

Investigation on estrogen receptor alpha gene polymorphisms in Iranian women with recurrent pregnancy loss

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Abstract

Background: Recurrent pregnancy loss (RPL) is a multifactorial disorder. Environmental factors and genetics can affect pregnancy outcomes.

Objective: Conflicting data suggest an association between estrogen receptor alpha (ESR1) gene polymorphisms and RPL. In this study, such association was investigated in Iranian women with RPL.

Materials and Methods: In this case control study, blood samples were collected from 244 women with a history of three or more consecutive pregnancy losses and 104 healthy women with at least two live births. Using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), we studied -397C/T and -351A/G polymorphisms on ESR1 gene in case and control subjects.

Results: The genotypic frequencies of -397C/T and -351A/G polymorphisms on ESR1 were not significantly different between RPL and control groups ($p=0.20$ and $p=0.09$, respectively). A significantly negative correlation was observed between -397C/T and -351A/G ($r=-0.852$, $p<0.001$) in RPL women and complete linkage disequilibrium between the investigated polymorphisms was found ($D': 0.959$; $r\text{-square}= 0.758$, $p<0.001$).

Conclusion: This investigation suggests that the analyzed polymorphisms on ESR1 gene are not associated with an increased risk of RPL in the studied population.

Key words: Estrogen receptor, Polymorphism, Abortion, Recurrent.

This article extracted from M.Sc. thesis. (Marzieh Mahdavi)

Introduction

Recurrent Pregnancy Loss (RPL) is defined as repeated occurrence of 3 or more miscarriages before 20th week of gestation and its occurrence affects about 1-5% of couples (1, 2). Diverse factors have been claimed to cause of RPL, including; uterine anomalies, chromosomal abnormalities, endocrine and immune defects as well as thrombophilia and infection. In most cases, however, a single cause for repeated abortions cannot be identified (3). However, the development and function of female reproductive system are controlled by hypothalamus-pituitary-ovarian (HPO) axis. In addition, fertility and pregnancy may be negatively affected by the levels of hormones

like follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2) and progesterone that are also controlled by the HPO axis (4, 5).

Estrogens modulate multiple reproductive functions, including progesterone production and uteroplacental blood flow (6). Factors like female secondary sexual characteristics, reproductive cycle, fertility, and maintenance of pregnancy may play a crucial role in embryonic and fetal development under the effect of estrogen (7, 8). Estrogen receptors (ERs), which in human consisted of ER α and ER β , coded by ESR1 and ESR2 genes, respectively, are the mediator of estrogen signaling and function as ligand-dependent transcription factors (9, 10). ER subtypes are members of nuclear hormone receptor

superfamily. The ESR1 gene (140 kb) which is located on chromosome 6q25.1 consists of 8 exons; likewise ESR2 gene is located on chromosome 14q22 and contains 8 exons.

Both ESR1 and ESR2 genes are expressed in the human ovary, within the oocyte, in granulosa cells, in ovarian epithelial cells and in endometrial cells of uterine (11-14). In recent years, increasing efforts have been made to describe genetic causes of recurrent miscarriage at the molecular level. Several polymorphic variants of the estrogen receptor genes have been described. More than 2200 single nucleotide polymorphisms (SNPs) have been identified in the ESR1 gene, while this assessment for ESR2 gene is 720 (www.snpper.chip.org). ESR1 and ESR2 gene polymorphisms have been linked to a higher risk of unexplained infertility and to the outcome of controlled ovarian stimulation, as well as to the ovarian response to FSH (15, 16). In the present study, we analyzed two SNPs (-397 C/T and -351 A/G) on the ESR1 gene in Iranian women with RPL.

Both these polymorphisms lie on intron 1 and are recognized by *PvuII* (-397C/T) and *XbaI* (-351A/G) restriction enzymes and have been reported to be associated with RPL in Spanish and Taiwanese Han population (17, 18). We therefore conducted a case-control study to examine the role of ESR1 SNPs and circulating concentrations of estradiol, FSH and LH with RPL risk. To our knowledge, there are no studies on the association of these polymorphisms and RPL in Iranian women. Also, in this study we compared Iranian haplotype with previous studies on different ethnic groups, to understand the analogy of different population.

Materials and methods

Patients

In this case control study, the case group consisted of 244 women with at least three consecutive miscarriages before 20th week of gestation who referred to Avicenna Infertility Clinic, Tehran, Iran, between the years of 2008-2010, for treatment of RPL. All patients had undergone standard diagnostic procedures, including a detailed history, chromosomal analyses of peripheral blood lymphocytes, ultrasonography and

hysterosalpinogography to detect uterine anomalies, hormone profiles on day 2-3 of the menstrual cycles; FSH, LH, E₂ as well as thyroid hormone studies, investigation of infections and immunological diagnostics (including anti-phospholipid and anti-cardiolipin antibodies). Women with uterine anomalies, karyotype abnormalities, hormonal, infection and immunological disorder were excluded from the study.

The control group consisted of 104 healthy ethnically matched women with two or more successful pregnancies and live births and no history of complicated pregnancies, miscarriages, still births, small for gestational age fetuses, preeclampsia, ectopic pregnancy or preterm delivery. The study was approved by the Avicenna Research Institute local ethics committee for medical research, and written consents were obtained from participating subjects before entry into the study.

Biochemical assays

LH and FSH were measured by chemiluminescent immune assays (CLIA) (Diasorin, Italy). E₂ was measured by enzyme immunoassay (EIA) (Axis Shield, Dundee, UK).

Isolation of genomic DNA

Peripheral blood from each case and control subject was collected in vacutainer tubes containing ethylenediamine tetra acetic acid (EDTA) as anticoagulant followed by DNA extraction using salting out method (19).

PCR-RFLP analysis of gene polymorphisms

PCR-RFLP for the -397C/T and -351A/G polymorphisms were performed using specific primers and restriction enzymes (Table I). PCR reaction mixture contained 1 µg of total DNA, 1X Master Mix (Taq DNA Polymerase Master Mix Red, Ampliqon, Denmark) and 10pM of each specific primer. The amplification cycles were 95°C for 5 min, and 35 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s, plus a final elongation at 72°C for 5 min. The PCR products were then digested by restriction enzymes *PvuII* (-397C/T), *XbaI* (-351A/G) (Fermentas, Lithuania) (Table I) followed by running on a

Gel Red (Biotium, USA) stained 2-2.5% agarose gel.

Statistical analysis

Comparisons between groups for distribution of each polymorphism were made by Chi-square test. Student's *t*-test was used to compare age, BMI and hormone levels between control and RPL groups. Correlations between different parameters were determined with Spearman's rank correlation test. Values of $p < 0.05$ were regarded as significant. All statistical analyses were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL). Haplotype analysis was determined with HaploView 4.2 (HaploView software Inc, USA).

Results

The average age of the case group was 33.4 years (ranging from 20-48) and of the control group was 39.2 years (ranging from 28-52). The genotype frequencies of all investigated polymorphisms in case and control groups are shown in table II. Allele frequency and genotype distributions of the

ESR1 polymorphisms were not significantly different between the control and case groups (-397C/T; $p=0.20$ and -351A/G; $p=0.09$). Heterozygous and homozygous polymorphic frequencies of -397C/T and -351A/G in the RPL group did not differ significantly from those in the controls.

A significantly negative correlation was observed between the two ESR1 gene polymorphisms, -397C/T and -351A/G ($r=-0.852$, $p < 0.001$) in RPL women. For this reason we conducted a study with inferred haplotypes (Figure 1 and Table III), (D' : 0.959; r -square= 0.758, $p < 0.001$) as a consequence of the short distance between them. Table IV summarizes endocrine parameters for the cases and controls. We found no statistically significant differences between groups in levels of E_2 and FSH. However, although women with increased the LH levels in the sera was excluded from the study, the level of LH (within the normal range) was significantly higher in RPL than that in controls (4.55 ± 0.22 vs. 3.04 ± 0.28 ; $p < 0.001$). In addition, genotype distribution analysis showed no correlation with the levels of E_2 , FSH and LH (data not shown).

Table I. Primer sequences and restriction enzymes used to detect ER gene polymorphisms

Polymorphism	Primer sequences	Amplicon size (bp)	Restriction enzymes	RFLP product size (bp)	Allele
ESR1 -397C/T	F: 5'- GATATCC AGGGTTATGTGGCA-3' [#]	346	PvuII	346 [#]	C
	R:5'-AGGTGTTGCCTATTATATTAACCTTGA-3'			103 + 243 [†]	T
ESR1 -351A/G			XbaI	148 + 198 [#]	A
				346 [‡]	G

ESR1; Estrogen receptor alpha gene,
[†]RFLP product for mutant allele.

[#]RFLP product for normal allele,

[‡]The same primer set was used to amplify for both polymorphisms variants

Table II. Genotype frequencies of RPL and control groups

Polymorphism	RPL n (%)	Controls (n=104) n (%)	p-value [†]
-397C/T- ESR1 (n=241)			
CC	41 (17.0)	10 (9.6)	0.20
TC	126 (52.3)	58 (55.8)	
TT	74 (30.7)	36 (34.6)	
-351A/G - ESR1 (n=244)			
AA	90 (36.9)	39 (37.5)	0.09
AG	121 (49.6)	59 (56.7)	
GG	33 (13.5)	6 (5.8)	

[†] Determined by Chi- Square test.

ESR1; estrogen receptor alpha gene

RPL; recurrent pregnancy loss

Table III. Haplotype frequencies of -397C/T and -351A/G polymorphisms in control and RPL groups

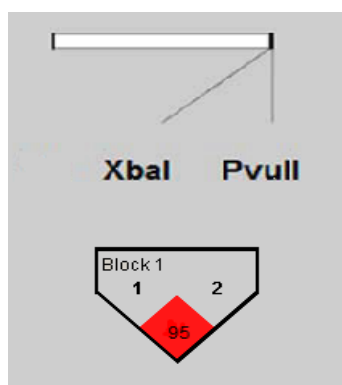
Haplotype	Control	RPL	Control+ RPL
Px (TA)	61.4%	55.9%	57.5%
PX (CG)	33.3%	37.3%	36.2%
Px (CA)	4.3%	6.0%	5.5%
pX (TG)	1.0%	0.0%	0.0%

Table VI. Endocrine parameters in women with RPL and healthy controls

Hormones	Controls	RPL	p-value [‡]
E ₂ (pg/ml)	45.95 ± 5.90	37.44 ± 3.56	0.22
FSH (IU/ml)	6.98 ± 0.41	7.50 ± 0.28	0.32
LH (IU/ml)	3.03 ± 0.28	4.54 ± 0.22	<0.001

[‡]Determined by student's *t*-test.

The data is presented as mean±SEM.

**Figure 1.** Linkage disequilibrium comparisons of, -397C/T and -351A/G polymorphisms in settlement order in the ER1 gene.

Discussion

Polymorphisms in the ERs influence vascular tone and flow and could result in a corresponding phenotypic difference in estrogen-dependent events, including those involved in pregnancy maintenance. Many studies have been performed to investigate the potential association between the -397T/C and -351A/G SNPs in ESR1 and recurrent miscarriage. In the present study, we found no significant differences in the allele distribution of the ESR1 gene polymorphisms between RPL and healthy Iranian women. Our finding is in line with studies by Aléssio *et al* and Hanna *et al* who did not find any association between these two SNPs and RPL in Brazilian and Canadian population, respectively (5, 20). These findings may suggest that these two polymorphisms have little or no effect on maintenance of pregnancy. However, a recent study in Spain found an association between increased number of miscarriages and the ESR1 haplotype composed of the -397T and -351A alleles (17).

These polymorphisms have opened an important area of research in reproductive disorders (RPL, infertility, endometriosis and age of menarche) and diseases such as osteoporosis, cardiovascular and cancer (10, 21-26). Conflicting results from different studies may be due to the differences in ascertainment of women with sporadic and

recurrent miscarriage. Moreover, the combinatorial effects of SNPs, particularly in extremely polymorphic genes, and gene environmental interactions is difficult to appropriately address in association studies. There are some hypotheses in the literature which describe functional significance of these two ESR1 gene polymorphisms. They are located on, 397 and 351 bp upstream of the start of exon 2, and possible functional mechanisms include changes in ESR1 expression due to altered binding of transcription factors and influence on alternative splicing of the ESR1 gene. These mechanisms can be caused directly by these polymorphisms or through linkage disequilibrium with a truly functional, but so far unknown sequence variation elsewhere in the ESR1 gene (27).

Schuit *et al* reported that the -397T/C polymorphism is associated with decreased plasma E₂ levels in an allele dose-dependent manner in postmenopausal women (27). They suggested that the potentially lower ESR1 expression caused by the T-allele leads to a lower expression of an enzyme, such as 17β-Hydroxysteroiddehydrogenases (HSD), in the estrogen synthesis pathway and subsequently, reduced E₂ synthesis. The fact that in the -351A/G polymorphism, the A-allele is also associated with E₂ levels may be due to linkage disequilibrium with the -397T/C SNP or another functional polymorphism, or due to functional significance of the -351A/G

polymorphism itself (27). These findings suggest that the genotype-dependent differences in E₂ levels created by these polymorphisms may be clinically significant.

Similar to our study, Zofkova *et al* showed no association between ESR1 gene polymorphisms and sex hormone levels they found that the -397T/C and -351A/G polymorphisms were not significantly associated with E₂ levels in a study of 114 postmenopausal women. In the present study, the distribution of -397T/C and -351A/G polymorphisms (Table IV) was very similar to what was previously reported in Caucasian populations of European ancestry and differed significantly from what was observed in populations of Asiatic (28).

Haplotype Px was detected, albeit in a low frequency whereas haplotype pX was not observed. This frequency of haplotypes may result from the disequilibrium which is not complete and may be due to recombination or multiple mutations which have occurred between/ at these two polymorphic sites. Endocrinological abnormalities are present in about a quarter of women with unexplained recurrent miscarriage (29). Several studies have reported high basal levels of LH, with or without polycystic ovaries, as a risk factor for miscarriage (30-32).

In the present study we found that LH levels were significantly higher in RPL compared to controls. No significant differences were observed in the levels of FSH and E₂ between the two groups which may be due to sample size. Larger studies may be needed to warrant a conclusion as to whether Iranian RPL women should be screened for the ESR1 gene polymorphisms.

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Conflict of interest

The authors declare that, there is no conflict of interest.

References

1. Stirrat GM. Recurrent miscarriage. II: Clinical associations, causes, and management. *Lancet* 1990; 336: 728-733.
2. Baek KH, Lee EJ, Kim YS. Recurrent pregnancy loss: the key potential mechanisms. *Trends Mol Med* 2007; 13: 310-317.
3. Li TC, Makris M, Tomsu M, Tuckerman E, Laird S. Recurrent miscarriage: aetiology, management and prognosis. *Hum Reprod Update* 2002; 8: 463-481.
4. Aksoy S, Celikkanat H, Senoz S, Gokmen O. The prognostic value of serum estradiol, progesterone, testosterone and free testosterone levels in detecting early abortions. *Eur J Obstet Gynecol Reprod Biol* 1996; 67: 5-8.
5. Hanna CW, Bretherick KL, Liu CC, Stephenson MD, Robinson WP. Genetic variation within the hypothalamus-pituitary-ovarian axis in women with recurrent miscarriage. *Hum Reprod* 2010; 25: 2664-2671.
6. Pepe GJ, Albrecht ED. Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr Rev* 1995; 16: 608-648.
7. Schindler AE. Endocrinology of pregnancy: consequences for the diagnosis and treatment of pregnancy disorders. *J Steroid Biochem Mol Biol* 2005; 97: 386-388.
8. Zulli K, Bianco B, Mafra FA, Teles JS, Christofolini DM, Barbosa CP. Polymorphism of the estrogen receptor beta gene is related to infertility and infertility-associated endometriosis. *Arq Bras Endocrinol Metab* 2010; 54: 567-571.
9. Herynk MH, Fuqua SA. Estrogen receptor mutations in human disease. *Endocr Rev* 2004; 25: 869-898.
10. Ayyaz OU, Ekmekci A, Baltaci V, Onen HI, Unsal E. Evaluation of in vitro fertilization parameters and estrogen receptor alpha gene polymorphisms for women with unexplained infertility. *J Assist Reprod Genet* 2009; 26: 503-510.
11. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, et al. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *J Clin Endocrinol Metab* 1997; 82: 4258-4265.
12. Wu TC, Wang L, Wan YJ. Detection of estrogen receptor messenger ribonucleic acid in human oocytes and cumulus-oocyte complexes using reverse transcriptase-polymerase chain reaction. *Fertil Steril* 1993; 59: 54-59.
13. Hurst BS, Zilberstein M, Chou JY, Litman B, Stephens J, Leslie KK. Estrogen receptors are present in human granulosa cells. *J Clin Endocrinol Metab* 1995; 80: 229-232.
14. Hillier SG, Anderson RA, Williams AR, Tetsuka M. Expression of oestrogen receptor alpha and beta in cultured human ovarian surface epithelial cells. *Mol Hum Reprod* 1998; 4: 811-815.
15. Altmae S, Haller K, Peters M, Hovatta O, Stavreus-Evers A, Karro H, et al. Allelic estrogen receptor 1 (ESR1) gene variants predict the outcome of ovarian stimulation in in vitro fertilization. *Mol Hum Reprod* 2007; 13: 521-526.
16. de Castro F, Moron FJ, Montoro L, Galan JJ, Hernandez DP, Padilla ES, et al. Human controlled

- ovarian hyperstimulation outcome is a polygenic trait. *Pharmacogenetics* 2004; 14: 285-293.
17. Pineda B, Hermenegildo C, Tarin JJ, Laporta P, Cano A, Garcia-Perez MA. Alleles and haplotypes of the estrogen receptor alpha gene are associated with an increased risk of spontaneous abortion. *Fertil Steril* 2010; 93: 1809-1815.
 18. Su MT, Lin SH, Chen YC. Association of sex hormone receptor gene polymorphisms with recurrent pregnancy loss: a systematic review and meta-analysis. *Fertil Steril* 2011; 96: 1435-1444.
 19. Rousseau F, Rehel R, Rouillard P, DeGranpre P, Khandjian EW. High throughput and economical mutation detection and RFLP analysis using a minimethod for DNA preparation from whole blood and acrylamide gel electrophoresis. *Hum Mutat* 1994; 4: 51-54.
 20. Alessio AM, Siqueira LH, de Carvalho EC, Barini R, Mansur Ade P, Hoehr NF, et al. Estrogen receptor alpha and beta gene polymorphisms are not risk factors for recurrent miscarriage in a Brazilian population. *Clin Appl Thromb Hemost* 2008; 14: 180-185.
 21. M'Rabet N, Moffat R, Helbling S, Kaech A, Zhang H, de Geyter C. The CC-allele of the PvuII polymorphic variant in intron 1 of the alpha-estrogen receptor gene is significantly more prevalent among infertile women at risk of premature ovarian aging. *Fertil Steril* 2012; 98: 965-967.
 22. Li Y, Liu F, Tan SQ, Wang Y, Li SW. Estrogen receptor-alpha gene PvuII (T/C) and XbaI (A/G) polymorphisms and endometriosis risk: a meta-analysis. *Gene* 2012; 508: 41-48.
 23. Manuck SB, Craig AE, Flory JD, Halder I, Ferrell RE. Reported early family environment covaries with menarcheal age as a function of polymorphic variation in estrogen receptor-alpha. *Dev Psychopathol* 2011; 23: 69-83.
 24. van Meurs JB, Schuit SC, Weel AE, van der Klift M, Bergink AP, Arp PP, et al. Association of 5' estrogen receptor alpha gene polymorphisms with bone mineral density, vertebral bone area and fracture risk. *Hum Mol Genet* 2003; 12: 1745-1754.
 25. Schuit SC, Oei HH, Witteman JC, Geurts van Kessel CH, van Meurs JB, Nijhuis RL, et al. Estrogen receptor alpha gene polymorphisms and risk of myocardial infarction. *JAMA* 2004; 291: 2969-2977.
 26. Weiderpass E, Persson I, Melhus H, Wedren S, Kindmark A, Baron JA. Estrogen receptor alpha gene polymorphisms and endometrial cancer risk. *Carcinogenesis* 2000; 21: 623-627.
 27. Schuit SC, de Jong FH, Stolk L, Koek WN, van Meurs JB, Schoofs MW, et al. Estrogen receptor alpha gene polymorphisms are associated with estradiol levels in postmenopausal women. *Eur J Endocrinol* 2005; 153: 327-334.
 28. Jakimiuk A, Nowicka M, Bogusiewicz M, Adamiak A, Skorupski P, Miotla P, et al. Prevalence of estrogen receptor alpha PvuII and XbaI polymorphism in population of Polish postmenopausal women. *Folia Histochem Cytobiol* 2007; 45: 331-338.
 29. Li TC, Spuijbroek MD, Tuckerman E, Anstie B, Loxley M, Laird S. Endocrinological and endometrial factors in recurrent miscarriage. *BJOG* 2000; 107: 1471-1479.
 30. Chandler CJ. Hypersecretion of luteinising hormone: a significant cause of infertility and miscarriage. *Br J Obstet Gynaecol* 1994; 101: 737-738.
 31. Clifford K, Rai R, Watson H, Regan L. An informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. *Hum Reprod* 1994; 9: 1328-1332.
 32. Watson H, Kiddy DS, Hamilton-Fairley D, Scanlon MJ, Barnard C, Collins WP, et al. Hypersecretion of luteinizing hormone and ovarian steroids in women with recurrent early miscarriage. *Hum Reprod* 1993; 8: 829-833.