

## Association of Polymorphism of Ser311cys Paraoxonase-2 Gene with Type 2 Diabetes Mellitus in Iran

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### ABSTRACT

**Background:** It is believed that paraoxonase-2 gene polymorphism is associated with type 2 diabetes. This study is aimed to investigate the association between paraoxonase-2 gene polymorphism and type 2 diabetes in an Iranian population.

**Methods:** This study was performed on 200 individuals including 100 diabetics and 100 healthy subjects. Genomic DNA was extracted from peripheral blood leukocytes, and PCR-RFLP was carried out. Palindromic sequence in PON2 gene was recognized by DdeI restriction endonuclease. In order to visualize restriction products, electrophoresis was carried out using polyacrylamide gel (8%) and ethidium bromide staining.

**Results:** The expected PCR product of 331 bp was obtained. Digestion of this product with DdeI showed four Ser homozygotes, three Cys homozygotes, and five Ser311 Cys heterozygotes. The gene frequency of Cys (C) in diabetic subjects was significantly higher than in healthy subjects.

**Conclusions:** This study suggests that an association exists between Ser311 Cys polymorphism and type 2 diabetes mellitus.

**Keywords:** Iranian population, paraoxonase-2 gene, polymorphism, type 2 diabetes

### INTRODUCTION

Type 2 diabetes (T2D) has been a common, serious, and costly public health problem. Millions of people have been diagnosed with T2D, and many more are unaware that they are exposed to T2D. Either the body does not make enough insulin or the cells ignore the insulin in T2D. Despite the impact of environmental components upon T2D, this disease clusters in families. To date, no specific genes causing T2D have been conclusively identified. Nonetheless, it is believed that gene families such as paraoxonase (PON) are associated with the high risk of T2D. PON gene family is located on the long arm of chromosome 7 (7q21-22) close to glucose absorption genes.<sup>[1]</sup> The paraoxonase gene family includes three known members as follows: PON1, PON2, and PON3. These genes encode enzymes, which are

responsible for breaking acetylcholinesterase inhibitors.<sup>[2]</sup> PON1 is synthesized in the liver and released along with HDL secretion in the plasma. It also poses antioxidant properties and prevents LDL oxidation. Inflammatory changes and levels of serum oxidized-LDL affect its concentration. PON2 protects cells against oxidative damage.<sup>[3]</sup> PON3 shares nearly 60% homology in the amino acid sequence with PON1 and its proteins are found on HDL. Effect of PON3 upon lipoprotein oxidation, which can retard aforesaid processes, has been demonstrated.<sup>[4,5]</sup> The effect of PON genes upon various disorders is well-documented in the literature. For instance, an association between the paraoxanase 192 Arg allele and coronary artery disease (CAD) was reported in French and Japanese patients with type 2 diabetes.<sup>[6,7]</sup> Paraoxonase polymorphism (Gln192-Arg) was found to be associated with coronary heart disease in Japanese non-insulin-dependent diabetes mellitus.<sup>[8]</sup> An association between paraoxanase-2 G148 variant with non-insulin-dependent diabetes was seen in aboriginal Canadians elsewhere.<sup>[9]</sup> Sureerut *et al.* stated that the role of PON genes in metabolic syndromes and cancers heightened the need for more investigation.<sup>[10]</sup> A correlation between paraoxanase and diabetes mellitus was found in Pima Indians.<sup>[11]</sup> The PON2 was associated with increased risk of T2D in Chinese population.<sup>[12]</sup> Sanghera *et al.* demonstrated that a variation at codon 311 (substitution of serine [S] for cysteine [C]) in the PON2 gene was associated with CAD in an Asian-Indian population.<sup>[13]</sup> A relationship between low serum PON activity and diabetes mellitus was reported elsewhere.<sup>[14]</sup> In spite of the fact that a large body of literature was published on the involvement of PON genes in T2D development in different populations, little is known about this association in Iranian population. Therefore, this study was designed to investigate the association between T2D and Ser 311 Cys variation of PON2 gene (rs17876171) in an Iranian population.

## METHODS

A total of 200 unrelated people including 100 diabetics and 100 nondiabetic individuals (average age of 51 years) referring to Sedigheh Tahereh Research Center (Isfahan, Iran) were

selected. T2D was clinically diagnosed (polyuria, polydipsia, and polyphagia) and confirmed by criteria of American Diabetes Association (ADA). More precisely, T2D was determined by (1) a fasting plasma glucose level of more than 7.0 mmol/L after a minimum 12 h fast or (2) a 2 h postglucose level [2 h oral glucose tolerance test (OGTT)] of more than 11.1 mmol/L. Impaired glucose tolerance (IGT) was determined by a fasting plasma glucose level of between 5.6 and 7.0 mmol/L or a 2 h OGTT of between 7.8 and 11.1 mmol/L. Normo-glycemic subjects were diagnosed by a fasting glycemia of less than 5.6 mmol/L or a 2 h glucose of less than 7.8 mmol/L. Biochemical and anthropometric parameters of participants are summarized in Table 1. Cholesterol, triglycerides, HDL, and glucose levels in blood were measured by standard enzymatic assays. LDL cholesterol level in blood was also derived by means of Friedewald equation.<sup>[15]</sup> Blood HbA1c level was measured using ion-exchange high performance liquid chromatography (normal reference range of 4.1-6.4%). Owing to having misleading results in analyses of the control group, people with

**Table 1:** Biochemical and anthropometric parameters of the type 2 diabetes group compared to the control group

Parameters	T2D group	Control group	P
Sex (men/women)	33/17	36/14	
Age (year)	51.98±5.5	51.12±8.5	0.551
Body mass index (kg/m <sup>2</sup> )	28.4±1.1	26.4±1.1	<0.001
Systolic blood pressure (mmHg)	140.8±10.3	122.8±9.9	<0.001
Diastolic blood pressure (mmHg)	89.6±10.2	78.7±6.5	<0.001
Fasting glucose (mmol/L)	8.03±0.14	5.1±0.2	<0.001
2 h plasma glucose (mmol/L)	12.6±0.2	6.7±0.2	<0.001
HbA1c (%)	7.5±0.1	5.2±0.1	<0.001
Total cholesterol (mmol/L)	4.96±0.1	4.83±0.1	0.410
HDL cholesterol (mmol/L)	1.3±0.1	1.2±0.1	0.003
LDL cholesterol (mmol/L)	3.4±1.1	3.2±0.1	0.689

HbA1c=Hemoglobin A1c, HDL=High-density lipoprotein, LDL=Low-density lipoprotein. Values are mean±SD, P values from Student's *t*-test, T2D=Type 2 diabetes, CG=Control group

positive family history of T2D and impaired glucose tolerance were excluded. Furthermore, individuals with impaired renal and hepatic function, previous diagnosis of blood disease, and those receiving pharmacological treatment for T2D, hypercholesterolemia, or hypertension were excluded to eliminate interference in biological variables. A written informed consent was obtained from all the subjects participated in the study.

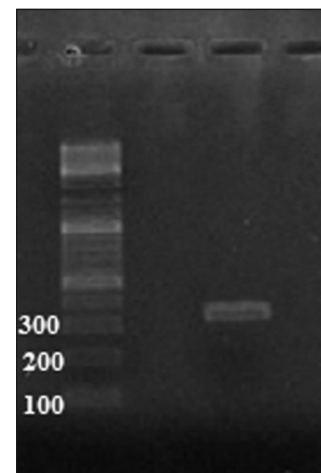
A blood sample (5 cc) was collected from each participant after an overnight fasting for PCR-RFLP. Extraction of genomic DNA was performed using a high pure PCR template preparation kit (Roche, Germany) following the manufacturer's instructions. PCR-RFLP was then carried out. A segment of PON2 gene (331 bp fragment) was PCR-amplified using the following primers: Forward primer: 5-TGGAAAACAGGGCTTATTGATGA-3 and reverse primer: 5-CTGGGTCAATGTTGCTGGT TAAA-3. Primers were selected according to a preceding study<sup>[12]</sup> and then were ordered (BIORON). The PCR mixture contained 100-200 ng DNA template, 0.2 mmol/L dNTPs, 1.5 mmol/L MgCl<sub>2</sub>, 0.3 mmol/L of each primer, and 1.25 IU Taq DNA polymerase with 5% dimethyl sulfoxide in a final volume of 50 µL. For warming up, an initial denaturation at 94°C for 3 min was carried out. Thereafter, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min were performed for 30 cycles. It was then followed by a postextension at 72°C for 10 min. In order to confirm the PCR amplification, gel electrophoresis (8% polyacrylamide gel and ethidium bromide staining) was carried out. The amplified products were then digested by DdeI restriction endonuclease enzyme at 37°C for 1 h. In order to visualize restriction products, electrophoresis was carried out using polyacrylamide gel (8%) and ethidium bromide stain. Four fragments were expected to see as follows: Ser 311 homozygotes (128, 105, 67, and 31 bp) and three fragments for Cys 311 homozygotes (172, 128, and 31 bp).<sup>[12]</sup> Therefore, Ser Cys heterozygotes had to yield five bands of 172, 128, 105, 67, and 31 bp. Inasmuch as the lower bands of 67 and 31 bp could not be separated from the bands of primer dimers, the presence of 172, 128, and 105 bp bands represented the polymorphism.

Data were analyzed using SPSS 17.0. More precisely, Student's *t*-test and  $\chi^2$ -test were applied for analyses of the variables. A *P* < 0.05 was

considered to be statistically significant. Odds ratio with 95% confidence interval was applied for determination of the association of the type of PON2 polymorphism with the risk of T2DM. Using the  $\chi^2$  results, the test for Hardy-Weinberg equilibrium and comparison of genotype and allele frequencies in the diabetic and nondiabetic subjects was carried out.

## RESULTS

Patients' biochemical and anthropometric blood parameters are summarized in Table 1. PCR amplification of PON2 gene produced the desired 331 bp band [Figure 1]. This product was subjected to digestion with DdeI restriction enzyme. As shown in Figure 2, different band patterns were observed due to the polymorphism of PON2 gene. As mentioned above, 67 and 31 bp bands were difficult to be observed owing to the presence of primer dimer bands. However, larger bands were sufficient for determination of the presence of Ser311 Cys polymorphism. Gene frequencies of PON2 gene are illustrated in Table 2. The obtained data showed that the frequency of C allele in diabetic subjects was significantly higher than in healthy subjects. The frequency of S allele in the healthy subjects was significantly high as compared to the C allele. Therefore, a correlation between diabetes and the presence of C allele



**Figure 1:** Confirmation of PCR amplification using gel electrophoresis. Lane 1: 100 bp DNA ladder, lane 2: PCR products from PON2 gene using forward and reverse primers mentioned in the "Methods" section. Extracted DNA from peripheral blood was used as a template

was demonstrated ( $P < 0.001$ ). Additionally, the genotype frequency of Ser/Ser was significantly associated with low frequency of T2D in diabetic subjects and genotype frequency of Cys/Cys and Cys/Ser was associated with high frequency of this disease [Table 3]. No significant difference was observed between the Cys311Cys and Cys311Ser genotypes in the healthy and diabetic groups.

Finally, observed genotype frequencies of the Cys311Ser polymorphism of paraoxonase-2 in diabetic and nondiabetic subjects were in

**Table 2:** Gene frequency of the PON2 gene in nondiabetic and type 2 diabetic subjects

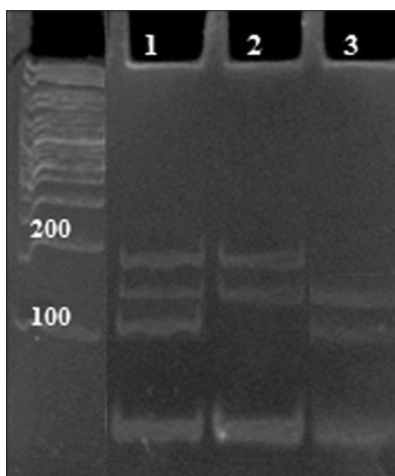
Allele	C allele (%)	S allele (%)
Nondiabetic subjects (total number of alleles=200)	66 (33)	134* (67)
Type 2 diabetic subjects (total number of alleles=200)	120* (60)	80 (40)

\* $P < 0.05$  by  $\chi^2$ -test (comparison between nondiabetic and type 2 diabetic subjects), PON2=Paraoxonase-2

**Table 3:** Genotype frequency of PON2 gene in nondiabetic and type 2 diabetic subjects

Genotype (n=100)	C/C (%)	C/S (%)	S/S (%)
Nondiabetic subjects	18 (18)	30 (30)	52* (52)
Type 2 diabetic subjects	44* (44)	32 (32)	24 (24)

\* $P < 0.05$  by  $\chi^2$ -test (comparison between nondiabetic and type 2 diabetic subjects), PON2=Paraoxonase-2



**Figure 2:** PCR-RFLP detection of the Ser/Cys polymorphism of PON2 gene using gel electrophoresis. Lane 1: Ser/Cys heterozygotes, lane 2: Cys 311 homozygotes, lane 3: Ser 311 homozygote. Extracted DNA from peripheral blood was used as a template

accordance with the Hardy–Weinberg equilibrium (data not shown).

## DISCUSSION

Complex diseases, such as T2D, arise from metabolic disruptions with genetic and environmental components. Multiple genes are believed to be associated with T2D. Paraoxonase has been found to exert antioxidant and protecting effects upon lipid peroxidation as well as its role in controlling oxidative stress<sup>[11,16]</sup> resulting in several human diseases.<sup>[10]</sup> PON2 polymorphisms include Ala148Gly and Cys311Ser which are associated with different pathological disorders.<sup>[17]</sup> It is believed that PON2 polymorphism is associated with the high risk of T2D. Some research studied this relationship in different countries. Thus, this study aimed to assess the association between PON2 polymorphism and T2D. This study showed that an association exists between Ser311Cys polymorphism with T2D; even so, a study investigating this association in a northern Chinese population was in agreement with our results.<sup>[12]</sup> In addition, a correlation between paraoxonase and T2D was demonstrated in Pima Indians elsewhere.<sup>[11]</sup> Hegele *et al.* stated that a relationship between PON2 and glycemic control existed inasmuch as PON1 is adjacent to PON2 gene on chromosome 7 and associated with complications of diabetes and glycemic control in T2D and insulin sensitivity.<sup>[9]</sup> Mackness *et al.* reported a relationship between PON2 variant and microvascular complications in diabetic patients.<sup>[18]</sup> Ikeda *et al.*<sup>[19]</sup> and Robertson *et al.*<sup>[20]</sup> demonstrated that patients with PON1 polymorphism encounter oxidative stress damage, which led to CAD and metabolic syndrome. Jalilian *et al.* showed that a variation in PON2 gene is associated with the risk of CAD in Iranian population.<sup>[21]</sup> Leus *et al.* showed an association between PON2 gene variants and clinical symptoms of cardiovascular disease in familial hypercholesterolemia patients.<sup>[22]</sup>

## CONCLUSION

We suggest that a Ser311Cys variation of PON2 gene is associated with type 2 diabetes. This change in amino acid may occur at an active site of PON2.<sup>[12]</sup> This can impair its antioxidation activity and change the metabolic potency of the cell.<sup>[12]</sup> Finally, it should

be mentioned that the most important limitation of this study is the relatively small sample size. This was due to difficulty of obtaining blood samples. In fact, the sample was regionally representative of center of Iran and thus would tend to miss people who were in marginal regions. Moreover, further investigation and experimentation into Ser311Cys variation of PON2 are strongly recommended to better understand the actual relationship between the risk of diabetes mellitus and PON2 polymorphism in Iranian populations.

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