Molecular Analysis of the Androgen Receptor Gene in Hong Kong Chinese Infertile Men

J. Y. M. Tse,^{1,2} V. W. S. Liu,¹ W. S. B. Yeung,¹ E. Y. L. Lau,¹ E. H. Y. Ng,¹ and P. C. Ho¹

Submitted July 31, 2002; accepted February 21, 2003

Purpose: To investigate the relationship between CAG repeat length in the androgen receptor gene and impaired spermatogenesis in Hong Kong Chinese population.

Methods: The CAG repeat region was amplified by polymerase chain reaction (PCR) in 85 nonobstructive azoospermic or severe oligozoospermic men, and 45 fertile males. The number of CAG repeat was analyzed by DNA sequencing. Serum FSH, LH, and testosterone levels were also determined in these men.

Results: Among nonobstructive azoospermic males, three men (5.7%) possessed short CAG repeats (<16), and three (5.7%) other men possessed long CAG repeats (>30). Short CAG repeats (<16) were also found in two severe oligozoospermic males (6.3%). The incidence of infertile men with short or long CAG repeats is significantly higher in the azoospermic group ($p = 0.03$) but not in the severe oligozoospermic group ($p = 0.17$) when compared with the fertile controls.

Conclusion: Our data suggest an association between CAG repeat lengths and impaired spermatogenesis in azoospermic males in our population.

KEY WORDS: Androgen receptor; CAG repeat; male infertility.

INTRODUCTION

Defective spermatogenesis is one of the common causes of male infertility. Genetic changes associated with male infertility include disease-related infertility (e.g., patients with cystic fibrosis), chromosomal abnormalities, and microdeletions of the Y chromosome (1,2). Spermatogenesis is exquisitely androgen-dependent, but most males with impaired spermatogenesis have normal serum androgen levels. Malfunction of AR is a possible cause of the impaired spermatogenesis. Androgen receptor (AR) is a structurally conserved member of the steroid/thyroid

receptor gene superfamily. It encodes a single copy gene in the X chromosome. The AR gene is composed of eight exons coding for 919 amino acids. It consists of three main functional domains: transactivation domain (exon 1) at the amino-terminal end, DNA-binding domain (exons 2–3), and hormone-binding domain (exons 4–8) at the carboxy-terminal end. Androgens, when bound to the hormone-binding domain, activate the receptor, causing nuclear translocation of the ligand-receptor complex and a series of molecular events leading to the transactivation of androgen-regulated genes (3).

Previous studies demonstrated that the length of CAG repeat can drastically change the properties of the androgen receptor (4–6). These changes have clinical implications for human diseases and could lead to abolition of AR function (7). Expansion of CAG repeats in different disease genes accounts for at least five inherited neurodegenerative disorders including spinal and bulbar muscular atrophy, Huntington's

¹ Department of Obstetrics and Gynaecology, The University of Hong Kong, Pokfulam Road, Hong Kong, People's Republic of China.

² To whom correspondence should be addressed; e-mail: jytmse@yahoo.com.hk.

disease, spinocerebellar ataxia, dentatorubral pallidoluysian atrophy, and Machado–Joseph disease. The expanded repeats are unstable and show a tendency to expand over generations (8). Clinical features of spinal and bulbar muscular atrophy include androgen insensitivity as evidenced by testicular atrophy, impotence, gynecomastia, oligo- or azoospermia, and elevated serum gonadotrophin levels (9,10). On the other hand, short CAG repeats are associated with increased risk of prostate cancer and the development of metastatic disease (11). The molecular mechanisms underlying these expansions or contractions are not yet known but are believed to involve either unequal crossover or single-strand slippage of the DNA polymerase during meiotic DNA replication (12).

Previous studies had provided endocrine-biochemical evidence linking oligozoospermia and azoospermia to abnormalities of AR (13–16). The expansion or contraction of CAG repeats may vary among infertile populations of different ethnic origin. Expansion or contraction of CAG repeats in the androgen receptor gene has been reported in men with defective sperm production in different populations including Japanese (17), Australian (18), Singaporean, and American (19) but not in European (20–23). The main objective of this study was therefore to determine the CAG repeat length analysis in a series of 85 Hong Kong Chinese infertile men with varying degrees of impaired spermatogenesis. It has been reported that the size of the CAG repeats in normal population also varies in a race-specific manner. For instance, the prevalence of short CAG repeats (<22 repeats) is high among African Americans (75%) but low among Asians (49%) (24). Therefore, 45 Hong Kong Chinese male volunteers of proven fertility were used as our normal controls in this study.

MATERIALS AND METHODS

Patients

Infertile men were recruited from the infertility clinic at the Department of Obstetrics and Gynaecology, The University of Hong Kong. This study was approved by the Ethics Committee, Faculty of Medicine, University of Hong Kong. Informed consent was obtained from each patient. Eighty-five patients of Chinese origin with either nonobstructive azoospermia ($n = 53$) or severe oligozoospermia (<5 million sperm per milliliter in the ejaculate, $n = 32$) were recruited. Semen analysis was performed ac-

cording to the World Health Organization guidelines (25). Forty-five healthy male volunteers with proven fertility and with no previous infertility history or treatment served as controls. Blood was taken from the recruited subjects for DNA extraction and for the determination of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone (T).

Hormone Assay

Serum concentrations of FSH and LH were measured by commercial chemiluminescence-based immunoassays (Chiron Diagnostics Corporation, East Walpole, U.S.A.). Serum concentration of T was determined by radioimmunoassay using ^{125}I -labelled T (Diagnostic Products Corporation, Los Angeles). To determine whether any patient with recognized androgen insensitivity on the basis of their hormone profile, the androgen insensitivity index (ASI) was calculated by multiplying the serum concentrations of LH and T (13). Since the normal hormone reference ranges and ASI range are not available from our Chinese population; therefore, these ranges were obtained from the assay kit manufacturers. Normal FSH: 1.5–10 IU/L; normal LH: 3.0–13.5 IU/L; normal T: 10–35 nmol/L; normal ASI: 15–270.

DNA Isolation and CAG Repeat Analysis

Genomic DNA was extracted from peripheral blood lymphocytes by using the DNA isolation kit for mammalian blood (Boehringer Mannheim Corp., TN.). The CAG repeats in exon 1 of the AR gene containing the CAG repeat region was amplified with primers flanking the repeat segment by PCR (17). PCR was carried out in a reaction volume of 25 μL containing 100–200 ng of genomic DNA, 1.5 mM MgCl_2 , 200 μM dNTP, 10 pmole primer, 1 $\times$ PCR buffer, and 1 U *Taq* polymerase. The PCR buffer, dNTP mix, and *Taq* polymerase were purchased from Boehringer Mannheim Corp. Thermocycling consisted of an initial denaturation of 2 min at 94°C, and of 35 cycles of 30 s at 94°C, 30 s at 49–50°C, and 30 s at 72°C. The amplified DNAs were sequenced by direct dideoxy terminator cycle sequencing using the dRhodamine Cycle Sequencing kit and an ABI Prism 310 automated sequencer (both from Perkin Elmer Applied Biosystems, Foster City, CA). The exact number of the CAG repeats located in exon 1 was then determined after sequencing. Those samples with abnormal CAG repeat were further confirmed after two successive sequencing from the same sample.

Statistical Analysis

Statistical analyses were performed by using SPSS software package, version 10.1 (SPSS Corp., Chicago, IL). The following statistical analyses were based on data from a previous study of the androgen receptor gene polymorphism (18). The Mann–Whitney test was used to evaluate the significance of the difference of the mean number of CAG repeats and the hormone levels between normal fertile control and infertile groups. The incidence of long or short CAG repeat length in infertile groups was compared with fertile control group by using Fisher's exact test. The locations and the shapes of the distribution of the CAG repeat lengths in normal fertile control and infertile patients were examined by Komogorov–Smirnov test. Statistical significance was defined as a two-sided *p* value of <0.05.

RESULTS

Analysis of the CAG Repeats Within the Androgen receptor Gene

The CAG repeat lengths in 53 nonobstructive azoospermic and 32 oligozoospermic patients were compared with those of the fertile control group (Table I). The mean CAG repeat length in azoospermic group was 23.3 ± 4.0 (mean $\pm$ SD) with a range of 15–36 and the severe oligozoospermic group was 22.7 ± 3.7 (mean $\pm$ SD) with a range of 14–28. In the fertile control group, the mean CAG repeat length was 23.0 ± 3.1 (mean $\pm$ SD) with a range of 16–30. There was no significant difference in the mean number of CAG repeat length among the three groups of men.

We defined those men having <16 and >30 CAG repeats as short and long CAG repeat, respectively, because they are outside the range of CAG repeat found in our fertile controls. In the azoospermic group, three patients (subject no. s40, s46, and s99) pos-

sessed short CAG repeat, and three patients (s44, s108, and s117) possessed long CAG repeat. In the severe oligozoospermic group, only two patients (s68 and s77) possessed short CAG repeat (Table II). Statistical analysis of the incidence of azoospermic men with short or long CAG repeat is significantly higher in the azoospermic group ($p = 0.03$) but not in the severe oligozoospermic group ($p = 0.17$) when compared with the fertile controls. The distribution of the CAG repeat lengths is shown in Fig. 1. Komogorov–Smirnov test showed no difference in the distribution of CAG repeats length when compared between the fertile control group and the two infertile groups.

Y chromosome microdeletion was performed in these fertile and infertile men as described previously (2). Among infertile males, five nonobstructive azoospermic and three severe oligozoospermic patients with Y chromosome microdeletion were also included in the analysis. However, their CAG repeat length was within the range in our fertile controls. No Y chromosome microdeletion was detected in all nonobstructive azoospermic and severe oligozoospermic patients that showed abnormal CAG repeats suggesting that Y chromosome microdeletions and abnormal CAG repeats are independent contributors to male infertility.

Hormone Analysis

As shown in Table I, high serum FSH alone is more commonly found in our nonobstructive azoospermic men as indicated by the high median FSH value when compared with the fertile controls ($p < 0.05$). High serum LH level was detected in about half of these patients with high serum FSH. High serum LH leads to an increase in ASI in these patients as indicated by the high median LH level and ASI index in the nonobstructive azoospermic group when compared with the other two groups.

Table I. Clinical and Molecular Data of Infertile and Fertile Groups

Patient group	N	Age (years)	Hormone concentration			ASI	CAG repeat
			FSH (IU/L)	LH (IU/L)	T (nmol/L)		
Nonobstructive azoospermic	53	29–48	25.4* (4–65.6)	9.4 (2.1–44)	11.6 (0.13–30)	85.9 (1.5–365.2)	23.3 $\pm$ 4.0 (15–36)
Severe oligozoospermic	32	23–49	6.8 (1.1–36.2)	3.5 (1.5–13.5)	13.2 (5.1–33.4)	45.3 (7.6–297.3)	22.7 $\pm$ 3.7 (14–28)
Fertile	45	17–54	4.5 (1.7–13.4)	3.9 (1.4–15.4)	15.9 (7.2–53.1)	60 (10–270)	23.0 $\pm$ 3.1 (16–30)

Note. The CAG repeat length is expressed as mean $\pm$ SD (range). Hormone and ASI values are expressed as median (range). FSH = follicle stimulating hormone; LH = luteinizing hormone; T = testosterone. The androgen insensitivity index (ASI) was calculated by multiplying the serum concentrations of LH and T.

* $p < 0.05$ significantly higher than normal fertile control group.

Table II. Clinical Data of Patients with CAG Repeat Contraction or Expansion

Patient no.	Age	FSH (IU/L)	LH (IU/L)	T (nmol/L)	ASI (LH-T)	CAG repeat length
<i>Nonobstructive azoospermia</i>						
s40	39	31.9 ^a	10.8	10.8	116.6	15
s46	44	27.6 ^a	12.7	9.6 ^b	121.9	15
s99	38	31.2 ^a	11.3	21.6	244.1	15
s44	35	14.4 ^a	3.8	10.5	39.9	32
s117	33	6.3	3.8	14	53.2	32
s108	33	21.8 ^a	4.0	19.9	79.6	36
<i>Severe oligozoospermia</i>						
s68	37	14.8 ^a	3.6	9.1 ^b	33	14
s77	34	5.8	1.6 ^b	16.1	26	14

Note. FSH = follicle stimulating hormone; LH = luteinizing hormone; T = testosterone. The androgen insensitivity index (ASI) was calculated by multiplying the serum concentrations of LH and T.

^a Above normal reference range.

^b Below normal reference range.

As shown in Table II, increased serum FSH concentration was observed in five azoospermic patients (s40, s44, s46, s99, s108) and in one severe oligozoospermic male (s68) with abnormal CAG repeat length. Since high FSH level alone was found in majority of nonobstructive azoospermic men with normal CAG repeats; therefore, elevated FSH levels was not di-

rectly associated with abnormal CAG repeats. In the nonobstructive azoospermic group, increased LH levels in the upper normal range and higher ASI were observed in three patients with short CAG repeats, and lower LH levels and ASI index were found in another three patients with long CAG repeats in nonobstructive azoospermic group.

Fig. 1. Distribution of CAG repeat length within the androgen receptor gene in normal fertile control group and in azoospermic and oligozoospermic patients.

DISCUSSION

The association between the length of CAG repeats in the AR gene and impaired spermatogenesis has been reported in some populations of azoospermic or oligozoospermic males (17,19,26,27). Similar reports on Chinese population are scarce. There are only two studies reporting the occurrence of long CAG repeats in a mixed population of Chinese–Indian and Australian–Chinese–Indian infertile patients when compared with their fertile controls of different ethnic origins (5,18). However, the relationship between expansion/contraction of CAG repeat and male infertility in Chinese is still unknown as these studies did not define the range of CAG repeats in each ethnic group. Moreover, our recent study demonstrated that different types of Y microdeletions exist between different Chinese populations (2). Therefore, it is important to examine the relationship between the length of CAG repeats and impaired spermatogenesis in our Chinese population.

In this study, both long and short CAG repeats are found in the azoospermic males. The incidence of men with abnormal CAG repeat is significantly higher in the azoospermic group (11.4%, $p = 0.03$) but not in the severe oligozoospermic group (6.3%, $p = 0.17$) when compared with that in the fertile controls. The lack of association in the severe oligozoospermic group could be due to small sampling size. Y chromosome microdeletions screening was also performed in the same group of nonobstructive azoospermic and severe oligozoospermic patients. The results are reported elsewhere (2). We found no Y chromosome microdeletion in any of the azoospermic and severe oligozoospermic patients with abnormal CAG repeats. Similar results were also observed in the study of Liow *et al.* (28). These data indicate that Y microdeletions and abnormal CAG repeat length are independent contributors to male infertility.

In comparison with our studies, higher incidence of abnormal CAG repeats was reported in other populations (Table III). We suggest that the variability of the results by various investigators is mainly attributed to regional variations and differences in ethnic origin of the studied subjects. This is because lower incidence of abnormal CAG repeats was reported in nonobstructive azoospermic men from Japan and the absence of abnormal CAG repeats found in European infertile populations (20–23). Other factor could be due to patient selection because patients with Y chromosome deletions were excluded in their studies (18,19,26,27). When we excluded the patients with Y chromosome

Table III. Incidence of Men With Abnormal CAG Repeats

Ethnic origin	CAG repeats	Incidence (%)	Reference
<i>Nonobstructive</i>			
<i>Azoospermia</i>			
Japanese	Long	9.8	26
Hong Kong	Long and short	11.4	This study
Singapore	Long	24	12
North American	Long	27	12
<i>Oligozoospermia</i>			
Hong Kong	Short	6.3	This study
Japanese	Short	15	11
North American	Long	—	27

deletions, the incidence of infertile men with abnormal CAG repeat becomes slightly higher, 12.5% (6/48) and 6.9% (2/29) in nonobstructive azoospermic and severe oligozoospermic men, respectively.

The expansion or contraction of CAG repeats may vary among populations of different ethnic origin. In oligozoospermic men, short CAG repeats are frequently found in Chinese and Japanese populations, whereas long CAG repeat is predominantly found in North American men (Table III). In azoospermic men, both long and short CAG repeats are found in our population, whereas only long CAG repeats are observed in other populations.

The ranges of normal for the hormone concentrations and ASI were slightly different from the range of normal for fertile men presented in Table I. This can be explained by two reasons. First, these normal ranges are not derived from Chinese men. Second, the reference ranges quoted from the manufacturer were obtained from a heterogeneous normal healthy population with unknown fertility status.

High FSH level alone is not directly associated with abnormal CAG repeats because high FSH level was observed in majority of our nonobstructive azoospermic men with normal CAG repeats. There is no correlation between abnormal CAG repeat length and serum *T* level in this study. However, we observed elevation of LH levels and higher ASI in azoospermic patients with short CAG repeats. An elevated ASI is regarded as an indication of androgen insensitivity, which leads to elevation of LH and *T* as a result of impaired negative feedback control of the hypothalamic–pituitary–testicular axis (29). Both LH levels and ASI were strikingly higher in azoospermic patients with short CAG repeats than in azoospermic patients with long CAG repeats. The serum LH levels in the upper normal range in the azoospermic patients with short CAG repeats may reflect resistance to androgen action. It has been suggested that short CAG

repeats affected the androgen receptor function by suppressing the expression of the AR gene in patients with complete androgen insensitivity syndrome (6). On the other hand, progressive expansion of the CAG repeat was shown to cause a linear decrease of trans-activation function and this may subsequently lead to a failure of spermatogenesis (5). Together, these data indicate that a suitable CAG repeat length is needed for the proper maintenance of the AR function.

In conclusion, our findings show abnormal CAG repeat length in our Chinese infertile population. However, how the changes in CAG repeat length of AR affect spermatogenesis in these infertile patients are still unknown.

ACKNOWLEDGMENTS

This research was funded by the Centre of Human Reproduction, and CRCG research grant (Grant No. 10201949/31846/20900/301/01) from The University of Hong Kong.

REFERENCES

- Dohle GR, Halley DJ, Van Hemel JO, van den Ouwel AM, Pieters MH, Weber RF, *et al.*: Genetic risk factors in infertile men with severe oligozoospermia and azoospermia. *Hum Reprod* 2002;17:13–16
- Tse JYM, Yeung WSB, Ng EHY, Cheng LN, Zhu HB, Teng XM, Liu YK, Ho PC: A comparative study of Y chromosome microdeletion in infertile males from two Chinese populations. *J Assist Reprod Genet* 2002;19:376–383
- Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS: Androgen receptor defects: Historical, clinical and molecular perspectives. *Endocr Rev* 1995;16:271–321
- Chamberlain NL, Driver ED, Miesfeld RLT: 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
- Tut TG, Ghadessy FJ, Trifiro MA, Pinsky L, Yong EL: Long polyglutamine tracts in the androgen receptor are associated with reduced trans-activation, impaired sperm production, and male infertility. *J Clin Endocrinol Metab* 1997;82:3777–3782
- Komori S, Tanaka H, Sakata K, Tsuji Y, Shima H, Koyama K: Shortage of glutamine (CAG) homopolymeric repeats suppresses the expression of the androgen receptor in familial cases with complete androgen insensitivity syndrome. *Gynecol Endocrinol* 1998;12:1–8
- McPhaul MJ: Molecular defects of the androgen receptor. *J Steroid Biochem Mol Biol* 1999;69:315–322
- Choong CS, Wilson EM: Trinucleotide repeats in the human androgen receptor: A molecular basis for disease. *J Mol Endocrinol* 1998;21:235–257
- Igarashi S, Tanno Y, Onodera O, Yamazaki M, Sato S, Ishikawa A: Strong correlation between the number of CAG repeats in androgen receptor genes and the clinical onset of features of spinal and bulbar muscular atrophy. *Neurology* 1992;42:2300–2302
- Arbizu T, Santamaria J, Gomez JM, Quilez A, Serra JP: A family with adult spinal and bulbar muscular atrophy, X-linked inheritance and associated testicular failure. *J Neurol Sci* 1983;59:371–382
- Giovannucci E, Stampfer MJ, Krithivas K, Brown M, Dahl D, Brufsky A, *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
- Hancock JM, Santibanez-Koref MF: Trinucleotide expansion diseases in the context of micro- and minisatellite evolution, Hammersmith Hospital, April 1–3, 1998. *EMBO J* 1998;17:5521–5524
- Morrow AF, Gyorki S, Warne GL, Burger HG, Bangah ML, Outch KH, *et al.*: Variable androgen receptor levels in infertile men. *J Clin Endocrinol Metab* 1987;64:1115–1121
- Aiman J, Griffin JE: The frequency of androgen receptor deficiency in infertile men. *J Clin Endocrinol Metab* 1982;54:725–732
- Bouchard P, Wright F, Portois MC, Couzinet B, Schaison G, Mowszowicz I: Androgen insensitivity in oligospermic men: A reappraisal. *J Clin Endocrinol Metab* 1986;63:1242–1246
- Smallridge RC, Vigersky R, Glass AR, Griffin JE, White BJ, Eil C: Androgen receptor abnormalities in identical twins with oligospermia. *Am J Med* 1984;77:1049–1054
- Komori S, Kasumi H, Kanazawa RI, 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
- Dowsing AT, Yong EL, Clark M, McLachlan RI, de Kretser DM, Trounson AO: Linkage between male infertility and trinucleotide repeat expansion in the androgen-receptor gene. *Lancet* 1999;354:640–643
- Mifsud A, Sim CKS, Boettger-Tong H, Moreira S, Lamb DJ, Lipshultz LI, *et al.*: Trinucleotide (CAG) repeat polymorphisms in the androgen receptor gene: Molecular markers of risk for male infertility. *Fertil Steril* 2001;75:275–281
- Legius E, Vanderschueren D, Spiessens C, Hooghe TD, Matthijs G: Association between CAG repeat number in the androgen receptor and male infertility in a Belgian study. *Clin Genet* 1999;56:166–167
- Giwerzman YL, Xu C, Arver S, Pousette A, Reneland R: No association between the androgen receptor gene CAG repeat and impaired sperm production in Swedish men. *Clin Genet* 1998;54:435–436
- Rajpert-De Meys E, Leffers H, Petersen JH, Andersen A-G, Carlsen E, Jorgensen N, *et al.*: CAG repeat length in androgen-receptor gene and reproductive variables in fertile and infertile men. *Lancet* 2002;359:44–46
- van Golde R, van Houwelingen K, Kiemeny L, Kremer J, Tuerlings J, Schalken J, *et al.*: Is increased CAG repeat length in the androgen receptor gene a risk factor for male subfertility? *J Urol* 2002;167:621–623
- Irvine RA, Yu MC, Ross RK, Coetzee GA: The CAG and GGC microsatellites of the androgen receptor gene are in linkage disequilibrium in men with prostate cancer. *Cancer Res* 1995;55:1937–1940

25. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. Cambridge, UK, Cambridge University Press, 1992
26. 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
27. Patrizio P, Leonard DG, Chen KL, 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
28. Liow SL, Ghadessy FJ, Ng SC, Yong EL: Y chromosome microdeletions, in azoospermic or near-azoospermic subjects, are located in the AZFc (DAZ) subregion. *Mol Hum Reprod* 1998;4:763–768
29. Aiman J, Griffin JE, Gazak JM, Wilson JD, MacDonald PC: Androgen insensitivity as a cause of infertility in otherwise normal men. *N Engl J Med* 1979;300:223–227