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Trinucleotide (CAG) repeat polymorphisms in the androgen receptor gene: molecular markers of risk for male infertility

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Objective: To determine whether changes in the polymorphic trinucleotide (CAG) tract of the androgen receptor gene are associated with spermatogenic defects in patients with male infertility.

Design: Case–control study of two ethnic groups.

Setting: University referral centers for male infertility at Baylor College of Medicine, Houston, Texas, and National University Hospital, Singapore.

Participant(s): Two hundred and fifteen patients with male infertility and depressed spermatogenesis and 142 fertile controls.

Main Outcome Measure(s): Size of androgen receptor CAG alleles according to fluorescent-labeled polymerase chain reaction and automated analysis using Genescan software (PE Biosystems Asia, Singapore), and statistical examination of its relation to clinical variables.

Result(s): In U.S. patients, the mean androgen receptor CAG length was significantly longer in infertile patients than in fertile controls (21.95 ± 0.31 vs. 20.72 ± 0.52). Logistic regression showed that each unit increase in CAG length was associated with a 20% increase in the odds of being azoospermic. The odds ratio for azoospermia was sevenfold higher for patients with $\geq$26 CAG repeats than in those with $<$26 CAG repeats. Although mean CAG length in Singapore patients was longer than in the U.S. samples, long androgen receptor CAG alleles were significantly related to male infertility in both populations.

Conclusion(s): Long ($\geq$26) androgen receptor CAG alleles, which are found in up to 25% of azoospermic men, are associated with male infertility and defective spermatogenesis. Conception in these men is possible with assisted reproductive technologies, as many have spermatozoa in their testes. (Fertil Steril® 2001;75: 275–81. ©2001 by American Society for Reproductive Medicine.)

Key Words: Androgen receptor gene, male infertility, polymorphic trinucleotide repeat

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Three to four percent of men have severe defects in sperm production that cause infertility. Most of these men are healthy, and the cause of impaired spermatogenesis is rarely identified with certainty. Obvious causes of male infertility, including mumps, orchitis, hypogonadotropic hypogonadism, obstruction, or infection of the ejaculatory ducts, account for $<$40% of cases (1). Androgens are essential for normal sperm production, and decreasing intratesticular androgens results in defective spermatogenesis (2). The physiologic androgens testosterone and dihydrotestosterone cause development of male internal and external genitalia in utero. At puberty, a surge of androgens leads to initiation of spermatogenesis and growth of accessory sex organs, including the prostate gland.

All androgens act through the androgen receptor, which encoded by a single-copy gene on the X chromosome. Disruption of the androgen receptor by genetic mutation results in complete androgen insensitivity syndrome and sexual reversal in otherwise healthy 46XY persons (3). Patients with complete androgen insensitivity syndrome have normal male levels of testosterone but are completely female in external appearance. Since the receptor is not essential to life, mutations in the androgen receptor gene are relatively common; more than

300 different mutations are documented in a mutational database (4), making it the steroid receptor with the largest number of mutations.

The androgen receptor, like other members of the steroid receptor superfamily, has three main functional domains: the transactivation domain, the DNA-binding domain, and the ligand-binding domain (3). The N-terminal transactivation domain harbors a polymorphic trinucleotide repeat segment, (CAG)n; this segment encodes a polyglutamine tract in which n normally ranges from 11 to 31 (5). This CAG repeat tract has been the source of unprecedented interest in recent years because it was found that CAG expansion to beyond 40 repeats leads to spinal bulbar muscular atrophy, a fatal neuromuscular disease (6). Trinucleotide repeat expansions are now implicated in many neuromuscular diseases, including the fragile X syndrome, Huntington disease, spinocerebellar ataxia type 1, dentatorubral-pallidoluysian atrophy, Machado–Joseph disease, and Friedrich ataxia (7). Of note, patients with spinal bulbar muscular atrophy have evidence of reduced androgen receptor function in the form of low virilization, oligospermia or azoospermia, testicular atrophy, and reduced fertility (8, 9).

In addition to the pathologic CAG repeat expansions in spinal bulbar muscular atrophy, short androgen receptor CAG alleles are associated with prostate cancer, an androgen-dependent tumor. Thus, short CAG repeats increase androgen receptor androgenicity, resulting in abnormally high stimulation of prostatic tissue and earlier age of onset of prostate cancer (10), increased tumor grade, and increased risk of extraprostatic extension (11). At the other end of the spectrum, preliminary data indicate that long CAG repeats, while still within the polymorphic range, can reduce receptor androgenicity and lead to male infertility (12, 13). No studies of U.S. patients are available. To further elucidate the relation between CAG repeats and male infertility, we examined the distribution of androgen receptor CAG alleles in a large infertile population from a U.S. center, compared it with that in a different ethnic group from Singapore, and explored clinical phenotypes associated with long androgen receptor CAG alleles.

MATERIALS AND METHODS

Patients

Patients were recruited from the Division of Male Reproductive Medicine and Surgery of the Scott Department of Urology, Baylor College of Medicine, Houston, Texas, and the Infertility Clinics, Department of Obstetrics and Gynecology, National University Hospital, Singapore. A complete history and physical examination were performed, and use of any medications or previous surgery was recorded. The size of both testes was measured by using a Prader orchidometer, and the presence of any associated varicoceles was noted. Patients who had hypopituitarism, hyperprolactinemia, or infective or obstructive syndromes of the genital tract were excluded.

Sperm variables were assessed by using standard criteria (14) and were the mean of at least two analyses done 3 months apart. Azoospermia was defined as the absence of any spermatozoa despite centrifugation of the semen specimen; oligospermia was defined as the mean sperm density $<20 \times 10^6$/mL. Controls were men with proven fertility who had no previous infertility history or treatment and no genetic disease. The project was approved by the Ethical Review Committee of the National University Hospital, Singapore, and the institutional review board of Baylor College of Medicine.

DNA Amplification and Genescan Analyses

Standard techniques were used to extract DNA from the peripheral blood of patients and control subjects. The CAG repeat segment was amplified by using sense (5′-TCCAGAATCTGTTCCAGAGCGTGC) and antisense (5′-GCTGTGAAGGTTGCTGTTCCTC) primers (Fig. 1A). The 30-μl reaction mix contained 500 ng of genomic DNA, 50μM of monodeoxyribonucleoside triphosphates, 0.25μM of each primer, fluorescent-labeled deoxycytosine triphosphate (dCTP) (R6G), and 0.5 U of Taq polymerase. A two-step 30-cycle amplification protocol was used, in which the denaturing temperature was 95°C for 45 seconds and the combined annealing and extension temperature was 68°C for 1.5 minutes. In the first cycle, the sample was denatured for 5 minutes. Amplified samples were mixed with formamide, loading buffer, and the Rox 500 size standard in a 1:10:2:2 ratio. This mixture was denatured for 5 minutes at 95°C and resolved on a 4% denaturing polyacrylamide gel.

The sizes of the samples was determined by using a 377 DNA Sequencer running Genescan 672 software (PE Biosystems Asia, Singapore). Androgen receptor CAG allele size automatically assigned with reference to internal size standards (Fig. 1B). Some samples were sequenced to obtain further accuracy in measurement of size. These samples of known length were inserted in every gel as controls.

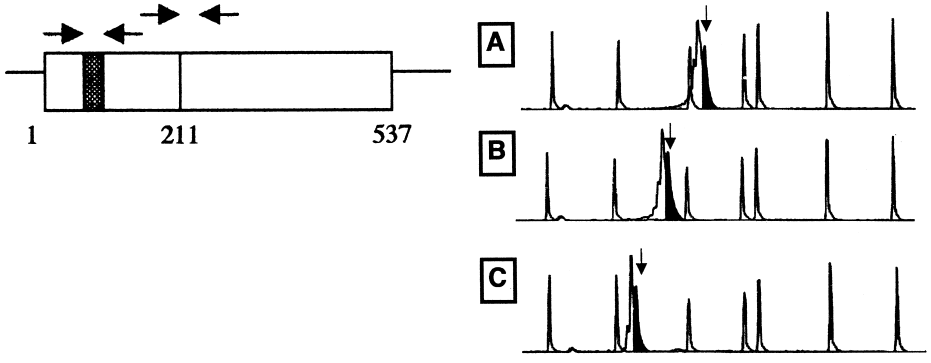
Androgen Receptor Point Mutation and Y-Microdeletion Screening

The remaining coding sequences of androgen receptor (exons 1 to 8) were examined by using polymerase chain reaction single-stranded conformation polymorphism (15) to screen for associated subtle mutations. This was done to ensure that our data were not confounded by exonic mutations that were linked to any particular trinucleotide repeat allele.

Multiplex polymerase chain reaction to assess Y microdeletions were also performed (16). In some cases, DNA was insufficient for full analysis of all androgen receptor exons and Y microdeletions. Patients with androgen receptor mutations and Y microdeletions were therefore not included in this report.

FIGURE 1

(**A**), Polymorphic segments in exon 1 of the androgen receptor gene. The polymorphic CAG repeat tract (*shaded box*) and fragment containing codon 211 were amplified with flanking primers (*arrows*). Codons were numbered according to the method of Lubahn et al. (26). (**B**), Genescan analyses of androgen receptor CAG length. Electropherograms show alleles with 29 (*A*), 22 (*B*), and 14 (*C*) CAG repeats from azoospermic men, oligospermic men, and fertile controls, respectively. Polymerase chain reaction fragments (*arrows*) were internally labeled with a fluorescent marker (dCTP, R6G), and their length was measured by using red internal size standards (ROX, 500).



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Hormonal Analysis

Radioimmunoassay for serum luteinizing hormone, prolactin, and testosterone were performed by using standard reagents from Diagnostic System Laboratories (Webster, TX). Follicle-stimulating hormone was assayed by using reagents from Diagnostic Products Corp. (Los Angeles, CA). The interassay and intraassay coefficients of variation were <15%.

Statistical Analysis

The mean number of CAG repeats and other polymorphisms from infertile patients were compared with those in fertile controls by using the two-sample independent *t*-test or Fisher exact test, as appropriate. Subsequently, multiple comparisons were performed comparing the mean number of CAG repeats in azoospermic and oligospermic patients with that in fertile controls by using analysis of variance and the Dunnett test. Logistic regression analysis was performed, using the number of CAG repeats as the exposure and clinical infertility as the outcome. We sought to determine whether the risk of infertility decreased progressively with shorter CAG repeat lengths by using patients with ≥26 CAG repeats as the reference group. This provided us with the ratios and corresponding confidence intervals. Odds ratios were also calculated for patients with ≥26 repeats and those with <26 repeats.

The association between number of CAG repeats and presence of varicocele was evaluated. Because low testicular volume has been associated with clinical infertility, we also examined the relation between low testicular volume and infertility and its relation to the number of CAG repeats.

Statistical analyses were performed by using SPSS software, version 9.01 (SPSS Corp., Chicago, IL). Statistical significance was defined as a two-sided *P* value <.05. Data are reported as means (±SE).

RESULTS

U.S. Patients

Ninety-five infertile men and 55 fertile controls at Baylor College of Medicine were analyzed. The racial distribution in the control and study groups was similar and consisted mostly of white persons (68% and 71%, respectively). The mean androgen receptor CAG was significantly longer in infertile subjects than in fertile controls (21.95 ± 0.31 vs. 20.72 ± 0.52; $P = .034$, *t*-test). Longer CAG lengths corre-

TABLE 1

CAG repeat lengths in U.S. and Singapore men with varying degrees of defective spermatogenesis.

	No. of patients	Mean ± SE CAG length	Range	*P* value[a]
Baylor College, Houston, Texas				
Fertile controls	55	20.72 ± 0.52	8–27	
Oligospermic men[b]	72	21.65 ± 0.34	14–29	.226
Azoospermic men	23	22.91 ± 0.73	17–31	.019
Singapore				
Fertile controls	87	22.38 ± 0.32	11–29	
Oligospermic men[b]	87	22.85 ± 0.33	14–32	.514
Azoospermic men	33	23.82 ± 0.52	18–33	.043

[a] By the Dunnett test after analysis of variance compared fertile controls.
[b] Sperm count $<20 \times 10^6$/mL.

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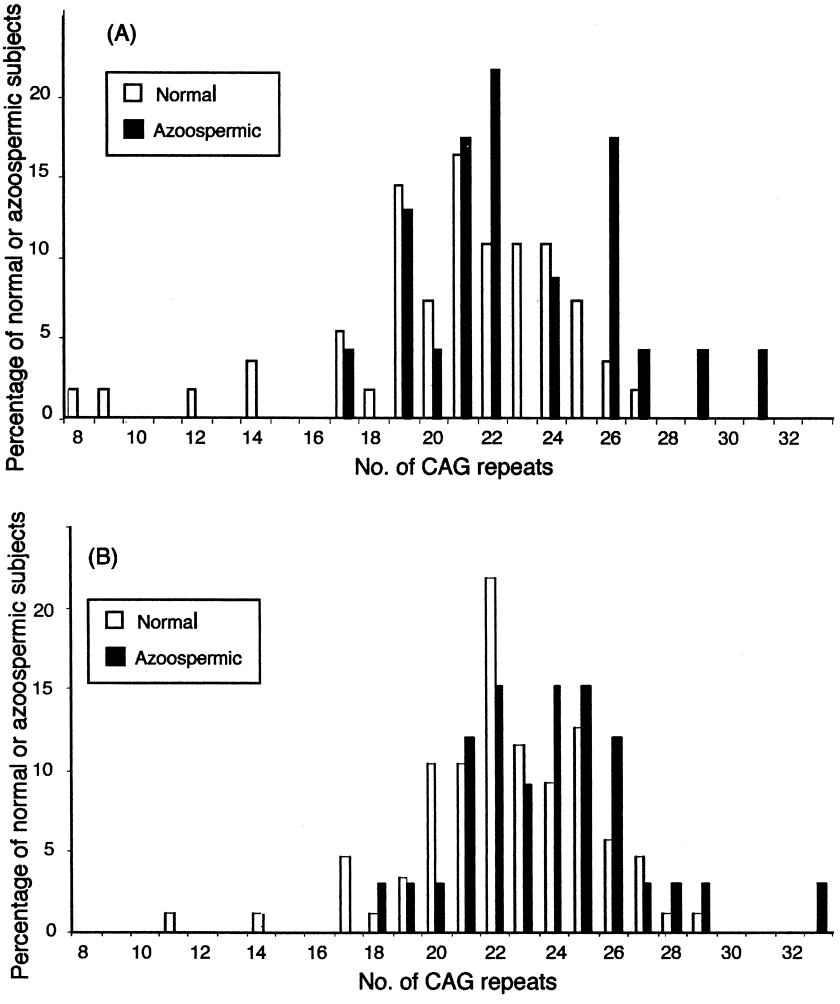
FIGURE 2

Androgen receptor CAG allele distribution in normal fertile controls and azoospermic men. (**A**), Patients from Baylor College of Medicine, Houston, Texas. (**B**), Patients from National University Hospital, Singapore.



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sponded to greater severity of spermatogenic defect. The CAG length was longer in oligospermic men than in fertile controls, and it was longer in azoospermic men than in oligospermic men (Table 1). The distribution of androgen receptor CAG repeats in azoospermic patients shifted to the right compared with controls (Fig. 2A); mean CAG length was longest in azoospermic patients, being about 2 CAGs longer than that in controls (22.91 vs. 20.72; $P=.019$) (Table 1).

When logistic regression was performed using the number of CAG repeats as a continuous predictor variable, the odds ratio for clinical infertility (defined as a composite of oligospermic and azoospermic men) was 1.11 (95% CI: 1.05–1.23) ($P=.038$) for each unit increase in CAG number (exp[β]). When the outcome was azoospermia, exp(β) increased to 1.20 (95% CI: 1.02–1.40) ($P=.0277$). Thus, each unit increase in CAG length was associated with a 20% increase in the odds of being azoospermic. For the categorical analysis, a threshold of 26 CAG repeats was chosen because this CAG length was about 1 SD longer than the mean. Patients with ≥26 CAG repeats were sevenfold more likely than those with >26 CAG repeats to be azoospermic (odds ratio 7.52 [95% CI: 1.75–32.78]; $P=.006$).

We studied the protective effect of having short CAG repeats. When men with ≥26 repeats were defined as the reference group, the likelihood of azoospermia decreased as the number of CAG repeats decreased (Table 2). For instance, if the number of CAG repeats was ≤19, the odds ration for azoospermia was 0.100 compared with men who had ≥26 CAG repeats ($P=.009$). Thus having 26 or more

TABLE 2

Protective effect of short CAG repeats on risk of male infertility in U.S. patients.[a]

	CAG length				
	≥26	≤22	≤21	≤20	≤19
Odds ratio	1.000	0.194	0.150	0.102	0.100
95% CI		0.043–0.887	0.031–0.723	0.019–0.540	0.017–0.572
P value		.018	.018	.007	.009

[a] Each category was compared with patients with ≥26 CAG repeats as the reference group.

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CAG repeats is an important and statistically significant marker for azoospermia.

In contrast, patients with and those without varicoceles did not differ in terms of CAG repeat length. Although mean testicular volume was significantly smaller in infertile patients than fertile controls (13.0 vs. 17.8 mL; $P<.001$), CAG length was not significantly correlated with testicular size.

Singapore Patients

To compare differences in CAG length by ethnic group, we analyzed 120 infertile men and 87 fertile controls from Singapore enrolled after April 1998. These patients were predominantly of Chinese ethnic origin.

Thirty-three men were azoospermic and 87 were oligospermic. Two patients with Y-chromosome microdeletions were not included in the analysis. Although the mean CAG length in these patients was 0.91 to 1.66 CAG longer than that in the corresponding U.S. samples (Table 1), the pattern of changes in CAG length with respect to male infertility was similar. The distribution of CAG length in azoospermic patients from Singapore was skewed to the right compared with that in fertile controls (Fig. 2B). Again, CAG length also was longer in oligospermic men than in fertile controls, and it was longer in azoospermic men than in oligospermic men (Table 1). Mean CAG length was significantly longer in azoospermic patients than controls (23.82 vs. 22.38) ($P=.043$).

Logistic regression in data from Singapore patients showed an exp(β) of 1.18 (95% CI: 1.01–1.37; $P=.032$), indicating that the risk for azoospermia increases by 18% for each unit increase in CAG length.

Polymorphism in Codon 211 Related to Ethnic Origin and Male Infertility

Several GAG→GAA polymorphisms were encountered in codon 211 (Fig. 1A), located in exon 1. This polymorphism, which did not change the sense of the codon, was observed only in U.S. patients. The polymorphism occurred in 4.3% (2 of 46) of fertile controls and 18% (13 of 70) of infertile men and was significantly related to infertility ($P=.027$, Fisher exact test). This polymorphism did not segregate with long androgen receptor CAG alleles, since all 15 men with the codon 211 polymorphism had <23 CAG repeats.

TABLE 3

Significant clinical variables in infertile patients with long (≥26) CAG repeats.

Variable	U.S. patients (n = 13)	Singapore patients (n = 24)
Azoospermia (%)	46	33
Nonazoospermic patients		
Sperm density (N: ≥20 × 10^6/ml)	6.5 ± 3.0	4.1 ± 0.7
Motility (N: ≥50%)	38.4 ± 9.9	32.0 ± 5.6
Testicular volume[a]	13.0 ± 1.6	13.1 ± 0.8
Variocele[b]	45	25
FSH level	16.4 ± 2.9	5.2 ± 0.9

Note: Data are percentages or means ± SE. FSH = follicle-stimulating hormone.

[a] Normal value 17.82 ± 0.61 mL, established in U.S. fertile controls.

[b] No significant association with CAG repeats.

[c] Normal level 0.8–4.7 IU/L.

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Clinical Characteristics of Patients With ≥26 CAG Repeats

To determine whether a particular phenotype is related to long CAG repeats, we analyzed the clinical characteristics of patients with ≥26 CAG repeats. About 24% of Singapore patients and 27% of U.S. patients had ≥26 CAG repeats, the point at which risk of infertility increased significantly. No patient with a long CAG repeat had any neurologic deficit or abnormal secondary sexual development; specifically, no patient had hypospadius, microphallus, gynecomastia, or abnormal distribution of pubic hair.

Among patients with ≥26 CAG repeats, 46% of U.S. patients and 33% of Singapore patients were azoospermic (Table 3). The mean sperm count in nonazoospermic patients was 6.5 × 10^6/mL in U.S. patients and 4.1 mil/mL in Singapore patients. Mean testicular volume was significantly reduced ($P < 0.001$) to 13 mL in both groups.

Varicoceles were present in 45% of U.S. patients and 25% of Singapore patients; however, this variable was not significantly associated with CAG length. The high prevalence of varicoceles in U.S. patients was observed because the clinic is a quaternary referral center for urological problems. The mean follicle-stimulating hormone was also increased, more so in U.S. patients than in Singapore patients.

Results of testicular biopsy in 14 patients with long CAG tracts were available. Histologic findings were nonspecific and included incomplete spermatocytic arrest, paucity of mature sperm, the Sertoli-cell-only syndrome, tubular atrophy, hyalinization, and fibrosis of seminiferous tubules. In 1

patient, all the above findings were observed in samples from seminiferous tubules obtained from different parts of the testes. Of the 7 azoospermic Singapore patients for whom biopsy results were available, 3 had complete absence of spermatogenesis and 4 had some evidence of spermatogenesis. Azoospermia associated with long CAG repeats therefore does not preclude the possibility of spermatogenesis in the tubules.

DISCUSSION

The major observation in this study is that some azoospermic men have significantly longer androgen receptor CAG alleles compared with fertile controls. The CAG length was measured accurately by using internal size standards in every lane, and allelic size was assigned objectively by Genescan software. Analysis of more than 350 patients and controls showed a significant direct correlation between CAG length and severity of spermatogenic defect. Patients with ≥26 CAG repeats have a sevenfold higher risk of azoospermia than those with ≥26 CAG repeats.

This study, the largest to date, independently replicates the findings of two earlier studies comprising Asian (12) and Australian populations (13). Ethnic differences in CAG length are well known; for instance, African-Americans have shorter CAG repeats than white Americans (5). We compared androgen receptor- AG allele distribution in two ethnic groups, one predominantly white and the other Chinese. A clear difference in CAG length according to ethnicity was seen; the mean CAG length in Singapore patients was 0.91 to 1.66 longer than that in U.S. patients. However, long CAG tracts were significantly associated with infertility in both populations, indicating that these differences were independent of ethnicity.

A conservative G→A polymorphism was detected in codon 211 on screening of the rest of the androgen receptor gene. This polymorphism was confined to U.S. patients, and unexpectedly, it seemed to be related to infertility. Since the polymorphism did not change the sense of the codon, its pathogenicity remains to be proven. Nonetheless, it is noteworthy that patients with the codon 211 (GAA) polymorphism do not have long CAG tracts, and the presence of this polymorphism does not confound the primary finding that long CAG tracts are significantly associated with male infertility.

Conversely, patients with short androgen receptor CAG alleles had a lower risk of male infertility, and the shorter the tract, the lower the risk. Men with ≤19 CAG repeats had only 10% the risk for infertility of men with ≥26 CAG repeats (Table 2). Of note, a CAG repeat length of 22 also marked the point at which risk for prostate cancer increased (17). Prostate cancer is an androgen-driven tumor, and androgen-ablation therapy is a commonly used treatment. Evidence indicates that spermatogenesis requires high concentrations of androgens (2). Patients with androgen receptor mutations that reduce intrinsic receptor activity have impaired spermatogenesis (18–20). Conceptually, prostate cancer and defective spermatogenesis may represent opposite ends of a spectrum of androgen receptor activity. In combination, these studies suggest that polymorphic changes in CAG length inversely regulate the function of the androgen receptor. This hypothesis implies that lower risk of defective spermatogenesis is associated with increased severity and earlier age of onset of prostate cancer, and vice versa. Long-term cohort studies are required to validate this model of androgen receptor function.

No clear phenotype was observed in patients with long androgen receptor CAG alleles. Long CAG tracts were not significantly associated with varicoceles (21). Infertile patients had a significantly smaller testicular volume than fertile controls, but this variable was not related to the size of the CAG tract. Serum follicle-stimulating hormone levels were slightly increased in the Singapore patients and more so in the U.S. patients; these differences are most likely attributable to referral patterns to the two centers.

Although about 33%–46% of patients with long CAG repeats were azoospermic, spermatozoa were present in the seminiferous tubules in most azoospermic men, suggesting that basal spermatogenic function was preserved in even the most severely affected patients. It is tempting to speculate that boosting intratesticular levels of androgens could improve spermatogenesis in these patients (22).

Long androgen receptor CAG alleles encoding long androgen receptor polyglutamine tracts are associated with low intrinsic androgen receptor activity in reporter gene assays (12, 23, 24). A possible molecular mechanism for this observation is linked to identification of a novel nuclear G protein, Ras-related nuclear protein/ARA24, which acts as a coactivator with the androgen receptor and can bind differentially with different lengths of androgen receptor polyglutamines (25). Interactions between androgen receptor CAG and ARA24 become stronger as the number of glutamines decreases, thereby increasing coactivation and androgen receptor transactivation capability. Although the probable increase in androgen receptor intrinsic activity with each reduction in androgen receptor CAG length is relatively small, these effects are genetically determined and therefore exert effects over a person's entire lifetime. Small changes can therefore have significant cumulative pathologic effects over time. Collectively, the evidence supports the hypothesis that the glutamine repeat plays a role in androgen receptor function by fine-tuning the balance between excess and deficient receptor function. The highly polymorphic nature of the glutamine repeat would imply a subtle gradation of androgen receptor function among individuals, possibly allowing alleles with evolutionary advantages to be rapidly selected and transmitted to future generations.

Our study, which involves the largest sample to date, is

the first to examine the relation of androgen receptor CAG polymorphisms with infertility in U.S. subjects. Long androgen receptor CAG alleles (≥26 CAG repeats), which affect up to 25% of azoospermic men, were significantly associated with male infertility and defective spermatogenesis. Modern assisted reproductive technologies that use sperm obtained by testicular biopsy and ICSI may result in viable pregnancies and transmission of this trait. Screening for long androgen receptor CAG tracts and appropriate genetic counseling would be important for men with infertility and defective spermatogenesis.

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