Journal of Andrology, Vol. 22, No. 3, May/June 2001
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Larger Trinucleotide Repeat Size in the Androgen Receptor Gene of Infertile Men With Extremely Severe Oligozoospermia

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ABSTRACT: Androgens are significant regulators of human spermatogenesis. Their action is mediated through the androgen receptor (AR), which binds to the androgen responsive element on DNA and regulates gene transcription. Men become infertile with spinobulbar muscular atrophy (Kennedy disease) caused by a trinucleotide repeat expansion, ≥40 CAG repeats, in the AR gene located on the X chromosome. In this prospective study, we investigated whether the variable size, larger repeats, of this trinucleotide could alter AR function and result in impaired spermatogenesis. A total of 69 infertile men were studied. Clinical and laboratory analysis showed idiopathic, nonobstructive azoospermia in 16 men, extremely severe oligozoospermia in 27 men (<1 million sperm/mL), and severe oligozoospermia in 26 men (1 to 5 million sperm/mL). Fertile control men (n = 45) were selected by documented paternity proven by linkage analysis. Leukocyte DNA was analyzed by polymerase chain reaction (PCR) amplification across the AR repeat region. Accurate size determination of the PCR product using an ABI 373 DNA sequencer allowed precise calculation of CAG repeat sizes. The AR gene was not analyzed for other types of mutations. The difference in CAG repeat size between infertile men and proven fertile controls was statistically significant, $P = .03$. Patients with extremely severe oligozoospermia had significantly longer CAG repeat tracts (mean, 25.4 ± 4.0; $P = .0005$; range 20–39) than controls (mean, 22 ± 2.8; range 12–30) or patients with severe oligozoospermia (mean, 22.2 ± 2.3; range 18–26). None of the 26 infertile men with sperm counts <1 million/mL had ≤19 CAG repeats compared with 6 out of 45 controls (13%; $P = .06$). This study suggests that some men with severe impairment of spermatogenesis have longer trinucleotide repeats in the AR gene. Although direct evidence is missing, lower affinity between androgen and the AR protein or decreased AR protein availability with longer repeats could be responsible for a diminished androgen effect on spermatogenesis. Two of the patients in the extremely severe oligozoospermia group had 35 and 39 CAG repeats, respectively (normal range is 11 to 33). Although not yet considered a mutation, longer trinucleotide repeats are unstable and might either expand or contract between generations. If they expand, conception through the use of intracytoplasmic sperm injection (ICSI), could result in the son of an ICSI daughter being affected not only by infertility but also by Kennedy disease.

Key words: ICSI, assisted reproduction, Kennedy disease, male infertility.

J Androl 2001;22:444–448

Today, many infertile men with a variety of spermatogenic disorders, ranging from severe oligoasthenozoospermia to nonobstructive azoospermia, can father children with the use of intracytoplasmic sperm injection (ICSI; Palermo et al, 1992). However, despite this remarkable clinical success, development of therapies to correct male infertility has been slow because the etiology for many of these disorders remains unknown.

Androgens, mainly testosterone and 5α-dihydrotestosterone, are essential regulators of human spermatogenesis. Their action is mediated by the androgen receptor (AR), a DNA-binding transcription factor protein encoded by a gene located on chromosome Xq11-12 (Lubhan et al, 1988). The binding of androgens to the AR induces hyperphosphorylation, conformational changes, and dimerization of the receptor, which then binds to the androgen responsive element located within promoter regions of androgen responsive genes. The DNA binding of the AR complex and other transcription factors and cofactors to the regulatory regions of genes, up-regulates or down-regulates the transcription of target genes (Ruijter et al, 1999). The AR belongs to the nuclear receptor superfamily. The AR gene has 8 exons that encode 3 protein domains, the transactivation domain at the *N*-terminus (exon 1), the DNA binding domain (exons 2 and 3), and the androgen ligand domain at the *C*-terminus (exons 4 to 8).

Two highly polymorphic CAG and GGN microsatellite

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Received for publication September 5, 2000; accepted for publication December 4, 2000.

repeats are present in exon 1 of the AR gene. An expansion of the CAG microsatellite repeat to greater than 40 repeats is the causative AR mutation in patients with Kennedy disease, an X-linked form of spinobulbar muscular atrophy, with onset in the third decade of life. These patients also become infertile due to testicular atrophy, resulting in marked oligozoospermia. Previous studies examining the number of CAG repeats in the AR gene of infertile men with unexplained oligozoospermia have reported conflicting results, with some (Komori et al, 1999; Dadze et al, 2000) showing no expansions or gross deletions of trinucleotide repeats within exon 1 of the AR gene, and others (Tut et al, 1995; Dowsing et al, 1999; Yoshida et al, 1999) reporting increased trinucleotide repeat size. The recent study by Dadze et al (2000) pointed out ethnic differences as a possible explanation for the contradictory findings.

The aim of this study was to assess the size of the AR CAG repeat in predominantly North American infertile white men with severely disturbed spermatogenesis, including patients with nonobstructive azoospermia and patients with sperm counts not exceeding 5 million/mL, compared with 45 proven fertile control males.

Materials and Methods

Patients

A total of 69 white, predominantly infertile men, referred to the Male Infertility Program at the Hospital of the University of Pennsylvania for ICSI, were prospectively enrolled in the study and gave written consent to carry out genetic testing of the AR CAG repeat size. Clinical and laboratory analysis (a minimum of 3 semen samples for each patient during a 6-month period) showed nonobstructive azoospermia in 16 patients, extremely severe oligozoospermia in 27 (sperm detectable but less than 1 million/mL), and severe oligozoospermia in 26 (sperm count between 1 and 5 million/mL). None of the patients studied had clinical features, by history or physical exam, of patients with androgen resistance or neurological symptoms seen in patients with high numbers of repeats. As part of the pre-ICSI genetic screening, the patients included in the study had normal karyotype analysis and were also negative for Y chromosome microdeletions (Reijo et al, 1996).

Fertile control men (n = 45) were selected from families tested for a variety of genetic diseases by linkage analysis. The linkage analysis results were used to document paternity in the selected controls. The DNA from these controls was then made anonymous prior to AR trinucleotide repeat testing.

Polymerase Chain Reaction Amplification

One hundred nanograms of genomic DNA was amplified using published primers 5′ forward primer Ky-int1 (5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′) and 3′ reverse Ky-int 2 (5′-TCC AGA ATC TGT TCC AGA GCG TGC-3′; La Spada et al, 1991). The reverse primer (Ky-int 2) was fluorescently labeled with hexachlorofluorescein at the 5′ end of the primer. The polymerase chain reaction (PCR) was performed in the presence of 0.2 μM of each primer, in a total reaction volume of 25 μL containing 200 μM each of dATP, dCTP, and dTTP, 150 μM dGTP (Amersham Pharmacia Biotec, Arlington Heights, Ill), 50 μM 7-deaza-dGTP (Boehringer-Mannheim, Indianapolis, Ind), 1.5 mM $MgCl_2$, 1× PCR Buffer I, and 0.3 units Ampli*Taq* (PE Applied Biosystems, Foster City, Calif). A GeneAmp PCR System 9600 (PE Applied Biosystems) was used for PCR thermal cycling as follows: 1 cycle at 95°C for 5 minutes, followed by 35 cycles at 95°C for 1 minute, at 60°C for 1 minute, 72°C for 1 minute, and a final cycle at 72°C for 10 minutes.

Gel Electrophoresis and PCR Product Sizing

PCR products were analyzed by gel electrophoresis using an ABI 373A automated sequencer (PE Applied Biosystems). One microliter of each PCR product was mixed with 5 μL of formamide plus reaction dye containing internal size standards (ROX-500; PE Applied Biosystems). The mixture was denatured at 95°C for 5 minutes, and electrophoresed through a 6% polyacrylamide/7.65 M urea DNA sequencing gel. The PCR product size was determined by comparison with the internal size standards using automated sequencer analysis software (676 GENESCAN and GENOTYPER software, *User's Manual,* PE Applied Biosystems).

Calculation of CAG Repeats

Each patient was tested once. The number of CAG repeats for each specimen was calculated by subtracting the nonrepeat region of the PCR product (107 base pairs; bp) from the PCR product size and dividing by 3 (the size of 1 repeat). The accuracy of the method is ± 1 repeat by comparison with sequence analysis of the PCR product (data not shown). Assessment of point mutations was not performed.

Statistical Analysis

The number of CAG repeats for each category of patients is expressed as the mean ± SD. Data were analyzed with STATA software (College Station, Tex) for the skewness/kurtosis test for normalcy. Data were not normally distributed, and comparisons of means were made with the nonparametric Mann-Whitney *U* test. The $\chi 2$ and the Fisher's exact test were used in comparing percentages between groups.

Results

Overall, the mean number of CAG repeats in exon 1 of the AR gene was 23.5 ± 3.4 (range 18–39) for infertile men and 22 ± 2.8 (range 12–30) for proven fertile controls. This difference was statistically significant, $P = .03$. When analyzed by category of spermatogenic disorders, the results showed that in men with nonobstructive azoospermia (n = 16) the mean number of CAG repeats was 23 ± 2.5 (range 19–26), in men with extremely severe oligozoospermia (n = 27) the mean number of CAG repeats was 25.4 ± 4.0 (range 20–39), and in men with

*Comparison of sperm count and the androgen receptor CAG-repeat size in the different groups of patients with controls**

	N (%)	Sperm Count	Mean CAG Size	*P*
Nonobstructive azoospermia	16 (23)	Undetectable	23 ± 2.5	. . .
Extremely severe oligozoospermia	27 (39)	<1 million/mL	25.4 ± 4.0	.005†
Severe oligozoospermia	26 (38)	1–5 million/mL	22.2 ± 2.3	. . .
All infertile males	69 (100)	0–5 million/mL	23.5 ± 3.4	.03†
Fertile controls	45	NA	22 ± 2.8	. . .

* NA indicates not available.
† Compared to fertile controls.

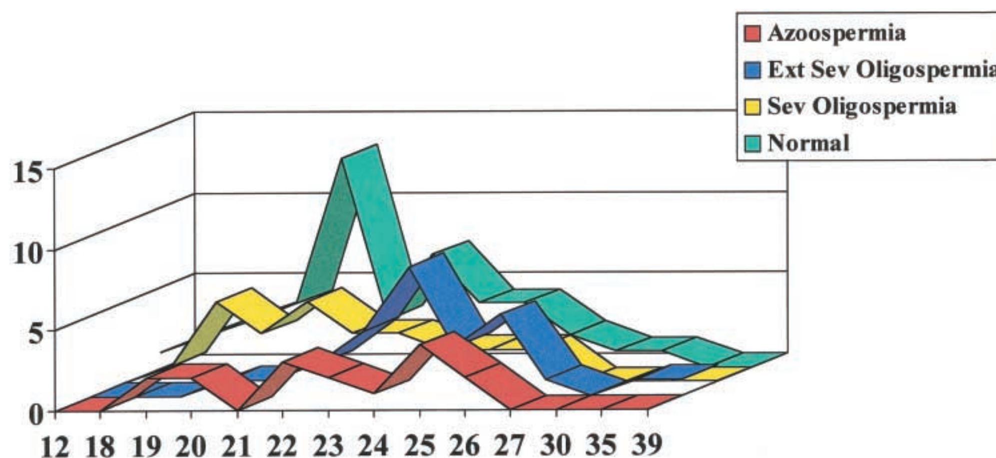
severe oligozoospermia (n = 26) the mean number of CAG repeats was 22.2 ± 2.3 (range 18–26). The difference between the CAG repeat length in men with extremely severe oligozoospermia compared with controls was statistically significant, $P = .0005$ (Table; Figure). This difference remained statistically significant even after removing the 2 patients with the largest CAG tracts, 35 and 39 repeats ($P = .003$). In addition, none of the infertile men with extremely severe oligozoospermia (sperm count ≤1 million/mL) had <19 CAG repeats, compared with 6 of the 45 controls (13%, $P = .06$).

Discussion

Since the cloning of the AR gene in 1988 (Lubhan et al, 1988), a number of mutations have been described in men with defective spermatogenesis and classified as partial or mild forms of androgen insensitivity syndrome (Ghadessy et al, 1999). These mutations range from complete androgen receptor deletion to point mutations (Marcelli et al, 1990; Saunders et al, 1992; Tsukuda et al, 1994; Brown et al, 1995).

In this study we examined the length of the CAG repeat in the *N*-terminal transactivation domain of the AR gene of predominantly North American white men with infertility due to severely reduced spermatogenesis and compared those results with control fertile men. Overall, the mean number of CAG repeats was found to be significantly larger in men with reduced spermatogenesis compared with fertile control men, and this difference was particularly striking for men with extremely severe oligozoospermia. These findings are in agreement with 2 recent similar studies from Singapore and Australia (Tut et al, 1995; Dowsing et al, 1999). In contrast, 2 recent studies from Japan and Germany failed to detect significant variations in the size of the CAG repeats (Komori et al, 1999; Dadze et al, 2000). Beside possible ethnic differences, it is interesting to note that in the work of Dadze et al (2000), the number of CAG repeats had a wider range of variations in the group of infertile males than in the controls.

The mechanism by which high normal or intermediate CAG repeat sizes may cause infertility is still unknown. Some of the hypotheses postulate a reduced amount of specific androgen binding (ie, lower affinity between androgens and the AR protein; Ruijter et al, 1999). Others claim that the length of CAG trinucleotide is inversely correlated with the transactivation function of the AR (Chamberlain et al, 1994). Alternatively, others have as-



Distribution of CAG-repeats size for each group of patients. Patients with extremely severe oligospermia had a larger size of CAG repeats than the

other groups.

sociated longer than normal CAG repeats to a reduced amount of AR messenger RNA and AR protein (Choong et al, 1996) or to low functional competence of the AR (Tut et al, 1995).

These longer but normal CAG repeat sizes may alter the activation function 1 (AF-1), because both are located in the *N*-terminal region, by altering interaction with coregulator proteins. Alteration of AF-1 function due to loss of a coregulator protein has been associated with androgen insensitivity in a recent case report (Adachi et al, 2000).

These minor but statistically significant deviations from the norm in the number of the CAG repeats could represent one genetic alteration in a multifactorial set of genetic polymorphisms or mutations that may lead to male infertility.

Variability in the CAG repeat length has also been studied in relationship to risk of prostate cancer. While the average length of the CAG repeat in the normal population is 21 ± 2 (range 11–35), shorter CAG repeat lengths, less than 19, have been associated with a higher risk of prostate cancer (Giovannucci et al, 1997), and longer repeats have been shown to decrease the risk of prostate cancer (Stanford et al, 1997). Furthermore, the frequency distribution of the AR gene CAG repeat length varies among different racial groups. Shorter alleles, associated with a higher risk of prostate cancer, are found more frequently in black American individuals; conversely, populations at the lowest risk, Asians and white men, have longer repeats.

Our findings are of great interest because the expansion of the same trinucleotide repeat (in excess of 38 to 40 repeats) is responsible for a neuromuscular disease, spinobulbar muscular atrophy or Kennedy disease (La Spada, 1991; Mac Lean et al, 1995), which appears later in life and is associated with a severe reduction in sperm count.

Because the AR gene is on the X chromosome, using ICSI to treat patients with extremely severe oligozoospermia and intermediate CAG trinucleotide repeats, has the potential risk for transmitting Kennedy disease in 2 generations. The mechanism of transmission of this fatal neuromuscular disease will involve first an expansion of the CAG repeat, with transmission to a daughter who will be a carrier (first generation), and then the subsequent risk (50%) of transmission of the expanded CAG repeat to a son (second generation) who will be affected.

A recent paper (Cram et al, 2000), evaluated the CAG trinucleotide repeats inheritance in female offspring conceived after ICSI. Although it was shown that 95% of the ICSI daughters had inherited an AR allele with the same number of CAG repeats of their fathers, it was also found that in the remaining 5% of the cases, the CAG trinucleotide underwent either contraction or expansion up to 8 unit base pairs. Expansions of CAG repeats can be dangerous.

We detected 2 patients in the extremely severe oligozoospermia group with CAG repeats of an intermediate size, between the normal range of 11 to 33 repeats, and the affected range of 40 to 62 repeats. These 2 men had 35 and 39 CAG repeat sizes. Because the stability of these intermediate size repeats is unknown, we recommend screening of infertile men with extremely severe oligozoospermia. Identification of men with intermediate repeat sizes will allow monitoring for expansions into the affected range in the next generation.

If future studies confirm these findings, screening and counseling for the length of the CAG trinucleotide repeat in the AR gene should become part of the pre-ICSI genetic testing for infertile men (Patrizio, 1995; Patrizio and Kopf, 1997).

Acknowledgment

The authors thank Kurt T. Barnhart, MD, MSCE, for statistical analysis of the data.

References

Adachi M, Takawanagi R, Tomura A, Imasaki K, Kato S, Goto K, Wanasi T, Ikuyama S, Nawata H. Androgen-insensitivity syndrome as a possible coactivator disease. *N Engl J Med.* 2000;343:856–862.

Brown TR. Human androgen insensitivity syndrome. *J Androl.* 1995;16: 299–303.

Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor *N*-terminal domain affect transactivation function. *Nucleic Acids Res.* 1994;22: 3181–3186.

Cram DS, Song B, McLachlan RI, Trounson AO. CAG trinucleotide repeats in the androgen receptor gene of infertile men exhibit stable inheritance in female offspring conceived after ICSI. *Mol Hum Reprod.* 2000;6:861–866.

Choong CS, Kemppainen JA, Zhou ZX, Wilson EM. Reduced androgen gene expression with first exon CAG repeat expansions. *Mol Endocrinol.* 1996;10:1527–1535.

Dadze S, Wieland C, Jakubiczka S, Funke K, Schroder E, Royer-Pokora B, Willers R, Wieacker PF. The size of the CAG repeat in exon 1 of the androgen receptor gene shows no significant relationship to impaired spermatogenesis in an infertile Caucasoid sample of German origin. *Mol Hum Reprod.* 2000;6:207–214.

Dowsing AT, Yong EL, Clark M, McLachlan R, De Kretser DM, Trounson AO. Linkage between male infertility and trinucleotide repeat expansion in the androgen receptor gene. *Lancet.* 1999;354:640–643.

Ghadessy FJ, Lim J, Abdullah AA, et al. Oligospermic infertility associated with an androgen receptor mutation that disrupts interdomain and coactivator (TIF2) interactions. *J Clin Invest.* 1999;103:1517–1525.

Giovannucci E, Stamper MJ, Kritnivas K, et al. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Proc Natl Acad Sci USA.* 1997;94:3320–3323.

Komori S, Kasumi H, Kanazawa R, Sakata K, Nakata Y, Kato H, Koyama K. CAG repeat length in the androgen receptor gene of infertile Japanese males with oligozoospermia. *Mol Hum Reprod.* 1999;5:14–16.

La Spada AR, Wilson EM, Lubhan DB, Harding AE, Fishbeck KH. Androgen receptor gene mutations in X linked spinal and bulbar muscular atrophy. *Nature.* 1991;352:77–79.

Lubhan DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM. Cloning of the human androgen receptor complementary DNA and localization to the X chromosome. *Science.* 1988;240:327–330.

Mac Lean HE, Warne CL, Zajic JD. Defect of the androgen receptor function: from sex reversal to motor neuron disease. *Mol Cell Endocrinol.* 1995;112:130–341.

Marcelli M, Tilley WD, Wilson CM, Griffin JE, Wilson JD, McPhaul MJ. Definition of the human androgen receptor gene structure permits the identification of mutations that cause androgen resistance: premature termination of the receptor protein at amino acid 588 causes complete androgen resistance. *Mol Endocrinol.* 1990;4:1105–1116.

Palermo GD, Joris H, Devroey P, Van Steirteghem A. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet.* 1992;340:17–18.

Patrizio P. Intracytoplasmic sperm injection (ICSI): potential genetic concerns. *Hum Reprod.* 1995;10:108–111.

Patrizio P, Kopf GS. Molecular biology in the modern work-up of the infertile male: the time to recognize the need for andrologists. *Hum Reprod.* 1997;12:879–893.

Reijo R, Alagappan RK, Patrizio P, Page DC. Severe oligospermia resulting from deletions of the azoospermia factor gene on the Y-chromosome. *Lancet.* 1996;347:1290–1293.

Ruijter E, Van De Kaa C, Miller G. Molecular genetics of prostate carcinoma. *Endocrinol Rev.* 1999;20:22–45.

Saunders PTK, Padayachi T, Tincello DG, Shalet SM, Wu FCW. Point mutations detected in the androgen receptor gene of three men with partial androgen insensitivity syndrome. *Clin Endocrinol.* 1992;37:214–220.

Stanford JL, Just JJ, Gibbs M, Wicklund KG, Neal CL, Blumenstein BA, Ostrander EA. Polymorphic repeats in the androgen receptor gene: molecular markers of prostate cancer risk. *Cancer Res.* 1997;57:1194–1198.

Tsukuda T, Inoue M, Tachibana S. An androgen receptor mutation causing androgen resistance in undervirilized male syndrome. *J Clin Endocrinol Metab.* 1994;79:1202–1207.

Tut TG, Ghadessy FJ, Trifiro MA, Pinsky L, Yong EL. Long polyglutamine tracts in the androgen receptor are associated with reduced transactivation, impaired sperm production and male infertility. *J Clin Endocrinol Metab.* 1997;82:3777–3782.

Yoshida KI, Masataka Y, Chiba K, Honda M, Kitahara S. CAG repeat length in the androgen receptor gene is enhanced in patients with idiopathic azoospermia. *Urology.* 1999;54:1078–1081.