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Male Infertility: No Evidence of Involvement of Androgen Receptor Gene Among Indian Men

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ABSTRACT: Spermatogenesis is collaboratively controlled by testosterone and follicle stimulating hormone. Testosterone and its immediate metabolite dihydrotestosterone affect their roles through the androgen receptor (AR). Mutations in the *AR* gene have been shown to cause partial to complete androgen insensitivity or infertility in otherwise normal males. The dependence of germ cells upon Sertoli and Leydig cells for their differentiation into sperms and deletion studies of the *AR* gene in animal models indicate a direct or indirect role of the *AR* gene in spermatogenesis. Although a few studies worldwide have reported *AR* mutations in male infertility, no similar study has been conducted on Indian populations. Therefore, we undertook this study to look at the contribution of *AR* mutations in male infertility among Indian men. We have sequenced the complete coding region of the *AR* gene in a total of 399 infertile samples, comprising 277 azoospermic, 100 oligozoospermic, and 22 oligoteratozoospermic samples. A total of 100 healthy males with proven fertility and the same ethnicity as the experimental group served as controls. Sequence analysis revealed no mutation in any of these samples. Our study suggests that mutations in the *AR* gene are less likely to cause azoospermia and oligozoospermia; however, it was difficult to rule out its effect in oligoteratozoospermia, as the sample size was small.

Key words: Azoospermia, oligozoospermia, oligoteratozoospermia, spermatogenesis.

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Androgens, testosterone and dihydrotestosterone, signal male secondary sexual differentiation and maturation through the androgen receptor (AR). The *AR* gene has been mapped to the long arm (Xq11–12) of the X chromosome (Lubhan et al, 1988; Yong et al, 1998). The gene has 8 exons and consists of transactivation domain, DNA-binding domain, and ligand-binding domain. Insensitivity to androgens due to mutations in the *AR* gene or its coactivators manifests in varying forms of androgen insensitivity in 46,XY individuals (Gottlieb et al, 1999). Complete androgen insensitivity is represented by female external genitalia and secondary sexual characteristics. Partial androgen insensitivity is associated with hypospadias and ambiguous genitalia (Quigley et al, 1995), while mild androgen insensitivity is characterized by undervirilization or infertility in otherwise normal males (Yong et al, 2003).

The androgen sensitivity index (ASI) is the multiplication product of leutinizing hormone (LH) and testosterone level values. Elevated ASI has been suggested as an indication of androgen insensitivity because the impaired feedback regulatory mechanism of the gonadotropin-androgen axis leads to an elevation of LH and testosterone levels (Aiman et al, 1979; Hiort et al, 2000). It is a well-known fact that germ cells are nurtured by Sertoli cells for their differentiation into sperm, and Sertoli cells are in turn dependent upon Leydig cells for androgens. Although germ cells themselves do not express *AR*, they are indirectly dependent upon androgens and hence AR for their differentiation into sperm. Studies of a hypomorphic and conditional allele of the *AR* gene have uncovered a dual postmeiotic requirement for androgen receptor activity during male germ cell differentiation.

Observations in *AR* hypomorphic animals demonstrate that terminal differentiation of spermatids and their release from the seminiferous epithelium is AR dependent and maximally sensitive to AR depletion within the testis. Cell-specific disruption of *AR* in Sertoli cells of hypomorphic animals further shows that progression of late-round spermatids to elongating steps is sensitive to loss of Sertoli cell *AR* function (Holdcraft and Braun, 2004). Considering this, ARs appear to play a role in the final stages of sperm differentiation to attain elongated morphology, and hence point mutations in the *AR* gene are more likely to result in dysmorphic sperm (teratozoospermia or oligoteratozoospermia) rather than complete absence of sperm (azoospermia).

Although a few studies carried out on infertile men

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Table 1. *Androgen sensitivity index (ASI) for different categories of samples*

Category of Samples	ASI Value (U × nmol/L²)		Number of Samples With		Total No. of Samples
	Range	Mean	Elevated ASI	Normal ASI	
Azoospermic	5.9–442.1	53.2	15	262	277
Oligoteratozoospermic	6.8–121.9	49.9	0	22	22
Oligozoospermic	5.2–505.1	58.7	20	80	100
Controls	6.3–125.2	51.9	1	99	100

from other parts of the world found mutations in the *AR* gene (Wang et al, 1998; Ghadessy et al, 1999; Hiort et al, 2000; Yong et al, 2003), no such study has been conducted on Indian populations. Therefore, we undertook this study to test the hypothesis that *AR* mutations are more likely to cause oligoteratozoospermia than oligozoospermia or azoospermia and that elevated ASI values are an indication of *AR* mutations and to look at the overall contribution of *AR* mutations in male infertility among Indian populations.

Materials and Methods

Subjects

A total of 399 infertile male samples, comprising 277 azoospermic, 100 oligozoospermic, and 22 oligoteratozoospermic men (Table 1) aged between 25 and 45 years were recruited for the study from the Institute of Reproductive Medicine, Kolkata. All patients had a long period (3 to 8 years) of infertility, and known causes of infertility, such as obstruction of sperm release, cystic fibrosis, congenital bilateral absence of the vas deferens, and any infection, had been ruled out by a team of expert andrologists. All patients had perfectly normal and fully mature male external genitalia with no symptoms of undervirilization. The patient population belonged to Indo-European, Austro-Asiatic, and Tibeto-Burman linguistic affiliations and were inhabitants of 6 states (West Bengal, Orissa, Bihar, Jharkhand, Utter Pradesh, and Assam) of India. A total of 100 healthy men from the same ethnic backgrounds as the patients and with proven fertility were recruited in the study as controls. Peripheral blood was obtained for karyotyping, hormone profiling, and molecular analysis; family history and informed written consents were also obtained from the subjects. Infertile men who had chromosomal abnormalities and Y chromsome microdeletions (Thangaraj et al, 2003a) were not included in the study.

Table 2. *List of primers used for amplification of* AR *exons**

Exon No.	Primer Sequence (5′-3′)	Product Size (bp)
1	AAGAGAAGGGGAGGCGGGGTAAG	521–584
	CAACGTGGATGGGGCAGCTGAG	
1	CCCGAGAGAGGTTGCGTCCC	592
	TCGCCTTCTAGCCCTTTGGTG	
1	AGCTTCGGGGGGATTGCATGTA	739
	GCCGCCAGGGTACCACACATC	
1	CCGCTTCCTCATCCTGGCACAC	617–668
	TCTGAAGGTGGCCCGTGCAATAG	
2	AATGCTGAAGACCTGAGACT	318
	AAAATCCTGGGCCCTGAAAG	
3	CTAGAAATACCCGAAGAAAG	257
	GAGAGACTAGAAAATGAGGG	
4	GTGATTTTCTTAGCTAGGGC	424
	ATCCCCCTTATCTCATGCTC	
5	GCTTTTCCCCACCACCCCTTAAT	527
	TTGGGTGTGAAAGGGGTGGTCTC	
6	CCAGCAGGAGAAACAGCAAGC	378
	GGGGAATGAAGAAGGGAAATGTC	
7	AGGCCCCAAGCACACAGACT	516
	CCTCCACCCCTTTCACAATATC	
8	GCCACCTCCTTGTCAACCCT	289
	AGAGGAGTAGTGCAGAGTTA	

* The product size variation in first and fourth sets of primers in exon 1 was due to the presence of CAG and GGN repeat motifs, respectively.

Hormone Assays and Testicular Biopsy Studies

Testosterone, leutinizing horomone (LH), and follicle stimulating hormone (FSH) levels were measured for both patient and control groups by radioimmunoassays. Absolute values of testosterone and LH were multiplied to calculate the ASI (Table 1). Testicular biopsy for azoospermic and oligozoospermic individuals was done wherever it was possible. A small incision was made in both the testicles under local anesthesia for taking the biopsies. Tissue sections were stained with hematoxylin-eosin for histological examination. The processed tissue sections were observed under an Axioplan 2 imaging system (Zieman, Zeiss, Germany).

Genetic Analysis

DNA was extracted from peripheral blood by a method described elsewhere (Thangaraj et al, 2002). Polymerase chain reaction primers covering the exon-intron splice junctions for the *AR* gene (Table 2) were designed using GeneTool software and synthesized using a 394 DNA/RNA oligosynthesizer (Applied Biosystems, Foster City, Calif). All 8 exons of the *AR* gene were amplified and subjected to direct sequencing using dideoxy chain terminator cycle sequencing protocol (BigDye V3.1, Foster City, Calif) (Thangaraj et al, 2003b) and ABI 3730 DNA Analyzer (Applied Biosystems). The first exon, being large in size, was amplified with 4 overlapping pairs of primers. Multiple alignment and sequence analysis was carried out using AutoAssembler software.

Results

Hormone Analysis and Testicular Biopsy Studies

FSH and LH levels were in the normal range for most of the patients; 35 patients, however, had high levels of tes-

Table 3. *Hormone levels for different categories of samples**

	FSH Value (mIU/mL)		LH Value (mIU/mL)		T Value (nmol/L)	
Category of Samples	Range	Mean	Range	Mean	Range	Mean
Azoospermia	1.8–89.0	8.2	0.6–36.0	3.9	5.8–66.1	19.9
Oligoteratozoospermia	1.4–12.7	3.7	1.1–7.8	3.7	5.1–34.3	15.9
Oligozoospermia	1.1–86.0	7.9	0.4–37.0	3.8	6.1–65.2	21.1
Controls	1.4–13.1	3.9	0.8–7.9	3.8	4.9–36.1	16.2

* FSH indicates follicle-stimulating hormone; LH, luteinizing hormone; and T, testosterone.

tosterone or LH and, hence, an elevated ASI (Tables 1 and 3). Testicular biopsies of azoospermic samples showed Sertoli cells only and very few or no germ cells in the seminiferous tubules. In oligozoospermic cases, varying numbers of germ cells were seen with an arrest of differentiation at the spermatocyte or round spermatid stage.

Genetic Analysis of the AR Gene

Sequences obtained from all 8 exons of the *AR* gene of all samples were compared with the reference sequence, and no mutations were found in any of the patients. Although CAG and GGN repeat motifs present in exon 1 of the gene showed repeat length variation, they were within the normal range.

Discussion

We have sequenced the complete coding region of the *AR* gene in a total of 399 infertile men, consisting of 277 azoospermic, 100 oligozoospermic, and 22 oligoteratozoospermic individuals (Table 1), who were otherwise normal and had known causes ruled out, to look at the role of the AR gene in male infertility. Interestingly, none of the samples showed any mutation in any of the 8 exons analyzed. Hundreds of mutations in the *AR* gene have been reported to be causative for androgen insensitivity resulting in female phenotype or undervirilization in 46,XY individuals (Gottlieb, 2005; Gottlieb et al, 1999), but relatively few mutations have been found to contribute to male infertility (Yong et al, 2003).

It is known that FSH regulates *AR* expression in Sertoli cells (Blok et al, 1992), and most studies suggest that ARs are not present in germ cells (Bremner et al. 1994). Tesarik et al (1998) demonstrated that testosterone potentiates the effects of FSH in spermatogenesis by preventing Sertoli cell apoptosis, and hence spermatogenesis can proceed in the presence of only FSH without testosterone. Thus, mutations in the *AR* gene are more likely to cause low or defective sperm production (oligozoospermia, teratozoospermia, or oligoteratozoospermia) rather than the complete absence of sperm (azoospermia). Hiort et al (2000) supported this hypothesis on the basis of their finding of a mutation in the AR gene in 3 oligoteratozoospermic cases out of a total cohort of 180 patients from a German population. Accordingly, most of the studies on the AR gene have found mutations in oligozoospermia or oligoteratozoospermia (Yong et al, 2003) and not in azoospermia (Tincello et al, 1997), except 2 studies in which a mutation was found in azoospermia (Akin et al, 1991; Wang et al, 1998).

Our study suggests that mutations in the *AR* gene are less likely to cause azoospermia and oligozoospermia. However, the sample size of oligoteratozoospermia was not large enough to make firm conclusions about the role of the *AR* gene in oligoteratozoospermia. Earlier studies have proposed that an elevated ASI is an indication of androgen insensitivity and hence *AR* mutations (Aiman et al, 1979), but our study shows that none of the azoospermic and oligozoospermic cases having elevated ASI values possessed an *AR* mutation. On the basis of this observation, we would like to revise the above hypothesis and propose that an elevated ASI value may be an indication of androgen insensitivity and, hence, *AR* mutations in teratozoospermic or oligoteratozoospermic cases but not in azoospermic or oligozoospermic cases. The reasons we did not find mutations in ARs in any of the oligoteratozoospermic cases may be because of the comparatively smaller number of these samples and because none of these samples had an elevated ASI value. However, the possibility of mutation in *AR* coactivators in any of these categories of infertile samples cannot be denied. A better understanding of the genetic makeup of different ethnic populations and the hormonal requirements of adult spermatogenesis is needed for proper diagnosis and development of rational treatments for male infertility and for designing contraceptive strategies.

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