Association between Serum Paraoxonase 1 Activities (PONase/AREase) and L55M Polymorphism in Risk of Female Infertility

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Abstract

Background: The risk of developing female infertility has been associated with gene polymorphisms that decrease the activity of enzymes involved in systemic Oxidative Stress (OS). In this study, PON1 L55M polymorphism for association with susceptibility to infertility was investigated among Iranian female population.

Methods: Samples from 120 Iranian females (20 endometriosis; 30 Polycystic Ovary Syndrome (PCO); 70 controls) were analyzed and PCR-RFLP assay was used to determine the PON1 rs854560 (L55M) frequencies. The paraoxonase (PONase) and arilesterase (AREase) activities of PON1 enzyme were also assessed in order to investigate the association between serum PON1 activities, female infertility, and PON1 L55M polymorphism.

Results: The women with a MM genotype (p=0.021; OR=2.55) showed more possibilities of experiencing infertility than those with a LM genotype (p=0.039; OR=1.91). According to LSD test, endometriosis subjects had significantly lower paraoxonase enzyme activity compared to control group (p=0.0024; CI=95%). No significant difference was found in women with PCOS for both PONase and AREase activity in comparison with control group (p=0.469; CI=95%). Furthermore, PON1 activities were the highest in LL genotype followed by LM and then MM genotype (MM<LM<LL) in both patients and controls.

Conclusion: PON1 L55M polymorphism may be associated with serum PON1 activity and the risk of developing female infertility.

Keywords: Endometriosis, Infertility, Polycystic ovary syndrome

Introduction

Female infertility is a complex disorder which may be caused by medical conditions including pelvic inflammatory disease, endometriosis, Polycystic Ovary Syndrome (PCOS), premature ovarian failure and uterine fibroids. PCOS is a heterogeneous female endocrine metabolic disorder affecting 5-10% of women characterized by its significantly complex clinical alignments. Endometriosis is a chronic, benign, estrogen-dependent condition characterized by the presence of endometrial tissue outside the uterus cavity associated with pelvic pain and infertility. The pathophysiologic mechanism of endometriosis is unknown and it affects 3-10% of women in reproductive years of their life and 20-50% of women with infertility. In fact, PCOS and endometriosis appear to have a complex and multifactorial etiology in which a variety of genes interact with environmental factors to produce these conditions. Recent biochemical and genetic studies on the pathogenesis of PCOS and endometriosis were focused on the Single Nucleotide Polymorphisms (SNPs) affecting Oxidative Stress (OS). The results of these studies suggest that genetic polymorphisms in antioxidant genes including the SNPs affecting the activities of paraoxonase 1 (PON1) may contribute to female infertility. Serum paraoxonase 1 is an antioxidant calcium-dependent esterase/lactonase, which circulates within High Density Lipoprotein (HDL) particles. PON1 possesses antioxidant, anti-atherogenic, and anti-inflammatory properties and is involved in hydrolysis of several organophosphorus insecticides and nerve agents, inhibition of Low Density Lipoprotein (LDL) oxidation and increase of macrophage-associated cholesterol efflux. The activities of PON1 are genetically regulated and the PON1 gene polymorphisms have potent influences on its activities. PON1 is a member of a multigene cluster including PON1, PON2, and PON3. Among all the SNPs of PON1 gene, -909G/C [rs854572], -162A/G [rs705381], -108C/T [rs705379] in the promoter region and Q192R [rs662], L55M [rs-854560] in the coding region are the most studied poly-morphisms. The L55M polymorphism influences the paraoxonase and arylesterase activities.
(ARE) activities and the stability of the protein \( \text{L55M} \) in
this case-control study, PONase and AREase activities
were examined and the association of coding L55M
polymorphism with female infertility in Iranian popula-
tion was investigated. The L55M polymorphism could
be a risk factor in determining susceptibility to female
infertility.

Materials and Methods

Subjects
This case-control study analyzed a group of 50 in-
fertile females including 20 with endometriosis who
were diagnosed by laparoscopy and 30 with PCOS
fulfilling the criteria for PCOS \(^{16}\), who attended the
Isfahan Fertility-Infertility Center and Royan Institute.
The control group consisted of 70 healthy age-matched
volunteers who visited the clinic for a regular health
check-up with proven fertility and no clinical or bio-
chemical hyperandrogenism, no menstrual cycle ir-
regularities, and no history of endometriosis and
PCOS. Women from different regions of Iran were
referred to this infertility center for check-up and they
made the population of this study. There was no evi-
dence of known paraoxonase-affecting diseases such as
CHD, liver diseases, atherosclerosis, diabetes, hyper-
tension, cancer and infections in all fertile and infertile
females who were not genetically related. Blood sam-
pling was done based on patient consent and an agree-
ment was signed between the University of Isfahan and
Isfahan Fertility-Infertility Center. Blood samples were
obtained in the morning after overnight fasting and
collected in EDTA and heparin coated tubes to analyze
PON1 L55M polymorphism and PON1 activities.
Blood and serum samples were stored at -20°C until
analysis.

Genotyping analyses
For genotype analysis, genomic DNA was extracted
from peripheral blood using a salting-out method de-
scribed by Miller \(^{17}\) and was stored at -20°C till geno-
typing could be performed. Coding L55M (rs 854560)
genotyping was carried out using polymerase chain
reaction-restriction fragment length polymorphism
(PCR-RFLP) method. The primers for SNP were de-
signed using the primer design software Oligo 7 and
were listed as follows:
Forward primer: 5’ TGAATTATCTGAACCTATTA
AGAAGA 3’
Reverse primer: 5’ AAGACTTAAACTGCCAGTCTC
AGA 3’

PCR was performed in 25 \( \mu l \) reaction mixture con-
taining 2 \( \mu l \) of each forward and reverse primers (10
\( \mu M \), 2.5 \( \mu l \) of \( \times 10 \) solution buffer (20 mM Tris-HCl
pH=8.6, 50 mM KCl, Cinnagen Inc, Iran), 0.5 \( \mu l \) of
each four mixed dNTPs (10 mM, Cinnagen Inc, Iran), 0.75
\( \mu l \) of MgCl2 (50 mM, Cinnagen Inc, Iran), 0.3 \( \mu l \) of
5\( \mu l /\mu l \) Taq DNA polymerase (Cinnagen Co., Iran),
and 2 \( \mu l \) (100 ng/\( \mu l \)) of genomic DNA. The PCR
program was as follows: initial denaturation for 4 min at
94°C followed by 35 cycles of 40 s at 94°C, 35 s at 54-
66°C, 72°C for 40 s, and a final extension step of 15
min at 72°C. The PCR products were run on 2% agarose gel and were visualized by ethidium bromide
staining. Moreover, a restriction analysis was per-
duced using 10 units of \( \text{Hin} \) III enzyme (Fermentas,
Vilnius, Lithuania) in buffer G (Fermentas) at 37°C for
16 hr. The restriction fragments were analyzed on 2%
arose gels and were visualized through ethidium bromide
staining. Digested DNA fragments of 146 and
103 \( \mu l \) were detected in MM homozygotes (restriction
site present), an undigested band of 249 \( \mu l \) was detect-
ed in LL homozygotes (restriction site absent) and het-
rozygous genotypes resulted in three different bands
(146, 103 and 249 \( \mu l \)) through PCR-RFLP technique
(Figure 1).

Analyses of PON1 activities
The serum was isolated by centrifugation at 800 \( g \)
for 10 min at 4°C and was immediately frozen at -80°C
in order to determine PON1 activities in blood sam-
ple. The PONase activity was measured using para-
oxon (Sigma Chem, USA) as substrate and the increase
in absorbance at 412 nm was determined, because of 4-
nitrophenol formation. The activity was measured at
25°C during 3 min after 5 \( \mu l \) of serum were added to
each well containing 100 \( \mu l \) of Tris/HCl (100 mM, pH=8.0) buffer including 2 \( \mu M \) CaCl2 and 5.5 \( \mu M \) of
paraoxon. All results are expressed in U/ml which is
defined as 1 \( \mu M \) of 4- nitrophenol formed per minute.
Arilesterase activity in serum was determined spectro-
photometrically using the phenylacetate (Merck-Sch-
brandt) as a synthetic substrate. The reaction started by
adding 100 \( \mu l \) of phenylacetate (10 mM) as substrate
solution to wells containing 5 \( \mu l \) of serum (prediluted
1:10) and 1 \( \mu M \) CaCl2 (Sigma, USA) in 50 mM Tris
buffer pH=8.0. The phenol production was measured
during 2 min at 270 nm and pH=8.0. The PON1’s
arilesterase activity is expressed as KU/ml which is
defined as 1 \( \mu M \) arilesterase per minute. PON1’s
paraoxonase and arilesterase activities were determined
in triplicate \(^{18}\).
Briefly, serum PON1 activity (PON1 phenotype) was determined by double substrate method. Phenotype is defined as the activity ratio of PONase activity divided by AREase activity.

Statistical analyses
Statistical analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) software. The Chi-square distribution ($\chi^2$) was used to evaluate the PON1 genotype frequencies between the patient and the control groups. Multiple comparisons were done by employing one way analysis of variance (ANOVA) followed by a LSD (least significant difference) test. All data are presented as means ± standard deviation (SD). Statistical significance was assessed using the 5% significance level.

Results

Distribution of PON1 L55M genotype
The association between PON1 L55M polymorphism and female infertility in case and control subjects is shown in table 1. The distribution of genotypes were in Hardy-Weinberg equilibrium (p=0.45). The results showed that there were significant differences regarding PON1 L55M polymorphism among fertile and infertile groups. The women with a MM genotype (p=0.021; OR=2.55) were statistically associated with infertility in comparison to those with a LM genotype (p=0.039; OR=1.91). There was no association between L55M polymorphism and each infertile group including patients with endometriosis and PCOS separately.

PONase, AREase activities and infertility
The group mean age, body mass index (BMI), and the serum PON1’s paraoxonase and arilesterase activities are presented in table 2 for fertile, PCOs, and endometriosis groups. Paraoxonase enzyme activity was highly variable among different samples. According to LSD test, endometriosis subjects had significantly lower paraoxonase enzyme activity compared to control groups (p=0.0024; CI=95%) (Table 3). No significant difference was found in women with PCOS for both PONase and AREase activity in comparison with control group (p=0.469; CI=95%).
Association between PON1 L55M polymorphism and serum PON1 activities

The association of PON1 L55M polymorphism with PON1 activities is shown in figure 2. Significant difference was observed among the genotypes of L55M polymorphism in patient and control groups. Serum PON1 activity was the highest in LL genotype followed by LM and then MM genotype (MM<LM<LL) in both patients and controls. It was found that in three genotypes, patients had significantly lower activity in comparison with controls.

Discussion

Serum PON1 is a esterase/lactonase which has significant roles in protection of LDL and HDL against oxidation. Recently, PON1 genotyping and analysis of serum PON1 activity has been comprehensively studied to investigate associations with variety of diseases including cardiovascular disease, diabetes, infertility, Alzheimer, cancer, and Parkinson. Genetic variants in PON1 gene may modulate OS and thus affect susceptibility to female infertility. OS which was defined as imbalance between pro-oxidants and antioxidants has critical roles in normal functioning of the female reproductive system and development of female reproductive disorders are generally unknown. In this case-control study, PON1 enzyme activities (PONase and AREase), and PON1 L55M coding polymorphism were investigated in Iranian infertile women population for the first time. The global minor allele frequency of PON1 L55M polymorphism (rs854560) is T=0.1827/915 according to the dSNP (www.ncbi.nlm.nih.gov/snp). This means that for rs854560, minor allele is 'T' and has a frequency of 18.27% in the 1000 genome phase 1 population. It was found that serum PON1 activity was significantly lower in endometriosis patients in comparison with controls. This result is similar to previous studies which have been performed in different populations. In another study, Verit et al reported a significant difference in two groups of endometriosis patients including women with moderate to severe endometriosis and women with minimal to mild endometriosis. PON-1 activity was significantly lower in women with moderate to severe endometriosis than in women with minimal to mild endometriosis and controls (p<0.0001). The association between PON1 activity and PCOS was suggested by Dursun et al for the first time. They reported a significant difference (p=0.027) in PON1 activity between PCOS patients and control groups. In the present study, there was no significant difference in serum PON1 activity of PCOS patients in comparison with the control group. The association of PON1 L55M polymorphism and PON1 activity was also evaluated in our study which indicated that PON1 activity was affected by L55M polymorphism. PONase activity was the highest in LL genotype and lowest in MM genotype in both patients and control groups (p=0.002; CI=95%) and in all the three genotypes (LL, LM, MM); patients had lower PONase activity in comparison with controls. PON's activity varies widely between individuals and is related to several other environmental factors. Our further analyses indicate that AREase activity was independent of L55M polymorphism which is similar to the observation by Chen et al. Briefly, serum PON1 activity or PON1 phenotype was the highest in LL genotype followed by LM and then MM genotype (MM<LM<LL) in both patients and controls. In addition, it was found that MM genotype was more common in infertile females as compared to the controls (OR=2.55; p=0.021; X² significance=0.029) and LM genotype was associated with low risk of infertility (OR=1.91; p=0.039) (Table 4). Furthermore, it was observed that the frequencies of PON1 L55M polymorphism in this Iranian population were similar to those for European Caucasians but different from those seen in other Asian populations. The findings of a case-control study which was performed by Sepehrvand et al confirmed this similarity of PON1 L55M polymorphism frequency in other Iranian population.

Conclusion

In conclusion, results of this study support the hypothesis that PON1 L55M polymorphism and female infertility is associated with low PON1 activity that may modulate OS and thus affect susceptibility to female infertility.

Table 4. Distribution of PON1 L55M genotypes in fertile and infertile groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fertile, N (f)</th>
<th>Infertile, N (f b)</th>
<th>OR c (CI d 95%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL</td>
<td>20 (28.6)</td>
<td>20 (40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LM</td>
<td>40 (57.2)</td>
<td>15 (30)</td>
<td>1.91 (CI=95%)</td>
<td>0.039</td>
</tr>
<tr>
<td>MM</td>
<td>10 (14.2)</td>
<td>15 (30)</td>
<td>2.55 (CI=95%)</td>
<td>0.021</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a: Number; b: Frequency; c: Odd ratio; d: 95% confidence interval.

Figure 2. L55M genotypes (LL, LM and MM) and serum PON1 activity in both controls and patients. Serum PON1 activity was the highest in LL genotype followed by LM and then MM genotype (MM<LM<LL) in both patients and controls.
pothesis that PON1 L55M polymorphism may have significant roles in serum PON1 activity and risk of developing female infertility. Our findings suggest further analyses are required to investigate other PON1 polymorphisms and their importance on the individual’s susceptibility to infertility in larger groups.

Acknowledgement

This study was completed at University of Isfahan and financially supported by Graduate Office Department of University of Isfahan; we would like to sincerely appreciate all the physicians and nurses at Isfahan Fertility-Infertility Center and Royan Institute.

Conflict of Interest

All authors have seen and agreed with the contents of the manuscript and there is no conflict of interest to report.

References

PON1 Activates, L55M Polymorphism and Female Infertility


