

ORIGINAL INVESTIGATION

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Estimates of sperm sex chromosome disomy and diploidy rates in a 47,XXY/46,XY mosaic Klinefelter patient

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Abstract A 47,XXY/46,XY male was investigated for the incidence of aneuploidy in sperm sex chromosomes using a three-colour X/Y/18 fluorescence in situ hybridisation (FISH) protocol. A total of 1701 sperm nuclei were analysed. The ratio of X-bearing to Y-bearing sperm did not differ from the expected 1:1 ratio although there were more 23,Y sperm than 23,X sperm (844 vs 795). There was a significantly increased proportion of disomy XY and XX sperm compared with normal controls (0.41% vs 0.10%, $P < 0.001$ and 0.29% vs 0.04%, $P < 0.01$). However, the incidence of YY sperm was similar to the controls (0.06% vs 0.02%). The diploidy rate was also significantly increased (1.7% vs 0.13%, $P < 0.0001$), as was disomy 18 (0.71% vs 0.01%) and 25,XXY (0.47% vs 0%). The results support the hypothesis that some 47,XXY cells are able to undergo meiosis and produce mature spermatozoa. Patients with mosaic Klinefelter syndrome with severe oligozoospermia have significantly elevated incidences of disomy XY and XX sperm and may be at a slightly increased risk of producing 47,XXX and 47,XXY offspring. Additionally, they may be at risk of producing offspring with autosomal trisomies. Hence, patients with Klinefelter mosaicism scheduled for intracytoplasmic sperm injection intervention should first undergo FISH analysis of their sperm to determine their risk.

Introduction

Klinefelter syndrome, first described by Klinefelter et al. (1942), is a progressive condition associated with hypogonadism and infertility resulting from complete spermatogenic arrest, leading to azoospermia or severe oligozoospermia. Males with a pure 47,XXY karyotype are al-

most invariably infertile but in the mosaic form oligozoospermia and normozoospermia have been reported (Cozzi et al. 1994; Chevret et al. 1996). Hence, the fertility status may be severely affected in some but not in other Klinefelter mosaics. In the case of those men whose sperm count is severely compromised, intracytoplasmic sperm injection (ICSI) may be the only realistic option open to them to father children.

It has been suggested that only the normal cell line is capable of undergoing meiotic division to produce spermatozoa (Kjessler 1966). However, evidence is accumulating in support of the hypothesis that such patients may face an increased risk of transmitting the extra sex chromosome to their offspring (Chevret et al. 1996; Guttenbach et al. 1997).

Nevertheless, embryos fertilised by ICSI with sperm from Klinefelter patients have been found to be karyotypically normal through preimplantation diagnosis (Staessen et al. 1996). Recently, Hinney et al. (1997) reported the first case of a pregnancy from sperm of a Klinefelter patient by ICSI. Unfortunately, this ended in a missed abortion. The abortus was found to have a normal 46,XX karyotype. Subsequently, karyotypically normal live births from sperm of non-mosaic Klinefelter patients following ICSI have been reported (Bourne et al. 1997; Reubinoff et al. 1998).

To date only a handful of reports have been published on the frequency of sex chromosome disomy in the sperm of men with Klinefelter syndrome and those with Klinefelter mosaicism (Cozzi et al. 1994; Chevret et al. 1996; Martini et al. 1996; Guttenbach et al. 1997). More data are still needed to confirm the observation of increased sex chromosome aneuploidy in the sperm of these men. Only then may the actual risk of transmitting an extra sex chromosome to the offspring be ascertained for the affected individual.

In this study, we report the case of a 47,XXY/46,XY male referred to our infertility clinic.

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Materials and methods

The 43-year-old patient was referred to our clinic for infertility. Seminal analysis showed severe oligoasthenoteratozoospermia according to WHO standards (World Health Organization 1992). His sperm density was only $0.2 \times 10^6/\text{ml}$. He was also leukocytospermic with a white blood cell count of $1.8 \times 10^6/\text{ml}$.

Cytogenetic analysis of his peripheral blood lymphocytes showed that he had a mosaic 47,XXY[17]/46,XY[39] Klinefelter karyotype, i.e., 30% of his lymphocytes belonged to the 47,XXY cell line.

A single semen sample was obtained by masturbation. After a smear had been made on a slide for morphological assessment the semen was washed with TRIS-buffered saline (TBS, pH 8.0). The supernatant containing the semen was discarded and the pellet resuspended with fresh TBS. A drop of the suspension was made on a pre-cleaned microscope slide over a circle etched by a diamond pen on the underside of the slide. The sperm density was adjusted to minimize overlap and overspreading of the sperm. A total of three slides were made. Two karyotypically normal fertile men (aged 28 years and 29 years) with normal semen parameters acted as controls. Sperm parameters were normal with densities of $80 \times 10^6/\text{ml}$. All slide preparations of sperm were made from undiluted semen and were stored in a slide box and kept at -20°C until further use.

A triple-colour fluorescence in situ hybridisation (FISH) protocol for chromosomes X, Y and 18 was employed (Cytocell, UK). The loci probed for were α -satellites DXZ1, DYZ1, and D18Z1, directly labelled by nick translation with fluorescein isothiocyanate (FITC), cyanine 3 (Cy3), and Cy3-FITC mix, respectively.

The slides were removed from the freezer and dehydrated through 70%, 85% and 100% ethanol for 2 min each. After air drying, the slides were treated with 10 mM dithiothreitol (DTT; Sigma) in 100 mM TRIS-HCl, pH 8.0 at 4°C for 8–30 min. They were further incubated in TRIS-HCl with 1 mM DTT and 10 mM lithium 3,5-diiodosalicylic acid (LIS; Sigma) for between 5 min and 1 h at room temperature, until the sperm heads were judged to be sufficiently decondensed. The slides were rinsed with two washes of TRIS-HCl and dehydrated through an alcohol series of 70%, 85% and 100% for 2 min each. They were then air dried and kept warm at 37°C in an incubator.

Hybridisation buffer (10 μl) was added to each slide over the encircled area and overlaid with a coverslip (Cytocell, UK). After bubbles that remained trapped under the coverslip had been gently removed, a layer of rubber solution was applied to seal the edges. When the rubber solution had dried, the slides were placed on a slide-heating tray at 75°C for 2 min to denature the DNA. Hybridisation was carried out in a dark moist box for 25 min at 37°C .

After removal of the coverslips, the slides were placed in a coplin jar containing 0.4xSSC, 0.3% NP-40 (Nonidet P-40; Sigma) for 2 min in a 73°C water-bath. They were next transferred into another jar containing $2 \times \text{SSC}$, 0.1% NP-40 at room temperature for 1 min and air-dried. 10 μl of 4,6-diamidino-2-phenylindole (DAPI) counterstain was applied to each slide, which was then coverslipped and sealed with rubber solution.

The slides were examined at 1000x magnification with a fluorescence microscope (Olympus BH2, Tokyo, Japan) equipped with a triple band pass filter for DAPI/FITC and tetramethylrhodamine isothiocyanate (Chroma Technology, Brattleboro, Vt., USA). The FITC-labelled X chromosome appeared green, the Cy3-labelled Y chromosome red, and the FITC-Cy3-labelled chromosome 18 yellow. The chromosome 18 probe was used as an internal control to distinguish between sex chromosome disomy and diploidy. Sperm nuclei were scored as having two signals when there were two discrete signals of equal intensity, separated by a minimum of one diameter of the domain of one signal (Martin and Rademaker 1995). Only nuclei with tails were analysed. Nuclei of the same size but without tails were considered spermatids. There were many spermatids and diploid cells present in the sample but these were not included in the analysis. Sperm with an extra sex chromosome but with only one chromosome 18 signal were classified as disomic. Sperm with two sex chromosome signals and two chromosome 18 signals were classified as diploid. Nuclei nullisomic for autosome 18 and sex chromosomes were excluded from the study.

The data were analysed by the χ^2 test and Fisher's exact test. A *P* value of < 0.05 was considered significant.

Results

Hybridisation efficiency was greater than 97% for all slides. A total of only 1701 sperm nuclei from this patient were scored owing to his low sperm count. There were slightly more Y- than X-bearing sperm (49.62% vs 46.74%) but the difference was not statistically different (Table 1). A total of 20,000 sperm from the two control specimens were analysed. The ratio of X- to Y-bearing sperm also did not differ significantly from 1:1. Statistical differences were observed in the incidences of 24,XY and 24,XX sperm compared with the controls ($P < 0.001$ and $P < 0.01$ respectively). The incidence of 24,XY was the highest among the sex chromosome disomies while no difference was detected in 24,YY disomy rates between the patient and the controls. The incidences of disomy 18 (0.71% vs 0.09%), diploid (1.70% vs 0.13%) and hyperhaploid 25,XXY (0.47% vs 0%) cells were also much elevated in the patient compared with the controls ($P < 0.0001$).

Discussion

Our patient showed significantly higher incidences of disomy XY and XX sperm than those of the controls (Table 1).

Table 1 Presumed karyotype of controls and patient by FISH (percentage in brackets)

Presumed karyotype by FISH	Control 1 46,XY	Control 2 46,XY	Mean controls*	Patient 47,XXY/46,XY**	<i>P</i> , * vs. **
23,X	4904 (49.04)	5041 (50.41)	4973 (49.73)	795 (46.74)	
23,Y	5079 (50.79)	4898 (48.98)	4989 (49.89)	844 (49.62)	
24,XY	4 (0.04)	16 (0.16)	10 (0.10)	7 (0.41)	< 0.001
24,XX	1 (0.01)	7 (0.07)	4 (0.04)	5 (0.29)	< 0.01
24,YY	0	4 (0.04)	2 (0.02)	1 (0.06)	> 0.05
24,X/Y,+18	8 (0.08)	11 (0.11)	9 (0.09)	12 (0.71)	< 0.0001
25,XXY	0	0	0	8 (0.47)	< 0.0001
Diploid	4 (0.04)	23 (0.23)	13 (0.13)	29 (1.70)	< 0.0001
Total	10000	10000	10000	1701	

Table 2 Comparison of FISH analysis results with previous studies (percentage in brackets)

Presumed Karyotype by FISH	Chevret et al. (1996) 47,XXY/46,XY	Guttenbach et al. (1997) 47,XXY	Current study (47,XXY/46,XY)
23,X	52.78	43.43	46.74
23,Y	43.88	48.82	49.53
24,XY	2.09	1.36	0.41
24,XX	0.11	1.22	0.29
24,YY	0.003	0.09	0.06
24,X/Y,+1 or +18	0.18	0.50	0.71
25,XXY	0	0.09	0.47
Diploid	0.33	0.23	1.70

Although the majority of 46,XY spermatogonia will give rise to 23,X and 23,Y sperm, non-disjunction of the sex chromosomes of primary spermatocytes at meiosis I will lead to the production of disomy XY and sex chromosome-nullisomic spermatozoa. If non-disjunction occurs at meiosis II, this will result in the formation of similar numbers of 24,XX and 22,-X sperm on the one hand, and 24,YY and 22,-Y sperm on the other hand. However in this study, the distribution of disomy XX and disomy YY was not found to be equal. In our patient, the rate of disomy YY was not significantly higher than that of the controls. This was similarly reported by Guttenbach et al. (1997) (Table 2). The elevated disomy XX rates suggest that some 47,XXY spermatogonia are able to undergo meiosis. Through both XX and XY bivalent pairing at meiosis I, normal segregation of 47,XXY germ cells can theoretically give rise to 24,XX and 24,XY cells, in addition to normal 23,X and 23,Y spermatozoa. This pathway will lead to further increases in the occurrence of disomy XX and XY sperm, in addition to disomy XX sperm that may arise from meiosis II non-disjunction and 24,XY sperm arising from meiosis I non-disjunction. Chevret et al. (1996) dismissed the possibility of XY bivalent pairing that could lead to 24,XX sperm production. However, their data actually showed elevated production of 24,XX sperm that was significantly higher than that of their controls (Table 2). Our results closely parallel those of Guttenbach et al. (1997), although at lower levels, presumably because their patient was non-mosaic. No increase in 24,YY sperm was observed in our study. However, a study by Martini et al. (1996) using *in situ* hybridisation with dual X/Y probes on a patient with low level 47,XXY mosaicism (2.5% by FISH) noted, in addition to elevated XX and XY sperm levels, a high level of 24,YY sperm, presumably as a result of meiosis II non-disjunction of 46,XY cells.

We postulate that disomy XY and XX sperm arise, respectively, not through a combination of meiosis I and II non-disjunction events of 46,XY cells alone, but through normal segregation of 47,XXY cells as well. Several authors have noted significant increases in the incidence of XY disomy and in disomy of some autosomes in the sperm of infertile men with oligoasthenozoospermia (Moosani et al. 1995; Lähdetie et al. 1997; Martin 1998). It would thus appear that being infertile further accentuates the risks of producing such abnormal sperm. The dis-

omy XX rate of our patient was the most elevated, possibly due to the additive effect of MII non-disjunction of 46,XY cells and normal segregation of 47,XXY cells. In order to account for the observed differences from control sperm, we hypothesise that sex-chromosome disomy in these men originates primarily from 47,XXY spermatogonia.

Thus, contrary to earlier suggestions that 47,XXY cells are incapable of undergoing meiosis (Steinberger et al. 1965; Sarkar and Marimuthu 1983), or are capable of completing meiosis but producing only normal haploid sperm (Benet and Martin 1988; Cozzi et al. 1994), it appears that some of these hyperdiploid cells are actually meiotically active.

What was surprising in our finding was the elevated level of diploid spermatozoa. The 1.7% incidence was higher than any previously reported figure. Diploid sperm are thought to arise by the failure of cytoplasmic division following nuclear duplication at the end of meiosis II. The use of two-colour X/Y FISH (Han et al. 1994; Flaherty et al. 1997) and *in vitro* penetration of zona-free hamster eggs (Cozzi et al. 1994) do not allow for the accurate estimation of the diploidy rate, if at all. Now, with the application of three-colour X/Y/autosome FISH protocols, diploidy rates can be determined quite precisely.

In addition, there was a high frequency of diploid 46,XY and 47,XXY somatic cells in the semen sample. It is believed that the majority of these cells were white blood cells as the patient was suffering from an episode of leukocytospermia. The remaining cells were probably spermatogonia and primary spermatocytes that were noted earlier in air-dried smears after Papanicolaou staining.

Equally unexpected was the increase in disomy 18. Newberg et al. (1998) also noted an increase in disomy 18 in the sperm of a 45,X/46,XY male in both whole sperm as well as sperm separated on Percoll fractions. The aetiology is not understood but they suggest that this increase might have been caused by a disturbance of meiotic segregation through hormonal imbalance unrelated to chromosomal constitution. Moosani et al. (1995) earlier reported increased incidences of autosomal disomies in oligozoospermic men. It may be that the increased incidence of disomy 18 is related to the severity of the condition.

Griffin et al. (1995), in a FISH study of over 390,000 sperm from 24 patients, provided evidence of an age-

related effect on the meiosis I and II non-disjunction rates in men resulting in elevated levels of disomy XY, XX and YY. Similarly, Robbins et al. (1995) reported an age effect that led to increased incidences of disomy XX and YY. The patient in the study of Martini et al. (1996), who had elevated sex chromosome disomy even though his mosaicism was very low level, was 45 years old. While it is possible that the differences in disomy rates between our control group (aged 28 and 29 years) and the patient (aged 43 years) might have been accentuated because of this age factor, the effect does not appear to be profound when we compare the differences between our patient and the patients (aged 32 and 28 years, respectively) of Chevret et al. (1996) and Guttenbach et al. (1997) (Table 2).

While Klinefelter patients are either azoospermic or severely oligozoospermic, mosaic cases are less affected. It has been suggested that in mosaic patients the severity of the syndrome increases with the degree of mosaicism (Sarkar and Marimuthu 1983). However, it appears that this varies from case to case. Only 10% mosaicism can result in oligozoospermia with sperm densities of around $9.6 \times 10^6/\text{ml}$ (Chevret et al. 1996). Our patient had a 30% 47,XXY cell line and his spermatogenesis was severely affected, resulting in a sperm density of only $0.2 \times 10^6/\text{ml}$. On the other hand, Cozzi et al. (1994) reported a mosaic Klinefelter individual who had a 40% hyperdiploid cell line but his sperm density was $100 \times 10^6/\text{ml}$, well within the range of normal fertile males (World Health Organization 1992). He had already fathered two children and had even volunteered himself as a sperm donor.

The variable effect is apparently similar with regard to the incidence of sex chromosome disomy in the sperm. Apparently, Klinefelter individuals and mosaics with higher percentages of the Klinefelter cell line can have a lower incidence of disomy XY sperm compared with an individual with only a 10% abnormal cell line (Chevret et al. 1996; Guttenbach et al. 1997; this study). These differences might be suggestive of differential degrees of mosaicism involving different organs.

The chance of transmitting the extra chromosome to the offspring of affected individuals is much reduced in those whose fertility is severely affected. However, with the intervention of ICSI, fertilisation with a sperm bearing sex chromosome disomy might result in an offspring with sex chromosome aneuploidy. Such has been the concern among reproductive scientists that several intense debates have resulted (Chandley and Hargreave 1996; Persson et al. 1996). Nevertheless, it is noteworthy that to date there have been very few reports of good fertilisation rates with ICSI from sperm of Klinefelter patients (Harari et al. 1995; Hinney et al. 1997).

Genetic counselling and prenatal diagnosis should be offered in the case of 47,XXY and 47,XYY mosaicism. Although 47,XXX and 47,XXY offspring are generally healthy and functionally normal, the latter are infertile (Gardner and Sutherland 1989). If prenatal diagnosis is declined, then couples seeking assisted reproduction, where the male partner is affected, should be prepared to accept such offspring. Nevertheless, it should be borne in

mind from this and previous studies that the elevated risks of producing an offspring with sex chromosome hyperdiploidy are in the order of less than 2% at the most. Of greater concern is the risk involving trisomy 18 and other autosomes. The recent reports of karyotypically normal pregnancies and births may be encouraging news.

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References

- Benet J, Martin RH (1988) Sperm chromosome complements in a 47,XYY man. *Hum Genet* 78:313–315
- Bourne H, Stern K, Clarke G, Pertile M, Speirs A, Baker HWG (1997) Delivery of normal twins following intracytoplasmic sperm injection of spermatozoa from a patient with 47,XXY Klinefelter's syndrome. *Hum Reprod* 12:2447–2450
- Chandley AC, Hargreave TB (1996) Genetic anomaly and ICSI. *Hum Reprod* 11:930–932
- Chevret E, Rousseaux S, Monteil M, Usson Y, Cozzi J, Pelletier R, Sele 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, Sele B (1994) Achievement of meiosis in XXY germ cells: study of 534 sperm karyotypes from an XY/XXY mosaic patient. *Hum Genet* 93:32–34
- Flaherty SP, Michalowska J, Swann NJ, Dmowski WP, Matthews CD, Aitken RJ (1997) Albumin gradients do not enrich Y-bearing human spermatozoa. *Hum Reprod* 12:938–942
- Gardner RJM, Sutherland GR (1989) Chromosome abnormalities and genetic counseling. Oxford University Press, New York, p 195
- Griffin DK, Abruzzo MA, Millie EA, Sheehan LA, Feingold E, Sherman SL, Hassold TJ (1995) Non-disjunction in human sperm: evidence for an effect of increasing paternal age. *Hum Mol Genet* 12:2227–2232
- Guttenbach M, Michelmann HW, Hinney B, Engel W, Schmid M (1997) Segregation of sex chromosomes into sperm nuclei in a man with 47,XXY Klinefelter's karyotype: a FISH analysis. *Hum Genet* 99:474–477
- Han TL, 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
- Harari O, Bourne H, Backer G, Gronow M, Johnston I (1995) High fertilization rate with intracytoplasmic sperm injection in mosaic Klinefelter's syndrome. *Fertil Steril* 63:182–184
- Hinney B, Guttenbach M, Schmid M, Engel W, Michelmann HW (1997) Pregnancy after intracytoplasmic sperm injection with sperm from a man with a 47,XXY Klinefelter's karyotype. *Fertil Steril* 68:718–720
- Kjessler B (1966) Karyotype, meiosis and spermatogenesis in a sample of men attending an infertility clinic. *Monogr Hum Genet* 2:1–92
- Klinefelter HF, Reifenstein FC, Albright F (1942) Syndrome characterised by gynaecomastia, aspermatogenesis, without a Leydigism and increased excretion of FSH. *J Clin Endocrinol* 2:615–627
- Lähdetie J, Saari N, Ajosenpää-Saari M, Mykkänen J (1997) Incidence of aneuploid spermatozoa among infertile men studied by multicolor fluorescence in situ hybridization. *Am J Med Genet* 71:115–121

- Martin RH, Rademaker RA (1995) Reliability of aneuploidy estimates in human sperm: results of fluorescence in situ hybridization studies using two different scoring criteria. *Mol Reprod Dev* 42: 89–93
- Martin RH (1998) Genetics in human sperm. *J Assist Reprod Genet* 15: 240–245
- Martini E, Geraedts JPM, Liebaers I, Land JA, Capitanio GL, Ramaeker FCS, Hopman AHN (1996) Constitution of semen samples from XYY and XXY males as analysed by in-situ hybridization. *Hum Reprod* 11: 1638–1643
- Moosani N, Pattinson HA, Carter MD, Cox DM, Rademaker AW, Martin RH (1995) Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescence in situ hybridization. *Fertil Steril* 64: 811–817
- Newberg MT, Francisco RG, Pang MG, Brugo S, Doncel GF, Acosta AA, Hoegerman SF, Kearns WG (1998) Cytogenetics of somatic cells and sperm from a 46,XY/45,X mosaic male with moderate oligoasthenoteratozoospermia. *Fertil Steril* 69: 146–148
- Persson JW, Peters GB, Saunders DM (1996) Is ICSI associated with risks of genetic disease? Implications for counselling, practice and research. *Hum Reprod* 11: 921–924
- Reubinoff BE, Abeliovich D, Werner M, Schenker JG, Safran A, Lewin A (1998) A birth in non-mosaic Klinefelter's syndrome after testicular fine needle aspiration, intracytoplasmic sperm injection and preimplantation genetic diagnosis. *Hum Reprod* 13: 1887–1892
- Robbins W, Baulch J, Moore D, Weier H, Blakey D, Wyrobek A (1995) Three probe FISH to assess chromosome X, Y and 8 aneuploidy in sperm of 14 men from two healthy groups: evidence for a paternal age effect on sperm aneuploidy. *Reprod Fertil Dev* 7: 799–809
- Sarkar R, Marimuthu KM (1983) Association between the degree of mosaicism and the severity of syndrome in Turner mosaics and Klinefelter mosaics. *Clin Genet* 24: 420–428
- Staessen C, Coonen E, Assche EV, Tournaye H, Joris H, Devroey P, Steirteghem AC Van, Liebaers I (1996) Preimplantation diagnosis for X and Y normality in embryos from three Klinefelter patients. *Hum Reprod* 11: 1650–1653
- Steinberger E, Smith KD, Perloff WH (1965) Spermatogenesis in Klinefelter syndrome. *J Clin Endocrinol* 25: 1325–1330
- World Health Organization (1992) WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction, 3rd edn. Cambridge University Press, Cambridge