Androgen Receptor CAGₙ Repeat Length Influences Phenotype of 47,XXY (Klinefelter) Syndrome

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Context: Klinefelter syndrome (KS; 47,XXY karyotype and variants) is characterized by tall stature and testicular failure, with marked variation in severity of the phenotype. Previous studies have proposed that genetic factors including mosaicism, parental origin of the supernumerary X-chromosome, skewed X inactivation, and androgen receptor (AR) polyglutamine repeat length may contribute to phenotypic variability in KS.

Objective: The objective of this study was to investigate the roles of these genetic factors in the variability of the KS phenotype.

Design: This was a cross-sectional study.

Setting: The study was performed at a pediatric endocrinology referral clinic.

Patients: Thirty-five KS boys and men, aged 0.1–39 yr, were studied.

Interventions: There were no interventions.

Main Outcome Measures: Auxological measurements, biological indices of testicular function, and clinical assessment of muscle tone were the main outcome measures. Genetic studies included karyotyping to detect mosaicism, genotyping of microsatellite markers to determine parental origin of the supernumerary X-chromosome, and genotyping and methylation studies to measure AR polyglutamine (AR CAGₙ) repeat length and X inactivation ratio.

Results: The only genetic factor that significantly influenced the KS phenotype was the AR CAGₙ repeat length, which was inversely correlated with penile length, a biological indicator of early androgen action. Mosaicism, imprinting, and skewed X inactivation did not account for the variability of the KS phenotype.

Conclusions: Normal genetic variation in the AR coding sequence may be clinically significant in the setting of early testicular failure and subnormal circulating testosterone levels, as occur in KS. (J Clin Endocrinol Metab 90: 5041–5046, 2005)
morphic CAG\(_n\) trinucleotide repeat in the coding sequence of the first exon. Translation of this repeat results in a polyglutamine tract in the N-terminal transactivation domain of the protein. The length of this polyglutamine tract is inversely related to receptor transactivation activity in vitro (22–25). AR CAG\(_n\) repeat length variation has been associated in vivo with androgen-related disorders, such as prostate cancer (26), male infertility (25, 27), or undermasculinized genitalia (28).

The goal of the present study was to investigate the roles of these genetic factors in the variability of the KS phenotype in a cohort of 35 KS boys and men, aged 1 month to 39 yr. The phenotype evaluation included auxological measurements, biological indices of testicular function, and clinical assessment of muscle tone. Genetic studies included karyotyping to detect mosaicism, genotyping of microsatellite markers to determine parental origin of the supernumerary X-chromosome, and genotyping and methylation studies to measure AR CAG\(_n\) repeat length and X inactivation ratio. The results indicated that one genetic factor examined influenced the KS phenotype: AR CAG\(_n\) repeat length inversely correlated with penile length, a biological indicator of early androgen action.

**Subjects and Methods**

All subjects or their parents gave informed consent or assent. This study was approved by the institutional review boards at Thomas Jefferson University and The University of Texas Southwestern Medical School. Individuals with KS variants with greater degrees of sex chromosome aneuploidy, e.g. 48,XXYY, were excluded because their phenotype is distinct from that of 47,XXY KS (29).

Subjects (n = 35) ranged in age from 0.1–39 yr (mean, 8.1 ± 8.9 yr). Twelve were infants (age, <2 yr), 21 were boys (age, 2–18 yr), and two were adults (age, >18 yr). Thirty-two were Caucasian, and three were African-American. Twenty-four subjects (69%) were ascertained by an unaffected father. All subjects were of adolescent age or older were receiving testosterone therapy at the time of evaluation and were not included in analyses of penile length, testicular volume, or muscle tone.

**Phenotypic assessment**

**Anthropometric measurements.** Subjects’ heights were measured with a stadiometer. Supine length was measured in boys less than 2 yr of age. Measured or reported parental heights were also recorded. Midparental height adjusted for the child’s sex (target height) was calculated using the formula 0.5 \(\times\) [father’s height (centimeters) + mother’s height (centimeters) + 13 cm] (30). The subjects’ target height sd scores were calculated from sex-adjusted midparental height obtained from the National Center for Health Statistics growth data (31). Body mass index was calculated as weight in kilograms divided by height in meters squared. Other measurements were also converted to sd scores where possible using age- and gender-specific norms (31–33).

**Genitalia.** Penile length was measured by one trained investigator (J.L.R.) and converted to sd score using available standards (34). Testicular size was assessed with the Prader orchidometer, and the measured volumes were converted to sd scores using published reference values (35). Only measurements from subjects who had received testosterone treatment for a total duration of less than 3 months and no treatment in the past year were used for analyses. For subjects with a discrepancy in testis size, the smaller measurement was used for analyses.

**Muscle tone.** Muscle tone was evaluated clinically as normal, mildly decreased, or severely decreased by one experienced clinician (J.L.R.), assessing resistance to passive movement at the elbow and knee. Also the degree of head lag in the infants less than 6 months of age was evaluated. Standing posture tone was also evaluated clinically in children able to bear weight on the lower extremities.

**Hormone measurements.** Serum testosterone, FSH, LH, and estradiol levels were measured by commercial assays (Esoterix Endocrinology, Calabasas Hill, CA) or as previously reported (21).

**Genetic studies**

**Karyotype.** A postnatal, G-band, peripheral blood karyotype was obtained for all subjects. Each karyotype included at least 20 cells. Antenatal karyotype reports were also obtained for most subjects diagnosed prenatally.

**Parental origin.** Parental origin of the supernumerary X-chromosome was determined by genotyping probands and parents with a panel of seven highly polymorphic microsatellite markers dispersed along the length of the chromosome. Markers were selected from the ABI Linkage Set 2.5 and analyzed using an ABI 3100 capillary electrophoresis instrument and GeneMapper software (Applied Biosystems, Foster City, CA). The panel included DXS987, DXS1001, DXS1047, DXS1214, DXS8091, DXS1060, and DXS1226. In seven cases a sample was not available from the father. For these cases if any of the proband’s marker alleles were nonmaternal, we assigned the origin of the supernumerary X to the father.

**X inactivation ratio and AR CAG\(_n\) repeat length.** Skewing of X-chromosome inactivation was measured using the AR methylation assay (36). One microgram of genomic DNA was either digested at 37 C for more than 6 h with 20 U restriction enzyme HpaII in buffer supplied by the manufacturer (New England BioLabs, Beverly, MA) or mock-digested in buffer alone, followed by incubation at 65 C for 30 min to inactivate the enzyme. DNA was then ethanol precipitated and redissolved in water, and 100 ng were used as template for PCR amplification of the AR CAG\(_n\) repeat. The primers were GCCGTGAAAGTTGCTGTTCCAT and TC-CAGAAATCGTGGTCCAGGC. One primer was labeled at its 5’ end with the fluorophor 6-carboxyfluorescein. Products were analyzed by capillary electrophoresis as described above for genotyping. Fragment lengths were determined by comparison with a reference sample containing pooled DNAs from individuals with CAG\(_n\) repeat lengths of 18, 19, 20, 21, 22, 23, or 25 copies, as determined by DNA sequencing. Peak areas were calculated using GeneMapper software. The percentage of each X-allele that was active (unmethylated) was determined from the ratio of peak areas in the HpaII-digested samples after correcting for unequal amplification of alleles in the mock-digested samples as previously described (37). Preferential inactivation favoring one allele more than 80% was considered skewed (38).

**Statistical analyses**

All results are presented as the mean ± sd. We calculated Pearson correlation coefficients and P values for height sd score vs. age, testosterone vs. estradiol levels, and penile length, testicular volume, and height, head circumference, and body mass index (BMI) sd scores vs. mean weighted AR CAG\(_n\) repeat length using PRISM (GraphPad, San Diego, CA). The same software was used to calculate Spearman’s correlation coefficient for the fraction of active alleles vs. AR CAG\(_n\) repeat length. Dichotomous variables were compared by Fisher’s exact test (two-tailed). The t tests were also two-tailed and assumed equal variance. All P values shown are nominal; P < 0.05 was considered statistically significant. We excluded postpubertal-aged KS males who had received testosterone treatment from certain analyses, including penile length, testicular volume, and muscle tone, because these phenotypes may be affected by the treatment.
**Results**

**Anthropometric and physical findings**

Subjects, on the average, were taller and heavier than normal, with proportional head circumference (Table 1). The height SD score tended to increase with chronological age, but the trend did not reach statistical significance (r = 0.27; P = 0.11; data not shown).

Gynecomastia, defined as the presence of at least Tanner stage 2 breast tissue, was present in six subjects: five with Tanner stage 2 breast tissue, ages 6.9, 10.9, 14, 13.9, and 39.9 yr, and one with Tanner stage 3 breast tissue, age 14 yr. Muscle tone was normal in 13 subjects, 16 had mild hypotonia, and six were severely hypotonic.

**Endocrine findings**

Hormone measurements, including testosterone, LH, and FSH, are presented in Table 2. Six of 32 nonandrogen-treated subjects had castrate gonadotropin levels; all were over 14 yr of age. There was a trend for testosterone and estradiol levels to correlate that did not achieve statistical significance (n = 20; r = 0.43; P = 0.06).

**Genetic data**

Only one subject had a mosaic karyotype (46,XY[14]/47,XXY[7]); the remaining 34 were 47,XXY. We were unable to assess the affect of clinically apparent mosaicism on the phenotype, because there was only one mosaic in our cohort.

Parental samples were available for 34 subjects. The extra X-chromosome was maternal in 19 subjects (56%) and paternal in 15 subjects (44%). There was no significant difference in any mean anthropometric measure or in penile length or testicular volume SD score in subjects with a maternal vs. a paternal extra X-chromosome (data not shown). Five patients had maternal X-chromosome isodisomy, as judged by homozygosity for all seven microsatellite markers tested. They did not show any significant differences from subjects with uniparental heterodisomy or biallelic inheritance of their X-chromosomes for any of the phenotypes listed in Table 1 (data not shown).

Thirteen of the 35 subjects (37%) were homozygous for the AR CAGn repeat polymorphism. The percentage of each X-allele that was active (unmethylated) was measured for the other 22 heterozygous subjects. Because the total percentage of active alleles (shorter plus longer) is 100%, only data for shorter alleles are shown (Fig. 1). The mean percentage of active shorter alleles was 48 ± 18%, which was not signifi-

**TABLE 1. Anthropometric and physical measurements of KS cohort**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>SD score</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>0.6 ± 1.3</td>
<td>35</td>
</tr>
<tr>
<td>Actual height minus expected</td>
<td>0.5 ± 1.3</td>
<td>35</td>
</tr>
<tr>
<td>Midparental adjusted height</td>
<td>0.6 ± 1.3</td>
<td>35</td>
</tr>
<tr>
<td>Weight</td>
<td>0.5 ± 1.3</td>
<td>35</td>
</tr>
<tr>
<td>BMI</td>
<td>0.3 ± 1.4</td>
<td>27</td>
</tr>
<tr>
<td>Head circumference</td>
<td>0.6 ± 1.6</td>
<td>35</td>
</tr>
<tr>
<td>Penile length</td>
<td>−1.4 ± 1.1</td>
<td>34</td>
</tr>
<tr>
<td>Testicular volume</td>
<td>−1.1 ± 1.5</td>
<td>31</td>
</tr>
</tbody>
</table>

Data are the mean ± SD.

**FIG. 1. X inactivation studies.** The percentage of shorter AR alleles that were active (unmethylated) vs. CAGn repeat length of shorter allele for each heterozygous subject is shown. The dashed line indicates the mean percent activity (48%) of shorter AR alleles for all 22 heterozygous subjects. n.s., Not significant.
both were 47,XmXmY. Their respective height SD scores were −0.58 and +0.12, penile length SD scores were −3.5 and +1.0, and testicular volume SD scores were both −1.5. Both subjects had mild hypotonia. The subject with the shorter penile length had AR CAGn repeat lengths of 21 and 26, with activities of 12% and 88%, respectively. The subject with the longer penile length had AR CAGn repeat lengths of 14 and 19, with respective activities of 88% and 12%.

AR gene CAGn repeat lengths ranged from 14–28, all within the normal range. We computed correlations of repeat length with penile length, testicular volume, and height, head circumference, and BMI SD scores for subjects who did not receive prior testosterone treatment. The weighted mean CAGn repeat length was calculated for heterozygotes to account for the effect of X inactivation (see Subjects and Methods). Measured CAGn repeat length was used for homozygotes. Penile length SD score showed a significant inverse correlation with AR CAGn repeat length (P < 0.01; Fig. 2A). In contrast, testicular volume and height SD scores did not correlate with CAGn repeat length (Fig. 2, B and C), nor did head circumference or BMI SD scores (data not shown). Similarly, the means of the AR CAGn repeat lengths did not differ significantly among untreated subjects with no (22.1 ± 2.3; n = 11), mild (22.1 ± 3.0; n = 15), or severe (23.2 ± 0.8; n = 6) hypotonia, although short repeats were conspicuously absent among subjects with severe hypotonia (Fig. 2D). There was also no significant difference in CAGn repeat length among untreated subjects with or without gynecomastia [21.4 ± 2.4 (n = 6) vs. 22.5 ± 2.5 (n = 26); P = 0.4, by t test].

**Discussion**

Previous studies have suggested that genetic factors involving the sex chromosomes may influence the phenotype of KS (19, 37). These studies were generally subject to ascertainment bias because most KS patients fail to be diagnosed in childhood (2). We analyzed a cohort of 35 KS patients, most ascertained by antenatal diagnosis for advanced maternal age. Our results should therefore show less phenotypic bias while admittedly introducing other potential biases, e.g., socioeconomic status and motivation to participate in research.

The proportion of mosaics in previous KS studies varied between 7–10% and 15% (2, 39). We identified only one mosaic patient (3%) in our cohort. Although in most cases only 20 cells were counted in postnatal karyotypes, most patients also had prior antenatal karyotypes that were non-mosaic. The difference in the prevalence of mosaicism could reflect sampling error and parental decisions about terminating pregnancies (40). Although mosaicism for a normal 46,XY cell line probably ameliorates the KS phenotype, mosaicism detected by standard clinical karyotyping did not account for the phenotypic variability of our cohort.

Iitsuka et al. (19) speculated that the KS phenotype might also reflect X chromosome imprinting effects, as has been proposed for 45,X Turner syndrome (41, 42). We therefore assayed the parental origin of the supernumerary X-chromosome in our KS cohort. This variable was not associated with the phenotypes of penile length, testicular volume, or height.

Isodisomy could result in the expression of X-linked recessive mutations in severely affected KS males, as has been suggested for females (43). Five of our 34 subjects for whom parental genotypes were available (15%) appeared to have maternal X isodisomy, as judged by the lack of heterozygosity of any microsatellite marker tested. These five subjects were not more severely affected than the 29 subjects with maternal heterodisomy or biparental disomy. Thus, isodisomy did not explain the variability of the KS phenotype in our cohort.

Skewed X inactivation has also been proposed to influence the severity of the KS phenotype (19). Only two of 22 (9%) of our patients who were informative for the AR CAGn repeat

![Fig. 2. Weighted mean AR CAGn repeat length vs. penile length SD score (A), testicular volume z-score (B), height SD score (C), or degree of hypotonia (D). n.s., Not significant.](https://academic.oup.com/jcem/article-lookup/doi/10.1210/jcem.90.9.5041)
length polymorphism showed highly skewed inactivation, defined as greater than 80% methylation of one allele. Zitzmann et al. (37) observed a similar prevalence of highly skewed inactivation (five of 46, 11%, see their Fig. 1A). Litsuka et al. (19) reported that five of 16 (31%) KS males who were informative for the AR polymorphism had skewed X-chromosome inactivation, but two of their subjects had karyotype 48,XXY. The proportion of 47,XXY subjects with highly skewed inactivation in their study was three of 14 (21%), which was not statistically significantly different from the 9% in our study (P = 0.28, by Fisher’s exact test).

AR CAG\textsubscript{n} repeat lengths greater than 40 are directly correlated with hormonal and biological indices of androgen resistance in patients with X-linked Kennedy’s disease or spinal and bulbar muscular atrophy (44). Repeat length varied from 40–62 within the Kennedy’s disease population and was directly correlated with the age at disease onset and the presence of testicular dysfunction and gynecomastia (44). All of our subjects had AR CAG\textsubscript{n} repeat lengths within the normal range of seven to 35. Even within this range, functional differences have been demonstrated in AR alleles in vitro, with proteins containing shorter polyglutamine repeats having greater transregulatory activity (26, 45). Variation in the AR CAG\textsubscript{n} repeat length in vivo may influence a variety of androgen-sensitive traits, including male fertility (25, 27), fused or unfused scrotum, and micropenis, compared with greater among 46,XY males with moderate to severe defects. Zitzmann et al. (19) recently reported an association between AR CAG\textsubscript{n} repeat lengths and multiple aspects of the KS phenotype in a cohort of 47,XXY men. They found that longer AR CAG\textsubscript{n} repeats were associated with increased height, decreased testicular volume, decreased bone density, presence of gynecomastia, decreased likelihood of being in a stable relationship, and less professional achievement. We found a highly significant negative correlation between AR CAG\textsubscript{n} repeat length and penile length SD score, but no significant correlation with testicular volume or height SD scores or the presence of gynecomastia. The youth of our cohort limited our ability to study this last phenotype. Although not statistically significant, there was a paucity of short AR CAG\textsubscript{n} repeat alleles among our severely hypotonic subjects.

Zitzmann et al. (37) also reported finding preferential inactivation of the shorter AR CAG\textsubscript{n} repeat alleles in their subjects, which would magnify the effect of CAG\textsubscript{n} repeat length on androgen action. We did not find any systematic trend toward preferential inactivation of the shorter or longer AR CAG\textsubscript{n} allele in our cohort. The reason for this discrepancy is not clear, but may relate to differences in ascertainment. Interestingly, of the two subjects in our cohort with highly skewed X inactivation, the one with preferential inactivation of his shorter AR CAG\textsubscript{n} allele had severely decreased penile length (−3.5 SD), whereas the one with preferential inactivation of his longer AR CAG\textsubscript{n} allele had a penile length SD score of +1.0.

Testicular volume during early childhood may reflect complex growth interactions of Leydig, Sertoli, and germ cells as well as pubertal development. The relationship of this phenotype to CAG\textsubscript{n} repeat length was therefore difficult to evaluate in our young cohort. Testicular biopsies of KS infants and boys demonstrated decreased or absent germ cells and abnormal seminiferous tubules (16, 50–52). Small testes in KS may be due more to germ cell loss than to interactions of testosterone deficiency and androgen activity, explaining the lack of correlation between testicular volume and AR CAG\textsubscript{n} repeat length.

In summary, AR CAG\textsubscript{n} repeat length variation was the only genetic factor we examined that was significantly correlated with an androgen-responsive aspect of the phenotype, penile length, in our cohort of KS males. The variability of other KS phenotypes could be due to allelic differences in (over)expression of X-linked genes that escape inactivation, differences in autosomal genes, or interactions between supernumerary X-linked genes and unknown environmental factors. Subsequent studies should examine other KS phenotypes, such as cognition and previously described learning deficits. Identifying genes that contribute to the variability of the KS phenotype may lead to new prognostic tools for counseling and targets for therapeutic interventions to improve the outcome of this common genetic disorder.

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