Association between Serum Paraoxonase 1 Activities (PONase/AREase) and *L*55*M* 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 L*55*M* 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* (*L*55*M*) frequencies. The paraoxonase (PONase) and arylesterase (AREase) activities of PON1 enzyme were also assessed in order to investigate the association between serum PON1 activities, female infertility, and *PON1 L*55*M* 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 L*55*M* polymorphism may be associated with serum PON1 activity and the risk of developing female infertility.

**Keywords:** Endometriosis, Infertility, Polycystic ovary syndrome
(ARE) activities and the stability of the protein 16. In this case-control study, PONase and AREase activities were examined and the association of coding L55M polymorphism with female infertility in Iranian population 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 infertile 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 biochemical hyperandrogenism, no menstrual cycle irregularities, 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 evidence of known paraoxonase-affecting diseases such as CHD, liver diseases, atherosclerosis, diabetes, hypertension, cancer and infections in all fertile and infertile females who were not genetically related. Blood sampling was done based on patient consent and an agreement 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 described by Miller 17 and was stored at -20°C till genotyping 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 designed using the primer design software Oligo 7 and were listed as follows: Forward primer: 5’ TGAATTATCTGAACCTATTA AAGAAGA 3’ Reverse primer: 5’ AAGACTAAACTGCAGTCCT AGA 3’

PCR was performed in 25 µl reaction mixture containing 2 µl of each forward and reverse primers (10 pM), 2.5 µl of 10× solution buffer (20 mM Tris-HCl pH=8.6, 50 mM KCl, Cinnagen Inc, Iran), 0.5 µl of four mixed dNTPs (10 mM, Cinnagen Inc, Iran), 0.75 µl of MgCl2 (50 mM, Cinnagen Inc, Iran), 0.3 µl of 5U/µl Taq DNA polymerase (Cinnagen Co., Iran), and 2 µl (100 ng/µl) 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 through ethidium bromide staining. Moreover, a restriction analysis was performed using 10 units of Hin1II enzyme (Fermentas, Vilnius, Lithuania) in buffer G (Fermentas) at 37°C for 16 hr. The restriction fragments were analyzed on 2% agarose gels and were visualized through ethidium bromide staining. Digested DNA fragments of 146 and 103 bp were detected in MM homozygotes (restriction site present), an undigested band of 249 bp was detected in LL homozygotes (restriction site absent) and heterozygous genotypes resulted in three different bands (146, 103 and 249 bp) 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 samples. The PONase activity was measured using paraoxon (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 µl of serum were added to each well containing 100 µl of Tris/HCl (100 mMol, pH=8.0) buffer including 2 mMol CaCl2 and 5.5 mMol of paraoxon. All results are expressed in U/ml which is defined as 1 nmol of 4-nitrophenol formed per minute. Arilesterase activity in serum was determined spectrophotometrically using the phenylacetate (Merck-Schardt) as a synthetic substrate. The reaction started by adding 100 µl of phenylacetate (10 mM) as substrate solution to wells containing 5 µl of serum (prediluted 1:10) and 1 mMol CaCl2 (Sigma, USA) in 50 mM Tris buffer pH=8. 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 µmol 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=0.024; 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%).

Table 1. Association between PON1 L55M polymorphism and female infertility

<table>
<thead>
<tr>
<th>1st Polymorphism</th>
<th>2nd Polymorphism</th>
<th>1st and 2nd mean difference</th>
<th>Standard deviation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM</td>
<td>LM</td>
<td>0.23214 *</td>
<td>0.09928</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>-0.02381</td>
<td>0.12924</td>
<td>0.854</td>
</tr>
<tr>
<td>LM</td>
<td>MM</td>
<td>-0.23214 *</td>
<td>0.09928</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td>LL</td>
<td>-0.25595 *</td>
<td>0.12272</td>
<td>0.039</td>
</tr>
<tr>
<td>LL</td>
<td>MM</td>
<td>-0.02381</td>
<td>0.09928</td>
<td>0.854</td>
</tr>
<tr>
<td></td>
<td>LM</td>
<td>0.25595 *</td>
<td>0.12924</td>
<td>0.039</td>
</tr>
</tbody>
</table>

* Significance difference with 95% confidence interval.

Table 2. Characteristics of cases and control samples (mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>PCOS</th>
<th>Endometriosis</th>
<th>Fertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.92±4.38</td>
<td>31.54±6.212</td>
<td>32.09±6.56</td>
</tr>
<tr>
<td>Body Mass Index (BMI)</td>
<td>28.03±5.41</td>
<td>24.89±4.02</td>
<td>24.66±3.86</td>
</tr>
<tr>
<td>Paraoxonase activity (U/l)</td>
<td>1682.88±2255.47</td>
<td>1259.90±1680.95</td>
<td>2529.02±3144.78</td>
</tr>
<tr>
<td>Arilesterase activity (KU/l)</td>
<td>227.67±131.11</td>
<td>212.88±119.79</td>
<td>255.58±145.53</td>
</tr>
<tr>
<td>Serum PON1 activity</td>
<td>7.39±6.89</td>
<td>5.91±6.78</td>
<td>9.89±5.71</td>
</tr>
</tbody>
</table>

Table 3. Association between infertility and paraoxonase enzyme activity

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Group 2</th>
<th>Mean difference between two groups</th>
<th>Standard deviation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometriosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertile</td>
<td>-3.86913 *</td>
<td>1.68803</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>PCO</td>
<td>-1.14816</td>
<td>1.58038</td>
<td>0.469</td>
<td></td>
</tr>
<tr>
<td>Fertile</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometriosis</td>
<td>4.86913 *</td>
<td>1.68803</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>PCO</td>
<td>2.72097</td>
<td>2.01152</td>
<td>0.179</td>
<td></td>
</tr>
<tr>
<td>PCO</td>
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<tr>
<td>Endometriosis</td>
<td>1.14816</td>
<td>1.58038</td>
<td>0.469</td>
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<tr>
<td>Fertile</td>
<td>-2.72097</td>
<td>2.01157</td>
<td>0.179</td>
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</table>

* Significance difference with 95% confidence interval.
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 an esterase/lactonase which has significant roles in protection of LDL and HDL against oxidation 19-23. 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 20-24. 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 27,28. 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) 29. 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 30. 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 31. 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; χ² 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 32. 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 33,34.

<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 (1.91 95%)</td>
<td>0.039</td>
</tr>
<tr>
<td>MM</td>
<td>10 (14.28)</td>
<td>15 (30)</td>
<td>2.55 (2.55 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.
hypothesis 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

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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

25. Ginsberg G, Neafsey P, Hattis D, Guyton KZ, Johns DO, Sonawane B. Genetic polymorphism in paraoxonase 1


