Human Reproduction vol.13 no.1 pp.124–127, 1998

Meiotic products of a Klinefelter 47,XXY male as determined by sperm fluorescence in-situ hybridization analysis

Anna M.Estop[1,5], Santiago Munné[2], Kathy M.Cieply[1], Kelly K.Vandermark[2], Allen N.Lamb[3] and Harry Fisch[4]

[1]Department of Human Genetics, Allegheny University of the Health Sciences, Allegheny General Hospital, 320 East North Avenue, Pittsburgh, PA 15212, [2]Institute for Reproductive Medicine and Science, Saint Barnabas Medical Center, West Orange, New Jersey, [3]Genzyme Genetics, Santa Fe, New Mexico and [4]Department of Urology, Columbia University College of Physicians and Surgeons, New York, New York, USA

[5]To whom correspondence should be addressed

The meiotic segregation of 24 spermatozoa obtained from a 47,XXY male is described. Three-colour fluorescence in-situ hybridization with probes for chromosomes X, Y and 18 was used. Five spermatozoa carried an X chromosome, seven carried a Y, six had an XY gonosomal complement, five were missing the sex chromosome and one spermatozoon was presumably diploid with an XX/1818 complement. Our results support the hypothesis that XXY cells are able to complete meiosis. In this patient, the percentage of spermatozoa with an abnormal number of sex chromosomes increased from 1/6 (17%) among spermatozoa with normal morphology to 11/18 (61%) in spermatozoa with abnormal morphology.

Key words: Klinefelter syndrome/meiosis/non-disjunction/sex chromosomes

Introduction

Klinefelter syndrome is characterized by gynaecomastia, hypogonadism, azoospermia or oligozoospermia, and increased excretion of urinary gonadotrophins. It occurs in one out of 600 newborn males. Affected patients have a 47,XXY, or a 46,XY/47,XXY mosaic karyotype. The clinical features are variable, and milder manifestations of the syndrome are found when a mosaic for a 46,XY cell line is present. Traditionally it has been accepted that patients with no evidence of mosaicism are sterile. However, exceptionally, focal spermatogenesis has been described in these patients (Steinberger *et al*., 1965; Foss *et al*., 1971) and more recently it has been shown that patients with a 47,XXY karyotype may have an occasional spermatozoon in their semen, and in wet preparations of testicular tissue (Tournaye *et al*., 1996)

The newer assisted reproductive procedures allow for the recovery of single spermatozoa from either ejaculates or testicular biopsies and their use in intracytoplasmic sperm injection (ICSI). There is some concern about the chromosome normality of the embryos generated through this infertility treatment. ICSI has already been applied to patients with Klinefelter syndrome, although no ongoing pregnancies have been achieved (Bourne *et al*., 1995; Harari *et al*., 1995; Staessen *et al*., 1996; Tournaye *et al*., 1996). Recent reports have shown an increased incidence of hyperhaploid 24,XY spermatozoa in males with 46,XY/47,XXY karyotypes by using either direct chromosome analysis of spermatozoa (Cozzi *et al*., 1994) or fluorescence in-situ hybridization (FISH) on sperm nuclei (Blanco *et al*., 1996; Chevret *et al*., 1996; Martini *et al*., 1996). These data support previous observations (Vidal *et al*., 1984) which indicate that germ cells with a 47,XXY chromosome complement might be able to complete meiosis and produce 24,XY spermatozoa. There are no reports of similar studies performed on patients with Klinefelter syndrome with absence of mosaicism.

This study presents FISH results on the sex chromosome segregation of 24 spermatozoa identified in two ejaculates of a male with a 47,XXY karyotype. Three-colour FISH was performed with probes specific for chromosomes X, Y and 18.

Materials and methods

Case history

The patient was referred for evaluation of a male factor in a primarily infertile marriage. The couple had been married for 4 years and had been attempting to initiate a pregnancy for a year and a half, without success. Neither the patient nor his wife had been involved in any prior pregnancies. A hormonal profile revealed an elevated follicle stimulating hormone of 39.2 ng/dl, a luteinizing hormone of 12.9 ng/dl and testoterone being low–normal at 247 ng/dl. Atrophic testicles, each measuring 3×1 cm, were present. A peripheral blood chromosome analysis showed a 47,XXY karyotype in 100 metaphases analysed, with no evidence of mosaicism. A skin biopsy was declined by the patient. Semen analyses were performed revealing an average volume of <1 ml, with an occasional motile spermatozoon present.

FISH studies

Two ejaculates obtained with a 1 month interval were used. The semen was mixed with test–yolk buffer (Bolanos *et al*., 1983), and shipped to the laboratory via overnight courier. Upon arrival, the samples were washed twice in modified sperm washing medium (Quinn *et al*., 1984) and resuspended in 1 ml of the same medium. Smears were made by dropping one drop of suspension onto a clean slide and smearing with the aid of another slide. The slides were air-dried and subsequently scored with phase contrast microscopy at ×20 to locate spermatozoa. After confirmation of cell type and evaluation of morphology by Kruger's strict criteria (Kruger *et al*., 1988) with a ×100 phase contrast oil objective, the location of the spermatozoa was recorded.

Sperm heads were decondensed for FISH following procedures described by Wyrobeck *et al*. (1990) with minor modifications. The

Table I. Results of the fluorescence in-situ hybridization (FISH) analysis of 26 spermatozoa recovered from a patient with a 47,XXY karyotype

Chromosomes	Number	Percentage
X/18	5	20.8
Y/18	7	29.2
XY/18	6	25.0
–/18	4	16.6
–/18 18	1	4.2
XX/18 18	1	4.2
FISH total	24	100.0
No hybridization	2	
Total	26	

slides were incubated in 1.0 mM dithiothreitol (Sigma, St Louis, MO, USA) for 30 min, followed by a second incubation in 20 mM lithium diiodosalicylate (Sigma) for 3 h and a brief rinse in 2×SSC. Immediately afterwards, the slides were processed for FISH.

Directly labelled probes from VYSIS (Downers Grove, IL, USA) were used. For chromosomes X and 18, the probes detected alpha satellite centromeric sequences, and for chromosome Y, satellite III sequences from Yq12 were highlighted. Three-colour hybridizations were performed with chromosome X labelled with d-UTP (orange), chromosome Y with d-CTP (green) and chromosome 18 (yellow) was a mixture of green and orange labelled probes. The use of an autosomal probe such as chromosome 18, in addition to the X and Y probes, allowed for the distinction between disomy and diploidy. Sperm slides were denatured in 70% formamide/2×SSC at 75°C for 10 min. Ten microlitres of the probe–hybridization mixture (7 µl hybridization buffer, 2 µl of H_2O, and 1 µl of each probe) were denatured at 75°C for 5 min, added to the slides and hybridized overnight at 42°C. The hybridization buffer contained 10% dextran sulphate and 55% formamide in 1×SSC. After post-hybridization washes at 50% stringency at 45°C, the slides were counterstained with DAPI.

The microscopic analysis was performed with a Zeiss Axiophot equipped with a Photometrics charge-coupled device camera and attached to an Imaging System, SmartCapture, from VYSIS. A triple band pass filter for FITC, Rodamine and DAPI allowed the simultaneous visualization of orange, green, yellow and blue. All identified spermatozoa were captured at the time of phase contrast analysis, prior to FISH, and after FISH was performed. Only individual, well-delineated sperm cells with a tail were scored. Two signals of the same colour were counted as disomy only if they were of the same size and intensity and separated by at least one domain. The presence of yellow signal(s) in the absence of orange and green was considered nullisomy for the sex chromosomes. No FISH signals in a spermatozoon showing DAPI stain was considered a no-hybridization.

Results

The results of the FISH analysis are depicted in Table I. A total of 26 spermatozoa were recovered from the two ejaculates. Two cells showed DAPI staining but no hybridization signals. Of these two, one had an abnormal morphology with a markedly constricted head under phase contrast and the other had a tapered head and an abnormal tail. The efficiency of hybridization was therefore 93.6%. Among the 24 cells with FISH positive signals, six different types of meiotic products were observed. Fifty per cent of the available spermatozoa had a normal chromosome constitution and 50% abnormal. Seven cells carried a Y (29.2%) and five carried an X chromosome (20.8%). Six spermatozoa (25.0%) showed an XY/18 complement, and five (20.8%) carried no sex chromosome. Of those five spermatozoa, four contained one chromosome 18 only and one had two chromosomes 18. One bicephalic spermatozoon with one tail and an XX/1818 complement was also seen. No 24,XX nor 24,YY spermatozoa were observed.

Six sperm cells presented with a normal morphology. Of those six, only one (17%) had an abnormal chromosome complement, namely the absence of the sex chromosome. Abnormal sperm forms seen were: slightly amorphous (four), vacuoles (seven), microcephalic (two), bicephalic (one), severe amorphous (three), neck abnormality (one). The rate of sex chromosome abnormality among spermatozoa with abnormal morphology was 61%.

Discussion

The meiotic segregation of the sex chromosomes in this patient with a 47,XXY karyotype resulted in 12 spermatozoa with a normal sex chromosome content and 12 spermatozoa with abnormal sex chromosomes. These results show that a male with a 47,XXY karyotype with no evidence of mosaicism may produce spermatozoa with normal sex chromosomes. Spermatozoa with an X or a Y were similarly represented: five and seven respectively. Six spermatozoa (25%) contained an XY/18 complement. This is a much higher frequency than found in our control subjects (0.18%; Estop *et al*., 1996) and also is much higher than found in spermatozoa of patients with evidence of 46,XY mosaicism. Several studies have reported frequencies of 24,XY spermatozoa in patients with 46,XY/47,XXY karyotypes ranging from 0.92% (Cozzi *et al*., 1994) to 1.3% (Martini *et al*., 1996) and 2.09% (Chevret *et al*., 1996). The meiotic observations of Skakkebaek *et al*. (1969) and Vidal *et al*. (1984) already suggested that 47,XXY cells could produce hyperhaploid 24,XY spermatozoa, as opposed to the observations of Luciani *et al*. (1970) which supported the hypothesis of only 46,XY cells being able to complete meiosis. Spermatozoa with an XY could be produced after pairing of XX chromosomes with random segregation of the Y univalent. This type of segregation should yield an equal number of spermatozoa with an XY and spermatozoa with an X chromosome, but no Y spermatozoa would be produced. This contrasts with our findings of similar numbers of nuclei with and X and a Y. The absence of XX cells (which would result after the segregation of XY bivalents in meiosis I) is also consistent with a preferential pairing of homologous sex chromosomes.

Chromosomes 18 with no sex chromosome signals were seen in five spermatozoa (20.8%), including one spermatozoon with two chromosomes 18. Again, spermatozoa without a gonosome were more common in this patient than in patients reported as mosaics for a 46,XY cell line (0.4%, Cozzi *et al*., 1994; 0.59%, Chevret *et al*., 1996; 1.5%, Martini *et al*., 1996) and in our control subjects (0.13%; Estop *et al*., 1996). An explanation for the presence of spermatozoa with no sex chromosome would be the pairing of the three gonosomes at the spermatocyte I stage and subsequent non-disjunction, so

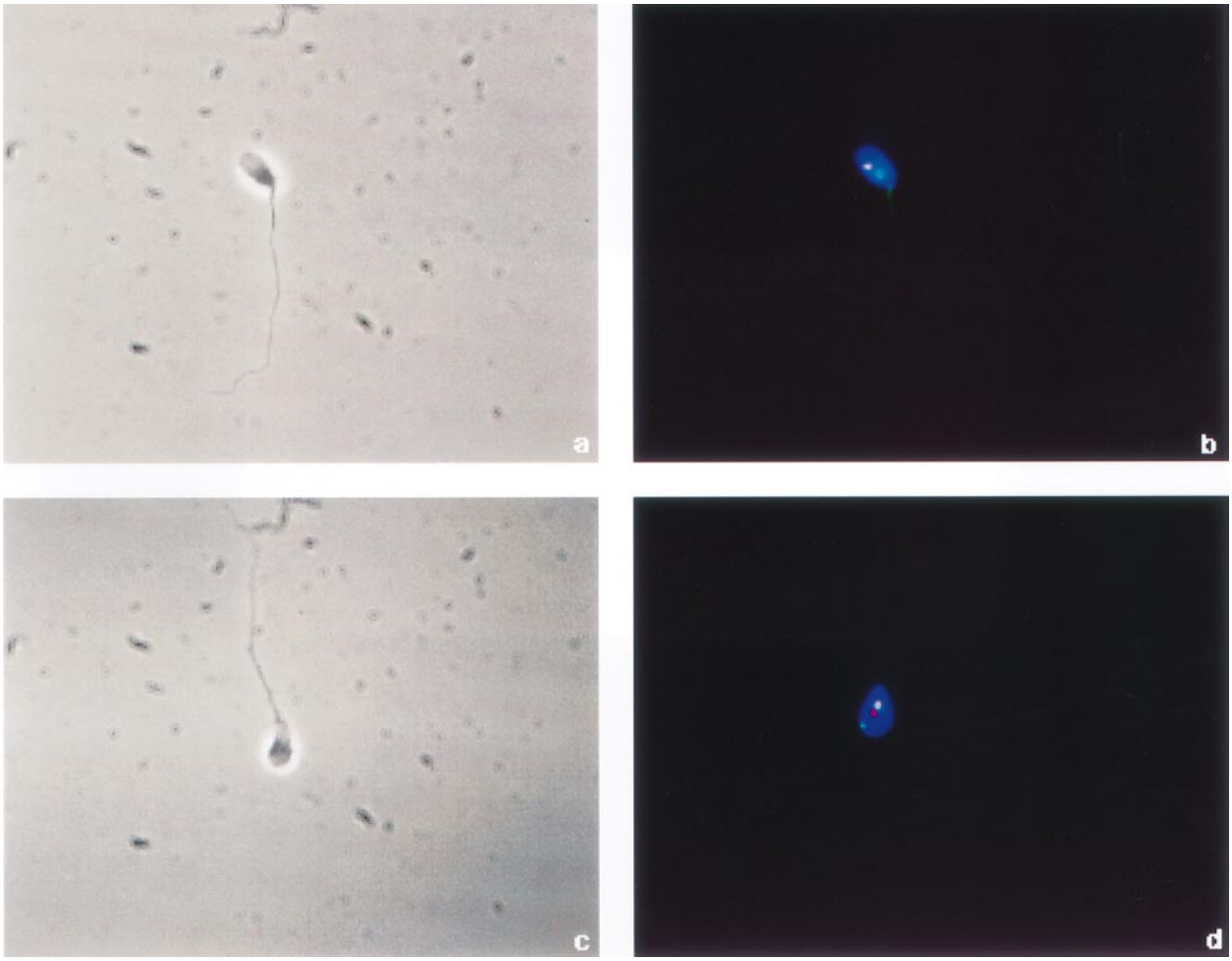

Figure 1. Sequential phase-contrast microscopy with a ×100 oil objective and fluorescence in-situ hybridization images of two spermatozoa. (**a**, **b**) Spermatozoa with normal morphology and a Y/18 chromosome complement; (**c**, **d**) spermatozoa with a neck abnormality and hyperhaploidy for the sex chromosomes (XY/18). Magnification ×1000.

that spermatozoa would be formed with no sex chromosome. This model implies the presence of immature cells with XXY trivalents which might not be able to complete meiosis. Only sequential studies of the whole spermatogenic process from spermatogonia to spermatozoa in the same individual could provide enough information to establish the true patterns of meiotic segregation in males with a 47,XXY karyotype.

Phase contrast microscopy with a ×100 oil objective allowed an accurate assessment of the sperm head, mid-piece and tail morphology, Kruger's strict criteria were followed as the boundaries between the nucleus and the acrosome were clearly visualized, as well as the mid-piece and tail. The FISH protocols established in our laboratory did not require any modifications when applied to preparations that had been observed under phase contrast oil microscopy. This type of analysis permits the capture of sequential images of all the analysed cells (Figure 1). Six spermatozoa out of 24 with FISH results presented with a normal morphology (25%). Only one of those (17%) had abnormal chromosomes. The remaining 18 cells with different types of abnormal morphology had an average of 61% of sex chromosome abnormality. This type of analysis, when applied to patients with oligoasthenozoospermia, might be of use in researching potential correlations between abnormal sperm forms and chromosome content.

Sperm genetic analysis in patients with infertility and a chromosome condition is of particular importance because of the availability of ICSI as an infertility treatment. ICSI bypasses all in-vivo sperm selection processes up to the events prior to pronuclear formation, and therefore may allow for the production of chromosomally imbalanced embryos that might have been selected against in an in-vivo environment. Indeed, a patient with a mosaic Klinefelter karyotype was reported to have a normal fertilization rate after ICSI; however, there was a high rate of cleavage arrest (44%) after pronuclei were observed (Bourne *et al*., 1995). Another study (Harari *et al*., 1995) reported similar findings with a mosaic Klinefelter patient with a high incidence of cleavage failure (50% of oocytes fertilized). On the other hand, Staessen *et al*. (1996) performed ICSI with testicular spermatozoa of three 47,XXY Klinefelter patients which yielded pronuclei in eight oocytes and five proceeded to the 6-cell stage. Preimplantation diagnosis of a normal sex chromosome was achieved in all five. However, four embryos were replaced with only one resulting biochemical pregnancy. Preimplantation diagnosis and/or pre-

natal diagnosis may be an option for patients carrying a chromosome abnormality and undergoing ICSI.

References

Blanco, J., Rubio, C., Simon, C. *et al*. (1996) A fluorescent in-situ hybridization study of the spermatogenic process and sperm production in a 47,XYY and a 47,XXY/46,XY male: implications for ICSI. *Abstracts of the 12th Annual Meeting of ESHRE, Hum. Reprod.*, **11**, 159–160.

Bolanos, J.R., Oversheet, J.N. and Katz, D.F. (1983) Human sperm penetration of zona-free hamster eggs after storage of semen for 48 h at 2°C to 5°C. *Fertil. Steril.*, **39**, 536–541.

Bourne, H., Richings, N., Harari, O. *et al.* (1995) The use of intracytoplasmatic sperm injection for the treatment of severe and extreme male infertility. *Reprod. Fertil. Dev.*, **7**, 237–245.

Chevret, E., Rousseaux, S., Monteil, M. *et al*. (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, R. *et al*. (1996) Achievement of meiosis in XXY germ cells: study of 543 sperm karyotypes from an XY, XXY mosaic patient. *Hum. Genet.*, **93**, 32–34.

Estop, A.M., Cieply, K. and Aston, C. (1996) Is there an interchromosomal effect in chromosome translocation carriers? Sperm FISH studies. *Am. J. Hum. Genet.*, **59**, A117.

Foss, G.L. and Lewis, J.W. (1971) A study of four cases with Klinefelter's syndrome showing motile spermatozoa in their ejaculates. *J. Reprod. Fertil.*, **25**, 401–408.

Harari, O., Bourne, H., Baker, G. *et al*. (1995) High fertilization rate with intracytoplasmatic sperm injection in mosaic Klinefelter's syndrome. *Fertil. Steril.*, **63**, 182–184.

Kruger, T.F., Acosta, A.A., Simmons, K.F. *et al*. (1988) Predictive value of abnormal sperm morphology in *in vitro* fertilization. *Fertil. Steril.*, **49**, 112–117.

Luciani, J.M., Mattei, A., Devictor-Vuillet, M. *et al*. (1970) Étude des chromosomes meiotiques dans un cas de maladie de Klinefelter avec spermatogènese et caryotype 46,XY/47,XXY. *Ann. Génét.*, **13**, 249–253.

Martini, E., Geraedts, J.P.M., Liebaers, I. *et al*. (1996) Constitution of semen samples from XYY and XXY males as analysed by in-situ hybridization. *Hum. Reprod.*, **11**, 1638–1643.

Quinn, P., Warnes, G., Kerin, J. and Kriby, C. (1984) Culture factors in relation to the success of human *in vitro* fertilization and embryo transfer. *Fertil. Steril.*, **42**, 202–209.

Skakkebaek, N.E., Philip, J. and Hammen, R. (1969) Meiotic chromosomes in Klinefelter syndrome. *Nature*, **221**, 1075–1076.

Staessen, C., Coonen, E., Van Assche, E. *et al*. (1996) Preimplantation diagnosis for X and Y normality in embryos from three Klinefelter patients. *Hum. Reprod.*, **11**, 1650–1653.

Tournaye, H., Staessen, C., Liebaers, I. *et al*. (1996) Testicular sperm recovery in nine 47,XXY Klinefelter patients. *Hum. Reprod.*, **11**, 1644–1649.

Vidal, F., Navarro, J., Templado, C. *et al.* (1984) Synaptonemal complex studies in a mosaic 46,XY/47,XXY male. *Hum. Genet.*, **66**, 306–308.

Wyrobeck, A.J., Alhorn, T., Balhorn, R. *et al*. (1990) Fluorescence *in situ* hybridization to Y chromosome in decondensed human sperm nuclei. *Mol. Reprod. Dev.*, **27**, 200–208.

Received on June 10, 1997; accepted on October 2, 1997