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Autosomal XX Sex Reversal Caused by Duplication of *SOX9*

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***SOX9* is one of the genes that play critical roles in male sexual differentiation. Mutations of *SOX9* leading to haploinsufficiency can cause campomelic dysplasia and XY sex reversal. We report here evidence supporting that *SOX9* duplication can cause XX sex reversal. A newborn infant was referred for genetic evaluation because of abnormal male external genitalia. The infant had severe penile/scrotal hypospadias. Gonads were palpable. Cytogenetic analysis demonstrated a de novo mosaic 46,XX,dup(17)(q23.1q24.3)/46,XX karyotype. Fluorescent in situ hybridization (FISH) with a BAC clone containing the *SOX9* gene demonstrated that the *SOX9* gene is duplicated on the rearranged chromosome 17. The presence of *SRY* was ruled out by FISH with a probe containing the *SRY* gene and polymerase chain reaction with *SRY*-specific primers. Microsatellite analysis with 13 markers on 17q23-24 determined that the duplication is maternal in origin and defined the boundary of the duplication to be approximately 12 centimorgans (cM) proximal and 4 cM distal to the *SOX9* gene. Thus, *SOX9* duplication is the most likely cause for the sex reversal in this case because it plays an important role in male sex determination and differentiation. This study suggests that extra dose of SOX9 is sufficient to initiate testis differentiation in the absence of *SRY*. Other *SRY*-negative XX sex-reversed individuals deserve thorough investigation of *SOX9* gene. Am. J. Med. Genet. 87:349–353, 1999.** © 1999 Wiley-Liss, Inc.

KEY WORDS: sex determination; sexual differentiation; 17q duplication; FISH; microsatellite analysis

INTRODUCTION

Human sex determination and differentiation are complex processes with the *SRY* (sex determining region, Y chromosome) gene considered the major determinant of testis development [Simpson et al., 1987; Berta et al., 1990; Jager et al., 1990; Koopman et al., 1991]. Female-to-male sex reversal in karyotypic XX individuals is most frequently due to the presence of *SRY*, either due to an X-Y translocation or low-level XXY/XX mosaicism [de la Chapelle et al., 1990; Fechner et al., 1993]. However, approximately 10–20% of the XX males do not have any detectable Y chromosome material [de la Chapelle et al., 1990]. These observations, along with the observations of XY females with an intact, nonmutated *SRY* gene, suggest the presence of autosomal genes in the male sexual differentiation pathway initiated by *SRY*. One of the genes in the sexual differentiation and developmental cascade, *SOX9*, was identified in individuals with campomelic dysplasia and XY sex reversal [Foster et al., 1994; Wagner et al., 1994]. *SOX9* mutations identified in these individuals result in loss-of-function products, suggesting that reduced gene dosage (haploinsufficiency) plays a role in altering the male developmental pathway. A dominant negative role of the mutated SOX9 protein was ruled out recently when a deletion of *SOX9* was reported in an XY sex reversed individual with campomelic dysplasia [Olney et al., 1999].

SOX9 haploinsufficiency can cause XY sex reversal. Supporting the hypothesis that *SOX9* duplication can cause XX sex reversal, we report here *SOX9* duplication in a phenotypically male infant with XX karyotype. To our knowledge, this is the first demonstration of XX sex reversal caused by an autosomal gene.

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MATERIALS AND METHODS

Clinical History

An infant boy was referred due to abnormal external genitalia. The infant had severe penile/scrotal hypospadias. The phallus was 0.5 cm in diameter and 1.2 cm long. The opening of the urethral meatus was at the base of the phallus. The perineal surface was closed between the urethral meatus and the anal opening. The scrotum was bifid with palpable gonads. Endoscopic or surgical visualization of the internal genitalia has not been done. Ultrasound examination did not identify a uterus. Full-body skeletal radiographs were normal. The remainder of the physical findings were normal. Congenital adrenal hyperplasia was not found as the serum electrolytes; 11-deoxycortisol and 17-hydroxyprogesterone were normal. Gonad biopsy was not performed at this time because of the young age.

Cytogenetic and Fluorescence In Situ Hybridization Studies

A peripheral blood specimen was collected at age 2 days and a skin biopsy specimen at age 1 month. Lymphocyte culture, skin fibroblast culture, harvest, and GPG banding were performed using standard methods.

Fluorescence in situ hybridization (FISH) studies with a chromosome 17 painting probe (Vysis, Downers Grove, Illinois) were performed according to the manufacturer's protocol. FISH studies with a probe containing *SRY* (Genzyme Genetics) and a BAC clone (RPCI-11 BAC clone 84E24, Research Genetics, Huntsville, Alabama) containing the *SOX9* gene were also performed following standard protocol. The presence of *SOX9* in this BAC clone was confirmed by polymerase chain reaction (PCR) with *SOX9* specific primers [Foster et al., 1994] and by verifying the sequence of the clone (GeneBank accession #AC007461).

Molecular Studies

To further determine whether *SRY* gene is present in this patient, multiplex PCR for *SRY* and β-actin were performed on DNA isolated from skin fibroblasts of the patient and blood of parents. The β-actin gene serves as internal positive control for PCR. *SRY* primers are as described [Lo et al., 1998]. Sequences of the forward and reverse primers of β-actin are ATCGTGATGGACTCCGGTGAC and GCTGATCCACATCTGCTGGA, respectively. PCR was performed in a Perkin-Elmer GeneAmp 2400 thermal cycler in a volume of 25 μl consisting of 10 pmol of each of the *SRY* and β-actin primers, 100 ng of genomic DNA, 200 μM of dNTPs, 1 U of AmpliTaq DNA polymerase in buffer containing 2 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris (pH 8.3). Following initial denaturing at 94°C for 3 min, amplifications were performed for 30 cycles of 94°C denaturing for 15 sec, 55°C annealing for 15 sec, and 72°C for 30 sec. PCR products were electrophoresed in 2% agarose gel, and bands were visualized under ultraviolet light after ethidium bromide staining.

Microsatellite PCR analyses were performed on the DNA isolated from skin fibroblasts of the patient and the blood specimens from both parents with 13 microsatellite markers on 17 q23-24. The markers studied are listed in Table I. The primer sequences, genetic map position, and distance were described previously [Foster et al., 1994; Dib et al., 1996]. PCR and denaturing polyacrylamide gel electrophoreses were performed as described previously [Christian et al., 1995].

RESULTS

Cytogenetic and FISH Analysis

Cytogenetic analysis demonstrated a mosaic 46,XX,dup(17)(q23.1q24.3)/46,XX karyotype. The 17q duplication was found in 34% (17/50) of the lymphocytes and 78% (39/50) of the skin fibroblasts. The chromosome 17 homologs are illustrated in Figure 1. Both parents had normal chromosomes.

FISH with a chromosome 17 painting probe (Vysis) hybridized to the entire length of both chromosome 17 homologs (data not shown), confirmed that the duplicated region is chromosome 17 in origin. The *SRY* probe did not hybridize to any of the chromosomes (data not shown). FISH with the BAC clone containing

TABLE I. Microsatellite Analyses of the Duplicated Region

Microsatellite markers	Map distance (cM)[a]	Genotypes			Result interpretation
		Father	Mother	Child	
D17S787	7.2	33	12	23	Not duplicated
D17S1604	1.3	12	23	23	Not duplicated
D17S794	10.6	12	12	112	Duplicated
D17S840	1.0	11	12	Homozygote 1	Uninformative
D17S1350	<1.4[b]	12	22	Homozygote 2	Uninformative
D17S970	<1.4[b]	22	12	Homozygote 2	Uninformative
D17S1351	2.1	13	23	223	Duplicated, mat
D17S1352	1.1	22	13	112	Duplicated, mat
D17S1807	0	12	12	112	Duplicated
D17S929	0	12	22	12	Not duplicated
D17S1864	4.3	22	13	23	Not duplicated
D17S1603	0	12	11	12	Not duplicated
D17S785	0	12	13	12	Not duplicated

[a]Sex-averaged distance to the next marker.
[b]D17S970 is mapped between D17S1350 and D17S1351.
Sex-averaged distance between D17S1350 and D17S1351 is 1.4 cM.
SOX9 gene is located between D17S970 and D17S1351.
mat, maternal in origin.

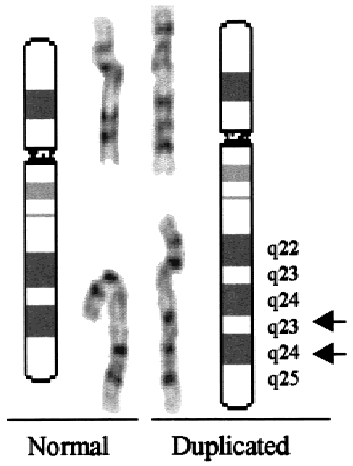

Fig. 1. Partial karyotype of two cells showing the normal (left) and the duplicated (right) chromosomes 17. Arrows indicate the duplicated region on the ideogram.

the *SOX9* gene showed one signal on the normal chromosome 17 and two signals on the rearranged chromosome 17 (Fig. 2), demonstrating that the *SOX9* gene is duplicated.

Molecular Studies

PCR with *SRY* specific primers failed to amplify the DNA from the patient and the mother but did amplify the *SRY* fragment from the father (Fig. 3). Therefore, the XX sex reversal in this case is unlikely to be caused by the presence of *SRY*.

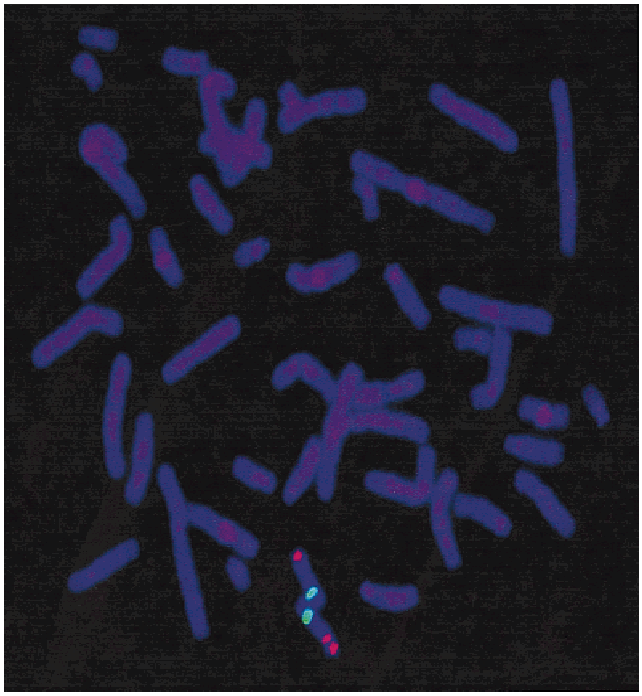

Fig. 2. FISH showing *SOX9* duplication. The chromosome 17 centromeric probe is shown in green and the *SOX9* probe shown in red. One *SOX9* hybridization signal was observed on the normal chromosome 17, and two signals were seen on the rearranged chromosome 17.

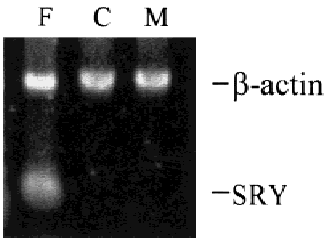

Fig. 3. *SRY* sequences not detected in the patient by PCR. F, father; C, child; M, mother. β-actin serves as the positive internal control for PCR.

To define the duplication boundary at the molecular level and to investigate the possible mechanism for this duplication, microsatellite analyses were performed. The results are illustrated in Figure 4 and Table I. The child inherited two copies of one allele from the mother, suggesting the duplication is maternal in origin. These results are consistent with a maternal mitotic unequal crossover event leading to mosaicism. The microsatellite analyses also demonstrated that the duplicated region extends approximately 12 cM proximal and 4 cM distal to the *SOX9* gene. The proximal and distal breakpoints were mapped between D17S1604 and D17S794 (1.3-cM genetic distance) and between D17S1807 and D17S929 (0-cM genetic distance), respectively.

DISCUSSION

XX sex reversal cases are relatively rare and mostly caused by the presence of the *SRY* gene [de la Chapelle et al., 1990; Fechner et al., 1993]. Most *SRY* negative XX males have a high incidence of genital ambiguity or hypospadias, which are not common in *SRY* positive XX males. XX males and XX true hermaphrodites may exist in the same family, and the inheritance pattern leading to XX males is consistent with either X-linked, autosomal dominant, or both modes of inheritance [Kasdan et al., 1973; Skordis et al., 1987; de la Chapelle et al., 1990]. These findings suggest the presence of autosomal sex determining genes "down-stream" to *SRY* that can lead to masculinization in XX individuals.

Human sex development is a complex process. Male sex differentiation requires delicate dosage balance and interaction among multiple genes including *SRY, SOX9, DAX-1, SF-1*, and *WT-1*, and female sex differentiation acts as a 'default' pathway in the absence of *SRY* [Capel, 1998; Parker and Schimmer, 1998; Parker et al., 1999]. Several lines of evidence suggest that *SOX9* plays an important role in male sex differentiation. Its expression is up-regulated in developing testes but absent in developing ovaries [Morais da Silva et al., 1996]. By direct interaction with *SF-1* gene, *SOX9* is also involved in the regulation of Sertoli cell-specific expression of anti-Müllerian hormone, which is required for Müllerian duct regression and phenotypic male differentiation [De Santa Barbara et al., 1998]. More importantly, *SOX9* haploinsufficiency caused by mutations, balanced chromosome rearrangements, and deletions can cause campomelic dysplasia and XY

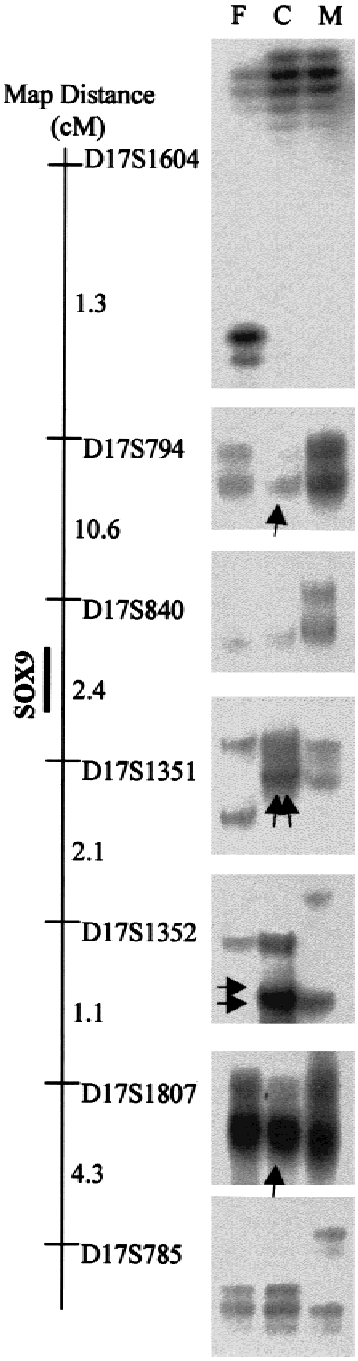

Fig. 4. Microsatellite analysis to define the boundary and the parental origin of the duplication. Single arrows identify duplicated alleles (alleles with higher intensity) and double arrows indicate the duplicated alleles are maternal in origin. The map (not to the scale) of the sex-averaged genetic distance between markers is illustrated with the position of *SOX9* indicated.

male-to-female sex reversal [Foster et al., 1994; Wagner et al., 1994; Olney et al., 1999].

In this report, we identified an *SRY*-negative female to male sex reversal patient with a duplication of chromosome band 17q23-24 including the *SOX9* gene. Because of its important role in testis determination and differentiation, *SOX9* duplication apparently is the most likely cause of the sex reversal in this case. However, not all XX individuals with a duplication of 17q23-24 have sex reversal, and several XX female patients with larger 17q duplications due to unbalanced translocations have been reported [Feldman et al., 1982; Lenzini et al., 1988; Caine et al., 1989]. The lack of sex reversal in these previously reported 17q duplication cases exemplifies the complex nature of mammalian sex determination and differentiation. The same phenomena have been observed in the other published findings of sex reversal cases. For example, not all XY patients with *SOX9* haploinsufficiency have sex reversal [Foster et al., 1994; Schafer et al., 1996]. In fact, an identical mutation in *SOX9* was found in two XY campomelic dysplasia patients, one with a male phenotype and the other with sex reversal [Kwok et al., 1995]. In another similar situation, duplication of *DAX-1* or *DSS* gene was demonstrated to cause XY sex reversal [Bardoni et al., 1994; Zanaria et al., 1994], but only a portion of XY patients with *DAX-1* duplication have sex reversal [Bardoni et al., 1994]. In a transgenic mouse study, it was found that high dose of *Dax1* caused complete male to female sex reversal only in mice with weak Sry alleles [Swain et al., 1998].

If replicated in other *SRY*-negative XX sex-reversal patients, our study has the following significance. First, it demonstrates that duplication of *SOX9* can cause XX sex reversal in absence of *SRY* and suggests that an extra dose of *SOX9* is sufficient to initiate testis differentiation. Second, as *DAX-1* is the first gene found to cause XY sex reversal when duplicated, *SOX9* is the first gene identified that may cause XX sex reversal when duplicated. Third, in the present case, the duplication of chromosome area 17q23-24 is present at a much lower percentage in lymphocytes than that in skin fibroblasts. This finding warrants study of other cell types besides peripheral blood lymphocyte in search for *SOX9* duplication in *SRY* negative XX sex reversal cases.

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