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A minority of 46,XX true hermaphrodites are positive for the Y-DNA sequence including SRY

Ken McElreavey[1], Raphaël Rappaport[2], Eric Vilain[1], Nacer Abbas[1], François Richaud[1], Stéphen Lortat-Jacob[2], Roland Berger[3], Maryvonne Le Coniat[3], Chafika Boucekkine[4], Kiran Kucheria[5], Samia Temtamy[6], Claire Nihoul-Fekete[2], Raja Brauner[2], and Marc Fellous[1]

[1] Immunogénétique Humaine, Institut Pasteur, 25, Rue du Dr. Roux, F-75015 Paris, France
[2] Hôpital des Enfants Malades, 149, Rue de Sèvres, F-75015 Paris, France
[3] Unité INSERM U301, Institut de génétique moléculaire, Paris, France
[4] Service d'Endocrinologie, Hôpital des Bains Romains, Alger, Algéria
[5] All India Institute of Medical Sciences, New Dehli 110029, India
[6] NRC, Tahrir Street, Dokki, Cairo, Egypt

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Summary. A total of 30 cases of 46,XX true hermaphroditism was analysed for Y-DNA sequences including the recently cloned gene for male testis-determination SRY. In 3 cases, a portion of the Y chromosome including SRY was present and, in 2 cases, was localised, to Xp22 by in situ hybridisation. Since previous studies have shown that the majority of XX males are generated by an X-Y chromosomal interchange, the Xp22 position of the Yp material suggests that certain cases of hermaphroditism can arise by the same meiotic event. The phenotype in the 3 SRY-positive cases may be caused by X-inactivation resulting in somatic mosaicism of testis-determining factor expression giving rise to both testicular and ovarian tissues. Autosomal or X-linked mutation(s) elsewhere in the sex-determining pathway may explain the phenotype observed in the remaining 27 SRY-negative cases.

Introduction

In mammals, male sex determination depends on the presence of the Y chromosome. However, certain rare individuals with a 46,XX karyotype develop testes. The majority, known as XX males (de la Chapelle 1987; Abbas et al. 1990), have normal male internal and external genitalia. Other XX males have genital ambiguities with incomplete masculinisation of external genitalia and persistence of Müllerian structures, a uterus and Fallopian tubes (Abbas et al. 1990). Moreover, 46,XX true hermaphrodites are clinically distinct from 46,XX males by having specific gonadal abnormalities together with both testicular and ovarian tissue in the same individual (Van Niekerk 1981; Ramsay et al. 1988).

Correspondence to: K. McElreavey

XX males without ambiguous genitalia have limited portions of the Yp chromosome on one X chromosome, generated by accidental X-Y terminal interchange during paternal meiosis (Petit et al. 1987; Bishop et al. 1983; Seboun et al. 1986). In contrast, most XX males with genital ambiguities and, apart from 4 exceptions, all XX true hermaphrodites have been described as Y-negative using available Yp probes (de la Chapelle 1987; Abbas et al. 1990; Waibel et al. 1987), including a previous candidate male testis-determining gene ZFY (zinc finger on the Y; Page et al. 1987).

The 4 exceptions are a ZFY-negative individual found positive for the pseudoautosomal region of the Y chromosome (PABY) region (Jäger et al. 1990), an individual found to be positive for the male testis-determining gene (SRY; Sinclair et al. 1990), an SRY- and ZFY-positive case (Berkovitz et al. 1992) and a 46,XX true hermaphrodite positive for the PABY and SRY regions (Nakagome et al. 1991). Here, we analysed 30 cases of 46,XX true hermaphroditism for the presence of Yp material. This included the 46,XX true hermaphrodite previously described by Sinclair et al. (1990). Three 46,XX true hermaphrodites were found to be positive for Yp sequences including SRY and the pseudoautosomal boundary.

Materials and methods

Patients

Thirty patients, aged between 1 month and 24 years were studied. They originated from Algeria, Brasil, Egypt, India and France and were referred to genetic, surgical and/or endocrinology clinics as part of a collaborative study. All presented with ambiguous external genitalia classified according to Prader (1954) as types III or IV.

Twenty three cases were sporadic. Three families presented with 2 affected siblings in each family. One additional, previously

reported family included two siblings (Sinclair et al. 1990; Palmer et al. 1989); one presented as a 46,XX true hermaphrodite (case 1 of the present study), the other as a 46,XX phenotypic male (not included in the present study). In all cases, the diagnosis was based on gonad histology.

The phenotypes of the three Y-DNA-positive 46,XX true hermaphrodites were similar to the phenotype of the 27 cases of Y-DNA-negative true hermaphrodites in relation to the degree of external genital masculinisation, persistence of Müllerian structures and gonadal differentiation. Case 1 from France was the third child of a sibship of 4 (Sinclair et al. 1990). He had a type III Prader genital ambiguity with 2 ovotestes and a uterus with two Fallopian tubes; case 2 from Algeria had the same presentation as case 1 except for an ovary (left) associated with a non-ectopic ovotestis (right); case 3 from France was more masculinised, with a type IV Prader genital ambiguity, 2 non-ectopic ovotestes composed of 90% testis and 10% ovarian tissue, a left hemiuterus and Fallopian tube, and a right vas deferens. No testes were observed in cases 1, 2 or 3.

Cytogenetics

Chromosome analysis was performed on peripheral blood lymphocytes using Q- and R-banding. Karyotypes were performed on 40–50 metaphases from each patient. Chimerism and mosaicism were not detected in any of the cases.

Histology

Gonadal biopsies were fixed in 10% formalin overnight. After dehydration in a graduated series of alcohol and toluene solutions, each specimen was embedded in paraffin. Tissue sections (5 µm thick) were cut on a microtome (Leitz). They were stained with haematoxylin and eosin, dehydrated and mounted in Entellan (Merck: rapid mounting medium containing xylene and allycrylate). True hermaphroditism was diagnosed when testicular tissue containing tubules and Sertoli cells, and ovarian tissue containing primary follicules were found in the same patient.

DNA analysis

DNA was prepared from peripheral blood lymphocytes of the patients, and Southern blotting was performed as described previously (Southern 1975).

Polymerase chain reaction analysis

The polymerase chain reaction (PCR) was performed with a 30-mer-oligonucleotide derived from pseudoautosomal sequences (Oligo C) in conjunction with either Y-specific (Oligo A) or X-specific (Oligo B) 30-mer-oligonucleotides (Ellis et al. 1990). Further details are described elsewhere (Ellis et al. 1990). Oligonucleotide sequences were: A, GTACTACCTTTAGAAAACTAGTATTTTCCC; B, CTGCAGAAACAAGCTCATCAGCGTGACTAT; C, GAATTCTTAACAGGACCCATTTAGGATTAA.

Human DNA probes: Southern blot analysis

Several human Y-derived sequences were used to analyse the DNA of the XX males. Probes HFO.2, P0.9, DYS104, DYS13 and ZFY have been described previously (Page et al. 1987; Palmer et al. 1989; Ellis et al. 1989; Affara et al. 1986; Weissenbach et al. 1989). The SRY probe (pY53.3) recognizing the testis-determining factor (TDF) gene of the human Y chromosome was kindly given by Dr. P. Goodfellow (Sinclair et al. 1990). The genetic organisation of the short arm of the Y chromosome containing the sex-determining region corresponds to the following map: telomere – PABY – SRY – P0.9 – DYS104 – DYS13 – ZFY – centromere (Sinclair et al. 1990).

In situ hybridisation on metaphase chromosomes

Chromosome preparations were obtained from phytohaemagglutinin-stimulated blood cultures of a normal male and from cases 1 and 3 of 46,XX true hermaphroditism. Slides were stored at room temperature at least one week before use.

The *Hin*dIII insert of plasmid pY53.3 was used for in situ hybridisation essentially as described by Harper and Saunders (1981) with slight modifications (Caubet et al. 1985). The slides were exposed for 1 and 2 weeks to Kodak NTB2 emulsion. G-bands were obtained with Wright's stain.

Results

Molecular analysis

The presence of X and Y boundary sequences was assayed using PCR amplification with oligonucleotides A, B and C for each patient. With DNA prepared from all cases of 46,XX true hermaphroditism, a fragment of 0.77 kb was amplified when using oligonucleotides B and C; this corresponds to the X pseudoautosomal boundary. A 0.95-kb fragment, which is characteristic of PABY, was amplified in DNA preparations from 3 patients (cases 1, 2 and 3; Fig. 1). The PABY boundary was also detected using Southern hybridisation blots with the HF0.2 probe (Ellis et al. 1989) only in these 3 individuals (data not shown). The 3 patients harboured both the X and Y pseudoautosomal boundaries.

The three DNA samples from PABY-positive individuals were hybridised with pY53.3, which recognises the SRY open reading frame (Fig. 2A). The size and signal intensity of the bands were the same as in a normal male control. The position of the X/Y exchange was mapped using the Y-DNA probes P0.9, DYS104, DYS13 and ZFY. Hybridisation was observed using only the probe P0.9 (Fig. 2B). Hybridisation was not observed using any of the three other probes (data not shown). Consequently, the break points of the translocations

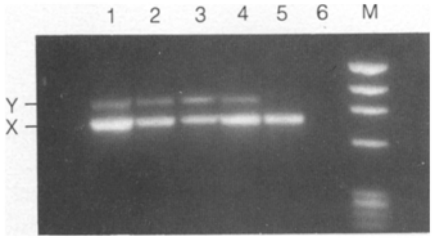

Fig. 1. PCR analysis of X- and Y-specific boundaries. Specific oligonucleotides for the X and the Y boundaries were used together with an oligonucleotide from the common pseudoautosomal region of the X and Y chromosomes. Specific amplification of the Y chromosome (noted *Y*; 0.95 kb) and X chromosome (noted *X*; 0.77 kb), were observed in 46,XX true hermaphrodite cases 1, 2 and 3 (*lanes 1, 2 and 3, respectively*). *Lanes 4 and 5* are male and female controls, respectively. *Lane 6* is a control with no DNA added; *lane M* is a molecular weight marker (PhiX174 *Hae*III fragments; 1353, 1078, 872, 603, 310, 271, 281, 234, 194, 118 and 72 bp)

were all localised between the Y-DNA sequences defined by P0.9 and DYS104. In all three 46,XX true hermaphrodites, the translocated region contains at least 30 kb, but no more than 60 kb of Y-DNA sequences extending from the pseudoautosomal boundary towards the centromere.

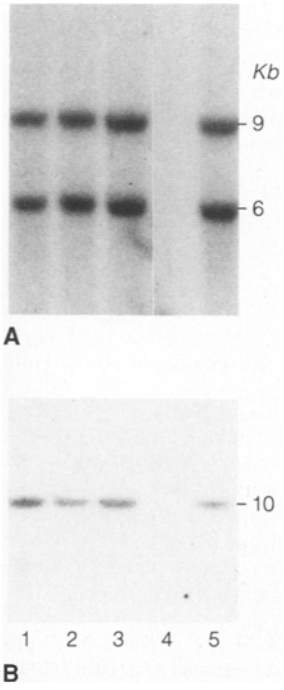

Fig. 2. Southern blot analysis of the 3 cases of 46,XX true hermaphroditism. DNA (15 µg) was digested by *Stu*I, and probed with the following Y-specific sequences: pY53.3 in **A** and P0.9 in **B**. *Lanes 1, 2 and 3* are 46,XX true hermaphrodite cases 1, 2 and 3, respecitvely. *Lanes 4 and 5* are female and male controls, respectively

In situ hybridisation

In situ hybridisation was performed on lymphocyte metaphasic chromosomes using pY53.3. In patient 1, a total of 155 mitoses was examined: of 325 silver grains, 50 (15.4%) were localised on the X chromosome and 13 (4% of the total and 26% of the grains on the X chromosome) lay on band Xp22 (Fig. 3). Similar results were found for patient 3 (data not shown). Lymphocytes from patient 2 could not be analysed. In a normal male control, 55 mitoses were examined: of 147 silver grains, 20 (13.7%) were on the Y chromosome, and 15 (10.2% of the total and 75% of the grains on the Y) lay on band Yp11. The distribution of grains on the other chromosomes including the X chromosome did not show any secondary peak.

Discussion

The present study demonstrates that 46,XX true hermaphroditism is a genetically heterogeneous condition. A limited portion of the Y chromosome, including the pseudoautosomal boundary and the male testis-determining gene SRY, was found in 3 individuals out of a total of 30 cases of 46,XX true hermaphroditism. SRY was mapped in 2 of these individuals by in situ hybridisation to the terminal region of Xp, which suggests an aberrant X/Y terminal meiotic exchange has occurred as previously described in 46,XX males (Petit et al. 1987). Some 30–60 kb of Y-specific sequences proximal to the

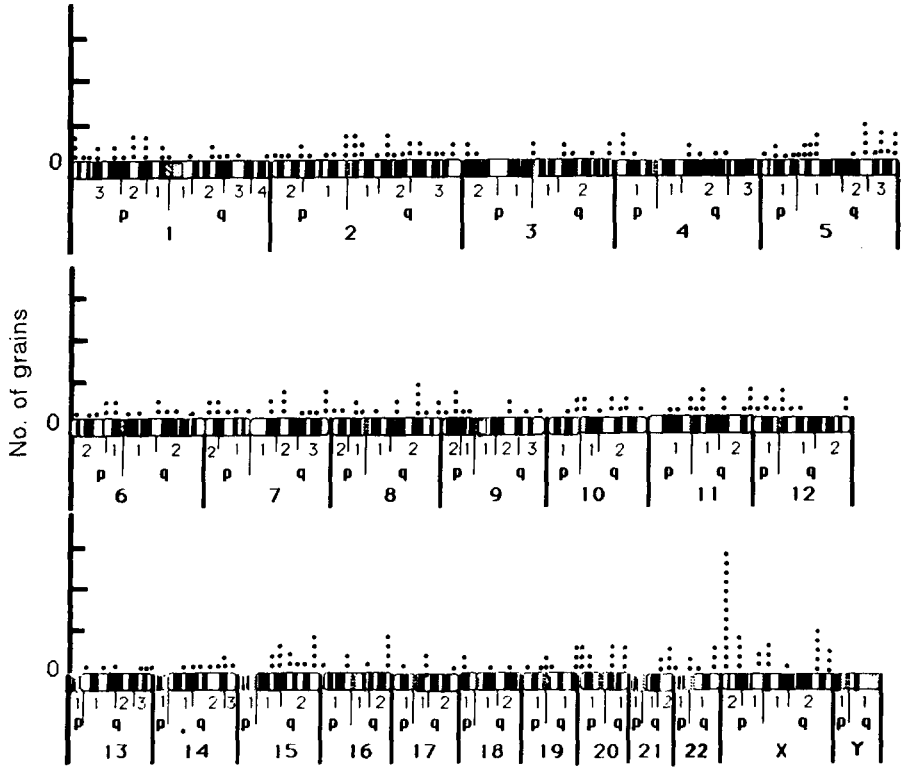

Fig. 3. Diagram of the grain distribution following in situ hybridisation of probe pY53.3 to SRY in 155 metaphases of case 1

pseudoautosomal boundary were present in each of the 3 individuals. SRY is probably complete since 14 kb of the equivalent mouse Y chromosome fragment containing *Sry* was capable of determining testis formation when introduced as a transgene into XX mouse embryos (Koopman et al. 1991).

Previous studies have demonstrated that 80% of XX males have Y chromosome material, including TDF, whereas in this study only 10% of XX true hermaphrodites had detectable Y chromosome sequences (de la Chapelle 1987; Abbas et al. 1990; Seboun et al. 1986; Vergnaud et al. 1986; Berkovitz et al. 1992). The amount of Y material or its position on the X may influence SRY expression, resulting in partial testicular formation; however, since the Y material was demonstrated on an X chromosome in 2 cases, the phenotype may be simply explained by X-inactivation. Hermaphroditism may occur when SRY expression is suppressed by X-inactivation such that a proportion of the cells in the fetal gonad develop along the ovarian pathway. If SRY escapes inactivation, the cells would differentiate as testicular tissue. This has been previously demonstrated in murine studies of the heritable sex-reversed condition Sxr, which involves a translocation of Y material including *Sry* on an X chromosome. XY male mice carrying Sxr give rise to 50% XX or XO progeny as sterile males rather than females. When partnered with an X/autosome translocation T16H, which results in preferential inactivation of the X^{Sxr} chromosome, the progeny are composed of sterile males, hermaphrodites or fertile females (Cattanach et al. 1982; Eicher and Washburn 1986). In man, loci located at the terminal Xp22 region normally escape X-inactivation; this would at first appear to weaken an inactivation hypothesis (Shapiro 1985). However, Mohandas et al. (1987) have characterised an abnormal X chromosome with a distal deletion from Xp22.3-Xpter and an Xq26-Xqter duplication inserted at the Xp22.3 locus. Xq genes inserted at the Xp22.3 locus were shown to be inactivated, despite their position between activated regions containing STS and MIC2.

The remaining SRY-negative cases including 3 familial cases may have been the result of mutations of X chromosomal or autosomal loci leading to a 'gain-of-function' in the male sex-determination pathway, which by itself is insufficient for complete testicular formation. Autosomal mutations involved in sex reversal have previously been described in mice (Eicher and Washburn 1986) and goats (Hamerton et al. 1969; Wolf 1988). It cannot be formally excluded that the phenotype of some of the remaining 27 SRY-negative cases may be explained by hidden XX/XY or XX/XXY mosaicism limited to the gonads. The frequency and distribution of Y-chromosome-bearing cells could influence the fate of the primitive genital ridge. This has been most clearly demonstrated in murine studies that show that ovotestes can develop in the presence of 20%–25% XY cells (McLaren 1991).

The data presented in this study and elsewhere brings to 6 the number of reported cases of 46, XX true hermaphroditism that have Y chromosome material. In all cases, with one exception (Berkovitz et al. 1992), the former candidate for TDF, viz. ZFY, was not detected. Similarly, XX males without ambiguities and having Y chromosome sequences have been described as ZFY-positive. This again raises the possibility that a Y locus, in addition to SRY, may be necessary to confer a fully masculinised phenotype (Burgoyne 1989; Jäger et al. 1990).

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