Electronic letter

Xp;Yp translocation inherited from the father in an *SRY*, *RBM*, and *TSPY* positive true hermaphrodite with oligozoospermia

EDITOR—The *SRY* gene (sex determining region of Y) and AZF (azoospermia factor), a gene from a gene family with multiple members, are both localised on the Y chromosome and are crucial for testis determination and spermatogenesis, respectively.[1-5] Sex reversal (XX males) may arise by translocation of sequences from Yp onto Xp owing to meiotic recombination. Eighty percent of XX males are *SRY* positive and in about one third of these the interchanges take place between the two *PRK* homologues, *PRKX* and *PRKY*, producing typical fusion fragments.[6] Usually, genes from Yq including the *AZF* locus are not present in these cases and no spermatogenesis is found. We have decided to review, using molecular methods, a previously described[7] male true hermaphrodite with a left scrotal testis, oligozoospermia, and no detectable Y chromosome or its fragments on standard karyotype analysis.

As described 24 years ago, the patient was referred to our laboratory because of short stature (1.5 m), right cryptorchidism, and gynaecomastia.[7] At the time of explorative laparotomy, the ovary and female internal genitalia on the right side were removed. Biopsy of the left scrotal gonad showed Sertoli cells only. Clinically, true hermaphroditism was established.

The patient was referred again when he was 44 years old. Because of increasing hardness and pain in the left epididymis and post inflammation changes observed by ultrasound, bacteriological analysis of semen was performed. Unexpectedly, spermatozoa were found. Their number ranged from a few to 500 000/ml in following tests. All spermatozoa were motionless; 70% of them were normal and 30% balloon headed. The semen samples were collected outside our clinic, so in order to confirm that they really were the patient's, the DNA profile was ascertained by VNTR (variable number of tandem repeats) testing with the probes MS1 (D1S7), MS31 (D7S21), MS43A (D12S11), and YNH24 (D2S44) and compared with the patient's lymphocyte DNA profile. The semen DNA profile was the same as the lymphocyte DNA with the statistical occurrence of the patient's profile being 1 per million in white population studies.

At the same time, the following hormonal results were observed: FSH 24.0 mIU/ml (normal 4.0-10.0 mIU/ml, BYK), LH 2.6 mIU/ml (normal 1.0-8.0 mIU/ml, BYK), 17-OH-progesterone 1.2 ng/ml (normal 0.3-2.2 ng/ml), testosterone 3.1 ng/ml (normal 3.0-10.0 ng/ml), DHEA 770.4 ng/ml (normal 2000-3350 ng/ml). These values are almost normal male levels.

When the patient was 47 years old, atrophy of the testis together with increasing hardness and thickness was reported. Because of the risk of neoplastic transformation of the gonadal tissue, orchidectomy was performed. Seminiferous tubules with Sertoli cells only were detected in serial sections of the whole gonad. In 32 of the 332 tubules analysed (9.62%), spermatogonia, spermatocytes, round spermatids, and mature spermatids with condensed nuclei were found. No spermatozoa were visible in the obliterated lumen (fig 1A). In three of the tubules analysed (3/332 =0.96%), single premeiotic cell divisions could be seen. The upper, small fragment of the scrotal gonad was an ovarian fragment (fig 1B).

In 100 peripheral blood lymphocytes analysed by GTG and QFQ banding and FISH, the mosaic karyotype 45,X[25]/46,XX[75] was confirmed. A similar result, 45,X[43]/46,XX[57], was observed in skin fibroblasts and in fibroblasts from the left testis, 45,X[37]/46,XX[63]. The marker chromosome observed previously[7] could not be detected any more using standard cytogenetic techniques and FISH.

DNA from the patient was extracted by a salt precipitation technique[8] from peripheral blood and from skin fibroblasts cultured after biopsy (DNA 864/FG 234) as well as from the removed left gonad (DNA 865/FG 270). Whole blood DNA preparations of the patient's parents, healthy females and males, as well as a 45,X female and two XX males were used as controls.

DNA samples were also obtained from histological slides of different parts of the left gonad.[9] Samples represent: DNA-1, small ovarian fragment at the upper tip of the gonad (for details see fig 1); DNA-2, more degenerated testicular part at the border of the ovarian fragment and from the middle part of the testis which is represented by DNA-3.
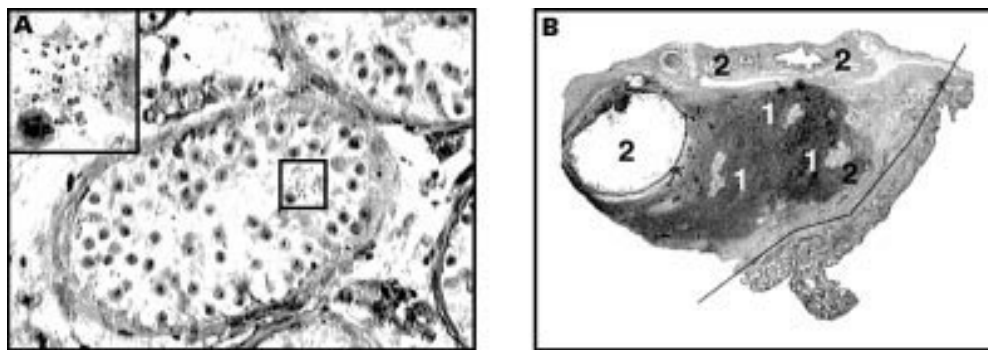

Figure 1   Histology of the left scrotal gonad. (A) Testicular fragment biopsy of the patient aged 44 years: spermatogonia, spermatocytes, round spermatids, and single mature spermatids with condensed nuclei but no spermatozoa were found in obliterated lumen. Single cell divisions could be seen with the approximately haploid chromosome number (square in the upper left corner). The lamina propria is not as thickened as in tubules with Sertoli cells only. (B) Three years later: upper end cross section of the scrotal gonad, stromal hyperplasia, corpora albicantia (1), and inclusion cysts lined by a single layer of well oriented columnar epithelial cells (2) are visible. The line separates small ovarian fragment and testis structures.

Y specific DNA probes or primers from Yp and Yq were applied to detect the presence of Y chromosome specific sequences as listed in fig 2. Probes pDP61, pDP34, and M1A (DXS31/DYS22) were used as described previously.[10] Probes pDP1007 and 47z were obtained from ATCC. The other probes were a gift from U Müller.[11–13] The *SRY* gene was analysed by application of two sets of primers: AP1/AP2 to amplify a 422 bp PCR fragment encompassing the HMG box conserved motif[14] and the TD-F/TD-R primers generating a product of 764 bp including the complete coding region.[15] In PCR analysis of *PABY*, *ZFY*, *TSPY*, *AMGL*, *DYZ3*, and *DYZ1*, the primers A;C (946 bp), 3,2;4,2 (650 bp), JA56;JA57 (2372 bp), AMGL-1;AMGL-2 (367 bp), DYZ3-1;DYZ3-2 (170 bp), Y1-6F;Y1-4R (1024 bp) were used, respectively. Primers F19;E355 (800 bp) and F20;E355 (800 bp) for the *RBM1*/*RBM2* gene family[16] were also included in this study for the interpretation of the sperm analysis results. We have additionally tested DNA samples from the patient's left gonad for the *DAZ*/*SPGY* locus (sY254/sY255, 107 bp/126 bp; DAZf/DAZr, 1300 bp; SPGYf/SPGYr, 460 bp), and the *AZFa* (sY113, 290 bp), *AZFb* (sY139, 120 bp; sY143, 311 bp), and *AZFc* (sY147, 100 bp; sY156, 950 bp; sY203, 157 bp) regions.[5]

Southern blotting was performed according to standard techniques.[17] PCR was carried out on 50 ng of genomic DNA of the patient and controls in a standard reaction mixture with *Taq* DNA polymerase (2 U/μl). Amplifications were carried out on a Gene-Tech-SPCR1 (Stuart Scientific, UK), Perkin Elmer Cetus (Perkin Elmer, Norwalk, CT, USA), or PTC-200 DNA (MJ Research Inc, Watertown, Mass, USA) thermocyclers. Amplified DNA fragments were separated on 2% agarose gels, stained with ethidium bromide, and photographed under UV illumination. A sequence was scored as absent after at least three independent amplification failures.

In order to define the breakpoint of the Xp region in the Xp;Yp translocation, DNA of the patient and his parents was analysed by PCR with the polymorphic markers DXS85, DXS1053, and DXS1241 localised in Xp22.3, Xp22.2, and Xp22, repectively. The PCR products of the sequences were resolved on 14% polyacrylamide gels stained with ethidium bromide.

Additionally, the dosage of genes from the DSS region (dosage sensitive sex reversal)[18] on Xp was analysed by quantitative Southern blotting as described by Baumstark *et al*,[19] and the X inactivation pattern was determined by means of methylation assay for the *AR* gene (androgen receptor) in Xq according to Green *et al*.[20]

A nested PCR reaction (35 cycles) was performed with *SRY*-PCR products from DNA prepared from gonadal slices with AP-1;AP-2 (422 bp) and TD-F;TD-R (764 bp) primers for the *SRY* gene.

The *SRY*-PCR products were screened for mutations by SSCP assay under the conditions described by Schmitt-Ney *et al*[21] using the entire 422 bp fragment as well as 246 bp and 176 bp subfragments obtained after *Hin*fI digestion. The 764 bp product of the complete coding region was subjected to SSCP analysis after *Hin*fI digestion into two subfragments of 340 bp and 424 bp.

For sequencing analysis of AP-1;AP-2 PCR products of the *SRY* gene, a USB Sequenase kit version 2.0 was used together with [α-^{35}S]dATP. The amplified DNA was sequenced directly with sequencing primers SP-1 and SP-2 (10 pmol) with double stranded DNA as template.[22 23]

FISH studies were performed using biotinylated libraries of the X and Y chromosome (Cambio, Cambridge, UK), digoxigenated *DXZ1* and *STS* probes (Oncor) as well as *SRY* (460 bp) and *TSPY* (2.8 kb) probes labelled by PCR with digoxigenin-16-dUTP (Boehringer Mannheim). All probes were first applied to metaphase preparations of a karyotypically normal man in order to check the effectiveness and verify the mapping position of the respective probes. The X and Y libraries were detected with FITC conjugated avidin and the signal was amplified with FITC antiavidin (Cambio, Cambridge, UK). The *STS* and *DXZ1* probes were detected with the standard procedure provided by Oncor. *SRY* and *TSPY* probes were detected by means of anti-digoxigenin conjugated to FITC and amplified with rabbit anti-sheep and fluorescein labelled anti-rabbit (Oncor). The slides were counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodine (PI).

Fluorescence microscopy was performed using a Nikon Microphot FXA microscope equipped with single (UV-2A for DAPI, B-2A for FITC) and dual (FITC/TRITC) bandpass filters (Nikon). Images were acquired using a Photometrix CCD camera, then converted and imported into Multiscan software for further evaluation. Hybridisation efficiency was defined as the ratio of the total number of hybridisation signals to the total number of chromosomal target sites. The *TSPY* probe showed a high hybridisation efficiency of up to 90% in metaphase spreads from normal peripheral blood, but the hybridisation efficiency of the *SRY* probe was low (8-10%) and limited owing to the small probe size (460 bp).

In the patient's genomic DNA, the Yp specific sequences distal to the *AMGL* locus are all present (fig 2), including copies of the *RBM* genes localised on Yp distal to the *TSPY* gene. The *AMGL* gene and sequences proximal to it within Yp, as well as centromeric and Yq specific sequences, are absent. Further analysis of the *DAZ*/*SPGY*
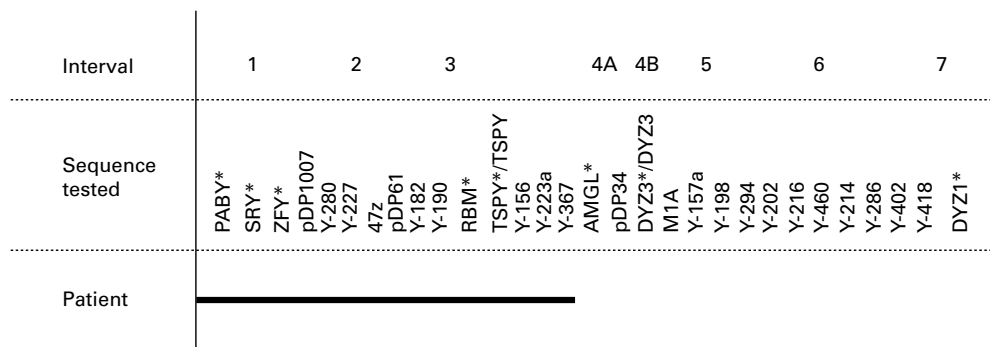

Figure 2 Molecular analysis of patient's genomic DNA with use of the Y specific DNA probes (without asterisks) or primers (analysed loci are marked by an asterisk) in seven intervals of the Y chromosome deletion map according to Vergnaud et al.[26] The list of the analysed sequences is generally in agreement with those of Müller and Lalande[27] and Vollrath et al.[28] Sequences distal to the AMGL locus on Yp were all present (solid line). Yq sequences including the DAZ/SPGY locus and AZFa-c regions (for details see text) were not detected.
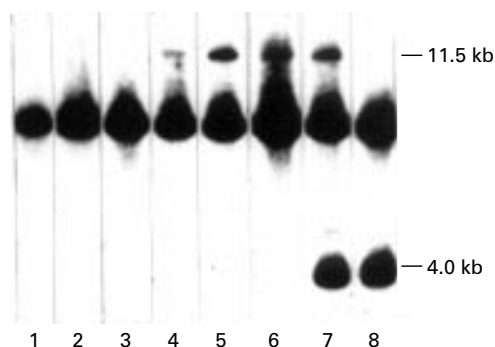
Figure 3 Blot of EcoRI digested genomic DNA hybridised with M1A probe. Lane 1 female 45,X; lane 2 normal female; lane 3 patient's mother, 46,XX; lanes 4 and 5 XX males; lane 6 patient; lane 7 patient's father, 46,XY; lane 8 normal male. The final wash was $1 \times SSC$ at $60°C$. In the reported patient, his father, and in two XX males, an additional band of 11.5 kb is present; there is no Yq specific 4.0 kb band in the patient or the XX males because of the absence of sequences from Yq. In all of the analysed DNA, the common 7.5 kb fragment from the X chromosome(s) is present.

locus and AZFa, AZFb, and AZFc regions in the DNA samples from the patient's lymphocytes and left gonad showed that the sequences tested were not detected by means of PCR.

After hybridisation of *Eco*RI digested genomic DNA with M1A, a 4 kb fragment was observed in the DNA of the patient's father and in a healthy control male. This band represents the Y chromosomal locus DYS22 in Yq11. Testing for the presence of DXS31 in Xp22.3, all subjects shared a common 7.5 kb fragment from their X chromosome(s) An additional band of 11.5 kb was observed in the patient, his father, and two control XX males (fig 3). The band is not found in controls, males, females, or a 45,X female.

There was no sign of loss of X chromosomal loci during meiotic recombination as seen after PCR with the markers DXS85, DXS1053, and DXS1241 in the patient and his father (not shown).

Chromosome painting with whole Y chromosome and X chromosome paint showed Y chromosome material to be localised on the tip of Xp of one of the two X chromosomes of the 46,XX cell line. Positive signals for the probes of *SRY* and *TSPY* in this region additionally proved the Y specificity of the detected material (fig 4). As the FISH signal of the *STS* probe is still present on the aberrant Xp, the breakpoint must be localised distal to the *STS* locus within Xp22.3, which is in complete agreement with the localisation of DXS31. The above results show that there has been no loss of important Xp material in the father.

In order to study whether a duplication of the region of dosage sensitive sex reversal (DSS) might have caused the severe azoospermia, the dosage of markers from this region was determined. Quantitative analysis of the DSS region of

the X chromosome did not show any hint of a duplication of this sex reversal region in the patient's DNA.

The *SRY*-PCR products of 422 bp and 764 bp were present in all gonadal tissue DNA samples of the patient (DNA-1-3) analysed by SSCP. No altered migration pattern of the *Hin*fI digested samples was seen in any of the DNA, thus indicating no mutations in the analysed *SRY*-PCR products. These findings show that there is no somatic mosaicism which could be invoked to explain the unusual phenotype of the patient. In the methylation assay, the digested and undigested DNA samples from the patient's lymphocytes, gonadal fibroblasts, and skin fibroblasts resulted in a pattern similar to female DNA and compatible with random X inactivation.

The combined results of cytogenetic and molecular analyses established the patient's karyotype as follows: 45,X/46,X,X.ish der(X)t(X;Y)(wcpX+, wcpY+, SRY+, TSPY+, DYZ3−, DYZ1−).

In most cases of true hermaphroditism (50-70%), a 46,XX karyotype is found. About 20% of these cases have chromosomal mosaicism with a Y chromosome positive cell line. More complex mosaic cases have also been reported. It is not known, however, how many of the hermaphrodites with an XX karyotype carry Y chromosomal material on the tip of one of the X chromosomes and thus represent a special manifestation of the condition usually known as XX males. The case reported represents this last category, but also exhibits a mosaic karyotype with a 45,X cell line. There is one similar published case with a non-mosaic 46,X,del(Xp) karyotype.[24] These authors suggested preferential inactivation of the Y bearing X chromosome possibly to be the cause of incomplete testicular differentiation. Preferential inactivation of the paternal X chromosome has been reported in another 46,XX true hermaphrodite by analysis of several X chromosomal loci.[25] Skewed X inactivation could be excluded in our patient by methylation sensitive PCR analysis of sequences of the androgen receptor gene.

The cytogenetic characterisation of our patient showed a translocation of a small distal segment from Yp to the tip of the short arm of one of the X chromosomes. This fragment contains an intact *SRY* gene as shown by PCR and sequencing. Furthermore, the presence of several sequences from Yp distal to *AMGL* could be shown by molecular techniques. The M1A probe is localised within the *PRKX* gene which has a homologue on the Y chromosome (*PRKY*). These genes have recently been reported to contain two hotspots of recombination leading to about 1/3 of XX males.[6] These translocations are likely to produce fusion fragments which can be detected in Southern blots using intragenic probes from the *PRKX* gene, like the M1A probe used here. In Southern blots of normal males and females, this probe detects fragments of 7.5 kb in length from the X and fragments of 4.0 kb in length from the Y
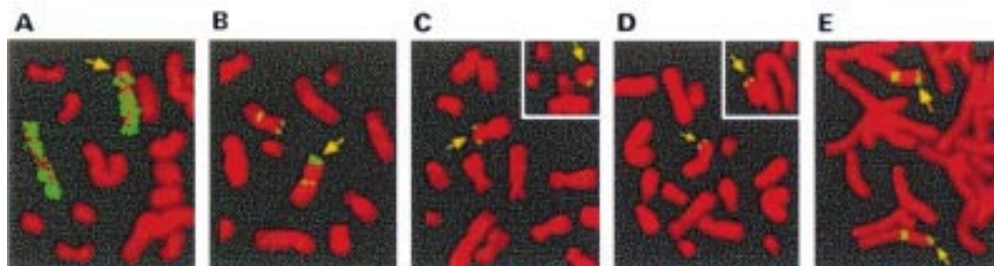
Figure 4 FISH results. Painting with X library probe (A) shows a pale tip on one of the X chromosomes (arrow); after hybridisation with the Y painting probe (B) a fragment from the Y chromosome translocated to Xp is clearly detectable (large arrow). Positive signals with PCR labelled SRY (C) and TSPY (D) probes confirmed translocation of Y specific fragment onto the X chromosome; squares in the upper right corners illustrate hybridisation results in a male control. Positive hybridisation with the STS probe and simultaneously with X centromere DXZ1 (E) shows in detail the breakpoint on the Xp distal to the STS locus (larger arrow indicates the translocated X).

chromosome. We observed an additional fragment of 11.5 kb in two XX males and also in our patient which may represent such a fusion fragment. Given the other information on the translocation in our patient, it seems most likely that he is indeed an XX hermaphrodite with a typical XY translocation and breakpoints in the *PRKY* and *PRKX* genes. Whether or not the incomplete masculinisation of the gonads is related to his mosaic status remains open to question.

The father of our patient was included as a reference in several of the molecular analyses, among them the Southern blot hybridised with the M1A probe. Very much to our surprise, the same fusion fragment as in the son and as in the two XX males was also seen in the father. In our opinion, the additional *Eco*RI DNA fragment reflects the predisposing situation for the translocation. To the best of our knowledge, this seems to be the first case in which a typical XY translocation chromosome has been passed from an XY father to his XX son. This observation indicates that in these translocation chromosomes all essential sequences of the X chromosome are present. It may well be that such cases occur much more frequently and have not previously been detected because nobody studied the fathers of XX males. Some, or many, of these fathers may represent germline mosaics, thus allowing for the generation of daughters and normal pedigree patterns. Germline mosaicism has been found with increasing frequency in several autosomal dominant disorders during recent years.

The other unusual feature in our patient was the observation of mature, albeit immotile, sperm. The production of sperm has been considered to depend strictly on the presence of functional *RBM* and *DAZ* genes. We could find only one copy of the *RBM* gene in our patient. Whether this is sufficient to allow spermatogenesis, as suggested by Ma *et al*,[2] remains questionable because of the 45,X cell line in our patient. Sterility of XX males could result from the presence of two X chromosomes and deletions of the Y chromosome in sterile XY males could have a structural effect in meiosis. The 45,X cell line in our patient could well be able to proceed to true meiosis and get sufficient support from surrounding cells with the copy of *RBM* to complete spermatogenesis. These questions can be answered only when the function of the spermatogenesis genes is resolved in detail.

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