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Influence of the *Xba*I polymorphism in the estrogen receptor-α gene on human spermatogenic defects

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ABSTRACT. Polymorphisms of estrogen receptor (ER) genes have been implicated in male infertility, but studies of this association have produced conflicting results. The present study was conducted to examine whether polymorphisms within the ERα and ERβ genes are susceptibility factors for human male idiopathic infertility in Chinese men. We investigated the association between the ERα gene and *Pvu*II and *Xba*I polymorphisms and the ERβ gene and *Rsa*I and *Alu*I polymorphisms and idiopathic male infertility in Han Chinese men. A total of 204 men with oligozoospermia (sperm count <20 x 10^6/mL) or azoospermia and 252 fertile control men were included in this study. The analysis revealed a strong association between the *Xba*I genotype distribution and impaired spermatogenesis (P = 0.0018). The frequency of the G allele was significantly lower in patients than in controls (P = 0.003). Furthermore, serum levels of follicle-stimulating hormone and luteinizing hormone in *Xba*I AA carriers were significantly higher than those in AG or GG carriers. Our findings further support a possible role of ERα in male infertility. Further studies are needed to replicate our

findings, as well as to elucidate more fully the biological mechanisms of the modulation of ERα on human spermatogenesis.

Key words: Male infertility; Estrogen; Estrogen receptor-α; Estrogen receptor-β; Spermatogenesis

INTRODUCTION

Infertility is a common medical condition, affecting 1 in 6 couples. Approximately half of these cases are related to male factors (Brugo-Olmedo et al., 2001). Idiopathic azoospermia and idiopathic oligospermia are common reasons for male infertility (Dohle et al., 2005), but little is known about the factors that cause these conditions.

The role of estrogens in the regulation of testicular function is widely accepted (Saunders, 2005). In males, the testes are the sites of estrogen biosynthesis (Saunders, 2005), and the estrogen concentration in semen is higher than serum estradiol concentration in women (Hess et al., 1997). Estrogen excess during adulthood can diminish sperm production and maturation (Atanassova et al., 2000). Estrogen signaling in the cell is mediated by estrogen receptors (ERs), of which at least 2 subtypes exist - ERα and ERβ (Rago et al., 2006) - encoded by 2 different genes in different chromosomes (6q25 and 14q23-24, respectively). ERα encodes a 595-amino acid protein (Menasce et al., 1993), whereas ERβ encodes a 530-amino acid protein (Ogawa et al., 1998). The steroid/thyroid hormone nuclear receptor in both subtypes shares a common structure but differs in C-terminal ligand-binding and N-terminal transactivation domains (O'Donnell et al., 2001). In addition to these 2 isoforms, a third membrane receptor called ERγ has been reported in various cellular models, including human spermatozoa. This receptor is similar to ERβ and may have resulted through the duplication of ERβ (Luconi et al., 2002).

The physiological role of estrogens in spermatogenesis is not clearly defined. ERα knockout and double-knockout mice are infertile from puberty, and the testicular tissue phenotype shows atrophy of the testes and seminiferous tubule dysmorphogenesis, which result in decreased spermatogenesis and sperm motility (Krege et al., 1998; Gould et al., 2007). Results from these studies indicated that ERα might play an important role in male reproduction. However, although messenger RNA and the protein of ERβ appear in the male reproduction tract, ERβ knockout mice are fertile and show normal testis histology.

Genetic screening for the ERα gene locus has revealed several polymorphic sites (Gennari et al., 2005). The most widely studied are the *Pvu*II (T397C, rs2234693) and *Xba*I (G351A, rs9340799) restriction fragment length polymorphisms. The *Pvu*II polymorphism is caused by a T/C transition in ERα intron 1, whereas the *Xba*I polymorphism is caused by a G/A transition located 50 bp downstream of the *Pvu*II polymorphic site (Shearman et al., 2003). Several sequence variants of the ERβ gene have been described, including 2 silent G/A polymorphisms, *Rsa*I (1082G>A, rs1256049) and *Alu*I (1730G>A, rs4986938) (Rosenkranz et al., 1998).

The association between male infertility and ERα and ERβ gene polymorphisms has been explored in several studies that have produced conflicting results (Kukuvitis et al., 2002; Suzuki et al., 2002; Aschim et al., 2005; Lazaros et al., 2010). For example, *Pvu*II and *Xba*I in ERα have been associated with azoospermia or severe oligozoospermia in 2 reports (Kukuvitis et al., 2002; Suzuki et al., 2002), but others have drawn different conclusions (Tuttelmann et al.,

2007; Lazaros et al., 2010). Moreover, *Rsa*I and *Alu*I in ERβ have been associated with male infertility in 2 studies (Aschim et al., 2005; Safarinejad et al., 2010), but the results could not be replicated (Khattri et al., 2009). A recent review article on the genetic causes of male factor infertility by O'Flynn et al. (2010) concluded that ER gene polymorphisms should be examined further to replicate the results of earlier uncoordinated studies and to elucidate more fully the impact of these polymorphisms on male fertility. Until now, few studies of ERα and ERβ polymorphisms in Chinese populations with azoospermia or severe oligozoospermia have been reported.

Accordingly, our aim was to investigate *Pvu*II and *Xba*I in ERα and *Rsa*I and *Alu*I in ERβ and their association with azoospermia or severe oligozoospermia. Moreover, by comparing sex hormone levels in subjects with different ER genotypes, we sought to examine whether these polymorphisms might play a role in ER function *in vivo*.

MATERIAL AND METHODS

Subjects

A total of 204 infertile men [including men with oligozoospermia (sperm count <20 x 10^6/mL) and men with azoospermia] aged 25-38 years (mean age, 32.1 ± 5.2 years) were recruited from the Assisted Reproduction Center, Maternal and Child Health Care Hospital of Shaanxi Province and Yanan University Affiliated Hospital. All of them received a comprehensive andrological examination, including medical history and physical examination, semen analyses, scrotal ultrasonography, serum determination of hormone analysis, karyotyping, and Y-chromosome microdeletion screening. Subjects with abnormal karyotypes, deletions of the Y chromosome, or other recognizable causes of male infertility such as a history of orchitis, varicocele, cryptorchidism, obstruction, congenital bilateral absence of the vas deferens, hypogonadotropic hypogonadism, and iatrogenic infertility were excluded.

Control subjects were recruited from among husbands of women who received regular prenatal care at the Yanan University Affiliated Hospital. All of the control subjects had fathered at least 1 child within 2 years without assisted reproductive technologies. The total of 252 fertile men aged 26-40 years (mean age, 34.2 ± 5.7 years) with normal sperm concentration and motility according to the criteria adapted from World Health Organization guidelines were included in the control group.

All the subjects were recruited from the Shaanxi Province in northwestern China. Subjects were excluded if their parents or grandparents were not of Han descent. Thus, all of the study subjects were of Han ethnicity, which makes up more than 90% of the Chinese population. Informed consent was obtained from all subjects, and the study was approved by the Local Medical Ethics Committee. Each subject donated 5 mL blood for genomic DNA extraction and hormone assays.

Semen analysis

Semen samples were collected via masturbation after at least 3 days of sexual abstinence. The assessment of concentration was performed as recommended by the World Health Organization (1992) using a modified Neubauer chamber and positive displacement pipettes for proper dilution of the ejaculate. Each sample was assessed twice successively.

Hormone assays

Blood samples were allowed to clot, and after centrifugation, serum was stored at -20°C until analysis. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, estradiol, progesterone, and testosterone levels in serum were determined via luminescence-based immunoassays with an ACS-180SE auto system (Ciba Corning Diagnostics Corp., Cambridge, MA, USA) according to manufacturer recommendations. Reagents and standards were provided by Chiron Diagnostics Corporation (Emeryville, CA, USA). The reference ranges were as follows: FSH, 1-7 IU/L; LH, 1-8 IU/L; estradiol, 8-36 ng/L; progesterone, <3.2 µg/L; testosterone, 9.1-46.2 nM; prolactin, 6.2-13.0 µg/L.

Genotyping

Genomic DNA was extracted using a TIANamp Blood DNA Kit (Tiangen, Beijing, China) according to manufacturer recommendations and stored at -20°C until use. Genotyping of *Pvu*II, *Xba*I, *Rsa*I, and *Alu*I was performed using a polymerase chain reaction (PCR)-restriction fragment length polymorphism method described elsewhere (Safarinejad et al., 2010). PCR amplification of the polymorphic regions was performed using oligonucleotide primers as described elsewhere (Safarinejad et al., 2010). PCR amplification was carried out in a total volume of 12 µL containing 50-300 ng genomic DNA, 200 µM deoxyribonucleotide triphosphate, 5 µM primer, 15 mM $MgCl_2$, and 0.5 U Taq polymerase (Tiangen). The PCR products and restriction enzyme fragments (New England Biolabs, Ipswich, MA, USA) were separated by 2% agarose gel electrophoresis before imaging analysis (Quantity One, Bio-Rad, Hercules, CA, USA) for genotype determination. The primers, PCR conditions, restriction enzymes, and DNA fragment sizes are listed in Table 1.

Table 1. Primers, PCR conditions and restriction enzymes for estrogen receptor SNPs.

Variants	PCR primer sequences	Annealing condition	Extension condition	Enzymes	Enzyme digests (bp)
*Pvu*II	F: 5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3'	62°C for 50 s	72°C for 50 s	*Pvu*II	T = 1372
	R: 5'-TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3'				C = 982+390
*Xba*I	F: 5'-CTGCCACCCTATCTGTATCTTTTCCTATTCTCC-3'	62°C for 50 s	72°C for 50 s	*Xba*I	G = 1372
	R: 5'-TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA-3'				A = 936+436
*Rsa*I	F: 5'-TCTTGCTTTCCCCAGGCTTT-3'	62°C for 1 min	72°C for 90 s	*Rsa*I	G = 409
	R: 5'-ACCTGTCCAGAACAAGATCT-3'				A = 110+299
*Alu*I	F: 5'-GACCTGCTGCTGGAGATGCT-3'	62°C for 1 min	72°C for 90 s	*Alu*I	G = 405
	R: 5'-AATGAGGGACCACACAGCA-3'				A = 163+242

All PCR experiments consisted of a pre-denaturation step of 4 min at 95°C, denaturation for 30 s at 95°C, 30 cycles and total extension for 15 min at 72°C.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows (SPSS, Chicago, USA). Hardy-Weinberg equilibrium (HWE) and allele and genotype frequencies for each polymorphism were evaluated using the chi-square test. The distributions of ER polymorphisms were compared between the patient groups and controls using the Fisher exact test or the chi-square test. Odds ratios at 95% confidence intervals were calculated. Laboratory parameters were compared with the Fisher exact test or analysis of covariance. A P value of

<0.05 denoted significant difference after Bonferroni's correction.

RESULTS

The genotype distribution of the 4 polymorphisms was consistent with HWE in the control group. The distribution of genotype and allele frequencies and statistical analysis of the 4 SNPs are listed in Table 2. A strong linkage was found between the *Xba*I genotype distribution and patients. The frequency of the G allele was significantly lower in patients than in controls (P = 0.003). We further assessed the relationship between 3 *Xba*I genotypes (AA genotype, N = 151; GG genotype, N = 11; and AG genotype, N = 42) and hormonal data among patients carrying these genotypes (see Tables 2 and 3). Serum FSH and LH levels in *Xba*I AA carriers were significantly higher than those in AG or GG carriers in the patient group.

Table 2. Genotype and allele frequencies of ER gene polymorphisms in azoospermia or oligospermia patients and control subjects.

Position	Genotyping	Control [252 (%)]	Azoospermia or oligospermia [204 (%)]
*Pvu*II	TT	82 (32.54)	83 (40.69)
genotype	CT	126 (50.0)	96 (47.06)
	CC	44 (17.46)	25 (12.25)
	χ^2, P		4.287, 0.117
*Pvu*II	T	290 (57.54)	262 (64.22)
allele	C	214 (42.46)	146 (35.78)
	χ^2, P, OR, 95%CI		4.206, 0.04, 0.755, 0.577-0.987
*Xba*I	AA	148 (58.73)	151 (74.02)
genotype	AG	89 (35.32)	42 (20.59)
	GG	15 (5.95)	11 (5.39)
	χ^2, P		12.595, 0.0018
*Xba*I	A	385 (76.39)	344 (84.31)
allele	G	119 (23.61)	64 (15.69)
	χ^2, P, OR, 95%CI		8.828, 0.003, 0.602, 0.429-0.843
*Rsa*I	GG	127 (50.39)	103 (50.49)
genotype	AG	102 (40.48)	91 (44.61)
	AA	23 (9.13)	10 (4.90)
	χ^2, P		3.236, 0.198
*Rsa*I	G	356 (70.63)	297 (72.79)
allele	A	148 (29.37)	111 (27.21)
	χ^2, P, OR, 95%CI		0.517, 0.472, 0.899, 0.672-1.202
*Alu*I	GG	193 (76.59)	155 (75.98)
genotype	AG	48 (19.05)	41 (20.10)
	AA	11 (4.36)	8 (3.92)
	χ^2, P		0.122, 0.941
*Alu*I	G	434 (86.11)	351 (86.03)
allele	A	70 (13.89)	57 (13.97)
	χ^2, P, OR, 95%CI		0.0013, 0.972, 1.007, 0.691-1.468

OR = odds ratio; 95%CI = 95% confidence interval.

Table 3. Relationship between different *Xba*I genotypes and serum levels of follicle-stimulating hormone (FSH), luteining hormone (LH), prolactin, estradiol, progesterone, and testosterone infertile men.

*Xba*I	FSH* mean ± SD (IU/L)	LH# mean ± SD (IU/L)	Prolactin mean ± SD (μg/L)	Estradiol mean ± SD (ng/L)	Progesterone mean ± SD (μg/L)	Testosterone mean ± SD (nM)
AA (151)	19.00 ± 4.50	18.20 ± 6.20	15.60 ± 8.90	27.90 ± 10.60	2.10 ± 0.80	20.50 ± 9.50
AG (42)	16.50 ± 5.60	14.80 ± 6.80	14.20 ± 12.50	26.20 ± 15.80	1.90 ± 1.10	18.50 ± 16.50
GG (11)	14.20 ± 6.40	11.60 ± 7.60	13.90 ± 13.60	24.80 ± 17.50	1.80 ± 1.30	16.20 ± 17.20

*Serum levels of FSH in patients were significantly higher in *Xba*I AA carriers compared to AG or GG carriers.
#Serum levels of LH in patients were significantly higher in *Xba*I AA carriers compared to AG or GG carriers.

DISCUSSION

Estradiol is a survival factor for germ cells (Pentikainen et al., 2000), and thus, an absence of or reduction in estrogen can lead to impaired sperm production. The effects of estrogen are mediated by at least 2 ER subtypes (ERα and ERβ), and these subtypes are expressed in various stages of human germ cells (Couse and Korach, 1999; Carreau et al., 2006). An important role for estrogen in male reproduction has been suggested by both animal models and the phenotypes of men with ERα and ERβ gene mutations (Krege et al., 1998; O'Donnell et al., 2001; Gould et al., 2007; Sinkevicius et al., 2009). Consequently, ERα and ERβ have become candidate spermatogenesis genes in humans.

In the present study, we analyzed the association between impaired spermatogenesis and the *Xba*I and *Pvu*II polymorphisms of the ERα gene and the *Rsa*I and *Alu*I polymorphisms of the ERβ gene. We found a significant association between the *Xba*I polymorphism and impaired spermatogenesis. However, no association of ERβ with impaired spermatogenesis was found in our study, which supports previous observations of fertility in ERβ spermatogenesis knockout mice (Krege et al., 1998).

Previous studies on the influence of the ERα and ERβ genes in male factor infertility have yielded conflicting results (Tuttelmann et al., 2007; O'Flynn et al., 2010). The ERα polymorphisms *Pvu*II and *Xba*I were recently studied in 29 men with oligozoospermia and 85 men with normozoospermia in a Greek population (Kukuvitis et al., 2002), and the *Xba*I polymorphism showed a significant association with infertility, whereas the *Pvu*II polymorphism did not, which is consistent with the results of our study. Significant differences have been observed in the frequency distributions of *Pvu*II and *Xba*I of the ERα gene and *Rsa*I and *Alu*I of the ERβ gene between male infertility patients and controls in the Iranian population (Omrani et al., 2010). Khattri et al. (2009) demonstrated that the ERβ polymorphism *Rsa*I is not associated with infertility in Indian men, which is consistent with the results of the present study. Aschim et al. (2005) demonstrated that the *Rsa*I AG genotype is associated with an approximately 3-fold increase in infertility in white men, a result similar to that of Safarinejad et al. (2010).

The contradictory results regarding polymorphisms of ER genes may be attributed to the following. 1) Genetic background of the participants: even a selected group of non-obstructive azoospermia patients represents a heterogeneous population in which many other (yet unknown) genetic anomalies might be the causative factors of spermatogenic failure. 2) Population and sample size (173, 199, and 31 in Greek, Spanish, and Japanese populations for polymorphisms of the ERα gene): notably, these sample sizes might be insufficient for some studies of genetic associations in multifactorial disorders (Guarducci et al., 2006). According to a meta-analysis by Ioannidis et al. (2001), a minimum of 150 subjects (controls and cases) is mandatory for association analyses. 3) Because of the polygenic nature of spermatogenic failure, we believe that additional loci might be involved in the development of the spermatogenic phenotype, either within or near the ERα gene or the other core genes involved in estrogenic and estrogen-related pathways. 4) Although we failed to find any association between 2 polymorphisms of the ERβ gene and impaired spermatogenesis in Chinese men, more studies are required to confirm our results. The screening of mutations specific to spermatogenesis in the ERβ genes of other populations is needed to substantiate or negate our finding that ERβ plays no role in spermatogenic failure.

The present study revealed a significant association between the *Xba*I polymorphism and impaired spermatogenesis. Moreover, plasma levels of FSH and LH in patients with the AA

genotype were significantly elevated compared with those in patients with the AG or GG genotypes. These findings indicate that specific allelic combinations of the ERα gene, which confer higher FSH and LH levels and thus a stronger estrogen effect, may negatively influence human spermatogenesis. However, the A/G change does not lead to amino acid changes in the protein. We speculated that *Xba*I is in linkage disequilibrium with other genetic variations that could affect gene expression or function. Furthermore, the *Xba*I polymorphism may undergo changes in messenger RNA syntheses, splicing, maturation, transport, translation, or degradation.

Of note, abnormal sperm production and reduced fertility have been reported in transgenic male mice lacking ERα, leading to the conclusion that ERα plays a key role in spermatogenesis. Our results support this view. Additional studies on the function of ERs in human spermatogenesis *in vivo* or *in vitro* are needed.

In conclusion, we found an association between the *Xba*I genotype of the ERα gene and spermatogenesis in the Chinese population, and that the AA genotype of *Xba*I was negatively related to effects on FSH and LH secretion. The mechanisms by which *Xba*I might be related to semen parameters and hormone levels warrant further investigation.

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