Hum Genet (1997) 99 : 407–412

RAPID COMMUNICATION

E. Chevret · S. Rousseaux · M. Monteil · Y. Usson
J. Cozzi · R. Pelletier · B. Sèle

Meiotic behaviour of sex chromosomes investigated by three-colour FISH on 35 142 sperm nuclei from two 47,XYY males

Received: 12 April 1996 / Revised: 26 August 1996

Abstract Meiotic segregation of sex chromosomes from two fertile 47,XYY men was analysed by a three-colour fluorescence in situ hybridisation procedure. This method allows the identification of hyperhaploidies (spermatozoa with 24 chromosomes) and diploidies (spermatozoa with 46 chromosomes), and their meiotic origin (meiosis I or II). Alpha-satellite probes specific for chromosomes X, Y and 1 were observed simultaneously in 35 142 sperm nuclei. For both 47,XYY men (24 315 sperm nuclei analysed from one male and 10 827 from the other one) the sex ratio differs from the expected 1:1 ratio ($P < 0.001$). The rates of disomic Y, diploid YY and diploid XY spermatozoa were increased for both 47,XYY men compared with control sperm (142 050 sperm nuclei analysed from five control men), whereas the rates of hyperhaploidy XY, disomy X and disomy 1 were not significantly different from those of control sperm. These results support the hypothesis that the extra Y chromosome is lost before meiosis with a proliferative advantage of the resulting 46,XY germ cells. Our observations also suggest that a few primary spermatocytes with two Y chromosomes are able to progress through meiosis and to produce Y-bearing sperm cells. A theoretical pairing of the three gonosomes in primary spermatocytes with an extra sex chromosome, compatible with active spermatogenesis, is proposed.

Introduction

The 47,XYY constitution is one of the most frequent sex chromosome anomalies at birth (close to 1/1000 according to Hecht and Hecht 1987). Despite this high rate, little is known about the meiotic behaviour of the extra Y chromosome.The hypothesis of meiosis peculiar to 47,XYY males was first introduced by Thompson et al. (1967), in order to explain the normal karyotype of seven children born to a 47,XYY father. With reference to an earlier study by Ohno et al. (1963), they proposed that XYY germ cells are able to eliminate the extra Y chromosome, before reaching meiosis. More recently meiotic observations on testicular biopsies from 47,XYY males have permitted the direct observation of premeiotic germ cells, meiotic I cells or to a lesser extent meiotic II cells (Thompson et al. 1967; Evans et al. 1970; Hsu et al. 1970; Skakkebaek et al. 1970; Baghdassarian et al. 1975; Chandley et al. 1976; Faed et al. 1976). From these studies it has been assumed that only normal 46,XY germ cells are able to progress through meiosis. Therefore, the loss of the extra Y chromosome must occur before XYY germ cells enter meiosis (Evans et al. 1970; Tettenborn et al. 1970) or during the first step of the meiotic process (Melnyk et al. 1969).

Since spermatozoa are the final products of meiosis, the direct cytogenetic analysis of spermatozoa from 47,XYY males is the most reliable way to analyse the meiotic behaviour of the extra Y chromosome. For almost two decades, the most informative analysis of human sperm chromosomes, has relied on in vitro fertilisation of hamster oocytes (Rudak et al. 1978); unfortunately, this technique is both difficult and time consuming. Only a few sperm karyotypes are available for analysis and only one study of the sperm chromosome complement in an 47,XYY man has been published (Benet and Martin 1988: 75 karyotypes analysed).

Fluorescence in situ hybridisation (FISH) on human interphase spermatozoa provides a powerful alternative technique for detecting aneuploid sperm nuclei. However, only one study has yet been reported on a subject with a 47,XYY constitution (Han et al. 1994). In this study, 3009 spermatozoa were analysed using two-colour FISH, but this does not allow a reliable distinction between diploidy and disomy.

In order to study the meiotic behaviour of the extra Y chromosome with more accuracy, we have extensively

E. Chevret[1] · S. Rousseaux · M. Monteil · Y. Usson · J. Cozzi
R. Pelletier · B. Sèle (✉)
Reproductive Biology Unit, DyOGen laboratory, U309 INSERM, Albert Bonniot Institute, Grenoble University Medical School, F-38706 La Tronche Cedex, France
Fax: +33-4-76-51-24-77

Present address:
[1] Human Cytogenetics Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Field, London, UK

studied the segregation of the sex chromosomes in 35 142 spermatozoa from two fertile 47,XYY men using a three-colour FISH procedure.

Materials and methods

Subjects

Subject 1, a 47,XYY male, was discovered during routine karyotyping of voluntary sperm donors. He was 32 years old and the father of two boys. Seminal analysis demonstrated normal parameters according to WHO standards (World Health Organization 1992) with a sperm density of 110×10^6/ml and 80% motility. Two semen samples were frozen and stored in liquid nitrogen.

Subject 2, was a 37 year old 47,XYY male, whose karyotype was performed prior to vasectomy. He was the father of three boys and one girl. Seminal analysis demonstrated normal parameters according to WHO standards with a sperm density of 75×10^6/ml and 70% motility. One semen sample was frozen and stored in liquid nitrogen.

Controls

Control men had normal sperm parameters according to WHO standards.

Subject A: sperm count 70×10^6/ml, 80% motility; 25 years old
Subject B: sperm count 50×10^6/ml, 70% motility; 34 years old
Subject C: sperm count 60×10^6/ml, 50% motility; 29 years old
Subject D: sperm count 50×10^6/ml, 60% motility; 20 years old
Subject E: sperm count 53×10^6/ml, 70% motility; 39 years old

Sperm preparation

Each fresh or thawed semen sample was centrifuged at 450 *g* on a three-layer discontinuous Percoll gradient (90%/70%/40%) in order to remove leukocytes. The 90% fraction of the Percoll was washed twice in 0.01 M TRIS, pH 8 for 5 min at 600 *g*. The spermatozoa were decondensed with 10 mM dithiothreitol (Sigma) at room temperature for 10–40 min (Rousseaux and Chevret 1995), dropped onto clean dry slides and fixed with ethanol-acetic acid (3:1).

Probes

Plasmid probes pUC1.77, pXBR2 and pHY2.1, which were specific for chromosomes 1, X and Y, respectively, were used. 1 and X were α-satellite probes specific for centromeres; the Y probe was specific for a repeat sequence on the long arm of the Y (Yq12).

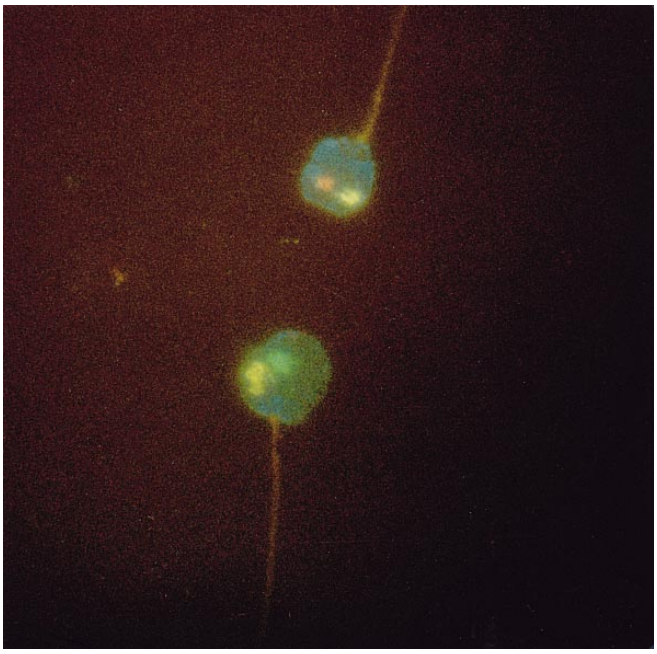

Fig. 1 Haploid X and haploid Y decondensed interphase human spermatozoa hybridised with probes specific for chromosome 1 (*yellow*), X (*red*) and Y (*green*)

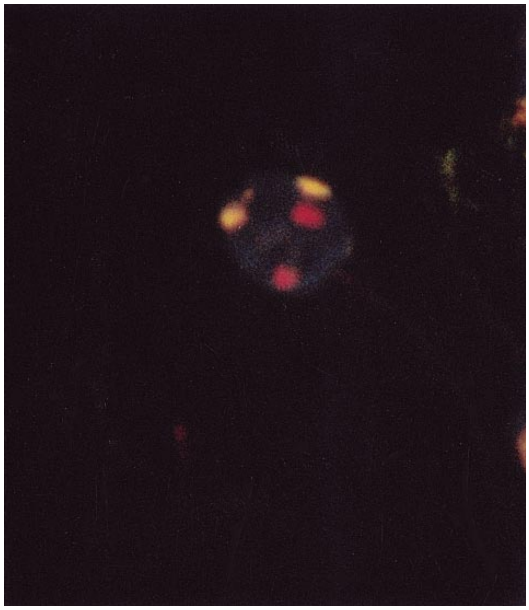

Fig. 2 Diploid XX decondensed interphase human spermatozoa showing two *yellow* signals (chromosome 1) and two *red* signals (chromosome X)

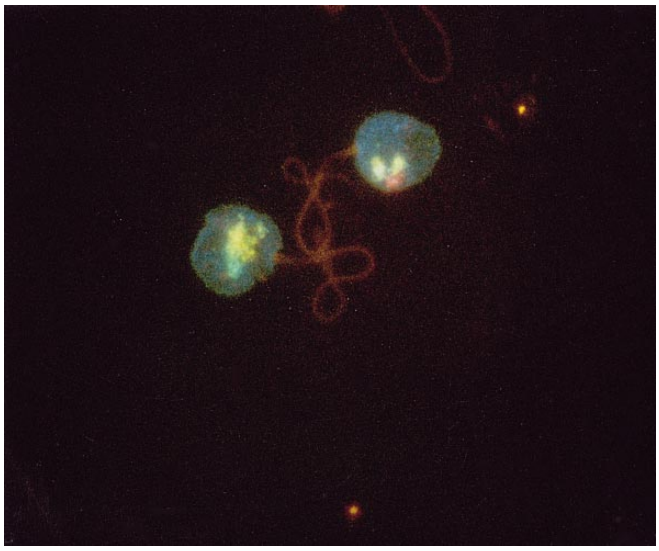

Fig. 3 Decondensed interphase human spermatozoa showing one *red* signal (chromosome X) and a split of the *yellow* signal (chromosome 1)

Table 1 Overall results of labelling of chromosomes X,Y and 1 in sperm of two 47,XYY males

Presumed karyotype	Subject 1		Subject 2	
	No. of sperm	%	No. of sperm	%
23,Y	13422	55.20	6086	56.21
24,YY	20	0.08	21	0.19
46,YY	10	0.04	2	0.02
23,X	10481	43.11	4630	42.76
24,XX	4	0.02	–	–
46,XX	5	0.02	–	–
24,XY	58	0.24	56	0.52
46,XY	42	0.17	12	0.11
24,Y + 1	38	0.16	12	0.11
24,X + 1	19	0.08	8	0.07
92,XXYY	2	0.008	–	–
No signal	0	–	0	–
Ambiguous	214	0.88	–	–
Total	24315	100	10827	100

Probe DNAs were isolated from an amplified phage library and then labelled by nick-translation with digoxigenin-11-dUTP (X probe) or biotin-16-dUTP (Y probe), or a mixture of digoxigenin-11-dUTP/biotin-16-dUTP (chromosome-1 probe). In order to determine their specificity, the probes were hybridised on lymphocyte spreads, and then co-hybridised in three-colour FISH, on the sperm preparation, as described below.

Hybridisation procedure

Sperm DNA was treated with RNase (100 µg/ml) (Eurogentec Seraing, Belgium) for 1 h at 37°C, dehydrated by passage through a cold ethanol series (70%/90%/100%), denatured for 2 min at 80°C in 70% formamide, 2xSSC, dehydrated again and air-dried.

A 20-µl sample of hybridisation mix (50% formamide, 0.5 × SSC, 0.5 × SSPE, 10% dextran sulphate) containing 100 ng of each probe and 10 µg of sonicated salmon sperm was heated at 75°C for 5 min to denature the probe, and then rapidly cooled on ice.

The mixture was applied to each sperm nuclei preparation and slides were covered with a 24 × 24-mm coverslip; this was sealed with rubber cement. Slides were placed in a moist chamber at 37°C for 20 h. Coverslips were then carefully removed and the slides were washed in 50% formamide, 2 × SSC at 45°C (three times for 5 min each), in 2 × SSC at 45°C (three times for 5 min each) and in 0.1 × SSC at 60°C (once for 3 min).

Biotinylated and/or digoxigenin-labelled probes were simultaneously detected with avidin-fluorescein isothiocyanate (FITC) (Vector Laboratories; Vector Biosys, diluted 1/300 in 4 × SSC, 5% skimmed milk) and anti-digoxigenin-rhodamine (Boehringer; diluted 1/200), respectively. A 20-min incubation at room temperature with avidin-FITC, followed by a 20-min incubation at room temperature with biotinylated anti-avidin-FITC (Vector Laboratories; diluted 1/200 in 4 × SSC, 5% skimmed milk) and a 30-min incubation at 37°C with avidin-FITC and anti-digoxigenin-rhodamine were performed. Between incubations, and following the last one, slides were washed three times for 5 min each in 4 × SSC, 0.1%Tween (Tween 20, Sigma) at room temperature. After the final incubation, slides were washed three more times for 3 min each in 4 × SSC at room temperature, and twice for 3 min each in phosphate-buffered saline. After the final wash, slides were air-dried in the dark. Then 10 µl of an antifade solution (Vector Laboratories) containing 50 ng/ml of the blue fluorescent dye DAPI (4′,6-diamidino-2-phenylindole) was applied to the slides under a 22 × 22-mm coverslip.

Scoring

The slides were screened using a × 100 objective in a Zeiss Axiophot equipped with a triple band pass filter, FITC/rhodamine/DAPI. Yellow, red and green spots detected chromosomes 1, X and Y, respectively (Fig. 1).

Two signals of the same colour were scored as two copies of the corresponding chromosome when they were the same intensity and size, and were clearly distinct. This was to ensure that they arose from two separate chromosomes (Fig. 2) and not from a split signal on a single chromosome (Fig. 3).

The slides were scored by two independent observers, who each counted approximately 3000 sperm nuclei per slide. No significant differences were detected between observers (results not shown). All sperm nuclei with split or superimposed signals were classified as ambiguous (Tables 1, 2).

The scores of the two XYY men were compared with those of control men because aneuploid sperm cells are present in normal men. Since data on aneuploidy frequencies differ between authors (Goldman et al. 1993; Han et al. 1993; Schattman et al. 1993; Williams et al. 1993; Bischoff et al. 1994; Chevret et al. 1995) it was necessary to establish the aneuploidy rates in control men.

A Students *t*-test was performed to compare results from 47,XYY males and controls, and a difference was considered significant at $P = 0.05$.

Results

For the three plasmid probes, using our decondensation procedure (Rousseaux and Chevret 1995), hybridisation efficiency was more than 99% (Tables 1, 2).

Table 2 Disomy and diploidy rates (%) of chromosomes X, Y and 1 in sperm of five controls observed by three-colour fluorescence in situ hybridisation (FISH)

	24,YY	46,YY	24,XX	46,XX	24,XY	46,XY	24,Y or X +1	Ambiguous	No signal
Subject A (32599 sperm analysed)	0.02	0.006	0.02	0.02	0.09	0.12	0.08	0.47	0.21
Subject B (11584 sperm analysed)	0.008	0.008	0	0.07	0.41	0.02	0.26	0.008	0
Subject C (73574 sperm analysed)	0.02	0.01	0.02	0.005	0.86	0.23	0.13	0.03	0
Subject D (13631 sperm analysed)	0	0	0.14	0.04	0.08	0.09	0.32	0.49	0
Subject E (10662 sperm analysed)	0.01	0	0.01	0.19	0.35	0	0.56	0.27	0

Table 3 Comparison of the behaviour of chromosomes X, Y and 1 from 47,XYY males and controls

Presumed karyotype	Subject 1 (%)	Subject 2 (%)	Controls [Mean (%) ± standard deviation]
23,Y	55.20	56.21	48.22 ± 2.23
24,YY	0.08	0.19	0.01 $\pm 6 \times 10^{-5}$
46,YY	0.04	0.02	0.005 $\pm 2 \times 10^{-5}$
23,X	43.11	42.76	50.10 ± 3.39
24,XX	0.02	–	0.04 $\pm 3 \times 10^{-3}$
46,XX	0.02	–	0.06 $\pm 4 \times 10^{-3}$
24,XY	0.24	0.52	0.36 ± 0.08
46,XY	0.17	0.11	0.09 $\pm 7 \times 10^{-3}$
24,X or Y +1	0.23	0.18	0.27 ± 0.03

In 47,XYY males, a total of 35 142 sperm nuclei (24 315 from subject 1 and 10 827 from subject 2) were analysed for the presence of chromosomes X, Y and 1 (Table 1). For the two subjects the sex ratio differs from the expected 1:1 ratio ($P < 0.001$).

Meiotic I errors

Frequencies of hyperhaploid XY spermatozoa were 0.24% for subject 1 and 0.52% for subject 2. Rates of diploidy XY were 0.17% for subject 1 and 0.11% for subject 2.

Meiotic II errors

Disomy Y was estimated to be 0.08% for subject 1 and 0.19% for subject 2. The frequency of disomy X was 0.02% for subject 1. No disomic X sperm nuclei were observed from subject 2. The frequency of diploid YY sperm nuclei was 0.04% for subject 1 and 0.02% for subject 2. Diploid XX sperm nuclei were only observed in subject 1, with a frequency of 0.02%. Chromosome 1 disomy was close to 0.20% for each 47,XYY male.

From subject 1, two tetraploid sperm cells were also observed (0.008%). In 212 spermatozoa (0.88%) the signal was ambiguous. Most of them (205) showed one yellow spot, but this was because the spots were superimposed on each other (yellow/green or yellow/red). It was therefore not possible to consider these spermatozoa as nullisomic for the gonosomes.

All these frequencies were compared with those of five control men (142 050 sperm nuclei analysed) (Tables 2, 3). The rate of disomy Y was significantly increased ($P < 0.001$ for both subjects). Increases of haploid Y, and diploid YY spermatozoa were also observed ($P < 0.02$). The rate of meiotic I diploid cells (46,XY) was slightly increased compared with controls ($P < 0.05$), whereas frequencies of hyperhaploidy XY, disomy X and disomy 1 were in the same range as in control spermatozoa.

Discussion

In the present study, the meiotic behaviour of the sex chromosomes from two 47,XYY fertile males was investigated by a three-colour FISH procedure. This method has allowed the scoring of a large number of sperm nuclei and the detection of small variations in frequencies. The rates of haploid Y, disomic Y, diploid YY and diploid XY spermatozoa were increased in both of the 47,XYY men, compared with controls, whereas hyperhaploidy XY, disomy X and disomy 1 were not significantly different from those of normal men. Neither Benet and Martin (1988), karyotyping 75 spermatozoa from one 47,XYY subject, nor Han et al. (1994), analysing 3009 sperm nuclei from one 47,XYY patient in two-colour FISH, have detected such increases. This could be related to the low numbers of sperm nuclei analysed in these studies.

The present results support the hypothesis of a process of meiosis peculiar to 47,XYY males, which had already been suggested by Thompson et al. (1967). In most cells, the extra Y chromosome would be lost before meiosis, resulting in 46,XY cells that would then progress through normal meiotic division. However, the slight but significant increases in haploidy Y, disomy Y and diploidy YY suggest that in a small proportion of spermatozoa the extra Y chromosome is not lost. In this latter case a YY bivalent with an X univalent would be formed and the X univalent could then be eliminated during the anaphase of the first meiotic division. The increase in disomic Y and diploid Y sperm nuclei would therefore be the consequence of the excess of 23,Y bearing spermatocytes II. This hypothesis is supported by previous observations in testicular biopsies from XYY males (Table 4) showing a few primary spermatocytes containing a YY bivalent with an X univalent, and secondary spermatocytes with 23 chromosomes (Hultén 1970; Tettenborn et al. 1970; Hultén and Pearson 1971; Berthelsen et al. 1981; Speed et al. 1991). Moreover, according to Burgoyne (1979) the presence of XY bivalents would result in meiotic I arrest, whereas a YY pairing associated with a free X chromosome would not affect the spermatogenic process.

Similarly, a slight but significant increase in hyperhaploid 24,XY spermatozoa associated with a normal rate of 22,0 hypohaploid sperm has been observed in 46XY/

Table 4 Meiotic studies of XYY males

	No. of 47,XYY males	Spermatogonia				Spermatocytes I				Spermatocytes II
		45 chromosomes	46 chromosomes	47 chromosomes	< 47 chromosomes	XY bivalent	X univalent + Y univalent	YY bivalent + X univalent	Others	
Thompson et al. (1967)	1	4	17	1[a]	–	155		–	–	34 with 23 chromosomes 5 with 24 chromosomes (no evidence of 2 Y chromosomes)
Melnyk et al. (1969)	6	1	56	8	–	145	–	–	–	–
Evans et al. (1970)	1	–	–	–	–	341	24	2 (not confidently identified)	–	47 with 23 chromosomes
Hsu et al. (1970)	1	–	3	5	6	20	6	–	8[b]	–
Hultén (1970)	2	–	18	9	–	61	49	6	7[c]	–
Tettenborn et al. (1970)	3	–	15	52	–	17	1	2	1[d]	–
Hultén and Pearson (1971)	1	–	–	–	–	34	2	31	2[c]	–
Berthelsen et al. (1981)	4	–	–	–	–	3	–	9	–	–
Speed et al. (1991)	1	–	8	2	–	88	6	5	–	20 with one X chromosome 20 with one Y chromosome

[a] Forty-seven chromosomes including only a single Y
[b] Two primary spermatocytes had 24 structures including a normal XY bivalent and 2 G univalent; six cells had 23 structures with no clear-cut XY bivalents
[c] Cells had a univalent X chromosome alone
[d] One primary spermatocyte with 24 elements including an extra bivalent of G-group size

47XXY males (Cozzi et al. 1994; Chevret et al. 1996) suggesting preferential XX pairing in the few XXY cells that were able to achieve meiosis.

Overall, we suggest that in these gonosomal constitutional abnormalities (XYY and XY/XXY) a few primary spermatocytes would carry an extra gonosome and in these preferential pairing would occur between the two homologous sex chromosomes. This pairing would allow active spermatogenesis, as opposed to XY pairing.

Acknowledgements We thank Dr. Rebecca Roylance for assistance with the preparation of this manuscript, Dr. Dominique Le Lannou CECOS ouest Rennes (Centre d'Etude et de Conservation du Sperme) for referring the 47,XYY males and Dr. Marie Guichaoua for her helpful comments on the manuscript. This research was supported by the Fondation pour la Recherche Médicale, and the Groupement de Recherches et d'Etudes sur les Génomes (grant 94122).

References

Baghdassarian A, Bayard F, Borgaonkard DS, Arnold EA, Solez K, Migeon CJ (1975) Testicular function in XYY men. Johns Hopkins Med J 136:15–24

Benet J, Martin RH (1988) Sperm chromosome complements in a 47,XYY man. Hum Genet 78:313–315

Berthelsen JG, Skakaebaek NE, Perboll O, Nielsen J (1981) Electron microscopic demonstration of the extra Y chromosome in spermatocytes from human XYY males. In: Byskov AG, Peters H (eds) Development and function of reproduction organs. Excerpta Medica, Amsterdam, pp 328–337

Bischoff FZ, Nguyen DD, Burt KJ, Schaffer LG (1994) Estimates of aneuploidy using multicolor fluorescence in situ hybridization on human sperm. Cytogenet Cell Genet 66:237–243

Burgoyne PS (1979) Evidence for an association between univalent Y chromosomes and spermatocyte loss in XYY mice and men. Cytogenet Cell Genet 23:84–89

Chandley AC, Flechter J, Robinson JA (1976) Normal meiosis in two 47,XYY men. Hum Genet 33:231–240

Chevret E, Rousseaux S, Monteil M, Pelletier R, Cozzi J, Sèle B (1995) Meiotic segregation of the X and Y chromosomes and chromosome 1, analyzed by three-color FISH in human interphase spermatozoa. Cytogenet Cell Genet 71:126–130

Chevret E, Rousseaux S, Monteil M, Usson Y, Cozzi J, Pelletier R, Sèle B (1996) Increased incidence of hyperhaploid 24,XY spermatozoa detected by three-colour FISH in a 46,XY/47,XXY male. Hum Genet 97:171–175

Cozzi J, Chevret E, Rousseaux S, Pelletier R, Benitz V, Jalbert H, Sèle B (1994) Achievement of meiosis in XXY germ cells: study of 543 sperm karyotypes from an XY/XXY mosaic patient. Hum Genet 93:32–34

Evans EP, Ford CE, Chaganti RSK, Blank CE, Hunter H (1970) XY spermatocytes in an XYY male. Lancet 1:719–720

Faed M, Robertson J, MacIntosh WG, Grieve J (1976) Spermatogenesis in an infertile XYY man. Hum Genet 33:341–347

Goldman ASH, Fomina Z, Knights PA, Hill CJ, Walker AP, Hultén MA (1993) Analysis of the primary sex ratio chromosome aneuploidy and diploidy in human sperm using dual-colour fluorescence in situ hybridisation. Eur J Hum Genet 1:325–334

Han TH, Ford JH, Webb GC, Flaherty SP, Correll A, Matthews CD (1993) Simultaneous detection of X- and Y-bearing human sperm by double fluorescence in situ hybridization. Mol Reprod Dev 34:308–313

Han TH, Ford JH, Flaherty SP, Webb GC, Matthews CD (1994) A fluorescent in situ hybridization analysis of the chromosome constitution of ejaculated sperm in a 47,XYY male. Clin Genet 45:67–70

Hecht F, Hecht BK (1987) Aneuploidy in humans: dimensions, demography, and dangers of abnormal numbers of chromosomes. In: (eds) Aneuploidy, part A: incidence and etiology. pp 9–49

Hsu YL, Shapiro LR, Hirschhorn K (1970) Meiosis in an XYY male. Lancet 1:1173–1174

Hultén, M (1970) Meiosis in XYY men. Lancet 1:717–718

Hultén M, Pearson PL (1971) Fluorescent evidence for spermatocytes with two chromosomes in an XYY male. Ann Hum Genet 34:273–276

Melnyk J, Thompson H, Rucci AJ, Hayes S (1969) Failure of transmission of the extra chromosome in subjects with 47,XYY karyotypes. Lancet 2:797–798

Ohno S, Jainchill J, Stenius C (1963) The creeping vole (*Microtus oregoni*) as a gonosomic mosaic 1 – the 0Y/XY constitution of male. Cytogenetics 2:232–239

Rousseaux S, Chevret E (1995) In-vitro decondensation of human spermatozoa for fluorescence in-situ hydridization. Mol Hum Reprod 10:2209–2213

Rudak E, Jacobs PA, Yanagimachi R (1978) Direct analysis of chromosome constitution of human spermatozoa. Nature 274: 911–913

Schattman GL, Munné S, Grifo JG, Carton L, Cohen J (1993) Aneuploidy in spermatozoa using fluorescence in situ hybridization. J Assist Reprod Genet 10:360–365

Skakkebaek NE, Philip J, Mikkelsen M, Hammen R, Nielsen J, Perboll O, Yde H (1970) Studies on spermatogenesis, meiotic chromosomes, and sperm morphology in two males with a 47,XYY chromosome complement. Fertil Steril 21:645–656

Speed RM, Faed MJW, Batstone PJ, Baxby K, Barnetson W (1991) Persistence of two Y chromosomes through meiotic prophase and metaphase I in an XYY man. Hum Genet 87: 416–420

Tettenborn U, Gropp A, Murken JD, Tinnefeld W, Fuhrmann W, Schwinger E (1970) Meiosis and testicular histology in XYY males. Lancet 1:267–268

Thompson H, Melnyk J, Hecht F (1967) Reproduction and meiosis in XYY. Lancet 2:831

Williams BJ, Ballenger CA, Malter HE, Bishop F, Tucker M, Zwingman TA, Hassold TJ (1993) Nondisjunction in human sperm – results of fluorescence in situ hybridization studies using two and three probes. Hum Mol Genet 2:1929–1936

World Health Organization (1992) Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambrige University Press