Incomplete masculinisation of XX subjects carrying the SRY gene on an inactive X chromosome

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Abstract
46,XX subjects carrying the testis determining SRY gene usually have a completely male phenotype. In this study, five very rare cases of SRY carrying subjects (two XX males and three XX true hermaphrodites) with various degrees of incomplete masculinisation were analysed in order to elucidate the cause of sexual ambiguity despite the presence of the SRY gene. PCR amplification of 20 Y chromosome specific sequences showed the Yp fragment to be much longer in XX males than in true hermaphrodites. FISH analysis combined with RBG banding of metaphase chromosomes of four patients showed that in all three true hermaphrodites and in one XX male the Yp fragment was translocated onto a late replicating inactive X chromosome in over 90% of their blood lymphocytes. However, in a control classical XX male with no ambiguous features, the Yp fragment (significantly shorter than in the XX male with sexual ambiguity and only slightly longer than in XX hermaphrodites) was translocated onto the active X chromosome in over 90% of cells.
These studies strongly indicate that inactivation on the X chromosome spreading into a translocated Yp fragment could be the major mechanism causing a sexually ambiguous phenotype in XX (SRY+) subjects.
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46,XX maleness is a rare abnormality of sex determination with an incidence of 1 in 20 000.[1] It is characterised by development of bilateral testes in spite of the lack of a cytogenetically identifiable Y chromosome. Many authors have described phenotypic heterogeneity in this genetic condition, and two main groups of patients have been distinguished. The first one contains the majority of 46,XX males (about 80%), who are characterised by normal male external genitalia. They are diagnosed in adulthood because of infertility.[2] In this group of XX(Y+) males, a portion of the Y chromosome is present,[3] mostly as a result of recombination between X and Y chromosomes during male meiosis.[4] Based on the size of a Y fragment present in this group, three classes of XX(Y+) males were distinguished: class 1 (interval 1, about 40% of cases),[5] class 2 (intervals 1 and 2), and class 3 (intervals 1, 2, and 3).[6] In the second, less frequent (20%) group of 46,XX(Y−) males, ambiguous external genitalia and a lack of Y chromosome material are typical.[7]

Another sex determination condition associated with genital ambiguity is 46,XX true hermaphroditism. These patients can be distinguished from 46,XX males at the histological level by the presence of both testis and ovary in the same person whose gonadal tissue frequently exists as ovotestis. It has been shown in cytogenetic and molecular studies that less than 10% of 46,XX true hermaphrodites contain Y chromosome DNA.[2 8]

Although 46,XX males and 46,XX true hermaphrodites are distinct types of sex determination defects and both are heterogeneous at the molecular level, they share several phenotypic features. (1) The endocrinological characteristics are similar in both groups; testosterone level is in the normal range during puberty, and lower in adults, and (2) the histology of the male part of the gonad, regardless of the presence or absence of the ovarian part, is similar, being normal in infancy with disappearance of spermatogonia at 5-8 years of age, leading to dysgenetic gonads.[7]

In this study, we present clinical, cytogenetic, and molecular data of a group of very rare cases of XX subjects (46,XX males and 46,XX true hermaphrodites) with various degrees of sexual ambiguity despite the presence of the SRY gene. There have been just six cases of XX(Yp+) males with evidence of genital ambiguity[4 7 9–11] and 15 cases of XX(Y+) true hermaphrodites[7 9 10–20] described so far. Here, we address the question of whether incomplete male differentiation in our group of patients could be related to the size of the translocated Y chromosome fragment and to the spreading of X inactivation into the translocated sex determining region.

Patients and methods

CLINICAL ANALYSIS

All patients studied were of Polish origin. Two 46,XX males with male genitalia and some features of sexual ambiguity as well as three 46,XX true hermaphrodites were submitted to clinical, histological, cytogenetic, and molecular study. None of the patients had a positive family history so all were sporadic cases. Plasma testosterone level was measured using radioimmunoassay (RIA). Testosterone stimulation test with human chorionic gonadotrophin (hCG) was performed in patients diagnosed at prepubertal age. Testosterone was measured after four hCG injections of 1500

Table 1 Description of the six cases of 46,XX males and 46,XX true hermaphrodites

Patient and age	*Diagnosis*	*External genitalia*	*Internal genitalia*	*Gonads*	*Testosterone*
JW (control) 15 y	XX male	Male	Male	Testes with no signs of spermatogenesis	3.9 ng/ml
MB 7 y	XX male	Male, penile hypospadias	Male	Testes	ND
KM 1 y	XX male	Male, penile hypospadias	Male	Testes, normal tissue adequate for age	1–2.7 ng/ml*
AK 17 y	XX true hermaphrodite	Ambiguous	Ambiguous	Left ovary, right testis with no signs of spermatogenesis	ND
RZ 14 y	XX true hermaphrodite	Ambiguous	Ambiguous	Left ovotestis with no signs of spermatogenesis in the testicular part, right ovary	0.6–5.5 ng/ml*
PG 6 mth	XX true hermaphrodite	Ambiguous	Ambiguous	Left ovotestis with signs of dysgenesis in the testicular part, right ovary	<0.1–0.2 ng/ml*

ND - not determined.
*Testosterone before and after stimulation with human chorionic gonadotrophin (hCG).

Table 2 Molecular analysis of patients through PCR amplification of selected Y specific STS sequences

Locus	*STS*	*Y localisation*	*Y interval*	*JW XX M*	*MB XX M*	*KM XX M*	*RZ TH*	*PG TH*	*AK TH*	*46,XY male*	*46,XX female*
PABY		Yp	1A1A	+	+	+	−	+	+	+	−
SRY		Yp	1A1A	+	+	+	+	+	+	+	−
DYS234	sY15	Yp	1A1B	+	+	+	+	−	−	+	−
DYS242	sY16	Yp	1A1B	+	+	+	−	−	−	+	−
DYS250	sY17	Yp	1A1B	+	+	+	+	−	−	+	−
DYS251	sY18	Yp	1A2	+	+	+	−	−	−	+	−
DYS252	sY19	Yp	1B	+	+	+	−	−	−	+	−
DYS257	sY57	Yp	3C2	−	+	+	−	−	−	+	−
DYF56S1	sY54	Yp,q	3C2 6C3 6F3	−	+	+	−	−	−	+	−
DYZ4	sY60	Yp	3C4 4A6	−	+	+	−	−	−	+	−
DYS260	sY65	Yp	3C7	−	+	+	−	−	−	+	−
DYS264	sY69	Yp	3G	−	+	−	−	−	−	+	−
DYS266	sY72	Yp	4A2	−	−	−	−	−	−	+	−
DYS268	sY76	Yp	4A5	−	+	+	−	−	−	+	−
DYZ3		cen	4B	−	−	−	−	−	−	+	−
DYS270	sY79	Yq	5A2	−	−	−	−	−	−	+	−
DYS273	sY84	Yq	5C2	−	−	−	−	−	−	+	−
DYS275	sY87	Yq	5D1	−	−	−	−	−	−	+	−
DYS278	sY90	Yq	5E2	−	−	−	−	−	−	+	−
DYS280	sY95	Yq	5H	−	−	−	−	−	−	+	−

XX male (XX *M*), true hermaphrodite (TH), presence of STS (+), absence of STS (−).

IU/m^2 daily. Ultrasonic imaging of the pelvis and abdomen was done to visualise Müllerian structures. Cystoscopy and genital examination were performed to determine the status of the urogenital sinus and structure of the internal ducts in patients with ambiguous genitalia. Gonadal tissue from gonadectomy or biopsy was examined using an optical microscope. The clinical description of patients is summarised in table 1.

KARYOTYPE ANALYSIS

Chromosome HRBT studies were performed on peripheral blood lymphocytes using GTG, FPG, CBG, and QFQ banding techniques. The karyotype of each patient was analysed in at least 100 metaphases.

DNA ANALYSIS

Genomic DNA was isolated from peripheral blood lymphocytes using standard techniques.[21] For polymerase chain reaction (PCR) amplification of DNA, we used primers complementary to Y specific sequence tagged sites (STSs) corresponding to interval 1, 3, 4, or 5: sY15 (59°C), sY16 (56°C), sY17 (58°C) sY18 (56°C), sY19 (58°C), sY57 (55°C), sY54 (60°C), sY60 (64°C), sY65 (56°C), sY69 (58°C), sY72 (53°C), sY76 (60°C), sY79 (52°C), sY84 (60°C), sY87 (55°C), sY90 (55°C), sY95 (60°C), as previously described.[22 23] For amplification of the whole SRY coding sequence, primers S1 5'-AAGC TTTT-GAGGGCGGAGAAATGCAA and S2 5'-GAATTCAAGGAGCATCTAGCTAGGTC (60°C) were used. Annealing temperatures are given in parentheses. Amplification reactions were performed in a volume of 20 µl, containing 50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.4, 5 mmol/l $MgCl_2$, 0.15 mmol/l each dNTP, 0.5 µmol/l each amplification primer, and 1 U *Taq* polymerase. The conditions were 10 minutes at 94°C, followed by 30 cycles at 94°C for 30 seconds, annealing 30 seconds, 72°C for 45 seconds, and 72°C for seven minutes. For analysis of the centromere of the Y chromosome, we amplified alphoid sequence using primers Y1 and Y2.[24 25] For amplification of the PABY region, we used primers described elsewhere.[12]

Table 3 Studies of activation/inactivation status of X chromosomes carrying the Yp fragment in XX males and true hermaphrodites

Patient	*Clinical phenotype*	*No of active X chromosomes (%)*	*No of inactive X chromosomes (%)*
JW (control)	XX male	48 (96)	2 (4)
MB	XX male	Not done	Not done
KM	XX male	4 (8)	46 (92)
RZ	True hermaphrodite	3 (6)	51 (94)
PG	True hermaphrodite	1 (2)	50 (98)
AK	True hermaphrodite	2 (3)	57 (97)

FLUORESCENCE IN SITU HYBRIDISATION (FISH)

FISH was performed using the plasmid pHu14 with the insert complementary to about 12 kb of the human Y chromosome sex determining region including the SRY gene. The insert was

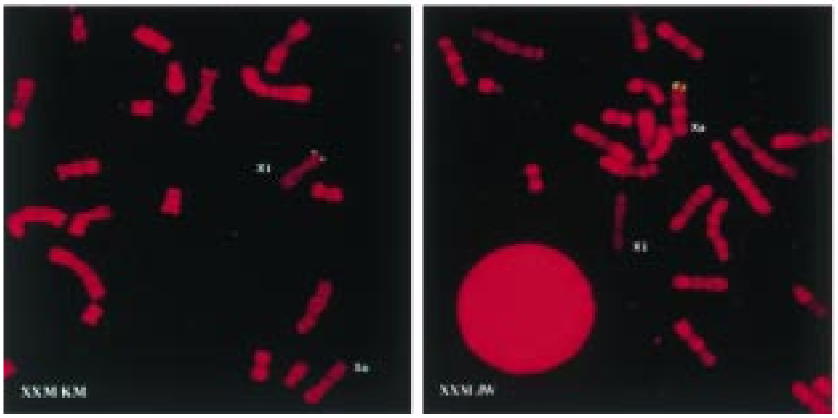

Figure 1 FISH analysis of chromosomes from true hermaphrodites (TH) RZ, PG, and AK, and XX males (XXM) KM and control JW. For localisation of the Yp fragment, the pHu14 probe containing the human SRY gene was used. Discrimination between the active and inactive X chromosome (Xa and Xi, respectively) was done by using RBG banding of metaphase chromosomes of the patients. Fragments of representative metaphases are shown for each patient.

derived from the cAMF cosmid as previously described.[26] Hybridisation of the biotin labelled probe to metaphase chromosomes from blood lymphocytes was performed on all three true hermaphrodite patients, RZ, PG, AK, and on XX males KM and JW and was followed by RBG banding. Detection and amplification of hybridisation signals was done using an immunocytochemical reaction. Hybridisation signals were analysed using a fluorescence microscope equipped with a CCD video camera and Meta-systems image analyser.

Results

Cytogenetic studies on blood lymphocytes showed a 46,XX karyotype in all male and true hermaphrodite patients. No structural aberrations of the autosomes or sex chromosomes were observed. Extensive chromosomal analysis and PCR amplification excluded mosaicism of a cell line containing a Y chromosome with a centromere. In patient PG, a 45,X karyotype was detected in 8% of metaphases.

Results of PCR analysis of the pseudoautosomal boundary Y (PABY), the SRY coding region, the centromere, and 17 other Y specific STS sequences in two 46,XX males with hypospadias and three 46,XX true hermaphrodites are presented in table 2. For comparison, clinical, molecular, and cytogenetic data of a completely male XX(Y+) patient (JW) are also included (tables 1, 2, and 3).

In two XX males (MB and KM) with ambiguity of the external genitalia (penile hypospadias), we detected a large portion of the Y chromosome containing STSs specific for intervals 1 and 2. Thus, we classified both patients as class 3 XX(Y+) males.[6] Interestingly, in the third control XX(Y+) male (JW), representing a classical XX male (no ambiguity of genitalia), a significantly smaller portion of the Y chromosome with no amplification product for interval 3 STSs was found. In all three XX males, the coding region of the SRY gene was found to be present (table 2).

Three XX true hermaphrodites, RZ, PG, and AK, were studied for the presence of the Y chromosome sequences. All of them were positive for the SRY gene in a very limited region of Y DNA corresponding to just a part of interval 1, namely only SRY, sY15, and sY17 in patient RZ, while only PABY and SRY sequences in patients PG and AK were detected (table 2).

The analysis of Yp (SRY+) specific FISH combined with X inactivation specific RBG banding was performed on metaphase chromosomes of all three true hermaphrodite patients, RZ, PG, AK, and on XX males KM and JW, the latter used in the study as a control carrying the SRY gene and with no sexual ambiguity. A blood sample for FISH analysis from patient MB was not available. In all cases studied, the Yp fragment was found to be translocated onto the X chromosome (fig 1). RBG banding of metaphase chromosomes was used in order to discriminate between the early and late replicating X chromosomes. The Yp fragment was found to be translocated onto the late replicating, that is, inactive, X chromosome in 92-98% of metaphases in all three true hermaphrodites (RZ, PG, AK) and in the XX male KM. However, the Yp was shown to be on the early replicating, that is, active, X chromosome in 96% of metaphases of the control classical XX male JW with complete masculinisation (fig 1, table 3).

Discussion

It has been established that the SRY gene is necessary and sufficient for the sex determining function of the Y chromosome in mammals.[27–29] This was supported by findings that XX(Y+) males have normal male genitalia, while XX(Y−) ones have genital ambiguity.[2 4 30 31] However, there are a small number of SRY positive XX males reported to have sexual ambiguity despite carrying the SRY gene.[4 7 9–20] Therefore, it was postulated that besides SRY there could be another gene on the Y chromosome, which contributed to complete male differentiation in XX males when translocated together with the SRY gene to the X chromosome.[15 32]

In this study, the SRY gene was detected in all five 46,XX(Y+) patients using PCR amplification and, as might be expected, the Yp in all three true hermaphrodites (RZ, PG, and AK) was found to be much shorter than in two XX males (MB and KM). Surprisingly, both of these two XX males, despite carrying large fragments of the Yp (class three XX males), had sexual ambiguity such as penile hypospadias, while the control XX male JW, carrying a Yp fragment only slightly larger than the XX true hermaphrodites, had no sexual ambiguity (tables 1 and 2).

In order to clarify whether this discrepancy could be explained by X inactivation spreading, as suggested previously,[33] we performed FISH analysis with a probe complementary to the human sex determining region followed by RBG metaphase banding to discriminate between active and inactive X chromosomes. Earlier studies showed preferential inactivation of the Y bearing X chromosome deleted for the steroid sulphatase gene in one XX male, while in another 11 XX males X inactivation was random. However, no information was presented as to whether any of these 12 XX(Y+) males had any sexual ambiguity.[34] In a more recent study of a single XX(Y+) true hermaphrodite, the Y chromosome fragment in the majority of cells was reported to be translocated onto the inactive but also partially deleted X chromosome.[35] Moreover, according to the authors, the positive selection of the inactivated X chromosome carrying Yp could be an artefact resulting from EBV transformation of lymphocytes used for analysis. In this study, we described X inactivation analysis performed on untransformed blood lymphocytes of three 46,XX males and three hermaphrodites.

We have shown that in all three true hermaphrodites the small Yp fragment was located on the inactive X chromosome in the majority of metaphases (table 3). Similarly, a translocation of the largest Yp fragment onto the inactive X chromosome in the majority of metaphases was also shown for XX male KM (table 3). However, in JW, a control classical XX male, the small Yp segment was translocated onto the active X chromosome in the majority of metaphases, presumably allowing sufficient SRY expression for complete masculinisation. The observed skewing (over 90%) of the random distribution of active or inactive Yp carrying X chromosomes could reflect cellular selection caused by rearrangements of X chromosomal genes close to the translocation breakpoint, as was shown for deletion of the steroid sulphatase gene.[34]

Assuming that the distribution of Yp bearing active and inactive X chromosomes in cells in the gonad resembles that found in blood lymphocytes of the studied patients, a likely explanation for a phenotype-genotype relationship in this group of disorders can be offered. We would argue that the presence of ovarian tissue in these true hermaphrodites could be the result of the X inactivation spreading into a relatively small Yp fragment leading to diminished expression of the SRY gene. Similarly, in XX male KM, the expression of the SRY gene might also be affected by translocation onto the inactive X chromosome, but possibly to a lesser extent owing to the larger Yp fragment yielding greater protection to the SRY gene against inactivation spreading, thus resulting in a less pronounced ambiguity.

In conclusion, our results strongly indicate that X inactivation spreading into a translocated Yp region containing the sex determining SRY gene and the selection of such a cell line could be the major mechanism causing phenotypic sexual ambiguity, including the presence of ovarian tissue in XX (SRY+) subjects.

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