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Association of polymorphisms in the estrogen receptors alpha, and beta (ESR1, ESR2) with the occurrence of male infertility and semen parameters

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ABSTRACT

Male infertility is a multifactorial condition with a strong genetic component. In the last decade a large number of investigations focused on the identification of gene variants affecting spermatogenesis in human. Polymorphisms of the estrogen receptor (ER) genes, have been implicated in male infertility, however, comprehensive data are lacking. We investigated the association between the ER-α gene (ESR1) PvuII and XbaI and ER-β gene (ESR2) RsaI and AluI polymorphisms and the idiopathic male infertility in Iranian males. Polymerase chain reaction (PCR) method and restriction fragment length polymorphism (RFLP) were used to detect the ER-α, and ER-β gene polymorphisms in 164 infertile men and 164 age-matched healthy controls. Reproductive hormones were measured and at least two semen analyses were performed in each subject. Significant differences were observed in the frequency distribution of PvuII and XbaI in the ESR-α gene and RsaI and AluI in the ER-β gene between patients and controls. The presence of the ER-α PvuII TC (OR = 0.56, 95% CI: 0.26–0.80; $P = 0.011$), ER-α XbaI AG (OR = 0.51, 95% CI: 0.31–0.84; $P = 0.017$), and ER-β AluI GG (OR = 0.48, 95% CI: 0.265–0.84; $P = 0.012$) genotypes suggest a protective effect for infertility. The ER-β RsaI AG (OR = 2.32, 95% CI: 1.61–3.22; $P = 0.012$) and ER-β AluI AG (OR = 2.76, 95% CI: 1.64–3.66; $P = 0.014$) genotypes are associated with increased infertility risk. Subjects (both fertile and infertile) with ER-α PvuII TT, ER-α XbaI AA, ER-β RsaI AG, and ER-β AluI AG genotypes had significantly lower levels of serum sex hormone binding globulin (SHBG), and luteinizing hormone (LH), but, higher serum levels of free estradiol and follicle stimulating hormone (FSH). The same genotypes had significantly lower values for sperm density, sperm motility, and percentage of sperm with normal morphology. Our results further suggest a possible role of ESR-α, and ER-β variants on male infertility. Further studies are needed to replicate our findings as well as to better elucidate the biological mechanisms of the modulation of ESR-α, and ER-β on male infertility.

1. Introduction

The cause of infertility is shared equally between male and female partners. At least 30% of cases of male infertility are still not determine and are considered as idiopathic infertility [1]. Estrogens can induce oxidative DNA damage. Oxidative DNA damage may be involved in estrogen induced effects on male reproduction [2]. Estrogen excess during the adulthood can deteriorate sperm production and maturation [3]. Estrogen acts both peripherally and in the central nervous system. The physiological responses to estrogens are modulated by the estrogen receptors α (ER-α, ER1) and β (ER-β, ER2) genes. ESR-α gene is located on chromosome 6q25 and is composed 8 exons separated by 7 intronic regions with a total size of 140 kb [4]. The ER-β gene resides on chromosome 14q22-24, comprises eight exons and spans approximately 40 kb [5]. ER-α encodes a 595 amino acid protein [6], while ERβ encodes a 530 amino acid protein [7]. A third ER, termed ER-γ, has been detected in teleosti, the most widespread subclass of vertebrate fish [8]. It is similar to ER-β and may have resulted through duplication of it. Various polymorphisms have been determined in ESR-α [9]. The most widely studied involve PvuII (rs2234693) and XbaI (rs9340799) in the ER-α gene, which are located in the first intron and separated by only 46 bp. The PvuII (T397C) polymorphism caused by a T/C transition in intron 1, while the XbaI (G351A) polymorphism is caused by a G/A transition located 50 base pairs downstream of the PvuII polymorphic site [10,11]. There are also several sequence variants of the ER-β gene, including two silent G/A polymorphisms, *RsaI* (rs1256049) and *AluI* (rs4986938) [12]. Both receptors are expressed in the testis and in the epididymis [13]. In human testis, ER-α and ER-β have also been found in ejaculated spermatozoa [14,15]. It has been shown that, the absence

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of ER-α leads to decreased epididymal sperm content, decreased sperm motility and fertilizing capacity [16,17].

Association between male infertility and polymorphisms in ER-α [18–22] and ER-β genes [23–25] has been shown in few studies. Polymorphisms in ER-α gene (*XbaI* and *Pvull*) have been shown to be associated with azoospermia or severe oligozoospermia [18,19]. In a study by Lazaros et al. [25] in men with oligozoospermia those with ER-α 397T/C and 397C/C genotypes had higher sperm motility while those with 397T/T genotype had lower sperm motility. Overall, studies regarding regarding the effect of ER genes on male fertility have produced conflicting evidence [26,27]. In a recent review article on the genetic causes of male factor infertility by O'Flynn O'Brien et al. [28], they concluded that the ER genes polymorphisms should be examined further to replicate the results of previous uncoordinated studies and to better elucidate the impact of these polymorphisms on male fertility. Therefore, the aim of the present study was to determine the importance of ER-α and ER-β polymorphisms in the etiology of unexplained male infertility and to find an association of these polymorphisms with sperm parameters. Moreover, by comparing sex hormone levels in subjects with different ER-α, and ER-β genotypes, we wished to examine whether these polymorphisms might play a role for ER-α, and β function *in vivo*.

2. Materials and methods

2.1. Subjects

This study included 328 subjects, in which 164 were infertile men (mean age 31.6 ± 4.8 years, range 25–40 years) with idiopathic oligoasthenoteratozoospermia (OAT). An equal number of age-matched fertile healthy men (n = 164) (mean age 32.1 ± 5.2 years, range 25–42 years) of the same ethnic group and drawn from the same geographical and linguistic lineage, and similar socioeconomic status were also recruited in the study and served as the controls. They were recruited in the study after screening for eligibility. Infertile men did not exhibit any known reason of infertility, such as obstructive azoospermia, endocrinological defect, karyotypic abnormality, and history of cryptorchidism, and varicocele. They were seeking work-up for couple infertility at our Urology facility. Infertility was defined as an attempt at conception by the couple that lasted more than 24 months. Their female partners were normal according to extensive investigation. All of the men in control group had fathered at least one child spontaneously. They exhibited normal semen parameters in accordance with WHO guidelines [29]. Also they possessed normal serum hormones, and inhibin B levels. Infertile and fertile men belonged to the same linguistic lineage and inhabitants of the same geographical regions.

At least two semen analyses were done one month apart in all the individuals participating in this study. To minimize the variability of semen analysis results, the duration of ejaculation abstinence was 3 days. Semen analyses were performed according to the World Health Organization guidelines [29]. The values for normal semen parameters were sperm density greater than 20×10^6/ml, sperm motility grade A + B greater than 50%, normal morphology greater than 30% and/or semen volume greater than 2 ml. Informed consent was obtained from all participants and local Medical Ethics Committee approved the study. The study protocol complied with the World Medical Association Declaration of Helsinki (1964, 2000 version).

2.2. Inclusion and exclusion criteria

An inclusion criterion for infertile subjects was a history of infertility for at least two years with no known reason for their infertility. Exclusion criteria for infertile cases included, history of varicocele or testicular torsion, azoospermia, urinary tract infections, iatrogen infertility, any endocrinopathy, karyotype anomalies, Y-chromosome microdeletions, use of cytotoxic drugs, immunosuppressants, anticonvulsives, androgens or antiandrogens; leukocytospermia (more than 10^6 white blood cells per millilitre), or positive mixed agglutination reaction test; tobacco use; and drug, alcohol or substance abuse were excluded. Patients were also excluded from study if they had concomitant medical problems known to be associated with decreased fertility; hepatobiliary disease; significant renal insufficiency; a body mass index (BMI) of 30 kg/m^2 or greater; and occupational and environmental exposures to potential reproductive toxins. None of the participants had ejaculatory disorder.

2.3. Evaluations

Peripheral venous blood samples were obtained from participants via an antecubital vein after 10 h fasting between 8 a.m. and 9 a.m. All of the subjects underwent comprehensive medical history, and andrological examination including medical history, semen analysis, scrotal ultrasound, hormonal analysis for the measurement of serum luteinizing hormone (LH), follicle stimulating hormone (FSH), total testosterone (T), free testosterone (free T), prolactin (PRL), estradiol (E2), free estradiol (free E2), sex hormone binding globulin (SHBG), thyroid stimulating hormone (TSH), free thyroxin (T4), free triiodothyronin (T3), and Inhibin B levels, karyotype and Y chromosome microdeletion screening. Hematological and routine biochemistry analyses were also done. The laboratory evaluation also included at least two semen analyses after 3 days of abstinence with an interval of 4 weeks between them. Values for semen parameters were calculated as means of two analyses.

2.4. Hormonal assays

Total serum T was measured using radioimmunoassay (RIA) kit (3H Testosterone, Biomerieux, Lyon, France) with a sensitivity of detection of 4 pg/ml. The normal reference range for T was 3.5–9.5 ng/ml. Serum LH and FSH levels were determined using time-resolved immunofluorometric assay kits (DELFIA hLH for LH and DELFIA hFSH for FSH; Wallac Co., Turku, Finland). The intra- and inter-assay coefficients of variation of the individual immunofluorometric assay method were below 9% within the reference ranges. The reference ranges were as follows: LH, 1.0–8.4 IU/l; and FSH, l.0–10.5 IU/l. SHBG concentrations were measured in serum using 1235 AutoDELFIA automatic system based on a time-resolved fluoroimmunoassay (AutoDELFIA SHBG, Wallac Co.). The between-assay coefficient of variation is 2.3–3.0%. The reference range for SHBG is 15–50 nmol/l. Serum E2 was quantified using a RIA (Esoterix, Inc., Calabasas Hills, CA, USA), in which intra- and inter-assay coefficients of variation were 5.2% and 8.0%, respectively. The normal reference range for E2 was 0–50 pg/ml. Serum levels of PRL were measured by commercial RIA kit. This commercial kit has been used previously with inter- and intra-assay variations of less than 10%. The reference ranges for PRL was 92–697 pmol/l. Serum Inhibin B was determined by ELISA method using kit reagents and inhibin B standard (Oxford Bio-innovation Ltd., Oxon, UK). The assay sensitivity was 4 pg/ml and the between-assay variation was 15%. Ciba Corning kits (Ciba Corning Diagnostics SA, Spain) were used to determine by chemoluminescence TSH (intra-assay < 4.7%, interassay < 6.25%), FT3 (intra-assay < 3.8%, interassay < 6.2%), and FT4 (intra-assay < 3.26%, interassay < 4.95%). The reference ranges were as follows: 0.35–5.5 mU/l for TSH, 3.5–6.5 pmol/l for FT3, and 10.3–23.2 pmol/l for FT4.

FT and free E2 (FE2) were determined using the method described by Vermeulen et al. [30] and van den Beld et al. [31] tak-

ing the blood levels of total T, total E2, and SHBG into account and assuming a fixed albumin concentration of 43 g/l.

2.5. ER-α genotype determination

Genomic DNA for genotyping was extracted from the buffy coat of EDTA blood samples, following a standard salting-out procedure [32]. Genotyping of the ER-α polymorphism was performed using a polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) analysis. For determining the PvuII (c.454–397 T > C) and XbaI (c.454–351 A > G) polymorphisms the following oligonucleotide primers were used: forward, 5′-CTG CCA CCC TAT CTGTAT CTT TTC CTATTC TCC-3′; and reverse, 5′-TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA-3′. The PCR amplification was performed in a total volume of 50 μl consisting of 1 U of Taq DNA polymerase, 100 ng template DNA, 50 ng of each primer, 0.2 mM deoxyribonucleotide triphosphate, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, and 3 mM $MgCl_2$. Each PCR amplification used the following profile: an initial denaturation step of 5 min at 95 °C temperature followed by 32 cycle of 95 °C/50 s (denaturation), an annealing step at 62 °C for 50 s, an initial extension at 72 °C for 50 s, and final extension step at 72 °C for 1 min. To differentiate c.454–397 TNC (PvuII) and c.454–351 ANG (XbaI) polymorphisms, the amplified PCR fragment of 1372 bp was digested overnight with XbaI and PvuII restriction enzymes, separately. The cleavage products were electrophoresed on 3% agarose gel. The alleles of the PvuII polymorphism were defined as T and C, denoting respectively the presence and the absence of the restriction site [33]. The genotypes for *PvuII* (397T → C) polymorphic sites were characterized as TT/TC/CC. Heterozygous TC exhibited fragments of 1372, 982, and 390 bp lengths, while the mutated homozygous variant CC produced fragments of 982, and 390 bp lengths. Wild-type CC exhibited one fragment of 1372 bp. The alleles of the XbaI polymorphism were defined as A and G, denoting respectively the presence and the absence of the restriction site [33]. The genotypes for XbaI (351A → G) polymorphic sites were characterized as AA/AG/GG. The mutated homozygous variant AA produced fragments of 936 bp, and 436 bp lengths, but heterozygote AG produced fragments of 1327 bp, 936 bp, and 436 bp lengths. Wild-type GG exhibited one fragment of 1327 bp.

2.6. ER-β genotype determination

The RsaI (1082G > A) and AluI (1730G > A) polymorphisms in the ER-β gene were detected by PCR amplification and RsaI and AluI digestion, respectively. The RsaI site is located in ligand-binding domain of exon 5 and the AluI site in 3′-untranslated region of exon 8. In both sites a G nucleotide was considered the wild-type sequence and was not digestible by *Rsa*I or *Alu*I. The primers for the RsaI and the AluI were, 5′-TCTTGCTTTCCCCAGGCTTT-3′ (forward) and 5′-ACCTGTCCAGAACAAGATCT-3′ (reverse), and 5′-GACCTGCTGCTGGAGATGCT-3′ (forward) and 5′-AATGAGGGACCACACAGCA-3′, respectively.

The PCR amplification was carried out in a total volume of 25 μl consisting of 1 U of Taq DNA polymerase, 1× Taq DNA polymerase buffer, 1 μl extracted DNA, 200 μM deoxyribonucleotide triphosphate, 1 mM $MgCl_2$, and 0.8 μM of each primer. Amplification condition included initial denaturation at 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1.30 min, and final extension step at 72 °C for 7 min. Each PCR product was then digested with restriction enzymes *RsaI* and *AluI*, respectively. The separation of The DNA fragments was accomplished by electrophoresis on 2% agarose gel electrophoresis, and the visualized under UV light after ethidiun bromide staining. *Rsa*I digestion will therefore result one uncleaved fragment of 409 bp in subjects carrying the homozygous wild-type GG genotype, two fragments of 110 bp and 299 bp in homozygous polymorphic AA subjects, and all three fragments in heterozygous AG carriers. *Alu*I digestion produced one fragment of 405 bp in the homozygous subjects carrying the wild-type GG polymorphism, two fragments of 163 bp and 242 bp in the homozygous polymorphic AA polymorphism, and all three fragments in heterozygous AG subjects. Three genotypes were constructed for RsaI (1082G → A) (GG/GA/AA) and AluI (1730A → G) (AA/AG/GG) polymorphic sites, based upon fragment patterns. The one who performed the genotyping was blinded to the identity of the participants and replicate quality control samples. To examine the accuracy of our genotyping protocols, we performed genotyping quality controls. Ten quality control samples were taken from the samples of randomly selected subjects of the study and tested three to five times per genotype. The consistency rate was 100% for quality control samples.

2.7. Statistical analysis

We had a sufficient power on the sample (0.80) to detect a medium-large effect size ($d = 0.60$) between the two groups (cases $n = 164$, controls $n = 164$). All results are expressed as the mean ± SD. The significance of differences in the genotype distribution and allele frequency were tested using the χ^2 test. The degree of subjects conforming to Hardy–Weinberg equilibrium, single genotype and the frequency of allele between each group were tested using the χ^2 test. Clinical variables were compared using the Student's *t*-test, whereas laboratory parameters were compared by Fisher's exact test or analysis of covariance. The normality of continuous variables was tested using the Shapiro–Wilk-test. We used the paired Student's *t*-test to compare continuous parametric variables between groups, while for categorical variables the Mann–Whitney *U*-test was used. An Odds Ratio (OR) and 95% confidence intervals (CI) were calculated with respect to the presence of the reference genotype using a logistic regression model. The multiple logistic regression model was constructed to evaluate the independent relationship between the various genotypes and the presence of infertility with control for age, BMI, duration of infertility, educational level, and occupational status, as the potential confounders. All analyses were conducted assuming an additive mode of inheritance. The association between the combined genotypes of the ER-α and ER-β genes polymorphisms and the risk of infertility was also evaluated. Haplotype construction and haplotypic frequencies were determined by using the Haploview software version 3.2 available at http://www.hapmap.org. Linkage disequilibrium analysis was also performed with the Haploview program. The level of significance was set at $P < 0.05$ and two-sided tests were carried out as the standard. Data were analyzed with SPSS 16.0 for Windows (SPSS, Chicago, USA) software.

3. Results

3.1. Patient characteristics

Baseline clinical and demographic characteristics of the participants are described in Table 1. Mean age, duration of infertility, duration of marriage and BMI were similar between the infertile and fertile groups.

3.2. ER-α genotypes

Strong linkage disequilibrium was found between the PvuII and XbaI polymorphisms of the ESR-α gene ($D' = 0.962$). There was no detectable deviation from the Hardy–Weinberg equilibrium for the PvuII TC polymorphism ($\chi^2 = 0.15$, $P = 0.76$), but there was a slight deviation for the XbaI AG polymorphism ($\chi^2 = 3.86$, $P = 0.046$). Allele and genotype frequencies for the ESR-α variant polymorphisms in

Table 1
Baseline demographics, serum hormones, and semen parameters of study groups.

Characteristics	Patients ($n=164$)	Controls ($n=164$)	P value
Age (year)	31.6 ± 4.8	32.1 ± 5.2	0.08
Duration of marriage	6.8 ± 3.1	7.1 ± 2.8	0.08
Infertility duration (year)	4.1 ± 2.2	4.2 ± 2.2	0.09
BMI (kg/m^2)	27.1 ± 2.6	26.8 ± 2.7	0.07
Occupational status No. (%)			
Employed	143 (87.2)	142 (86.6)	0.1
Unemployed	21 (12.8)	22 (13.4)	0.1
Educational level			
None	0	0	–
Primary school	8 (4.9)	8 (4.9)	0.08
High school	104 (63.4)	103 (62.8)	0.09
Graduate	52 (31.7)	53 (32.3)	0.07
Serum hormones			
Testosterone (ng/ml)	5.2 ± 1.31	6.3 ± 2.12	0.06
Free testosterone (pg/ml)	149.6 ± 49.2	134.8 ± 47.1	0.06
LH (IU/l)	6.4 ± 2.3	6.9 ± 2.1	0.08
FSH (IU/l)	7.1 ± 2.4	6.5 ± 2.2	0.08
Estradiol (pg/ml)	18.7 ± 6.4	20.6 ± 7.1	0.04
Free estradiol (pg/ml)	0.43 ± 0.22	0.37 ± 0.17	0.04
PRL (pmol/l)	367 ± 121	376 ± 119	0.09
TSH (mIU/ml)	2.2 ± 1.2	2.1 ± 1.2	0.1
Free thyroxine (pmol/l)	14.3 ± 2.7	14.4 ± 2.7	0.08
Free triiodothyronine (pmol/l)	3.6 ± 1.2	3.7 ± 1.1	0.08
SHBG (nmol/l)	21.1 ± 5.3	25.4 ± 5.6	0.032
Inhibin B (pg/ml)	170.6 ± 19.7	185.2 ± 22.7	0.06
Semen parameters			
Ejaculate volume (ml)	2.7 ± 1.3	2.8 ± 1.2	0.1
Total sperm/ejaculate ($\times 10^6$)	44.3 ± 11.2	185.4 ± 24.2	0.001
Sperm density ($\times 10^6$/ml)	13.7 ± 3.4	60.4 ± 11.8	0.001
Motility (%motile)	21.2 ± 4.5	63.6 ± 8.2	0.001
Morphology (%normal)	20.2 ± 4.2	57.7 ± 8.4	0.001

Key: BMI = body mass index, LH = luteinizing factor, FSH = follicle stimulating hormone, PRL = prolactin, TSH = thyroid stimulating hormone, SHBG = sex hormone binding globulin.

Table 2
Frequency distribution of the ER-α, and ER-β alleles, genotypes and haplotypes in study subjects, No. (%).

Variables	All participants ($n=328$)	Cases ($n=164$)	Controls ($n=164$)	P value
ER-α PvuII				
TT	82 (25.0)	49 (29.9)	33 (20.1)	0.024
TC	156 (47.6)	70 (42.7)	86 (52.4)	0.012
CC	90 (27.4)	45 (27.4)	45 (27.4)	1.0
T	320 (48.8)	168 (51.2)	152 (46.3)	0.076
C	336 (51.2)	160 (48.8)	176 (53.7)	0.073
ER-α XbaI				
AA	103 (31.4)	62 (37.8)	41 (25.0)	0.014
AG	172 (52.4)	77 (47.0)	95 (57.9)	0.018
GG	53 (16.2)	25 (15.2)	28 (17.1)	0.086
A	378 (57.6)	201 (61.3)	177 (54.0)	0.048
G	278 (42.4)	127 (38.7)	151 (46.0)	0.032
ER-β RsaI				
GG	294 (89.6)	142 (86.6)	152 (92.7)	0.078
GA	29 (8.8)	21 (12.8)	8 (4.8)	0.011
AA	5 (1.5)	1 (0.61)	4 (2.4)	0.087
G	617 (94.1)	305 (93.0)	312 (95.1)	0.091
A	39 (5.9)	23 (7.0)	16 (4.9)	0.087
ER-β AluI				
AA	38 (11.6)	17 (10.4)	21 (12.8)	0.82
AG	145 (44.2)	82 (50.0)	63 (38.4)	0.011
GG	145 (44.2)	65 (39.6)	80 (48.8)	0.012
A	221 (33.7)	116 (35.4)	105 (32.0)	0.088
G	435 (66.3)	212 (64.6)	223 (68.0)	0.074
ER-α haplotypes				
PvuII T–XbaI A	318 (48.5)	166 (50.6)	152 (46.3)	0.076
PvuII C–XbaI A	60 (9.1)	35 (10.7)	25 (7.6)	0.082
PvuII C–XbaI G	276 (42.1)	125 (38.1)	151 (46.1)	0.015
PvuII T–XbaI G	2 (0.3)	2 (0.6)	0 (0.0)	0.84
ER-β haplotypes				
RsaI G–AluI A	183 (27.9)	94 (28.7)	89 (27.1)	0.67
RsaI G–AluI G	434 (66.1)	211 (64.3)	223 (68.0)	0.084
RsaI A–AluI A	38 (5.8)	22 (6.7)	16 (4.9)	0.076
RsaI A–AluI G	1 (0.2)	1 (0.3)	0 (0.0)	0.92

Key: ER = estrogen receptor.

the fertile and infertile subjects are shown in Table 2. For the PvuII polymorphism, T allele frequency in cases (51.2%) was higher than those of in controls (46.3%); however, the difference did not reach statistical significance ($P=0.074$). The PvuII genotype distributions were significantly different between fertile and infertile groups ($P=0.014$). The genotype frequencies were TT-20.1%, CC-52.4%, and TC-27.4% for fertile men, and TT-29.9%, CC-42.7%, and TC-27.4%, for infertile subjects. Subjects carrying the PvuII CC genotype had decreased risk of infertility compared to the subjects carrying the PvuII TT genotype (OR = 0.56, 95% CI: 0.26–0.80; $P=0.011$) (Table 3).

The genotype and allelic distributions of ESR-α XbaI gene in infertile and fertile men are given in Table 2. For the XbaI polymorphism, A allele frequency in normal controls was lower than infertile men (54.0% vs. 61.3%). This difference was statistically significant ($P=0.048$). The XbaI G allele frequency is significantly lower in infertile subjects (38.7%) than in controls (46.0%) ($P=0.032$). The homozygous AA genotype of the ESR-α XbaI was more prevalent in the infertile group (37.8%) than in the controls (25.0%) ($P=0.014$). Multivariate analysis with the logistic regression model revealed that the presence of XbaI AG genotype was independent protective factor against infertility in men (OR = 0.51, 95% CI: 0.31–0.84; $P=0.017$) (Table 3). The risk of XbaI A allele for infertility is 1.72 times higher (95% CI: 1.21–2.66; $P=0.042$). Regarding XbaI G allele, carriers have more than 30% decreased risk for development of infertility (OR = 0.68, 95% CI: 0.47–0.89; $P=0.032$).

3.3. ER-β genotypes

The study of the haplotype frequencies for the two polymorphisms showed that RsaI and AluI polymorphisms were not in linkage disequilibrium ($\chi^2=4.78$, $P>0.3$. The genotype frequencies observed for the ER-β RsaI and ER-β AluI polymorphisms among the study groups did not differ significantly from those expected under Hardy–Weinberg equilibrium ($\chi^2=0.18$, $P=0.72$). The allele frequencies for ER-β RsaI were 94.1% for G and 5.9% for A alleles; for ER-β AluI the frequencies were 33.7% for A and 66.3% for G alleles (Table 2). The allele frequencies between two groups were not significantly different (all $P\geq 0.05$). In logistic regression analyses, the genotype distribution of ER-β RsaI polymorphism in the infertile group was significantly different from that of the controls (GG/GA/AA rates were 86.6%/12.8%/0.61% and 92.7%/4.8%/2.4% for the cases and control groups, respectively; $P=0.011$) (Table 2). As the frequency of the homozygous RsaI AA genotype was very rare both in the cases (0.61%) or the controls (2.4%), the RsaI GA, and RsaI AA genotypes were combined together and are referred to as variant genotypes (RsaI GA + RsaI AA) of ER-β. There was a significant difference in genotype distribution between the infertile and control groups [RsaI GA + RsaI AA rates were 13.4% for the infertile patients and 7.3% for controls, respectively (OR = 2.30, 95% CI, 1.61–3.22: $P=0.011$)]. The adjusted OR for infertility was significantly increased in individuals with RsaI GA genotype compared to individuals with the RsaI GG genotype (OR = 2.32, 95% CI 1.61–3.22, $P=0.012$) (Table 3).

There was no significant difference in the ER-β AluI A and ER-β AluI G allele frequencies between the infertile group and the control group (35.4% vs. 32.0%, and 64.6% vs. 68.0%, respectively) (Table 2). When OR were adjusted for confounding factors, individuals with ER-β AluI GG genotypes had more than 50% decreased risk to develop infertility compared to the ones with ER-β AluI AA genotype (OR = 0.48, 95% CI 0.25–0.84, $P=0.012$). In contrast, com-

Table 3
Frequency distribution of the ER-α, and ER-β alleles, genotypes and haplotypes, and their associations with the risk of infertility.

Genotype frequency	Controls (n = 164)	Cases (n = 164)	Crude OR (95% CI)	P value	Adjusted OR[a] (95% CI)	P value
ER-α PvuII						
TT	33 (20.1%)	49 (29.9%)	1.0 (Referent)			
CC	86 (52.4%)	70 (42.7%)	0.54 (0.27–.82)	0.016	0.56 (0.26–0.80)	0.011
TC	45 (27.4%)	45 (27.4%)	1.0 (0.64–1.27)	1.0	1.0 (0.66–1.29)	1.0
T	152 (46.3%)	168 (51.2%)	1.0 (Referent)			
C	176 (53.7%)	160 (48.8%)	0.82 (0.65–1.17)	0.072	0.89 (0.67–1.21)	0.074
ER-α XbaI						
AA	41 (25%)	62 (37.8%)	1.0 (Referent)			
AG	95 (57.9%)	77 (47.0%)	0.49 (0.29–0.81)	0.011	0.51 (0.31–0.84)	0.017
GG	28 (17.1%)	25 (15.2%)	0.81 (0.43–1.24)	0.081	0.87 (0.51–1.20)	0.088
A	177 (54.0%)	201 (61.3%)	1.0 (Referent)			
G	151 (46.0%)	127 (38.7%)	0.64 (0.42–0.88)	0.039	0.68 (0.47–0.89)	0.032
ER-β RsaI						
GG	152 (92.7%)	142 (86.6%)	1.0 (Referent)			
GA	8 (4.8%)	21 (12.8%)	2.34 (1.67–3.46)	0.010	2.32 (1.61–3.22)	0.012
AA	4 (2.4%)	1 (0.61%)	0.84 (0.63–1.44)	0.074	0.87 (0.67–1.26)	0.086
AG + AA	12 (7.3%)	22 (13.4%)	2.46 (1.71–3.72)	0.010	2.30 (1.61–3.22)	0.011
G	312 (95.1%)	305 (93.0%)	1.0 (Referent)			
A	16 (4.9%)	23 (7.0%)	1.57 (0.81–1.84)	0.075	1.52 (0.77–1.76)	0.086
ER-β AluI						
AA	21 (12.8%)	17 (10.4%)	1.0 (Referent)			
AG	63 (38.4%)	82 (50.0%)	2.81 (1.72–3.75)	0.010	2.76 (1.64–3.66)	0.014
GG	80 (48.8%)	65 (39.6%)	0.43 (0.22–0.83)	0.011	0.48 (0.25–0.84)	0.012
A	105 (32.0%)	116 (35.4%)	1.0 (Referent)			
G	223 (68.0%)	212 (64.6%)	0.82 (0.43–1.45)	0.067	0.87 (0.51–1.28)	0.072
ER-α haplotypes						
PvuII T–XbaI A	152 (46.3)	166 (50.6)	1.0 (Referent)			
PvuII C–XbaI A	25 (7.6)	35 (10.7)	1.76 (1.22–2.56)	1.82	(1.28–2.67)	0.032
PvuII C–XbaI G	151 (46.1)	125 (38.1)	0.54 (0.32–0.78)	0.49	(0.28–0.72)	0.003
PvuII T–XbaI G	0 (0.0)	2 (0.6)	1.12 (0.87–1.34)	1.04	(0.89–1.28)	0.87
ER-β haplotypes						
RsaI G–AluI A	89 (27.1)	94 (28.7)	1.0 (Referent)			
RsaI G–AluI G	223 (68.0)	211 (64.3)	0.68 (0.36–0.81)	0.64	(0.30–0.76)	0.027
RsaI A–AluI A	16 (4.9)	22 (6.7)	1.74 (0.78–2.46)	1.80	(0.81–2.40)	0.058
RsaI A–AluI G	0 (0.0)	1 (0.3)	1.18 (0.82–1.79)	1.03	(0.85–1.67)	0.91

Key: OR = odds ratio, CI = confidence interval, ER = estrogen receptor.
[a] Adjusted OR: adjusted in multivariate logistic regression models including age, duration of infertility, body mass index, occupational status, educational level and ER genotypes.

parison of the ER-β AluI AA, and ER-β AluI AG genotype groups showed that men with AluI AG genotype had a 2.76-fold higher risk to have infertility compared to individuals with AluI AA genotype (95% CI 1.64–3.66, P = 0.014).

3.4. Association of combined ER-α PvuII/ER-αXbaI and ER-β RsaI/ER-βAluI polymorphisms with infertility

The combined ER-α PvuII TC + ER-α XbaI AA variant genotype was significantly higher in the cases (24.4%) than the controls (18.3%, P = 0.024) (Table 4). When we used the ER-α PvuII TT + ER-α XbaI AA variant genotype as the reference, we found that the ER-α PvuII TC + ER-α XbaI AA variant genotype was associated with a higher risk of infertility (OR = 2.42, 95% CI 1.68–4.24, P = 0.021), while the ER-α PvuII TT + ER-α XbaI AG variant genotype was associated with a borderline increased infertility risk (OR = 1.87, 95% CI 0.89–2.68, P = 0.058) (not significant). We also found that the combined ER-α PvuII TC + ER-α XbaI AG variant genotype was associated with a more than 30% decreased risk of infertility (OR = 0.64, 95% CI 0.42–0.87, P = 0.017). Similarly, the frequency of the combined ER-β RsaI GG + ER-β AluI AG variant genotype was higher in the cases (49.4%) than the controls (36.0%, P = 0.014). When we used the ER-β RsaI GG + ER-β AluI AA variant genotype as the reference, we found the ER-β RsaI GG + ER-β AluI AG variant genotype was associated with a higher risk of infertility (OR = 2.76, 95% CI 1.82–4.54, P = 0.018). The combined ER-β RsaI GA + ER-β AluI GG variant genotype increased more than 3-fold the risk of infertility (OR = 3.24, 95% CI 2.25–5.49, P = 0.003). The ER-β RsaI GG + ER-β AluI GG variant genotype occurred at greater frequencies in controls vs. cases, suggesting that it is protective for infertility (OR = 0.45, 95% CI = 0.28–0.68, P = 0.002).

3.5. ESR-α PvuII–XbaI haplotype analysis

A high degree of linkage disequilibrium was seen between the ESR-α PvuII T/C and XbaI A/G polymorphisms (D' = 0.962), resulting in 3 common and 1 rare haplotypes: haplotype 1 (T-A) 48.8%, haplotype 2 (C-A) 9.1%, haplotype 3 (C-G) 42.1%, and two subject with haplotype 4 (T-G, 0.3%) (Table 2). In logistic regression analyses adjusting for the same covariates used in the genotype models, a decreased risk of infertility among men with the PvuII C-XbaI G haplotype was observed (OR = 0.49, 95% CI, 0.28–0.72, P = 0.003). Furthermore, simultaneous presence of the ESR-α PvuII C-XbaI A was associated with increased infertility risk (OR = 1.82, 95% CI, 1.28–2.67, P = 0.032) (Table 3).

3.6. ESR-β RsaI–AluI haplotype analysis

The most common haplotype (RsaI G-AluI G) had a frequency of 66.1%, while 27.9% were RsaI G-Alu A, 5.8% were RsaI A-AluI A and 0.2% were RsaI A-AluI G (Table 2). In logistic regression analyses, adjusting for the same potential confounders used in the genotype models, a significant association was observed with decreased risk of infertility, among men with the RasI G-AluI G haplotype (OR = 0.64, 95% CI 0.30–0.76, P = 0.027) compared to referent haplotype (RsaI G-AluI A) (Table 3). On contrary, individuals with RsaI A-AluI A haplotype had a borderline increased risk for infertility (OR = 1.80, 95% CI 0.81–2.40, P = 0.058) (not significant).

Table 4
Combined genotype distribution for ER-α PvuII/ER-α XbaI, and ER-β RsaI/ER-β AluI polymorphisms in all cases and controls.

ER genotypes	Cases (n = 164) (%)	Controls (n = 164) (%)	Odds ratio (OR)[a]	95% CI	P value
ER-α PvuII/ER-α XbaI					
TT + AA	20 (12.2)	11 (6.7)	1.0 (Referent)		
TT + AG	17 (10.4)	13 (7.9)	1.87	0.89–2.68	0.058
TT + GG	12 (7.3)	9 (5.5)	1.84	0.85–2.58	0.071
TC + AA	40 (24.4)	30 (18.3)	2.42	1.68–4.24	0.021
TC + AG	25 (15.3)	47 (28.7)	0.64	0.42–0.87	0.017
TC + GG	5 (3.1)	9 (5.5)	0.83	0.62–1.64	0.078
CC + AA	2 (1.2)	0 (0.0)	1.24	0.87–1.86	0.086
CC + AG	35 (21.3)	35 (21.3)	1.0	0.89–1.42	1.0
CC + GG	8 (4.8)	10 (6.1)	0.85	0.61–1.46	0.076
ER-β RsaI/ER-β AluI					
GG + AA	17 (10.4)	21 (12.8)	1.0 (Referent)		
GG + AG	81 (49.4)	59 (36.0)	2.76	1.82–4.54	0.018
GG + GG	44 (26.8)	72 (43.9)	0.45	0.28–0.68	0.002
GA + AA	0 (0.0)	0 (0.0)	NA		
GA + AG	0 (0.0)	0 (0.0)	NA		
GA + GG	21 (12.8)	8 (4.9)	3.24	2.25–5.49	0.003
AA + AA	0	0 (0.0)	NA		
AA + AG	1 (0.6)	4 (2.4)	0.76	0.54–1.87	0.069
AA + GG	0	0 (0.0)	NA		

Key: OR = odds ratio, CI = confidence interval, ER = estrogen receptor, NA = not applicable.
[a] Adjusted OR: adjusted in multivariate logistic regression models including age, duration of infertility, body mass index, occupational status, educational level and ER genotypes.

3.7. Compared between ER-α, and ER-β gene polymorphisms and reproductive hormones

All hormone measurements were within the normal limit, although subclinical changes were determined in some hormones. Table 1 shows the mean concentrations of reproductive hormones as compared between the infertile and control groups. The mean levels of total E2, free E2, and SHBG were statistically significantly decreased in infertile group compared with the control group (all $P < 0.05$), but the levels of testosterone, LH and FSH showed no statistically significantly difference. The association analysis of the two ER genes with the reproductive hormones was carried out using one-way ANOVA (Table 4). The following results were observed both in controls and infertile men.

3.8. ER-α genotypes

3.8.1. ER-α PvuII polymorphism

The SHBG (24.80 ± 4.2 nmol/l), total T (6.5 ± 2.4 ng/ml), and total E2 (20.8 ± 6.4 pg/ml) for individuals with ER-α PvuII TC genotype, was higher than for individuals with ER-α PvuII TT (21.40 ± 3.7 nmol/l, 4.8 ± 2.2 ng/ml, and 18.1 ± 6.2 pg/ml, respectively), and ER-α PvuII CC (22.24 ± 4.1 nmol/l, 5.3 ± 2.4 ng/ml, and 19.1 ± 6.4 pg/ml, respectively) genotypes. However, there were only significant differences between ER-α PvuII TT, and ER-α PvuII TC genotypes in the plasma concentrations of the above mentioned hormones (Tables 5 and 6). In individuals with ER-α PvuII TT genotype, the plasma free T (158 ± 39 pg/ml), and free E2 (0.45 ± 0.2 pg/ml) concentrations were significantly higher than that found in individuals with ER-α PvuII TC (134 ± 37 pg/ml, and 0.38 ± 0.2 pg/ml, respectively; both $P = 0.004$), and ER-α PvuII CC (142 ± 39 pg/ml, and 0.40 ± 0.2 pg/ml; $P = 0.006$, and $P = 0.005$, respectively) genotypes. The same picture was observed for serum FSH levels, where the value in ER-α PvuII TT genotype (7.8 ± 2.2 IU/l) was significantly higher than that found in ER-α PvuII TC (6.2 ± 2.1 IU/l), and ER-α PvuII CC (7.0 ± 2.1 IU/l) genotypes ($P = 0.004$, and $P = 0.006$, respectively). Regarding serum LH levels, the ER-α PvuII TT individuals (5.8 ± 2.2 IU/l) showed blood levels significantly lower than that observes in individuals with ER-α PvuII TC (7.2 ± 2.2 IU/l), and ER-α PvuII CC (6.4 ± 2.1 IU/l) genotypes ($P = 0.002$, and $P = 0.008$, respectively).

3.8.2. ER-α XbaI polymorphism

The ER-α XbaI AG genotype resulted significantly higher serum levels of SHBG (24.72 ± 4.1 nmol/l), total T (6.7 ± 2.3 ng/ml), and total E2 (21.8 ± 6.7 pg/l) compared with ER-α XbaI AA genotype (21.27 ± 4.2 nmol/l, 4.5 ± 2.1 ng/ml, and 16.6 ± 5.4 pg/l, corresponded with $P = 0.004$, $P = 0.007$, and $P = 0.002$, respectively) (Tables 5 and 6). In subjects carrying ER-α XbaI GG genotype the plasma level of total E2 (18.6 ± 6.2 pg/l) was also significantly higher than subjects who carry ER-α XbaI AA (16.6 ± 5.4 pg/l) genotype ($P = 0.006$). Subjects with ER-α XbaI AG genotype were found to have significantly lower serum levels of free T (134 ± 38 pg/ml), and free E2 (0.37 ± 0.2 pg/ml), when compared to subjects with ER-α XbaI AA (157 ± 41 pg/ml, and 0.46 ± 0.2 pg/ml, respectively) ($P = 0.004$, and $P = 0.002$, respectively), and ER-α XbaI GG (139 ± 40 pg/ml, and 0.40 ± 0.2 pg/ml, respectively) ($P = 0.006$, and $P = 0.005$, respectively) genotypes. The men with ER-α XbaI AG genotype had a higher LH (7.8 ± 2.1 IU/l) and lower FSH levels (6.1 ± 2.2 IU/l) compared with the ER-α XbaI AA genotype (5.2 ± 1.8 IU/l, and 8.0 ± 2.2 IU/l; corresponded with $P = 0.002$, and $P = 0.003$, respectively). Similar correlations were found when ER-α XbaI AG genotype was compared with ER-α XbaI GG genotype (Table 4).

3.9. ER-β genotypes

3.9.1. RasI polymorphism

Univariate analysis revealed that there was a statistically significant interaction between the ER-β RsaI genotypes and, SHBG, testosterone, and free T concentrations. In men with the GA genotype of ER-β RsaI gene, the level of SHBG (19.13 ± 4.8 nmol/l), and total T (4.0 ± 2.1 ng/ml) significantly decreased, but the level of free T (166 ± 42 pg/ml) significantly increased compared with the GG genotype of ER-β RsaI gene (23.64 ± 4.7 nmol/l, 5.9 ± 2.2 ng/ml, and 140 ± 38 pg/ml; corresponded with $P = 0.002$, $P = 0.008$, and $P = 0.003$, respectively) (Tables 5 and 6). In the GA genotype of ER-β RsaI gene, the total E2 concentration (16.4 ± 5.8 pg/ml) was decreased compared with the GG genotype of ER-β RsaI gene

Table 5
Relationship between different ER-α, and ER-β genotypes and serum levels of SHBG, LH, FSH, total and free testosterone, and estradiol in normal fertile men.

Variables	*n*	Sex hormone binding globulin[a]			Total testosterone (mean)			Free testosterone (mean)			Total estradiol (mean)			Free estradiol (mean)			LH (mean)			FSH (mean)		
		nmol/l	95% CI[b]	*P* value	ng/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value	IU/l	95% CI[b]	*P* value	IU/l	95% CI[b]	*P* value
ER-α PvuII																						
TT	33	21.92	1.0 (Referent)		5.0	1.0 (Referent)		155	1.0 (Referent)		18.4	1.0 (Referent)		0.42	1.0 (Referent)		6.1	1.0 (Referent)		7.5	1.0 (Referent)	
TC	86	24.86	20.81–28.91	0.004	6.7	4.6–8.4	0.004	131	95–171	0.004	21.1	14.7–27.5	0.004	0.35	0.33–0.37	0.004	7.5	4.8–8.5	0.002	5.9	3.8–8.1	0.004
CC	45	22.29	18.67–26.64	0.066	5.7	4.0–6.8	0.061	138	105–193	0.006	19.4	13.5–25.8	0.062	0.37	0.35–0.39	0.005	6.7	4.0–8.2	0.007	6.7	4.9–8.6	0.005
ER-α XbaI																						
AA	41	21.76	1.0 (Referent)		4.8	1.0 (Referent)		154	1.0 (Referent)		16.9	1.0 (Referent)		0.43	1.0 (Referent)		5.5	1.0 (Referent)		7.7	1.0 (Referent)	
AG	95	25.22	20.83–29.23	0.004	6.9	4.8–8.9	0.007	131	89–179	0.004	22.2	15.7–27.7	0.002	0.33	0.31–0.35	0.002	7.5	5.3–8.8	0.002	5.8	3.9–7.9	0.003
GG	28	22.84	19.02–26.92	0.073	5.3	3.6–6.5	0.064	135	92–190	0.006	19.0	13.1–25.7	0.005	0.36	0.35–0.38	0.005	5.4	3.6–7.5	0.006	6.7	4.7–6.8	0.005
ER-β Rsa																						
GG	152	24.14	1.0 (Referent)		6.1	1.0 (Referent)		137	1.0 (Referent)		20.2	1.0 (Referent)		0.37	1.0 (Referent)		6.5	1.0 (Referent)		6.1	1.0 (Referent)	
GA	8	19.67	16.14–22.65	0.002	4.2	2.9–5.5	0.008	163	118–198	0.003	16.7	12.6–20.4	0.002	0.41	0.39–0.43	0.007	4.5	2.8–6.1	0.002	7.5	5.9–8.8	0.006
AA	4	24.54	20.87–28.72	0.062	7.1	4.8–8.9	0.061	137	90–185	1.0	24.7	19.1–28.5	0.001	0.34	0.32–0.35	0.005	7.4	5.1–8.8	0.005	5.1	3.6–7.0	0.006
GA + AA	12	20.32	17.02–24.21	0.002	4.6	3.3–5.8	0.062	158	121–196	0.002	17.9	13.1–23.84	0.005	0.40	0.38–0.42	0.008	5.2	3.4–7.1	0.005	7.2	6.9–8.5	0.005
ER-β AluI																						
AA	21	23.14	1.0 (Referent)		5.5	1.0 (Referent)		138	1.0 (Referent)		19.6	1.0 (Referent)		0.33	1.0 (Referent)		6.2	1.0 (Referent)		6.6	1.0 (Referent)	
AG	63	21.55	17.74–25.04	0.01	5.0	3.5–6.4	0.062	151	98–193	0.074	18.2	12.9–24.1	0.006	0.37	0.36–0.40	0.005	5.1	3.3–6.8	0.007	7.4	5.1–8.7	0.007
GG	80	26.01	21.92–29.79	0.004	7.0	4.9–7.1	0.063	127	88–174	0.091	21.8	16.2–27.2	0.007	0.30	0.28–0.31	0.008	7.1	5.0–8.5	0.006	5.6	3.5–8.69	0.006

Key: ER = estrogen receptor, LH = luteinizing hormone, FSH = follicle stimulating hormone.
[a] Geometric least-squares means adjusted for, age, BMI, duration of infertility, occupational status, and educational level.
[b] 95% CI for mean.

Table 6
Relationship between different ER-α, and ER-β genotypes and serum levels of SHBG, LH, FSH, total and free testosterone, and estradiol in infertile subjects.

Variables	*n*	Sex hormone binding globulin[a]			Total testosterone (mean)			Free testosterone (mean)			Total estradiol (mean)			Free estradiol (mean)			LH (mean)			FSH (mean)		
		nmol/l	95% CI[b]	*P* value	ng/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value	pg/ml	95% CI[b]	*P* value	IU/l	95% CI[b]	*P* value	IU/l	95% CI[b]	*P* value
ER-α PvuII																						
TT	49	21.11	1.0 (Referent)		4.5	1.0 (Referent)		161	1.0 (Referent)		17.8	1.0 (Referent)		0.48	1.0 (Referent)		5.5	1.0 (Referent)		8.1	1.0 (Referent)	
TC	70	23.90	20.02–28.12	0.004	6.2	4.1–7.9	0.004	137	102–178	0.004	20.5	14.1–26.9	0.004	0.41	0.39–0.43	0.004	6.9	6.8–8.5	0.002	6.5	4.4–8.7	0.004
CC	45	22.04	17.87–25.83	0.057	5.0	3.5–6.3	0.064	145	111–199	0.005	18.8	13.2–25.3	0.062	0.43	0.41–0.45	0.005	6.1	4.0–8.2	0.006	7.3	5.5–9.2	0.005
ER-α XbaI																						
AA	62	20.87	1.0 (Referent)		4.2	1.0 (Referent)		161	1.0 (Referent)		16.3	1.0 (Referent)		0.49	1.0 (Referent)		4.9	1.0 (Referent)		8.3	1.0 (Referent)	
AG	77	24.44	20.15–28.44	0.004	6.3	4.3–8.5	0.007	137	95–185	0.004	21.5	15.1–27.0	0.002	0.40	0.38–0.42	0.002	7.5	5.3–6.8	0.002	6.5	4.5–8.5	0.004
GG	25	22.11	18.16–26.13	0.072	4.7	3.1–6.0	0.061	142	98–197	0.006	18.2	12.5–25.1	0.007	0.43	0.42–0.45	0.005	5.3	3.6–7.5	0.007	7.4	5.4–7.5	0.007
ER-β RsaI																						
GG	142	23.32	1.0 (Referent)		5.6	1.0 (Referent)		143	1.0 (Referent)		19.5	1.0 (Referent)		0.43	1.0 (Referent)		6.5	1.0 (Referent)		6.7	1.0 (Referent)	
GA	21	18.84	15.36–21.80	0.002	3.7	2.4–5.1	0.007	169	124–205	0.003	16.1	12.0–19.8	0.002	0.47	0.45–0.49	0.007	4.4	2.8–6.1	0.002	8.3	6.5–9.4	0.004
AA	1	23.73	20.34–27.91	0.068	6.5	4.2–8.3	0.067	143	97–193	1.0	24.1	18.5–27.9	0.002	0.40	0.38–0.41	0.004	7.4	5.1–8.8	0.005	5.7	4.2–7.6	0.007
GA + AA	22	19.54	16.54–23.45	0.002	4.1	2.8–5.2	0.062	165	127–202	0.004	17.3	12.5–23.1	0.005	0.46	0.44–0.48	0.008	5.3	3.4–7.1	0.007	7.8	5.5–9.2	0.005
ER-β AluI																						
AA	17	22.63	1.0 (Referent)		5.0	1.0 (Referent)		143	1.0 (Referent)		19.0	1.0 (Referent)		0.39	1.0 (Referent)		6.2	1.0 (Referent)		7.3	1.0 (Referent)	
AG	82	20.84	16.91–24.25	0.01	4.5	3.0–6.9	0.071	158	104–202	0.072	17.5	12.3–23.4	0.006	0.44	0.42–0.46	0.007	5.1	3.4–6.8	0.006	8.0	5.8–9.4	0.006
GG	65	25.22	21.17–29.14	0.003	6.5	4.4–6.4	0.062	133	94–181	0.087	21.2	15.5–26.5	0.007	0.36	0.34–0.38	0.008	7.1	5.0–8.5	0.007	6.2	4.1–9.2	0.007

Key: ER = estrogen receptor, LH = luteinizing hormone, FSH = follicle stimulating hormone.
[a] Geometric least-squares means adjusted for, age, BMI, duration of infertility, occupational status, and educational level.
[b] 95% CI for mean.

(19.9 ± 6.2 pg/ml, $P = 0.002$). In contrast, free E2 concentration in the ER-β RsaI GA genotype (0.44 ± 0.2 pg/ml) significantly increased compared with ER-β RsaI GG (0.40 ± 0.2 pg/ml, $P = 0.008$) genotype.

In both controls and infertile men, the heterozygous polymorphic RsaI GA genotype was associated with significant decreased serum LH (4.7 ± 1.8 IU/l), and significant increased serum FSH levels (7.9 ± 2.1 IU/l) compared with the homozygous wild-type RsaI GG genotype (6.8 ± 1.9 IU/l, and 6.4 ± 1.8 IU/l; $P = 0.002$, and $P = 0.005$, respectively).

3.9.2. ER-β AluI polymorphism

The mean serum SHBG was significantly low in heterozygous ER-β AluI AG genotype individuals (21.04 ± 4.6 nmol/l) in comparison with AluI AA (22.98 ± 4.7 nmol/l, $P = 0.01$), and AluI GG (25.52 ± 4.8 nmol/l, $P = 0.004$) genotype individuals. The mean serum total T decreased, and the mean free T increased in subjects with ER-β AluI AG genotype (4.8 ± 1.5 ng/ml, and 155 ± 44 pg/ml), compared with AluI AA (5.3 ± 1.8 ng/ml, and 140 ± 42 pg/ml), and AluI GG (6.8 ± 2.1 ng/ml, and 130 ± 40 pg/ml), individuals. However, the differences did not reach statistical significance (all $P > 0.05$). With regard to E2 it was found that ER-β AluI AG genotype individuals had significantly lower serum E2 levels (17.8 ± 6.2 pg/ml), and significantly higher serum free E2 levels (0.41 ± 0.2 pg/ml) in comparison with ER-β AluI AA (19.3 ± 6.4 pg/ml, 0.36 ± 0.2 pg/ml; $P = 0.007$, and $P = 0.006$, respectively), and ER-β AluI GG (21.6 ± 6.8 pg/ml, 0.33 ± 0.2 pg/ml; $P = 0.008$, and $P = 0.009$, respectively) individuals. The mean serum LH levels were significantly low, and the mean serum FSH levels were significantly high in ER-β AluI AG genotype (5.4 ± 1.6 IU/l, and 7.7 ± 2.1 IU/l) with respect to ER-β AluI AA (6.5 ± 2.1 IU/l, and 6.9 ± 2.2 IU/l; $P = 0.006$, and $P = 0.007$, respectively) and ER-β AluI GG genotype (7.4 ± 2.2 IU/l, and 5.9 ± 2.1 IU/l; $P = 0.006$, and $P = 0.007$, respectively) individuals (Tables 5 and 6).

3.10. Association with semen parameters

We further assessed the relationship between ESR-α, and ER-β genotypes and semen parameters. There were statistically significant differences between the sperm density, sperm motility, and sperm with normal morphology among infertile (Table 7) and healthy fertile men (Table 8) with different ER-α, and ER-β genotypes. The following results were achieved both in fertile and infertile subjects (for details see Tables 7 and 8).

3.10.1. ER-α genotype

The mean sperm density, sperm motility, and sperm morphology was significantly higher in the ER-α PvuII TC genotype, compared with ER-α PvuII TT, and ER-α PvuII CC homozygotes. There was a statistically significant trend towards higher percentage of sperm density, sperm motility, and percentage of the sperm with normal morphology in the ER-α XbaI AG genotype carriers, compared with ER-α XbaI AA, and ER-α XbaI GG carriers.

3.10.2. ER-β genotype

ER-β RsaI GA, and ER-β RsaI GA + AA genotypes had significantly lower levels of sperm density, sperm motility, and percentage of sperm with normal morphology than homozygous ER-β RasI GG individuals. The mean of sperm density, sperm motility, and percentage of sperm with normal morphology in individuals with ER-β AluI AG genotype, was significantly lower than those with ER-β AluI AA, and ER-β AluI GG genotypes. The significant differences were noticeable both in the fertile and infertile men.

3.11. Correlations

3.11.1. Serum hormones

A positive correlation has been found between ER-α PvuII TC genotype and serum levels of SHBG ($r = 0.65$, $P = 0.001$), total T ($r = 0.58$, $P = 0.004$), total E2 ($r = 0.70$, $P = 0.002$), and LH ($r = 0.74$, $P = 0.001$) in our study. A negative correlation was found between ER-α PvuII TC genotype and serum levels of free T ($r = -0.66$, $P = 0.001$), free E2 ($r = -0.75$, $P = 0.001$), and FSH ($r = -0.64$, $P = 0.002$). There was a positive correlation between ER-α XbaI AG genotype and serum levels of SHBG ($r = 0.66$, $P = 0.001$), total T ($r = 0.57$, $P = 0.004$), total E2 ($r = 0.68$, $P = 0.002$), and LH ($r = 0.75$, $P = 0.001$).

A negative correlation was found between ER-α XbaI AG genotype and serum levels of free T ($r = -0.68$, $P = 0.001$), free E2 ($r = -0.73$, $P = 0.001$), and FSH ($r = -0.69$, $P = 0.002$). A negative correlation between serum levels of SHBG ($r = -0.71$, $P = 0.001$), total T ($r = -0.60$, $P = 0.002$), total E2 ($r = -0.78$, $P = 0.001$), and LH ($r = -0.74$, $P = 0.001$) was also demonstrated in subjects with ER-β RsaI GA genotype. In contrast, there was a positive correlation between serum levels of free T ($r = 0.64$, $P = 0.002$), free E2 ($r = 0.72$, $P = 0.001$), and FSH ($r = 0.68$, $P = 0.001$) and ER-β RsaI GA genotype.

Our study showed a negative correlation between serum levels of SHBG ($r = -0.67$, $P = 0.001$), total T ($r = -0.61$, $P = 0.004$), total E2 ($r = -0.74$, $P = 0.002$), and LH ($r = -0.79$, $P = 0.001$) in carriers of the ER-β AluI AG genotype. A positive correlation was found between the circulating serum free T ($r = 0.63$, $P = 0.001$), free E2 ($r = 0.73$, $P = 0.001$), and FSH ($r = 0.65$, $P = 0.002$) in subjects from ER-β AluI AG genotype group.

3.11.2. Semen parameters

In the present study, we found a significant positive correlation between sperm density ($r = 0.49$, $P = 0.004$), sperm motility ($r = 0.52$, $P = 0.004$), and percentage of sperms with normal morphology ($r = 0.44$, $P = 0.006$) values and ER-α PvuII TC carriers. A trend for positive correlation was also found between sperm density ($r = 0.47$, $P = 0.004$), sperm motility ($r = 0.55$, $P = 0.004$), and percentage of sperms with normal morphology ($r = 0.46$, $P = 0.005$) and presence of ER-α XbaI AG genotype. The presence of ER-β RsaI GA genotype was negatively correlated with sperm density ($r = -0.67$, $P = 0.001$), sperm motility ($r = -0.68$, $P = 0.001$), and percentage of sperms with normal morphology ($r = -0.73$, $P = 0.001$). A highly strong negative correlation also existed between the ER-β AluI AG genotype and sperm density ($r = -0.77$, $P = 0.001$), sperm motility ($r = -0.78$, $P = 0.001$), and percentage of sperms with normal morphology ($r = -0.77$, $P = 0.001$).

4. Discussion

In a case-control study we analyzed the association between the XbaI and PvuII polymorphisms of the ESR-α gene and, RsaI and AluI polymorphisms of the ESR-β gene and fertility status. We found significant associations between these four polymorphisms and infertility. The ER-α PvuII CC and ER-α XbaI AG genotypes were associated with a significantly lower infertility rates as compared to subjects with ER-α PvuII TT and ER-α XbaI AA genotypes. According to our results, the XbaI polymorphism is in strong linkage disequilibrium with the PvuII polymorphism. On the other hand, we found significant association between increased infertility risk and ER-β RsaI GA and ER-β AluI AG genotypes as compared to subjects carrying ER-β RsaI GG and ER-β AluI AA genotypes. In addition, higher sperm could be noted in subjects carrying ER-α PvuII TC, ER-α XbaI AG, ER-β RsaI GG, and ER-β AluI GG genotypes. The effect of these polymorphisms was found to be similar in both fertile and infertile men, which was consistent with the findings

Table 7
Relationship between ER-α, ER-β gene polymorphisms and semen parameters in infertile men.

Genotypes	*n*	Density × 10^6/ml	95% CI[a]	*P* value	Motility (%)	95% CI[a]	*P* value	Morphology (%)	95% CI[a]	*P* value
ER-α PvuII										
TT	49	10.4	1.0 (Referent)		17.4	1.0 (Referent)		16.5	1.0 (Referent)	
TC	70	16.3	14.1–18.8	0.001	24.5	21.2–26.4	0.001	23.6	19.4–27.3	0.001
CC	45	13.4	10.5–16.4	0.004	20.1	17.2–24.4	0.008	19.2	15.8–23.1	0.008
ER-α XbaI										
AA	62	10.6	1.0 (Referent)		17.2	1.0 (Referent)		16.2	1.0 (Referent)	
AG	77	16.4	13.2–19.2	0.001	24.5	20.7–28.1	0.001	23.4	19.2–27.4	0.001
GG	25	13.4	10.1–16.4	0.004	20.5	16.4–24.7	0.008	20.7	16.7–24.3	0.007
ER-β RsaI										
GG	142	14.1	1.0 (Referent)		21.8	1.0 (Referent)		20.9	1.0 (Referent)	
AG	21	11.2	10.2–12.7	0.004	16.8	15.7–18.2	0.006	15.8	14.1–18.7	0.006
AA	1	16.4	13.5–19.2	0.008	24.1	20.4–28.3	0.007	23.2	19.2–27.4	0.008
AG + AA	22	11.0	9.8–12.3	0.004	17.0	15.8–18.7	0.008	16.0	14.2–18.1	0.008
ER-β AluI										
AA	17	14.7	1.0 (Referent)		21.3	1.0 (Referent)		20.1	1.0 (Referent)	
AG	82	11.2	10.2–12.8	0.004	18.3	16.3–21.7	0.008	17.5	14.4–20.2	0.008
GG	65	16.7	13.2–18.9	0.008	24.8	21.4–28.5	0.008	23.7	19.3–27.6	0.008

Key: ER = estrogen receptor, CI = 95% confidence interval.
[a] 95% CI for mean.

in Italian men, although in that study the result was not statistically significant in the fertile population [22]. Previous studies on the effect of ER-α, and ER-β genes in male factor infertility have resulted conflicted evidence [26–28]. In a recent study in Greek population, the role of ER-α, and ER-β has been examined in 29 oligozoospermic and 85 normozoospermic men [25]. In contrast to our study, ER-α gene polymorphisms affected in different manners fertile and infertile men. In addition ER-β gene polymorphisms, had no significant associations with sperm concentration or motility. The ESR-α also includes the AGATA haplotype, which is caused by five single nucleotide polymorphisms (SNPs) located within the gene [34]. This haplotype has been reported as a risk factor for cryptorchidism [35] in Japanese population, however this finding was not replicated in Italian and Spanish populations [36]. In contrast to our study, Khattri et al. reported that, the SNPs and mutations in ER-β gene are not a common cause of spermatogenesis failure in Indian men [23]. Aschim et al. have demonstrated that the infertile men have approximately three times higher frequency of the heterozygous ER-β RsaI AG genotype than controls [24]. This is in consistent with our finding. Furthermore, we have observed statistically significant effect of studied polymorphisms on the three principal sperm parameters (concentration, motility and morphology). The analysis of the ESR-α (TA)n polymorphism and SNP12, failed to demonstrated such a correlation in either the Italian infertile or the control groups [36]. The observed differences could be due to the different genetic backgrounds, different study population and design, small sample size, or environmental difference. An example for significant ethnic differences has also been demonstrated for other polymorphisms such as the DAZLA gene exon 2, which was accounted as a risk factor for male infertility in the Chinese population but was completely lacking in European populations [37,38]. In addition, it is worthy to note that the sample sizes are probably insufficient in most studies dealing with genetic associations in multifactorial disorders [22]. According to a meta-analysis by Ioannidis et al. [39], a minimum of 150 subjects (controls and cases) is mandatory for association analyses.

We cannot fully explain the mechanism involved in ER-α, and ER-β polymorphisms induced change in the sperm parameters. Previous studies on bone tissue signify that long TA repeats would augment estrogen action, whereas short TA repeats would cause the opposite [20]. We believe that the effects of ER-α, and ER-β polymorphism on reproductive hormones, are mediated via alterations in serum SHBG concentrations. SHBG is involved in both delivering reproductive hormones to target tissues and controlling the concentration of androgens and estrogens in the serum and tissues [40]. A lower SHBG concentration allows for a higher fraction of T to circulate unbound, but it may emphasize the negative feedback effect of increased free E2 levels. In humans, a decrease

Table 8
Relationship between ER-α, ER-β gene polymorphisms and semen parameters in normal fertile men.

Genotypes	*n*	Density × 10^6/ml	95% CI[a]	*P* value	Motility (%)	95% CI[a]	*P* value	Morphology (%)	95% CI[a]	*P* value
ER-α PvuII										
TT	33	46.4	1.0 (Referent)		49.2	1.0 (Referent)		41.5	1.0 (Referent)	
TC	86	67.4	56.7–77.8	0.001	71.2	63.4–79.2	0.001	65.2	57.2–73.3	0.001
CC	45	57.4	48.3–66.8	0.002	59.8	52.4–67.6	0.002	55.2	47.5–64.1	0.001
ER-α XbaI										
AA	41	45.6	1.0 (Referent)		48.4	1.0 (Referent)		43.4	1.0 (Referent)	
AG	95	67.7	57.1–78.4	0.001	71.7	63.2–79.6	0.001	64.4	56.6–72.3	0.001
GG	28	57.5	46.6–67.2	0.001	58.6	51.4–65.7	0.002	55.8	48.8–63.4	0.002
ER-β RsaI										
GG	152	59.7	1.0 (Referent)		63.9	1.0 (Referent)		57.8	1.0 (Referent)	
AG	8	49.9	38.7–60.4	0.002	53.1	46.7–60.2	0.002	48.1	41.2–55.7	0.002
AA	4	71.4	59.8–83.4	0.002	75.0	67.7–83.4	0.002	72.2	64.4–80.9	0.003
AG + AA	12	57.4	49.4–65.6	0.063	59.6	52.4–66.1	0.065	54.8	66.3–61.8	0.067
ER-β AluI										
AA	21	60.2	1.0 (Referent)		63.5	1.0 (Referent)		56.1	1.0 (Referent)	
AG	63	46.7	38.455.7	0.003	49.8	42.7–57.4	0.002	44.1	37.8–51.4	0.002
GG	80	71.3	60.6–82.7	0.002	74.6	66.8–82.5	0.002	68.8	60.1–79.4	0.002

Key: ER = estrogen receptor, CI = 95% confidence interval.
[a] 95% CI for mean.

in testosterone/estrogen ratio has been reported to be associated with infertility. Pavlovich et al. [41] demonstrated that infertile men with severe oligozoospermia had significantly lower T and higher E2 concentrations than fertile control subjects, resulting in an elevated T/E2 ratio. Lower levels of SHBG have also been reported with less than 20CA repeats in the ESR-β gene [42]. Lower SHBG can result in increased risk of breast cancer through higher estrogen bioavailability [43]. One study in women has shown that ER-β gene polymorphisms are associated with the levels of androgens and SHBG [42]. The finding of decreased LH levels in men with the ER-α PvuII TT, ER-α XbaI AA, ER-β RsaI AG, and ER-β AluI AG genotypes, despite decreased T and E2 concentrations, might indicate that these genotypes result an increased ER-α, and ER-β activity, leading to elevated estrogen sensitivity. The finding that the above mentioned genotype groups presented with lower LH concentrations in fertile as well as in the infertile men makes it more possible that these are biologically relevant findings and not just a effect of multiple testing.

As already mentioned, the two ESR-α polymorphisms investigated in the present study are in strong linkage disequilibrium, hence it is possible, that the association seen between these polymorphisms and infertility is caused only by one of them. In addition, the association can be caused by a third polymorphism as well, which might also be in strong linkage disequilibrium with XbaI and PvuII. A multifactorial condition such as infertility is likely to depend on several polymorphisms. Therefore, the possibility that the examined polymorphisms affect the correct splicing of RNA, producing alternatively spliced mRNA variants or that they are in linkage disequilibrium with another polymorphism in the ESR-α gene, which is relevant for protein expression, may not be rolled out.

Of the polymorphisms determined in the ESR-α, and ER-β genes, two SNPs in ER-α (PvuII and XbaI) and two SNPs in ER-β (RsaI and AluI) are most widely investigated. These four polymorphisms have been identified to influence fertility status in both men and women. Because of the large size of the ER gene locus, a thorough examination of the entire gene was not possible. Therefore, we sought to examine the ESR-α, and ER-β SNPs that have been implicated in previous studies. More extensive analyses of the entire locus are necessary to determine whether other regions of ESR-α and ER-β may also have functional significance.

While the precise role of estrogen receptors in male fertility status is understood, our findings suggest that specific polymorphisms of the ER-α, and ER-β genes which confer a lower SHBG and thus a stronger unbound estrogen effect, may adversely affect human spermatogenesis. This study has several advantages and limitations. It is one of the largest reported studies addressing the relationships between the both ESR-α, and ER-β gene polymorphisms and semen parameters in fertile and infertile men. Of note, our analyses were restricted to white Iranian population and thus cannot necessarily be applied to other racial or ethnic groups. Although we have demonstrated significant associations between studied ESR-α, and ER-β gene polymorphisms and fertility status in men, we have no accurate explanation of causative association for these special polymorphisms. We had limited power to determine interactions or carry out subgroups analyses. But, the current data do replicate findings of some previous studies. In addition, we did not gathered data regarding mood disorders such as anxiety or depression. Finally, we do not completely exclude the possibility that the reported results may be false-positive because of the multiple testing. However, the ER-α PvuII and XbaI, and ER-β RsaI and AluI polymorphisms are in strong linkage disequilibrium, and serum hormones and semen parameters are correlated. Therefore, in this case, each new test would not have provided a completely independent opportunity for a type I error, and the Bonferroni correction for multiple testing would have been too conservative in genetic case control studies [44]. Therefore, in the present study, the simple original statistical analysis are adopted with the aim of reporting some potentially important associations that are likely to be worthwhile pursuing further.

5. Conclusion

In conclusion, we found a significant inverse association between the ER-α PvuII CC, and ER-α XbaI AG polymorphism, and a significant positive association between the ER-β RsaI GA, and ER-β AluI AG polymorphisms and prevalent infertility in men. Carriers of the ER-α XbaI G allele had a 32% lower and carriers of ER-α XbaI A allele had a 1.72-fold higher prevalence of infertility. The mechanisms by which the ESR-α, and ER-β genes might be related to infertility and semen parameters warrant further investigation.

Conflict of interest

None.

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