

ORIGINAL INVESTIGATION

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Segregation of sex chromosomes into sperm nuclei in a man with 47,XXY Klinefelter's karyotype: a FISH analysis

Received: 6 November 1996

Abstract Meiotic segregation of the sex chromosomes was analysed in sperm nuclei from a man with Klinefelter's karyotype by three-colour FISH. The X- and Y-specific DNA probes were co-hybridized with a probe specific for chromosome 1, thus allowing diploid and hyperhaploid spermatozoa to be distinguished. A total of 2206 sperm nuclei was examined; 958 cells contained an X chromosome, 1077 a Y chromosome. The ratio of X:Y bearing sperm differed significantly from the expected 1:1 ratio ($\chi^2 = 6.96$; $0.001 < P < 0.01$). Sex-chromosomal hyperhaploidy was detected in 2.67% of the cells (1.22% XX, 1.36% XY, 0.09% YY) and a diploid constitution in 0.23%. Although the frequency of 24,YY sperm was similar to that detected in fertile males, the frequencies of 24,XX, 24,XY and diploid cells were significantly increased. A sex-chromosomal signal was missing in 4.26% of the spermatozoa. This percentage appeared to be too high to be attributed merely to nullisomy for the sex chromosomes and was considered, at least partially, to be the result of superposition of sex-chromosomal hybridization signals by autosomal signals in a number of sperm nuclei. The results contribute additional evidence that 47,XXY cells are able to complete meiosis and produce mature sperm nuclei.

Introduction

With an incidence of about 1/600 newborn males, Klinefelter's syndrome is one of the most common sex-chro-

mosomal abnormalities in man (Nielsen and Wohler 1991). In general, mosaics are less affected than non-mosaics. The severity of the syndrome in mosaics increases with the increase in the affected cell population (Sarkar and Marimuthu 1983). Klinefelter's syndrome is usually associated with azoospermia or severe oligozoospermia. Although a few cases of fertility and even of proven paternity (Warburg 1963; Laron et al. 1982; Terzol et al. 1992) have been reported, patients in general are not able to father children of their own. With the introduction of ICSI (intracytoplasmic sperm injection), however, a way has been opened to treat male infertility caused by highly reduced semen parameters. Harari et al. (1995) obtained fewer than 100 motile spermatozoa from a patient with mosaic Klinefelter's syndrome for ICSI. In two treatment cycles, they achieved a high fertilization rate but, in both cases, a pregnancy test carried out 16 days after embryo transfer was negative. In this context, the question arises regarding sex-chromosome aneuploidies in sperm cells from Klinefelter patients. To date, only two studies on sperm-chromosome complements and fluorescence in situ hybridization (FISH) analysis of spermatozoa in mosaic Klinefelter patients, respectively, have been published (Cozzi et al. 1994; Chevret et al. 1996). In both cases, a significantly increased frequency of 24,XY sperm nuclei (0.92% and 2.09%) compared with fertile controls has been reported, i.e. Klinefelter patients may have an increased risk of producing sex-chromosomally abnormal offspring. In the present study, 2206 sperm nuclei from a patient with Klinefelter's syndrome referred for ICSI therapy have been investigated by three-colour FISH with DNA probes specific for chromosomes 1, X and Y in order to reveal the distribution of the sex chromosomes.

Materials and methods

The 28-year old patient came to the infertility clinic because of fertility problems. Analysis of 50 metaphases from blood lymphocytes revealed a 47,XXY constitution in all cells. Semenograms performed on four different occasions showed severe oligoasthenoteratozoospermia (Table 1). For classification of the semenograms,

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Table 1 Semen parameters of the Klinefelter patient

| Sample | Volume (ml) | Sperm concentration ($\times 10^6/\text{ml}$) | Progressive motility (%) | Normal morphology (%) |
|--------|-------------|---|--------------------------|-----------------------|
| 1 | 3.3 | 0.38 | 0 | 20 |
| 2 | 6.6 | 0.4 | 0 | 26 |
| 3 | 7.7 | 1.3 | 20 | 29 |
| 4 | 3.9 | <<1 | 30 | ? |

the World Health Organization (WHO) references of 1992 were used as the standard (WHO 1992). Since the semen parameters were highly reduced and hardly met the criteria requested for in vitro fertilization (IVF) therapy (Michelmann 1995), ICSI was suggested to the patient.

A small remainder of semen sample no. 4 (Table 1) was used to prepare slides for in situ hybridization as described elsewhere (Guttenbach and Schmid 1990). Aged slides (2–5 days old) were denatured in 2 M NaOH for 2–4 min before being passed through a chilled alcohol series. Sperm morphology was controlled under phase contrast optics. Optimally decondensed sperm heads showed a homogeneously grey appearance with a distinct increase in size. If the swelling of sperm was not pronounced enough, i.e. sperm heads were still bright and compact, further treatment with NaOH was applied.

Sequences pUC1.77 (Cooke and Hindley 1979), pXBR (Willard et al. 1983) and pHY2.1 (Cooke 1976) were used as chromosome 1-, X- and Y-specific probes, respectively; they were labelled by nick translation with biotin-16-dUTP (pHY2.1, pUC1.77) and digoxigenin-11-dUTP (pXBR, pUC1.77). For in situ hybridization, equal amounts of biotin- and digoxigenin-labelled pUC1.77 were mixed and added to the hybridization mixture together with the labelled X- and Y-specific probes (final concentration of probes: 5 ng, 10 ng and 20 ng/slide, respectively).

The hybridization mixture containing 50% formamide, $2 \times \text{SSC}$ ($1 \times \text{SSC} = 150 \text{ mM NaCl}$, 15 mM sodium citrate pH 7.0), 10% dextran sulphate and labelled probes was denatured for 5 min at 70°C and chilled on ice. Aliquots of 20 μl were applied to each slide and distributed with a $24 \times 30 \text{ mm}$ coverslip. Hybridization was performed overnight at 37°C in a humidified chamber. The slides were washed consecutively in 50% formamide/ $2 \times \text{SSC}$, $2 \times \text{SSC}$ and $1 \times \text{SSC}$ at 45°C for 5 min each, followed by a 5-min wash in $0.1 \times \text{SSC}$ at 60°C .

Probe detection of the biotin- and/or digoxigenin-labelled probes was performed by simultaneous application of mouse anti-digoxigenin-FITC (fluorescein isothiocyanate, Sigma) at a dilution of 1 : 100 in $4 \times \text{SSC}/1\%$ bovine serum albumin (BSA), and avidin-TRITC (tetramethylrhodamine isothiocyanate, Sigma) at a dilution of 1 : 200 in $4 \times \text{SSC}/1\%$ BSA. After 30 min incubation at 37°C , the slides were washed in $4 \times \text{SSC}/0.1\%$ Tween, stained with 4,6-diamidino-2-phenylindole (DAPI) for 5 min and mounted with antifade solution.

Slides were analysed on a Zeiss Axiophot equipped with a DAPI filter and an FITC/rhodamine double-band pass filter. Chromosome 1 yielded a yellow hybridization signal, chromosome X a green signal, and the Y chromosome a red signal. Sperm cells with indistinct margins or ambiguous hybridization signals were excluded from analysis. A sperm nucleus was regarded as disomic for a specific chromosome if two distinct, clearly separated signals of the same size and colour were present.

Results

Hybridization efficiency in all hybridized preparations was greater than 98%. A total of 2206 sperm nuclei from the Klinefelter patient was evaluated by three-colour FISH (Table 2); 92.25% of the spermatozoa were presumed to have a normal haploid karyotype (23,X or 23,Y), 43.43% were X-bearing sperm cells (Fig. 1a) and 48.82% con-

Table 2 Data on 2206 sperm nuclei from the Klinefelter patient; simultaneous hybridization with probes specific for chromosomes 1, X and Y

| Hybridization signals | Presumed karyotype | No. of sperm | % of sperm |
|-----------------------|--------------------|--------------|------------|
| 1/X | 23,X | 958 | 43.43 |
| 1/Y | 23,Y | 1077 | 48.82 |
| 1/X/X | 24,XX | 27 | 1.22 |
| 1/X/Y | 24,XY | 30 | 1.36 |
| 1/Y/Y | 24,YY | 2 | 0.09 |
| 1/1/X | 24,X,+1 | 2 | 0.09 |
| 1/1/Y | 24,Y,+1 | 9 | 0.41 |
| 1/X/X/Y | 25,XXY | 2 | 0.09 |
| 1/1/X/X | 46,XX | 1 | 0.05 |
| 1/1/X/Y | 46,XY | 2 | 0.09 |
| 1/1 | 44,-XX,-XY or -YY | 2 | 0.09 |
| 1 | 22,-X or -Y | 94 | 4.26 |
| Total | | 2206 | 100 |

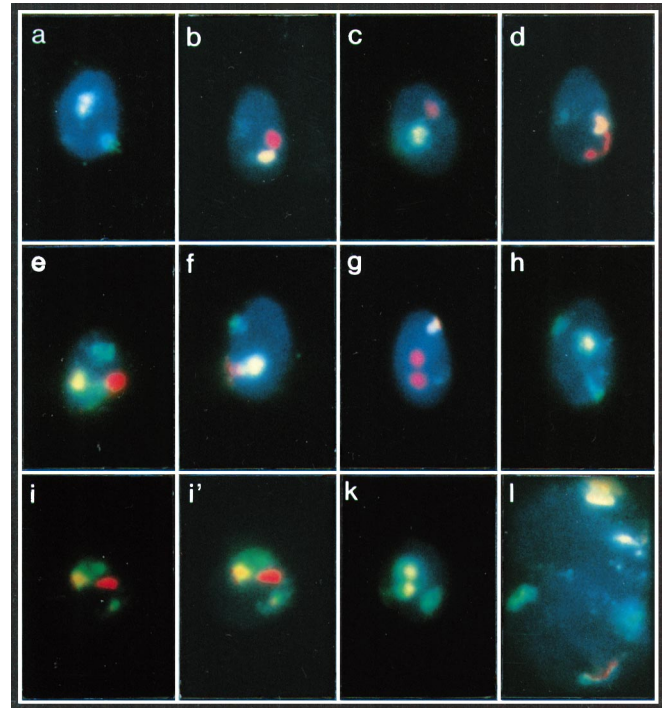


Fig. 1 a–l Sperm nuclei from the Klinefelter patient; simultaneous hybridization with probes specific for chromosomes 1 (yellow), X (green), and Y (red). Sperm cells with **a** 23,X, **b–d** 23,Y, **e, f** 24,XY, **g** 24,YY, **h** 24,XX, **i, i'** 25,XXY and **k** 24,X,+1 karyotypes. **i, i'** The same cell, which is overexposed in **i'** to show the margins of the sperm nucleus. **l** Diploid immature germ cell or somatic cell with a 47,XXY karyotype

tained a Y chromosome (Fig. 1 b–d). The ratio of X-bearing sperm to Y-bearing sperm nuclei differed significantly from the expected 1:1 distribution ($\chi^2 = 6.96$; $0.001 < P < 0.01$). Sex-chromosomal hyperhaploidy was detected in 2.67% of sperm nuclei. Although the frequency of 24,YY spermatozoa (0.09%, see also Fig. 1 g) was similar to that detected in fertile males (0.11%), the incidence of 24,XX (Fig. 1 h) and 24,XY (Fig. 1 e, f) nuclei was significantly increased (1.22% vs 0.12% and 1.36% vs 0.17%). Similarly, the rate of diploid cells (0.23%) was distinctly higher than that in fertile controls (0.05%). Disomy 1 (Fig. 1 k) was observed at 0.5% and two hyperhaploid spermatozoa with XXY gonosomes were found (Fig. 1 i, i').

In as many as 4.26% of the sperm cells, no sex-chromosomal hybridization signals were detectable. This rate appeared to be too high to represent nullisomy of the sex chromosomes. We considered that the smaller sex chromosomal signals (especially X-signals) were superpositioned by the larger signals of chromosome 1 in these nuclei. Only a few immature germ cells or somatic cells were present in the sperm preparations, all of them presenting an 47,XXY karyotype (Fig. 1 l).

Discussion

In a review of the karyotypes of 9207 infertile males reported in several studies, de Braekeleer and Dao (1991) have found a total of 424 47,XXY males (4.6%). This rate is approximately 44 times higher than the frequency detected in (unselected) newborn males. Although sperm nuclei have been investigated in a number of infertile males by *in situ* hybridization and sperm karyotyping (Miharu et al. 1994; Moosani et al. 1995; Guttenbach et al. 1997), up to now, only two studies analysing the sperm chromosome constitution and the segregation of sex chromosomes into sperm nuclei in Klinefelter patients have been performed (Cozzi et al. 1994; Chevret et al. 1996). The patient investigated by Cozzi et al. (1994) exhibited a mosaic of 60% 46,XY and 40% 47,XXY in peripheral blood lymphocytes and normal semen parameters. Chevret et al. (1996) detected 90% 46,XY cells and 10% 47,XXY cells in lymphocyte cultures of their patient. Semen analysis showed oligoasthenoatozoospermia and a sperm count of $9.6 \times 10^6/\text{ml}$. In comparison, our patient appeared to be more affected: no mosaic constitution was detectable in an analysis of 50 metaphases from lymphocyte cultures, and semen parameters were strongly reduced with sperm counts of $0.38\text{--}1.3 \times 10^6/\text{ml}$ and progressive motility of 0%–30%. Since the patient did not agree to a skin biopsy, a possible mosaic chromosome constitution in tissues other than the testis cannot be excluded. Early investigations (Kjessler et al. 1966) presumed that, in mosaic Klinefelter patients, only normal 46,XY cells could complete meiosis. Meanwhile, evidence has arisen that 47,XXY cells are also able to go through meiosis and produce sperm cells. Foss and Lewis (1971) analysed testicular biopsies from four Klinefelter patients with motile spermatozoa in their ejaculates. In one patient, who was later revealed as having

a mosaic 46,XY/47,XXY constitution, one tubule showed full spermatogenesis; in the other cases, where no mosaicism could be detected, no active spermatogenesis was seen in the tubules observed in the sections. However, the authors conceded that there may have been a minor 46,XY cell line responsible for sperm production in the testis.

Only a small number of analyses exists on meiotic pairing of the sex chromosomes in Klinefelter patients. Skakkebaek et al. (1969) studied testicular biopsy material from four patients with Klinefelter's syndrome. In one male, they were able to identify eight cells in diakinesis. Three of these were not informative, three had 22 autosomal bivalents and an XY bivalent, and two exhibited 22 autosomal bivalents, an XX bivalent and a separate Y chromosome. Vidal et al. (1984) conducted studies on synaptonemal complexes (SCs) of a mosaic Klinefelter patient. Out of 32 pachytene cells, 14 exhibited normal SCs and a normal sex vesicle (SV; i.e. 46,XY cells), 7 showed fragmented SCs and a normal SV, and 11 presented a 24(?) constitution without an SV (47,XXY cells: XX pairing and free Y chromosome). Thus, both studies agreed with respect to finding preferential pairing of homologous sex chromosomes in 47,XXY cells, with a free Y chromosome. This observation is also supported by Cozzi et al. (1994) and Chevret et al. (1996). Analysing sperm karyotypes and interphase nuclei from Klinefelter mosaics, the authors found only the frequency of 24,XY hyperhaploid sperm cells to be significantly increased (0.92% and 2.09%) compared with normal controls, whereas the incidence of sex-chromosomal disomies and diploid spermatozoa was within the normal range. This phenomenon was explained by regular segregation of 47,XXY cells during meiosis, with the XX chromosomes being paired.

Because of the scarcity of material available for *in situ* hybridization, only 2206 sperm nuclei of the Klinefelter patient could be analysed in the present study. When about 1000 sperm heads were analysed, the frequencies of disomic and diploid nuclei were estimated. These relative frequencies did not change when 2206 spermatozoa had been screened. It can therefore be assumed that there is no decisive variation in the X:Y ratio and the frequency of hyperhaploid cells when analysing a larger number of spermatozoa. In accordance with the results of Cozzi et al. (1994) and Chevret et al. (1996), we have found the frequency of 24,XY spermatozoa (1.36%) to be significantly increased. However, in contrast to the authors mentioned above, we have also observed a highly increased rate of 24,XX spermatozoa (1.22%). These frequencies surely cannot be explained by meiotic nondisjunction in any normal 46,XY cells possibly present in the testis: nondisjunction during meiosis I should result in equal numbers of XY spermatozoa and spermatozoa nullisomic for the sex chromosomes, whereas nondisjunction in the second meiotic division would lead to equal numbers of 24,XX and 22,-X or 24,YY and 22,-Y nuclei. Only 0.09% of the sperm heads analysed revealed a 24,YY constitution, whereas 1.22% were 24,XX. Since nondisjunction would be expected with similar frequencies in X and Y sperma-

toocytes, our results cannot be explained in this way. It appears more likely that the hyperhaploid cells are the products of regular segregation in 47,XXY cells. This, however, requires that XY bivalents are formed together with XX bivalents during prophase I, a combination that has not been described in the few studies on meiotic chromosome pairing in Klinefelter patients. Nevertheless, XY bivalents might not have been identified in these investigations, because of the scarcity of material. The lack of 24,XX cells in the studies of Cozzi et al. (1994) and Chevret et al. (1996) might be attributable to the mosaic constitutions of their patients.

Two presumably haploid XXY-cells were found in the 2206 spermatozoa analysed. Since no such cells have been described in normal controls (Bischoff et al. 1994; Spriggs et al. 1995, 1996), these sperm nuclei probably arose from 47,XXY spermatogonia. Similarly, XYY spermatozoa were detected by Han et al. (1994) in a 47,XYY male.

In summary, our data obtained by three-colour FISH on sperm cells of a Klinefelter patient provide further strong evidence that 47,XXY cells are able to complete spermatogenesis and further indicate that Klinefelter patients may have an increased risk of producing gonosomally abnormal offspring. Thus, ICSI therapy should always be followed by prenatal diagnosis.

Acknowledgements This study was supported by the Bundesministerium für Forschung und Technology (grant 01KY9104).

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