

Link between PON 1 gene mutation and recurrent miscarriage among women exposed to pesticides in North India is insignificant

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Abstract:

Recurrent miscarriage is a loss of disconcerting disorder characterized by RPL (recurrent pregnancy loss) of pregnancy, affecting around 1-2% of couples trying to conceive. Exposure to pesticide affects spontaneous abortion, and infertility in women. Placental oxidative stress is often linked to miscarriage. Therefore, it is of interest to link PON1 (Q1922R) polymorphism with recurrent pregnancy loss. We selected 200 subjects in which 100 patients with RPL having consecutive 2 or more miscarriages and 100 healthy controls from the northern India for this study. Blood samples were collected for DNA isolation and further assessment. Genotyping of the Q1922R polymorphism was completed using the RFLP markers. The digested PCR product size was 99 bp (control). The heterozygous fragments were found to be 66 and 33 bp homozygous mutants. It was observed that allele frequency homozygous (TT) was more prevalent among control than the case groups among the healthy north-Indian population. However, heterozygous group (Tt) was more in cases compared to control groups as well as homozygous mutant was observed high in control in than case (CI-0.3 to 1.3).

Keywords: Recurrent pregnancy loss, Polymorphism, Pesticides, PON1, RFLP.

Background:

Recurrent miscarriage or habitual abortion is loss of disconcerting disorder characterized by recurrent spontaneous loss of pregnancy, affecting around 1% to 2% of couples trying to conceive [1, 2, 3]. It is defined as the loss of pregnancy before the fetus reaches the viability, which is from the time of conception until 24 weeks of gestation period [4]. It can be caused by multiple factors such as hormonal imbalances, nutritional deficiencies, physiological trauma, menstrual disorders, hematologic disorders, chromosomal aberration, endocrinological disorders, uterine fibroid, uterine pathology, Parental chromosomal anomalies, Heritable thrombophilia, and uterine abnormalities [5, 6, 7, 8, 9] in which exposure of pesticides is one of the most common causes of recurrent miscarriages. Heavy metals such as organochlorine pesticides (OCPs) and organophosphate pesticides (OPPs) are confirmed environmental pesticides and their exposure could contribute to pregnancy loss [10]. The induced oxidative stress level as a consequence due to the exposure of selected pesticides play a key role in the recurrent miscarriage loss [11-12]. A significantly higher level of organochlorines ($p < 0.04$) and organophosphate ($p < 0.02$) pesticides in the patients of recurrent abortions is observed. The gene polymorphism studies have crucial role in disease identification. Paraoxonase (PON1) gene encoded a high-density lipoprotein (HDL)-linked enzyme that inhibits oxidation of low-density lipoproteins (LDL). It also involved in detoxification from organophosphate and organochlorine pesticides [13]. The important common genetic variation PON1-Q192R was identified by the molecular studies in the coding region of the PON1 gene at the position 192 [14, 15]. Various studies has been identified in association between the polymorphism and risk of different diseases such as in previous reports it was suggested that there is a high variability in PON1 enzymes related to various problems including recurrent pregnancy loss [16, 13, 17], female infertility [18], Coronary heart disease [19, 20, 21, 22, 23], Cancer [24, 25], Interleukin [26], hypercholesterolemia [27], Osteonecrosis [28], Metabolic Syndrome [29], atherosclerosis [30]. Few studies have been the interaction between assessed on (PON1) gene Q192R polymorphism and pesticide exposure on early pregnancy loss [16, 13]. Therefore, it is of interest to link PON1 (Q192R) polymorphism with recurrent pregnancy loss.

Materials and Method:

Patient Selection/Genotyping:

A case-control study was performed with the comparison of polymorphism of *Q192R* genotypes of randomly selected 100 (RPL) case and 100 control subjects (Healthy women) age between 25-40 Years. Controls were matched to cases with regard to ethnicity, gender, age, and a low-risk working environment. In the present study, healthy women with one or more healthy kids were enrolled

as control, whereas women with two or more consecutive pregnancy loss were subjected as cases of RPL [12]. To investigate the recurrent miscarriage problems among the group of females in northern region in India were divided into 5 groups (Table 1). Data collection was done for each patient on clinical variables including age, rural area, urban area, some of the samples were collected from the women who belongs to high exposure of pesticides regions, some of the patients belongs to central area of the cities where the contact of pesticides was more. The patient's history hormonal disorders, thyroid abnormalities, uterine abnormalities, hypertension, bacterial infection, uterus fibroid, tuberculosis infection and smokers were excluded from all the groups. Before enrolment in the study each subject written informed consent was obtained in response to a fully written and verbal explanation of the nature of study. The study was approved by the Ethical committee's from the respective departments, earlier to the recruitment of subjects in this study.

Blood sample collection:

To extract the genomic DNA, blood samples (5 mL) were collected from both cases and control from the patients admitted in the department of obstetrics and gynecology, KGMU, Lucknow (India). The samples were collected in tubes containing anticoagulant Ethylene-diamine tetra acetic acid (EDTA) (1 mg/mL). The plasma was immediately separated from the blood tissues by centrifuge at 4000 rpm for 15 min at 4 °C. The separated plasma was stored at -80 °C for further analysis [16].

Total genomic DNA isolation:

Peripheral blood was collected from all the subjects in 0.5M EDTA tubes. The genomic DNA was isolated by conventional phenol chloroform extraction method followed by ethanol precipitation and re-suspended in Tris-EDTA buffer [31, 32, 33].

Quantification Qualitative Analysis of isolated DNA:

The amount of total genomic DNA isolated from the samples was estimated with the help of UV Spectrophotometer 2000 at A260/280 and A260/230 ratio for the purity of the DNA. The quality of the concentrated DNA samples was checked on the 0.8% agarose gel with the 100 bp ladder (Fermentas Pvt. Ltd).

PON 1 (Q192R) primer and Restriction Enzyme:

The screening of PON1 (*Q192R*) mutations/polymorphisms in selected genotypes was determined by polymerase chain reaction and restriction fragment length polymorphism (PCR- RFLP) analysis. The *Q192R* polymorphism was analyzed by the PCR followed by restriction fragment length polymorphism (RFLP). The

primers used for amplification of the *Q192R* gene polymorphisms were listed in (Table 2) [34, 30, 24].

Standardization of PCR conditions:

Genomic DNA was amplified (Applied Biosystems, Veriti, Singapore) using the following PCR conditions: 94 °C for 5 min, 35 cycles at 94 °C for 45 s, 56 °C for 50 s, 72 °C for 45 s, and finally 72 °C for 7 min (Simsek et al. 2001) (35). Amplification was performed with 25 µL PCR reaction mixture containing 100 ng template DNA [27], 10 pmol of reverse and forward primers with 2X of PCR master mix containing 10X PCR buffer, nuclease free water, dNTPs, MgCl₂ and 0.5U/µL taq polymerase, (Fermentas, Germany). Amplification success of samples was monitored on EtBr containing 2.0% agarose gel by Gel electrophoresis.

Restriction digestion:

The PCR products were further digested using BspP1 enzyme (NEB, UK) to screen for *Q192R* polymorphism. The enzymatic mixture contained 1µL restriction enzyme, 1 µL 10X buffer, 1 µL PCR products and 2 µL distilled water; the mixture was incubated overnight at 56 °C for digestion.

Separation and detection of amplified PCR products:

The digested product was electrophoresed on 3.5% high-resolution agarose gel [36] stained in Ethidium Bromide solution in 1X TAE buffer at 90 V current for 1 h. To visualize the bands, gel was observed in UV light produced by Trans-illuminator in Syngene G: Box) gel documentation system. The size of amplification products was estimated using the standard 100 bp ladder (Fermentas Thermofisher Pvt. Ltd.). These digested PCR product size were 99 bp wild types, 99, 66, 33 bp heterozygous type and 66 and 33 bp homozygous mutant type.

Statistical analysis:

Statistical analysis data was conducted by using the statistical tools SPSS (version 2015) SPSS: (2015) to analyze the obtained data. *Q192R* genotype and allele frequencies in the patients of recurrent pregnancy loss were compared to the respective frequencies of the control groups using the chi-square (χ²) tests and Odds ratios (ORs) were calculated to estimate relative risk conferred by a particular allele and genotype. ORs were given with 95% confidence interval (CI) were calculated by multiple logistic regression for genotype frequencies. Differences with P value was considered significant at <0.05.

Table 1: Classification of selected patients of RPL based on the exposure of pesticides

| Sl. No. | Number of patients |
|-----------|---|
| Group I | N=30 cases with diagnosis of three consecutive abortions |
| Group II | N=20 cases with the history of three or more consecutive events |
| Group III | N=20 cases with the history of high exposure of pesticides |
| Group IV | N= 20 cases with the history of low income women working in agriculture fields or |
| Group V | N= 10 labours in pesticides production industries |

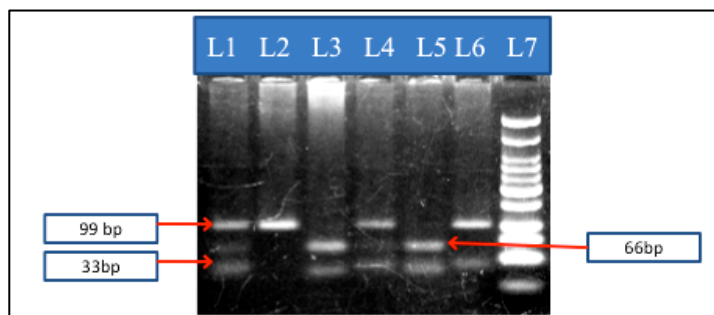


Figure 1: Gel documentaion for genotyping analysisfor *Q192R* polymorphism different groups. L1 (Control) L2: Group I; L3: Group II; L4: Group III; L5: Group IV; L6: Group V; L7: (Ladder 100 bp Fermentas)

Results:

Genotyping PON1 (*Q192R*):

Paraoxonase1 is an A-esterase capable of hydrolyzing the active metabolites of a number of pesticides. It is an important xenobiotic metabolizing enzyme and polymorphism of this gene exhibit ethnic and racial variation. It has a critical role in the metabolism and detoxification of pesticides. Majorities of studies were primarily focused on either miscarriages or polymorphism while the literatures on exposure of pesticide on recurrent miscarriages risk were scarce. Hence, this study evaluates the interaction between exposure to pesticides and PON1 gene polymorphism in recurrent miscarriages in northern Indian females. In the present investigation, it was recorded that allele frequency of wild homozygous (TT) (Q) was more prevalent among control group in case (65%) and control (72%) consists of healthy north-Indian population. However, heterozygous group (Tt) (R) (35%) was more in cases compare to control groups (28%) (CI-0.3 to 1.3) as well as homozygous mutant (S) was observed high in control (12%) in

than case (7%) at marginal significance ($p=0.06$). Frequencies of PON1 gene polymorphisms among recurrent miscarriage and their respective controls subjects are presented in (Table 3). Wild homozygous TT was more in prevalent among control group. The homozygous allele i.e., Q allele was confirmed in control case (L2) when bands corresponding to 99 bp genotypes. The heterozygous

alleles were observed in Group I, Group II and Group III with 66 +33 bp bands in L1, L3 and L4. L5 that represented group IV showed heterozygosity (R allele). The homozygous alleles (Q) was also observed in was observed in-group number V i.e., L7 (Figure 1).

Table 2: RFLP primer and enzyme

| Polymorphisms | Primers | Annealing Temperature | PCR fragment length (bp) | RFLP Fragment (bp) | Restriction Enzyme |
|---------------|---|-----------------------|--------------------------|--|--------------------|
| Q192R | F:5'TATGTGCTGTGGGACCTGAG3' R:5'CACGCTAAACCCAAATACATCTC3' | 56 °C | 99 bp | Wild Homozygous (Q) allele: 99 bp; Heterozygous (R) allele: +99 +66+ 33 bp; Homozygous mutant (S): 66 +33 bp | BspPI |

Discussion:

There are many studies have investigated the role of the respective gene products in human physiology and pathology. However, emerging evidence from biochemical and genetic experiments is providing clues about the role of the products of these genes, which indicates that PON1 acts as important guardian against cellular damage from toxic agents, such as organophosphates and oxidized lipids in the plasma low-density lipoproteins [6]. Animals with low PON1 activity were more sensitive to the toxic effects of organophosphate pesticides [37]. From the above results authors were able to suggest that PON1 gene polymorphisms may not be associated with risk of miscarriage and it was more prone to mixed results of possible association of PON1 gene polymorphism and recurrent miscarriage. Similar to our result there was no significant differences was found for PON1 activity between normal and obese in Portuguese women [38] and similar result was observed in patients with Idiopathic Recurrent Early Pregnancy Loss [17]. In another study on environmental workers there was poor association was observed between poison and PON1 polymorphism [39]. Some genetic studies in Turkish population have confirmed that there were no significant association was found between pulmonary embolism and PON1 gene Q192R polymorphism [40]. Similarly allele frequency was observed more homozygous (77.6%) in the patients of pulmonary embolism and genetic polymorphism in paraoxonase1 [40]. In another study, Allele frequency was high in homozygous [18]. In contrast to our study, R allele frequency was found to be high (61.4%) in women exposed to pesticides during pregnancy in Mexico [16]. In contrast to our study, PON1 L55M polymorphism associated with serum PON1 activity and the risk of developing female infertility [18]. Some other genetic studies have confirmed that the Q192 polymorphism may be risk factor for other diseases 192RR (homozygous) genotypes were found more susceptible to develop reproductive toxic effect by pesticide exposure such as in

agricultural workers from southern Mexico [41]. Q192R polymorphism was associated with Familial Hypercholesterolemia and adverse cardiovascular risks [27, 42, 43, 44, 20, 45]. Prenatally exposed children to pesticides showed adverse cardio-metabolic health profile were found to be associated with PON1 192R-allele polymorphism [46]. PON1 (Q192R) polymorphism and gastric cancer significantly increased in the R allele in the patients group compared with the control ($P= 0.0006$) while the Q Allele was more frequent in the control group [47]. In this study we investigated the genetic polymorphism and allele frequency of PON1 gene in northern Indian females with high risk of miscarriage due to high exposure of pesticides. We found that there was no significant association among genotypes and Q192R analysis.

Table 3: Genotypic analysis for paraoxonase 1 case and control groups

| Genotypes | Case (n=100) | | Control (n=100) | | OR (CI 95%) | p value |
|------------------|--------------|----------|-----------------|----------|-------------|---------|
| | Observed | Expected | Observed | Expected | | |
| TT | 41 | 42.25 | 54 | 51.6 | (0.45-1.68) | 0.05 |
| Tt | 48 | 45.5 | 36 | 39.7 | (0.3-1.3) | 0.06 |
| tt | 11 | 12.25 | 10 | 7.6 | 0.26-1.78) | 0.44 |
| Tt+tt | 59 | - | 46 | - | - | - |
| Allele frequency | | | | | | |
| Q | 130 (65%) | | 144 (72%) | | | |
| R | 70 (35%) | | 56 (28%) | | | |
| S | 11 (12%) | | 32 (7%) | | | |

Conclusion:

We show that there is no significant association observed in genetic maternal gene PON1 and the risk of recurrent miscarriage due to pesticide exposure in the population of northern part of India. Further studies are needed with large sample size with different states, as the present study was limited relatively small number of genotypes of one state in India.

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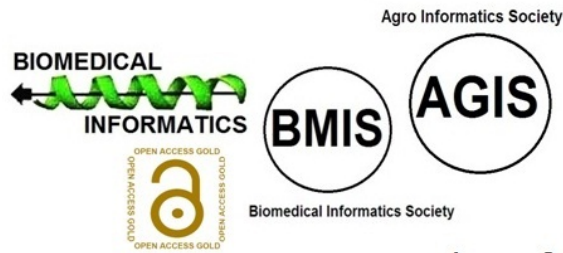
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