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Significance of Mutations in the Androgen Receptor Gene in Males with Idiopathic Infertility*

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ABSTRACT

Abnormal human spermatogenesis is caused by a variety of genetic and acquired conditions. Because spermatogenesis is dependent on androgens, some males may have a minimal form of androgen insensitivity that does not inhibit virilization but impairs fertility. This has lead us to investigate the possibility of abnormalities in the androgen receptor (AR) gene in a large cohort of males suffering from infertility of unknown cause.

We studied 180 males with variable impairment of spermatogenesis. In all patients, serum levels of testosterone and gonadotropins were analyzed to define an androgen sensitivity index (ASI). Single-strand conformation analysis and direct DNA sequencing of PCR-amplified blood leukocyte DNA were used to identify mutations within the whole coding region of the AR-gene. Endocrine and molecular investigations were compared with 53 normal males with proven fertility.

In three infertile males, mutations in the AR were identified. Two unrelated males had the same variation within the first exon encoding for the transactivation domain of the receptor (Pro390Ser), whereas, in the third, a mutation in the hormone-binding region was characterized (Gln798Glu). All identified mutation carriers had a significantly elevated ASI.

A proportion of males with idiopathic infertility carry relevant variations within the AR-gene. These males may be distinguished on the basis of hormone levels, calculating the ASI, although this index lacks specificity. (*J Clin Endocrinol Metab* **85:** 2810–2815, 2000)

INFERTILITY IN otherwise healthy males may be caused by a variety of disorders. These include acquired, as well as heritable conditions. Genetic causes of male infertility are of special interest to reproductive biology, because these disorders can be passed on to the offspring with assisted reproductive techniques (1). Defects in the androgen receptor (AR) gene, a nuclear transcription factor, draw attention for two reasons. First, the AR-gene is localized on the X-chromosome and is therefore carried in hemizygous fashion by males and may be inherited from healthy female transmitters. Second, androgens are known to have a direct effect on Sertoli cells and sperm maturation.

Germline mutations in the AR-gene usually lead to a defect of virilization in karyotypic males despite normal, or even supraphysiologic, androgen levels. The phenotype of affected individuals varies considerably, depending on the type and localization of these mutations. It ranges from unequivocally female, without any androgenic effects over partial androgen insensitivity characterized by genital ambiguity, to males with undervirilization (2). Because of an impaired AR-mediated regulatory mechanism of the gonadotropin-androgen axis, both LH and testosterone (T) in serum may be elevated, leading to a distinctively high value of the LH × T multiplication product. This value has previously been termed as the androgen sensitivity index (ASI) (3). The majority of alterations within the AR-gene are point mutations with subsequent amino acid substitutions, destructing AR-function and diminishing androgen response. However, selective mechanisms of androgen action, *i.e.* specific support of external virilization or spermatogenesis, have not been demonstrated yet to be altered conversely by such mutations *in vivo*. This may be different for changes in the size of one of the two trinucleotide repeat polymorphisms within the transactivation domain of the receptor. Expansion of the polyglutamine tract above the normal range is associated with adult onset of spinal and bulbar muscular atrophy (Kennedy's disease). Affected males also demonstrate signs of androgen insensitivity and infertility. Two reports recently proposed that longer polyglutamine tracts within the normal range increased the risk of impaired spermatogenesis in otherwise normal males (4, 5). These data would indicate a direct relation between the length of this AR-specific variable polymorphic region and defective sperm maturation caused by decreased functional competence of the AR. However, these findings have been challenged both by us and others (6, 7), indicating that a correlation between the variable CAG (polyglutamine)-repeat length in the AR-gene and male infertility cannot be made in Caucasians.

Moreover, the importance of infertility in males, caused by defects in the AR, has been discussed quite controversially on the ground of biochemical analysis of specific androgen

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binding in genital skin fibroblasts. These older studies proposed, as a cause for male infertility, that between 0% (irrelevance of androgen insensitivity) and 40% of all infertile males are affected by defective androgen binding (3, 8–12). With the knowledge of the genetic structure of the AR-gene, molecular analysis based on PCR methodology has been reported for males with idiopathic infertility in newer research papers; however, most of these publications were either single-case reports or covered only part of the AR-gene (13–21). The aim of our present study was to investigate a large cohort of males with infertility and impaired spermatogenesis of unknown cause, for variations within the coding region of the AR-gene. Results were correlated with biochemical endocrinological findings and spermatologic values and compared with a normal control group.

Subjects and Methods

Patients and controls

One hundred eighty patients, 20.9–54.4 yr old, were referred, between 1993 and 1997, from the Department of Andrology at the University Hospital Hamburg-Eppendorf and the Department of Obstetrics and Gynecology at the Medical University of Lübeck (both regional centers of andrology) for molecular analysis. All patients had had a period of at least 2 yr of unfulfilled fertility. Clinical investigations by experienced andrologists covered the exclusion of known causes of male infertility as overt virilization disorders (*e.g.* hypospadias, gynecomastia) and other genetic (*e.g.* chromosomal aberrations, such as Klinefelter syndrome and genetic abnormalities as cystic fibrosis, endocrine (*e.g.* hypogonadotropic hypogonadism and prolactinoma), infectious, or obstructive causes (*e.g.* congenital bilateral aplasia of the vasa deferentia). Clinical work-up also covered questions regarding libido and shaving frequency, as well as a family history.

Routinely, hormonal status was requested and included values for LH, FSH, and T by commercially available RIAs. Ejaculates were investigated at least 2 times on different occasions, according the guide lines of the World Health Organization (22). One hundred ten patients showed oligoteratozoospermia (<10 million sperm/mL ejaculate, <30% normal forms), 57 had teratozoospermia (<30% normal sperm), and 13 patients suffered from azoospermia. Informed consent was obtained from all patients for experimental molecular investigations, and EDTA-blood was drawn at routine venopuncture.

Fifty-three Caucasian males served as controls. Fertility was assured, because all had fathered at least one child. Spermiograms were not available in these cases. These males consented to a single blood draw for serum analysis of LH, FSH, and T , as well as molecular genetic studies from blood leukocyte DNA.

DNA analysis

The whole coding region of the AR gene, including intron/exon junctions, was amplified from blood leukocyte-derived DNA, by PCR, in 14 segments as described previously (23). While exons 2–8 were separately amplified and each amplification product spanned completely a single exon, primers were designed to amplify the first exon in 7 overlapping segments. All PCR-products were subjected to nonisotopic single-strand conformation analysis for the detection of point mutations as described previously (23). In addition to the negative control DNA samples of males with proven fertility, 65 positive control samples with previously detected point mutations within one of the exons or segments were included in the study. Sequencing of variations was performed on an automated sequencer (ALF Express II, Amersham Pharmacia Biotech, Freiburg, Germany) employing cycle sequencing protocols provided by the manufacturer. To assess the length of the variable CAG (polyglutamine) and GGN (polyglycine) trinucleotide polymorphisms within the first exon of the AR, additional PCR products were electrophoresed on nondenaturing polyacrylamide gels with adequate size markers and visualized with silver staining. Additionally, selected samples were sequenced to determine the exact number of CAG or GGN-repeats, and these control samples were included in each electrophoresis. Determination of the repeat-sizes was performed on a computerized analysis system (ImageMaster, Amersham Pharmacia Biotech).

Assessment of mutant AR function in vitro

The two mutations Pro390Ser and Gln798Glu were introduced into the AR expression vector pSVAR0 (a kind gift of Dr. A. O. Brinkmann, Erasmus University, Rotterdam, The Netherlands), employing the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the instructions supplied by the manufacturer and as described previously (24).

Cultured Chinese hamster ovary cells were maintained at 37 C in 5% CO_2 with DMEM F12 (Life Technologies, Karlsruhe, Germany), 5% FCS, and antibiotics. Transfections were performed using the Ca^{2+} phosphate precipitation method. Then, 50 ng/mL of either wild-type or mutant AR expression plasmid was transfected. In addition, 2 μg/mL of mouse mammary tumor virus (MMTV)-luciferase reporter plasmid (Organon, Oss, The Netherlands), 10 ng/mL of constitutively expressed pRL-SV40 Renilla luciferase plasmid (Promega Corp., Madison, WI), and 17.9 μg pTZ19 carrier plasmid were cotransfected. Firefly luciferase counts were corrected for transfection efficiency by Renilla luciferase activity, both determined using the Dual Luciferase Reporter gene assay (Promega Corp.). For investigation of transactivation activity, cells were incubated with increasing concentrations of either dihydrotestosterone or T (see Fig. 3). Each transfection was done in triplicate, and three independent experiments were performed. The two mutant plasmids and the wild-type AR plasmid were always assayed in parallel, for means of comparability of results.

Results

Hormone analysis

Hormonal values within the group of infertile males were as follows. The mean for FSH was 8.4 U/L (range, 1.3–94.0); for LH, 3.5 U/L (range, 0.4–29.0); and for T, 20.5 nmol/L (range, 6.2–69.3). Absolute values for serum T and LH were multiplied with each other to determine the ASI. The mean for this index was 58.9 U $\times$ nmol/L^2 (range, 6.2–499.2). Within the control group, the mean for LH was 3.7 U/L (range, <0.1–8.5); for FSH, 4.8 U/L (range, 1.0–14.5); and for T, 14.7 nmol/L (range, 5.9–34.7). The mean for the ASI was found to be 54.1 U $\times$ nmol/L^2 (range, 6.7–138.7). In comparison with the normal control group, 39 of the 180 infertile males had an ASI above the normal range.

Molecular genetic analysis of the AR-gene

PCR-amplification was successfully achieved for the whole coding region in all patients and controls investigated, and no gross abnormalities were detected in any of the patients' DNA samples. Employing single-strand conformation analysis, several variations were encountered and subjected to sequencing. Within the second fragment of exon 1, a similar abnormality was found in 33 of 180 patients (18.3%), as well as in 11 of 53 normal fertile males (20.8%). Sequencing revealed a nucleotide substitution in codon position 211 (GAG-GAA), which does not alter the encoded amino acid glutamate (data not shown). This variation has been well described by us and others as a naturally occurring polymorphism (25).

In three patients, nucleotide substitutions were characterized that alter the predicted amino acid sequence of the AR (Table 1). In two unrelated patients (B 386 and B 236), a cytidine-to-thymidine substitution in exon 1 leads to an ex-

TABLE 1. Endocrine data and sperm counts of the three patients in whom mutations within the AR-gene were identified

Patient	AR-gene ↑ mutation	Sperm count ↑ (mill./mL)	Morphology ↑ (percent normal)	FSH ↑ (U/L)	LH ↑ (U/L)	T ↑ (nmol/L)	Androgen ↑ sensitivity index
B 236	Pro390Ser	0.6	None	17.0	5.5	45.1	247.9
B 386	Pro390Ser	0.6	22%	27.0	13.0	31.2	405.6
B 388	Gln798Glu	0.9	6%	7.8	7.4	31.2	230.9

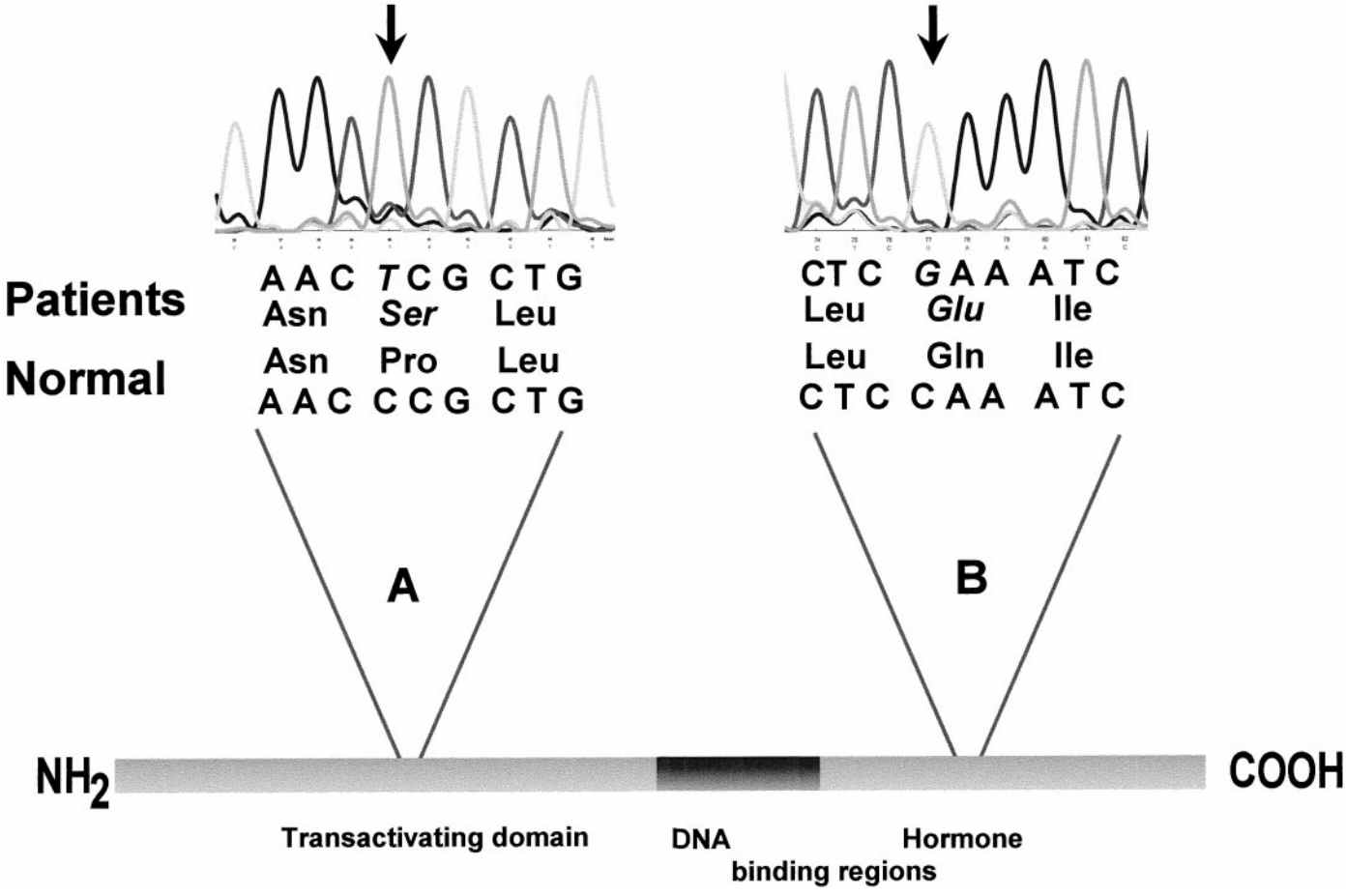

FIG. 1. Mutational analysis of the AR gene in patients B 388 and B 236. Patient B 236 carries a cytidine-to-thymidine substitution (CCG-TCG) in exon 1, predictively altering the normally present proline to serine in codon position 390 within the transactivation domain (A). In patient B 388, a cytidine-to-guanine conversion in exon 6 leads to an exchange of glutamic acid for the normally present glutamine in codon position 798 within the hormone binding domain.

change of the normally present proline by serine (*C*CG-*T*CG) (Fig. 1). Both patients with this abnormality had a decreased sperm count with a high percentage of abnormal sperm (Table 1). A third patient (B 388) exhibited an abnormality in exon 6, where a cytidine-to-guanine substitution leads to a predicted glutamine-to-glutamic acid exchange in codon position 798 (CAA-GAA) (Fig. 1). In all three patients, the ASI was highly elevated above the range of the normal control group (normal < 138 U $\times$ nmol/L^2).

Analysis of polymorphic repeat regions within the AR-gene

We had determined both the length of the CAG- and the GGN-repeats within the first exon of the AR-gene in both the patient and the control group. Investigation of the CAG-repeat had revealed a mean of 23 repeats (range, 13–30) in the patient group; whereas in the control group, the mean was 24 repeats (range, 17–39) (see Ref. 6). Also for the GGN-repeat, no significant difference was found between the patient and control groups. For all infertile males, the mean was 22 GGN-repeats (range, 13–27); in the control group, the mean was 23 repeats (range, 17–27) (Fig. 2). The Wilcoxon/Mann-Whitney test was used for statistical analysis and revealed no statistical difference between either group for both the length of the CAG and GGN-repeat.

Assessment of AR function in vitro

The mutated Glu^{798}AR displayed a differential pattern of partial transcriptional deficiency in the luciferase assay. Incubation of the cells with dihydrotestosterone showed only minimal receptor impairment, compared with the wild-type

AR. However, in the presence of T , reporter gene induction was reduced and could not be overcome by increasing ligand concentrations (Fig. 3). In contrast to the $Glu^{798}AR$, the $Ser^{390}AR$ did not lead to gross alterations of transcriptional activity, compared with the wild-type AR in the presence of both hormones (Fig. 3). AR protein expression levels, as assessed by Western immunoblotting of transfected Chinese hamster ovary cells, displayed no apparent differences among the three constructs (data not shown).

Discussion

This study constitutes the survey of the complete coding region of the AR gene in a large cohort of infertile males. Our findings support the suggestion that some men suffering from infertility of otherwise unknown cause may carry relevant abnormalities in the AR gene.

An elevated ASI has been suggested previously as an indication of androgen insensitivity, because the elevation of both LH and T was interpreted as an impaired negative feedback control of the hypothalamic-pituitary-testicular axis (3). In all 3 patients with mutations of the AR-gene in our study, the ASI was between 230.9 and 405.6 U $\times$ nmol/L^2, thus, strikingly higher than in the control group (normal range in the control cohort, <138 U $\times$ nmol/L^2). We believe that the determination of the ASI can be useful in identifying patients at risk for a minimal form of androgen insensitivity caused by mutation of the AR. Our results may be interpreted to mean that patients who do not display an elevated ASI are not likely to carry mutations in the AR gene. In contrast, 39 infertile males of our cohort had an ASI above the determined normal range in the control group. Thus, only a minority of infertile males with an elevated ASI above the normal range had mutations in the AR-gene. Most likely this is because of the low specificity of this endocrine abnormality, as, for instance, in estrogen insufficiency, LH and T are also elevated as a sign of a defective feedback control on gonadotropins (26). However, only 9 patients had an ASI more than 200 U $\times$ nmol/L^2. Thus, we propose that the higher the ASI, the more likely an abnormality within the AR-gene.

All three mutation carriers had oligoteratozoospermia with severely deformed sperm (Table 1). Recently, Tesarik *et al.* (27) demonstrated that T potentiates the effects of FSH because of prevention of Sertoli cell apoptosis. It is also known, that FSH regulates AR expression in Sertoli cells (28). These experiments support our clinical findings that variations of the AR may interfere with mechanisms of sperm maturation rather than cause complete absence of spermatogenesis.

Two different mutational mechanisms within the AR gene are of interest in infertile males: variable polymorphisms, and mutations in the conserved regions. In contrast to previous studies (3, 4), we found no abnormalities within either trinucleotide repeat polymorphism of the AR, in comparison to the control group, nor did Giwercman and co-workers in their recent study on a Swedish population (5, 6). Thus, the role of polyglutamine repeat expansion within the AR gene in infertile males remains questionable. Moreover, in our study, no presymptomatic patients with Kennedy's disease

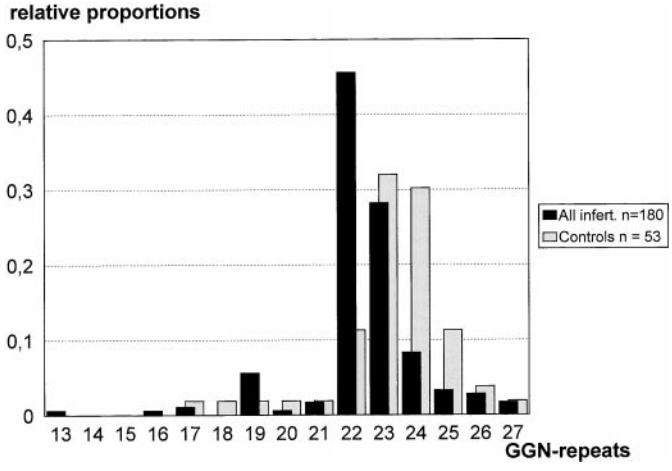

FIG. 2. Evaluation of polymorphic repeat region for the GGN (polyglycine)-repeat numbers. The repeat numbers from the infertile males (n = 180) did not vary significantly from the control group (n = 53).

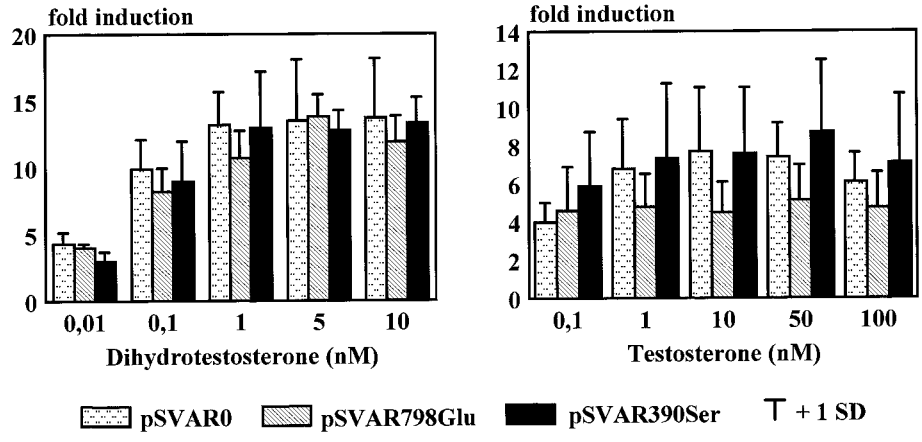

FIG. 3. Transcriptional activity attributable to wild-type (pSVAR0) and mutant AR constructs (pSVAR798Glu and pSVAR390Ser), respectively. *Bars* represent the relative induction of MMTV luciferase reporter gene activity in the presence of increasing concentrations of either T or dihydrotestosterone related to basal activity in the absence of hormone. Data are based on three independent cotransfection experiments, consisting of triplicate transfections each. Whereas the pSVAR798Glu construct leads to a significantly lower transcriptional activity after incubation with T , the luciferase induction was almost normal with dihydrotestosterone. With the pSVAR390Ser construct, the transcriptional activity, using the MMTV luciferase reporter gene, was not significantly altered, in comparison with the wild-type plasmid construct.

were detected. Also, investigations of the polyglycine repeat revealed no deviation from the normal control group. Thus, elongation or reduction of this variable repeat region within the AR does not play a significant role in male infertility.

Point mutations leading to amino acid substitutions represent the most important mechanism of AR abnormalities in all patients with androgen insensitivity syndrome. Though two unrelated patients with similar ASI and impairment of spermatogenesis carry a previously unreported point mutation within the first exon altering the amino acid structure of the transactivation domain (Pro390Ser), the mutation Gln798Glu within the hormone binding region has been described by us and others previously (1, 15, 28). Cotransfections of the mutant AR construct Glu^{798} displayed a partial loss of androgen-induced transcriptional activity. However, the phenotype of patients with this mutation is highly variable. Bevan *et al.* and we ourselves (1, 29) have described patients carrying the Glu798Gln mutation who presented with a severe virilization defect characterized by predominantly female external genitalia. The occurrence of completely normal external virilization with only severely impaired spermatogenesis caused by this mutation is much less clear with respect to the transactivation deficit demonstrated *in vitro* (Fig. 3b). Thus, the *in vitro* studies can hardly reflect the changes induced *in vivo*, because the *in vitro* model is highly oversimplified to explain the differential pattern leading to defective spermatogenesis. The differential response to dihydrotestosterone, compared with T, suggests that the Gln798Glu mutation may cause a more subtle defect, particularly impairing spermatogenesis attributable to selective response to different androgens.

Correlation of *in vitro* function of the Ser^{390} AR with the phenotype of impaired spermatogenesis in the two affected subjects is also limited. Interestingly, this mutation is located within a region of the AR that is important for transcriptional activity (30). Moreover, a Pro390Asp mutation of the AR gene has been reported in association with complete AIS, marking the importance of this amino acid residue (31). The endocrine abnormalities of the two patients with this mutation, both characterized by the elevated ASI, suggest a functional defect of the Ser^{390}AR *in vivo*. We may assume that interactions of currently unknown cofactors of the AR that are important for normal receptor function *in vivo* could be affected by the Pro390Ser mutation characterized in our cohort; however, this remains to be proven.

Thus, the *in vivo* mechanism leading to such highly variable phenotypes of the described mutations, and their molecular impact on spermatogenesis, still have to be further elucidated. However, the assessment of mutations of the AR gene is of severe importance in clinical medicine: The transmission of an AR defect in reproductive procedures may lead to a more severely affected offspring in future generations. Therefore, infertile males carrying genetic variations have to be identified and counseled accordingly before *in vitro* fertilization procedures (5).

We conclude that AR gene mutations may play an important role as genetic determinants of male infertility. In contrast to other genetic markers of male infertility, patients with AR-gene mutations can be preselected on the basis of endocrine evaluation. Molecular analysis of the AR gene should be instituted in males with a highly abnormal ASI. Whether infertile men with AR gene mutations generally respond favorably to conservative treatment options remains to be investigated. Future identification of molecular abnormalities of the AR gene in normally virilized males with impairment of spermatogenesis will give exciting new insights into different receptor mechanisms and alternatively mediated androgen action and their relevance in human disease.

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