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Original Article

Is There Any Association Between ERβ Gene Polymorphism and Infertility in Iranian Men?

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ABSTRACT

Introduction: A significant proportion of infertile men with azoospermia and severe oligoazoospermia have a genetic etiology for their reproductive failure. Genetic analysis has major effects on finding the causes of infertility in last decade, but still in some cases, we still do not have clear answer for our patients. During last years it has become evident that endogenous estrogens and estrogen receptors (ER) play role in the regulation of testicular function. Present study was performed to evaluate the significance of RsaI and AluI single nucleotide polymorphism in the *ERβ* gene in infertile patients in comparison with normal fertile male control.

Methods: From 120 infertile men referred to our center after ruling out all the known causes of the infertility such as chromosomal abnormalities, Y-chromosome microdeletion, and other pathologic disorders, 5 ml peripheral blood were obtained for DNA extraction. PCR amplification of the polymorphic region was carried out and after running the PCR products on 1.5% agarose gel, the frequency of the polymorphism were calculated.

Results: A 3 times higher frequency of the heterozygous RsaI genotype was found in men with low sperm concentration compared to control (P=0.003). In contrast, the proportion of homozygous AluI genotype was only 1/3 in severely oligoazoospermic men in comparison with control (P=0.03).

Conclusion: Our results could suggest that *ERβ* and RsaI and AluI single nucleotide polymorphisms on this gene are important for spermatogenesis in humans, and could play an important role in the spermatogenesis process in males. Also it is possible to conclude that different conditions of infertility may not have genetic predisposition in common.

Key words: estrogen receptor, polymorphism

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The physiological and pathological roles of estrogens in men have gained increasing attention which have profound effects on the male reproductive system. In men, estrogens are synthesized from testosterone mainly in testis, through the action of aromatase cytochrome P450 [1, 2]. Estrogens seem to play an important role for male fertility, which was demonstrated by the finding that aromatase deficiency caused progressive infertility in adult mice [3] and reduced sperm production and sperm motility in humans [4, 5]. In contrast, increased levels of estrogens in utero have been shown, at least in some studies, to lead to TDS-like conditions in both mice [6-9] and men [10, 11]. It is important to emphasize, however, that the hypothesized relationship between estrogens and TDS development is still a matter of controversy.

It has been suggested that increased exposure to estrogenic and anti-androgenic endocrine disruptors, or other changes in the androgen-estrogen balance, in utero could lead to disruption of embryonic programming and

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gonadal/sexual development during fetal life, resulting in symptoms of TDS [12]. The role of estrogens in male reproductive organs is complex and although the underlying cause of TDS is unknown, genetic variants in strategic genes might predispose to this syndrome under particular circumstances. Recently, several sequence variants of the ERβ gene have been described, in different study groups [13]. Among these genetic variants, a common AluI polymorphism in the 3' nontranslated region at position 1730 (A_G) as well as a G_A polymorphism at position 1082 (RsaI), in the ligand binding domain of the ERβ were described.

Both polymorphisms have been overrepresented in ovulatory dysfunctions [14]. However, studies on genetic variants of ERβ with respect to male infertility are still lacking. Such information might add to our knowledge regarding the role of estrogens in the physiology and pathophysiology of male reproductive systems.

Accordingly, our aim was to investigate the two ERβ polymorphisms with respect to male infertility.

Subjects and Methods

Patients

One hundred twenty infertile men, presenting with sperm concentrations below $5x10^6$ /ml in at least two ejaculates, were included in this study. Men with known genetic causes of infertility, e.g. Klinefelter syndrome or Y-chromosome microdeletions, were excluded from this study. Cases with low sperm concentration were divided into three subgroups of: azoospermic (0 spermatozoa), cryptoazoospermic (<1 $x10^6$ spermatozoa/ml), and severe oligoazoospermic (1-5 $x10^6$ spermatozoa/ml). Also, 204 fertile men, without genital abnormalities and at least one healthy child, served as control.

Informed consent was obtained from all subjects, according to protocol of ethical review board of Uromia university.

ER - polymorphism analysis

In all subjects, allele-specific PCR was performed to detect the RsaI and AluI variants of ERβ. For each polymorphism two reactions per subject were used, containing either one mutant (mut) or one wild-type (wt) specific primer, together with an upstream and a downstream primer (Cinnagen company, Iran). PCR conditions were established to generate a short, allele-specific band in the presence of the variant and only a long control fragment in its absence.

Allele specific PCR of the RsaI polymorphism was performed in total volume of 25µI containging 25 ng of genomic DNA, 45mM of KCI, 10mM of Tris HCI (pH 9.1), 0.2mM of dNTP, 1.5mM of MgCI2, 1 U of Taq DNA polymerase, and 0.5 µM of each of the primers RsaI Fw, RsaI Rew, and either RsaI RevA or RsaI RevG (all the materials were provided from Cinnagen company, Iran).

Primer sequences are presented in table 1. Amplification was performed in a Mastercycler gradient thermocycler (eppendorf, Germany) for 35 cycles; each cycle including denaturation for 1 min at 96◦C, primer annealing for 30 sec at 58◦C, and a primer extention for 3 min at 72◦C, with an initial denatuartion step for 3 min at 96◦C and a final extention step for 7 min at 72◦C. Ten I of the PCR products were analyzed on 1.5% agarose gel. Primer RsaI RevG was considered the wild type primer, whereas RsaI RevA was regarded as the mutant primer.

For AluI polymorphism, an annealing temperature of 54◦C for 30 sec was used and other conditions were same as for RsaI reaction. Primer AluI RevG was considered the wild type primer, whereas AluI RevA was regarded as the mutant primer. Primers were designed using the primer 3 program by standard selection criteria. (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)

The control fragment and the allele-specific fragment were 409 and 127 bp, respectively, for the RsaI polymorphism, and 405 and 258 bp, respectively, for the AluI polymorphism.

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Restriction fragment length polymorphism (RFLP)

Both the RsaI and the AluI polymorphisms are RFLPs, and digestion with the respective restriction enzymes was performed according to the manufacturer (Fermentas, Helsingborg, Sweden) to verify the results from the allele-specific PCR. In the RsaI polymorphism, a G to A nucleotide exchange at nucleotide 1082 in exon 5 created a recognition site for RsaI, and in the AluI polymorphism an exchange of G to A at nucleotide 1730 in the noncoding end of exon 8 introduced a recognition site for

AluI (nucleotide numbering according to GeneBank accession no. AB006590). In both positions a G nucleotide was considered the wild-type sequence, and was not digestible by RsaI or AluI.

RsaI digestion produced one uncleaved band of 409 bp in subjects with the homozygous wild-type GG genotype, two bands of 110 bp and 299 bp in homozygous polymorphic AA subjects, and all three bands in heterozygous AG carriers. AluI digestion yielded one band of 405 bp in the uncleaved homozygous wild-type GG polymorphism, two bands of 163 bp and 242 bp in the homozygous polymorphic AA polymorphism, and all three bands in heterozygous AG subjects.

Statistical analysis

The distributions of ER_polymorphisms were compared between the patient groups and controls using Fisher's exact test. All statistical tests were two sided. $P<0.05$ was considered statistically significant.

Table 1. the sequence of the primers used in the projects.

primer	Sequence 5'-3'	Fragment length
RsaI Fw	5'-ACT TGC CAT TCT GTC TCT ACA-3'	
RsaI Rev	5'-CAC AGG ACC CTG AAT CCT-3'	409 (control)
RsaI RevA	5'-AGC TCT CCA AGA GCC GT-3'	127 (A-variant)
RsaI RevG	5'-AGC TCT CCA AGA GCC GC-3'	127 (G-variant)
AluI Fw	5'-TTT TTG TCC CCA TAG TAA CA-3'	
AluI Rev	5'-CCT CTG CTA ACA AGG GAA A-3'	405 (control)
AluI RevA	5'-GAG TTC ACG CTT CAG CT-3'	258 (A-variant)
AluI RevG	5'-GAG TTC ACG CTT CAG CC-3'	258 (G-variant)

Results

The distribution of the RsaI and AluI polymorphism alleles and genotypes in different study groups are presented in table 2. When analyzing the distribution of the RsaI polymorphism in the different study groups, we found that all men with low sperm concentration had 3 times higher incidence of the heterozygous AG RsaI genotype than control (P=0.003). Also, the findings, revealed that azoospermic and cryptoazoopermic men had 4 times higher incidence of the AG genotype than controls (P=0.006 and P=0.02, respectively), whereas severely oligoazoopermic men did not differ from the control group (P=0.16). Regarding the AluI polymorphism, the incidence of homozygous AA AluI genotype was only one third in severely oligoazoospermic men compared to controls (P=0.03). Conclusive with this, men with severe oligoazoospermia had a significantly lower frequency of the AluI A allele than controls (P=0.04) and cryptoazoospermic men (P=0.01), but not with azoospermic men (P=0.14).

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Table 2. Incidence of the RsaI and AluI genotypes (AA, AG, and GG) and the frequency of RsaI and AluI A alleles in the ER_gene in different study groups.

Study group	Genotype						Frequency	
	RsaI			AluI			RsaI	AluI
	AA	AG	GG	AA	AG	GG	A allele	A allele
Controls (n=204)	1 (0.5)	9 (4.5)	194 (95)	30 (15)	88 (43)	86 (42)	0.027	0.363
All men with oligoazoospermia (n=120)	0 (0)	17* (14)	103* (86)	12 (10)	57 (47.5)	51 (42.5)	0.071*	0.338
Azoospermia (n=39)	0 (0)	7* (18)	32* (82)	4 (10)	20 (51)	15 (39)	0.090*	0.359
Cryptoazoospermia(n=30)	0 (0)	5* (17)	25* (83)	6 (20)	15 (50)	9 (30)	0.083*	0.450
Severe oligozoo (n=51)	0 (0)	5 (10)	46 (4)	2* (4)	22 (43)	27 (53)	0.049	0.255*

*P<0.05 compared with controls (Fisher's exact test)

Discussion

The RsaI polymorphism has an approximate frequency of 5% of heterozygotes in the normal Caucasian population [15, 16]. The frequency of heterzygotes for the AluI polymorphism is close to 50% depending on the study group[16,17].

In this study, increased incidence of the RsaI and decreased incidence of the AluI polymorphisms was observed in men with oligoazoospermia in general, and in severely oligospermic men in particular. This may indicate that ERβ polymorphisms is associated with spermatogenesis, which in accordance with the fact that ERβ is widely distributed in somatic and spermatogenic cells in human testis.

The mechanisms behind altered ERβ function in subjects with RsaI and AluI polymorphisms remain to be elucidated. The G to A change does not lead to amino acid changes in the protein. It can be speculated, however, that this polymorphism is in linkage disequilibrium with other genetic variations that could affect gene expression or function. A recent study showed that the RsaI polymorphism was in complete linkage disequilibrium with a polymorphism located at the splice acceptor site just prior to exon 8 in ERβ [18]. This may potentially affect the splicing of this exon, leading to proteins with different properties than the wild-type ERβ [19, 20]. The RsaI polymorphism could also have a direct effect through changing the nucleotide sequence and thereby the secondary structure of the ERβ mRNA, possibly leading to changes in mRNA syntheses, splicing, maturation, transport, translation, or degradation [21, 22].

As controls, we included fertile men with at least one child and no genital abnormalities that can be considered as representative for the general population.

In conclusion, our results could suggest that ERβ and RsaI and AluI single nucleotide polymorphisms on this gene are important for spermatogenesis in humans, and could play an important role in the spermatogenesis process in males.

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