Ploidy of spermatogenic cells of men with non-mosaic Klinefelter's syndrome as measured by a computerized cell scanning system

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Abstract

Purpose This study aims to characterize the origin of testicular post-meiotic cells in non-mosaic Klinefelter's syndrome (KS).

Methods The study included testicular tissue specimens from 11 non-mosaic KS patients, with (6 positive) and without (5 negative) spermatozoa presence. The obtained testicular cells were affixed and stained for morphology followed by fluorescence in situ hybridization (FISH) for centromeric probes X, Y, and 18. We used a computerized automated cell scanning system that enables simultaneous viewing of morphology and FISH in the same cell.

Results A total of 12,387 cells from the positive cases, 11,991 cells from the negative cases, and 1,711 cells from the controls were analyzed. The majority of spermatogonia were 47, XXY in both the positive and negative KS cases ($88.9 \pm 4.76\%$ and $90.6 \pm 4.58\%$) as were primary spermatocytes ($76.8 \pm 8.14\%$ and $79.6 \pm 7.30\%$). The respective rates of secondary spermatocytes and post-meiotic cells (round, elongating spermatids and sperm cells) were $1.1 \pm 1.39\%$ in the positive cases, $2.9 \pm 3.33\%$ in the negative cases, compared to $67.6 \pm 6.22\%$ in the controls ($P < 0.02$). Pairing of both 18 and XY homologous chromosomes in 46,XY primary spermatocytes was $2.5 \pm 2.31\%$ and $3.4 \pm 2.39\%$, respectively, compared to $19.8 \pm$

8.95% in the control group ($P < 0.02$) and in 47,XXY primary spermatocytes in $2.4 \pm 3.8\%$ in the positive group and $3.2 \pm 2.26\%$ in the negative group.

Conclusions This study presents data to indicate that the majority of primary spermatocytes in the testes of non-mosaic KS patients are 47,XXY and could possibly develop into post-meiotic cells.

Keyword Ploidy · Testicular suspension · Klinefelter's syndrome · Spermatogenic cells

Introduction

Klinefelter's syndrome (KS) is the most common sex chromosome abnormality in human males. In the non-mosaic KS type, all peripheral blood lymphocytes contain a 47,XXY chromosomal constitution, while testicular mosaicism may be present in some of them [1–3]. Testicular mosaicism has been proposed as having a high prognostic value for spermatogenesis [4], and sperm has been retrieved through testicular sperm extraction (TESE) in over 50 % of KS patients (KS positive). In the other 50 %, no germ cells at all or different stages including post-meiotic cells with no mature figures might be present (KS negative). Although higher rates of sex and autosomal aneuploidy have been reported in positive KS patients [5–11], most offspring had a normal karyotype [12, 13]. To date, only one fetus with the 47,XXY karyotype has been conceived and reduced in the 14th gestational week (from a triplet pregnancy) [14], and two other fetuses have been diagnosed as mosaic 46,XY/47,XXY karyotype by pre-implantation genetic diagnosis (PGD) [15]. The question of meiotic completion of spermatogenic cells and the origin of euploid and uneuploid spermatozoa in KS men has intrigued researchers for years. Some studies suggest that XXY cells are

Capsule A computerized system enables the follow-up of progenitor cells giving evidence that majority of primary spermatocytes in the testes of non-mosaic KS patients are 47,XXY and could possibly develop into post-meiotic cells.

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unable to enter and complete meiosis, possibly because of the presence of two functional X chromosomes [16], and assume that the origin of spermatozoa is from normal 46, XY germ cells that are located in the testes [4, 17, 18]. Others believe that mitotic and meiotic progression of these cells is possible [5, 18]. Researchers have used various ways to find out the cause for the presence or absence of these euploid and aneuploid spermatozoa and to investigate their origin and precursor cells. Since in vitro experiments are not yet available, some have looked at progenitor germ cells and the meiotic process itself in vivo, for example, using fluorescence in situ hybridization (FISH) on pachytene figures (the meiotic stage during which crossing-over occurs) [4, 17, 1, 19–23]. Others [5–7, 11, 24] have used an indirect approach and have deduced the most likely hypothesis from FISH results on spermatozoa, i.e., the “end product” of the meiotic process. The conclusion remains controversial, especially due to the small number of available testicular cells and the difficulties in their identification.

A unique computerized cell scanning system (CCSS) technique based on corresponding morphology and FISH images enables screening of maximal amounts of cells from the extracted testicular tissue and their accurate identification might help to provide a clearer answer [25, 26].

Materials and methods

Infertile patients

Testicular tissue specimens were obtained from 11 men with non-mosaic KS who were undergoing TESE. Six of them had spermatozoa in their testicular tissue (positive) and the remaining five had none (negative). The mean age was 33.2 years (range 27–38) for the positive group and 33.0 years (range 27–38) for the negative group. All 11 men had been diagnosed as being azoospermic after at least two consecutive semen analyses, with an abnormal 47,XXY constitutional blood karyotype. All subjects had small testis volume (estimated between 4 and 9 ml). The mean plasma FSH levels were 37.2 ± 19.94 IU/l in the positive group and 37.7 ± 17.82 IU/l in the negative group.

Normal controls

Testicular tissue specimens were obtained from the autopsy of three accident victims with proven fertility, whose autopsy specimens were donated by their relatives. The mean age of the healthy controls was 23.7 years (range 18–28).

Testicular cell retrieval

Testicular sperm extraction was performed under general anesthesia. Testicular biopsies were excised and collected in a Petri dish (3801, Falcon) filled with 3 ml modified HEPES-buffered Earle's medium with heparin (Sigma). One small tissue specimen per testis was fixed in Bouin's fixative for histological examination. The rest of the biopsy was shredded using two sterile microscopic slides in order to obtain a testicular cell suspension. Initial microscopic examination of the wet preparation was performed at $\times 400$ magnification under an inverted microscope with the Hoffman Modulation Contrast System (Modulation Optics Inc., Greenvale, NY, USA). The testicular tissue was centrifuged at $300 \times g$ for 7 min and the pellet was suspended with 2 ml of erythrocyte-lysing buffer [27] for 10 min. The small testicular tissue specimen underwent enzymatic treatment with 1,000 IU/ml collagenase type IV (Sigma-Aldrich, C5138) in order to completely digest the testicular tissue [28].

An extended sperm preparation [29] was performed in order to increase the chance of visualizing any spermatozoa that were present. At this stage, the case was determined as a positive or negative case. In the positive cases, an intracytoplasmic sperm injection (ICSI) procedure was performed with selected sperm. The rest was frozen for the patient's use in a subsequent cycle. A minimal amount of specimen was taken from the remaining tissue for study. In the negative cases, an unrestricted specimen was used.

Assay characteristics

The method employed an automated cell-scanning system that had been originally designed for diagnosis and follow-up of pathological cells in hematological diseases. It enables multi-sequential staining of cell preparations (Duet™, BioView Ltd, Nes-Ziona, Israel). This method provides large-scale automated bright field and fluorescence scanning for simultaneous classification of morphology and FISH in the same cell and for many cells [25, 26].

Bright field scanning

The obtained testicular cells were affixed on glass slides (Super Frost® Plus, $25 \times 75 \times 1.0$ mm, Menzel-Glaser, Germany) using a Cytospin centrifuge (Shandon Cytospin 4) at $600 \times g$ for 5 min.

Each slide was first analyzed and scanned automatically by bright field microscopy using $\times 20$ or $\times 40$ objectives. Coordinates and images of cells confined to an area of a circle with a 900- μ m radius on the slide were digitally recorded for future analysis.

Destaining

The Giemsa-May Grunwald staining was removed after immersion in ice-cold methanol/acetic acid 3:1 solution for 1 h and washed with phosphate-buffered saline (PBS). The slides were washed in digestion solution (containing 25 µl digesting enzyme solution and 50 ml HCl 10 mM) for 15 min at 37 °C and then washed with PBS for 5 min. The slides were then washed twice with formaldehyde 1 % [1.5 ml formaldehyde (36.5 %) in 50 ml PBS] for 5 min at room temperature (18–25 °C), dehydrated through an ethanol series, and dried for 5 min at 37 °C.

FISH

In situ hybridization using a chromosome X centromeric sequence probe (Spectrum Green; Vysis, Downer's Grove, IL, USA), a chromosome Y centromeric sequence probe (Spectrum Orange; Vysis), and a chromosome 18 centromeric sequence probe (Spectrum Aqua; Vysis) was performed on the same slide according to Harper et al. [30]. Slides and probes were co-denatured at 74 °C for 5 min and hybridized for 2 h at 37 °C using a HYBrite™ system (Vysis). Washings were performed for 5 min with 60 % formamide/2×SSC and 5 min with 2×SSC at 42 °C, followed by two additional 5-min washes at room temperature with 0.001 % NP-40/2×SSC. Finally, slides were mounted in Blue View Counter stain (Bio-Blue™) (BioView Ltd).

Fluorescence scanning

Following the FISH procedure, the slides were searched automatically by Bio View™ for target cells with fluorescence signals. Fluorescent illumination was achieved by a standard 100-W mercury lamp (HBO103/W2; Osram, Munchen, Germany), a triple color filter (V6200; Chroma, Brattleboro, VT, USA), and an aqua color filter (61008; Chroma, Cc/YFP/dsR). The high-speed automated scanning (~5,000 cells/h) was performed in full color using ×63 objective (0.16 m per pixel). A color camera enabled the system to capture a single image that contained all of the color details of each cell, and the image was analyzed without further digital processing. Because the system gave a combined picture of four separate images taken from four different planes with a difference of 0.6 µm between each of them, a single image measuring 2.4 µm and covering all the possibilities for a signal was screened. Ploidy was based on number of chromosomes number (*n*).

Only clear hybridization signals that were similar in size and separated from each other by at least one signal domain and clearly positioned within the sperm head were considered as a single chromosome. However, for the autosomal 18 chromosome bivalent (centromeres synapse), when one signal or

two signals of similar size were located at a distance of less than one diameter of a signal domain, a paired bivalent was recorded. The signal size was varied depending on the dimension and cross section that was taken. X and Y chromosomes, only centromeric signals a maximum of two signal diameters apart, were recognized as close bivalents and marked as X-Y. Since the DNA probes are very close to the centromere, it was impossible to distinguish the number of chromatids that comprised the chromosome [31].

Combined analysis of morphology and FISH

Each cell was represented by a pair of images on a screen: one for morphology and the other for FISH results. Cells could be observed and definitively identified by the combination of the two images. Nomarski optics was utilized to sort the germ cells according to size, shape, chromatin pattern of nuclei, and presence and shape of the acrosome and flagella [31, 32]. Two kinds of somatic cells were identified. Both were distinguishable from spermatogonia. Diploid Leydig cells were large in diameter (15–20 µm) and had a highly refractive surface. The cytoplasm was strongly acidophilic and finely granular. The nucleus was large, round, and often located in an eccentric pattern in the cell. Diploid Sertoli cells had an irregular cell boundary: the nucleus was ovoid or angular, large and lightly stained, and often contained a large nucleolus. Diploid spermatogonia were identified by a rounded nucleus with chromatin granules and one or two nucleoli. Primary spermatocytes were identified as the largest cells (19–24 µm in diameter). They had a low cytoplasm/nuclear ratio and a granular surface with clumped chromatin masses. Haploid secondary spermatocytes and early spermatids (which are intricate to distinguish) were 8.0–12 µm in diameter and had very light homogeneous nucleus often eccentric. Advanced spermatids were spherical cells (6–8 µm in diameter), with clear cytoplasm, a smaller but distinctive round nucleus that stained darker, and a prominent central nucleolus.

Statistical analysis

Statistical analysis was performed using Stats Direct statistical software (Version 1.9.14, Cheshire, UK). The Mann–Whitney *U* test was applied to compare different parameters between and within the control and study groups. Differences were considered as significant when $P < 0.05$.

Ethical approval

Written consent was obtained from all the study participants and from the families of the normal controls, and the study was approved by our Institutional Ethics Committee (Approval Number: 37/06.)

Results

A total number of 28,839 cells were screened following bright field scanning and FISH. Among them, 2,750 had either over-detection or under-detection of signals or low Giemsa staining quality (FISH efficiency 95.5 %) and were excluded from analysis. A total of 12,387 cells (mean $2,064 \pm 1,380.59$ nuclei per case) from positive KS cases, 11,991 cells (mean $2,398.2 \pm 1,126.30$ nuclei per case) from negative KS cases, and 1,711 cells (mean 570.3 ± 187.28 nuclei per case) from the controls were successfully analyzed. The distribution between somatic and germ cells was as follows: 95 (1.1 % ± 0.85) for positive cases, 254 (3.3 % ± 5.01) for negative cases, and 133 (11 % ± 15.21) in the controls.

The rate of secondary spermatocytes and post-meiotic cells [round spermatids (>8 and ≤ 8 μm), elongating spermatids, and spermatozoa] was significantly lower in the positive and negative KS cases: 3.0 ± 3.27 % (235/12,387 cells) and 1.2 ± 1.43 % (160/11,991 cells), respectively, compared to the controls: 67.6 ± 6.22 % (1,168/1,711 cells); $P < 0.02$. Spermatogonia and primary spermatocyte rates, however, were significantly higher among the KS positive cases [47.2 ± 6.00 % (5,625/12,387) and 48.7 ± 19.82 % (7,036/12,387), respectively] and the KS negative cases [61.1 ± 24.75 % (5,852/11,991) and 34.6 ± 27.59 % (4,541/11,991), respectively] compared to the controls [6.7 ± 5.27 % (122/1,711) spermatogonia and 14.7 ± 10.82 % (288/1,711) primary spermatocytes, respectively, $P < 0.05$].

The distributions of 46,XY and 47,XXY spermatogonia and primary spermatocytes are represented in Table 1. The majority of the spermatogonia and primary spermatocytes were aneuploid 47,XXY in both the positive and negative cases.

The chromosomal patterns of secondary spermatocytes (which are intricate to distinguish from early spermatids, however not often to be seen) and round spermatids (>8 and ≤ 8 μm) are shown in Table 2. The prevalence of euploid primary spermatocytes in the KS positive cases (14.6 ± 9.30 ,

Table 1) was lower than the rate of haploid secondary spermatocytes and spermatids (44.4 ± 46.42 , Table 2), $P < 0.002$.

Almost all (99.5 %) of the spermatocytes II cells and round spermatids in the control group were haploid and equally distributed between chromosomes 18 and X and chromosomes 18 and Y (50.8 ± 9.48 % and 48.8 ± 9.69 %, respectively). The ratio of cells with the chromosomes 18 and X to cells with chromosomes 18 and Y in the positive KS group was 34.5 ± 29.07 % vs 22.5 ± 16.94 % ($P = \text{NS}$). There was some dominance of cells diploid for chromosomes 18 and X (18/18 X/X) over cells diploid for chromosomes 18 (18/18) bearing XY among spermatocytes II cells and round spermatids in the positive KS group (17.8 ± 21.84 % vs 1.8 ± 4.30 %; $P = \text{NS}$). A few spermatocytes II and round spermatids were identified in only two negative KS cases: four of five haploid cells were 18X in one of them and all spermatocytes II and round spermatids were aneuploid in the other one.

The pairing of chromosomes 18 and XY in 46,XY and 47,XXY primary spermatocytes (Fig. 1) is presented in Tables 3 and 4. The majority of the 46,XY and 47,XXY primary spermatocytes did not display either autosomal or sex chromosome pairing in any of the KS cases (18/18 XY and 18/18 XXY). Simultaneous pairing of both 18 and XY chromosomes (18 X-Y bivalents) was identified in both 46XY and 47,XXY primary spermatocytes. There was no difference in the percentage of pairing between the two KS groups. The presence of these autosomal (chromosome 18) and sex chromosome bivalents (18 X-Y) in 46,XY cells was however lower in the positive and negative cases compared to the control group (Table 3).

The percentage of 46,XY spermatocytes with paired 18 chromosomes and unpaired X and Y chromosomes (18 XY) in the control group was significantly higher than that of 46,XY spermatocytes with unpaired 18 chromosomes and paired X and Y chromosomes 18/18; X-Y. There was no significant difference between these two alignments in the two KS groups (Table 3). The same relation was observed between the 18

Table 1 Distribution of 46,XY and 47,XXY spermatogonia and primary spermatocytes

	No. patients	No. per case (average)	1818XY (%)	1818XXY (%)	18 aneuploidy (%)	Others (%)
Analyzed spermatogonia						
Control	3	55.5 ± 34.65	99.4 ± 0.88	0	0.1 ± 0.02	0.5 ± 0.78
KS positive	6	$1,034.2 \pm 894.63$	$8.37 \pm 4.54^*$	$88.9 \pm 4.7^*$	0.9 ± 0.85	0.9 ± 0.55
KS negative	5	$1,368.0 \pm 1,005.68$	$5.2 \pm 2.14^{**}$	$90.6 \pm 4.58^{**}$	2.0 ± 0.91	2.2 ± 1.80
Analyzed primary spermatocytes						
Control	3	96.0 ± 75.54	$95.4 \pm 5.79^{***}$	$0.7 \pm 1.19^{***}$	0.5 ± 0.41	3.3 ± 5.24
KS positive	6	975.3 ± 659.20	$14.6 \pm 9.30^\#$	$76.8 \pm 8.14^\#$	4.0 ± 2.15	4.6 ± 2.96
KS negative	5	908.2 ± 679.11	$9.64 \pm 2.14^{###}$	$79.6 \pm 7.30^{###}$	3.3 ± 1.91	7.5 ± 3.50

KS Klinefelter's syndrome, KS positive sperm were retrieved, KS negative sperm were not found

* $P < 0.002$; ** $P < 0.007$; *** $P < 0.05$; # $P < 0.002$; ### $P < 0.007$

Table 2 Chromosomal patterns of secondary spermatocytes and round spermatids (>8 and ≤8 μm)

Group	No. cells	Haploid cells			Aneuploid cells					
		No. total haploid cells (%)	18X	18Y	No. total aneuploid cells	18XX	18XY	1818XX	1818XY	Others
Control	456	454 (99.5±0.12)	226	228	2 (0.5±0.12)	1	—	—	—	1
*KS positive	88	46 (44.4±46.42)	27	19	42 (26.4±34.82)	1	1	30	2	8
Case no. 1		18	10	8	21	—	—	17	—	4
Case no. 2		17	11	6	5	—	—	4	—	1
Case no. 3		3	1	2	16	—	1	9	2	4
Case no. 4		2	1	1	—	—	—	—	—	—
Case no. 5		4	3	1	—	—	—	—	—	—
Case no. 6		2	1	1	—	—	—	—	—	—
**KS negative	26	5 (14.3±31.94)	4	1	21 (25.7±43.33)	—	2	—	—	19
Case no. 1		—	—	—	—	—	—	—	—	—
Case no. 2		—	—	—	19	—	2	—	—	17
Case no. 3		—	—	—	—	—	—	—	—	—
Case no. 4		5	4	1	2	—	—	—	—	2
Case no. 5		—	—	—	—	—	—	—	—	—

The percentages of average euploid and aneuploid cells in KS positive as well as in KS negative patients do not add up to 100 % since only a few if any cells were identified in both, resulting in a variety of percentages for the individuals in each group (wide SD). The numbers of euploid and aneuploid cells in both groups reached a total number of secondary and round spermatids as recorded in the second column of the table: 42+46 equaled 88 and 5+21 equaled 26

KS Klinefelter's syndrome, *KS positive* sperm were retrieved, *KS negative* sperm were not found

XXY and 18/18 XX-Y body bearing aneuploid 47,XXY primary spermatocytes (Table 4).

Discussion

With the TESE procedure, sperm can be recovered from the testes of non-mosaic KS (47,XXY) men in about 50 % of the cases [33, 34]. However, it is widely reported that men with KS have higher rates of sex and autosomal aneuploidy [5–11]. Two approaches on the ability of 47,XXY testes to yield post-meiotic cells exist. According to some researchers, 47,XXY spermatogonia have the potential to complete meiosis, resulting in both the increase in sex chromosomal aneuploidy rates as well as the presence of normal (haploid) spermatozoa [6, 7, 19, 21, 23]. According to others, primary 47,XXY spermatocytes are unlikely to complete meiosis, and spermatozoa of KS men arise from the patches of 46,XY spermatogonial stem cells in the testes that are affected by a compromised testicular environment and produce a high rate of aneuploid sperm due to meiotic errors. Bergere et al. [4], Blanco et al. [17], and Sciarano et al. [20], who looked at pachytene figures of positive KS patients, found that almost all of them (94.5–100 %) were 46,XY and deduced that these cells are the only active cells. They were reinforced by Egozcue et al. [35] who identified the absence of 46,XY spermatogonia in patients negative for spermatids and spermatozoa, and by Mroz et al. [36] who showed a clear absence of meiotic competence of

47,XXY cells in mice. Small quantities of studied germ cells such as 20–100 spermatogonia and primary spermatocytes in very few biopsies [1–5, 8], as well as technical shortcomings, preclude a definite conclusion [1, 4, 17, 19, 20, 23] (the study by Yamamoto et al. [23] (2002) included 12 biopsies, but lacked information regarding the number of meiotic cells).

The CCSS technique, which is based on corresponding size and morphology of the cells and their FISH images, enabled an extended study at three levels:

1. CCSS enables a maximal sampling of the whole extracted tissue while screening dozens of testicular cell, including post-meiotic cells, with a high level of confidence [25, 26] and a screening of more than 1,000 spermatogonia and 900 primary spermatocytes for each of the 11 biopsy samples. As expected, based on lymphocyte karyotypes in non-mosaic KS men, and similar to Rives et al. [11] and Yamamoto et al. [23], who identified between 88.5 and 100 % 47,XXY spermatogonia in testicular biopsies, we found around 90 % spermatogonia with 47,XXY and 10 % with 46,XY, independently of whether or not patients produced testicular sperm. It needs to be specified that Yamamoto et al. [23] did not observe any testicular XY cells in negative cases. Similar to spermatogonia, we found that the majority of primary spermatocytes in positive and negative KS cases was 47,XXY, (76.8 and 79.6 %, respectively). These results are in line with Foresta et al. [7], who found a 47,XXY karyotype in 20/

Fig. 1 Morphology of primary spermatocyte and chromosome location in the 46,XY and 47,XXY nuclei. Green and red signals represent X and Y centromeres, respectively. The aqua color signals represent chromosome 18 centromere. Four chromosomal signatures were documented: (A1, A2) unpaired 18 and X; Y chromosomes. (B1, B2) Paired 18 homologous represented by one aqua signal, unpaired X; Y

chromosomes. (C1, C2) Unpaired 18 chromosomes, green and red signals in proximity represent paired X and Y chromosomes X-Y. (D1, D2) The simultaneous presence of paired homologous chromosomes 18 and sex chromosome (X-Y). In all 47,XXY primary spermatocytes signatures (A2, B2, C2, and D2), an extra single X chromosome is seen

Table 3 Pairing of 18 and XY chromosomes in 46,XY primary spermatocytes

Group	Average primary spermatocyte no.	18/18 XY (%)	18/18 X-Y (%)	18 XY (%)	18 X-Y (%)
Control	94.3±74.85	32.8±10.56a,b	1.5±1.07c	45.9±2.68c	19.8±8.95d,e
KS positive	91.3±34.12	75.3±10.14a,f	8.6±2.31	13.7±8.34	2.5±2.31d,f
KS negative	85.4±69.83	64.4±25.61b,g	6.2±4.16	25.9±21.60	3.4±2.39e,g

a,b,c ($P<0.05$); d, e ($P<0.02$); f, g ($P<0.001$)

KS Klinefelter's syndrome, 18/18 XY unpaired 18 chromosomes as well as X and Y chromosomes, 18/18 X-Y unpaired 18 chromosomes, but paired X and Y chromosomes, 18 XY paired 18 chromosomes and unpaired X and Y chromosomes, 18 X-Y paired 18 chromosomes and paired X and Y chromosomes

Table 4 Pairing of 18 and XY chromosomes in 47,XXY primary spermatocytes

	Average primary spermatocyte no.	18/18 XXY (%)	18/18 X/X-Y (%)	18 XXY (%)	18 X/X-Y (%)
KS positive	789.0±570.57	73.3±5.49*	13.3±2.28	11.0±5.25	2.4±1.38*
KS negative	716.0±535.31	69.4±10.24**	10.4±2.24	16.9±10.66	3.2±2.26**

KS Klinefelter's syndrome, 18/18 XXY unpaired 18 chromosomes as well as 2×X and Y chromosomes, 18/18 X/X-Y unpaired 18 chromosomes, but paired X and Y chromosomes, one extra X chromosome, 18 XXY paired 18 chromosomes and unpaired 2×X and Y chromosomes, 18 X/X-Y paired 18 chromosomes and paired X and Y chromosomes, one extra X chromosome

* $P<0.002$; ** $P<0.008$

20 (100 %) primary spermatocytes of KS men and are in favor of the approach that 47,XXY primary spermatocytes might be active in meiosis.

- The quintessential feature of our study includes graphic evidence for 46,XY and 47,XXY pachytene figures, in which pairing of 18 as well as of X and Y chromosomes can be seen, both in positive and negative KS patients (Fig. 1D1, D2). This graphic evidence suggests that 47,XXY spermatocytes might be active similarly to XY cells and both are able to enter meiosis (we did not find any evidence for XX pairing). It is well recognized that successful recombination between homologous chromosomes followed by segregation (desynapsis) is essential for accurate spermatogenic progression through meiosis [37–40], while altered pairing and recombination lead to germ cell aneuploidy or even infertility [38, 41–43]. In this aspect, there was a significantly lower rate of bivalent formation in the KS cases compared to the healthy controls in 46,XY and 47,XXY primary spermatocytes. We could not detect any difference in the percentage of pairing of either the autosomes or sex chromosomes between 46,XY and 47,XXY primary spermatocytes in both KS groups (Tables 3 and 4). It must however be emphasized that there is no data presented to demonstrate that 47,XXY spermatogonia undergo meiosis to form spermatid.

- Some evidence in favor of the ability of 47,XXY spermatocytes to complete meiosis comes also from the indirect approach—FISH results on post-meiotic cells.

Theoretically, if 46,XY primary spermatocytes were to undergo meiotic errors caused by a compromised testicular environment, one would expect the presence of 18YY disomy or nullisomy in the testes in addition to equal X and Y distribution. However, we did not detect any of them while screening KS biopsies (Table 2). If segregation of XXY occurs, one would expect an increase in both XY and XX disomy in the spermatozoa (with a 2:1 ratio if segregation occurs randomly), and that an excess of X-bearing euploidy in the post-meiotic cells would be observed [44]. We found an elevation of X-bearing euploid secondary spermatocytes and round spermatids ($\leq 8 \mu\text{m}$) together with a high rate of diploid 18/18 XX

round cells. There was a high rate, albeit not significant, of diploid 18/18 XX round cells. The latter could be an accumulation of secondary spermatocytes as a result of incomplete secondary meiosis. This assumption is based on the work of Chevre et al. [45] in which they speculated that 47,XXY primary spermatocytes experience difficulties in completing their progression through the meiotic process. In this case, there might be a situation where sparse normal haploid cells are mostly originated from XY primary spermatocytes while XXY primary spermatocytes contribute to the aneuploid ones. Since, however, post-meiotic cells were sparse and varied individually in their presence and ploidy among the KS patients, the results did not reach significance level and thus our deductions are restricted. Theoretically, the expected rate of aneuploidy in the setting of 47,XXY meiosis is up to 50 % [46, 47]. However, the percentages of aneuploidy values in the testes of KS men reported by others reached 10–20 % [5, 8, 9]. We found an elevation in the percentage of euploid cells after the first meiotic division along the final stages of spermatogenesis (from 8.4 % for spermatogonia, and 14.7 % in primary spermatocytes to 44.4 % post-meiotic round cells for positive cases). We did not, however, study testicular spermatozoa cells as these, if present, were used or frozen (Table 2).

Preferential apoptosis of abnormal germ cells after the pachytene stage [48–50] or dominance of the few normal spermatocytes [23] could explain this elevation.

In summary, the use of the CCSS technique enabled us to include and evaluate hundreds of spermatogenic cells throughout the entire spermatogenesis process in KS patients. Our study has provided graphic evidence for at least 900 progenitor cells with 46,XY and mainly 47,XXY karyotype in the form of pachytene figures in each KS patient, both of which could yield haploid and aneuploid post-meiotic cells. Similarities in the first meiotic stage of spermatogenesis between positive and negative KS patients were shown. Thus, although several investigators claim to have proven strong evidence to support either the 46,XY or the 47,XXY progenitor cell hypothesis, it would seem that there are stronger arguments to believe that these two hypotheses may actually coexist. We should however consider that there is no direct data presented to demonstrate that 47,XXY spermatogonia completed meiosis to form spermatids.

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