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Evidence for Increased Prevalence of *SRY* Mutations in XY Females with Complete Rather than Partial Gonadal Dysgenesis

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Summary

The Y chromosome gene *SRY* (sex-determining region, Y gene) has been equated with the mammalian testis-determining factor. The *SRY* gene of five subjects with 46,XY complete gonadal dysgenesis (46,XY karyotype, completely female external genitalia, normal Müllerian ducts, and streak gonads) was evaluated for possible mutations in the coding region by using both single-strand conformation polymorphism (SSCP) assay and DNA sequencing. Mutations were identified in three subjects, of which two gave altered SSCP patterns. Two of them were point mutations causing amino acid substitutions, and the third was a single-base deletion causing a frameshift. All three mutations caused alterations in the putative DNA-binding region of the SRY protein. Genomic DNA was obtained from the fathers of two of the three mutant patients: one mutation was demonstrated to be de novo, and the other was inherited. The presence of *SRY* mutations in three of five patients suggests that the frequency of *SRY* mutations in XY females is higher than current estimates.

Introduction

Sex differentiation follows the commitment of the embryonic undifferentiated gonadal ridges to form testes or ovaries (Jost et al. 1973). The mammalian Y chromosome carries a gene known as the testis-determining factor (*TDF* in humans, *Tdy* in mice) that is required for testis formation and, hence, for normal male sexual differentiation. In the absence of the Y chromosome (and *TDF*), the fetal gonads follow the ovarian pathway, with ensuing female development (Ford et al. 1959).

Subjects with 46,XY chromosomal constitution and failed testis development (streak gonads) are presumed to have a mutation either in *TDF* or in another step in the sex determination and differentiation pathways. Individuals with 46,XY complete or "pure" gonadal dysgenesis (also called "XY females") suffer rapid and early degeneration of their gonads (gonadal dysgenesis), which are present in the adult as streak gonads consisting mainly of fibrous tissue and variable amounts of ovarian stroma. The external genitalia in these subjects are completely female, and Müllerian structures are normal. By contrast, subjects with 46,XY partial gonadal dysgenesis have ambiguous genitalia, a mix of Müllerian and Wolffian structures, and dysgenic gonads. These gonads usually consist of disorganized seminiferous tubules admixed with ovarian stroma.

The Y-located gene *SRY* (sex-determining region, Y gene) has been equated with *TDF* (Berta et al. 1990; Gubbay et al. 1990; Jäger et al. 1990; Koopman et al. 1990, 1991; Sinclair et al. 1990). A total of 41 subjects with an XY karyotype and abnormalities of sex differentiation were examined for mutations in *SRY*, and four de novo mutations have been identified, as

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well as one inherited mutation (Berta et al. 1990; Jäger et al. 1990; Hawkins et al. 1992; Ogata et al. 1992). The phenotypes of the 41 patients varied, some having complete dysgenesis and others having ambiguous genitalia of uncertain etiology. Some patients had isolated anomalies of sex differentiation, while others had additional somatic anomalies such as Turner stigmata. The five mutations identified were in patients with complete 46,XY gonadal dysgenesis without Turner stigmata.

We describe here the mutational analysis of five subjects with 46,XY karyotype, who are classified by anatomical and histological studies as having complete gonadal dysgenesis. The open reading frame of *SRY* (Sinclair et al. 1990) was screened for mutations by using both the SSCP (Orita et al. 1989) assay and DNA sequencing. Mutations were identified in three of the five patients. These mutations—two point mutations and a single-base deletion—like all the previously described *SRY* mutations, alter the putative DNA-binding region of the SRY protein. It is surprising that three of the five patients described here are mutant for *SRY,* given that previous studies suggested that only 12% of subjects with 46,XY gonadal dysgenesis were mutant for *SRY*.

Patients and Methods

Patients

The clinical presentation of the five patients in this study was described in a previous report (Berkovitz et al. 1991). Patients 207, 208, 212, 213, and 217 in the present report correspond to patients 5, 6, 3, 4, and 1, respectively, of the earlier study. The patients in this study presented for evaluation between the ages of 14 and 25 years. Patients 208, 212, and 213 were evaluated for delayed puberty; patient 207 sought help for dyspareunia, and patient 217 for tall stature. All subjects had female external genitalia, and none of them had the stigmata of Turner syndrome. The karyotype of four patients was 46,XY, while patient 208 had a 46,XY9qh + karyotype, the 9qh + chromosome being considered a normal variant. At laparotomy, all subjects had bilateral streak gonads, well-formed Müllerian structures including normal fallopian tubes and uterus, and no Wolffian structures.

Mutation Analysis

Genomic DNA was extracted from peripheral leukocytes by standard techniques (Sambrook et al. 1989). PCR amplification, PCR primers, cloning of PCR products, cloned DNA sequencing, SSCP analysis, and paternity testing were performed as described elsewhere (Hawkins et al. 1992). For SSCP analysis, three restriction digests were performed: *Dde*I, *Hpa*II/*Pst*I, and *Hin*fI/*Taq*I. Direct sequencing of *SRY* PCR products was performed using a sequencing kit (U.S. Biochemicals) with double-stranded DNA as template (Casanova et al. 1990; Hawkins et al. 1992).

Allele-specific Hybridization

Allele-specific oligonucleotide hybridizations were performed using PCR amplified genomic DNA and primers XES2 and XES7 (Hawkins et al. 1992). One-tenth of a 50 μl PCR reaction was denatured in 0.4 M NaOH and 25 mM EDTA and was spotted onto a Hybond-N+ membrane (Amersham) by using a Schleicher and Schuell SRC-96 dot blotter. The membrane was hybridized overnight with a ^{32}P kinase-labeled oligonucleotide at approximately 0.1 pmol/ml in the buffer of Church and Gilbert (1984) containing 500 μg denatured sheared salmon sperm DNA/ml. Dot blots were probed with both mutant and wild-type oligonucleotides for each mutation. The oligonucleotide sequences were as follows: 213 wild-type, 5′-CTCAGAGATCAGCAAGCAGC; 213 mutant, 5′-CTCAGAGATGAGCAAGCAGC; 207 wild-type, 5′GCCGAAAAATGGCCATTCTTC; and 207 mutant, 5′GCCGAAATATGGCCATTCTTC. The 213 oligonucleotides were hybridized at 56°C and washed at 58°C. The 207 oligonucleotides were hybridized at 56°C and washed at 62°C. Filters were stripped by boiling in 0.6% SDS.

Results

SSCP analysis of *SRY* revealed mutations in patients 208 and 213. Three restriction digests were performed prior to SSCP analysis to increase the chance of mutation detection. The mutation in patient 208 was detected in all three digests. The mutation in patient 213, however, was only detected in the *Dde*I digest (fig. 1).

For all five patients, a single clone from the XES10/XES11 PCR amplification was sequenced, in case mutations were missed by the SSCP assay. The fragment sequenced included the entire *SRY* open reading frame and 61 bp 5′ to the open reading frame. cDNA cloning and RNase protection experiments (A. H. Sinclair, personal communication; J. R. Hawkins, unpublished data) indicate that the two stop codons spanning the open reading frame are transcribed, implying that all

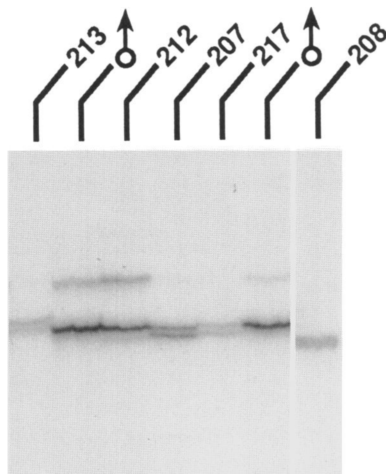

Figure 1 SSCP analysis of *SRY* DNA from XY females 207, 208, 212, 213, and 217. Normal male DNA (♂) derived from the lymphoblastoid cell line PGF (Goodfellow et al. 1989) is interspersed with the patient samples as a control. Patients 208 and 213 show abnormal mobilities.

the coding DNA of *SRY* was sequenced in this study. Sequence differences were observed in three patients (fig. 2, top panel): patient 208 had a deletion of nucleotide 734 of the pY53.3 sequence (Sinclair et al. 1990), causing a frameshift; patient 213 had a C-to-G transversion at nucleotide 680, causing an isoleucine-to-methionine amino acid substitution; and patient 207 had an A-to-T transversion at nucleotide 727, causing a lysine-to-isoleucine substitution. To check that the mutations were not artifacts due to *Taq* polymerase infidelity, the variant region was sequenced directly from the PCR product, and in all cases the variants were confirmed.

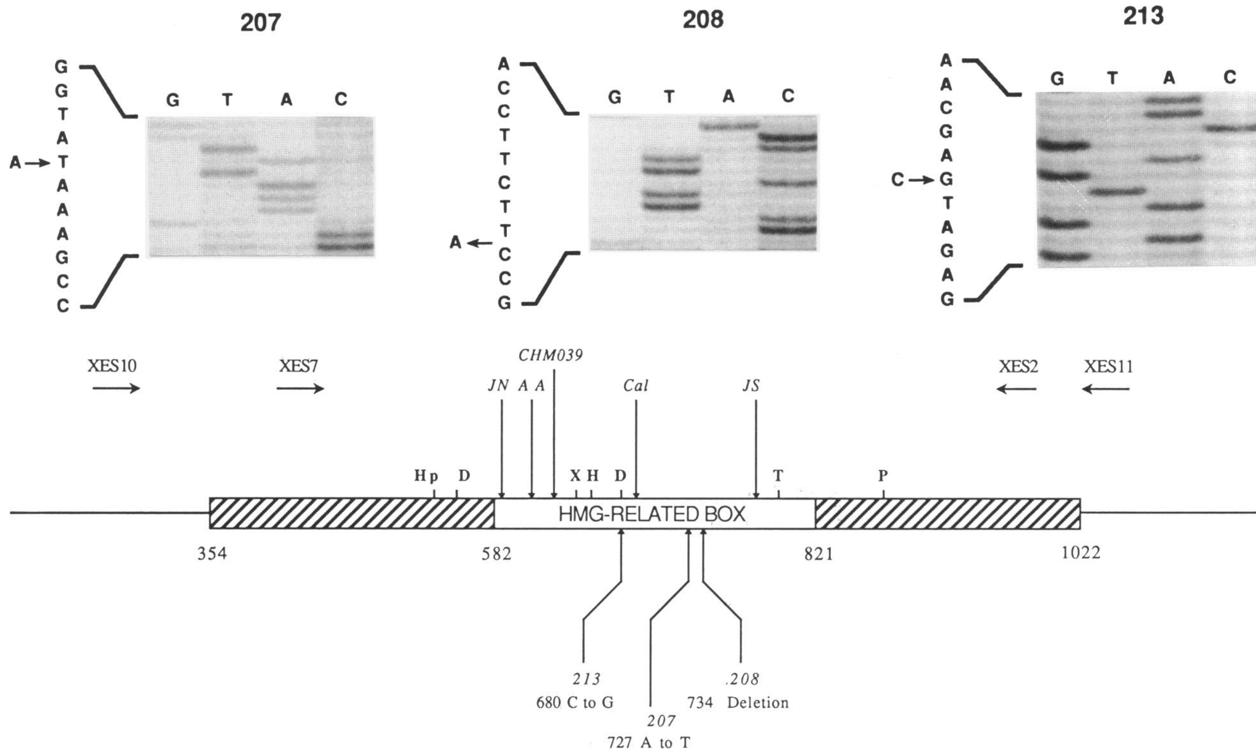

Figure 2 *Top,* Direct sequencing of the mutations in patients 207, 208, and 213. Patient 207 has suffered an A-to-T transversion at position 727, causing a lysine-to-isoleucine amino acid substitution. Patient 208 is deleted for a single adenine nucleotide from position 734, causing a frameshift. Patient 213 has suffered a C-to-G transversion at position 680, causing an isoleucine-to-methionine amino acid substitution. *Bottom,* Schematic map of *SRY*. The boxed region represents the open reading frame extending from nucleotide position 354 to nucleotide position 1022 of the genomic clone pY53.3. The HMG-related box, which lies between positions 582 and 821, is marked as an open box. The positions of oligonucleotide PCR primers are indicated by horizontal arrows. The positions of restriction endonuclease sites used for SSCP analysis are marked between primers XES7 and XES2. The cleavage sites are *Dde*I (D), 536 and 672; *Hin*fI (H), 654; *Hpa*II (Hp), 495; *Pst*I (P), 874; *Taq*I (T), 798; and *Xba*I (X), 648. The patient numbers and mutations in this study are indicated below the gene. Above the gene are the positions of *SRY* mutations described elsewhere: JN and AA from Berta et al. (1990); CHM039 and Cal from Hawkins et al. (1992); and JS from Jäger et al. (1990).

The father and normal brother of patient 208, as well as the father and normal brother of patient 213, were checked for the presence of the *SRY* sequence variation, by direct sequencing of the *SRY* fragment. The father and brother of patient 208 do not share the deletion, indicating that this mutation is de novo. The father and brother of patient 213, however, both share the mutation. Familial relationships were confirmed by Southern blotting with minisatellite probes MS1, MS31, MS32, MS41, and pλg3 (Wong et al. 1986, 1987) (data not shown). Analysis of the father of patient 207 was not possible, as DNA was unavailable. To confirm that the mutations in patients 207 and 213 are not polymorphisms, 78 unrelated normal Caucasian males were tested for the presence of these alterations, by allele-specific oligonucleotide hybridization. The mutations were not present in any of these control samples (data not shown).

Discussion

Both the SSCP assay and DNA sequencing were used for the analysis of mutations in the *SRY* open reading frame, in this study and in another study by Hawkins et al. (1992). Of the five *SRY* mutations identified by these two studies, those of patients 207 (described here) and CHM039 (described in the other study) failed to give SSCP band shifts in the three digests performed. Patient CHM039 did show a subtle band shift on a further digest, so it may be that the mutation in CHM039 and 207 are at the edge of SSCP detectability or that conditions employed were not optimal.

Three of the five subjects in this study had mutations in *SRY*. Patient 208 suffered a de novo mutation causing a long frameshift with a termination codon at nucleotide positions 949–951, presumably resulting in a grossly abnormal protein. The point mutation of patient 207, resulting in a lysine-to-isoleucine amino acid substitution, could not be confirmed as being de novo, as the father was unavailable for study. Patient 213 presented a point mutation that caused an isoleucine-to-methionine amino acid substitution.

The base change in patient 213 was also carried by the patient's normal father and brother, suggesting that this variant is not the cause of sex reversal. The paternal lineage of the family is of European descent. To address the question of whether this variant is a neutral polymorphism, 78 unrelated British males were tested for the variant, but none were positive. These data combined with the sequence analysis of 22 additional XY female patients (Hawkins et al. 1992, and present study; Ogata et al. 1992) rule out the 213-type base change in 100 Caucasian Y chromosomes. Indeed, the *SRY* region of the Y chromosome is known to have a very low level of polymorphism (Jakubiczka et al. 1989; Ellis et al. 1990; Malaspina et al. 1990). It remains possible that the 213 mutation is a low-frequency variant with no phenotypic consequence; however, the recent findings of Harley et al. (1992), who demonstrated that the SRY in vitro DNA-binding activity is reduced in patient 213, support the hypothesis that the 680 C-to-G mutation is the cause of sex reversal. Variable penetrance of disease-causing mutations can be due to the pleiotropic effects of other genes, and one explanation for the behavior of the 213 mutation is SRY–target site variation. The 213 mutation may be analogous to the mouse *poschiavinus* Y-linked inherited mechanism of sex reversal (Eicher et al. 1982), in which an autosomal locus (*Tda-1*) is believed to be influencing gonadal development (Eicher and Washburn 1983). Palmer and Burgoyne (1991) have proposed that the *poschiavinus Sry/Tdy* allele is late acting. It may therefore be that the inherited human *SRY* mutations are late acting because it takes longer to reach the threshold required to trigger testis determination. In the case of sex reversal the threshold may not be reached before ovarian development ensues.

The father of patient 207 was unavailable for study. This patient is of African-American origin, and polymorphism cannot be ruled out despite the base change not being present on 100 Caucasian Y chromosomes. However, the data of Harley et al. (1992) indicate that the in vitro DNA-binding activity of this patient is drastically reduced, strongly suggesting that the mutation is responsible for the phenotype.

It is interesting that the three *SRY* mutations described here and the five described elsewhere (Berta et al. 1990; Jäger et al. 1990; Hawkins et al. 1992) all lie in the HMG-related box (see fig. 2, bottom panel) and presumably reduce SRY DNA-binding activity. Even conservative amino acid substitutions in the HMG-related box have been correlated with sex reversal. Complete loss of function of the 3′ end of the box and the region 3′ to the box (the frameshift mutations in patients 208 and JS; Jäger et al. 1990) produces a phenotype similar to that produced by more subtle changes in the box. The absence of any mutations that affect the 5′ end of the gene from bp 354 to bp 582 (fig.

2, bottom panel) leaves open the question of whether mutations in this region are unimportant or possibly lethal.

It is noteworthy that three (60%) of five patients with 46,XY complete gonadal dysgenesis in our study had mutations in *SRY*, whereas only 5 (12.2%) of the 41 subjects with various forms of 46,XY gonadal dysgenesis evaluated in earlier studies were demonstrated to be mutant for *SRY* (Berta et al. 1990; Jäger et al. 1990; Hawkins et al. 1992; Ogata et al. 1992). The higher frequency of *SRY* mutations in our study may be due to the small sample size or to differences in criteria used for patient selection. However, the probability of obtaining three or more *SRY* mutations in a sample of five patients on the basis of published and unpublished frequencies is significantly low, and it is therefore unlikely that this result is due to chance. The five patients described here had complete gonadal dysgenesis, confirmed by histological examination. The other 41 subjects previously tested for *SRY* mutations included a majority of patients not investigated at the histological level; it is conceivable that some of them presented other abnormalities of sex differentiation. Such patients would have a normally functioning *SRY* gene but would be mutant in other genes required for normal testis differentiation. By selecting 46,XY subjects with histologically confirmed complete gonadal dysgenesis, it is likely that we enriched for mutations in *SRY*. Our studies underscore the importance of careful phenotypic evaluation of subjects with abnormalities of sex differentiation.

The *SRY* genes of two of the five patients described here were apparently normal. These patients could be either mutant for other genes in the testis-determining pathway or mutant in sequences flanking that examined here. The data from patients 212 and 217 indicate that the same clinical phenotype might arise from mutations in genes other than *SRY*. It is likely, then, that these two patients are mutant for genes close to *SRY* in the testis-determining pathway. Analysis of additional patients with the complete and partial forms of gonadal dysgenesis may ultimately provide information about the other genes involved in testis determination and differentiation.

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