Androgen Receptor Gene CAG and GGN Repeat Polymorphisms in Chilean Men With Primary Severe Spermatogenic Failure

EDUARDO CASTRO-NALLAR,* KETTY BACALLAO,* ALEXIS PARADA-BUSTAMANTE,*
MARÍA C. LARDONE,* PATRICIA V. LÓPEZ,† MARCIA MADARIAGA,* RAÚL VALDEVENITO,‡
ANTONIO PIOTTANTE,§ MAURICIO EBENSPERGER,† AND ANDREA CASTRO*

From the *Institute of Maternal and Child Research, School of Medicine, University of Chile, Santiago, Chile; the †San Borja Arriarán Clinical Hospital, Santiago, Chile; the ‡José Joaquín Aguirre Clinical Hospital, Department of Urology, School of Medicine, University of Chile, Santiago, Chile; and the §School of Medicine, Andrés Bello University, Santiago, Chile.

ABSTRACT: There is ample documentation supporting the fact that androgens are required for normal spermatogenesis. A minority of infertile men have abnormal testosterone blood levels or mild androgen receptor mutations. We investigated the androgen receptor CAG and GGN repeat lengths in Chilean men with spermatogenic impairment. We studied 117 secretory azoospermic/oligozoospermic men (93 idiopathic and 24 excryptorchidic), without Y-chromosome microdeletions, and 121 controls with normal spermatogenesis (42 obstructive and 79 normozoospermic men). Peripheral blood was drawn to obtain genomic DNA for polymerase chain reaction and automated sequencing of CAG and GGN repeats. Testicular characterization included hormonal studies, physical evaluation, and seminal and biopsy analysis. The CAG and GGN polymorphism distributions were similar among idiopathic men, excryptorchidic men, and controls and among the different types of spermatogenic impairment. However, the proportion of the CAG 21 allele was significantly increased in idiopathic cases compared to controls (P = .012 by Bonferroni test, odds ratio = 2.99, 95% confidence interval, 1.27–7.0) and the CAG 32 allele only was observed in excryptorchidic patients (P < .0002, Bonferroni test). Idiopathic cases with Sertoli cell–only syndrome showed the highest proportion of the CAG 21 allele (P = .024, χ^2 test). On the other hand, in idiopathic cases and controls the most common GGN allele was 23, followed by 24, but an inverse relation was found among excryptorchidic cases. The joint distribution of CAG and GGN in control, idiopathic, and excryptorchidic groups did not show an association between the 2 allele repeat polymorphisms (P > 0.05, χ^2 test). Our results suggest that the CAG 21 allele seems to increase the risk of idiopathic Sertoli cell–only syndrome. Moreover, the GGN 24 allele could be contributing to deranged androgen receptor function, associated with cryptorchidism and spermatogenic failure.

Key words: Glutamine and glycine repeat polymorphisms, male infertility, Sertoli cell–only syndrome.

J Androl 2010;31:552–559

Failure of spermatogenesis is largely responsible for male infertility, but its etiology remains unknown in nearly half of all cases (Bhasin, 2007; Krausz and Giachini, 2007). Until now, Y-chromosome microdeletions have constituted the most important known etiological factor for spermatogenic failure. Several studies indicate a prevalence of 5% to 20% in subjects with azoospermia or severe oligozoospermia (Vogt, 1998; Krausz et al, 1999), and only a few reports have found a higher prevalence in patients with severe testiculopathies, such as hypospermatogenesis, maturation arrest (MA), and Sertoli cell–only syndrome (SCOS; Foresta et al, 1998; Foresta, 2001; Ferlin et al, 2007).

Development of male phenotype and the initiation of spermatogenesis leading to production of male gametes are dependent on cellular events that respond to androgens. In fact, mutations in the androgen receptor (AR) gene cause a variety of defects, known collectively as the androgen insensitivity syndrome (AIS), which range from XY patients with female phenotype and high serum levels of testosterone and estradiol, known as complete insensitivity syndrome, to subjects with a mild AIS who have infertility as their primary or even sole symptom (Davis-Dao et al, 2007). Furthermore, a significant proportion of infertile males have a history of cryptorchidism, which may constitute an additional phenotypical expression of AIS. This is the most frequent congenital birth defect in males and represents the best-characterized risk factor for infertility and testicular cancer in adulthood, but its etiology remains mostly unknown (Ferlin et al, 2008; Foresta et al, 2008).

The AR contains 4 main functional domains: the amino-terminal transactivation domain (TAD), the centrally positioned DNA-binding domain, the hinge region, and the carboxyl-terminal ligand binding domain. Within TAD are 2 segments consisting of amino acid repeats, glutamine (encoded by CAG) and glycine (encoded by GGN). These repeat tracts are polymorphic, in that their size varies among individuals from a normal population (Lundin et al, 2003, 2007; Palazzolo et al, 2008). The CAG repeat lengths span from approximately 12 to 25 repeats, with a median number of 22, and in rare cases more than 35 contiguous CAGs (Palazzolo et al, 2008).

Longer CAG repeat lengths result in reduced AR transcriptional activity both in vivo and in vitro (Tut et al, 1997; Beilin et al, 2000; Crabbe et al, 2007). In fact, the CAG repeat tract has been the source of unprecedented interest in recent years because it was found that CAG expansion beyond 37 repeats leads to spinal bulbar muscular atrophy (also known as Kennedy disease), an adult-onset X-linked neurodegenerative disease that shows an inverse correlation between repeat length and the age of onset of gynecomastia, as well as clinical and hormonal evidence of androgen insensitivity (La Spada et al, 1991; Dejager et al, 2002; Palazzolo et al, 2008).

Even though CAG tract lengths correlate inversely with sperm concentration in normal men (von Eckardstein et al, 2001), several studies involving infertile men have reported conflicting results, in part related to ethnicity, sample size, and inclusion criteria, with some showing no increase (Dadze et al, 2000; Sasagawa et al, 2001; von Eckardstein et al, 2001; Ferlin et al, 2004; Martinez-Garza et al, 2008; Westerveld et al, 2008), and others reporting an increased length with respect to controls (Tut et al, 1997; Dowsing et al, 1999; Mifsud et al, 2001; Patrizio et al, 2001; Wallerand et al, 2001). In 2007, Davis-Dao et al provided support for an association between the CAG length and idiopathic male infertility by a meta-analysis, but recommended measurement of additional AR length polymorphisms, such as GGN repeat length sequence. Moreover, a recent study investigated different CAG lengths in the normal range (16, 22, and 28) together with the GGN 23 allele and found that the highest AR activity was confined to CAG = 22 and not to CAG = 16, indicating some CAG alleles into the normal range may show no linearity between length and sensitivity of the AR (Nenonen et al, 2010).

The functional consequences of variations in the GGN repeat are even less clear, and epidemiological investigations of the association between the number of GGN repeats in male infertility have produced inconsistent results (Tut et al, 1997; Lundin et al, 2003; Ferlin et al, 2004). In general, the GGN repeats span from 10 to 27 and the predominant allele has 23 repeats (Lundin

et al, 2003). In addition, in vitro data has indicated that ARs with glycine numbers other than 23 have low transactivating capacity in response to both testosterone and 5-α dihydrotestosterone (DHT; Lundin et al, 2007).

Recently, other studies have investigated the distribution of different CAG/GGN combinations in infertile men and controls (Ferlin et al, 2004, 2005; Ruhayel et al, 2004). In particular, the same 2 CAG/GGN haplotypes (CAG = 21/GGN = 24 and CAG $\geq$ 21/GGN $\geq$ 24) showed an increased susceptibility to idiopathic secretory infertility (Ferlin et al, 2004) and to cryptorchidism (Ferlin et al, 2005), associated with spermatogenic damage in an Italian population. Similar results were found in a Swedish population who showed evidence for a protective effect in <21 CAG and GGN = 23 length repeat carriers (Ruhayel et al, 2004).

Therefore, our aim was to study the CAG and GGN repeat lengths alone and in combination in Chilean men with primary spermatogenic failure, idiopathic or with a history of cryptorchidism, compared to controls with normal spermatogenesis.

Materials and Methods

Subjects

This study was approved by the Ethical Review Board of the Central Metropolitan Health Service, Santiago, Chile, and all subjects gave their informed consent. We studied 159 selected Chilean infertile patients who consulted for infertility at the Institute of Maternal and Child Research, San Borja Clinical Hospital, or at the José Joaquín Aguirre Hospital, Santiago, Chile. One hundred forty-two infertile patients were referred for testicular biopsy for spermatic recuperation by testicular sperm extraction (TESE). Patients undergoing TESE had a minimum of 1 year of infertility and sperm count $\leq$5.0 $\times$ 10^6/mL. Patients underwent an evaluation that included complete physical examination, hormonal studies, and karyotype. Testis volume was measured by ultrasonography and/or Prader orchidometer. Seventeen of the 159 infertile patients did not undergo a testicular biopsy, but they were included in the study because they were azoospermic and they had a high serum follicle-stimulating hormone (FSH) associated with a reduced testicular volume (<12 mL). Subjects were excluded if they had hypogonadotropic hypogonadism, hyperprolactinemia, chronic diseases, clinical varicocele, retractile testis, male accessory gland infections, orchitis, genital trauma, drug consumption, or concomitant hormonal treatment. Moreover, all infertile men had a normal karyotype and they did not have Y-chromosome microdeletions (Castro et al, 2004). Cryptorchidism or history of cryptorchidism was absent from controls.

Among 159 infertile patients, 117 had spermatogenesis failure and 42 had normal spermatogenesis (obstructive oligospermic/azoospermic controls). Among 117 patients with spermatogenic failure, 93 were idiopathic based on the absence of infertility contributing factors (n = 65), or when the only

andrological abnormality was subclinical (nonpalpable) varicocele, detected by ultrasonography (n = 15), or grade II varicocele operated more than 3 years before (n = 13). Twenty-four of the patients with spermatogenic failure had a diagnosis of cryptorchidism based on the self-reported history of the patients that was concordant with the parent's report (when required) and signs in the physical examinations (inguinal scar). The precise location of the testes at the time of orchidopexy could not be determined in most cases. Persistent cryptorchidism had been bilateral in 13 of 24 (54%) or unilateral in 11 of 24 (46%) of the patients, and orchidopexy was performed in all cases between 2 and 12 years of age (excryptorchidic).

All obstructive controls had a normal spermatogenesis and all of them had a positive spermatic recuperation on TESE. Among normozoospermic men, 34 (43%) reported fertility; the other 45 normozoospermic men had never tried to achieve paternity. We studied an additional 79 control men from the same geographic region as normozoospermic volunteers.

Hormonal Measures

Serum concentrations of luteinizing hormone (LH), FSH, and sex hormone-binding globulin (SHBG) were measured by immunoradiometric assay (Siemens Medical Solutions Diagnostics, Los Angeles, California). Intra-assay coefficients of variation (CVs) were 6.5%, 3.6%, and 3.9%, and interassay CVs were 7.6%, 6.2%, and 6.9% for LH, FSH, and SHBG, respectively. Total testosterone was measured by radioimmunoassay (Diagnostic System Laboratories, Webster, Texas); intra-assay and interassay CVs were 5.1% and 6.4%, respectively. Blood samples were collected between 8 and 10 AM. Absolute values for serum testosterone were multiplied by those for LH to determine the androgen sensitivity index (ASI; Hiort et al, 2000).

Semen Analysis

Semen analysis was performed according to the World Health Organization (1999) criteria. The diagnosis of azoospermia was based on the absence of sperm in at least 2 separate semen analyses after centrifugation of semen samples (1000 × g, 5 minutes). Infertile patients and normozoospermic volunteers underwent at least 2 semen analyses. Sperm morphology evaluation using Kruger strict criteria (Kruger et al, 1987) was also performed in normal controls.

Testicular Biopsy

The testicular biopsy procedure was performed between March 2003 and October 2009 in men in whom previous semen analyses had shown azoospermia or low numbers of viable spermatozoa that implied a high risk of a futile IVF/ICSI procedure if relying on ejaculated spermatozoa only.

A small piece of testicular tissue was fixed in Bouin solution during 6 hours for histopathological evaluation. Testicular histology assessment included a qualitative and quantitative analysis of germinal epithelium in 20–25 tubules, and the modified Johnsen score (JS) was calculated (Johnsen, 1970; Jezek et al, 1998). According to this score, the tissues were classified in SCOS, complete (JS = 2) or incomplete (some foci of spermatogenesis); MA (germ cells until spermatogonia or spermatocyte, which may be complete or incomplete); hypospermatogenesis (proportional and quantitative reduction of the different types of germ cells); severe atrophy (SA; hyalinization of seminiferous tubules and lack Sertoli and germ cell, JS = 1); mixed atrophy (mixture of the above mentioned types of tubular histology); and normal spermatogenesis (all the tubules evaluated had complete spermatogenesis or elongated spermatids at least, JS ≥ 8).

Determination of the CAG and GGN Repeat Number

Standard automated sequencing was performed using 2 different amplicons that contained CAG or GGN repeats. The CAG and GGN amplicons were obtained after polymerase chain reaction (PCR) reactions with CAG (A0: GTG GTTGCTCCCGCAAGTTTCC and A5: TAATTGTCCTTG-GAGGAAGTGGGAGC) and GGN pairs of primers (A3n: CAGCAAGAGACTAGCCCCAG and A10: CCAGAACA-CAGAGTGACTCTGCC) as described previously (Ferlin et al, 2004; Lubahn et al, 1989). Amplification was performed in 25 µL reaction volume containing 130 ng of DNA, 200 µM of each deoxynucleotide triphosphate (Invitrogen, San Diego, California), 1X Optimized DyNAzime EXT Buffer (FINN-ZYMES OY, Espo, Finland), 8% DMSO (FINNZYMES OY), 150 nM of each sense or antisense primer, and 1 U of DyNAzime EXT DNA polymerase (FINNZYMES OY). Both PCR reactions were performed under the same conditions previously described (Ferlin et al, 2004): 37 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute; initiated with a denaturation step of 94°C for 3 minutes; and terminated with an extension step at 72°C for 10 minutes.

The CAG repeat contained in the amplicon was sequenced with the internal primer A2.2: GCTGTGAAGGTTGCTG TTC, and the GGN repeat was sequenced with the primer A8.2: GGACTGGGATAGGGCA. Sequence analyses were performed with the gap4 software of the Staden package (Staden, 1996; Ferlin et al, 2004), which is available at the UK Human Genome Mapping Project Web page (www.hgmp.mrc.ac.uk/).

Statistical Analysis

Statistical calculations were performed using SPSS 11.5 for Windows (SPSS Inc, Chicago, Illinois). Pearson's χ^2 and Fisher's exact test were applied for testing differences in proportions between groups. Differences among groups were compared by the Kruskal–Wallis test and the Mann-Whitney U test. The odds ratio was used to estimate relative risk among different subsets of cases and controls. A Bonferroni test was performed to correct for multiple comparisons. P values less than .05 (2-sided) were considered statistically significant.

Results

Patients and Hormonal Evaluation

Among 117 secretory infertile patients, 100 underwent testicular biopsy analysis that showed severe spermato-

Table 1. *Hormonal profile in cases and controls[a]*

	Age, y	FSH, mIU/mL	LH, mIU/mL	T, ng/mL	ASI, IU × nmol/L^2	SHBG, nmol/L
Cases	33 (20–45)	13 (1.8–41)[b]	4.8 (1.6–17)[b]	3.5 (1.7–7.2)	57 (13.5–184)[b]	33 (14–76)
Excryptorchidic (n = 24)	33 (21–42)	14 (4.0–59)[b]	5.7 (1.2–19)[b]	3.6 (1.9–5.3)	58 (17–197)[b]	30 (16–58)
Idiopathic (n = 93)	33 (21–46)	13 (1.6–35)[b]	4.8 (1.6–15)[b]	3.4 (1.5–8.1)	57 (14–182)[b]	34 (12–77)
Controls	33 (19–49)	2.9 (1.2 –7.8)	2.4 (1.6–6.5)	3.6 (2.1–6.3)	31 (8.3–92)	42 (14–77)
Azoospermic/oligozoospermic (n = 42)	35 (25–45)	2.9 (1.5–8.3)	2.3 (1.0–7.4)	3.5 (1.9–5.7)	28 (6.9–84)	33 (14–73)
Normozoospermic (n = 79)	32 (19–49)	3.0 (1.0–7.1)	2.5 (1.1–6.2)	3.9 (2.4–6.3)	35 (11–92)	52 (36–77)

Abbreviations: ASI, androgen sensitivity index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; T, testosterone.
[a] Values represent median (2.5–97.5 percentile). Reference values: FSH, 1.0–9.0; LH, 1.0–8.0; T, 2.0–8.0; SHBG, 10–80.
[b] $P < .01$ vs controls, Mann-Whitney *U* test.

genic impairment (60 SCOS, 17 MA, 11 mixed atrophy, 8 hypospermatogenesis, and 4 SA). There was no significant difference in the prevalence of histological phenotypes between idiopathic and excryptorchidic cases (data not shown). The chronological ages were similar in cases and controls (Table 1).

A complete hormonal evaluation was performed in all infertile patients. In normozoospermic controls, FSH, LH, and total testosterone serum levels were measured in all subjects and SHBG in only 11 men. Table 1 shows the hormone serum levels and the age of the subjects. Comparison of hormonal levels between excryptorchidic and idiopathic cases did not show statistical differences. However, cases with SCOS, mixed atrophy, MA, and SA showed higher levels of FSH and LH compared to controls ($P < .01$, Mann-Whitney *U* test, data not shown). The median ASI was significantly higher in cases with a history of cryptochidism and idiopathic cases compared to controls ($P < .01$, Mann-Whitney *U* test). When taking into account the highest level reported in a control group of 53 fertile Caucasian men (range: 6.7–138.7 IU × nmol/L^2; Hiort et al, 2000), ASI was above the normal range in only 9 cases (range: 155–293 IU × nmol/L^2): 5 idiopathic patients (2 SCOS, 2 MA, and 1 SA) and 4 excryptorchidic patients (1 MA, 1 SA, and 2 secretory azoospermic patients without diagnosis by testicular biopsy) .

CAG and GGN Analysis

The statistical analysis revealed that neither CAG nor GGN repeat lengths differed significantly among cases, among case subgroups, or among the different spermatogenic impairments and controls. Table 2 shows the medians and ranges of the repeat lengths for idiopathic cases, excryptorchidic cases, and controls.

The Figure shows the distribution of CAG and GGN alleles repeat in case subgroups and controls. The Figure (A) shows that the CAG repeat total distribution was not different among the 3 groups studied. Despite this fact, we observed that the CAG <19 alleles were almost

absent from the excryptorchidic cases. In addition, the frequency comparison of each CAG allele showed a statistical difference in the allele CAG 21 when we compared idiopathic cases, excryptorchidic cases, and controls ($P = .015$ by χ^2 test), showing a higher proportion of the CAG 21 allele in the idiopathic cases compared to controls (26% vs 11%, odds ratio [OR] = 2.99, 95% confidence interval [CI], 1.27–7.0), which was statistically different when the Bonferroni correction was applied ($P = .012$).

We observed that the GGN 23 was the predominant allele in controls and also in idiopathic cases (65%, $P = .002$, and 62%, $P = .028$, respectively, χ^2 test), and GGN 24 was the second most common allele in both groups (33% and 38% respectively, $P < .001$, χ^2 test; B in Figure). In contrast, patients with a history of cryptorchidism had an inverse relation of these alleles (GGN 23 vs GGN 24, $P = .048$, χ^2 test), showing a lower proportion of the GGN 23 allele (42%). The distribution of GGN alleles was statistically different among idiopathic cases, excryptorchidic cases, and controls by χ^2 test ($P = .017$). However, no statistical significance was found after applying the Bonferroni test ($P > .05$).

In Table 3 we show the joint distribution of alleles CAG and GGN in each subgroup of subjects. We analyzed the different types of combinations of CAG and GGN, considering the less frequent alleles as single categories (CAG ≤ 20 and ≥ 24; GGN ≤ 22). Because the GGN 25 allele was absent from the excryptorchidic cases and was detected only once in the other groups, it

Table 2. *CAG and GGN repeat length in cases and controls*

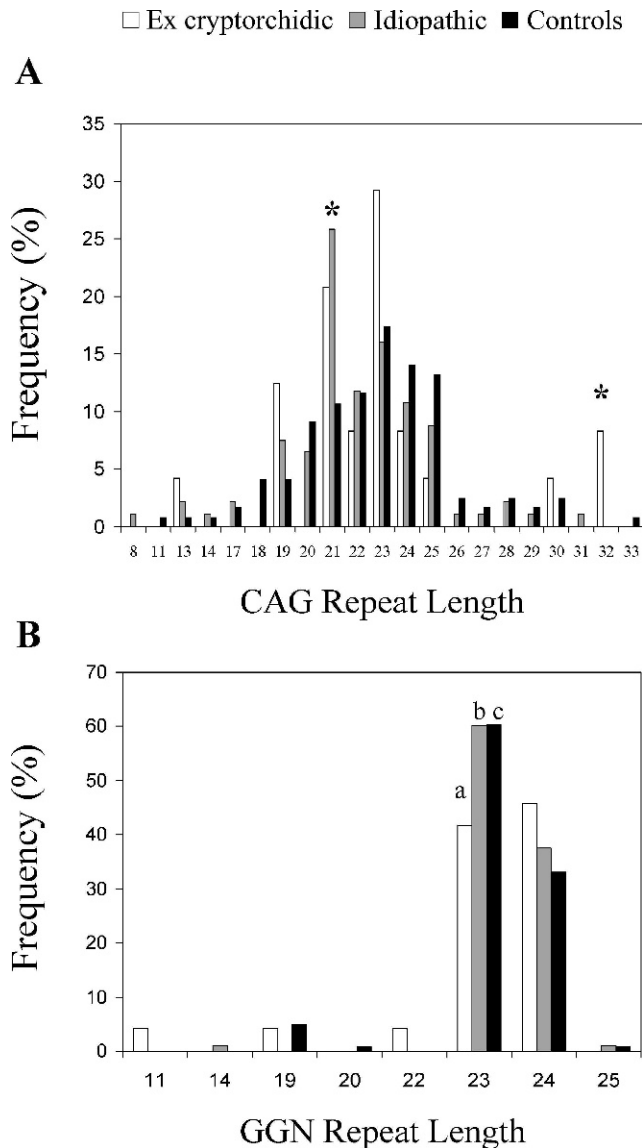|	CAG		GGN	
	Median	Range	Median	Range
Cases				
Excryptorchidic (n = 24)	23	13–32	23	11–24
Idiopathic (n = 93)	22	8–31	23	14–25
Controls (n = 121)	23	11–33	23	19–25

Figure. Bar charts displaying distributions of the CAG **(A)** and GGN repeat lengths **(B)** in controls (n = 121), idiopathic cases (n = 93), and excryptorchidic cases (n = 24). Letter a indicates GGN 24 is the most common allele in excryptorchidic cases; b and c, allele 23 is the most common in idiopathic cases and controls respectively; *, a higher proportion of CAG 21 allele was observed in idiopathic cases compared to controls (P = .006 by Fisher exact test) and CAG 32 was present only in excryptorchidic cases (P = .0002 by Bonferroni test).

was considered in the category GGN ≥ 24. The distribution of the different combinations showed independence (P < .05, χ^2 test) and therefore, there was no association between the 2 repeat polymorphisms in controls, cases with a history of cryptorchidism, and, in a lesser degree, in idiopathic cases (P = .43, P = .12, and P = .092, respectively). However, we observed that the combination CAG = 21/GGN = 23 was higher in idiopathic cases compared with controls (P = .028 by

Bonferroni test); this combination gave about 3-fold greater risk of idiopathic spermatogenic failure, but somewhat less than CAG 21 (OR = 2.89, 95% CI, 1.38–6.01 vs OR = 2.99, 95% CI, 1.27–7.0, respectively). When we compared the different testicular impairments, we observed a statistically higher prevalence of the CAG 21 allele in idiopathic SCOS cases (n = 52) compared to controls (P = .008, 26.9% vs 10.7%, respectively), and found a >3-fold greater risk (OR = 3.06, 95% CI, 1.32–7.09). The combination CAG = 21/GGN = 23 was similar among idiopathic cases with SCOS compared to controls (P = .09).

On the other hand, in excryptorchidic cases we observed a trend for higher prevalence of GGN = 24/CAG > 22, but this difference did not reach statistical significance (P = .16, χ^2 test).

All of the variables studied were similar among obstructive and normozoospermic controls.

Discussion

Normal levels of androgens and a functional receptor are essential for development and maintenance of the male phenotype and for spermatogenesis (Quigley et al, 1995; Hiort and Holterhus, 2000). A number of genetic factors that include chromosomal aberrations, Y-chromosome microdeletions, mutations in the CFTR gene, and several types of mutations in the AR gene may be responsible for about 15% of infertile men (Vogt, 1998; Foresta, 2001; Ferlin et al, 2006; Bhasin, 2007). To our knowledge, this is the first report of CAG and GGN polymorphisms in a South American group of patients with primary severe spermatogenic failure.

The distribution of CAG and GGN repeats in our cases and controls was within the normal range, and this was consistent with findings in Caucasian populations (Lumbroso et al, 1997; Sasaki et al, 2003; Ferlin et al, 2004; Ruhayel et al, 2004). Even though we did not observe significant differences in the distribution of all CAG or GGN alleles, we observed that CAG 21 was significantly more frequent in idiopathic cases than in controls. Although CAG 21 may be within the normal range, it can be associated with a 3-fold increased risk for idiopathic SCOS (OR = 2.99, 95% CI, 1.27–7.0). However, the mechanism by which this allele seems to increase susceptibility for this severe spermatogenic impairment is not clear.

The number of GGN repeats in idiopathic cases and controls showed that GGN 23 was the predominant allele and GGN 24 was the second most common allele. Conversely, an inverse relation was found in cases with a history of cryptorchidism, where GGN 24 was the prevalent allele compared to GGN 23. Our findings are

Table 3. *Joint distribution of CAG and GGN repeat lengths[a]*

| | GGN | | | | | | | | | | | |
| | Control | | | | Idiopathic | | | | Excryptorchidic | | | |
	≤22	23	≥24	Total	≤22	23	≥24	Total	≤22	23	≥24	Total
CAG												
≤20	2 (1.7)	15 (12.4)	9 (7.4)	26 (21.5)	0 (0)	11 (11.8)	8 (8.6)	19 (20.4)	2 (8.3)	1 (4.2)	1 (4.2)	4 (16.7)
21	1 (0.8)	9 (7.4)	3 (2.5)	13 (10.7)	1 (1.1)	18 (19.4)	5 (5.4)	24 (25.8)	1 (4.2)	3 (12.5)	1 (4.2)	5 (20.8)
22	0 (0)	10 (8.3)	4 (3.3)	14 (11.6)	0 (0)	7 (7.5)	4 (4.3)	11 (11.8)	0 (0)	2 (8.3)	0 (0)	2 (8.3)
23	1 (0.8)	8 (6.6)	12 (9.9)	21 (17.4)	0 (0)	4 (4.3)	11 (11.8)	15 (16.1)	0 (0)	2 (8.3)	5 (0)	7 (29.2)
≥24	3 (2.5)	31 (25.6)	13 (10.7)	47 (38.8)	0 (0)	16 (17.2)	8 (8.6)	24 (25.8)	0 (0)	2 (8.3)	4 (16.7)	6 (25)
Total	7 (5.8)	73 (60.3)	41 (33.9)	121 (100)	1 (1.1)	56 (60.2)	36 (38.7)	93 (100)	3 (12.5)	10 (41.7)	11 (45.8)	24 (100)

[a] Data are expressed as No. (%) of subjects.

similar to those of Aschim et al (2004), who found the same relationship in a similar group of Swedish excryptorchidic men compared to controls. In vitro characterization has showed a lower transactivating capacity for the GGN 24 allele and GGN 27 or GGN 10, compared to GGN 23, with a constant CAG repeat number of CAG 22, in response to testosterone analogs (R1881) and DHT (Lundin et al, 2007). Therefore, our results and those mentioned above suggest that the GGN 24 allele can increase susceptibility to cryptorchidism and infertility. In order to obtain more conclusive results, however, more patients with primary testiculopathies and a history of cryptorchidism should be studied. We were not able to assess the contribution of cryptorchidism to spermatogenic damage, because our subjects underwent orchidopexy at a relatively late age.

Recently, Foresta et al (2008) reviewed the role of genetic, hormonal, and environmental factors regarding human cryptorchidism. Evidence of possible genetic causes includes chromosomal alterations or mutations in insulinlike factor 3 (INSL3), INSL3 receptor (also known as RXFP2 or LGR8), and AR gene (Ferlin et al, 2008; Foresta et al, 2008). The first transabdominal phase of testicular descent is essentially INSL3-dependent. The role of AR in normal testis descent is related to the second phase of a 2-step process, the inguinoscrotal phase, in which testes move from the inguinal region to the scrotum. However, it has been suggested that the involvement of AR point mutations in isolated cryptorchidism is unclear (Ferlin et al, 2008; Foresta et al, 2008).

Genetic alterations, including mutations in the INSL3 receptor and Klinefelter syndrome, have been associated with bilateral persistent cryptorchidism and with progressive testicular damage, whereas early orchidopexy may reduce the risk for these sequelae (Ferlin et al, 2008). Likewise, studies regarding CAG polymorphisms and alterations in the AR gene are not associated with idiopathic azoospermia (Sasagawa et al, 2001) or cryptorchidism (Sasagawa et al, 2000), and the combined contribution of both polymorphisms has been poorly studied.

In this report, patients with a history of cryptorchidism showed a trend for a higher proportion of the combination GGN 24/CAG > 22. This may be explained because, besides a reduced transactivating capacity of the GGN 24 allele, CAG alleles above 22 have shown decreased in vivo and in vitro transactivation (La Spada et al, 1991; Tut et al, 1997).

One report by other authors (Ferlin et al, 2005) comparing cryptorchidic patients, with or without spermatogenic damage, and normal fertile men have found that 2 CAG/GGN haplotypes (CAG = 21/GGN = 24 and CAG ≥ 21/ GGN ≥ 24) were more frequent in men with bilateral cryptorchidism (with and without spermatogenic impairment), who frequently had severe spermatogenic failure. In another report from the same authors (Ferlin et al, 2004), they studied men with idiopathic infertility and observed that the CAG = 21/ GGN = 24 combination appeared to increase susceptibility to infertility. In those studies the combination CAG = 21/GGN = 24 was associated with a higher risk for both idiopathic spermatogenic impairment (21 of 163 cases vs 6 of 115 controls; OR = 2.7, 95% CI, 1.05–6.9) and cryptorchidism (8 of 50 cases vs 6 of 115 controls; OR = 3.4, 95% CI, 1.13–10.6). In contrast, our study showed that allele CAG = 21 per se was associated with a 3-fold greater risk for idiopathic SCOS (OR = 3.06, 95% 95% CI, 1.32–7.09) and not for spermatogenic impairment in excryptorchidic cases.

The possible implication of the CAG 21 allele on AR activity, which could be related to severe spermatogenic impairment and infertility in our patients, is not clear. In this regard, no reports had documented an increased frequency for this allele in patients with idiopathic SCOS. Several in vitro analyses to determine the effect of CAG length in AR transcriptional activity have been reported in the literature, with most of them showing that a progressive expansion of the CAG repeat in

human AR caused a linear decrease of transactivation function. However, none of them determined the effect of CAG = 21. Tut et al (1997) compared the effect of CAG = 15, CAG = 20, and CAG = 31, determining that CAG = 20 had a mean activity between CAG = 15 (high activity) and CAG = 31 (lower activity), whereas Beilin et al (2000) compared the effect of CAG = 15, CAG = 24, and CAG = 31, and observed similar results, because CAG = 24 had a mean activity between CAG = 15 (high activity) and CAG = 31 (lower activity). These results would indicate that CAG = 21 probably does not have a transcriptional activity very different from that of other similar alleles. However, a recent study (Nenonen et al, 2010), investigated different CAG lengths in the normal range (16, 22, and 28) together with the GGN 23 allele observed that the highest AR activity was confined to CAG = 22 and not to CAG = 16, suggesting that subtle differences in the number of CAG repeats close to CAG = 21 can produce differences in transcriptional activity of the AR.

On the other hand, our results may be chance findings that may not allow firm conclusions regarding the biological importance of these combinations in men with spermatogenic defects. Therefore, we suggest that further studies of these polymorphisms should be performed, including in vitro transactivation studies using appropriated models for different tissues. In this regard it has been reported that some AR mutations observed in infertile patients showed a diminished transactivational response using extensive analysis with relevant in vitro systems, in particular with the PEM promoter (Zuccarello et al, 2008).

Even though we studied a relatively small number of patients with a history of cryptorchidism, our findings distinguished 2 different types of patients, excryptorchidic and those with idiopathic spermatogenic impairment. We observed a higher prevalence of CAG 21 in idiopathic cases and an inverse relation of the GGN 23 and GGN 24 in excryptorchidic cases. Moreover, we performed a detailed biopsy analysis in most of our patients that allowed us to select only subjects with severe spermatogenic impairment, finding a higher prevalence of the CAG 21 allele among idiopathic infertile patients with SCOS.

In summary, we suggest that the CAG 21 allele seems to increase the susceptibility for idiopathic SCOS, and the GGN 24 allele may contribute to deranged AR function, associated with cryptorchidism and spermatogenic failure.

Acknowledgments

References

Aschim EL, Saether T, Wiger R, Grotmol T, Haugen TB. Differential distribution of splice variants of estrogen receptor beta in human testicular cells suggests specific functions in spermatogenesis. *J Steroid Biochem Mol Biol.* 2004;92:97–106.

Beilin J, Ball EM, Favaloro JM, Zajac JD. Effect of the androgen receptor CAG repeat polymorphism on transcriptional activity: specificity in prostate and non-prostate cell lines. *J Mol Endocrinol.* 2000;25:85–96.

Bhasin S. Approach to the infertile man. *J Clin Endocrinol Metab.* 2007;92:1995–2004.

Castro A, Codner E, Kaune H, Lopez P, Vantman D, Cassorla F. Absence of Y chromosome microdeletions in patients with cryptorchidism and hypospadias. *J Pediatr Endocrinol Metab.* 2004;17:143–148.

Crabbe P, Bogaert V, De Bacquer D, Goemaere S, Zmierczak H, Kaufman JM. Part of the interindividual variation in serum testosterone levels in healthy men reflects differences in androgen sensitivity and feedback set point: contribution of the androgen receptor polyglutamine tract polymorphism. *J Clin Endocrinol Metab.* 2007;92:3604–3610.

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.

Davis-Dao CA, Tuazon ED, Sokol RZ, Cortessis VK. Male infertility and variation in CAG repeat length in the androgen receptor gene: a meta-analysis. *J Clin Endocrinol Metab.* 2007;92: 4319–4326.

Dejager S, Bry-Gauillard H, Bruckert E, Eymard B, Salachas F, LeGuern E, Tardieu S, Chadarevian R, Giral P, Turpin G. A comprehensive endocrine description of Kennedy's disease revealing androgen insensitivity linked to CAG repeat length. *J Clin Endocrinol Metab.* 2002;87:3893–3901.

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.

Ferlin A, Arredi B, Foresta C. Genetic causes of male infertility. *Reprod Toxicol.* 2006;22:133–141.

Ferlin A, Arredi B, Speltra E, Cazzadore C, Selice R, Garolla A, Lenzi A, Foresta C. Molecular and clinical characterization of Y chromosome microdeletions in infertile men: a 10-year experience in Italy. *J Clin Endocrinol Metab.* 2007;92:762–770.

Ferlin A, Bartoloni L, Rizzo G, Roverato A, Garolla A, Foresta C. Androgen receptor gene CAG and GGC repeat lengths in idiopathic male infertility. *Mol Hum Reprod.* 2004;10:417–421.

Ferlin A, Garolla A, Bettella A, Bartoloni L, Vinanzi C, Roverato A, Foresta C. Androgen receptor gene CAG and GGC repeat lengths in cryptorchidism. *Eur J Endocrinol.* 2005;152: 419–425.

Ferlin A, Zuccarello D, Zuccarello B, Chirico MR, Zanon GF, Foresta C. Genetic alterations associated with cryptorchidism. *JAMA.* 2008;300:2271–2276.

Foresta C. Y chromosome microdeletions and alterations of spermatogenesis. *Endocr Rev.* 2001;22:226–239.

Foresta C, Ferlin A, Garolla A, Moro E, Pistorello M, Barbaux S, Rossato M. High frequency of well-defined Y-chromosome deletions in idiopathic Sertoli cell-only syndrome. *Hum Reprod.* 1998;13:302–307.

Foresta C, Moro E, Ferlin A. Y chromosome microdeletions and alterations of spermatogenesis. *Endocr Rev.* 2001;22:226–239.

Foresta C, Zuccarello D, Garolla A, Ferlin A. Role of hormones, genes, and environment in human cryptorchidism. *Endocr Rev*. 2008;29:560–580.

Hiort O, Holterhus PM. The molecular basis of male sexual differentiation. *Eur J Endocrinol*. 2000;142:101–110.

Hiort O, Holterhus PM, Horter T, Schulze W, Kremke B, Bals-Pratsch M, Sinnecker GH, Kruse K. Significance of mutations in the androgen receptor gene in males with idiopathic infertility. *J Clin Endocrinol Metab*. 2000;85:2810–2815.

Jezek D, Knuth UA, Schulze W. Successful testicular sperm extraction (TESE) in spite of high serum follicle stimulating hormone and azoospermia: correlation between testicular morphology, TESE results, semen analysis and serum hormone values in 103 infertile men. *Hum Reprod*. 1998;13:1230–1234.

Johnsen SG. Testicular biopsy score count—a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. *Hormones*. 1970;1:2–25.

Krausz C, Bussani-Mastellone C, Granchi S, McElreavey K, Scarselli G, Forti G. Screening for microdeletions of Y chromosome genes in patients undergoing intracytoplasmic sperm injection. *Hum Reprod*. 1999;14:1717–1721.

Krausz C, Giachini C. Genetic risk factors in male infertility. *Arch Androl*. 2007;53:125–133.

Kruger TF, Ackerman SB, Simmons KF, Swanson RJ, Brugo SS, Acosta AA. A quick, reliable staining technique for human sperm morphology. *Arch Androl*. 1987;18:275–277.

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

Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS. Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. *Proc Natl Acad Sci U S A*. 1989;86:9534–9538.

Lumbroso R, Beitel LK, Vasiliou DM, Trifiro MA, Pinsky L. Codon-usage variants in the polymorphic (GGN)n trinucleotide repeat of the human androgen receptor gene. *Hum Genet*. 1997;101:43–46.

Lundin KB, Giwercman A, Dizeyi N, Giwercman YL. Functional in vitro characterisation of the androgen receptor GGN polymorphism. *Mol Cell Endocrinol*. 2007;264:184–187.

Lundin KB, Giwercman A, Richthoff J, Abrahamsson PA, Giwercman YL. No association between mutations in the human androgen receptor GGN repeat and inter-sex conditions. *Mol Hum Reprod*. 2003;9:375–379.

Martinez-Garza SG, Gallegos-Rivas MC, Vargas-Maciel M, Rubio-Rubio JM, de Los Monteros-Rodriguez ME, Gonzalez-Ortega C, Cancino-Villarreal P, de Lara LG, Gutierrez-Gutierrez AM. Genetic screening in infertile Mexican men: chromosomal abnormalities, Y chromosome deletions, and androgen receptor CAG repeat length. *J Androl*. 2008;29:654–660.

Mifsud A, Sim CK, Boettger-Tong H, Moreira S, Lamb DJ, Lipshultz LI, Yong EL. Trinucleotide (CAG) repeat polymorphisms in the androgen receptor gene: molecular markers of risk for male infertility. *Fertil Steril*. 2001;75:275–281.

Nenonen H, Björk C, Skjaerpe PA, Giwercman A, Rylander L, Svartberg J, Giwercman YL. CAG repeat number is not inversely associated with androgen receptor activity in vitro. *Mol Hum Reprod*. 2010;16:153–157.

Palazzolo I, Gliozzi A, Rusmini P, Sau D, Crippa V, Simonini F, Onesto E, Bolzoni E, Poletti A. The role of the polyglutamine tract in androgen receptor. *J Steroid Biochem Mol Biol*. 2008; 108:245–253.

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.

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.

Ruhayel Y, Lundin K, Giwercman Y, Hallden C, Willen M, Giwercman A. Androgen receptor gene GGN and CAG polymorphisms among severely oligozoospermic and azoospermic Swedish men. *Hum Reprod*. 2004;19:2076–2083.

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. *Mol Hum Reprod*. 2000;6:973–975.

Sasaki M, Kaneuchi M, Sakuragi N, Fujimoto S, Carroll PR, Dahiya R. The polyglycine and polyglutamine repeats in the androgen receptor gene in Japanese and Caucasian populations. *Biochem Biophys Res Commun*. 2003;312:1244–1247.

Staden R. The Staden sequence analysis package. *Mol Biotechnol*. 1996;5:233–241.

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.

Vogt P. Human chromosome deletions in Yq11, AZF candidate genes and male infertility: history and update. *Mol Hum Reprod*. 1998;4:739–744.

von Eckardstein S, Syska A, Gromoll J, Kamischke A, Simoni M, Nieschlag E. Inverse correlation between sperm concentration and number of androgen receptor CAG repeats in normal men. *J Clin Endocrinol Metab*. 2001;86:2585–2590.

Wallerand H, Remy-Martin A, Chabannes E, Bermont L, Adessi GL, Bittard H. Relationship between expansion of the CAG repeat in exon 1 of the androgen receptor gene and idiopathic male infertility. *Fertil Steril*. 2001;76:769–774.

Westerveld H, Visser L, Tanck M, van der Veen F, Repping S. CAG repeat length variation in the androgen receptor gene is not associated with spermatogenic failure. *Fertil Steril*. 2008;89: 253–259.

World Health Organization. *Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*. 4th ed. Cambridge, United Kingdom: Cambridge University Press; 1999.

Zuccarello D, Ferlin A, Vinanzi C, Prana E, Garolla A, Callewaert L, Claessens F, Brinkmann AO, Foresta C. Detailed functional studies on androgen receptor mild mutations demonstrate their association with male infertility. *Clin Endocrinol (Oxf)*. 2008;68: 580–588.