Journal of Andrology, Vol. 23, No. 6, November/December 2002

CAG Repeat Expansion in the Androgen Receptor Gene Is Not Associated With Male Infertility in Indian Populations

KUMARASAMY THANGARAJ,* MANJUNATH B. JOSHI,* ALLA G. REDDY,* NALINI J. GUPTA,† BAIDYANATH CHAKRAVARTY,† AND LALJI SINGH*

*From the *Centre for Cellular and Molecular Biology, Hyderabad, India; and the †Institute of Reproductive Medicine, Kolkata, India.*

ABSTRACT: CAG repeat expansion in exon 1 of the androgen receptor (AR) gene has been reported to be associated with male infertility in some but not all populations. Until now, studies have not been carried out to examine this among Indian populations. For the first time, we have analyzed the CAG repeat motif in the AR gene in 280 men with azoospermia and in 201 men with normal fertility. The mean number of CAG repeats in the AR gene of men with azoospermia was 21.7 ± 0.18, with a high incidence of repeat number 22. Among fertile-control men, the mean number of CAG repeats was 22.4 ± 0.19, with a predominance of repeat number 23. The highest number of CAG repeats (32) was found with low frequency in both fertile and azoospermic groups. Comparison of fertile men and those with azoospermia on the basis of CAG repeats revealed that the number of CAG repeats in both groups were similar, as revealed with a paired *t* test ($t = 0.04$; $P = .967$). Expansion of the CAG repeat in the AR gene is therefore not associated with male infertility in Indian populations. This suggests that what is true for one population may not be true for other populations.

Key words: X chromosome, azoospermia.

J Androl 2002;23:815–818

Approximately 15% of couples are infertile, and in these couples, male factor infertility accounts for approximately 50% of causes (Mosher, 1985). Y chromosome microdeletion has been considered an important genetic factor in male infertility (Fujisawa et al, 2001). Androgen is an important steroid for maintaining sperm production and growth of the prostate gland. The androgen receptor (AR) gene has been mapped on the long arm (Xq11-12) of the X chromosome (Lubhan et al, 1988; Yong et al, 1998). The AR gene consists of 8 exons, of which exon 1 contains a CAG repeat motif, resulting in a polyglutamine stretch. About 20% of infertile men have reduced androgenicity caused by a longer CAG tract (Yong et al, 1998). Several studies have demonstrated that expansion of CAG repeats in the AR gene causes azoospermia (Sasagawa et al, 2001), oligospermia (Patrizio et al, 2001), testicular atrophy, spinal bulbar muscular atrophy (SBMA; La Spada et al, 1991), and a greater risk for breast cancer (Haiman et al, 2002). Expansions of CAG repeats over generations have been reported to lead to an increase in the severity of SBMA pathology and a concomitant decrease in the age of disease onset (Choong and Wilson, 1998). Reductions in CAG repeats have been found to be associated with prostate cancer (Giovannucci et al, 1997). Mutation in the *N*-terminal of the AR has been found to be associated with a lower transmission of a transactivation signal from the activation function 1 region (Adachi et al, 2000), and other studies have deduced that CAG repeat tracts are polymorphic among infertile men in various ethnic populations (Dadze et al, 2000; Sasagawa et al, 2000). Therefore, we undertook an analysis of the CAG repeat in the AR gene among infertile and fertile Indian men in order to assess its association with infertility and its distribution pattern in Indian populations.

Materials and Methods

Patients and Control Subjects

A total of 280 men with azoospermia from different parts of India were subjected to CAG repeat analysis. They were selected from among 750 azoospermic men who were attending the infertility clinic at the Institute of Reproductive Medicine (IRM) in Kolkata, India. An experienced urologist at IRM performed a detailed history and clinical examination of each patient. Blood samples were taken for endocrinological assays (follicle-stimulating hormone, luteinizing hormone, testosterone, prolactin, thyroid-stimulating hormone), and tests were performed for postprandial blood sugar, human immunodeficiency virus, and syphilis; and patients also underwent a vasogram and karyotyping. Testicular pathology was carried out whenever it was possible to do so, and results varied from Sertoli cell–only syndrome to

Supported by grant from the Indian Council of Medical Research, and the Council of Scientific and Industrial Research.

Correspondence to: Dr Lalji Singh, Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India. (e-mail: lalji@gene.ccmbindia.org).

Received for publication January 28, 2002; accepted for publication May 23, 2002.

maturation arrest. Patients who did not exhibit obstruction, endocrinological defect, pelvic injury, major illness, or karyotyping abnormality were included in the study. Two-hundred-one fertile Indian men were included in the study as controls. Blood samples (5.0 mL) from each man with azoospermia and fertile controls were collected with their informed written consent.

DNA Isolation

DNA was isolated from the samples using the following protocol. Erythrocytes were lysed with 15.0 mL of erythrocyte lysis buffer (10 mM Tris pH 8.0, 320 mM sucrose, 5 mM $MgCl_2$, 1% Triton X-100; Sigma Chemical Company, St Louis, Mo) for 5 minutes. After complete lysis of erythrocytes, leukocytes were pelleted by centrifugation at 500 × *g* for 5 minutes. The leukocyte pellet was dissolved in 8.0 mL of leukocyte lysis buffer (400 mM Tris, 60 mM EDTA, 150 mM NaCl, and 1% SDS; Sigma) and mixed thoroughly. To this, 2.0 mL of 5 M sodium perchlorate (E. Merck, Damstadt, Germany) was added and mixed thoroughly for 2–3 minutes. DNA was precipitated after extracting once with phenol:chloroform and once with chloroform. DNA was washed with 70% ethanol and dissolved in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA).

PCR Assay for CAG Repeat Analysis

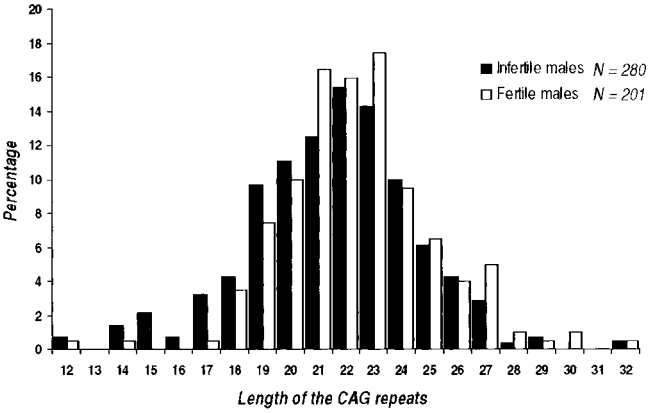
CAG repeat motif of exon 1 in the AR gene was amplified using a pair of primers flanking the CAG repeat motif (Cram et al, 2000). Primers were synthesized in an ABI 392 Oligo synthesizer (Perkin Elmer, Foster City, Calif). The forward primer was synthesized with 5′ FAM (carboxy-fluorescein) label (Perkin Elmer) in order to analyze the PCR product in the automated DNA sequencer (ABI 377). The forward primer was also synthesized without a fluorescent label for sequencing. Polymerase chain reaction (PCR) of each sample was performed in a 0.2-mL thin-wall tube using 5.0 ng of DNA, 10 pM of each primer, 200 μM deoxynucleotide triphosphates, 1× PCR buffer containing 1.5 mM $MgCl_2$, and 2 units of AmpliTaqGold (Perkin Elmer). Amplification was carried out in a GeneAmp9600 thermal cycler (Perkin Elmer) at 94°C for 10 minutes, and then 30 cycles at 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 5 minutes.

GeneScan and Genotyping

Samples (PCR products) were prepared by mixing 1.0 μL of PCR products, 1.5 μL of loading dye (formamide:blue dextran; 5:1) and 0.5 μL of GS-ROX500 (0.5 μL/sample). After denaturation (94°C for 2 minutes), samples were electrophoresed in 5% Long Ranger (FMC) gel using an ABI 377 automated DNA sequencer (Perkin Elmer). Raw data were analyzed using GeneScan and Genotyping software programs (Perkin Elmer) to obtain the allele (repeat) size (Thangaraj et al, 1999).

Automated DNA Sequencing

To confirm the CAG repeat numbers (allele), 2 samples (PCR products obtained using nonfluorescent primers) from each repeat size were sequenced after treating them with exonuclease I and shrimp alkaline phosphatase (Amersham) at 37°C and 80°C (15 minutes each). Sequencing of PCR products was carried out using 50 ng (2 μL) of PCR product and 4 pM (1 μL) of non-



Distribution of CAG repeat range in men with azoospermia (closed bar) and in men with normal fertility (open bar).

fluorescent primer (forward and reverse separately), 4 μL of BigDye Terminator ready reaction kit (Perkin Elmer), and 3 μL of double-distilled water to adjust the volume to 10 μL. Cycle sequencing was carried out in a GeneAmp9600 thermal cycler (Perkin Elmer) for 30 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Extended products were purified by alcohol precipitation followed by washing with 70% alcohol (Thangaraj et al, 1999). Purified samples were dissolved in 10 μL of 50% Hi-Di formamide and analyzed in an ABI 3700 automated DNA Analyzer (Perkin Elmer).

Statistical Analysis

Means, standard deviations, and standard errors were calculated for both the azoospermic and fertile groups. A paired *t* test was carried out to find the difference in CAG repeats between azoospermic and fertile men (Systat, version 10.0, from SPSS Inc, Chicago, Ill).

Results

A total of 18 different CAG repeat sizes (alleles), ranging from 12 to 32 were observed in a total of 280 infertile men. Among 201 fertile men, 17 different repeat sizes (alleles) ranging from 12 to 32 were observed (Figure). The mean number of CAG repeats in the infertile group was 21.7 ± 0.18. In the fertile group the mean repeat size was 22.4 ± 0.19 (Table). The smallest repeat size (12) was found with very low frequency in both the groups (0.71% in azoospermic and 0.49% in fertile groups). The highest repeat size (32) was also found with very low frequency in both azoospermic (0.35%) and fertile men (0.49%). Repeat sizes 13 and 31 were absent in both groups. Alleles 15 and 16 were absent in fertile men, and allele 30 was absent in men with azoospermia (Table). Among men with azoospermia, repeat size 22 was observed with high frequency (15.35%), whereas in fertile men, repeat 23 was observed with high frequency (17.41%). In the lower range of CAG repeat size (12–

Comparative data on CAG repeats in men with azoospermia and normal fertility

CAG Repeat Size/ Allele	Azoospermic Men		Fertile Men	
	Number	Percentage	Number	Percentage
12	2	0.71	1	0.49
14	4	1.42	1	0.49
15	6	2.14	0	0
16	2	0.71	0	0
17	9	3.21	1	0.49
18	12	4.28	7	3.48
19	27	9.64	15	7.46
20	31	11.07	20	9.95
21	35	12.50	33	16.42
22	43	15.35	32	15.92
23	40	14.28	35	17.41
24	28	10.00	19	9.45
25	17	6.07	13	6.46
26	12	4.28	8	3.98
27	8	2.85	10	4.98
28	1	0.35	2	0.99
29	2	0.71	1	0.49
30	0	0.00	2	0.99
32	1	0.53	1	0.49
Total	280	100	201	100
Mean	21.7		22.4	
SE	±0.18		±0.19	

17), men with azoospermia were found to have high frequency (8.2%) compared with fertile men (1.5%). In the middle and higher repeat sizes (21 to 32), 77.58% of men with normal fertility was higher than in men with azoospermia (67%; Figure). However, the mean variation in the CAG repeats between fertile (mean 22.4 ± 0.19) and infertile (mean 21.7 ± 0.18%) men was found to be statistically nonsignificant, as evident from the paired *t* test ($t = 0.04$; $P = .967$).

Discussion

Our study on CAG repeats in the AR gene of infertile men revealed a total of 18 alleles, ranging from 12–32 repeats, with a mean CAG repeat of 21.7 ± 0.18. Of these, the most frequent CAG repeat number observed was 22 (15.35%). Control studies showed a total of 17 alleles ranging from 12 to 32 repeats, with a mean CAG repeat of 22.4 ± 0.19. The most common CAG repeat, 23, was found in 17.4% of controls (Figure). It is interesting to note from our study that the distribution of various repeat sizes (19–27) in both infertile and fertile men were almost similar (85%). However, Yong et al (1998) and Dowsing et al (1999) demonstrated that the size of the CAG repeat was higher in infertile men. Giovannucci et al (1997) showed that the size of the CAG repeat was smaller in patients with prostate cancer. Studies among Chinese and Japanese infertile men with defective spermatogenesis and idiopathic azoospermia, respectively, demonstrated the presence of significantly longer CAG repeats compared with those in fertile men (Tut et al, 1997; Yong et al, 1998; Yoshida et al, 1999). These reports contradict our present study, which clearly revealed no direct association of CAG repeat expansion in the AR gene with infertility in Indian populations. However, our present observation is in agreement with that of Dadze et al (2000) who observed 17 alleles in a population of infertile German men, ranging from 16 to 34 repeats, with the predominance of allele 21, whereas in the control group, the predominant allele was 23 (22.7%), which was slightly higher than that of infertile men. A similar study conducted by Sartor et al (1999) among American black men and white men showed that the CAG repeat expansion was polymorphic in a race-specific manner (ie, it was shorter in black men than it was in white men). The recent study on European and Japanese populations in which no significant difference in the CAG repeat length was found between normal and infertile men (Sasagawa et al, 2001; Meytes et al, 2002) is also in agreement with our present observation in Indian populations.

The CAG repeat and its association with infertility has been debatable, as some studies reported the presence of a longer stretch of CAG repeat in infertile men (Patrizio et al, 2001) and others showed no significant increase in CAG repeats in infertile men (Dadze et al, 2000). Patrizio and Leonard (2001) suggested in their editorial commentary that more data from different ethnic groups with a large number of controls are required to determine the association of expanded CAG repeat in the AR gene in male infertility. For the first time we assessed the association of CAG repeat with male infertility in Indian populations. Our study clearly demonstrates no association between CAG repeat length and infertility in Indian populations as is evident from the paired *t* test ($t = 0.04$; $P = .967$). A recent report by Meytes et al (2002) supports our conclusions. It clearly shows that what is true for one population may not be true for other populations and suggests that it would be detrimental to extrapolate such findings to other populations, particularly for diagnostic purposes.

References

Adachi M, Takayanagi R, Tomura A, et al. Androgen-insensitivity syndrome as a possible coactivator disease. *N Engl J Med.* 2000;343: 856–862.

Choong CS, Wilson EM. Trinucleotide repeats in the human androgen receptor: a molecular basis for disease. *J Mol Endocrinol.* 1998;21: 235–257.

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.

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 show 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 RI, De Krester DM, Trounson AO. Linkage between male infertility and trinucleotide repeat expansion in the androgen receptor gene. *Lancet.* 1999;354:640–643.

Fujisawa M, Shirakawa T, Kanzaki M, Okada H, Arakawa S, Kamidono S. Y-chromosome microdeletion and phenotype in cytogenetically normal men with idiopathic azoospermia. *Fertil Steril.* 2001;76:491–495.

Giovannucci E, Stamper MJ, Kritnivas K, Brown M, Brufsky A, Talcott J, Hennekens CH, Kantoff PW. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Proc Natl Acad Sci USA.* 1997;94:3320–3323.

Haiman CA, Brown M, Hankinson SE, Spiegelman D, Colditz GA, Willett WC, Kantoff PW, Hunter DJ. The androgen receptor CAG repeats polymorphism and risk of breast cancer in the nurses' health study. *Cancer Res.* 2002;62:1045–1049.

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

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

Meytes DER, Leffers H, Petersen JH, Andersen AG, Carisen E, Jorgensen N, Skakkebaek NE. CAG repeat length in androgen receptor gene and reproductive variables in fertile and infertile men. *Lancet.* 2002;359: 44–46.

Mosher WD. Reproductive impairments in the United States, 1965–1982. *Demography.* 1985;22:415–430.

Patrizio P, Leonard DGB. Expansion of the CAG trinucleotide repeats in the androgen receptor gene and male infertility: a controversial association. *J Androl.* 2001;22:748.

Patrizio P, Leonard DGB, Chen K-L, Hernandez-Ayup S, Trounson AO, Larger trinucleotide repeat size in the androgen receptor gene of infertile men with extremely severe oligozoospermia. *J Androl.* 2001; 22:444–448.

Sartor O, Zheng Q, Eastham JA. Androgen receptor gene CAG repeat length varies in a race specific fashion in men without prostate cancer. *Urology.* 1999;53:378–380.

Sasagawa I, Suzuki Y, Ashida J, Nakada T, Muroya K, Ogata T. CAG repeat length analysis and mutation screening of the androgen receptor gene in Japanese men with idiopathic azoospermia. *J Androl.* 2001; 22:804–808.

Sasagawa I, Suzuki Y, Tateno T, Nakada T, Muroya K, Ogata T. CAG repeat length of the androgen receptor gene in Japanese males with cryptorchidism. *Hum Mol Reprod.* 2000;6:973–975.

Thangaraj K, Ramana GV, Singh L. Y-chromosome and mitochondrial DNA polymorphisms in Indian populations. *Electrophoresis.* 1999; 20:1743–1747.

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.

Yong EL, Ghadessy F, Wang Q, Mifsud A, Ng SC. Androgen receptor transactivation domain and control of spermatogenesis. *Rev Reprod.* 1998;3:141–144.

Yoshida KI, Yano M, 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.