Androgen receptor gene CAG and GGC repeat lengths in cryptorchidism

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Abstract

Objective: Cryptorchidism is the most common congenital birth defect in male children, and accumulating evidence suggests that genetic abnormalities may be associated with it. The androgen receptor has two polymorphic sites in exon 1, with different numbers of CAG and GGC repeats, resulting in variable lengths of polyglutamine and polyglycine stretches. Longer CAG repeats result in a reduced androgen receptor transcriptional activity, but the role of the GGC triplets is less clear. In this study we analysed CAG and GGC repeat lengths in men with a history of cryptorchidism, associated or not with impairment of sperm production, in comparison with normal fertile subjects.

Methods: We analysed CAG and GGC repeat lengths in a group of 105 ex-cryptorchid men in comparison with 115 fertile non-cryptorchid men.

Results: No difference was found between patients and controls in the mean and median values, and in distribution of CAG and GGC, when considered separately. However, the analysis of the joint distribution of CAG and GGC showed that some combinations are significantly more frequent in men with bilateral cryptorchidism (who frequently presented severe testiculopathies), in a manner similar to that found in idiopathic infertile subjects.

Conclusions: Although further studies are needed to elucidate the possible role of specific CAG/GGC combinations as a causative factor, these data suggest a possible association between androgen receptor gene polymorphisms and cryptorchidism.

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Introduction

Cryptorchidism is the failure of the testes to descend into the scrotal sacs; it is the most common congenital birth defect in boys. Although its frequency may vary among different countries (1), a figure of 2–4% in full-term male births is generally accepted (2). The aetiology of cryptorchidism remains unknown for a large proportion of cases, reflecting our limited knowledge of the mechanisms regulating testicular descent from abdomen to scrotum during embryonic development. A growing body of evidence suggests that genetic abnormalities may be associated with cryptorchidism (3), such as mutations in the INSL3/LGR8 hormonal system (4–14). Mutations in the androgen receptor (AR) gene, causing the androgen insensitivity syndrome, are known to be associated with variable development of the Wolffian duct and with micropenis, hypospadia and cryptorchidism (15, 16). However, screening for mutations in the AR gene in patients with isolated cryptorchidism failed to find any abnormality (17, 18), even if clear conclusions cannot be drawn from the limited number of subjects studied. Nevertheless, androgens, together with Mullerian inhibiting substance (MIS) and 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) (3). In fact, the critical role of androgens in testicular descent is supported by numerous clinical and animal evidence. The AR is a member of the steroid/nuclear receptor superfamily of ligand-activated transactivation factors, and it is encoded by a gene located on chromosome Xq11-12 (19). The gene exhibits two polymorphic sites in exon 1, characterized by different numbers of CAG and GGC repeats, resulting in variable lengths of polyglutamine and polyglycine stretches in the N-terminal region of the AR protein. Longer CAG repeats result in reduced AR transcriptional activity (20, 21), and there is evidence that an inverse correlation between CAG number and androgenicity exists. Consistent with this, expansion of the tract to > 40 CAG repeats results in Kennedy’s syndrome, a rare motoneuron disorder also characterized
by low masculinization, testicular atrophy, reduced sperm production and infertility (22, 23). However, cryptorchidism is not a major feature of Kennedy’s syndrome; therefore, the role of CAG expansion in cryptorchidism is not clear. On the other hand, shorter AR polyglutamine tracts have been associated with increased prostate cancer risk (24–30), but this is still controversial. Although polymorphisms in CAG tract length correlate with sperm concentration in normal men (31), numerous studies examining CAG repeats in infertile men have reported conflicting results only in part justifiable by ethnicity, some (32–38) showing no expansion, and others (39–46) reporting increased length (but still in the normal range) with respect to fertile control men. To the best of our knowledge, only two studies have examined the CAG repeat length in men with cryptorchidism (47, 48), and they found no difference with respect to controls. The functional consequences of variations in the GGC repeat are even less clear, and epidemiological investigations of the association between the number of GGC repeats and prostate cancer risk (25, 27, 49–51) or male infertility (38, 39, 52) produced inconsistent results. However, we have recently shown that, instead of the crude number of CAG or GGC triplets examined separately, the distribution of particular CAG/GGC combinations is significantly different between infertile men and controls (38), although these data should be further confirmed. In this study we analysed CAG and GGC repeat lengths in men with a history of cryptorchidism, associated or not with impairment of sperm production, in comparison with normal fertile subjects.

**Subjects and methods**

**Subjects**

Patients and controls were prospectively recruited for this study with the approval of the hospital ethics committee, and informed consent was obtained from each subject. Among adult subjects referred to our centre for semen analysis, we recruited 105 men who were orchiopexied in childhood and who presented no other obvious causes of testicular damage. Men who reported spontaneous descent of the testes at the time of orchidopexy could not be determined in all cases, even if the majority of them were in the inguinal region. A complete medical history and a physical examination were undertaken. Semen analysis was repeated at least twice and was performed according to WHO guidelines (53). When semen analysis repeatedly revealed azoospermia (absence of sperm) or oligozoospermia with sperm concentrations of $< 5 \times 10^9$/ml, bilateral testicular fine-needle aspiration cytology (FNAC) (54) was performed. In all subjects, we had ultrasound examination of the testes (for testis morphology and volume) and plasma determination of follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin and testosterone concentrations. Exclusion of known causes of male infertility was done by careful history (excluding, for example, varicocele, orchitis and testicular trauma), sperm antibody determination, endocrine profile (excluding, for example, hypogonadotrophic hypogonadism and hyperprolactinaemia), karyotype analysis, Y chromosome microdeletion analysis (55–57), cystic fibrosis transmembrane regulator gene mutation analysis, and INSL3 and LGGR gene mutation analysis (13). Furthermore, AR gene mutations were excluded by PCR and direct sequencing, using a set of 11 oligonucleotide primers covering exons 1–8 (58). Ex-cryptorchid men were classified by type of cryptorchidism (bilateral versus unilateral) and presence or absence of a history of infertility with spermatogenic defect, as evaluated by semen and testicular cytological parameters. Therefore, of the 50 subjects with bilateral cryptorchidism, 40 had spermatogenic damage (sperm count of $< 20 \times 10^9$/ml) with infertility, and 10 had normozoospermia. Of the 55 subjects with unilateral cryptorchidism, 33 had spermatogenic damage with infertility, and 22 had normozoospermia. A total of 115 men with proven fertility and normozoospermia, without history of cryptorchidism, and recruited from men whose wives were in the first trimester of pregnancy, served as controls. All patients and controls were of Caucasian origin and came from different Italian regions.

**Determination of the CAG and GGC repeat number**

The number of CAG and GGC triplets was determined as previously reported (38). Genomic DNA was extracted from peripheral blood leucocytes with a DNA isolation kit (Roche, Milan, Italy). The AR exon 1 was amplified from genomic DNA in two different PCR reactions, giving overlapping amplicons. Both reactions were performed under the same conditions (standard conditions with 8% dimethylsulphoxide) and with the same cycle (94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, repeated 37 times). The CAG repeat is contained in the amplicon produced with the primers A0 GGTGTTCCTCCGACAGTTTCC and A5 GCTCCACTTCTCCAAGGA-CATTA. It is sequenced with the primer A2 GCTGGAGGTTGGTTGCTT, using standard conditions for automated sequencing. The GGC repeat is amplified with the primers A3n CAGCAAGACTAGC-CCCAG and A10 CCCAGACACAGAGTGACTCTGCC, and it is sequenced with the primer A8 GAGTGGGATA-GGCGACTCTGCTCAA, Primers A2, A5, A8 and A10 are from Lubahn et al. (58), whereas we designed the new primers A0 and A3n. Sequence analyses were performed with the gap4 software of the Staden package.

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(59), which is available at the UK Human Genome Mapping Project Web page (www.hgmp.mrc.ac.uk/).

**Statistical analysis**

Differences in CAG and GGC mean repeat length were tested by Wilcoxon’s rank sum test. Differences among frequencies were calculated with both the chi-square test and Fisher’s exact test. Relative risks and the corresponding 95% confidence intervals were calculated on the basis of the asymptotic normal distribution of these quantities. Fisher’s exact test was used to analyse independence in two-way contingency tables. $P < 0.05$ was considered statistically significant. Computations were performed with the open-source statistical software ‘R’.

**Results**

Overall, the mean number of CAG and GGC repeats in exon 1 of the AR gene was $21.6 \pm 3.3$ (range 9–31) and $17.0 \pm 1.7$ (range 8–21) respectively, in proven fertile control men, and $21.8 \pm 2.9$ (range 12–29) and $17.4 \pm 1.4$ (range 10–21) in cryptorchid men (Table 1). These differences were not statistically significant, and no difference was evident when comparing the median value of CAG and GGC number. The subgrouping of cryptorchid men in bilateral and unilateral forms, and in those with or without spermatogenic damage also showed no differences in terms of mean and median values of CAG and GGC repeat number with respect to controls (Table 1). The distribution of CAG and GGC allele frequencies (Fig. 1) was not different between cryptorchid men and controls. Although there was an apparent trend toward a shift to GGC=$18$ or GGC $>18$ in men with cryptorchidism especially in the bilateral form, no significant difference was observed with respect to controls. Our previous work on AR triplet number (38) showed that some haplotypes (combination of CAG and GGC repeat number) could be associated with male infertility, suggesting that both CAG and GGC could modulate AR function. Therefore, we assume the joint distribution of CAG and GGC. As reported previously (38), for this analysis, the data were collected in two-way tables (Tables 2 and 3) reporting frequencies for each CAG/GGC haplotype. CAG numbers of $<20$ and $>24$, corresponding to the first and third quartiles of the distribution for controls, were considered in single categories. In the GGC distribution, most of the observations belong to categories 17 and 18 (Fig. 1); therefore, we considered in a single category the GGC numbers under or equal to 16 and those over or equal to 19. Analysis of the percentages of the two-way tables showed that there were no differences between controls and cryptorchids considered as a whole. However, analysis in the different subgroups of cryptorchid men showed a difference between controls and men with bilateral cryptorchidism concerning the cell CAG $=21$ GGC $=18$ (prevalence of 16.0% in bilateral cryptorchid men versus 5.2% in controls; $P < 0.05$), and those corresponding

| Table 1 CAG and GGC values in cryptochid men and controls. All data not statistically significant ($P > 0.05$). |
|---|---|---|---|---|---|---|
| | CAG | | |
| | No. of alleles | Range | Mean $\pm$ S.D. | Median | |
| Controls ($n = 115$) | 17 | 9–31 | $21.6 \pm 3.3$ | 22 | |
| Cryptorchid men ($n = 105$) | 14 | 12–29 | $21.8 \pm 2.9$ | 22 | |
| With spermatogenic damage ($n = 73$) | 12 | 13–27 | $21.7 \pm 28$ | 21 | |
| Without spermatogenic damage ($n = 32$) | 11 | 12–29 | $21.9 \pm 3.1$ | 22 | |
| Bilateral ($n = 50$) | 12 | 13–27 | $21.8 \pm 2.7$ | 22 | |
| Unilateral ($n = 55$) | 13 | 12–29 | $21.8 \pm 3.0$ | 21 | |
| | GGC | | |
| | No. of alleles | Range | Mean $\pm$ S.D. | Median | |
| Controls ($n = 115$) | 10 | 8–21 | $17.0 \pm 1.7$ | 17 | |
| Cryptorchid men ($n = 105$) | 9 | 10–21 | $17.4 \pm 1.4$ | 17 | |
| With spermatogenic damage ($n = 73$) | 9 | 10–21 | $17.3 \pm 1.5$ | 18 | |
| Without spermatogenic damage ($n = 32$) | 5 | 13–20 | $17.4 \pm 1.1$ | 17 | |
| Bilateral ($n = 50$) | 6 | 12–20 | $17.4 \pm 1.4$ | 18 | |
| Unilateral ($n = 55$) | 8 | 10–21 | $17.3 \pm 1.5$ | 17 |
Cryptochid men (Control) versus 20.9% in controls; the associated relative risks (RR) and 95% confidence intervals (CI).

Variables are analysed separately. However, we found significant differences when the joint distribution of CAG and GGC triplets in a group of men with a history of cryptorchidism more frequently presented the combination CAG$^{18}$/GGC$^{18}$ (prevalence of 38.0% in bilateral cryptorchid men versus 20.9% in controls; P < 0.05). The calculated relative risks and the corresponding confidence intervals of these combinations are shown in Table 4.

Discussion

Accumulating evidence suggests that a genetic component may be associated with cryptorchidism. However, a direct cause–effect relationship between specific genetic alterations and cryptorchidism is not evident in many cases. For example, Y chromosome long-arm microdeletions removing one or more ‘azoospermia factors’ (AZFα, β and γ) (60) may be found in cryptorchid men (57, 61, 62). In these cases, AZF deletions seem not to be implicated in the pathogenesis of cryptorchidism itself; rather, they are responsible only for the severe spermatogenic damage that is frequently associated with this condition. On the other hand, the numerous data from animal models suggesting a role for some genes in testicular descent and cryptorchidism have not been confirmed in humans, as we recently showed for the CgRP system (63). Other genetic abnormalities, such as those involving MIS or HOXA-10, may lead to cryptorchidism (3), but their clinical importance is not so evident (64). Therefore, mutations in INSL3/LGR8 or AR genes may be considered the only genetic factors known to be responsible for human cryptorchidism with specific frequency. The role of CAG triplets in exon 1 of the AR in cryptorchidism is less clear, and the combined effect of CAG and GGC repeat numbers is completely unknown.

In this study we analysed for the first time both CAG and GGC triplets in a group of men with a history of cryptorchidism, with or without associated spermatogenic impairment, and found that there is no difference with respect to normozoospermic fertile controls when these variables are analysed separately. However, we found significant differences when the joint distribution of CAG and GGC and the specific combinations of CAG and GGC were analysed. In particular, men with a history of bilateral cryptorchidism more frequently presented the combination CAG$^{18}$/GGC$^{18}$ and CAG$^{18}$/GGC$^{21}$ and CAG$^{21}$/GGC$^{18}$ in cryptorchid men and controls with associated spermatogenic impairment. For the first time, we also analysed CAG$^{18}$/GGC$^{18}$ triplet analysis in idiopathic infertile males (38). In fact, in that study, we found that the same two combinations were significantly more frequent in idiopathic infertile patients with respect to controls.

First of all, the present study confirms the findings of Sasagawa et al. (48) in Japanese subjects regarding the absence of an association between CAG length and cryptorchidism. For the first time, we also analysed the GGC polyglycine tract length and found that it is not related to cryptorchidism. Furthermore, our data suggest that CAG and GGC number is not different between normal, idiopathic infertile, and cryptorchid men (38). Secondly, the finding that two CAG/GGC combinations seem to increase susceptibility to

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**Table 2** Joint distribution of CAG and GGC percentages (number in parenthesis) for the 115 fertile control men. P values with respect to that found in cryptorchid men (Table 3) are reported in Table 4.

<table>
<thead>
<tr>
<th>CAG</th>
<th>GGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤20</td>
<td>3.5 (4)</td>
</tr>
<tr>
<td>21</td>
<td>2.9 (3)</td>
</tr>
<tr>
<td>22</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>≥24</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>6.3 (7)</td>
</tr>
</tbody>
</table>

**Table 3** Joint distribution of CAG and GGC percentages (number in parenthesis) for the 105 cryptorchid men. P values with respect to that found in controls (Table 2) are reported in Table 4.

<table>
<thead>
<tr>
<th>CAG</th>
<th>GGC</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤20</td>
<td>2.9 (3)</td>
</tr>
<tr>
<td>21</td>
<td>2.9 (3)</td>
</tr>
<tr>
<td>22</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>≥24</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>6.3 (7)</td>
</tr>
</tbody>
</table>

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**Table 4** Analysis of distribution of combinations CAG$^{21}$/GGC$^{18}$ and CAG$^{21}$/GGC$^{18}$ in cryptorchid men and controls with the associated relative risks (RR) and 95% confidence intervals (CI).

<table>
<thead>
<tr>
<th>Combination</th>
<th>Control (n = 115)</th>
<th>Cryptorchid men (n = 105)</th>
<th>Bilateral (n = 50)</th>
<th>Unilateral (n = 55)</th>
<th>%</th>
<th>RR (95% CI)</th>
<th>%</th>
<th>RR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG$^{21}$/GGC$^{18}$</td>
<td>5.2</td>
<td>13.3</td>
<td>16.0*</td>
<td>10.9</td>
<td>20.9</td>
<td>NS</td>
<td>30.5</td>
<td>38.0*</td>
</tr>
<tr>
<td>CAG$^{21}$/GGC$^{18}$</td>
<td>13.3</td>
<td>13.3</td>
<td>16.0*</td>
<td>16.0*</td>
<td>NS</td>
<td>30.5</td>
<td>38.0*</td>
<td>1.82 (1.10–3.01)</td>
</tr>
</tbody>
</table>
cryptorchidism, particularly of the bilateral form, could be interpreted in different ways. In fact, although we found a difference between the bilateral cryptorchid men and the controls, these could be chance findings, and no conclusion regarding the biological importance of these combinations can be drawn. We have also to consider that the great majority of bilateral cases (40/50, 80%) actually present a spermatogenetic impairment, and idiopathic severely infertile men showed the same higher frequency of these particular combinations (38). Therefore, further analyses on a larger group of cryptorchid men without spermatogenetic damage should be performed to clarify this aspect. For example, one hypothesis is that the presence of these combinations causes a testicular alteration whose consequences are impairment of spermatogenesis (germ cell damage) and cryptorchidism (altered testicular responses to mechanisms regulating testicular descent), in a manner similar to that proposed for Y chromosome microdeletions (57, 61). Another hypothesis is that these two CAG/GGC combinations directly cause the cryptorchid phenotype by preventing normal testicular descent. The finding of this association only in bilateral cases seems to support this hypothesis, since unilateral cases are less likely to be explained by genetic alterations. Therefore, although cryptorchidism and infertility may frequently be associated, they could also represent two distinct clinical phenotypes of the same genetic alteration. However, again in these cases, a larger number of subjects are needed to verify this hypothesis. We have also to keep in mind that cryptorchidism is a heterogeneous and variable condition, and hence is likely to be multifactorial (3). Strict clinical and pathological criteria are therefore important when analysing putative genetic aetiologies. Even if the association between CAG/GGC haplotypes and the clinical phenotype is confirmed, the mechanism by which they cause cryptorchidism or spermatogenetic impairment can only be speculative. We can hypothesize that different CAG/GGC combinations may modulate AR function, and that the combinations CAG = 21/GGC = 18 and CAG ≥ 21/GGC ≥ 18 could determine a reduction in the transcriptional activity of the receptor. However, CAG = 21/GGC = 18 is one of the most common alleles among Caucasians, and it is the fourth most frequent haplotype found in our fertile control group. Therefore, its role as a causative factor of cryptorchidism is less understandable. In vitro transactivation studies with the AR constructed with different CAG/GGC combinations and expression analyses are under way to verify this hypothesis and clarify the molecular mechanisms involved in the modulation of AR activity. In our series, the precise location of the cryptorchid testes at the time of orchidopexy could not be recorded in all cases, even if the majority of them were in the inguinal region. A more detailed analysis of CAG and GGC lengths in relation to the severity of cryptorchidism and a prospective study in childhood would be interesting. In conclusion, we found no association between CAG or GGC repeat number in the AR gene and cryptorchidism. Taken together with previous studies (34), this suggests that expansion of these tracts is unlikely to constitute a major cause of cryptorchidism. The possible relationship of specific CAG/GGC haplotypes to cryptorchidism, as well as idiopathic male infertility, seems to suggest that some combinations of CAG and GGC may modulate AR function, but this needs to be verified in other studies.

Acknowledgements

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