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Molecular, cytogenetic, and clinical characterisation of six XX males including one prenatal diagnosis

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Abstract

Cytogenetic analysis, fluorescent in situ hybridisation (FISH), and molecular amplification have been used to characterise the transfer of Yp fragments to Xp22.3 in six XX males. PCR amplification of the genes SRY, RPS4Y, ZFY, AMELY, KALY, and DAZ and of several other markers along the Y chromosome short and long arms indicated the presence of two different breakpoints in the Y fragment. However, the clinical features were very similar in five of the cases, showing a male phenotype with small testes, testicular atrophy, and azoospermia. All these patients have normal intelligence and a stature within the normal male range. In the remaining case, the diagnosis was made prenatally in a fetus with male genitalia detected by ultrasound and a 46,XX karyotype in amniocytes and fetal blood. Molecular analysis of fetal DNA showed the presence of the SRY gene. FISH techniques also showed Y chromosomal DNA on Xp22.3 in metaphases of placental cells. To our knowledge, this is the second molecular prenatal diagnosis reported of an XX male.

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Keywords: XX males; Xp-Yp interchanges; SRY; prenatal diagnosis

The genetic analysis of sex reversed subjects, with a phenotypic sex different from that expected based on the karyotype, has been crucial for the identification of the SRY gene (sex determining region on Y chromosome). This gene has been proven to be the testicular determinant that causes a switch to the male sex differentiation pathway during fetal life.[1–3] SRY mutations have been found in 15% of XY females and 80% of XX males are SRY positive. 46,XX males occur with an incidence of 1/20 000 male neonates.[4] Since the first patient was described,[5] numerous additional cases have been reported, most of them sporadic.[6–10] The clinical manifestations of XX males are usually hypogonadism, gynaecomastia, azoospermia, and hyalinisation of seminiferous tubules, with altered hormonal levels at puberty. Less frequently, some sexual ambiguities are found in these patients, ranging from cryptorchidism and hypospadias to a vaginal pouch, always with sterility owing to reduced testicular development.

The mechanism proposed to explain 46,XX males is the presence of a Y chromosome fragment transferred to the X chromosome short arm by unequal interchange between homologous regions in the short arms of the sex chromosomes during paternal meiotic division.[11] This Y fragment would be a carrier of testicular determinants and would give male sexual characteristics to apparently XX subjects. In other patients, the presence of a low level mosaicism for an XY cell line accounts for the ambiguous phenotype.[12] The existence of Y chromosome material in non-mosaic XX males was first detected as a heteromorphism in the short arm of one X chromosome.[13 14] More recently, studies on metaphase chromosomes by in situ hybridisation techniques located Y sequences to distal Xp in XX males, supporting the initial hypothesis of Ferguson-Smith.[9 11 15–17] Subsequently, the use of molecular techniques has allowed the detection of Y sequences in most XX males either by Southern blot analysis[6 18–20] or by PCR analysis,[10 21 22] confirming the presence of the SRY gene. Some XX male cases have also been reported lacking any Y sequences.[19 23] These cases usually have sexual ambiguities or hermaphroditism or both that may have arisen from a different mechanism, probably by a recessive mutation in a gene controlling the testicular pathway, since some cases are familial. This mutation would allow testicular development in the absence of SRY.[24] Alternatively, Y sequences might be confined to testicular tissue, and therefore escape detection by the analysis of leucocyte DNA.[25]

Here we present the characterisation of five new cases of XX males, not previously reported. Additionally, we provide new data for a previously reported case[10] that was partially characterised. PCR amplification using markers along the Y chromosome has allowed the Y sequences present in each patient to be defined and correlations with the phenotype to be established. Subsequently, the location of Y fragments has been further determined by in situ hybridisation on metaphase chromosomes.

Methods

CASES

We included in this study five patients either attending our Genetics Service or referred from other centres for karyotyping, FISH, or molecular studies. The patients had pubertal hypogonadism, testicular atrophy, and azoospermia as the main clinical manifestations

Table 1 Clinical, cytogenetic, and molecular data obtained from the six cases

			Y markers										
Case	*Clinical features*	*Karyotype*	*PABY*	*SRY*	*Y53*	*RPS4Y*	*ZFY*	*SY59*	*AMELY*	*SY78*	*KALY*	*sY130*	*DAZ*
1	Testicular atrophy, azoospermia, small testes	46,XX,t(X;Y)(p22.3;p11.3)	+	+	+	+	+	+	–	–	–	–	–
2	Testicular atrophy, azoospermia, small testes	46,XX,t(X;Y)(p22.3;p11.2)	+	+	+	+	+	–	–	–	–	–	–
3	Testicular atrophy, azoospermia, small testes, gynaecomastia, obesity	46,XX,t(X;Y)(p22.3;p11.2)	+	+	+	+	+	–	–	–	–	–	–
4	Testicular atrophy, azoospermia, gynaecomastia, obesity	46,XX,t(X;Y)(p22.3;p11.2)	+	+	+	+	+	–	–	–	–	–	–
5	Testicular atrophy, azoospermia	46,XX,t(X;Y)(p22.3;p11.2)	+	+	+	+	+	–	–	–	–	–	–
6	Male genitalia (internal and external)	46,XX,t(X;Y)(p22.3;p11)	n	+	n	n	n	n	n	n	n	n	n

n: not tested.

Table 2 Y specific primer pairs used in the PCR analysis of genomic DNA

Primer pair	*Gene/locus*	*Localisation (deletion interval)*	*Band (bp)*	*Reference*
PABY	Pseudoautosomal boundary	Yp11.31	947	26
PABX	Pseudoautosomal boundary	Xp22.3	771	26
SRY	SRY, sex determining region	Yp11.31 (1A)	336	3
Y53	SRY, sex determining region	Yp11.31 (1A)	470	1
sY274	RPS4Y, ribosomal protein S4	Yp11.31 (1)	350	36
sY238	ZFY, zinc finger protein	Yp11.31 (1)	350	36
sY59	DYF59S1	Yp11.3 (3)	267	22
sY276	AMELY, amelogenin	Yp11.2 (3)	200	36
sY78	DYZ3	Y centromere (4B)	170	22
sY90	KALY	Yq11.21 (5E)	176	36
sY130	DYS221	Yq11.23 (6A)	173	22
sY254	DAZ, deleted in azoospermia	Yq11.23 (6D)	107	35

(table 1). Some of the cases (Nos 3 and 4) also had gynaecomastia and obesity. Testicular histology was available for cases 1 and 4 and showed atrophy and hyalinisation of seminiferous tubules, with a complete absence of germinal cells. In all five cases, growth had been normal, with spontaneous pubertal development. Their stature was within the normal male range. Intelligence was normal in all cases. The remaining case (No 6) was a fetus with a female karyotype (detected after cytogenetic prenatal diagnosis because of advanced maternal age, 39 years) but with male genitalia detected by ultrasound at 20 weeks of gestation. The 46,XX karyotype was confirmed afterwards in a second amniocentesis and on fetal blood. Subsequently, placental cell cultures were referred to our centre for molecular and FISH studies in order to confirm the diagnosis.

CYTOGENETIC ANALYSES

Karyotypes were performed by standard procedures from peripheral blood lymphocytes in patients 1-5 (GAG and CBG banding). In the prenatal diagnosis, two successive cytogenetic analyses were performed on amniotic fluid cells at 12.4 and 20 weeks of gestation and confirmed on fetal lymphocytes.

MOLECULAR CHARACTERISATION

DNA was extracted from leucocytes by standard protocols, and from placental cells in the case of fetal DNA. Molecular amplification was performed by PCR using 12 different primer pairs from both arms and the centromere of the Y chromosome (table 2). The reactions contained 100 ng of DNA, 10 mmol/l tris-Cl, pH 8.3, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 200 μmol/l dNTPs, 10 pmol of each primer, and 1 unit of *Taq* polymerase in a final volume of 25 μl. Amplification conditions were initial denaturation for five minutes at 94°C, and 30 cycles for 20-30 seconds at 94°C, 30 seconds at 52-65°C, and 30-45 seconds at 72°C (depending on the primer pair used), with a final extension for five minutes at 72°C. After electrophoresis, PCR products were visualised under a UV transilluminator on a 2% agarose gel stained with ethidium bromide. Three oligonucleotides, PAB A, B, C,[26] were simultaneously used in the reaction to detect both X specific and Y specific sequences from the proximal border of the pseudoautosomal region, giving two bands of different size on electrophoresis in normal males and PABY(+) XX males.

FLUORESCENT IN SITU HYBRIDISATION

FISH analysis was performed using a WCP® chromosome painting system Y probe and SpectrumCEP® alpha satellite probes for X and Y (Vysis), labelled with different fluorophores (Spectrum Green™ or Spectrum Orange™, Vysis). Hybridisations were performed on chromosome metaphases from lymphocyte culture or placental cell culture. Hybridisation conditions were: after an initial slide denaturation (five minutes in 70% formamide/2 × SSC at 73°C) and dehydratation (70/85/100% ethanol), 10 μl of the labelled probe hybridisation buffer mix were applied to each slide before sealing it with a coverslip. Hybridisation took place overnight in a humidified box at 37°C. After a rapid wash in 0.4 × SSC at 73°C for two minutes and 2 × SSC/0.1% NP-40 at room temperature for one minute, 10 μl of DAPI II counterstain was used. Slides were studied with a fluorescence microscope equipped with a CCD video camera and the Cytovision (Applied Imaging) image analyser.

Results

CYTOGENETIC ANALYSES

G banded chromosome spreads showed a 46,XX karyotype in all patients. In the prenatal diagnosis (case 6), a male phenotype was detected prenatally by ultrasonography while the chromosome study on amniocytes showed a female karyotype. Therefore, additional cytogenetic analyses were performed on a second amniotic fluid sample and on fetal blood. After the discrepancy between the karyotype and the phenotype was confirmed, the parents decided to terminate the pregnancy. The examination of the conceptus showed normal external and internal male genitalia, with presence of prostate, epididymis, testes, vas deferens, and cavernous bodies, with no signs of congenital adrenal hyperplasia. The global fetal development was appropriate for the gestational age.

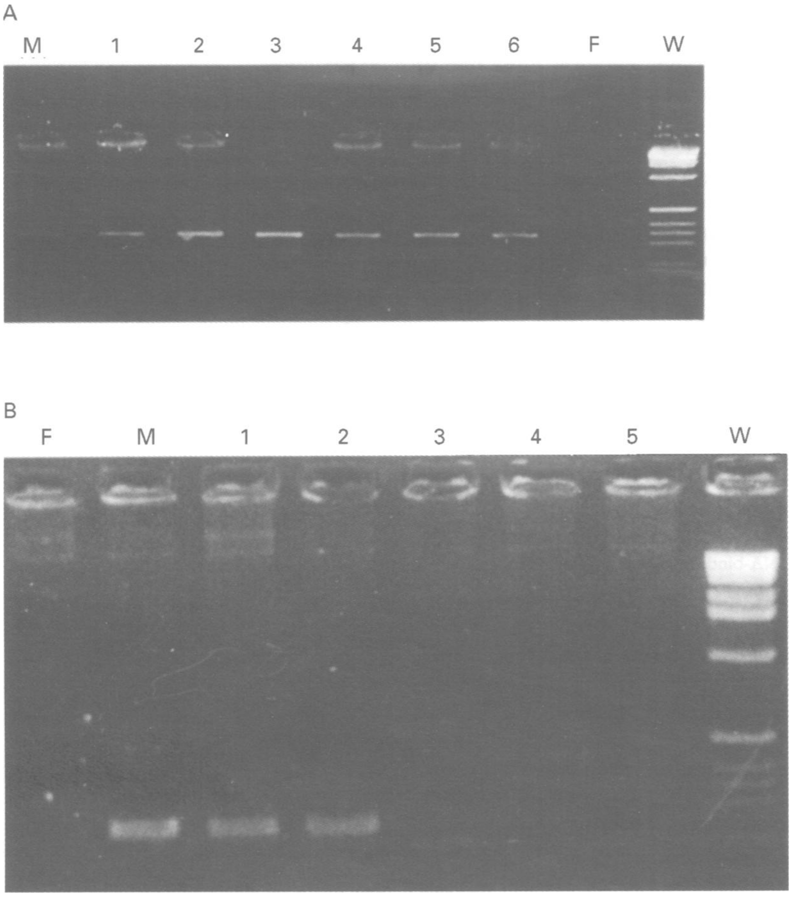

Figure 1 Molecular characterisation of 46,XX males: M, normal male. F, normal female. W, size marker (1 kb, Gibco BRL). (A) PCR analysis of genomic DNA from the six patients (lanes 1-6) using the SRY primer pair, obtaining a product of 336 bp in all cases. (B) Four of the five cases tested with the primer pair sY59 are represented on the 2% agarose electrophoresis gel. Only patient 1 (lane 2) showed a positive amplification, displaying a band of 267 bp. The sample in lane 1 is not related to this work.

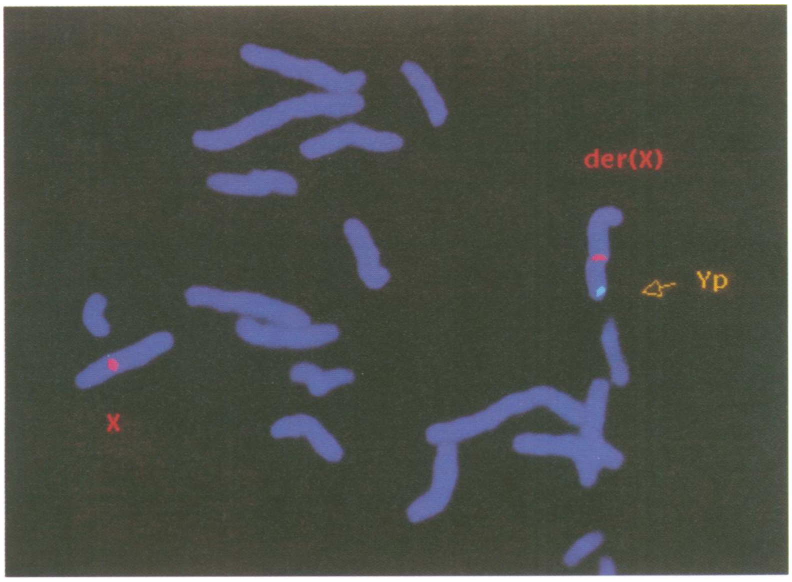

Figure 2 FISH analysis of chromosomes from a 46,XX male. A partial metaphase of patient 1 shows both X chromosomes labelled in red with an X centromere specific probe. The extra material on the derivative X chromosome is painted in green by a probe specific for the whole Y chromosome.

MOLECULAR STUDIES

PCR analysis proved that the SRY gene was present in all six cases (fig 1A). Other Yp genes or markers (PABY, RPS4Y, ZFY) were also detected in the five cases tested (cases 1-5) (table 1). The marker sY59, in contrast, was present in only one of the XX males tested, indicating the presence of different breakpoints on the Yp chromosome in different patients (fig 1B). As expected, the amplification using markers from the Y centromere (sY78) and from the long arm (KALY, sY130, DAZ) was negative in all patients.

FLUORESCENT IN SITU HYBRIDISATION

In all six cases, the Y chromosome fragment was located at the tip of the short arm of the X chromosome (Xp22.3), using FISH with a painting Y probe and an X centromere probe (fig 2). Hybridisation with a Y centromere probe was negative in all samples, indicating that only the short arm of the Y chromosome is implicated in the X-Y interchange.

Discussion

In this work, we have used cytogenetic analysis, FISH, and molecular amplification to characterise the Y chromosome sequences present in six XX males. All the cases in this study are positive for the SRY gene and other Y specific sequences, which is the most frequent type of XX male reported.[7,8] The phenotype described in these cases is that of males with sterility owing to the absence of germinal cells and hypogonadism, without sexual ambiguities, consistent with the clinical data detected in our patients (table 1).

All our patients have the Y chromosome DNA transferred to Xp22.3, as found in all other reported cases.[9,15,16,29] This confirms the relative frequency of an Xp/Yp interchange originating through unequal crossing over between homologous pseudoautosomal regions in the paternal meiosis, extending the exchange to sex specific sequences.[11,27] Some cases of Y;autosome translocation have been described, conferring maleness to 45,X subjects.[28-31] However, to our knowledge, no 46,XX male with a Y;autosome translocation has been reported.

In four cases (2, 3, 4, 5), PABY, SRY, RPS4Y, and ZFY sequences were amplified, while markers sY59, AMELY, sY78, and Yq were negative. In case 1, the sY59 marker was also positive, indicating a Yp breakpoint more proximally. Some authors have reported that the Y sequences present in the XX males extend from distal Yp to the Y centromere, showing a variation in the size of the fragment exchanged from less than 40 kb[1] to more than 11 Mb.[7,32] The similarity of the clinical features in all cases confirms that the SRY gene is responsible for testicular determination, and that other genes on the Y chromosome are not so important in defining the phenotype.

The case of a prenatally diagnosed XX male is especially interesting as an example of the problems that may arise in a laboratory when the sexual phenotype of a fetus is different from that expected based on prenatal cytogenetic analysis. In the present case, FISH and molecular studies allowed the confirmation of the XX male diagnosis in the fetus. To our knowledge, this is the first molecular prenatal diagnosis of a Y positive XX male, as the only other prenatal case reported was an XX male fetus lacking any Y sequence.[33] Other cases were detected only after the birth of a male following a cytogenetic prenatal diagnosis of 46,XX,[34] and where postnatal studies allowed the possibility of a sample error or maternal cell contamination to be ruled out.

Molecular and FISH techniques are very useful for detecting and locating Y sequences in cytogenetically XX males, allowing an accurate diagnosis and correct management of the patient. Testing new Y chromosome markers in XX males will make it possible to narrow the breakpoints further in each case and to establish correlations with the clinical features, identifying the Y regions implicated in the definition of the phenotype.

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