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The *Rsa*I Polymorphism in the Estrogen Receptor-β Gene Is Associated with Male Infertility

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Context: Hypospadias, cryptorchidism, testicular cancer, and low semen quality have been proposed as being parts of the testicular dysgenesis syndrome (TDS) hypothetically due to changes in the androgen-estrogen balance *in utero*. Estrogens and estrogen receptors (ERs) play a role in regulating testicular function. ERβ contains two silent polymorphisms, *Rsa*I (G1082A) and *Alu*I (G1730A).

Objective: We investigated the significance of these polymorphisms in the etiology of disorders being part of TDS.

Setting: The patients were recruited consecutively through university hospital clinics.

Participants: Four groups of Caucasian patients were included: 106 men from infertile couples with a sperm concentration less than 5×10^6 spermatozoa/ml, 86 testicular cancer patients, 51 boys with hypospadias, and 23 cases with cryptorchidism. Military conscripts (n = 186) with sperm concentration higher than 5×10^6 spermatozoa/ml served as controls.

Main Outcome Measures: ERβ polymorphisms *Rsa*I and *Alu*I were determined by allele-specific PCR. In addition, reproductive hormone analyses were performed in controls and infertile men.

Results: Compared with the controls, the frequency of the heterozygous *Rsa*I AG-genotype was three times higher in infertile men (13.2 *vs.* 4.3%; $P = 0.01$). The heterozygous *Rsa*I AG genotype was associated with an approximately 20% reduction in LH concentration, compared with the wild-type *Rsa*I GG genotype in both controls and infertile men. Subjects with testicular cancer, hypospadias, or cryptorchidism did not differ from controls regarding the frequency of any of the polymorphisms.

Conclusions: Polymorphisms in ERβ may have modulating effects on human spermatogenesis. The phenotype of TDS seems to be, at least partly, determined by the genotype. **(*J Clin Endocrinol Metab* 90: 5343–5348, 2005)**

IN RECENT YEARS there have been reports on increasing prevalence of hypospadias, cryptorchidism (1), and testicular germ cell cancer (TGCC) (2, 3) as well as declining semen quality (4, 5), recently referred to as the testicular dysgenesis syndrome (TDS) (6). It has been hypothesized that changes in the androgen-estrogen balance *in utero* could lead to impairment of embryonic programming including the development of male reproductive organs during fetal life, resulting in one or more of the above-mentioned conditions. The hormonal balance could, for example, be disturbed by increased exposure to estrogenic or antiandrogenic endocrine disruptors (6, 7). Alterations in genes involved in androgen or estrogen action or metabolism could predispose for the development of some TDS conditions, by rendering some individuals more sensitive to an exogenously or endogenously derived disruption of the androgen-estrogen balance.

In men, estrogens are synthesized from testosterone via the action of aromatase cytochrome P450. 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 (8) and reduced sperm production and sperm motility in humans (9, 10). In contrast, increased levels of estrogens *in utero* have, at least in some studies, been shown to lead to TDS-like conditions in both mice (11–14) and men (15, 16). It is important to emphasize, however, that the hypothesized relationship between estrogens and TDS development is still a matter of controversy.

Estrogen signaling in the cell is mediated by estrogen receptors (ERs), of which at least two subtypes exist, ERα and ERβ. Two silent polymorphisms in ERα have been associated with azoospermia or severe oligozoospermia (17, 18), whereas no associations between ERα polymorphisms and cryptorchidism or TGCC have been found (19). Recently several sequence variants of the ERβ gene have been described (20), including two silent G⇒A polymorphisms, *Rsa*I and *Alu*I. Both polymorphisms have been overrepresented in ovulatory dysfunctions (21). However, studies on genetic variants of ERβ with respect to TDS 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, TGCC, hypos-

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Abbreviations: ER, Estrogen receptor; Fw, forward; Rev, reverse; TDS, testicular dysgenesis syndrome; TGCC, testicular germ cell cancer.

padias, and cryptorchidism. Furthermore, by comparing reproductive hormone concentrations in subjects with different ERβ genotypes, we wished to evaluate whether these polymorphic forms might play a role for ERβ function *in vivo*.

Patients and Methods

Infertile men

One hundred six consecutive Caucasian men from infertile couples were included in the study. All men presented with sperm concentrations lower than 5×10^6/ml in all (at least two) ejaculates, delivered for examination. Although the fertility of their female partners was not explored, these men are for the sake of simplicity referred to as infertile throughout this paper. Men with known genetic causes of infertility, *e.g.* Klinefelter syndrome or Y-chromosome microdeletions, as well as those with a history of cryptorchidism were excluded.

TGCC patients

During the period March 2001 to August 2002, all patients under the age of 50 yr with the diagnosis of TGCC passing through the outpatient clinic of the Department of Oncology, Lund University Hospital (Lund, Sweden) were asked to participate in a study of reproductive function. Seventy-nine percent of the patients accepted to participate. Blood samples for DNA analysis were drawn from 86 of them, all being Caucasians. Among these, 28 presented with a diagnosis of seminoma and 58 were nonseminomas, the latter group including 14 that had tumors with a seminoma component. The distribution of histological tumor types and clinical stages in those who denied taking part in the study, was the same as among the participants (data not shown).

Boys with hypospadias

Fifty-one consecutive Caucasian boys with normal male 46, XY karyotype, referred for surgery due to isolated hypospadias, were included in the study. The study group included glanular (n = 21), penile (n = 13), and penoscrotal (n = 17) hypospadias cases.

Men with a history of cryptorchidism

Twenty-three men with a history of cryptorchidism were included in the study. Of these, 15 were originally excluded from the group of infertile men, whereas eight were from the military conscripts group (see below).

Controls

Normal controls served a group of 186 Swedish military conscripts without genital abnormalities and with sperm concentration higher than 5×10^6 spermatozoa/ml. All underwent scrotal ultrasound, which did not reveal testicular microlithiasis indicative of carcinoma-*in situ* of the testis (22). This cohort was derived from an original group of 203 conscripts with Swedish parents. Subsequently, eight men were excluded due to cryptorchidism (see above) and eight due to sperm concentration lower than 5×10^6 spermatozoa/ml.

Informed consent was obtained from all subjects or their parents, according to protocols approved by the ethical review boards of Karolinska Institutet and Lund University.

Allele-specific PCR

In all subjects, allele-specific PCR was performed to detect the *Rsa*I and *Alu*I variants of ERβ. For each polymorphism two reactions per subject were run, using a specific primer for either the polymorphic A variant or for the wild-type G variant, together with an upstream and a downstream primer. PCR conditions were established to generate both a control fragment and a shorter, allele-specific band in the presence of the variant and only the control fragment in its absence.

Allele-specific PCR of the *Rsa*I polymorphism was performed in a total volume of 25 μl containing 25 ng genomic DNA, 45 mmol/liter KCl, 10 mmol/liter Tris HCl (pH 9.1), 0.1% Tween 20, 0.2 mmol/liter deoxynucleotide triphosphate, 1.5 mmol/liter $MgCl_2$, 1 U Dynazyme Taq polymerase (Finnzymes Oy, Espoo, Finland), and 0.5 μmol/liter of each of the primers *Rsa*I forward (Fw), *Rsa*I reverse (Rev), and either *Rsa*I RevA or *Rsa*I RevG. Primer sequences are presented in Table 1. Amplification was performed for 35 cycles; each cycle including denaturation for 1 min at 96 C, primer annealing for 30 sec at 58 C, and primer extension for 3 min at 72 C, with an initial denaturation step for 3 min at 96 C, and a final extension step for 7 min at 72 C. For the *Alu*I polymorphism, an annealing temperature of 54 C for 30 sec was used. Other conditions were the same as for the *Rsa*I reaction. The sequences of the primers are presented in Table 1.

The control fragment and the allele-specific fragment were 409 and 127 bp, respectively, for the *Rsa*I polymorphism and 405 and 258 bp, respectively, for the *Alu*I polymorphism.

Analysis of restriction fragment length polymorphisms

Both the *Rsa*I and *Alu*I polymorphisms are restriction fragment length polymorphisms, 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 *Rsa*I polymorphism, a G to A nucleotide exchange at nucleotide 1082 in exon 5 created a recognition site for *Rsa*I, and in the *Alu*I polymorphism an exchange of G to A at nucleotide 1730 in the noncoding end of exon 8 introduced a recognition site for *Alu*I (nucleotide numbering according to GenBank accession no. AB006590). In both positions a G nucleotide was considered the wild-type sequence and was not digestible by *Rsa*I or *Alu*I.

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

Hormone analysis

Inhibin B levels were assessed using a specific immunometric assay, as previously described (23), with a detection limit of 15 ng/liter and total assay variation coefficients less than 7%. In military conscripts, circulating levels of FSH, LH, SHBG, testosterone, and estradiol were measured by an automated fluorescence detection system (Autodelfia, Wallac Oy, Turku, Finland) at the routine clinical chemistry laboratory, Uppsala University Hospital. Intraassay and total assay variation was below the level of 4 and 7.5%, respectively.

In infertile men, analyses were performed at Malmö University Hospital (Malmö, Sweden) using immunometric ELISAs with a commercially available kit (Oxford Bio-Innovation Ltd., Oxfordshire, UK). Laboratory total assay variation was 12.4% at 25 ng/liter and 12.8% at 305 ng/liter. Testosterone levels were measured using an immunoassay (Access; Beckman Coulter Inc., Fullerton, CA). Laboratory total assay variation was 2.8% at 2.9 nmol/liter and 3.2% at 8.1 nmol/liter. Plasma FSH and LH concentrations were measured by means of immunoassays (Immuno 1; Bayer Diagnostics Division, Tarrytown, NJ). Laboratory total assay variation for FSH was 2.5% at 2.9 IU/liter and 1.4% at 15 IU/liter, and for LH it was 2.6% at 3.0 IU/liter and 1.7% at 15 IU/liter. Serum SHBG was measured using an immunoassay (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA). Total assay variation was

TABLE 1. Primer sequences

Primer	Sequences (5′–3′)	Fragment length (bp)
*Rsa*I Fw	ACT TGC CAT TCT GTC TCT ACA	
*Rsa*I Rev	CAC AGG ACC CTG AAT CCT	409 (control)
*Rsa*I RevA	AGC TCT CCA AGA GCC GT	127 (A-variant)
*Rsa*I RevG	AGC TCT CCA AGA GCC GC	127 (G-variant)
*Alu*I Fw	TTT TTG TCC CCA TAG TAA CA	
*Alu*I Rev	CCT CTG CTA ACA AGG GAA A	405 (control)
*Alu*I RevA	GAG TTC ACG CTT CAG CT	258 (A-variant)
*Alu*I RevG	GAG TTC ACG CTT CAG CC	258 (G-variant)

3.7% at 29 nmol/liter and 6.7% at 85 nmol/liter. Because the methods of hormonal measurement applied to samples from controls and infertile men were not the same, we did not compare the hormone levels between the two groups but instead compared the hormone concentrations between the genotypes within each group.

Semen analysis

The ejaculate was obtained by masturbation after a minimum 48 h of sexual abstinence. The assessment of concentration was performed as recommended by the World Health Organization's recommendations by use of a modified Neubauer chamber and positive displacement pipettes for proper dilution of the ejaculate (24). Three laboratory technicians performed the analyses of the ejaculates. The interobserver coefficient of variation was found to be 8.5% for concentration assessment. The laboratory participates in an external Quality Control Program, organized by Nordic Association for Andrology and European Society of Human Reproduction and Embryology.

Statistical analysis

The distributions of ERβ polymorphisms were compared between the patient groups and controls using Fisher's exact test. Median hormone levels for the different genotypes, in controls and infertile men, separately, were compared by Mann-Whitney *U* test applying the SPSS statistical software (version 11.0; SPSS, Inc., Chicago, IL). All statistical tests were two sided. $P < 0.05$ was considered statistically significant.

Results

Infertile men

The *Rsa*I AA genotype was found in one control subject only. When analyzing the distribution of the *Rsa*I polymorphism, we found that the infertile men had approximately three times higher frequency of the heterozygous *Rsa*I AG genotype than controls ($P = 0.01$; Table 2 and Fig. 1). The infertile men did not differ from the controls regarding the *Alu*I polymorphism.

TGCC patients

The total group of TGCC patients did not differ from the controls with regard to any of the two ERβ polymorphisms (Table 2). Patients with nonseminomas with a seminoma component had five times higher frequency of the *Rsa*I AG genotype ($P = 0.03$) and a 68% higher frequency of the heterozygous *Alu*I AG genotype ($P = 0.05$), compared with controls.

Boys with hypospadias

No significant differences between controls and patients with hypospadias were found with respect to the two ERβ polymorphisms (Table 2).

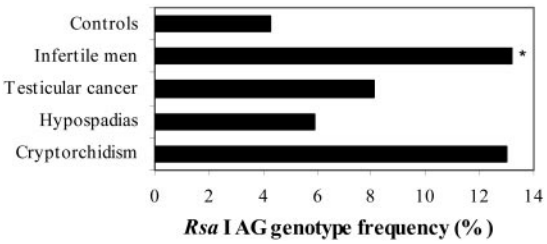

FIG. 1. Proportion of *Rsa*I AG genotype in the different study groups. Significant difference from controls is marked with an *asterisk*.

Men with cryptorchidism

The *Rsa*I AG genotype was three times more frequent among the men with a history of cryptorchidism, compared with the controls, although this difference did not reach statistical significance ($P = 0.11$). This group of men with cryptorchidism did not differ from the controls regarding the frequency of *Alu*I genotypes.

Hormone analysis

In both controls and infertile men, the heterozygous polymorphic *Rsa*I AG genotype was associated with an approximately 20% reduction in the median concentration of LH ($P = 0.05$ and $P = 0.04$, respectively), compared with the homozygous wild-type *Rsa*I GG genotype (Table 3 and Fig. 2). No such differences were found for FSH, inhibin B, testosterone, estradiol, or SHBG.

Regarding the *Alu*I genotypes, controls with AA had a nonsignificant 17% reduction in the median LH level, compared with those with GG ($P = 0.10$), whose median LH level was similar to individuals with AG (Table 4). In the infertile men, the median LH levels were 19% higher in both AA and AG, compared with GG ($P = 0.07$ and $P = 0.02$, respectively; Table 4). No significant differences were found for the other hormones.

Discussion

The main finding of our study was a significantly increased frequency of the ERβ *Rsa*I AG genotype among infertile men, compared with controls. This is to our knowledge the first study showing an association between ERβ genetic variants and male infertility. No statistically significant differences regarding any of the two ERβ polymorphisms were found between the patients with cryptorchidism or hypospadias or the total group of men with TGCC and the controls. However, in particular with regard to the

TABLE 2. Distribution of the *Rsa*I and *Alu*I genotypes AA, AG, and GG

Study groups	*Rsa*I			*Alu*I		
	AA	AG	GG	AA	AG	GG
Controls (n = 186)	1 (0.54)	8 (4.30)	177 (95.2)	25 (13.4)	79 (42.5)	82 (44.1)
Infertile men (n = 106)	0 (0)	14 (13.2)[a]	92 (86.8)	11 (10.4)	48 (45.3)	47 (44.3)
Testicular cancer (n = 86)	0 (0)	7 (8.14)	79 (91.9)	8 (9.30)	43 (50.0)	35 (40.7)
Hypospadias (n = 51)	0 (0)	3 (5.88)	48 (94.1)	9 (17.6)	21 (41.2)	21 (41.2)
Cryptorchidism (n = 23)	0 (0)	3 (13.0)	20 (87.0)	3 (13.0)	11 (47.8)	9 (39.1)

For all groups, the numbers of subjects and the proportion (in *brackets*) presenting with the specific genotype are presented.
[a] Heterozygous polymorphic genotype AG *vs.* homozygous wild-type genotype GG $P < 0.05$, compared with controls (Fisher's exact test).

TABLE 3. Hormone concentrations[a] in *Rsa*I genotypes

Hormone	Controls				Infertile men			
	*Rsa*I AG		*Rsa*I GG		*Rsa*I AG		*Rsa*I GG	
	n	Median (range)	n	Median (range)	n	Median (range)	n	Median (range)
Inhibin B (ng/liter)	8	180 (113–272)	177	206 (100–415)	9	81.0 (10.0–187)	56	83.5 (10.0–252)
T (nM)	8	22.5 (7.30–32.0)	177	23.0 (6.10–38.0)	11	12.8 (6.90–19.2)	78	13.0 (2.70–31.9)
FSH (IU/liter)	8	2.88 (1.25–6.95)	177	3.10 (0.60–9.95)	11	9.50 (1.70–27.5)	77	7.80 (2.10–48.7)
LH (IU/liter)	8	3.12[b] (1.92–4.62)	177	3.96 (1.14–9.72)	11	3.00[b] (1.40–8.90)	77	3.70 (1.20–15.9)
SHBG (nM)	8	35.0 (17.6–59.0)	177	28.0 (7.20–67.0)	11	2.80 (20.2–38.0)	78	26.8 (10.7–77.0)
E2 (pM)	8	76.5 (52.0–110)	177	76.0 (49.0–144)	7	86.0 (11.0–109)	42	75.5 (49.0–171)

There was only one person with *Rsa*I AA genotype in this study; thus, this genotype is omitted from the table.

[a] Reference values for controls (infertile men): inhibin B, 100–240 mg/liter (100–240); FSH, 1.0–10 IU/liter (1.0–10); LH, 1.2–9.6 IU/liter (1.0–10); testosterone (T), 8.7–33 nmol/liter (10–35); SHBG, 13–50 nmol/liter (13–71); estradiol (E2), 60–150 pmol/liter (<130).

[b] $P < 0.05$, compared with the corresponding GG group (Mann-Whitney *U* test).

former group, in which the proportion of *Rsa*I AG was at the same level as in the infertile group, the lack of statistical significance might be due to low statistical power resulting from small sample sizes.

The finding of decreased LH levels in men with the *Rsa*I AG genotype, despite unchanged testosterone and estradiol concentration, might indicate that this genotype implies an increased ERβ activity, leading to increased estrogen sensitivity. The finding that the *RsaI* AG group presented with lower LH values in controls as well as in the infertile men makes it more probable that this is a biologically relevant observation and not just a result of multiple testing. Correspondingly, decreased LH levels were reported in Chinese patients with ovulatory dysfunction presenting with combined *Rsa*I and *Alu*I AA genotypes (21).

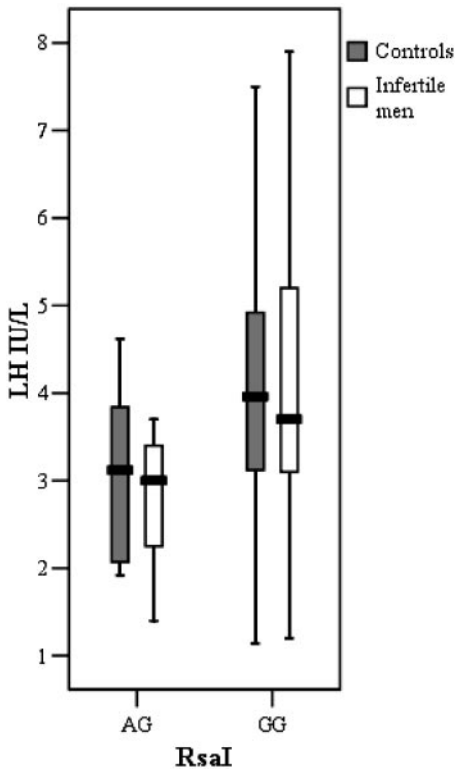

FIG. 2. Box plots showing median and interquartile range of the LH levels according to *Rsa*I genotype in controls and infertile men. The median LH level was significantly lower in *Rsa*I AG, compared with *Rsa*I GG in both groups.

With regard to increased estrogen sensitivity in *Rsa*I AG subjects, our findings are in accordance with the observation of increased risk of reduced sperm quality in men exposed to the potent estrogen diethylstilbestrol *in utero* (25). The latter finding, however, might be a chance observation because it was not confirmed in a later study (26). Furthermore, it is well known that spermatogenic arrest occurs when men are on a long-term estrogen replacement therapy before sex change surgery (27, 28), and hence, it seems plausible to assume that increased exposure to estrogens hampers spermatogenesis. This effect might be indirect, mediated through lower gonadotropin secretion and as one of the consequences of a decreased testosterone synthesis by the Leydig cells. In our study, however, *Rsa*I AG and *Rsa*I GG subjects had similar circulating testosterone levels, suggesting that this was not the mechanism behind the low sperm concentration in our infertile subjects. An additional direct role of estrogen in spermatogenesis is indicated by the detection of ERβ in human testis in the Sertoli cells as well as germ cells, including round spermatids (29) and the fact that the splicing of this gene seems to be cell-dependent within the human testis (28).

The mechanisms behind altered ERβ function in subjects with *Rsa*I 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 *Rsa*I polymorphism was in complete linkage disequilibrium with a polymorphism located at the splice acceptor site just before exon 8 in ERβ (30). This may potentially affect the splicing of this exon, leading to proteins with different properties than the wild-type ERβ (31, 32). The *Rsa*I polymorphism could also have a direct effect through

TABLE 4. Hormone concentrations[a] in *Alu*I genotypes

Hormone	Controls						Infertile men					
	*Alu*I AA		*Alu*I AG		*Alu*I GG		*Alu*I AA		*Alu*I AG		*Alu*I GG	
	n	Median (range)	n	Median (range)	n	Median (range)	n	Median (range)	n	Median (range)	n	Median (range)
Inhibin (ng/liter)	25	200 (113–415)	79	200 (102–353)	82	209 (100–415)	6	47.5 (15.0–181)	31	79.0 (10.0–252)	28	99.5 (10.0–236)
T (nM)	25	23.0 (14.3–32.0)	79	23.0 (6.10–37.0)	82	23.0 (7.30–38.0)	10	11.9 (6.30–16.9)	42	12.6 (4.90–23.8)	37	13.3 (2.70–31.9)
FSH (IU/liter)	25	2.95 (1.10–6.95)	79	3.15 (0.85–9.95)	82	3.08 (0.60–8.00)	10	8.80 (3.20–37.2)	42	8.70 (1.70–48.7)	36	7.15 (2.80–35.0)
LH (IU/liter)	25	3.36 (1.68–6.24)	79	3.96 (1.92–8.10)	82	4.08 (1.14–9.72)	10	3.80 (2.70–13.5)	42	3.85[b] (1.40–15.9)	36	3.25 (1.20–11.0)
SHBG (nM)	25	29.0 (13.1–59.0)	79	28.0 (7.20–67.0)	82	28.0 (8.60–59.0)	10	23.8 (12.0–41.0)	42	28.2 (10.7–77.0)	37	26.8 (12.0–71.0)
E2 (pM)	25	77.0 (60.0–144)	79	77.0 (52.0–121)	82	74.0 (49.0–134)	5	62.0 (49.0–120)	24	77.5 (49.0–145)	20	76.0 (11.0–171)

[a] Reference values for controls (infertile men): inhibin B, 100–240 mg/liter (100–240); FSH, 1.0–10 IU/liter (1.0–10); LH, 1.2–9.6 IU/liter (1.0–10); testosterone (T), 8.7–33 nmol/liter (10–35); SHBG, 13–50 nmol/liter (13–71); estradiol (E2), 60–150 pmol/liter (<130).

[b] $P < 0.05$, compared with the corresponding GG group (Mann-Whitney U test).

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 (33, 34).

We found no clear association between ERβ polymorphisms and hypospadias or cryptorchidism, although the latter group presented with equally high prevalence of the *Rsa*I AG polymorphism as the infertile men. This finding was possibly linked to the infertility status of the majority of these men rather than to cryptorchidism *per se*.

Nonseminomatous TGCC with seminoma components was associated with an increased frequency of both *Rsa*I and *Alu*I AG genotypes. However, because no such association was found in patients with pure nonseminomas or seminomas, this might be a chance finding.

As controls we included military conscripts, who can be considered as representative for the general population of Swedish adolescents (35). None of them presented with genital abnormalities, and men with a history of cryptorchidism were excluded from this group. Although their age was below the peak incidence of TGCC, scrotal examination gave no indication of carcinoma *in situ* of the testis in any of these men. However, although we selected the controls based on sperm concentration above 5×10^6 spermatozoa/ml, it cannot be excluded that some of these men in the future might experience infertility problems. On the other hand, we believe that any misclassification would introduce a nondifferential bias and result in an underestimation of the association between genotype and infertility or TGCC rather than produce false-positive results. Furthermore, because we used consecutive cases, we believe that the results of this study can be generalized to the respective groups of patients.

The distribution of *Rsa*I and *Alu*I genotypes in our controls was similar to that in previous studies in Caucasians (20, 36–38). This indicates that the comparison of Swedish controls with case groups including a fraction of non-Swedish Caucasians in our study is justifiable.

In conclusion, we found an association between the *Rsa*I genotype of the ERβ gene and male infertility, which may be related to effects on LH secretion. Our findings demonstrate that genetic polymorphisms in the ERβ gene might modify the phenotypic outcome in TDS.

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