligomer 5’→3’</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF</td>
<td>TAAACCCCAAATACATCTCCCAGGCTT</td>
<td>57.8</td>
</tr>
<tr>
<td>IR</td>
<td>ATCACTATTTTCTTGACCCCTACTTCCG</td>
<td></td>
</tr>
<tr>
<td>OF</td>
<td>TACATTTGAGAGGTTCATCATGCTGCCA</td>
<td></td>
</tr>
<tr>
<td>OR</td>
<td>TTTTGGAAATAGACATGGAATGCCA</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Different genotypes in tetra-ARMS PCR method. A) Primer map in the \textit{tetra}-ARMS PCR; B) Electrophorese patterns of genotypes in 2% agarose gel; C) Electropherograms of flanking nucleotides in the three loci.
In silico analysis

In silico analyses were performed to evaluate the potential biological functions of two protomerindel polymorphisms, rs662, located in the coding region of the PON1 gene. So, the coding sequence of the PON1 gene was screened by Polyphen-2, I-Mutant, Panther, PhD-SNP, SNP&GO, and PROVEAN prediction tools.

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) was calculated for groups. All data were analyzed using SPSS software version 16. Differences in the frequency of alleles and genotypes were analyzed using the chi-square test or Fisher’s exact test. The association between PON1 gene polymorphisms and male infertility was estimated by computing the Odds Ratio (OR) and 95% Confidence Intervals (CI) from a logistic regression analysis model after adjustment for age. The p<0.05 was considered statistically significant.

Results

Extraction of the human genome from blood was done by salting out method and to check the quality of extracted DNA, it was run on the agarose gel. Also, the concentration of samples was measured by the spectrophotometric method. Polymorphism genotyping was done by tetra-ARMS PCR method. The desired PCR product used four inner and outer primers was run on a 2% gel with an optimal binding temperature of 57.8°C. The 317 bp band is specific to the T allele, which is the wild allele, and which can be seen in all samples. The 174 bp band is the result of two Outer primers, which can be seen in all samples. The bp band 197 bp is specific to the T allele, which is the wild allele, and the 174 bp band is specific to the C allele, which is the mutant allele. A heterozygous individual has all three bands. The wild allele homozygous individual has two 174 bp bands and the mutant allele homozygous individual has two 317 bp and 174 bp bands (Figure 1).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Normal (n=100)</th>
<th>DwR (n=100)</th>
<th>OR (95% CI)</th>
<th>p-value</th>
<th>DR (n=100)</th>
<th>OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>47 (47%)</td>
<td>49 (49%)</td>
<td>Ref</td>
<td></td>
<td>21 (21%)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>22 (22%)</td>
<td>21 (21%)</td>
<td>0.916</td>
<td>0.446-1.880</td>
<td>0.810</td>
<td>62 (62%)</td>
<td>3.307</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.07-12.83</td>
</tr>
<tr>
<td>CC</td>
<td>31 (31%)</td>
<td>30 (30%)</td>
<td>0.928</td>
<td>0.489-1.764</td>
<td>0.820</td>
<td>17 (17%)</td>
<td>1.227</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.56-2.688</td>
</tr>
<tr>
<td>CT+CC</td>
<td>53 (53%)</td>
<td>51 (51%)</td>
<td>0.923</td>
<td>0.530-1.608</td>
<td>0.777</td>
<td>79 (80%)</td>
<td>3.336</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.79-6.208</td>
</tr>
<tr>
<td>T- allele</td>
<td>116 (58%)</td>
<td>119 (59.5%)</td>
<td>Ref</td>
<td></td>
<td>104 (52%)</td>
<td>Ref</td>
<td></td>
</tr>
<tr>
<td>C- allele</td>
<td>84 (42%)</td>
<td>81 (40.5%)</td>
<td>0.940</td>
<td>0.631-1.4</td>
<td>0.761</td>
<td>96 (48%)</td>
<td>1.275</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.859-1.892</td>
</tr>
</tbody>
</table>

Table 2. Genotypic and allelic frequency of PON1 gene in C>T rs662 region for healthy and diseased groups

Table 3. Results from bioinformatics servers

<table>
<thead>
<tr>
<th>RsID</th>
<th>Polyphen2</th>
<th>I-Mutant3.0</th>
<th>Provean</th>
<th>SNP&amp;GO</th>
<th>PhD-SNP</th>
<th>Panther</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs662 (Q192R or T&gt;C)</td>
<td>Benign</td>
<td>Large decrease instability</td>
<td>Neutral</td>
<td>Neutral</td>
<td>Neutral</td>
<td>Neutral</td>
</tr>
</tbody>
</table>

The analysis of allele frequency distribution of rs662 T>C showed that the frequency of CC, TT, and CT genotypes in healthy groups is 31, 47, and 22%, respectively. The frequency of CC, TT, and CT genotypes in the diabetic group without retinopathy was 30, 49, and 21%, respectively. Moreover, the frequency of CC, TT, and CT genotypes in the DR group was 17, 21, and 62%, respectively (Table 2).

Investigations of the SIFT database, which examines protein function when another amino acid is substituted, indicated that Q192R may affect the protein which requires further investigation. The results of the GO & SNP database showed that Q192R polymorphism is a neutral polymorphism. The Mutant-I database determines protein stability based on energy changes released by amino acid substitutions. According to the result of this database, which reported 88.0:DDG, replacing the amino acid arginine instead of glutamine at position 129, causes a significant decrease in protein stability. This neutral polymorphism was also reported in the PROVEAN database. However, considering that the Polyphen database examined the changes caused by replacing the amino acid arginine instead of glutamine at position 129 on the protein, it does not consider these changes dangerous and has introduced this polymorphism as benign (Table 3).

Discussion

In this study, the relationship between rs662 polymorphism and DR was evaluated in the population of Qom. The results of our study showed that there was a significant relationship between rs662 polymorphism and diabetic retinopathy in the Qom’s population. Our finding showed a significant relationship between CT allele and DR. According to our findings, there was no study on the relationship between rs662 polymorphism and DR. DR is the most severe diabetic microvascular...
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ties and hippocampus, this factor can be a reason for the development of DR. The limitation of our study can be the relatively small sample size.

Conclusion

The findings of this research showed that there is a significant relationship between the mutant C allele in three groups: healthy, diabetic without retinopathy, and DR causing such a result in this research. Therefore, larger population in the future research are recommended to conduct such a study.

Acknowledgement

We appreciate all the colleagues who have worked with us in this study. We would like to extend our thanks to the University of Mazandaran (Iran) for the financial support, dedicated to the MSc candidate of Fatemeh Sabbaghian Bidgoli (#IrranDoc1602456). Thanks to our colleagues: Mr. Mammadkazem Heydari & Ms. Zahra Shirzad (from the Molecular and Cell biology lab, University of Mazandaran).

Conflict of Interest

There is no conflict of interest to declare.

References
