

# High Incidence of Sperm Sex Chromosomes Aneuploidies in Two Patients with Klinefelter's Syndrome

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## ABSTRACT

In this study we have investigated the arrangement of sex chromosomes in sperm from two severe oligozoospermic patients, apparently affected by the classic form of Klinefelter's syndrome (KS). Multicolor fluorescence *in situ* hybridization has been used to recognize chromosomes X, Y, and 8 in sperm from patients and 10 fertile men with normal 46,XY karyotype. In patients affected by KS, we detected important numerical sex chromosome abnormalities (~20%). In all normal fertile men, X- and Y-bearing spermatozoa were present in a 1:1 ratio. On the contrary, in our patients the frequency of 23,Y-bearing sperm was strongly reduced compared with that of

both 23,Y sperm in the controls and 23,X sperm in the same subject affected by KS, resulting in a 23,X-/23,Y-bearing sperm ratio of 2:1. Moreover, the frequency of 24,XY disomic sperm was significantly higher in the absence of the 22,0 hypoploidy expected from a common origin from a nondysjunction during the first meiosis in a normal 46,XY cell.

In conclusion, the results of the present study demonstrate a peculiar distribution of sex chromosomes in sperm from two patients with KS, in agreement with the hypothesis that 47,XXY germ cells are able to complete the meiotic process by producing mature spermatozoa. (*J Clin Endocrinol Metab* 83: 203–205, 1998)

**K**LINFELTER'S syndrome (KS) is the most frequent sex chromosome aneuploidy in human males, occurring in 0.1–0.2% of newborn infants (1, 2) and in 3.1% of infertile subjects (3). Patients affected by the classic form (~80%) present a 47,XXY karyotype; higher grade aneuploidy (48,XXX; 48,XXYY; 49,XXXXY), 46,XY/47,XXY mosaicism, and structurally abnormal X-chromosomes distinguish the remaining 20% (4).

In KS a picture of primary testicular failure was observed, characterized by small gonads, elevated FSH and LH plasma levels, and normal or low serum testosterone levels. Usually these patients are azoospermic, and the seminiferous tubules appear fibrotic and hyalinized. Some studies have reported the presence of severe oligozoospermia in mosaicism 46,XY/47,XXY with motile sperm in the ejaculate (5), and in rare cases, a proved paternity has been described (6).

In oligozoospermic patients affected by KS, meiotic studies have shown different alterations: arrest of meiosis at primary spermatocyte or spermatid stages and foci of normal spermatogenesis in few seminiferous tubules (7–9). In patients with mosaicism (46,XY/47,XXY), it has been assumed that only 46,XY germ cells can complete meiosis even if recently it has been proposed that some XXY germ cells can go through meiosis and produce spermatozoa (10). This hypothesis arises from the results obtained by sperm karyotyping and, more recently, by DNA *in situ* hybridization (11, 12), which allows rapid identification of spermatozoa with specific chromosomal aberrations.

The prevalence of sperm sex chromosome numerical aberrations in these KS mosaics is significantly higher than that

observed in normal fertile subjects, but is relatively low, being, on the average, not higher than 3%. These findings suggest that few 47,XXY spermatogonia complete the meiotic process and produce spermatozoa.

To this date studies have been carried out on a limited number of patients, and in all cases in 46XY/47XXY mosaic subjects; therefore, the actual constitution of spermatozoa in KS remains to be better clarified. In this study we report for the first time the meiotic distribution of sex chromosomes, investigated by multicolor fluorescence *in situ* hybridization (FISH), on sperm nuclei from two severe oligozoospermic subjects apparently affected by the classic KS, showing a very high incidence of sperm sex chromosome alterations.

## Subjects and Methods

### Patients

We studied two subjects, aged 37 and 25 yr, who consulted our clinic because of infertility and were found to have nonmosaic KS. This pathology was demonstrated by peripheral lymphocyte karyotyping on 200 metaphases (performed by GTG and QFQ banding) and by FISH (using X- and Y-specific probes) that revealed a 47,XXY constitution in all examined cells. Ten normal fertile men with normal 46,XY karyotype represented the control group.

Physical, hormonal, and seminal analysis were performed in both patients and control subjects; FSH, LH, and testosterone plasma levels were measured by RIA using a double antibody RIA (Ares-Serono, Milan, Italy). Semen samples were collected on two different occasions, separated by a 3-week interval, after 3 days of sexual abstinence and analyzed as recommended by WHO (13).

The study was approved by the hospital ethical committee, and informed consent was obtained from all subjects.

### FISH

Numerical alterations of sperm chromosomes were evaluated by multicolor FISH. Soon after standard seminal analysis, sperm were selected by means of a mini-Percoll technique (14) to remove somatic cells and debris and then were fixed overnight in methanol-acetic acid solution (3:1) at –20°C. Samples were transferred on cleaned, degreased

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slides and air-dried, and the sperm nuclei were decondensed according to the method proposed by Martini *et al.* (11). This technique permits a certain sperm nuclei identification based on limited head swelling and complete preservation of the tail, the latter being often visible as a weak fluorescence and, in any case, by phase contrast microscopy. After decondensation, slides were immediately used for the successive steps or were stored in a refrigerator (2–4 days, 4 C).

DNA hybridization was performed using human  $\alpha$ -satellite probes specific for chromosomes X, Y, and 8 (Amersham Life Sciences) directly labeled using fluorochromes FluorX (chromosome X, green) and Cy3 (chromosome Y, orange): for the detection of chromosome 8, a mixture (1:1) of FluorX and Cy3 directly labeled specific probes was used, resulting in a yellow signal.

DNA denaturation of sperm and probes, incubation, and posthybridization washing were performed following the Amersham protocol. Sperm nuclei were successively counterstained (1 min at room temperature) in a Coplin jar containing a phosphate-buffered saline (pH 7.4)-4',6'-diamidine-2'-phenylindole dihydrochloride solution (20 ng/mL). Slides were then rinsed in distilled water, air-dried in the dark, mounted using an antifade solution (glycerol-distilled water, 9:1-1,4 diazabicyclo-[2.2.2]octane, 2%, wt/vol), and stored (1–4 days, 4 C) or immediately observed using a Leica Diaplan epifluorescence microscope (Leica, Wetzlar, Germany) fitted with a 100-watt mercury lamp and a triple bandpass filter suitable for the fluorochromes in use. This procedure allows the detection of all probes as bright, compact, and uniformly sized spots.

Each spot was evaluated and scored as specific for the chromosome corresponding to its color only when the intensity and size were similar to those of spots of the same color in the surrounding cells. Furthermore, if two spots of the same color were located in the same cell, the distance had to be more than their diameter for them to be considered distinct chromosomes (15, 16). For each patient, 10,000 cells have been scored.

DNA probes were provided by Amersham Life Sciences (Milan, Italy). 4',6'-Diamidine-2'-phenylindole dihydrochloride was purchased from Boehringer Mannheim (Milan, Italy). All other chemicals were purchased from Sigma Chemical Co. (Milan, Italy).

### Statistical analysis

Student's *t* test was performed to compare results from 47,XXY males and controls, and a difference was considered significant at  $P < 0.05$ .

## Results

Table 1 reports clinical, hormonal, and seminal parameters in our patients affected by KS compared with those in the controls. Both patients showed lower testicular volume. Plasma FSH and LH levels were significantly higher, and plasma testosterone levels were reduced. Seminal analysis revealed severe oligozoospermia, with percentages of forward motility and normal morphology significantly lower than those found in the controls.

Table 2 reports the results of chromosomal arrangement analysis relative to chromosomes X, Y, and 8 in the controls and in our patients with KS. For each subject, 10,000 spermatozoa were scored, and an elevated FISH efficiency (rate of hybrid/nonhybrid cells) was obtained (average, 98.1%). Moreover, in control subjects, X- and Y-bearing spermatozoa were present in a regular 1:1 proportion in all analyzed samples. These results support the reliability of the findings obtained in the two patients. In these subjects the frequency

of 23,X-bearing cells was slightly, although not significantly, increased with respect to that in controls, whereas the rate of 23,Y-bearing sperm was strongly reduced compared to that in both controls ( $P < 0.05$ ) and 23,X sperm of patients themselves ( $P < 0.001$ ), resulting in a 23,X/23,Y-bearing sperm ratio of about 2:1. The frequency of 24,XY hyperaploid sperm was significantly higher than that observed in the fertile men ( $P < 0.05$ ). Furthermore, the frequency of 24,XX disomic sperm cells was greatly increased compared with that in the normal controls, although the increase was not statistically significant. The frequencies of 46,XY diploid sperm and the other chromosomal constitutions observed in our patients with KS were statistically similar to those in the controls.

## Discussion

Oligozoospermia has seldom been reported in subjects affected by KS, above all in 46,XY/47,XXY mosaicism, and is associated with various degrees of tubular alterations: arrest of the maturative process at primary spermatocyte or spermatid level and the presence of spermatogenesis only in rare seminiferous tubules (7–9). It has been suggested that in KS, spermatogenesis is related to the presence of normal 46,XY germ cells (17, 18), and the different degrees of testicular alteration depend on the proportion of normal (46,XY) tubular cells, including germ cells and cells surrounding them (19).

However, since 1969, Skakkebaek *et al.* (8) as well as others suggested that 47,XXY germ cells may achieve meiosis and produce mature spermatozoa (10–12). From these studies, obtained by sperm karyotyping or DNA *in situ* hybridization, it appears that in 46,XY/47,XXY mosaicism there is a significant increase in hyperaploid, 24,XY-bearing sperm in the absence of the corresponding 22,0 hypoaploid cells expected from meiosis I nondysjunction in a 46,XY cell, suggesting that 47,XXY spermatogonia are able to complete spermatogenesis and produce hyperaploid spermatozoa. In all cases these studies were performed in patients with peripheral 46,XY/47,XXY mosaicism, and the low proportion of spermatozoa showing numerical sex chromosomal abnormalities (~3%) suggests that few 47,XXY germ cells are able to complete meiosis.

In the present study, three-color FISH was performed on sperm nuclei of two severe oligozoospermic subjects affected by the classic form of KS. The analysis of lymphocytes from both patients showed a 47,XXY constitution in all examined cells, but the presence of spermatogenesis strongly suggests a mosaicism confined to testicular tissue.

The three-color FISH was performed using X and Y DNA probes to study the percentages of sex chromosome aneuploidy and a DNA probe for chromosome 8 as a parameter to evaluate the hybridization efficiency and to distinguish diploidy and disomy.

The fertile control subjects present X- and Y-bearing sper-

**TABLE 1.** Clinical parameters of two Klinefelter subjects and controls

	Testicular vol (mL)	Hormonal			Seminal			
		FSH (IU/L)	LH (IU/L)	T (nmol/L)	Vol (mL)	Conc. ( $\times 10^6$ /mL)	Motility (%)	Morphology (%)
Patients								
1	6.0	18.0	7.0	12.8	2.5	1.9	15	28
2	6.8	16.0	6.5	13.5	3.0	2.3	20	20
Controls (mean $\pm$ SD)	14.5 $\pm$ 3.4	3.2 $\pm$ 1.2	2.8 $\pm$ 0.8	16.1 $\pm$ 3.1	3.6 $\pm$ 1.3	45.8 $\pm$ 7.4	62.0 $\pm$ 13.2	61.9 $\pm$ 7.8

**TABLE 2.** Frequencies of sperm sex chromosome set in two XXY males and control

	23,X	23,Y	22,O	24,XY	24,XX	24,YY	46,XY	45,X	45,Y
Patients									
1	51.87	24.60 <sup>a</sup>	1.70	14.58 <sup>b</sup>	6.92	0.21	0.05	0.03	0.04
2	56.00	28.63 <sup>a</sup>	1.82	10.03 <sup>b</sup>	3.34	0.09	0.03	0.02	0.04
Controls (mean ± SD)	49.39 ± 1.60	49.04 ± 1.56	1.05 ± 0.23	0.19 ± 0.52	0.10 ± 0.52	0.12 ± 0.98	0.05 ± 0.02	0.03 ± 0.03	0.03 ± 0.03

<sup>a</sup>  $P < 0.05$  vs. 23,X- and 23,Y-bearing sperm of the controls;  $P < 0.001$  vs. 23,X-bearing sperm of Klinefelter patients.

<sup>b</sup>  $P < 0.05$  vs. 24,XY-bearing sperm of the controls;  $P < 0.001$  vs. 22,0-bearing sperm of the controls and of Klinefelter patients.

matozoa in the expected 1:1 ratio in all analyzed samples. On the contrary, in patients with KS the frequency of 23,Y cells was strongly reduced compared to that of 23,X cells, with a 23,X-/23,Y-bearing sperm ratio of 2:1. These findings provide evidence that the majority of spermatozoa do not originate from 46,XY spermatogonia. This hypothesis is further supported by the presence of a high proportion of spermatozoa showing numerical sex chromosome abnormalities (~20%), suggesting that a large number of 47,XXY germ cells are able to complete the spermatogenetic process.

Regular meiosis in a 47,XXY spermatogonium with XX pairing should lead to the same proportion of 23,X- and 24,XY-bearing sperm cells. In this study we observed a high incidence of hyperaploid 24,XY spermatozoa (14.58% and 10.03%), but not the same proportion of 23,X forms (51.87% and 56.0%, respectively). These findings may be related to an impaired maturation process of XY-bearing germ cells. In XYY males, it has been suggested that XY pairing associated with univalent Y would result in a high level of primary spermatocyte death, which, in turn, would lead to a secondary damage (20, 21).

On the other hand, 47,XXY spermatogonia with XY pairing and univalent X should lead to 24,XX- and 23,Y-bearing spermatozoa in the same proportion, considering a regular segregation both of bivalents and in meiosis II. In our patients, Y-bearing spermatozoa represent 24.60–28.63%, whereas XX sperm represent only 6.92–3.34%, respectively. Also in these cases the lower incidence of XX-bearing with respect to Y-bearing spermatozoa may be related to an alteration along the progress through meiosis to secondary spermatocyte characterized by an anomalous chromosomal set. The prevalence of 23,X-bearing with respect to 23,Y-bearing sperm confirms the preferential pairing of homologous sex chromosomes in spermatogonia with three gonosomes (8, 12, 22). The incidence of sperm sex chromosome abnormalities in our patients with KS is much higher than that reported previously. In a mosaic 46,XY/47,XXY, Cozzi *et al.* (10), using spermatozoa karyotyping, found an incidence of 0.92% hyperaploid 24,XY sperm. Chevret *et al.* (12) and Martini *et al.* (11), using *in situ* hybridization, showed in two mosaics an incidence of these aneuploidies of 2.09% and 1.3%, respectively. The high increase in numerical sperm sex chromosomal abnormalities found in our study may be related to the apparently complete 47,XXY alteration and suggests that the majority of ejaculated spermatozoa may originate from 47,XXY germ cells.

In conclusion, the results of this study strongly suggest that in oligozoospermic subjects affected by KS, XXY germ cells are able to complete spermatogenesis and produce mature spermatozoa, frequently bearing sex chromosome aneuploidy. The major problem pointed out in this study is that intracytoplasmic sperm injection using spermatozoa of these subjects will pass

sex chromosome numerical abnormalities on to the children. Therefore, analysis of the sex chromosome status of sperm from oligozoospermic subjects affected by KS must be performed before application of an artificial reproductive technique, and genetic counseling should be provided.

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