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Association of Long Polyglycine Tracts (GGN Repeats) in Exon 1 of the Androgen Receptor Gene With Cryptorchidism and Penile Hypospadias in Iranian Patients

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ABSTRACT: Hypospadias (a urethral orifice located along the ventral side of the penis) and cryptorchidism (failure of the testes to descend into the scrotal sacs) are the 2 most common congenital malformations in males, affecting 0.3%–0.7% and 2%–4%, respectively, at birth. To study the association of CAG/GGN trinucleotide repeats in the androgen receptor gene with cryptorchidism and hypospadias in an Iranian population, we performed a case-control study of 76 cryptorchid and 92 hypospadiac (divided into subgroups of glanular, penile, and penoscrotal hypospadias) Iranian males. The length of the CAG/GGN repeat segment was evaluated by using polymerase chain reaction (PCR) sequencing in exon 1 and PCR–single-strand conformation polymorphism (PCR-SSCP) in exons 2–8. There were no significant differences in CAG lengths between the cases and controls, but GGN numbers were found to be significantly higher (median, 24 vs 22) among both subjects with penile hypospadias (P = .018) and those with a history of cryptorchidism (P = .001) compared with controls. In addition, the GGN numbers among subjects with penile hypospadias were significantly different compared with the 2 other subgroups of hypospadias (P = .001). We were able to identify 12 different CAG alleles and 8 different GGN alleles in the cryptorchid group. Although further studies are needed to elucidate the possible role of specific CAG/GGC combinations as a susceptible factor, our data suggested the possible association between polyglycin tract polymorphism in androgen receptor gene and cryptorchidism.

Key words: CAG repeats, testicular dysgenesis syndrome, molecular diagnosis.

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Hypospadias (a urethral orifice located along the ventral side of the penis) and cryptorchidism are the 2 most common congenital malformations in males, affecting 0.3%–0.7% and 2%–4%, respectively, at birth (Sharpe, 2003). The etiology of cryptorchidism is probably multifactorial, related to extrinsic (extragonadal) or intrinsic (gonadal) causes disrupting testicular descent (Foresta et al, 1996). In patients with hypospadias, the phenotype ranges from a slight anomaly in which the urethral orifice is on the ventral side of the glans (glanular hypospadias) via a penile form to a third subtype (penoscrotal hypospadias) in which the orifice is in the perineum, often combined with a curvature of the penile shaft (chordee) (Sadler and Langman, 2004).

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Cryptorchidism and hypospadias are together with testicular cancer and low sperm quality considered to be features of the so-called testicular dysgenesis syndrome (TDS) (Skakkebæk et al, 2001). The etiology of the clinical components of the TDS remains unknown in most cases but is believed to be multifactorial and at least partly due to a genetic predisposition combined with a certain adverse hormonal milieu of the fetus.

The presence of androgens and androgen receptor (AR) together with insulin-like peptide 3 (INSL3) are thought to be important in testicular descent, inducing the involution of the cranial suspensory ligament and the second migration step from the groin to the scrotum (transinguinal descent) (Ivell and Hartung, 2003). The human *AR* gene is located on chromosome Xq11-12 (Lubahn et al, 1988). This gene exhibits 2 polymorphic sites in exon 1 characterized by different numbers of CAG and GGN repeats. The AR is highly polymorphic due to a glutamine repeat encoded by (CAG)nCAA and a glycine repeat encoded by (GGT)3(GGG)(GGT)2(GGC)n. Abnormal expansion of the CAG segment to 44 repeats, which is known to reduce AR function both in vivo and in vitro (La Spada

Table 1. *Seminal and hormonal analysis of patients and fertile controls*

Study Groups	No.	Seminal Pattern (× 10^6 mL)	Hormone Concentration (mean ± SE [range])		
			Luteinizing Hormone (IU/L)	Follicle-Stimulating Hormone (IU/L)	Testostrone (nmol/L)
Cryptorchidism	76	2.9 ± 0.9	5.8 ± 0.9 (1.9–6.4)	15.3 ± 1.7* (7.2–17.1)	13.5 ± 1.8 (8.1–14.9)
Bilateral	49	1.4 ± 0.7	4.7 ± 0.7 (2.3–5.1)	13.7 ± 0.8 (7.2–16.8)	14.1 ± 0.7 (10.3–14.8)
Unilateral	27	3.2 ± 1.1	5.9 ± 1.1 (1.9–6.4)	14.9 ± 1.1 (8.3–17.1)	13.1 ± 2.4 (8.1–14.9)
Hypospadias	92	11.9 ± 1.8	5.7 ± 0.6 (1.7–6.2)	15.3 ± 2.2* (5.9–18.1)	14.5 ± 2.3 (7.6–16.8)
Glaular hypospadias	42	7.5 ± 0.7	4.5 ± 0.7 (2.0–5.3)	14.3 ± 1.7 (6.3–15.5)	12.7 ± 3.4 (7.6–16.8)
Penile hypospadias	19	10.4 ± 2.1	5.2 ± 1.3 (1.7–6.2)	15.6 ± 2.2 (7.2–18.1)	14.9 ± 0.9 (8.5–15.9)
Penoscrotal hypospadias	31	12.3 ± 2.7	5.8 ± 0.8 (2.2–5.9)	14.7 ± 1.1 (5.9–16.7)	12.3 ± 1.1 (7.8–14.7)
Healthy fertile controls	190	42.6 ± 3.4	5.3 ± 1.3 (1.5–6.1)	5.5 ± 0.7 (2.1–6.9)	16.7 ± 2.6 (12.1–20.5)

* Statistically different from fertile controls ($P < .05$).

et al, 1991; Tut et al, 1997), has been found in 1 patient with hypospadias (Ogata et al, 2001). A more modest expansion of CAG repeat lengths, although within the normal range (approximately 10–30), has also previously been reported in 78 males with varying undermasculinization, including hypospadias (Lim et al, 2000). The CAG repeat length has also been assessed in males with cryptorchidism, but no association between CAG repeat length and undescended testes has been found (Sasagawa et al, 2000; Lim et al, 2001). Although the polymorphic GGN region of the *AR* also plays a role in the receptor function (Gao et al, 1996), studies on this polymorphism in relation to hypospadias or cryptorchidism are still not clear.

The purpose of this study was to examine the association between cryptorchidism or hypospadias with polymorphisms in the *AR* gene involved in the androgen pathway, the CAG/GGN repeat length polymorphisms. Therefore, we conducted a case-control study of 76 cryptorchid and 92 hypospadiac patients. To eliminate other mutations in the *AR* gene that might be implicated in cryptorchidism and hypospadias, exons 2–8 of the *AR* gene were screened.

Materials and Methods

Cases

Blood samples were collected from 76 unrelated Iranian males with cryptorchidism and 92 males with hypospadias. A complete medical history and a physical examination were undertaken. Men who reported spontaneous descent of the testes were excluded. As control subjects, we studied 190 infertile males from the general population of Iran who had fathered at least 1 child by natural conception. Semen parameters were evaluated according to published recommendations (World Health Organization, 1999). Semen analysis of the patients was performed at least twice at 4- to 8-week intervals after at least 2 days of abstinence and the mean result recorded. The 76 cryptorchid patients were divided into 2 subgroups (bilateral and unilateral), and 92 hypospadiac patients were divided into 3 subtypes according to the position of the urethral orifice and degree of chordee. The classifications were glanular, penile, and penoscrotal hypospadias. Ultrasound examination of the testes (for testis morphology and volume) and plasma determination of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone concentrations (Table 1) were conducted. Exclusion of known causes of male infertility was done by careful history (excluding, for example, varicocele, orchitis, and testicular trauma), endocrine profile (excluding, for example, hypogonadotrophic hypogonadism and hyperprolactinemia), karyotype analysis, Y chromosome microdeletion analysis (Vogt et al, 1996; Lahn and Page, 1997; Peterlin et al, 2002), cystic fibrosis transmembrane regulator (CFTR) gene mutation analysis (Radpour et al, 2006), and INSL3/LGR8 gene mutation analysis (Ferlin et al, 2003).

Genotyping of CAG/GGN Trinucleotide Repeats

The genomic DNA samples of all the subjects were prepared from peripheral blood lymphocytes according to standard protocols, and CAG/GGN repeat motifs of exon 1 in the *AR* gene were determined by analyzing the size of a polymerase chain reaction (PCR) product containing the polymorphic microsatellites. The *AR* exon 1 was amplified from genomic DNA in 2 different PCR reactions, giving overlapping amplicons by previously reported primers A2, A5, A8, and A10 (Lubahn et al, 1989) and primers A0 and A3n (Ferlin et al, 2005). Both reactions were performed with the same cycle (94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, repeated 37 times). The CAG repeat was amplified with the primers A0 and A5 (Table 2). The GGN repeat is amplified with the primers A3 and A10 (Table 2). The reaction components for each PCR consisted of a total volume of 50-µL reaction mixture containing 100 ng DNA, PCR buffer (Applied Biosystems, Norwalk, CT), 1.5 mM $MgCl_2$ (Applied Biosystems), 200 µmol deoxynucleotide triphosphate (Roche Diagnostics, Vienna, Austria), 20 pmol of each primer, and 1.5 units of *Taq* Polymerase (Applied Biosystems).

Sequencing was carried out by using A2 for CAG and A8 (Table 2) for GGN repeats by VBC-Genomics (VBC-

Table 2. *Sequences and polymerase chain reaction (PCR) parameters of primers used to amplify* AR *exons*

Exon No.	Sequencing and PCR Primers: Forward Primer (5′ → 3′)	Reverse Primer (5′ → 3′)	Annealing Temperature (°C)	Product Size (bp)
1	A0: GTGGTTGCTCCCGCAAGTTTCC A2: GCTGTGAAGGTTGCTGTTCCTC	A5: GCTCCCACTTCCTCCAAGGACAATTA	59	...
	A3: CAGCAAGAGACTAGCCCCAG A8: GGACTGGGATAGGGCACTCTGCTCAACC	A10: CCAGAACACAGAGTGACTCTGCC	58	...
2	Normal: CCTATTTCTGCCATTCAGTG Mutated: CCTATTTCTGCTATTCAGTG	AGAAAGGGAAAGAGAAGTGC	57	244
3	Normal: TCTGGAAACTCATTATCAG Mutated: TCTGGAAACTAATTATCAG	AGAGGAAGGAGGAGGAAGAG	58	203
4	Normal: TTGTGTGTTTTTGACCACTG Mutated: TTGTGTGTTTTCGACCACTG	CCCACTTCCCTTTTCCTTAC	58	362
5	Normal: TCAGTACCCAGACTGACCAC Mutated: TCAGTACCCAGACTGATCAC	GCCAAGCTGCTGTATTTTAG	57	235
6	Normal: TGTAAACTTCCCCTCATTCC Mutated: TGTAAACTTCCCATCATTCC	TCCTCTCTGAATCTCTGTGC	57	234
7	Normal: CTGTCTTCATCCCACATCAG Mutated: CTGTCTTCATCCCATATCAG	GGCTGTTCTCCCTGATAAAG	58	219
8	Normal: TGTCAACCCTGTTTTTCTCC Mutated: TGTCAACCCTGTTTTTATCC	AAATTCCCCAAGGCACTG	60	298

Table 3. *Androgen receptor CAG/GGN repeats; number of patients and fertile controls*

Study Groups	No. (%)	CAG Repeat: Mean (± SD)	CAG Repeat: Median (range)	GGN Repeat: Mean (± SD)	GGN Repeat: Median (range)
Cryptorchidism	76 (100)	21.9 (2.5)	21 (17–30)	24.5* (2.4)	24 (18–26)
Bilateral	49 (64.5)	22.4 (1.6)	22 (19–30)	24.6* (1.7)	24 (22–26)
Unilateral	27 (35.5)	21.5 (0.8)	21 (17–29)	23.8* (1.6)	23 (18–24)
Hypospadias	92 (100)	22.5 (3.2)	22 (16–29)	22.2 (3.1)	22 (13–25)
Glaular hypospadias	42 (45.7)	22.6 (2.8)	22 (16–27)	21.6 (0.8)	21 (14–23)
Penile hypospadias	19 (20.6)	22.7 (0.9)	23 (19–29)	23.9* (1.9)	23 (18–25)
Penoscrotal hypospadias	31 (33.7)	21.3 (1.6)	21 (17–25)	22.7 (1.6)	22 (13–24)
Healthy fertile controls	190	21.8 (2.8)	21 (16–28)	22.3 (2.1)	22 (11–24)

* Statistically different from fertile controls ($P = .001$).

Genomics Bioscience Research, Vienna, Austria) using 50 ng (2 µL) of PCR product and 4 pM (1 µL) of nonfluorescent primer (forward and reverse separately), 4 µL of BigDye Terminator ready reaction kit (Perkin Elmer, Wellesley, Mass), and 3 µL of double-distilled water to adjust the volume to 10 µL. Genotyping was done blinded to case-control status, and 25% of the samples were randomly repeated for quality control, where no discrepancies were observed. Sequencing results were analyzed with DNASIS MAX software version 2.6 (Hitachi Software Engineering Co, Tokyo, Japan).

AR Gene Point Mutation Screening

The remaining coding sequences of *AR* gene (exons 2–8) were examined by PCR–single-strand conformation polymorphism (PCR-SSCP) using new primers (Table 2) to screen for associated subtle mutations. Each primer was localized in intron sequence, allowing amplification of the corresponding exon coding sequence and the associated intron-exon junctions. A positive control of mutations was artificially created by using a primer containing a point mutation (Table 3). This mutated control was performed for each exon by using DNA of a participant belonging to the control population. For SSCP, the PCR products were heated at 95°C for 10 minutes and placed on ice. Single-strand DNA separation was done by using 12.5% polyacrylamide gels according to the size of the amplified fragments. For each exon, SSCP was performed at 4°C and compared with the mutated control. This was done to ensure that our data were not confounded by exonic mutations that were linked to any particular trinucleotide repeat allele.

Statistical Analysis

Means, standard deviations, and standard errors were calculated for both the patients and healthy fertile groups. The mean number of CAG/GGN repeats from patients (cryptorchidism and hypospadias) was compared with those in healthy fertile controls by 2-sample independent *t*-test (using

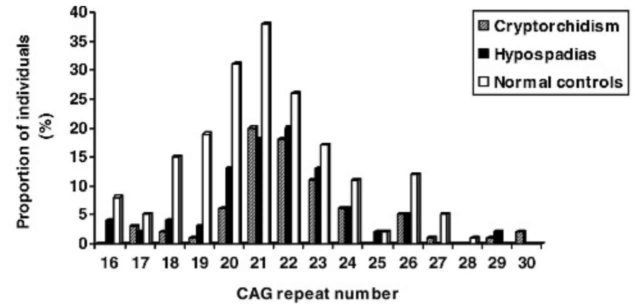
the computer software SPSS for Windows version 13.0; SPSS Inc, Chicago, Ill). Subsequently, multiple comparisons were performed comparing the mean number of CAG/GGN repeats in cryptorchid and hypospadiac patients with fertile controls by analysis of variance. Logistic regression analysis was performed using the number of CAG/GGN repeats as the exposure and clinical infertility as the outcome. All P values were 2-sided; P less than .05 was considered statistically significant.

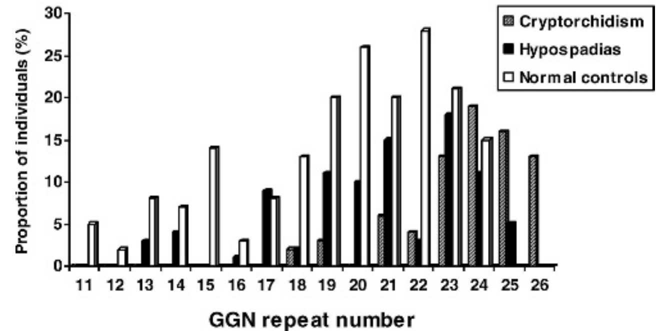
Results

The mean level of FSH in patient groups (cryptorchidism and hypospadias) was significantly higher than in the control group ($P = .012$) (Table 1). In contrast, the mean level of serum LH and testosterone in the patient groups did not significantly different from the control group ($P > .05$) (Table 1). All studied patients were 46,XY males (data not shown). Patients with visible cytogenetic aberrations were excluded from the study. None of the studied patients had *AR* mutations or Y chromosome microdeletions. In the subsequent molecular genetic investigation, the means of the *AR* gene CAG and GGN repeat length in the cryptorchidism and hypospadias were analyzed and compared with CAG/GGN repeat length in the control group (Table 3). The mean number of CAG and GGN repeats in exon 1 of the *AR* gene was 21.8 ± 2.8 (range, 16–28) and 22.3 ± 2.1 (range, 11–24) respectively, in proven healthy fertile controls, 21.9 ± 2.5 (range, 17–30) and 24.5 ± 2.4 (range, 18–26) in cryptorchid males, and 22.5 ± 3.2 (range, 16–29) and 22.2 ± 3.1 (range, 13–25) in males with hypospadias (Table 3).

GGN numbers were found to be significantly higher (median, 24 vs 22; Table 3) among both subjects with penile hypospadias ($P = .018$) and those with a history of cryptorchidism ($P = .001$) compared with controls. In addition, the GGN numbers among subjects with penile hypospadias were significantly different compared with the 2 other subgroups of hypospadias combined ($P = .001$). The highest mean length of the *AR* gene GGN number in patients was observed in the cryptorchid group (24.5), but there were no significant differences between the 2 subgroups (bilateral and unilateral) (Table 3). In addition, the frequency of having a GGN repeat number (at least 22) was 85.5% in healthy infertile men and 33.7% in fertile controls ($P = .001$) (Figure). Also, our findings showed no significant differences between CAG repeat numbers in patients and control groups.

The distribution of GGN allele frequencies (Figure) was different between cryptorchid men and controls, and there was an apparent trend toward a shift to





Distribution of CAG and GGN repeat sizes in exon 1 of the *AR* gene in fertile controls and patient males (cryptorchidism and hypospadias).

a GGN of 22 or a GGN of more than 22 in males with cryptorchidism, but no significant difference was observed with the hypospadiac group with respect to controls. We were able to identify 12 different CAG alleles and 8 different GGN alleles in the cryptorchid group, with a range of 17–30 CAG repeats corresponding and a range of 18–26 GGN repeats. In the hypospadiac group, the CAG repeat range was 16–29 and the GGN repeat range was 13–25. The most common alleles in the cryptorchid group consisted of 24 GGN repeats (25.1%) corresponding to 24 glycine residues and 23 GGN repeats (19.6%) corresponding to 23 glycine residues in the hypospadiac group, whereas the most common alleles in fertile control group consisted of 22 GGN repeats (14.8%) corresponding to 22 glycine residues.

In summary, we report that the mean GGN repeat length increased with the cryptorchidism and penile hypospadias. However, statistical analysis showed no significant differences between the cryptorchidism subgroups (bilateral or unilateral) ($P > .05$).

Discussion

Although there are various hypotheses about the mechanisms responsible for testicular descent, it is generally accepted that endocrine factors, especially

androgens, play a major role in promoting the descent of testes into the scrotum (Heyns and Huston, 1995). The possible genetic background of hypospadias and cryptorchidism still remains unresolved. In this study we analyzed CAG and GGC repeat lengths in males with a history of cryptorchidism and hypospadias, associated or not with impairment of sperm production, in comparison with healthy fertile subjects. The results from analyses of reproductive hormones with elevated LH and FSH levels and normal or low levels of testosterone suggested partial impairment of testicular function.

We found a significant increase (P = .001) in the mean length of the GGN trenucleotide repeat (24.5 ± 2.4) in cryptorchid males and males with penile hypospadias (23.9 ± 1.9) compared with healthy fertile controls (22.3 ± 2.1). Whereas a GGN length of 22 is the most prevalent in men from the general population, most individuals with a history of cryptorchidism presented with GGN numbers of 24 or more. The same allele distribution was also found in patients treated for penile hypospadias. Until now, few studies have examined the CAG repeat length in men with cryptorchidism (Sasagawa et al, 2000; Lim et al, 2001; Aschim et al, 2004; Ferlin et al, 2005), and they found no difference with respect to controls. The functional consequences of variations in the GGN repeat are even less clear, and epidemiologic investigations of the association between the number of GGN repeats and prostate cancer risk (Hakimi et al, 1997; Irvine et al, 1997) or male infertility (Tut et al, 1997; Lundin et al, 2003; Ferlin et al, 2004) produced inconsistent results. However, we showed that an increased number of GGN repeats significantly linked with cryptorchidism and some forms of hypospadias, and our findings confirmed previous data (Aschim et al, 2004), but these data should be further confirmed in other populations. This finding is in agreement with the available data on the embryologic development of external male genital organs.

Androgens are known to play a crucial role for the development of male external genital organs as well as the testicular descent, and previous studies have pointed to partly shared etiology of these 2 conditions (Akre et al, 1999). The finding of an identical switch in the GGN repeat lengths in patients with penile hypospadias as well as in those with a history of cryptorchidism indicates that our results point to true biological associations rather than being chance findings. Despite the alteration in the distribution of the GGN lengths among subjects, they were still within the range found in controls. Therefore, it can be hypothesized that any increase in GGN length above the most common length of 22 causes a slight impairment of AR function, which, combined with insufficient function of the Leydig cells and/or increased exposure to environmentally derived antiandrogens, leads to undervirilization of male genital organs. The significance of the GGN repeats on the function of the AR has been demonstrated previously (Gao et al, 1996).

Generally, the estimated effects of CAG or GGN repeats on infertility risk in most of studies are relatively small and reached significance only in subgroups with higher grade of disease. But different analyses make these studies difficult to compare. Additionally, the studied populations of these studies vary in size. Another point that must be considered is the population heterogeneity, where disease incidence varies with ethnic background. In this Iranian population study, this problem can be excluded because the study population consisted exclusively of Iranian males. Furthermore, the selection of a control group is a critical point for the final outcome of molecular epidemiologic studies. In our study, the control group consisted of fertile men with a normal range of sperm count.

In this case-control study, our data provides an association between cryptorchidism and GGN repeat length and confirmed 1 similar study suggested that the chance to find an association between the polyglycine tract length and the cryptorchidism in a given population. It is obvious that studies from a variety of different ethnic and genetic backgrounds using comparable patient subgroups are extremely valuable to further evaluate this association. The stability of the CAG/GGN repeats is still unknown. It has been shown that about 5% of daughters conceived through intracytoplasmic sperm injection (ICSI) have an inherited *AR* allele with either contraction or expansion up to 8 bp (Cram et al, 2000). Because expansions of CAG or GGN repeats can be deleterious, it should be further considered in ICSI candidates for cryptorchidism, hypospadias, or idiopathic male infertility. The possible relationship of specific CAG/GGN haplotypes to cryptorchidism suggests that some combinations of CAG and GGN may modulate *AR* function, but this needs to be verified in other studies.

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