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Hypospadias and the androgen receptor gene: mutation screening and CAG repeat length analysis

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We report on mutation screening and CAG repeat length analysis of the androgen receptor (*AR*) gene in 21 patients with hypospadias. The urethral meatus was located at the glandular region in six patients (glandular type), at the penile shaft in seven patients (penile type), and at the scrotal/perineal region in eight patients (scrotal/perineal type). Mutation screening was performed for exons 1–8 and their flanking introns (except for the CAG and GGC repeat regions at exon 1) by the heteroduplex detection method and showed no abnormal chromatograms. The CAG repeat length analysis was carried out using 50 normal boys and 50 fertile males as controls, and demonstrated no statistically significant difference in the median of CAG repeat lengths or in the frequency of long CAG repeats (≥26 or ≥28) between the controls and the patients with the three different types of hypospadias. The results suggest that *AR* gene abnormalities do not constitute a major factor in the development of hypospadias.

Key words: androgen receptor/CAG repeat length/hypospadias/mutation screening

Introduction

Hypospadias is a relatively common genital anomaly with a prevalence of ~0.5% (Grumbach and Conte, 1998). It may be classified into glandular, penile, and scrotal/perineal types on the basis of the anatomical location of the urethral meatus. Glandular and penile types often appear as an isolated anomaly and account for the majority of hypospadias, whereas a scrotal/perineal type frequently occurs in association with other genital anomalies such as microphallus, bifid scrotum, and cryptorchidism. The aetiology is poorly understood, and both genetic and environmental factors have been implicated in the development of hypospadias.

The androgen receptor (AR) plays a crucial role in male sex differentiation by mediating the biological effects of androgens (Grumbach and Conte, 1998). The *AR* gene resides on chromosome Xq11-12 and consists of eight exons; exon 1 encodes the transactivation domain, exons 2 and 3 encode the DNA binding domain, the 5′ portion of exon 4 encodes the hinge domain, and the 3′ portion of exon 4 and exons 5–8 encode the ligand binding domain (Quigley *et al*., 1995). In addition, exon 1 contains a highly polymorphic CAG repeat encoding a polyglutamine tract, and function studies with different CAG repeat numbers have indicated an inverse relationship between the CAG repeat length and transactivation function or expression level of the *AR* gene (Chamberlain *et al*., 1994; Choong *et al*., 1996).

Thus, the *AR* gene has been examined in patients with undermasculinized genitalia. Consequently, mutations of the *AR* gene have been identified in at least six males from four families with hyopspadias (Batch *et al*., 1993; Alléra *et al*., 1995; Sutherland *et al*., 1996), and a weak but significant expansion of the CAG repeat lengths has been reported in 78 males with undermusculinization including hypospadias (Lim *et al*., 2000). However, the *AR* gene mutations are infrequent in hypospadias, and it remains uncertain whether the CAG repeat lengths are expanded in other patient populations with hypospadias. Here, we report on mutation screening and CAG repeat length analysis in patients with hypospadias.

Materials and methods

Patients

This study consisted of 21 Japanese patients with hypospadias. Genital findings of each patient are summarized in Table I. The urethral meatus was located at the glandular region in cases 1–6, at the penile shaft in cases 7–13, and at the scrotal (penoscrotal junction) or perineal region in cases 14–21. Twelve cases had microphallus (below –2.5 SD of the penile length in age-matched normal Japanese boys) (Fujieda and Matsuura, 1987b), four cases had undescended and/or small testis (below –2 SD of the testis size in age-matched normal Japanese boys) (Fujieda and Matsuura, 1987a), two cases had scrotal abnormalities, and 14 cases had chordee of various degrees.

Table I. Genital findings in each patient

Case	Age (years)	Urethral meatus	Other genital features
1	5	Glandular	Microphallus, inguinal testes (B)
2	6	Glandular	Microphallus, chordee
3	8	Glandular	Chordee
4	9	Glandular	Absent
5	11	Glandular	Chordee
6	20	Glandular	Chordee
7	2	Penile	Microphallus, inguinal testis (L)
8	4	Penile	Microphallus, chordee
9	4	Penile	Microphallus, chordee
10	6	Penile	Microphallus, small testes (B)
11	9	Penile	Microphallus, chordee
12	10	Penile	Microphallus, unpalpable testis (L), small testis (R)
13	29	Penile	Chordee
14	0.9	Perineal	Microphallus, bifid and hypoplastic scrotum
15	1	Perineal	Microphallus, bifid and hypoplastic scrotum
16	2	Scrotal	Chordee
17	8	Scrotal	Microphallus, chordee
18	15	Scrotal	Chordee
19	15	Scrotal	Microphallus, chordee
20	18	Scrotal	Chordee
21	22	Scrotal	Chordee

L = left; R = right; B = bilateral.
Type of hypospadias: cases 1–6, glandular; cases 7–13, penile; and cases 14–21, scrotal/perineal.

Pubic hair was at Tanner stage 1 in cases 1–5, 7–12, and 14–17, at Tanner stage 4 in cases 18–20, and at Tanner stage 5 in cases 6, 13, and 21. Extragenital features included left ureteral duplication and epilepsy in case 7, right hydrocele in case 11, and epilepsy in case 14. Basal serum LH, FSH, and testosterone were age-appropriate in all cases. All the 21 cases had a normal 46,XY karyotype. Informed consent was obtained from all patients or their parents.

Mutational screening

Leukocyte genomic DNA of each patient was amplified for exons 1–8 and their flanking intron sequences, except for the CAG and GGC repeat regions at exon 1, by polymerase chain reaction (PCR). The primer sequences and the PCR conditions were based on the previous reports (Lubahn *et al*., 1989; Zhu *et al*., 1999), with minor modifications. The PCR products were mixed with those of a control male with intact *AR* gene sequences, and were subjected to denaturing high-performance liquid chromatography (DHPLC), a heteroduplex detection method with a proven sensitivity and specificity of >95% (O'Donovan *et al*., 1998), on an automated instrument (WAVE; Transgenomic, San Jose, CA, USA). (This method is not applied to the polymorphic triplet repeat regions because of complicated heteroduplex formation.) For comparison, a normal male with intact *AR* gene sequences and a patient with androgen insensitivity resulting from an L812F (CTC to TTC) mutation at exon 6 were similarly analysed. The DHPLC melting temperature was calculated by WAVE Maker software version 3.44, and three different temperatures (the calculated temperature and ± 1–2°C of that temperature) were used for the DHPLC analysis.

CAG repeat length analysis

Leukocyte genomic DNA was amplified by PCR with a fluorescently labelled forward primer and an unlabelled reverse primer flanking the CAG repeat region at exon 1. The primer sequences and the PCR condition have been described (Allen *et al*., 1992). The PCR products were mixed with size standards, and were electrophoresed on an autosequencer (ABI PRISM 310; Applied Biosystems, Norwalk, CT, USA). The size of the PCR products was determined by GeneScan software version 2.1. Furthermore, to confirm the precise CAG repeat length, a total of 15 PCR products with different sizes on GeneScan analysis were subjected to direct sequencing on the autosequencer. For controls, 50 boys with normal external genitalia who were seen by us because of short stature (age range 3-16 years, mean 8.5 years) and 50 adult males with proven fertility (age range 25-48 years, mean 38.5 years) were similarly analysed with permission; all the 100 control subjects had a 46,XY karyotype.

The normality of the distribution of CAG repeat numbers was examined by the χ^2-test. The CAG repeat length was analysed for the median as an indication of the overall distribution (Dowsing *et al*., 1999; Lim *et al*., 2000) and for the frequency of CAG repeats $\geqslant$26 or $\geqslant$28 regarded as a threshold for untoward effects on transactivation function (Rebbeck *et al*., 1999; Young *et al*., 2000). The statistical significance of the median of the CAG repeat lengths was analysed by the Mann-Whitney *U*-test, and that of the frequency of long CAG repeats ($\geqslant$26 or $\geqslant$28) was examined by the χ^2-test or Fisher's exact probability test, depending on the data size. $P < 0.05$ was considered significant.

Results

Mutational screening

Representative results are shown in Figure 1. Mutation screening of genomic DNA from the 21 men with hypospadias using DHPLC did not detect any heteroduplex formation indicative of point mutations in exons 1–8 and flanking introns of the *AR* gene. By contrast, a heteroduplex formation was found in the patient with the *AR* gene mutation.

CAG repeat length analysis

The distribution of the CAG repeat lengths is shown in Figure 2, and the results are summarized in Table II. The CAG repeat lengths did not follow the normal distribution. There was no statistically significant difference in the median of CAG repeat lengths or in the frequency of long CAG repeats ($\geqslant$26 or $\geqslant$28) between the patients with hypospadias of three different types, between the 50 normal boys and the 50 fertile males, and between the patients and the control males of any combinations. However, the CAG repeat lengths tended to be a little short in patients with scrotal/perineal hypospadias and the long CAG repeats were absent in glandular hypospadias.

Discussion

Mutation screening failed to identify an abnormal chromatogram. In this regard, it might be possible that a mutation remained undetected as an abnormal chromatogram by the heteroduplex detection method, or existed in an unexamined region(s) such as the CAG and GGC repeat regions, the promoter region, or the intron sequences. Overall, however, the results suggest that a mutation of the *AR* gene occurs rarely, if ever, in males with hypospadias. Consistent with this, other authors have detected mutations of the *AR* gene in only a few patients with hypospadias (Batch *et al*., 1993; Alléra *et al*., 1995; Sutherland *et al*., 1996).

The median CAG repeat length was not expanded, nor was the frequency of long CAG repeats increased in this study.

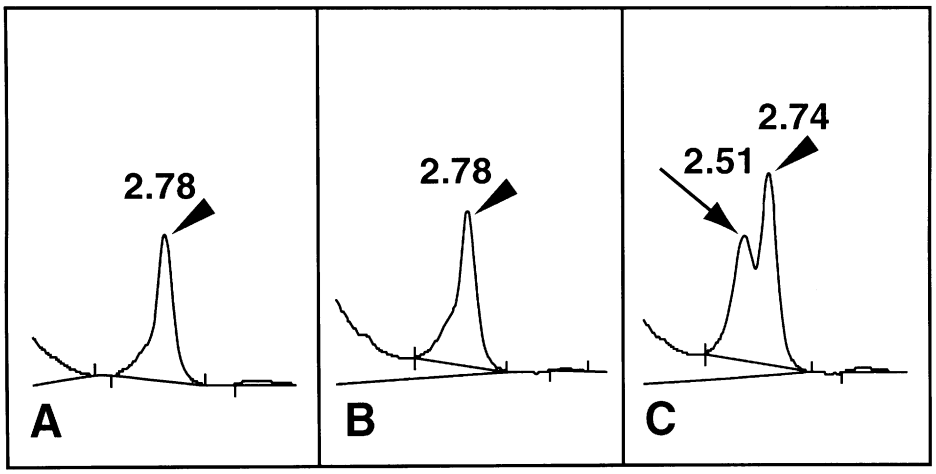

Figure 1. Chromatograph patterns for exon 6 and its flanking introns, obtained under the denaturing high-performance liquid chromatography melting temperature of 60°C. The numbers indicate the retention time (min). (**A**) A chromatograph pattern in a normal fertile male with intact *AR* gene sequences, indicating a single homoduplex peak (arrowhead). (**B**) A chromatograph pattern in case 14 with perineal hypospadias, indicating a single homoduplex peak (arrowhead), as shown in the normal fertile male. (**C**) A chromatograph pattern in a patient with androgen insensitivity caused by an L812F (CTC to TTC) mutation in the exon 6, indicating both homoduplex (arrowhead) and heteroduplex (arrow) peaks.

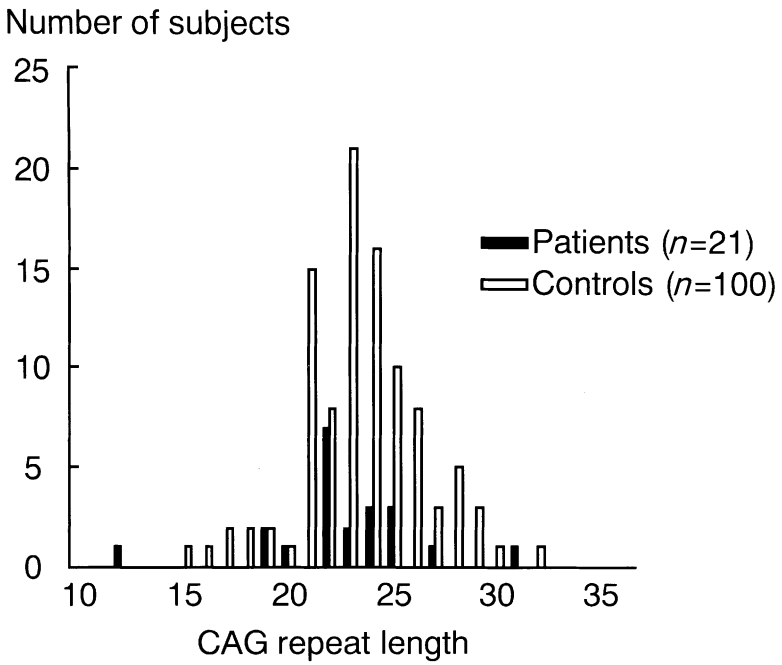

Figure 2. The distribution of the CAG repeat lengths at exon 1 of the *AR* gene in patients with hypospadias and in control males.

Table II. Results of the CAG repeat length analysis

CAG repeat length	Patients with hypospadias				Control subjects		
	Glandular (n = 6)	Penile (n = 7)	Scrotal/perineal (n = 8)	Total (n = 21)	Normal boys (n = 50)	Fertile males (n = 50)	Total (n = 100)
Normality	No	No	No	No	No	No	No
Mean ± SE	23.2 ± 0.60	24.0 ± 0.65	21.0 ± 1.87	22.6 ± 0.79	23.7 ± 0.46	23.2 ± 0.23	23.5 ± 0.29
Median	22.5	24	21	22	23	23	23
Range	22–25	22–27	12–31	12–31	16–32	17–28	16–32
Frequency							
(CAG), n = 26	0	14.3	12.5	9.5	24	18	21
(CAG), n = 28	0	0	12.5	4.8	14	6	10

This suggests that the CAG repeat length genotype has no discernible effect on the development of hypospadias in the 21 patients. However, this would not necessarily imply that the CAG repeat length genotype is irrelevant to the development of hypospadias in general. It has been reported (Lim *et al*., 2000) that both the median CAG repeat length and the frequency of long CAG repeats are increased in 78 males with undermasculinization including 73 males with moderate to severe hypospadias. Thus, CAG repeat lengths could be variable among different patient populations with hypospadias. In addition, since the extent of undermasculinization appears to be more severe in patients described in another study (Lim *et al*., 2000) than in those examined here (the frequency of scrotal/perineal hypospadias: 62/73 versus 8/21), longer CAG repeats may be more prevalent in patients with severe undermasculinization.

One patient (case 16 in Table I) had a short CAG repeat length ($n = 12$), which was absent in the 100 normal males. However, the repeat length of 12 has been detected in a normal Japanese subject (Kishida and Tamaki, 1997), and short CAG repeat lengths should increase rather than decrease the AR function (Chamberlain *et al*., 1994; Choong *et al*., 1996). Thus, it is unlikely that the short CAG repeat in case 16 is involved in the development of hypospadias.

Two points should be made for the CAG repeat length analysis. First, the CAG repeat length in the *AR* gene is believed to act as one of a multiple of susceptibility factors, rather than a major determining factor, relevant to the development of androgen-related disorders. Thus, although a long CAG repeat genotype could raise susceptibility to androgen-related diseases, the diseases themselves would not result from a long CAG repeat genotype alone and could occur with a short CAG repeat genotype depending on other genetic and/or environmental conditions. Indeed, patients with spinal and bulbar muscular atrophy caused by markedly expanded CAG repeats ($n \geqslant 40$) (La Spada *et al*. 1991), have no undermasculinized genitalia, although they often exhibit clinical features of mild androgen resistance such as gynaecomastia, reduced fertility, and testicular atrophy from adulthood (Quigley *et al*., 1995). Second, the CAG repeat length ranges widely in patients with androgen-related disorders as well as in the control subjects. Thus, patients with relatively long or short CAG repeats may occur more frequently by chance in a given study. It is likely, therefore, that expansion of the CAG repeat length can be detected as a positive modifying factor in some patient populations but not in other patient populations, and that unexpectedly short CAG repeat lengths can be found in several patients. Consistent with this, previous studies in azoospermic males have shown both positive and negative results for the association between expanded CAG repeat lengths and infertility (Tut *et al*., 1997; Giwercma *et al*., 1998; Dowsing *et al*., 1999; Yoshida *et al*., 1999; Dadze *et al*., 2000).

In summary, the present study suggests that *AR* gene abnormalities do not constitute a major factor in the development of hypospadias. However, further studies in different patient populations are necessary to draw final conclusions on the relevance of the *AR* gene abnormalities to hypospadias.

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