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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 paraoxanase1 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 is known as a serum esterase/lactonase which is synthesized in liver [8]. PON1, a polymorphic protein

prevents low-density lipoprotein oxidation in diabetes [9]. PON1 is an HDL-associated protein that hydrolyzes oxidized LDL-cholesterol and exerts potential atheroprotective effects [10]. However, few studies reported a relationship between *PON1* polymorphisms with DR. Hampe *et al* demonstrated that the *PON1* R allele is associated with susceptibility to DR [11]. Another study showed that genotype L/L was significantly associated with DR [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 (#IR. 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. Ex-

tracted 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$_2$, 0.25 *μl* of 5u/*μl 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 (Bio-Rad). The amplified PCR products were checked using a primer map in the *tetra*-ARMS, which is shown in figure 1.

Table 1. PCR primer sequences of *PON1*-rs662 locus

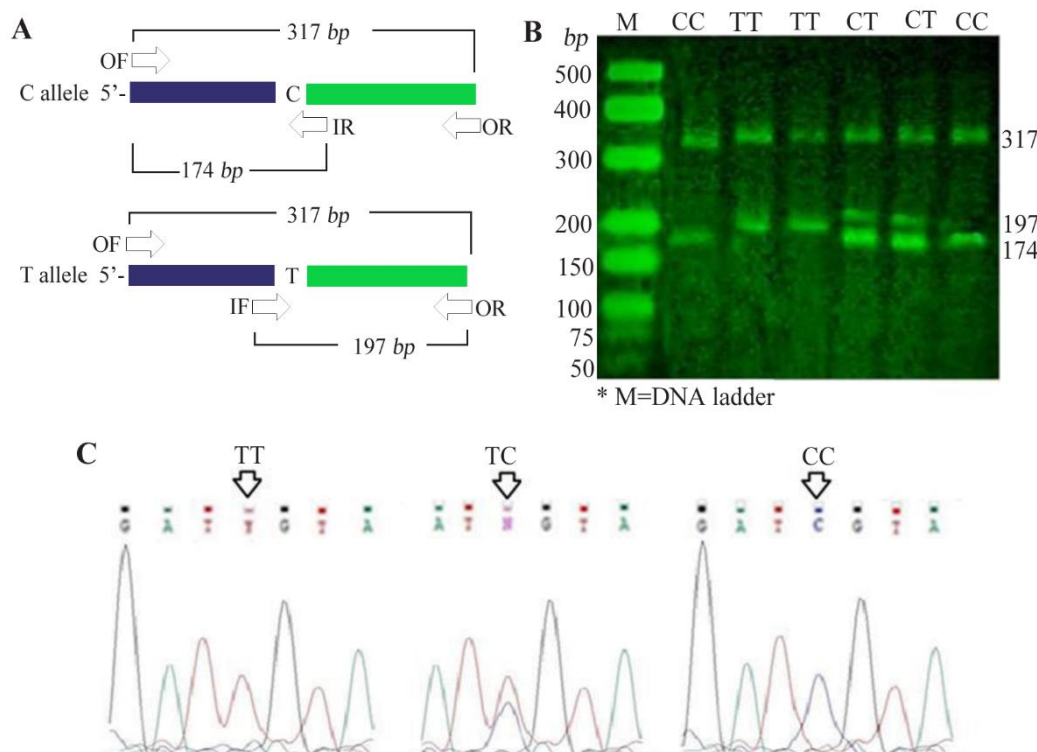| Primer name	Oligomer 5'→3'	Tm (°C)
IF	TAAACCCAAATACATCTCCCAGGCTT	57.8
IR	ATCACTATTTTCTTGACCCCTACTTCCG	
OF	TACATTTCAGAGAGTTCACATACTTGCCA	57.8
OR	TTTTAGGAATAGACAGTGAGGAATGCCA	

Figure 1. Different genotypes in tetra-ARMS PCR method. A) Primer map in the *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 protomeric indel 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 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 317 *bp* and 197 *bp* bands and the mutant allele homozygous individual has two 317 *bp* and 174 *bp* bands (Figure 1).

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

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

Genotype	Normal (n=100)	DwR (n=100)	OR (95% CI)	p-value	DR (n=100)	OR (95% CI)	p-value
TT	47 (47%)	49 (49%)	Ref	-	21 (21%)	Ref	-
CT	22 (22%)	21 (21%)	0.916 (0.446-1.880)	0.810	62 (62%)	6.307 (3.107-12.8.3)	0.001
CC	31 (31%)	30 (30%)	0.928 (0.489-1.764)	0.820	17 (17%)	1.227 (0.560-2.688)	0.609
CT+CC	53 (53%)	51 (51%)	0.923 (0.530-1.608)	0.777	79 (80%)	3.336 (1.793-6.208)	0.001
T- allele	116 (58%)	119 (59.5%)	Ref	-	104 (52%)	Ref	-
C- allele	84 (42%)	81 (40.5%)	0.940 (0.631- 1.4)	0.761	96 (48%)	1.275 (0.859-1.892)	0.228

OR: Odds Ratio, CI: Confidence Interval, DwR: Diabetic without retinopathy, DR: Diabetic retinopathy.

Table 3. Results from bioinformatics servers

RsID	Polyphen2	I-Mutant3.0	Provean	SNP & GO	PhD-SNP	Panther
rs662 (Q192R or T>C)	Benign	Large decrease instability	Neutral	Neutral	Neutral	Neutral

complication. Therefore, in line with the results, some studies showed the relationship between polymorphism and vascular diseases. The immunohistochemical analysis in a study indicated that gene expression of *PON1* was decreased in atherosclerotic arteries compared to normal arteries. There was a significant relationship between PON1 Q192R (rs662) polymorphism and the risk of Coronary Artery Disease (CAD). Moreover, 192R allele carriers had a higher risk of CAD compared with other allele carriers [13]. Deng *et al* reported that rs662 (G>A) was markedly associated with Congenital Heart Defects (CHD) susceptibility compared with healthy subjects. In addition, a G allele was related to an increased risk of CHD [14]. Another study demonstrated that C and R alleles were associated with T2DM susceptibility. In addition, the frequency of CC and RR genotype was markedly higher in patients with T2DM compared with healthy subjects. Moreover, CC and RR genotypes were associated with low HDL and higher LDL levels [15]. GG genotype of PON1 rs662 was related to an increased risk of CAD in an Indian population. In addition, a higher frequency of the G allele was also observed in CAD patients compared with control subjects [16]. However, a study reported that there was no association between *PON1* (rs662) polymorphisms and diabetic dyslipidemia [17]. The relationship between the PON1 rs662 polymorphism and CHD may be mediated by abnormal Ox-LDL and lipid levels caused by the R allele [18]. The inconsistent findings in the relationship between Q192R polymorphism with the increased risk of vascular impairment may be due to variations in genotyping methods, sample size, pathological states, and diverse environmental effects. Oxidative stress plays an important role in chronic inflammatory diseases such as CAD, diabetes, and DR. PON1 has a protective function due to its anti-inflammatory and antioxidant role in the body [19, 20]. PON1 is mainly synthesized in liver. It is associated with HDL. Decreased PON1 levels lead to increasing ox-LDL in blood, thereby increasing the susceptibility to vascular dysfunction [21,22]. PON1 rs662 gene polymorphism is involved in modulating the PON1 enzyme [23]. The R allele variant is implicated in the less active isoform of PON1 against lipoprotein oxidation, and higher ox-LDL, triglycerides, and LDL-C, which lead to an increased risk of vascular dysfunction [24,25]. Moreover, the inter-relationship between the *PON1* and *PON2* genes may affect glycaemic control in patients with diabetic retinopathy [26].

The analysis of bioinformatics software showed that this polymorphism causes a large decrease in protein stability. This decrease in stability by affecting the function of the PON1 enzyme can be a factor for the lack of hydrolysis of organophosphates. PON1 enzyme has an antioxidant role. Since the high level of Reactive Oxygen Species (ROS) plays a role in the pathogenesis of various diseases such as diabetes and heart failure and causes the destruction of the retinal capillar-

ies 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.

Conflict of Interest

There is no conflict of interest to declare.

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