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GENETICS

Evaluation of in vitro fertilization parameters and estrogen receptor alpha gene polymorphisms for women with unexplained infertility

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Abstract
Purpose Association of *ESR*1 gene *Pvu*II, *Xba*I and (TA)n microsatellite polymorphisms and woman infertility was evaluated.
Methods Infertile(n=104) and fertile(n=107) women were included in this study. We performed polymerase chain reaction-restriction fragment-length polymorphism analysis for detecting *ESR1* polymorphisms.
Result(s) PvuII and XbaI polymorphisms confered risk for infertility in a simple dominant manner in which a significant relationship was observed between infertile and control women. Infertile women had fewer(<18) short repeat alleles in promotor region. *ESR1* genotypes were compared concerning maturation, fertilization, pregnancy rates and embryo quality. Although no difference was found in terms of pregnancy rates, maturation and fertilization rates were significantly smaller in *pp and xx* genotypes. Also, pp genotypes had significantly lower number of good quality embryos. Long TA repeat in promotor was found to be associated with low fertilization rate.
Conclusion(s) Polymorphisms at the *ESR*1 gene are associated with infertility in this Turkish infertile women population.

Keywords Estrogen receptor gene · Infertility · IVF · Microsatellite polymorphism · Pregnancy rate

Capsule In this study role of *ESR*1 gene polymorphisms on infertility was focused and a significant association between *ESR*1 genotypes and some IVF parameters was reported.

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Introduction

Estrogen is synthesized by granulosa cells under the control of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Estrogen and FSH act in synergism in the ovary to increase the number of FSH receptors in the granulosa cells, resulting in follicular growth and maturation [1, 2]. It is believed that estrogen plays crucial roles in oocyte maturation and fertilization [3].

Estrogen signaling is mediated via binding to estrogen receptors (ERs), which are ligand-dependent transcription factors. Two subtypes of estrogen receptors exist in humans; ERα [4] and ERβ [5], coded by *ESR*1 and *ESR*2 genes, respectively. ERα and ERβ are both members of nuclear hormone receptor family. The *ESR*1 gene (140 kilo base) is located on chromosome 6q25.1 and consists of 8 exons; and intron 1 contains two single-nucleotide polymorphisms (SNPs) named the *PvuII* (T/C) and *Xba*I (A/G) [6].

Recent studies have tried to evaluate the distribution of various *ESR*1 gene polymorphisms, associated with female and male infertility. *ESR*1 *Pvu*II polymorphism in women was found to affect pregnancy rate following in vitro fertilization (IVF) [7, 8], whereas in males *ESR*1 *Xba*I polymorphism was suggested to have an effect on azoospermia or idiopathic severe oligospermia [9].

ER genes harbour several polymorphisms that may influence the risk for certain infertility-associated gynecological disorders and IVF outcome [10]. *Pvu*II polymorphism is reported to be associated with the susceptibility to endometriosis [11] and controlled ovarian hyperstimulation (COH)/pregnancy outcome of IVF [7, 8].

An additional *ESR*1 promoter (TA)n dinucleotide repeat polymorphism is suggested to increase the risk of premature ovarian failure (POF) in a simple dominant manner in which women carrying a long (TA)n repeat allele were suggested to have approximately 10 times the risk of POF compared to women homozygous for short *ESR*1 (TA)n repeats [8]. These previous findings indicate that improving our understanding of ER gene polymorphisms may be important associations for infertility diagnoses and treatments [7, 8, 10]. Therefore, the purpose of the present study was to determine the importance of *ESR*1 *Pvu*II, *Xba*I and (TA)n polymorphisms in the etiology of unexplained infertility and to find an association of these polymorphisms with oocyte maturation, fertilization, pregnancy rates and embryo quality.

Materials and methods

Subjects

One hundred and four women who underwent an IVF-ET procedure were retrospectively recruited for this study. Serum FSH levels (≤8.0 IU/ml) were measured for all participants between day 3 and 5 of the spontaneous menstrual cycle using chemiluminescence immunoassay (Immulite 2000w station, Diagnostic Products Corporation, Los Angeles, CA, USA). Females with infertility because of unexplained factor between 28 and 35 ages were enrolled as study group. The age-matched control group consisted of 107 proven fertile healthy females with a history of regular menstrual cycle. Informed consent was obtained from all participants and Gazi University Medical Faculty Local Ethics Committee, the Institutional Review Board (IRB) approved the study.

Stimulation protocol and oocyte retrieval

All the IVF women were administrated the same ovulation stimulation protocol [12] in Gen-Art Woman Health and Reproductive Biotechnology Center. When the leading follicle reached 18 mm in mean diameter with a serum estradiol (E2) level of 200 pg/ml per mature follicle, 10,000 U of hCG (Profasi, Serono, Switzerland) was administered. Oocyte retrieval was performed 36 h after the human chorionic gonadotropin (hCG) administration injection. The ICSI intracytoplasmic sperm injection) was performed according to conventional protocols and the number of mature oocytes was calculated. The oocytes were considered mature if they reached MII stage by 2–3 h after oocyte retrieval. The total number of embryos was calculated by counting the embryos with two pronuclei (2PN-embryos). Routine examination of embryo quality included the number of blastomeres, the degree of fragmentation, and the uniformity of the blastomeres. Embryos were classified according to a simplified system based on Veeck's morphological criteria: Grade I embryos have equal-sized blastomeres and no cytoplasmic fragmentation, grade II embryos have blastomeres of equal size and minor cytoplasmic fragmentation covering 10% of the preembryo surface, grade III embryos have blastomeres of distinctly unequal size and variable fragmentation, grade IV embryos have blastomeres of equal or unequal size and moderate-to-significant cytoplasmic fragmentation covering >10% of the preembryo surface, and grade V embryos have few blastomeres of any size and severe fragmentation covering >50% of the preembryo surface. None of the embryos were classified as grade V in this study. Depending on the woman's age and the embryo quality up to three embryos were transferred on the third day after retrieval. Biochemical pregnancy was established when serum ß-HCG was found >20 IU/L on the 12th day of embryo transfer, and clinical pregnancy was defined as the presence of a gestational sac on ultrasound at six gestational weeks.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using Invisorb DNA extraction kit (Invitek, Berlin, Germany). Patients and controls were genotyped for *Pvu*II (T/C, rs2234693, c.454-397) and *Xba*I (A/G, rs9340799, c.454-351 A>G) SNPs in *ESR*1 intron 1, using restriction fragment length polymorphism (RFLP) analysis. For the *ESR*1 *Pvu*II and *Xba*I SNPs, the forward and reverse primers were: 5′-CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC-3′ and 5′-TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA-3′, respectively. The total volume of the polymerase chain reaction (PCR) reaction mixture was 50 μL and contained 0.2 mM dNTPs (MBI Fermentas, Vilnius, Lithuania), 2 mM $MgCl_2$, 1X PCR buffer (MBI Fermentas, Vilnius, Lithuania), 10 pmol of primers (MWG Biotech, Martinsried, Germany) and 1.5 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania). PCR was performed using Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). Following an initial denaturation step (5 min at 94°C), samples were subjected to 30 cycles of PCR at 94°C for 30 sec, 62°C for 1 min, and 72°C for 1.5 min with a final extension of 5 min at 72°C.

The PCR products were digested overnight with *Pvu*II (MBI Fermentas, Vilnius, Lithuania) and *Xba*I (MBI

Fermentas, Vilnius, Lithuania) restriction enzymes as described previously [13, 14]. After digestion with *PvuII*, PCR product was cut into 850 and 450 bp fragments in the presence of p allele, whereas P allele was undigested (1300 bp) (Fig. 1). After digestion with *XbaI* PCR product was cut into 900 and 400 bp fragments in the presence of x allele, whereas X allele was undigested (1300 bp) (Fig. 2). The PCR products and the restriction fragments were separated in 2% agarose gel stained with ethidium bromide, and were visualized by Gel Logic 100 Imaging System (GL 100) (Kodak, NY, USA). To confirm the genotypes obtained by PCR-RFLP method, DNA sequencing was carried out 5% of the samples using ABI Prism 310 Genetic Analyzer. (Applied Biosystems, Foster City, CA, USA).

The (TA)n (rs3138774) repeat polymorphism in the *ESR*1 promoter region was investigated by PCR using a FAM labelled forward primer 5′ GACGCATGATATACTT CACC 3′ and reverse primer 5′ GCAGAATCAAATATC CAGATG 3′ in a 25 ml PCR reaction containing: 1X PCR buffer, 20 μM of dNTP, 2 mM $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania), 20 pmol of primers (MWG Biotech, Martinsried, Germany) and 1 U Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania). The reaction volume was made up to 25 ml using deionised water. PCR was performed using Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). Following an initial denaturation step (5 min at 94°C), samples were subjected to 30 cycles of PCR at 94°C for 30 sec, 62°C for 1 min, and 72°C for 1 min with a final extension of 5 min at 72°C. The fluorescence-labelled PCR products were analysed for size using an ABI Prism 310 Genetic Analyzer. (Applied Biosystems, Foster City, CA, USA). The sizes of the PCR products were determined by Genescan v.3.0 software (Applied Biosystems, Foster City, CA, USA). ROX-500 (Applied Biosystems, Foster City, CA, USA) was used as an internal size standard.

Statistical analysis

Allele frequencies were determined by the gene counting method. Hardy-Weinberg equilibrium was tested using Genepop Version 4.0 [15]. The relationship between *ESR*1 genotypes and infertility was analyzed using the $\chi 2$ test. Reference genotypes/alleles were used to calculate crude odds ratios (ORs) and 95% confidence intervals (CIs). Clinical characteristics of the study and control subjects were compared using one way analysis of variance (One way-ANOVA) and Kruskall-Wallis. The Linkage Disequilibrium (LD) values for the three pairs of SNPs have been calculated using Haploview Version 4.0 (Website: http://www.broad.mit.edu/mpg/haploview) [16]. Haplotype frequencies were estimated by web based haplotype analysis tool (HAP) (Website: http://research.calit2.net/hap). The most common haplotype was used as the reference, and association between other haplotypes and infertility risk was calculated by using the $\chi 2$ test. *P* values of <0.05 were regarded as statistically significant. SPSS system 15.0 version was used for calculation.

Results

The *ESR*1 genotypes of the three polymorphisms were in Hardy-Weinberg equilibrium in the fertile group. With the exception of (TA)n microsatellite repeat, other *ESR*1 polymorphisms were in agreement with the Hardy-Weinberg equilibrium in the infertile group. Genotype distributions and allele frequencies of three *ESR*1 polymorphisms are shown in Table 1. For the *Pvu*II polymorphism, a noteworthy association was observed between infertile and fertile groups ($p<0.001$). Moreover, compared with the *PP* genotype, the ORs for the *Pp* and *pp* elevated 2.25 (95% Cl, 1.22–4.17) and 4.10 (95% Cl, 1.80–9.34) times, respectively. In addition, *Pvu*II alleles, present in the

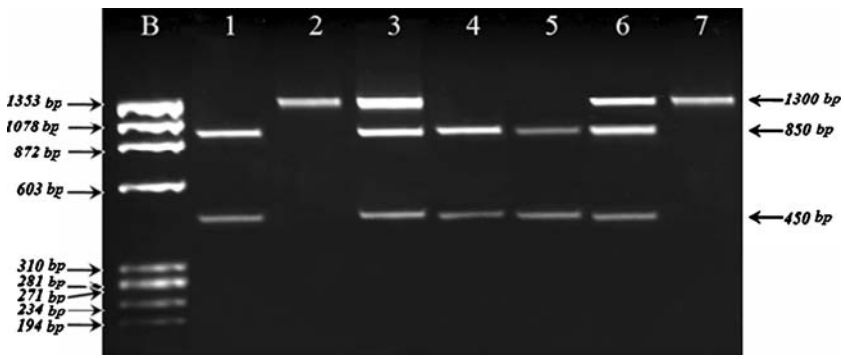

Fig. 1 RFLP analysis of the *PvuII* polymorphism. *Lane 1, 4, 5*: Two fragments of 850 bp and 450 bp for *pp* genotype, *Lane 2*: undigested PCR product of 1300 bp for *PP* genotype, *Lane 3, 6*: Three fragments of 1300 bp, 850 bp and 450 bp for *Pp* genotype, *Lane 7*: PCR product of external PCR. **b:** ϕX174 molecular size marker

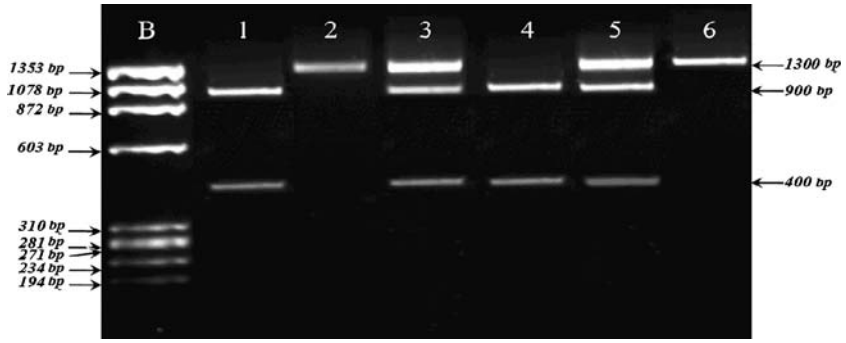

Fig. 2 RFLP analysis of the *XBaI* polymorphism. *Lane 1, 4*: Two fragments of 900 bp and 400 bp for *xx* genotype, *Lane 2*: undigested PCR product of 1300 bp for *XX* genotype, *Lane 3, 5*: Three fragments of 1300 bp, 900 bp and 400 bp for *Xx* genotype, *Lane 6*: PCR product of external PCR. **b:** ϕX174 molecular size marker

infertile group had also significant difference with the control group ($p<0.001$) (Table 1).

There is also an outstanding correlation revealed between *Xba*I polymorphism and the risk of infertility ($p=0.002$). The *xx* genotype (OR 6.75 [95% CI: 2.11–21.59]) was an approximately sevenfold increased predisposition compared with the carriers of the homozygote common allele (*XX* genotype), whereas in heterozygotes (*Xx* genotype) no significant disposition was detected (OR 1.53 [95% CI: 0.86–2.73]). Besides, we found a considerable difference in the allele frequencies of the *Xba*I polymorphism among groups ($p<0.001$) (Table 1).

For the (TA)n repeat polymorphism, we observed 18 different alleles with (TA)n repeat number ranging between 8 and 25 (Fig. 3). This polymorphism showed a bimodal distribution, with two peaks at 13 repeats (35.3% of alleles) and 21 repeats (10.9% of alleles), and a breakpoint at 18 TA repeats as reported previously [17–19]. This cutoff point was used to divide alleles into categories of either short (S<18 TA repeats) or long alleles (L≥18 TA repeats).

Table 1 Incidence of *PvuII, XbaI* and (TA)n repeat dinucleotid genotypes in infertile and control groups

	Controls		Cases		*p* values	OR (95% CI)	*p* values
Genotypes	*n*	(%)	*N*	(%)			
PvuII					**$p<0.001$**		
PP	53	49.5	28	26.9		1[a]	
Pp	42	39.3	50	48.1		2.25 (1.22–4.17)	**0.010**
pp	12	11.2	26	25		4.10 (1.80–9.34)	**$p<0.001$**
Alleles							
P	148	69.2	106	51.0		1[a]	
p	66	30.8	102	49.0		2.16 (1.45–3.21)	**$p<0.001$**
Genotypes	*n*	(%)	*N*	(%)			
XbaI					**0.002**		
XX	54	50.5	36	34.6		1[a]	
Xx	49	45.8	50	48.1		1.53 (0.86–2.73)	0.148
xx	4	3.7	18	17.3		6.75(2.11–21.59)	**$p<0.001$**
Alleles							
X	157	73.4	122	58.7		1[a]	
x	57	26.6	86	41.3		1.94 (1.29–2.93)	**$p<0.001$**
Genotypes	.*n*	(%)	*N*	(%)			
(TA)n repeat					**0.004**		
SS	58	54.2	35	33.6		1[a]	
SL	41	38.3	50	48.1		2.02 (1.12–3.64)	**0.019**
LL	8	7.5	19	18.3		3.94 (1.56–9.94)	**0.004**
Alleles							
S	157	73.4	120	57.7		1[a]	
L	57	26.6	88	42.3		2.02 (1.34–3.04)	**0.001**

OR odds ratio, *CI* confidence interval

[a] Reference genotype/allele

P values <0.05 are shown in **bold**

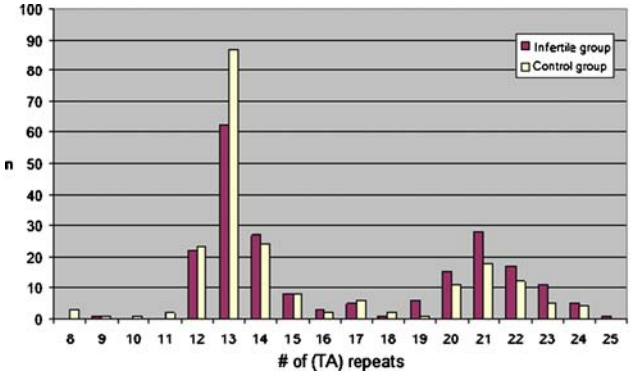

Fig. 3 Frequence distribution of dinucleotide TA repeats in ESR1 gene

When divided upon this basis, for the infertile group the (TA)n repeat genotype frequencies were *SS*=35 (33.6%); *SL*=50 (48.1%); and *LL*=19 (18.3%). For the control group same frequences were *SS*=58 (54.2%); *SL*=41 (38.3%); and *LL*=8 (7.5%) (Table 1). A significant relationship was observed between infertile and control group in terms of (TA)n repeat microsatellite polymorphism ($p<0.001$). It had been revealed that longer ESR (TA)n was linked with a higher risk for infertility.

Linkage disequilibrium (LD) was only observed between *Pvu*II and *Xba*I polymorphisms. No LD was detected between the (TA)n microsatellite polymorphism and *Pvu*II-*Xba*I (Fig. 4). The distributions of *ESR*1 haplotypes with estimation of ORs in infertile women and controls are presented in Table 2.

Table 3 summarizes number of follicles and retrieved oocytes, maturation, fertilization and pregnancy rates in each *Pvu*II, *Xba*I and (TA)n genotype groups. A statistically significant difference was found between maturation and the fertilization rates in women with different *ESR*1 *Pvu*II genotypes. *ESR*1 *pp* genotype tended to be associated with a lower number of fertilized oocyte ($p=0.003$). Each *Pvu*II genotype was significantly related with each other in terms

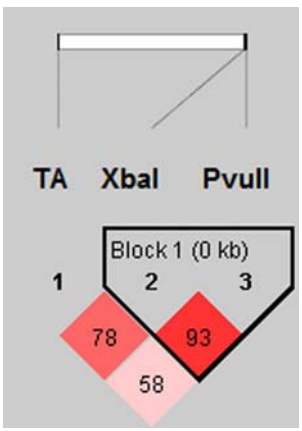

Fig. 4 Linkage disequilibrium comparisons of TA, *Pvu*II and *Xba*I polymorphisms in settlement order in the *ESR*1 gene

Table 2 Haplotype frequencies of PvuII and XbaI polymorphisms in control and study groups

Haplotype	Control (2*n*=214) (%)	Infertile (2*n*=208) (%)	OR (%95 CI)	*p*[a]
PX	145 (67.8)	104 (50.0)	1[b]	
Px	54 (25.2)	84 (40.4)	2.17 (1.42–3.32)	**0.0001**
pX	12 (5.6)	18 (8.7)	2.09 (0.97–4.53)	0.057
px	3 (1.4)	2 (0.9)	0.93 (0.15–5.66)	1.000

OR Odds Ratio, *CI* Confidence Interval

[a] χ^2 test was used to compare the groups. Statistically significant results were shown in bold

[b] Reference haplotype

of maturation and fertilization rates. Both maturation and fertilization rates and also number of high quality embryos were higher for women with the *ESR*1 *Pvu*II *PP* genotype; lower for women with the *ESR*1 *Pvu*II *Pp* genotype and the lowest for women with the ESR1 PvuII *pp* genotype ($p<0.001$).

The associations between the *ESR*1 *Xba*I polymorphism and IVF parameters were assessed and a statistically significant difference was found between fertilization rate. *XX* genotypes showed a higher fertilization rate and difference was significant between *XX* and *Xx* or *xx* genotypes ($p<0.001$) (Table 3).

Differences in the outcomes of IVF according to *ESR*1 (TA)n genotypes were also observed. Three *ESR*1 (TA)n genotypes were also differed with each other significantly in terms of fertilization rates ($p=0.011$ for *SS* and *SL* genotypes, $p<0.001$ for *SS* and *LL* genotypes and $p=0.013$ for *SL* and *LL* genotypes). The mean fertilization rates were higher in *SS* genotypes (%80.0), lower in *SL* genotypes (%71.1) and the lowest in *LL* genotypes (%58.8) (Table 3).

The mean clinical pregnancy rate for all study patients was 33.7% (35/104). The associations between the ESR1 genotypes and the occurence of clinical pregnancy were examined but none of the ESR1 variants included in this study predicted the probability for clinical pregnancy per embryo transfer.

Discussion

Since the first application of assited reproduction techniques, many factors have been associated with the outcome of IVF treatment [20]. Although all the patients were exposed to the same IVF protocol in follicular stimulation, follicular responses among the patients differed significantly. The alterations in the genotype of the estrogen and/or its receptor may be one of the factors that contribute toward such observed variability. Since the estrogen hormone affects maturation of oocytes and provides an

Table 3 Follicle number, oocyte number, follicle:oocyte ratio, maturation rate and fertilization rate in each of the three genotypes for PvuII, XbaI and TA dinucleotide polymorphisms

	Genotypes of ESR1, PvuII				Genotypes of ESR1, XbaI				Genotypes of ESR1,(TA)n repeat			
	PP (n=28)	Pp (n=50)	pp (n=26)	p value	XX (n=36)	Xx (n=50)	xx (n=18)	p value	SS (n=35)	SL (n=50)	LL (n=19)	p value
# of follicles Median(Min-max)%	18.5 (15.25–23.00)	19.0 (15.00–23.25)	19.0 (16.00–25.00)	0.579[a]	18.5 (16.00–23.75)	19.0 (15.00–24.00)	19.0 (16.00–23.25)	0.972[a]	18.0 (15.00–24.00)	19.5 (16.00–23.00)	19.0 (15.00–24.00)	0.894[a]
# of collected oocyte Median(Min-max)%	18.5 (14.00–22.00)	17.5 (15.00–22.25)	19.0 (15.00–22.25)	0.812[a]	18.0 (15.00–22.00)	17.5 (15.00–23.00)	18.5 (15.00–22.00)	0.938[a]	17.0 (14.00–23.00)	18.0 (16.00–22.25)	19.0 (14.00–22.00)	0.836[a]
Maturation rate Median(Min-max)%	94.0 (87.09–100.0)[b]	93.5 (86.76–96.03)[c]	82.0 (63.38–89.83)[b,c]	p<0.001[a]	94.3 (87.84–100.00)[d]	91.7 (80.75–95.50)[e]	86.5 (65.00–91.39)[d,e]	p<0.001[a]	92.9 (83.33–95.55)	92.5 (85.80–95.59)	86.4 (66.67–92.86)	0.075[a]
Fertilization rate Median(Min-max)%	86.6 (72.1–94.02)[b,f]	71.0 (59.58–81.25)[f]	60.0 (48.86–75.48)[b]	p<0.001[a]	84.0 (72.61–94.36)[d,g]	68.8 (59.58–78.33)[g]	59.5 (39.78–79.56)[d]	p<0.001[a]	80.0 (70.0–92.85)[h,j]	71.1 (60.0–81.53)[i,j]	58.8 (40.00–71.43)[h,i,j]	p<0.001[a]
# of grade 1–2 embryos Median (Min-max)%	10.0[b] (6.00–14.00)	9.5[c] (4.00–16.00)	8.0[b,c] (4.00–12.00)	p<0.001	9.0 (4.50–17.00)	10.0 (6.00–14.00)	8.5 (3.50–14.00)	0.668	10.0 (7.00–12.00)	12.0 (6.00–17.00)	10.0 (5.00–14.00)	0.772
# of grade 3–4 embryos Median (Min-max)%	6.0[b] (3.00–10.00)	7.0[c] (2.00–10.00)	7.5[b,c] (3.00–11.00)	p<0.001	7.0 (2.00–11.00)	8.0 (2.00–7.00)	8.0 (3.0–15.00)	0.645	6.5 (3.00–10.00)	5.5 (2.00–11.00)	6.0 (2.00–12.00)	0.739
Frequency (%) of clinical pregnancies	35.7	34.0	30.8	0.556[k]	33.3	36.0	27.8	0.895[k]	31.4	36.0	31.5	0.712[k]

[a] Kruskal Wallis test

[b] Significant relationship was found between the groups when PP and pp genotypes were compared p<0.001

[c] Significant relationship was found between the groups when Pp and pp genotypes were compared p<0.001

[f] Significant relationship was found between the groups when PP and Pp genotypes were compared p<0.001

[d] Significant relationship was found between the groups when XX and xx genotypes were compared p<0.001

[e] Significant relationship was found between the groups when Xx and xx genotypes were compared p=0.028

[g] Significant relationship was found between the groups when XX and Xx genotypes were compared p<0.001

[h] Significant relationship was found between the groups when SS and LL genotypes were compared p<0.001

[i] Significant relationship was found between the groups when SS and SL genotypes were compared p=0.011

[j] Significant relationship was found between the groups when SL and LL genotypes were compared p=0.013

[k] Logistic regression analysis

optimal oocyte cytoplasm and oolemma maturation [3], it counts as an important factor that determines the quality of oocyte. A good quality oocyte is essential for maturation, fertilization and post-embryonic development. Edwards et al. (1984) reported that fertilization rates were higher with mature oocytes and Tesarik et al. (1995) found that addition of estradiol to human oocyte maturation medium increased the fertilization and cleavage rates of in-vitro matured oocytes [20, 21].

In the present study we have analysed three polymorphisms of the *ESR*1 gene in patients undergoing ovarian stimulation for in-vitro fertilization and embryo transfer in order to assess the numbers of follicles and oocytes produced by individuals having different *ESR*1 genotypes as well as the maturation, fertilization and pregnancy rates obtained from these patients. Throughout the study we investigated whether these polymorphisms affect the response and the incidence of a polymorphism is different between women undergoing IVF and controls.

*Pvu*II and *Xba*I polymorphisms located in intron 1 are approximately 50 bp apart from each other. Their location in the intron makes it unlikely that the polymorphisms may affect ESR expression. Neither PvuII nor XbaI polymorphism cause amino acid substitutions. However they may be in linkage disequilibrium with other *ESR*1 mutations which may affect both the estrogen receptor gene expression and function [13].

The *ESR*1 gene promoter has a very complex genomic organization. It contains multiple promoter regions with alternative splice sites, resulting in expression of alternative first exons and different estrogen protein transcripts [22]. Thus (TA)n dinucleotide repeat lenght may affect alternative promoter and first exon usage resulting in different expression patterns [17]. Different expression patterns may affect the function of ESR protein and also the amounts of *ESR1* protein that is produced.

We first evaluated the genotype distribution and allele frequencies of the three polymorphisms in Turkish women both in the controls as well as in patients undergoing IVF program. For the *Pvu*II, *Xba*I and (TA)n polymorphisms, significant relationship was observed between infertile and fertile groups (p=0.001, p=0.002 and p=0.004 respectively). Furthermore we found a considerable difference in the allele frequencies in every three polymorphisms among the groups (p=0.001, for each three). These results indicate that these polymorphisms may have impact on infertility.

Role of *ESR*1 polymorphisms on human fertility was indicated previously [23] and it has been reported that *ESR*1 genotype manifest in modern societies as successful outcome in women undergoing IVF [7, 8]. According to some previous findings polymorphism can affect the outcome of IVF by affecting folliculogenesis, oocyte maturation, embryo quality and endometrial receptivity [8]. In the present study strong negative associations were found between severity of *PvuII* polymorphism in the ESR1 gene with embryo quality (p<0.001).

Sundarrajan et al. examined the relationship of *Pvu*II and a rare *BstUI* polymorphism in the *ESR*1 gene to the mean numbers of follicles and oocytes, their mean ratios, mean number of embryos and pregnancy rates [8]. They reported that the mean follicular number, oocyte number, embryo number, follicular size and prengancy rate were significantly smaller in patients homozygous for *Pvu*II polymorphism.

Sundarrajan et al. (1999) also investigated 72 pregnant patients and found that the number of obtained and replaced embryos in each of the three *Pvu*II genotypes showed a highly significant negative correlation with the severity of the polymorphism [8]. In our study fertilization rate was found to have a strong negative correlation with the severity of each *Pvu*II, *Xba*I and (TA)n repeat polymorphisms. Moreover a significant relationship was observed between maturation rates and *Pvu*II & *Xba*I genotypes (p<0.001).

Recently, Altmae et al. (2007) evaluated the impacts of ESR1 *Pvu*II, *Xba*I and (TA)n genotypes on the the etiology of female infertility, as well as their contributions to the COH and pregnancy outcome of IVF in 159 infertile women undergoing IVF-ET [10]. They concluded that *ESR*1 variants predict the chance for clinical pregnancy rate per COH rather than per single embryo transfer. Contrary to findings in this study, Georgiou et al. (1997) and Sundarrajan et al. (1999) showed that there was a relationship between some *ESR*1 variants and clinical pregnancy rate per embryo transfer [7, 8]. In our study the impact of polymorphism on maturation rate, fertilizaton rate and post-embryonic development was shown nevertheless no statistical relationship was detected between any of the *ESR1* variants and pregnancy rate.

When a haplotype analysis was made in order to interrogate the linkage between these three polymorphisms, linkage disequilibrium was only detected between *PvuII* and *XbaI* polymorphisms. For the infertile and fertile group four haplotypes were observed and the most frequent haplotype; *PX* in the control group was assigned as the reference. Infertility risk was estimated by comparing other haplotype frequencies against the reference and infertility risk for women having *Px* genotype was found to be approximately 2 times higher than that for the women having *PX* genotype (p<0.001).

Conclusions

In conclusion, this study showed a significant association between *ESR*1 genotypes and risk for infertility and some IVF parameters. Still, further studies are needed to confirm our findings in larger scale studies, which will probably

reveal more significant results. The expression of *ESR*1 could be regulated depending on the *ESR*1 genotypes and *Pvu*II, *Xba*I and (TA)n polymorphisms may serve as markers in predicting the risk for infertility, ovarian response of IVF patients and success rates of IVF treatment. Nevertheless further studies are necessary to determine whether it is possible to apply this relationship to the pre-cycle evaluation of individual genetic predisposition.

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References

1. Goldenberg RL, Vaitukaitis JL, Ross GT. Estrogen and follicle-stimulating hormone interactions on follicle growth in rats. Endocrinology. 1972;90:1492–8.
2. Ireland JJ, Richards JS. Acute effects of estradiol and follicle-stimulating hormone on specific binding of human [125I] iodofollicle-stimulating hormone to rat ovarian granulosa cells in vivo and in vitro. Endocrinology. 1978;102:876–83.
3. Filicori M, Cognigni GE, Taraborrelli S, Spettoli D, Ciampaglia W, de Fatis CT, et al. Luteinizing hormone activity supplementation enhances follicle-stimulating hormone efficacy and improves ovulation induction outcome. J Clin Endocrinol Metab. 1999;84 (8):2659–63.
4. Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, et al. Cloning of the human estrogen receptor cDNA. Proc Natl Acad Sci U S A. 1985;82(23):7889–93.
5. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS Lett. 1996;392(1):49–53.
6. Aléssio AM, Siqueira LH. de Carvalho ECCouto, Barini R, Mansur AP, 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;14:180–5.
7. Georgiou I, Konstantelli M, Syrrou M, Messinis IE, Lolis DE. Oestrogen receptor gene polymorphisms and ovarian stimulation for in-vitro fertilization. Hum Reprod. 1997;12(7):1430–3.
8. Sundarrajan C, Liao W, Roy AC, Ng SC. Association of oestrogen receptor gene polymorphisms with outcome of ovarian stimulation in patients undergoing IVF. Mol Hum Reprod. 1999;5:797–802.
9. Kukuvitis A, Georgiou I, Bouba I, Tsirka A, Giannouli CH, Yapijakis C, et al. Association of oestrogen receptor alpha polymorphisms and androgen receptor CAG trinucleotide repeats with male infertility: a study in 109 Greek infertile men. Int J Androl. 2002;25(3):149–52.
10. Altmäe 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(8):521–6.
11. Hsieh YY, Wang YK, Chang CC, Lin CS. Estrogen receptor alpha-351 XbaI*G and -397 PvuII*C-related genotypes and alleles are associated with higher susceptibilities of endometriosis and leiomyoma. Mol Hum Reprod. 2007;13(2):117–22.
12. Baltaci V, Şatıroğlu H, Kabukçu C, Ünsal E, Aydınuraz B, Üner Ö, et al. Relationship Between Embryo Quality and Aneuploidies. Reprod BioMed Online. 2006;12:77–82.
13. Yaich L, Dupont WD, Cavener DR, Parl FF. Analysis of the PvuII restriction fragment length polymorphism and exon structure of the estrogen receptor gene in breast cancer and peripheral blood. Cancer Res. 1992;52:77–83.
14. Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Orimo H. Association of bone mineral density with polymorphism of the estrogen receptor gene. J Bone Miner Res. 1996;11:306–11.
15. Raymond M, Rousset F. GENEPOP (version 4.0): an updated version of GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Heredity. 1997;86:248–9.
16. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: Analysis and visualization of LD and haplotype maps. Bioinformatics. 2005;21:263–5.
17. Becherini L, Gennari L, Masi L, Mansani R, Massart F, Morelli A, et al. Evidence of a linkage disequilibrium between polymorphisms in the human estrogen receptor alpha gene and their relationship to bone mass variation in postmenopausal Italian women. Hum Mol Genet. 2000;9(13):2043–50.
18. Albagha OM, Pettersson U, Stewart A, McGuigan FE, MacDonald HM, Reid DM, et al. Association of oestrogen receptor alpha gene polymorphisms with postmenopausal bone loss, bone mass, and quantitative ultrasound properties of bone. J Med Genet. 2005; 42:240–6.
19. 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(14):1745–54.
20. Edwards RG, Fishel SB, Cohen J, Fehilly CB, Purdy JM, Slater JM, et al. Factors influencing the success of in vitro fertilization for alleviating human infertility. J In Vitro Fert Embryo Transf. 1984;1(1):3–23.
21. Tesarik J, Mendoza C. Nongenomic effects of 17 beta-estradiol on maturing human oocytes: relationship to oocyte developmental potential. J Clin Endocrinol Metab. 1995;80(4):1438–43.
22. Kos M, Reid G, Denger S, Gannon F. Genomic organization of the human ERα gene promoter region. Mol Endocrinol. 2001;15:2057–63.
23. Corbo RM, Ulizzi L, Piombo L, Martinez-Labarga C, De Stefano GF, Scacchi R. Estrogen receptor alpha polymorphisms and fertility in populations with different reproductive patterns. Mol Hum Reprod. 2007;13(8):537–40.