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Clinical Report

45,X/47,XXX/47,XX, del(Y)(p?)/46,XX Mosaicism Causing True Hermaphroditism

Karem Nieto,[1] Rocío Peña,[2] Icela Palma,[1] Luis M. Dorantes,[2] Luis Eraña,[2] Rebeca Alvarez,[1] Ricardo García-Cavazos,[3] Susana Kofman-Alfaro,[1] and Gloria Queipo[1]*

[1]*Department of Genetics, Hospital General de Mexico-Facultad de Medicina, UNAM, Mexico City, Mexico*
[2]*Department of Pathology, Endocrinology and Urology, Hospital Infantil de Mexico Federico Gomez, Mexico City, Mexico*
[3]*Biomedical Research Department, Instituto Nacional de Perinatologia, Mexico City, Mexico*

Sex differentiation in humans depends on the presence of the Y-linked gene SRY, which is activated in the pre-Sertoli cells of the developing gonadal primordium to trigger testicular differentiation. Occasionally testicular formation can take place in subjects lacking a Y chromosome resulting in a 46,XX sex reversal condition. True hermaphroditism (TH) is a rare form of intersexuality characterized by the presence of testicular and ovarian tissue in the same individual. Genetic heterogeneity has been proposed as a cause of dual gonadal development in some cases and recently, hidden mosaicism was reported to cause TH in some 46,XX SRY negative patients. Here we report a TH case in which hidden mosaicism for the Y and X chromosome was detected by PCR and FISH in peripheral blood and gonadal tissue, supporting the fact that mosaicism may cause TH and that molecular analysis of gonadal tissue should be performed in all 46,XX cases.

KEY WORDS: sex reversal; true hermaphroditism; SRY gene; gonadal mosaicism; sex differentiation

INTRODUCTION

True hermaphroditism (TH) is a form of intersexuality, characterized by the presence of well-differentiated testicular and ovarian elements in the same individual [Van Nierkerk and Retief, 1981]. Approximately 60% TH individuals have a 46,XX karyotype, 33% are mosaics with a second cell line containing a Y chromosome, while the remaining 7% are 46,XY [Krob et al., 1994]. Molecular analyses have demonstrated that SRY is present in only 10% of THs with a 46,XX karyotype [Berkovitz et al., 1992; McElreavey et al., 1993; Boucekkine et al., 1994; Damiani et al., 1997] therefore, in the remaining 90% mutations at unknown X-linked or autosomal sex determining loci have been proposed as factors responsible for testicular development [McElreavey et al., 1993; Jimenez et al., 1996]. TH presents considerable genetic heterogeneity as several molecular anomalies may lead to gonadal development in a single subject: (a) 46,XX SRY positive cases; (b) in three 46,XY unrelated cases, point mutations in the SRY coding region were observed [Braun et al., 1993; Hiort et al., 1995; Maier et al., 2003]; and c) Hadjiathanasiou et al. [1994] and Inoue et al. [1998] proposed gonadal SRY mosaicism to explain in two sporadic 46,XX patients who were SRY negative in peripheral blood leukocytes but positive in gonadal tissue.

In 1998 we described a 46,XX TH in which SRY was not detectable in leukocytes and epithelial oral cells, but present and partially deleted in DNA obtained from the testicular portion of the ovotestis [Jimenez et al., 2000]. Two years later, the presence of mosaicism for Y-derived sequences was confirmed as cause of TH etiology in these patients [Ortenberg et al., 2002; Queipo et al., 2002]. Here we report on a new TH case in which mosaicism for the Y and X chromosome was detected by PCR and FISH in peripheral blood and gonadal tissue, supporting the fact that mosaicism may cause TH and that molecular analysis of gonadal tissue should be done in all 46,XX cases.

SUBJECTS AND METHODS

The propositus was evaluated at 12 years for ambiguous genitalia; he had a 1.7 cm phallus, labioscrotal folds and urogenital sinus without previous operation. Growth was normal and no other physical abnormalities were found during clinical examination. Karyotype using a conventional GTG technique in 100 metaphases was 46,XX. An hCG stimulation test (1,500 IU/24 hr/4 days) yielded a serum testosterone of 0.89 ng/ml (reference value 3–10 ng/ml). Exploratory surgery demonstrated a right Fallopian tube and hypoplastic uterus. Gonadal biopsies showed an ovary on the left side and contralateral ovotestis. Both gonads were removed.

Genomic DNA was isolated from peripheral blood leukocytes and formalin-fixed paraffin embedded ovotestis, according to standards protocols [Sambrook et al., 1989; Banerjee et al., 1995].

PCR Amplification

PCR analysis for the Y-specific sequences PABY, SRY, AMELY, Ycen, and Yqh were performed in DNA obtained from leukocytes and ovotestis. We analyzed the SRY open reading frame (615 bp) using two sets of primers: (1) SRY 6-forward (5′-GTG GTC TCG CGA TCA GA-3′) and XES-11-reverse (5′-GTA GCC AAT GTT ACC CGA TTG TC-3′) that amplifies a 432-bp fragment from nucleotide 207 to nucleotide 638 with respect to the initial SRY ATG codon; (2) XES10 forward (5′-GGT GTT

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*Correspondence to: Gloria Queipo, Department of Genetics, Hospital General de México-Facultad de Medicina, UNAM. Dr. Balmis 148 Col. Doctores CP 06726, Mexico City, Mexico.
E-mail: skofman@servidor.unam.mx

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GAG GGC GGA GAA ATG C-3′) and SRY-8 reverse (5′-TAG AGC CAT CTT GCG CCT-3′), which amplifies a 380-bp fragment from nucleotide −140 to nucleotide 240. Additionally, we used an extra set of primers SRY 6-forward (5′-GTG GTC TCG CGA TCA GA-3′) and SRY-3 reverse (5′-CTT CCG ACG AGG TCG ATA C-3′) amplifying a 196-bp fragment from nucleotide 206 to nucleotide 402. The total PCR volume was 25 μl; all amplifications include 50 ng of DNA, 100 ng of each primer, 1 U of Taq polymerase, 1.5 mM $MgCl_2$, and 80 μM of each dNTP. The amplification program for SRY was: 94°C (5 min), 94°C (1 min), 68°C (1 min), and 72°C (2 min) for 35 cycles and then 72°C (10 min). All the assays were performed in triplicate including a blank as negative control and DNA from a normal male as a positive control. An X-chromosome centromeric alphoid repeat was also used as an internal control.

FISH Analysis

FISH analysis was carried out in metaphase and interphase lymphocytes and on histological sections of routine-processed ovotestis, according to standard protocols [Pinkel et al., 1986a,b]. The probes used during the protocol were for the centromeric regions of X, Y and chromosome 18 labeled with spectrum green™, spectrum red™, and spectrum aqua™, respectively (Vysis). For Y chromosome painting, we utilized a whole chromosome painting probe labeled with spectrum orange. As an internal control, we used a centromeric region labeled with spectrum green.

RESULTS

PCR amplification of Y-derived sequences from the DNA of leukocytes and ovotestis demonstrated the presence of Ycen and Yqh; all Yp sequences (PABY, SRY, and AMELY) were absent (Fig. 1). These PCR data suggest the presence of a second cell line containing a Y chromosome with a short arm deletion distal to AMELY at Yp11.2. In order to verify the presence of this second cell line, we performed a new chromosome analysis with GTG and C banding, no second cell line could be identified in 200 metaphases.

To confirm the absence of an SRY (Sex Region on Y chromosome) cell line, we used FISH analysis to compare the gonadal tissue of our patient with normal embedded testicular tissue and ovotestis from a previously reported TH patient 46,XX-SRY negative in peripheral blood but positive in ovotestis [Queipo et al., 2002]. No SRY hybridization signal was observed in metaphases from peripheral blood and in the gonadal tissue of our patient, ruling out presence of an SRY containing cell line (Fig. 2A–C).

Six hundred interphase nuclei were analyzed by FISH using centromere probes X, Y, and 18. The results showed that most of the cells had two XX hybridization signals; however, a cell line with a Y chromosome was recognized in 1% of the nuclei. In all Y-positive cells, two X centromere signals were present indicating an XXY complement. We also found unexpected 45,X and 47,XXX cell lines (Fig. 2D), making the final interphase nuclei count: *nuc ish* Xcen(DXZ1 × 1)[9]/Xcen(DXZ1 × 3)[3]/Xcen(DXZ1 × 1),Ycen(DYZ3 × 1)[3]/Xcen(DXZ1 × 2)[231], suggesting a 45,X[9]/47,XXX[3]/47,XXdel(Y)(p?)[3]/46,XX [231] final karyotype. To rule out the possibility of a Y/autosome translocation, we performed FISH analysis using a whole Y chromosome painting probe. Analysis of 150 metaphases failed to show an isolated Y chromosome signal or a Y chromosome translocation (Fig. 2E).

The FISH analysis of gonadal tissue from different sections of the ovotestis demonstrated the same *nuc ish* result observed in peripheral blood and similar cell line proportions (Fig. 2F).

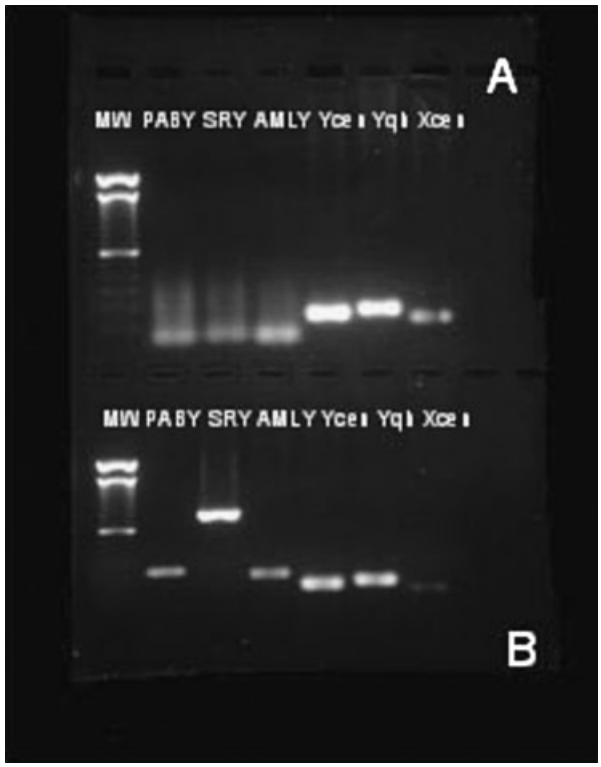

Fig. 1. PCR analysis of Y-derived sequences in leukocytic DNA from a 46,XY normal male (**A**) and patient (**B**). All Y sequences tested were present in the male control, while only Ycen and Yqh were observed in our patient.

DISCUSSION

We report on a new 46,XX-SRY negative TH patient in whom PCR analysis showed absence of all the Yp sequences analyzed, including the sex determining gene SRY. However, amplification showed presence of Ycen and Yqh. FISH analysis performed in peripheral blood and gonadal tissue of this patient documented the presence of several cell lines involving the X chromosome (45,X and 47,XXX) and a 47,XX,del(Y)(p?) cell line. Although, in most reports, patients with 46,XX/46,XY karyotypes are a result of chimerism; in this particular case, the observation of a 47,XX,del(Y)(p?) cell line may represent a mosaicism originating from a 47,XX,del(Y)(p?) zygote, as originally proposed by Wit et al. [1987]. The different cell lines observed in our patient 45,X,47,XX,del (Y)(p?),47,XXX,46,XX could be the result of a sequence of events during cell division in early embryogenesis, where anaphase lag of the abnormal Y chromosome in a 47,XX,del(Y)(p?) zygote, led to 47,XX,del(Y)(p?) and 46,XX cell lines. This aneuploid product predisposed to later sequential non-disjunction of the X chromosome, leading to the low level mosaicism observed. Since Yp sequences PABY, SRY, and AMELY were not observed by PCR, a short arm deletion is suggested. These data were confirmed by the absence of an SRY signal using FISH analysis. Therefore, testicular determination in this patient cannot be attributed to the SRY gene, rather, as it was previously proposed, to abnormalities either in X-linked or autosomal sex determining loci participating in testicular development [McElreavey et al., 1993; Vilain, 2002]. On the other hand, the presence of X polysomy in our patient is an interesting finding that may be related to the gonadal phenotype. Recently, Aviv et al. [2001], studying a 46,X,del

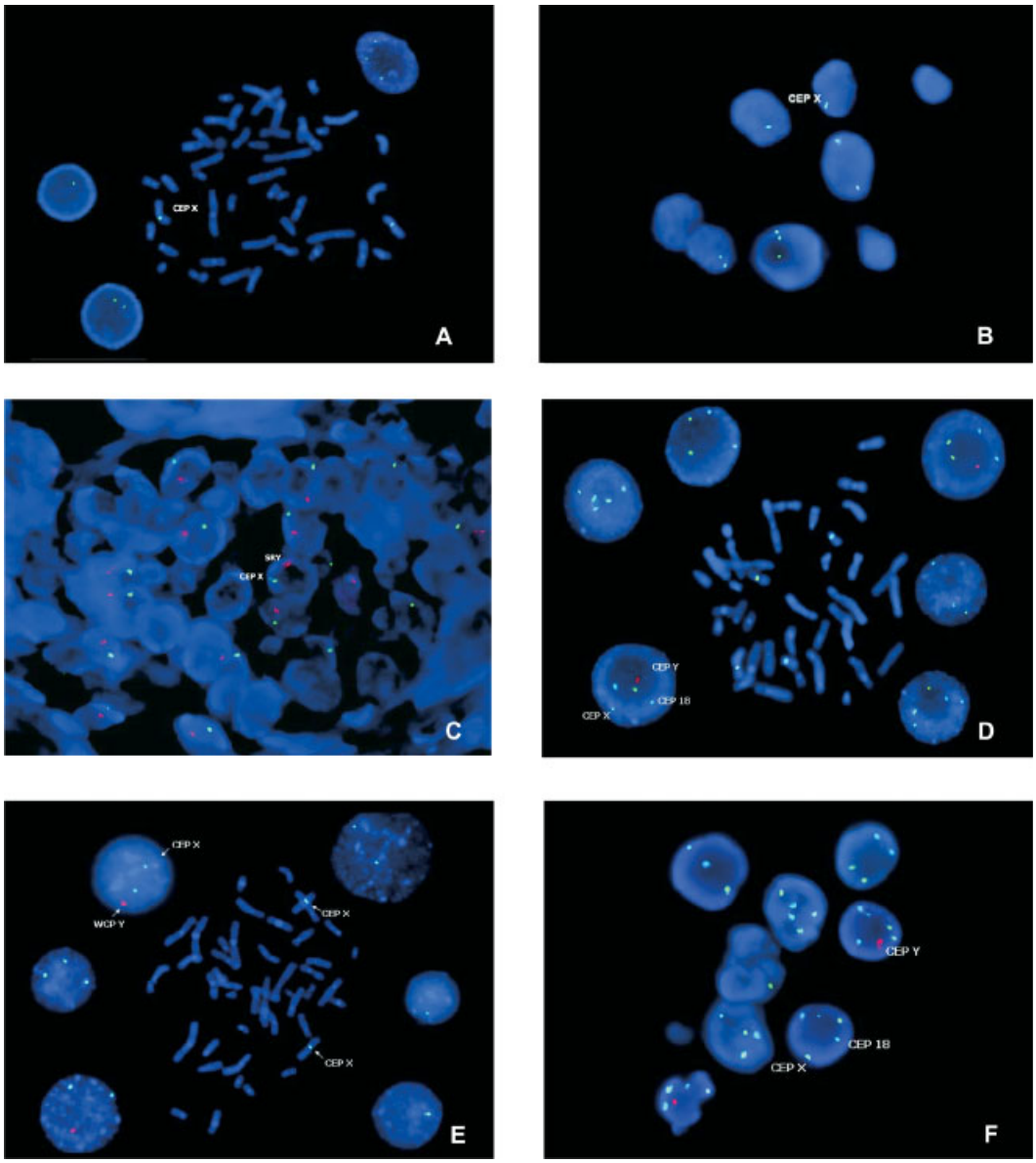

Fig. 2. FISH analysis using an SRY probe labeled in red and X centromere probe labeled in green. **A**: Metaphase and interphase nuclei in peripheral blood of the patient; showing only the X hybridization signal. Notice the absence of SRY hybridization in all cells. **B**: The analysis of gonadal tissue shows the same pattern as (A). **C**: Normal testicular tissue used as control showing both SRY an X centromere hybridization signals. **D**: FISH analysis using a Y, X, and 18 chromosome centromeric probes label with spectrum red, green, and aqua, respectively. Metaphase and interphase nuclei show different cell lines involving the X chromosome. **E**: FISH analysis using a whole Y-chromosome painting probe (red) and an X-chromosome centromeric probe (green). The metaphase shows only two X cen signals (46,XX), while the nuclei exhibit the different cell lines observed in (D). **F**: Formalin fixed paraffin embedded gonadal tissue shows the pattern observed in (D) and (E).

(Yp) SRY negative fetus, observed the presence of X polysomy restricted to the ovary, and suggested that this represents a postzygotic event which facilitates ovarian development in the absence of the necessary factors for normal gonadal development [Aviv et al., 2001]. Using transgenic mice, Burgoyne et al. [2001] showed that the presence of non-dosage compensated gene(s) on the X chromosome appear to participate in the testis-determining pathway. Our findings support the idea that mosaiscm for Y-derived sequences participate in producing the abnormal gonadal phenotype in 46,XX SRY negative TH patients and suggest that X polysomy may also be involved in gonadal development.

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