Association of ESR-α and ESR-β gene polymorphisms with implantation failure in IVF-treated women in northwest of Iran: A case-control study

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Abstract

Introduction: Estrogen, a crucial hormone during pregnancy, acts through two types of receptors. The estrogen receptor alpha and beta (ESR-α and ESR-β) are more abundant and exists in all human reproductive systems. Association of ESR-α and ESR-β genes polymorphisms has been reported in some reproductive problems such as spontaneous abortion, endometriosis-related infertility, and *in vitro* fertilization failure. In the present study, we investigated association between single nucleotide polymorphisms rs9340799 and rs2234693 (ESR-α) and rs1256049 and rs4986938 (ESR-β) with implantation failure in Iranian women.

Materials and Methods: In this case-control study, we collected 60 women with implantation failure as case group and 60 age and ethnically matched IVF-treated women with successful implantation as controls. Extraction of genomic DNA of both case and control members was performed using salting out method. The case and control groups genotyping was performed using tetra-primer amplification refractory mutation system-polymerase chain reaction (Tetra-ARMS PCR) method.

Results: There were no significant differences in the frequencies of genotype and allele frequency in ESR-β gene rs4986938 polymorphism between patients and control groups ($p>.005$). In contrast, we observed a significant difference in the frequencies alleles and genotypes of rs9340799 and rs2234693 (ESR-α) and rs1256049 (ESR-β) polymorphisms between patients and control groups.

Conclusion: We demonstrated that rs9340799 and rs2234693 (ESR-α), and rs1256049 (ESR-β) polymorphisms may play important role in implantation failure in women in northwest of Iran. However, more studies on different geographic areas, races and ethnicities are required to determine exact role of ESR-α and ESR-β genes polymorphisms in implantation failure.

Keywords: Polymorphisms, ESR-α, ESR-β, Implantation failure

Introduction

During implantation process, a receptive uterus is an essential factor that guaranties success of implantation and pregnancy (1). Factors, involved in the process, prepare the uterine luminal epithelium to its best situation for blastocyst reception that finally makes a cross-link between uterus and blastocyst (2). Here, estrogen hormone plays a crucial role throughout the entire pregnancy, from regulating the production of progesterone to fetal development. This important role of estrogen has been proven in

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the uterine preparation for the implantation in mice (3).

Estrogen action is observed both on peripheral and central nervous system which is mediated by estrogen receptors (ER). Estrogen receptors α (ESRα, ESR1) and β (ESRβ, ESR2) genes are involved in controlling the physiological response to estrogen (4). ESRα gene is present on chromosome 6q25.1-q25.2. It consists of 8 exons which are parted by 7 intronic regions having a complete size of 140 kb. The ESRβ gene is located on chromosome 14q23.2-q23.3, comprising 8 exons and covers approximately 40 kb (5). ESRα codes for a 595 amino acid protein, while ESRβ codes for a 530 amino acid protein (6).

Most widely studied polymorphisms of ESRα gene is rs2234693 and rs9340799, and ESRβ gene is rs1256049 and rs4986938. The rs2234693 polymorphism occurs due to T/C transition in first intron, while the rs9340799 polymorphism is caused by a G/A transition located 50 base pairs downstream of the polymorphic site (7). Several sequence variants of the ESRβ gene have also been identified which include 2 silent polymorphisms, rs1256049 and rs4986938. The expression of both these receptors has been recorded in testis and epididymis (8).

The aim of present study was to investigate association between ESR-α gene single nucleotide polymorphisms rs9340799 and rs2234693, and ESR-β gene single nucleotide polymorphisms rs1256049 and rs4986938 with implantation failure in Iranian northwest infertile women who were submitted for conventional in vitro fertilization (IVF) procedure and had no blastocyst implantation.

Materials and Methods

Collection of Patients and Samples

In this case-control study, we recruited 120 women (20-50 years old) referred to Medical Genetics Laboratory, Tbriz, Iran, from July 2019 to Apr 2020. In this among, 60 IVF-treated women with implantation failure were recruited as case groups with confirmed idiopathic. Also, 60 IVF-treated women with successful implantation recruited as control group. The demographic information includes age, body mass index (BMI), alcohol drinking, tobacco smoking, and family history of case and control groups were collected by questionnaires and interviews from studied subjects (Table 1). All studied subjects were informed about the study and signed consent form, according to the Declaration of Helsinki ethical standards.

Primer Design

The ESR-α and ESR-β genes sequence were obtained from National Center for Biotechnology Information (NCBI) database. The Primer3 software were used to primer design for amplification of ESR-α gene rs2234693 and rs9340799 polymorphisms, and ESR-β gene rs4986938 and rs1256049 polymorphisms. The designed sequences of primers were synthesized in SinaClon Company, Iran. The sequences and characteristics of primers used amplify studied genes polymorphisms were presented in Table 2.

DNA Extraction and Genotyping

The peripheral blood samples (5 ml) were drawn from all subjects, and genomic DNA extraction was performed using salting out method. The quantity and quality of extracted DNA were investigated using nanodrop instrument and electrophoresis on 1% agarose gel. The genotype determination was performed by tetra-primer amplification refractory mutation system-polymerase chain reaction (Tetra-ARMS PCR) method. A 25 μL total volume was used as following: PCR buffer (2.5 μL), 1 μL template DNA, 2.5 μL dNTP (2 mM), 0.1 μL each primera (25 pmol), 0.8 μL Mgcl2 (50 mM), and Taq DNA

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polymerase (0.25 μL). PCR condition was as following: 1 cycle initial denaturation (94°C for 4 minutes), 40 cycles denaturation (94°C for 40 seconds), annealing (30 seconds), and extension (72°C for 25 seconds), and 1 cycle final extension (72°C for 5 minutes). The amplified PCR products were electrophoresed on 1% agarose gel with 50bp size marker. A gel documentation instrument was used to visualize the bands of PCR products.

Statistical Analysis

The statistical analysis was performed using SPSS software (version 19.0). The association of gene rs2234693, rs9340799, rs4986938, and rs1256049 polymorphisms and implantation failure was evaluated by logistic regression. Moreover, the chi-square ($\chi 2$) test and Fisher's exact test were used to evaluation of Hardy-Weinberg equilibrium (HWE). The difference between clinical and demographic features between healthy controls and patients were analyzed using independent sample t-test. The statistically significant was considered as $p<0.05$.

Results

The obtained results showed no significant difference between patients and healthy controls in terms of family history, alcohol drinking, age, body mass index (BMI), and tobacco smoking (Table 1).

Table 1. The clinical features and demographic variables of cases and controls.

Variables	Patients (n=60)	Controls (n=60)	P value
Age (year)	34.19 ± 1.20	32.34 ± 3.76	0.659
Body mass index (kg/m)	25.09 ± 4.09	23.18 ± 3.11	0.398
Tobacco smoking			0.118
Never	49 (81.66%)	52 (86.66%)	
Ever	11 (18.33%)	8 (13.33%)	
Alcohol drinking			0.326
Never	41 (68.33%)	43 (71.66%)	
Ever	19 (31.66%)	17 (28.33%)	
Family history			0.211
Negative	56 (93.33%)	58 (96.66%)	
Positive	4 (6.66%)	2 (3.33%)	

Data are shown as mean ± SD or number (percent).

According to the $\chi 2$ tests, the rs9340799 A/G, rs2234693 T>C, rs1256049 G>A, and rs4986938 G>A polymorphisms was in HWE in women with implantation failure and healthy controls (p>0.05). The genotypes and alleles frequencies in women with implantation failure presented in Table 3.

We observed a significant difference between case and controls in term of rs9340799, rs2234693, rs1256049 polymorphisms; whereas rs4986938 polymorphism showed no significant association between case and healthy controls (Table 3).

Moreover, the frequency of C allele of rs2234693 in patients and healthy controls were 34% and 18%, respectively. Also, the frequency of A allele of rs1256049 in patients and healthy controls were 76% and 45%, respectively. The statistical analysis of C allele (rs2234693) and A allele (rs1256049) frequencies showed a significant difference between patients and healthy controls (p>0.05); whereas alleles frequencies of rs9340799 and rs4986938 polymorphisms showed no significant association between case and healthy controls (Table 3).

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Table 2. The sequences and characteristics of used primers.

Gene	Polymorphism	Primer sequence	Genotype
ESR-α	rs2234693	Forward Outer: 5′-ACCACCATGCTCAGTCTCTAC-3′	553+379 bp (TT)
		Reverse Outer: 5′-CAAAACATGCACTCTCTGGGA-3′	221+553 (CC)
		Forward Inner: 5′-ATCTGAGTTCCAAATGTCCCATCT-3′	221+379+553 bp (CT)
		Reverse Inner: 5′-GAAACAGAGACAAAGCATAAAGCG-3′	
	rs9340799	Forward Outer: 5′-ACCACCATGCTCAGTCTCTAC-3′	262+553 bp (AA)
		Reverse Outer: 5′- CAAAACATGCACTCTCTGGGA-3′	330+553 (GG)
		Forward Inner: 5′-CAGAGACCCTGAGTGTGGTATG-3′	262+330+553 bp (AG)
		Reverse Inner: 5′-CAATGCTCATCCCAACGCT-3′	
ESR-β	rs4986938	Forward Outer:5′- GTATGACCTGCTGCTGGAGA-3′	191+486 bp (GG)
		Reverse Outer: 5′-ATTCGCAGCCCTTCCAAGT-3′	333+486 (AA)
		Forward Inner:5′- TGGCCCACAGAGGTCAAAA-3′	191+333+486 bp (GA)
		Reverse Inner: 5′-CTGGAGTTCACGCTTCATCC-3′	
	rs1256049	Forward Outer: 5′-GGCAGCCAAGCATCAACATTCTCAG-3′	360+552 bp (GG)
		Reverse Outer: 5′-ATTGCAGCACCCAGGACTTTGTTC-3′	198+552 (AA)
		Forward Inner: 5′-GGAGCTCAGCCTGTTCGACCAATTA-3′	198+360+552 bp (GA)
		Reverse Inner: 5′-ATCCAACAGCTCTCCAAGAGCAGC-3′	

ESR: estrogen receptor alpha; bp: base pair

Table 3. Genotype and allele distribution of ESR-α and ESR-β genes polymorphisms.

Gene	Polymorphism	Genotype and Allele	Patients (n=60)	Controls (n=60)	OR (95% CI)	P value
ESR-α	rs9340799	AA	15 (25%)	36 (60%)	Ref =1	Ref
	(A>G)	AG	42 (70%)	18 (30%)	1.003 (0.108-1.645)	**0.001***
		GG	3 (5%)	6 (10%)	1.342 (0.229-1.664)	0.14
		A	72 (60%)	90 (75%)	Ref =1	Ref
		G	48 (40%)	30 (25%)	1.022 (0.963-2.284)	0.54
	rs2234693	TT	21 (35%)	39 (65%)	Ref =1	Ref
	(T>C)	TC	37 (62%)	21 (35%)	1.233 (0.130-2.372)	**0.003***
		CC	2 (3%)	0 (0%)	1.332 (0.988-1.235)	0.32
		T	79 (66%)	99 (82%)	Ref =1	Ref
		C	41 (34%)	21 (18%)	1.544 (0.887-1.356)	**0.001***
ESR-β	rs1256049	GG	3 (5%)	30 (50%)	Ref =1	Ref
	(G>A)	GA	23 (38%)	6 (10%)	1.943 (0.199-2.874)	**0.001***
		AA	34 (57%)	24 (40%)	1.123 (0.984-1.454)	0.23
		G	29 (24%)	66 (55%)	Ref =1	Ref
		A	91 (76%)	54 (45%)	1.107 (0.121-1.118)	**0.005***
	rs4986938	GG	45 (75%)	48 (80%)	Ref =1	Ref
	(G>A)	GA	13 (22%)	12 (20%)	1.989 (0.112-2.322)	0.344
		AA	2 (3%)	0 (0%)	1.170 (0.567-1.777)	0.19
		G	103 (86%)	108 (90%)	Ref =1	Ref
		A	17 (14%)	12 (10%)	1.656 (0.340-1.544)	0.32

*Statistically Significant; OR: Odds Ratio; CI: Confidence Interval

Discussion

Even in the best assisted reproductive technology units, pregnancy rate is not more than 30% (9). Different factors may be involved in failed pregnancy during the cycle, such as inappropriate ovarian stimulation, unsuitable uterus milieu and suboptimal laboratory culture conditions (10). Implantation failure is the most common cause of lack of pregnancy after embryo transfer in IVF procedure. It takes place in about of 40% of IVF experiments (11). Some evidences indicate that genetic factors regulate implantation process. Thus, genetic defects and polymorphisms influence increasing susceptibility to process failure both directly and indirectly (12, 13). Among

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the factors involved in the pregnancy, estrogen hormone has a crucial role. Fetal growth and development during both extra- and intra-uterine periods of life depends on estrogen produced by placenta (14). As it was mentioned before, null female mice with knockout ESR1 gene are infertile, with no corpus luteum formation and altered gonadotropin levels (15).

Considering the pivotal role of estrogen and its receptors during pregnancy, some studies have been done to find out whether there is any association between ESR-α and ESR-β variants and factors involved in pregnancy. The single nucleotide polymorphisms rs9340799, rs2234693, rs1256049, and rs4986938 is possible that it can influence ESR-α and ESR-β genes expression and subsequently pregnancy fate. In a study by Liaqat et al. reported that ESRα (rs2234693 and rs9340799) polymorphisms were strongly associated with risk of infertility in Pakistanian papulation; whereas ESRβ (rs1256049) polymorphism no association with infertility risk (16). In another study by Ganesh et al. suggested that ESRα gene rs2234693 and rs9340799 polymorphisms are involved in increase of implantation failure in IVF-treated women in Indian papulation (17). Zulli et al. showed that ESRβ gene rs4986938 polymorphism can be associated with risk of infertility in Brazilian women (18). Moreover, it has been revealed that ESR-α and ESR-β polymorphisms have association with ovarian response to follicle stimulating hormone in a person who undergoes IVF procedure (19). Based on an investigation in Turkish infertile women regarding IVF parameters such as numbers of collected oocyte, maturation and embryo quality, it has been suggested that these parameters could be associated with estrogen receptors variants (20).

In this study we tried to find an association between XbaI variant in ESR1 gene and blastocyst implantation failure in women who undergo conventional IVF cycles. Our results showed that there is some association though not significant between the two. Effect of ESR1 gene variants over expression level of ESR1 gene may be useful to clear the potential role of these variations, but the exact mechanism behind the association remains unclear so far (21).

The reasons for the difference of reported results among the above mentioned studies could be due to several other related genes and environmental factors, and difference in studied samples size, ethnicity, race, and geographical area (22-25).

Considering the high medical cost and mental burden of couples who suffer from infertility, doing genetics screening along with clinical screening seems necessary before using artificial techniques. Beside other genomic markers, high valuable single nucleotide polymorphisms that have a significant association with infertility can be promising to avoid unpleasant results from failure cycles.

Conclusion

Generally, our study indicated more details of implantation failure, and suggested that rs9340799 and rs2234693 (ESR-α) and rs1256049 (ESR-β) polymorphisms may be associated with implantation failure in the Iranian northwest population. On the other hands, our study showed no association between rs4986938 (ESR-β) polymorphism and implantation failure in Iranian northwest population. Nevertheless, to reach the better understanding about implantation failure, in view of its multi-genetic background, identification of gene variants would determine the treatment strategies of the subjects.

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Conflict of Interests

The authors declare no conflict of interest.

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