

# Paraoxonase 1 activities and its gene promoter single nucleotide polymorphisms (-108, -126, and -162) in diabetes mellitus

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**Abstract:** *Background:* Paraoxonase 1 (PON1) enzyme is known enzyme with, aryl esterase, phosphatase, peroxidase, and lactonase activities. According to some studies, the activity of PON1 enzyme is decreased in type 2 diabetic patients. We analyzed the enzyme activity and its single nucleotide polymorphisms (SNPs) distribution on promoter regions (-108, -126, and -162) in type 2 diabetic patients compared with non-diabetic individuals to reveal the likely relationship between PON1 activity and its gene promoter polymorphisms. *Methods:* On the whole, 98 diabetic and 104 non-diabetic individuals were examined in this study. The enzyme activity and the genotypes were studied using spectrophotometry, real-time PCR-HRM, and sequencing techniques, respectively. *Results:* There was no meaningful difference in enzyme activity between two under-studied groups (P.V = 0.671). Moreover, no meaningful difference was also seen between two groups in terms of the frequency of polymorphism -108 (P.V = 0.277). The frequencies of SNPs -126 and -162, however, showed a meaningful difference between two groups (P.V = 0.000 and P.V = 0.017, respectively). *Conclusions:* We indicated PON1 activity could be similar in DM-2 patients and non-DM-2 individuals. The significant role of SNP -108 in PON1 activity in DM-2 patients compared with non-DM-2 individuals was confirmed in the study too. On the other hand, the role of -162 and -126 SNPs in causing diabetes cannot be easily overlook because of a meaningful difference of their distribution in understudied groups. However, they may be attributed to DM-2-associated genes.

**Keywords:** type 2 diabetes, paraoxonase 1 enzyme activity, PON1 gene polymorphisms, diabetes mellitus, type 2, Aryldialkylphosphatase, paraoxonase-1, polymorphism, genetic

## Introduction

Diabetes mellitus type 2 (DM-2) is caused by environmental elements, lifestyle and genetic factors. Numerous studies have been conducted to explore the molecular pathology of diabetes through molecular studies by which hundreds of genes have been reported as DM-2-associated [1, 2]. According to some studies, the activity of an enzyme called paraoxonase 1 (PON1) is decreased in patients suffering from DM-2 [3, 4]. PON1 is known with hydrolysis, aryl esterase, phosphatase, peroxidase, and lactonase activities [5, 6]. It is synthesized in liver,

goes into blood circulation, and binds to high-density lipoprotein (HDL) during the blood circulation [7–11]. It also hydrolyzes metabolites of pesticides and organophosphates including parathion, soman, and sarin [12, 13]. Given the different populations, various values of PON1 activity could be observed in individuals. The aforementioned differences may be attributed to the polymorphisms of coding region and promoter of PON1 gene or environmental factors affecting on the gene expression. C-108T SNP in promoter and Q192R in coding region could be regarded as significant polymorphisms leading to mentioned differences [14, 15].

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However, the simultaneous analysis of all factors affecting enzyme activity is impossible. Given the different frequencies of PON1 gene polymorphisms in various populations, in present study we analyzed enzyme activity and the polymorphisms of its promoter region (-108, -126, and -162) in DM-2-affected patients in comparison with those not suffering from the disease. We try to figure out the relationship between PON1 activity and the SNPs of its promoter region (-108, -126, and -162) to determine their likely effects on diabetes incidence. The Ethical Committee of Yazd University of Medical Sciences approved the current study.

### Subjects

The samples included those reference to Sedighe Tahereh clinic for endocrine and metabolic diseases, Isfahan. Samples comprised of 202 individuals, 98 cases with DM-2 and no history of cardiovascular disease, and 104 non-diabetic individuals. As some studies have indicated lower activity of the enzyme in cardiovascular patients [6, 11, 16, 17], the diabetic patients with cardiovascular diseases were excluded from the study. The fact of including criteria for affected group was FBS higher than 126 mg/dl or HbA1c higher than 6.5%. Non-diabetic individuals did not have parents or siblings suffering from diabetes. The sex ratio was almost equal in both groups and all females were not pregnant. There was no meaningful difference among participants in the case of age and gender in both groups (P.V = 0.421 and P.V = 0.751, respectively).

### Methods

The present study is a descriptive cross-sectional study. To measure enzyme activity, Eserin ( $10^{-5}$  M) was firstly added to each serum and it was incubated for 10 min. The obtained solution was, then, added to paraoxon 2 mM that contained paraoxon and Tris/HCl buffer (Tris/HCl, Sigma-Aldrich 100 mM, pH 8.00, and 2M  $\text{CaCl}_2$ ). Spectrophotometry technique was employed at 405 nm wavelength and each sample underwent 5 min of reading [9, 18]. Then to determine polymorphisms -108, -162, and -126 of PON1, DNA was extracted using Accue Prep Genomic DNA Extraction Kit (Bioneer Inc., Korea). Having determined SNPs through NCBI data bank, the required primers were designed by Premier Biosoft International, USA and synthesized subsequently (Bioneer Inc.). Real-time polymerase chain reaction (PCR) and high-resolution melt (HRM) techniques were achieved using Rotor Gene 6000 (Corbett Life Science Australia) device. Real-time PCR technique was applied using Type-it HRM™ PCR kit (QIAGEN, R-GERMANY), PCR HRM buffer, nucleotides, Evagreen dye, HotstarTaq plus DNA polymerase enzyme, and DNA (25 ng). The forward primer was

5'-TGTCTTCTCTCTTTACAGTTGG-3' and reverse primer was 5'-GGGATAGACAAAGGGATCG-3'. First of all, enzyme activation step of HotstarTaq plus DNA polymerase (existing in Master Mix) was performed at 95 °C temperatures for five minutes. Real-time PCR reactions were programmed as an initial denaturation-activation step at 95 °C for 15 min, followed by a 40-cycle program (denaturation at 95 °C for 15 s, annealing conditions 55 °C for 10 s, and 72 °C for 15 s). After the completion of PCR, HRM technique was applied using HRM Kit. HRM Kit contains Polymerase Taq compounds, X10, dNTP, Band sharpener, EvaGreen Dye, and H<sub>2</sub>O. The required quantities are stated by the Kit. Then, the temperature was decreased to 65 °C, whereas it was increased by 0.1 °C every 2 s thereafter. An increase in the temperature leads to the separation of the two DNA strands formed at PCR stages and the connecting dye to the two-strand DNA is released and consequently the fluorescence value recorded by the device is gradually decreased. Upon continuing the process up to 95 °C, the melting graph at this temperature is drawn based on the recorded fluorescence changes by the software. Two homozygote and one heterozygote curves are easily detectable; but, identification of homozygote forms is still difficult. Accordingly, the difference graph is drawn by the device's software. Figure 1 shows the difference graph regarding polymorphism -162. The aforementioned diagram sheds a lighter curve on the differences among three graphs so that they could be better differentiated. At this stage, the software draws the fluorescence changes of two graphs regarding the other graph in terms of temperature. There is one normalized graph per each mutation versus three graphs per each normalized one in which one genotype is as regarded the base line and the other genotypes are measured and drawn with respect to it (one graph is presented here, graph 1). Having conducted Real-time PCR HRM, the graph regarding each sample was drawn and the samples were categorized into three groups (two groups of homozygote and one group of heterozygote). From each group, some samples were chosen and sent to Takapoozist Co., Tehran for DNA sequencing where DNA sequencing was done using Applied Biosystems Device 3730/3730 XI. Then distribution of natural homozygote, heterozygote, and mutant alleles of polymorphisms

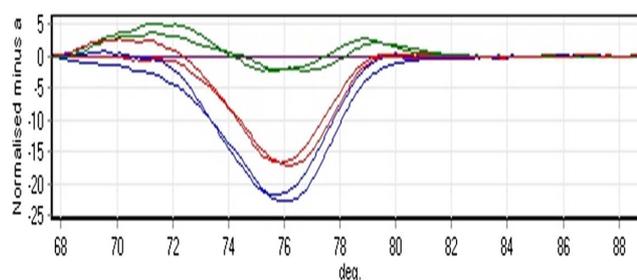


Fig. 1. Difference graph of polymorphism -162 of paraoxonase 1 gene

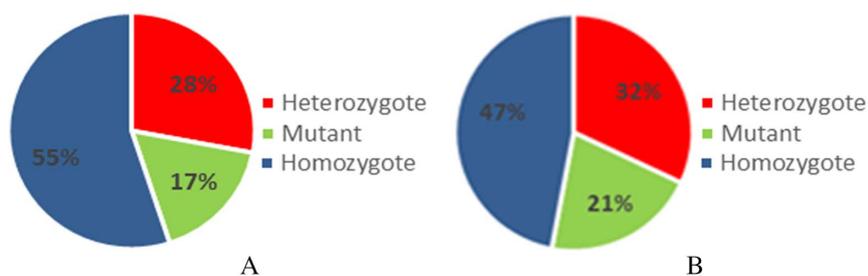


Fig. 2. Chart A represents the abundance of natural alleles (C/C), heterozygote alleles (C/T), and mutant alleles (T/T) of -108 polymorphism of paraoxonase 1 gene with respect to control group, whereas chart B represents them with respect to diabetic patients

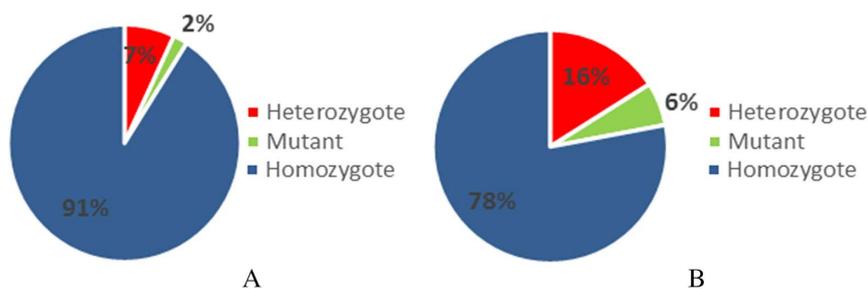


Fig. 3. Pie charts, chart A represents the abundance of natural alleles (C/C), heterozygote alleles (C/G), and mutant alleles (G/G) of -126 polymorphism of paraoxonase 1 gene with respect to control group, whereas chart B represents them with respect to diabetic patients

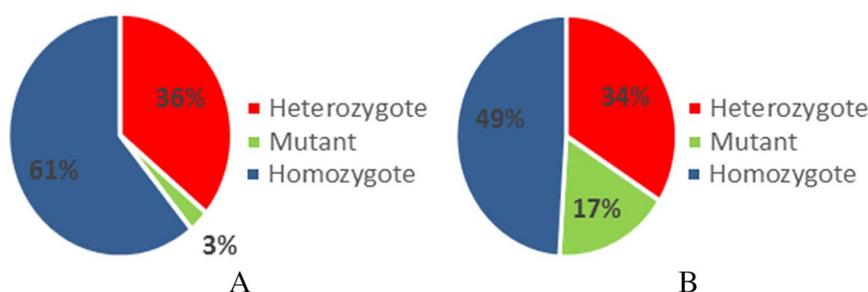


Fig. 4. Chart A represents the abundance of natural alleles (A/A), heterozygote alleles (A/G) and mutant homozygote alleles (G/G) of -162 polymorphism of paraoxonase 1 gene with respect to non-diabetic participants, whereas chart B represents them with respect to diabetic patients

-162, -108, and -126 was assessed in diabetic patients compared with non-diabetic individuals (Figs 2-4).

#### Statistical analysis

The results regarding the enzyme activity were analyzed using SPSS 16 software and *t*-test in terms of means  $\pm$  standard deviation and 95% of reliability. The level of significance was considered 0.05. The results of polymorphisms distribution were statistically analyzed using the  $\chi^2$  test.

#### Results

Overall, 98 male and female patients with DM-2 and 58.13  $\pm$  12.87 average age and 104 male and female individuals

without DM-2 and 59.75  $\pm$  12.29 average age were examined in this study. The enzyme activity of PON1 in DM-2 group was estimated to be 122.7  $\pm$  66.44 nmol/min/ml versus 126.92  $\pm$  76.18 nmol/min/ml in non-DM-2 group. The enzyme activity of PON1 showed no meaningful difference between the two understudied groups ( $P.V = 0.671$ ). SNP -108, C/T heterozygote alleles were more common among participants of both groups compared with C/C and T/T homozygote alleles [47% in DM-2 and 55% in non-DM-2 groups, ( $P.V = 0.277$ ) (Fig. 2)]. Moreover, in SNP -126, 91% of non-DM-2 participants had C/C natural allele versus 78% of DM-2 patients. Compared with 2% of non-DM-2 participants having G/G mutant alleles, 6% of DM-2 patients had mutant alleles ( $P.V = 0.000$ ) (Fig. 3). According to SNP -162, 61% of non-DM-2 participants had A/A natural homozygote alleles compared with 49% of DM-2 patients.

Compared with 3% of non-DM-2 participants having G/G mutant alleles, 17% of DM-2 patients had mutant alleles ( $P.V = 0.017$ ) (Fig. 4).

## Discussion

Different values of PON1 enzyme activity have been reported in various populations. PON1 activity could be up to 40 times different in a given population. These differences are caused by polymorphisms of coding region and PON1 gene promoter as well as environmental factors affecting both concentration and activity of the enzyme [11, 14, 15]. Polymorphisms of C-108T promoter and Q192R on coding region are among the most important factors causing these differences [15]. A 200-bp sequence containing polymorphisms -162 and -108 plays a significant role in transcription of PON1 gene. A (-162) G site has a far less effect (by 1.1%) on enzyme activity [11, 19]. As the site -108 had been the most important factor in determining PON1 serum value and plays a significant role in PON1 serum expression of human being, it has been the subject of numerous studies [11, 19]. Studies have indicated that individuals in a given population who have the same genotype might show totally different PON1 activities too [11]. Rainwater et al. analyzed the effects of genetic and environmental factors on the activity of PON1 enzyme using three different substrates. They concluded the effects of genetics and environmental factors on enzyme activity depending on the type of the substrate. In case paraoxon is used as the substrate, environmental, demographic, and metabolic factors reflecting HDL and LDL trivially affect enzyme activity and compared with other factors, genotype plays a significant role in this regard and might affect enzyme phenotype up to 92% [12]. However, simultaneous analysis of all factors affecting enzyme activity is impossible. PON1 polymorphisms are associated with some disease. Q192R SNP in coding region has been widely studied [11]. According to some studies, PON1 polymorphisms in coding region are associated with cardiovascular diseases, retinopathy, and neuropathy in DM-2 patients [11, 20–22]. However, the likely role of promoter polymorphisms in DM-2 has not been specified yet [11]. Some studies have reported a decrease in PON1 enzyme activity regarding DM-2 even in patients under treatment [6, 21, 23]. In 2010, Poh and Muniandy [17] claimed that PON1 activity generally decreases in diabetic patients compared with the control group, but other studies on PON1 activity in DM-2 have concluded differently [6]. In 2003, Kopprasch [22] stated that no difference could be observed in terms of PON1 activity among individuals suffering from impaired glucose tolerance, healthy people, or those who have been afflicted with diabetes recently. In 2010, Poh and Muniandy [17] concluded that healthy people and diabetic ones without diabetic symptoms

shared the same level of PON1 activity, whereas it was significantly decreased merely in patients with diabetic symptoms. In 2007, Hashim and Zarina [24] reported the same results. On the contrary, some studies indicated that no considerable decrease was observed in terms of PON1 activity in diabetic patients exhibiting cardiovascular consequences or neuropathy [6, 17]. In 2002, Rahmani et al. [25] reported that no meaningful difference in terms of PON1 activity was observed between healthy people and cardiac artery diseases patients suffering from diabetes. In 2006, Hofer concluded that healthy people and diabetic patients exhibiting diabetic symptoms had the same PON1 activity [26]. In this study, PON1 activity in DM-2 patients compared with non-DM-2 individuals was analyzed through applying paraoxon as the substrate, whereas distribution of some promoter SNPs were examined as well. We used a new technique of HRM to obtain an accurate and flawless  $T_m$ . In case the technique is applied, melting behavior is analyzed meticulously and homozygote and heterozygote genotypes are accurately separated as well. The obtained results in this study were similar to Rahmani and Hofer's work in which compared with the control group, patients suffering from DM-2 did not exhibit any decrease in PON1 activity ( $P.V = 0.671$ ). On the contrary, subsequent to the analysis of promoter SNPs, it was revealed that SNP C-108T has the same distribution in both understudied groups ( $P.V = -0.277$ ). The same enzyme activity in both groups is likely due to the aforementioned same polymorphism distribution in two understudied groups. Previous studies have revealed that compared with other polymorphisms, SNP C-108T plays a more significant role in PON1 enzyme activity [11, 19]. This study also confirms its considerable role in PON1 activity in DM-2 patients compared with non-DM-2 individuals. Previous studies have also stated that SNPs C-126G and A-162G could be regarded among polymorphisms affecting PON1 gene expression and A-162G plays a trivial role [11, 19]. The two understudied groups showed statistically meaningful difference in distribution of A-162G and C-126G SNPs ( $P.V = 0.017$  and  $P.V = 0.000$ , respectively). Apparently, these two polymorphisms could increase the risk of DM-2 as diabetes genetic predisposition factors. Given the different distributions of PON1 polymorphisms among various populations [3, 19], further studies must be conducted so that distribution of the aforementioned polymorphisms and their effects on "enzyme activity and risk of affliction with diabetes" could be clearly identified.

## Conclusions

We indicated PON1 activity can be similar in DM-2 patients and non-DM-2 individuals. Some studies demonstrated the most important factor to determine PON1 serum is SNP -108 that highly affects the expression of PON1

serum [11, 15, 19]. The significant role of SNP -108 in PON1 activity in DM-2 patients compared with non DM-2 individuals was confirmed in the study too. On the other hand, the role of -162 and -126 SNPs in causing diabetes cannot be easily overlook because of a meaningful difference of their distribution in understudied groups. However, they may be attributed to DM-2-associated genes. Apparently, the distribution of these SNPs and enzyme activity needs to be analyzed in different populations to clarify their interactions. Moreover, promoter polymorphisms require particular attention given fewer studies than coding region. It seems, more molecular studies are needed to clarify the likely role of polymorphisms and even their regulators on susceptibility to DM-2 and using them to find more efficient ways to control diabetes.

\* \* \*

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## Abbreviations

bp	: base pairs
DM-2	: diabetes mellitus type-2
DNA	: deoxyribonucleic acid
FBS	: fasting blood sugars
HbA1c	: glycated hemoglobinA1c
HDL	: high-density lipoprotein
HRM	: high-resolution melt
LDL	: low-density lipoprotein
PCR	: polymerase chain reaction
PON1	: paraoxonase I
SNP	: single nucleotide polymorphism
Tm	: melting temperature

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