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Biopsied testis cells of four 47,XXY patients: fluorescence in-situ hybridization and ICSI results

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BACKGROUND: A testis biopsy was performed for four non-mosaic 47,XXY azoospermic patients. Spermatozoa were found in three cases and frozen before ICSI. We analysed the various cells found in the four samples by multicolour fluorescence in-situ hybridization (FISH), to evaluate the meiosis and spermatogenesis possibilities of the 47,XXY and 46,XY testis cell lines, and to estimate aneuploidy rate in the resulting spermatids and spermatozoa. METHODS AND RESULTS: Testis diploid cells (either somatic or premeiotic), meiotic, and post-meiotic haploid germ cells were hybridized with probes for chromosomes X, Y and 18. The only patient with no spermatozoa had a homogeneous diploid XXY constitution in the testis; the three other patients presented two cell populations (46,XY and 47,XXY) among their diploid testis cells. All the observed pachytene figures were XY; no XXY pachytene figure was found. The aneuploidy rate among post-meiotic cells for chromosomes X,Y and 18 was 6.75% (5/74). This rate was 1.5% (2/133) for control. Three couples underwent ICSI; four attempts were made, one healthy baby was born. CONCLUSION: FISH results suggest that only 46,XY cells can undergo meiosis.

Key words: aneuploidy/FISH/ICSI/Klinefelter's syndrome/testis biopsy

Introduction

Testicular biopsy has recently begun to be widely offered to non-mosaic XXY patients. Since 1997 several authors have reported positive testis biopsy with testicular sperm extraction (TESE) facilitating subsequent ICSI attempts; 12 live births resulted from 22 reported attempts (Tournaye *et al*., 1996; Bourne *et al*., 1997; Palermo *et al*., 1998; Reubinoff *et al*., 1998; Hinney *et al*., 1999; Nodar *et al*., 1999; Ron-El *et al*., 1999; Boucher *et al.*, 2000; Zaki *et al.*, 2000).

We performed testicular biopsy and studied the chromosomal constitution of the testicular cells obtained from four azoospermic homogeneous Klinefelter patients. The aims of the study were: (i) to analyse the chromosome constitution of diploid Klinefelter testicular cells and investigate possible linkage between germline mosaicism (with both Klinefelter 47,XXY and normal 46,XY cell lines) and the preservation of spermatogenesis; (ii) to analyse the chromosomal constitution of the meiosis figures observed, and particularly to study the number and pairing of X and Y chromosomes in pachytene cells to evaluate the meiotic possibilities of 46,XY and 47,XXY cells; and (iii) to evaluate the aneuploidy rate of post-meiotic germ cells (spermatids and spermatozoa) to make the genetic counselling more precise.

In all the cases in which spermatozoa were found the possibility of ICSI was discussed with the couple, and in all cases then performed.

Materials and methods

Patients

Four azoospermic patients were diagnosed with homogeneous Klinefelter's syndrome on lymphocyte karyotype with at least 40 cells analysed. FISH of lymphocytes for X and Y on more than 200 nuclei was performed to confirm this diagnosis. Biochemical values were determined: 26 IU/l to 33.7 IU/l for plasma FSH levels and 5 pg/ml to 22 pg/ml for plasma inhibin B levels. All patients had a small testis volume (estimated at between 4 and 6 ml). We offered testis biopsy to all four patients, so that testis tissue could be frozen for a further ICSI attempt if spermatozoa were found. All patients agreed and underwent testis surgery. We compared the FISH results with those for two other azoospermic patients with normal karyotype and with spermiogenesis failure diagnosed after testis biopsy.

Biopsy and sperm cryopreservation

Multiple site (between four and eight) bilateral testis biopsies were performed under local anaesthesia. Biopsy tissue was rinsed and divided into two parts; a small sample was used for cytopathological analysis, and the rest was minced. Cells were carefully examined under an inverted microscope (Nikon Diaphot 300®) to detect spermatozoa. In all cases a few isolated testis cells were smeared on two glass slides and stored at –20°C for FISH. If any spermatozoa were found in the observed sample, the rest of the minced tissue was placed at 37°C for 4–5 h, the supernatant and pellet were frozen separately [using Spermfreeze™ (Fertipro) v/v and usual sperm freezing programme: from 20°C to –8°C at a rate of 5°C/min, from

–8°C to–25°C at a rate of –10°C/min; and then to –140°C at a rate of 25°C/min]; finally straws were plunged in liquid nitrogen. Between two and 10 straws containing a small volume (around 50 µl of cell suspension) were prepared. Due to the very small number of spermatozoa available, morphological examination was not possible after conventional Schorr staining, but examination under an inverted microscope just before sample freezing or just before injection revealed that most of the spermatozoa were abnormal.

ICSI procedure

Four ICSI attempts were made for three couples. Ovaries were stimulated with FSH after down-regulation by GnRH agonist. Oocytes were collected 36 h after HCG injection. A sperm straw was thawed to obtain the live spermatozoa required. Modified hypo-osmotic swelling (HOS) medium (Ahmadi *et al.*, 1997) was required for all four ICSI attempts because all recovered spermatozoa were immobile. If there were too few live spermatozoa in the first straw to inject all the mature oocytes, another straw was thawed. The oocyte treatment by hyaluronidase and the ICSI were then done as described by Van Steirteghem *et al.* (Van Steirteghem *et al.*, 1993*).* Pronuclei were checked 15–18 h after injection. The criteria of Steer (Steer *et al.*, 1992) were used to evaluate embryo quality.

FISH procedure

Frozen sperm smears were thawed and fixed with methanol/acetic acid 3:1, washed with ×2 SSC and dehydrated through a graded ethanol series. No decondensation treatment was applied. Slides were denatured for 4 min 30 s with probes, and hybridized overnight. We used a set of three probes, X, Y and 18 Chromoprobe® (Cytocell) to evaluate abnormalities in sex chromosome number, the ploidy and cytological type of the observed cells (somatic cells, spermatocytes I, spermatocytes II, spermatids, or testicular spermatozoa). Probes were directly labelled: X with fluorescein isothiocyanate (FITC), Y with Cyanin 3 (cy3), 18 with FITC/cy3. On the second day unbound probe was eliminated by washing in formamid-free buffer, slides were counterstained with 4′-6 diamidino 2-phenylindole (DAPI) and observed with an Olympus BX 60® epifluorescence microscope equipped with single, double and triple band filters. Various hybridization patterns were captured on an applied imaging system equipped with the Macprobe® program. Cultured lymphocytes were analysed using the same protocol.

FISH interpretation criteria

We scored the number of autosomal spots and the number of sex chromosomes spots for each cell. Interphase diploid nuclei may belong to premeiotic spermatogonia, Sertoli cells or other somatic cells. Pachytene meiotic spermatocytes were identified by pairing of the two homologous chromosomes 18 and of XY. Spermatocytes II were identified on the basis of labelling of single autosome and of single sex chromosomes in each in a duplicated manner (two signals of the same intensity close to one another) and according to the size of the nucleus (around twice that of a spermatid).

Haploid nuclei are post-meiotic and may belong to spermatids or spermatozoa. Sperm nuclei were taken into account only if they showed a non-overlapping normal shaped nucleus and a flagellum. Disomy for a given chromosome was diagnosed if the two signals had the same intensity, were separated by a minimum of one diameter of the domain of one signal, and required the same focus.

Results

Results of the karyotype and the FISH analyses of blood cells

All the four cases were diagnosed as homogeneous 47,XXY by conventional karyotyping (40 mitoses analysed) and by interphasic FISH analysis of 200 nuclei. The two control cases with spermiogenesis failure had undergone blood karyotyping before the testis biopsy, with a normal 46,XY result obtained for the 15 mitoses analysed.

Cytological and FISH results for the testis biopsy

For one patient (number 1), no spermatozoa were observed on examination of the minced tissue under the inverted microscope. Cytopathological diagnosis was germ cell aplasia and Sertoli cells only syndrome (SCOS). In all the hybridized nuclei of this biopsy, FISH revealed two chromosomes 18, indicating the diploid status of those nuclei, and an XXY sex chromosome complement. The patient was informed of these results and of the lack of appropriate assisted reproduction techniques in his case.

Two patients (numbers 2 and 3) had very few motile sperm and patient 4 had a very small number of immobile sperm at only one of the sites biopsied. The samples were cryopreserved. FISH revealed, in all cases, the presence of two premeiotic or somatic diploid cell lineages: one XY and one XXY. For two of these three patients pachytene figures were found, and were all observed to be XY. Spermatids and spermatozoa were found and labelled on the hybridized slides of the three patients, with a 6.7% (5/74) overall aneuploidy rate among haploid cells for chromosomes X, Y and 18; the aneuploidy rate among haploid cells for X and Y was 2.7% (2/74) and that for chromosome 18 was 4.0% (3/74) (Table I). FISH was 95% efficient in the analysable area (that in which cell dissociation and spreading were satisfactory, ensuring the preservation of cell morphology). The respective incidences of X,18 and of Y,18 post-meiotic cells were similar.

For the two control patients with spermiogenesis failure all the pachytene figures ($n = 103$) were, as expected, XY1818 labelled, the frequency of aneuploidy rate in post-meiotic haploid cells was 1.5% (2/133), the two aneuploid post-meiotic cells found were both XY18, no meiotic error involving chromosome 18 disjunction was observed.

ICSI results

Four ICSI attempts led to one biochemical pregnancy and one clinical pregnancy with a healthy baby. The details of the attempts are given in Table II.

Discussion

Two main questions were raised by the recent successes reported for Klinefelter patients in ICSI programmes: does a homogeneous XXY karyotype for lymphocytes contraindicate testis biopsy? and what are the post-meiotic aneuploidy rates and subsequent risk for embryos obtained by ICSI performed with testicular spermatozoa from Klinefelter patients?

Klinefelter's syndrome patients do not seem to be a homogeneous group. The karyotype of the lymphocyte cell lineage does not predict the chromosomal constitution of testis cells and the presence or absence of spermatogenesis (Westlander *et al.*, 2001). In the cases studied here, three of four patients had testicular mosaicism. Our results suggest that testicular mosaicism has a high prognostic value for spermatogenesis.

Table I. Results of the FISH on the testicular cells smears

Case	Hybridized cells	Diploid cells: premeiotic or somatic	Pachytene	Spermatocytes II	Spermatids	Spermatozoa
1	56	Total = 56 47,XXY = 56	None found	0	0	0
2	26	Total = 8 46,XY = 7 47,XXY = 1	None found	XX1818 = 1	Total = 14 X18 = 8 Y18 = 5 XY18 = 1	Total = 3 X18 = 3
3	68	Total = 29 46,XY = 17 47,XXY = 12	Total = 7 46,XY = 7	XX1818 = 2	Total = 14 X18 = 5 Y18 = 9 X1818 = 2	Total = 16 X18 = 6 Y18 = 8
4	93	Total = 52 46,XY = 37 47,XXY = 15	Total = 13 46,XY = 13	XX1818 = 1	Total = 23 X18 = 11 Y18 = 12	Total = 4 X18 = 1 Y18 = 1 YY18 = 1 Y1818 = 1
Total XXY patients	243	145	20 46,XY = 20	4	51	23
Control patients	236	46,XY = 100%	103 46,XY = 103	-	133 X18 = 73 Y18 = 58 XY18 = 2	0

Table II. ICSI results

Case	Number of straws thawed/ total straws cryopreserved	MII oocytes injected	2PN	Transferred embryos	Pregnancy	Following events
2						
1st attempt	2/3	9	2	2 = 2 grade II	No	New testicular biopsy scheduled
2nd attempt	1/3	9	6	3 = 1 grade I and 2 grade II	No	
3	1/7	3	1	1 = 1 grade I	Healthy boy born	No preimplantation diagnosis, no chromosomal prenatal diagnosis
4	1/10	9	4	2 = 1 grade I and 1 grade II	Biochemical pregnancy	

The difficulty with such studies is that the number of testicular cells available is very small: Klinefelter patients have small testes. Biopsy samples are therefore also small and the spermatozoa in these samples are preferentially reserved for assisted reproduction treatment, leaving very few for diagnostic purposes.

Testicular sex chromosome mosaicism may owe its importance for spermatogenesis to the fact that XY rather than XXY cell lines enter meiosis.

All 20 observed meiotic pachytene figures had an XY1818 constitution. We observed no pachytene meiosis with an unpaired or eliminated extra X has been reported for the extra Y in the case of the XYY constitution (Blanco *et al.*, 1997, 2001; Bergère *et al.*, 1998). On these pachytene figures, we observed a single X signal that was not large enough to correspond to two closely associated chromosomes X. In patient 1, the only patient for whom no spermatozoa were recovered and for whom histological analysis showed no spermatogenesis, all the testis nuclei smeared were diploid XXY. These results suggest that, for the patients we studied, only XY cells entered meiosis. This hypothesis was also the conclusion of previous studies on XXY subjects' testis cells by FISH: one recent report using FISH (Blanco *et al.*, 2001) observed only 46,XY pachytene spermatocytes I among spermatocytes of two Klinefelter's syndrome patients and by histology and electron microscopy (Luciani *et al.*, 1970; Rajendra *et al.*, 1981) which reported that only XY cells entered and completed meiosis. Foresta (Foresta *et al.*, 1999) described two patients with a small number (respectively six and four) of XXY spermatogonia and residual spermatogenesis, leading the authors to conclude that the meiotic progression of these XXY cells is possible. These differences may be due to the heterogeneity of the syndrome between individuals and the small number of testicular cells analysed. Since 1994 various studies (Cozzi *et al.*, 1994; Chevret *et al.*, 1996; Guttenbach *et al*., 1997; Estop *et al*., 1998; Hennebicq *et al.*, 1999; Bielanska *et al.*, 2000) have also suggested that XXY cells may enter and complete meiosis, based on indirect evidence (the presence of 24,XY or 24,XX spermatozoa in ejaculate potentially issued from XXY spermatogonia) and on the basis of a distorted sex ratio (Chevret *et al.*, 1996), leading to an excess of 23,X versus 23,Y. Our results were not consistent with this: the frequencies of 23,X and 23,Y in post-meiotic cells were similar. We think that the aneuploidy rate reported by these authors could be accounted for by meiotic errors of normal XY spermatogonia in an impaired testicular

Table III. FISH studies performed on testis cells of non mosaic 47,XXY men and of controls

Study	Number of patients	Number of hybridized post-meiotic cells	% post-meiotic sex chromosomes aneusomy	% post-meiotic autosomal aneusomy	Number and % of XY spermatocytes I	Number and % of XXY spermatocytes I
Studies on XXY non-mosaic patients						
Levron *et al*., 2000	5	112	4.4	1.7	Not mentioned	Not mentioned
Blanco *et al*., 2001	1	120	18.3	Not mentioned	n = 36, 94.7%	0%
Blanco *et al*., 2000	1	Not mentioned	18	Not mentioned	100%	
Levron *et al*., 1999	Not mentioned	Not mentioned	7.5	7.5	Not mentioned	Not mentioned
Foresta *et al*., 1999	2	79	43	Not mentioned	0%	n = 20, 100%
Our study	4	74	2.7	4.05	n = 20, 100%	0%
Control studies						
Martin *et al*., 2000	3	3324	0→1.87	0 →0.5	–	–
Huang *et al*., 1999	20	6380	42	–	–	–

environment (Finkelstein *et al*., 1998) though we cannot exclude the possibility that XXY cells enter meiosis.

In a murine Klinefelter model it was recently shown that spermatozoa aneuploidy for X or Y are likely to arise from abnormal meiosis of normal XY spermatogonia if these spermatogonia are in a 'compromised testicular environment' (Mroz *et al*., 1999). In our study several lines of evidence suggest that not only meiosis but also spermiogenesis were altered: first, spermatozoon labelling efficiency was high (over 90%) though FISH was done without any prior decondensation. Such a protocol would generally result in law labelling efficiency if applied to normal ejaculated spermatozoa (around 2% according to our personal data). Chromatin compaction therefore seemed to be affected in the three Klinefelter patients with spermatozoa studied.

A compromised testicular environment affecting spermiogenesis is also suggested by the high rate of teratozoospermia among non-azoospermic Klinefelter's syndrome patients (Foresta *et al*., 1998; Okada *et al*., 1999; Morel *et al*., 2000; Rives *et al*., 2000). The small number of spermatozoa recovered here made it difficult to quantify teratozoospermia but inverted microscopy before injection revealed that most of the spermatozoa were abnormal. Further evidence for an altered spermiogenesis was provided by the very high number of observed spermatids, exceeding the number of spermatozoa in patients 2 and 4 by a factor of 5 and 6. This may be due to post-meiosis cell differentiation wastage. The high frequency of spermatids which presumably degenerate was also noted by Kruse (Kruse *et al.*, 1998), working on ejaculated sperm samples of patients with Klinefelter's syndrome, and by Blanco (Blanco *et al*., 2001) on two testicular samples.

We evaluated the aneuploidy rate of post-meiotic cells to guide the genetic counselling of patients with testicular spermatozoa.

We observed an overall aneuploidy rate of 6.7% (5/74) with a rate of 2.7% (2/74) for sex chromosomes among post-meiotic cells of XXY subjects. Errors affected chromosome 18 more frequently than XY chromosomes in this series. Meiosis II alterations resulting in a raised autosomal aneusomy rate are reported by different studies (Estop *et al.*, 1998; Lim *et al.*, 1998; Levron *et al.*, 1999; Rives *et al.*, 2000; Bielsanska *et al.*, 2000) and recently by others (Hennebicq *et al.*, 2001) who reported too a higher risk (6.2%) of aneusomy for a control studied autosome (21) than that observed for the XY pair, on ejaculated spermatozoa of a non-mosaic Klinefelter patient.

Other authors studying ejaculated spermatozoa from Klinefelter's syndrome patient have reported an aneuploidy rate of 0.9% (543 karyotyped spermatozoa; Cozzi *et al.*, 1994) to 25% (24 spermatozoa studied with X,Y, and 18 probes; Estop *et al*., 1998). To our knowledge, FISH analysis of testis samples from non-mosaic subjects has been reported in only five recent publications (Foresta *et al*., 1999; Levron *et al.*, 1999, 2000; Blanco *et al*., 2000, 2001). Results of these five studies are summarized in Table III. An 18.3% rate of post-meiotic XY disomy on 120 post-meiotic cells of one Klinefelter's syndrome patient has been reported (Blanco *et al*., 2000, 2001). Levron reported in the first abstract he published (Levron *et al.*, 1999) an aneuploidy rate of 7.5% for chromosomes X, Y, and 18 (the number of spermatozoa scored was not stated), and of 6.3% (110 testicular spermatozoa analysed for five patients) in an article (Levron *et al*., 2000).

All these authors concluded that meiosis errors of normal XY spermatocytes probably accounted for the high rate of aneuploidy in post-meiotic cells. However Foresta (Foresta *et al.*, 1999) observed XXY primary spermatocytes and obtained a much higher post-meiotic aneuploidy rate (30% hyperhaploidy rate). Control studies of testicular cells from azoospermic patients with normal karyotype are few and controversial (Table III). Some studies (Martin *et al.*, 2000) reported a slightly high post-meiotic aneuploidy rate that was not statistically significant, whereas others (Huang *et al.*, 1999) reported a large excess of aneuploidy (statistically significant). Though the results of those two studies were established on a high number of hybridized cells the two conclusions do not coincide and do not allow any consensual estimation concerning the control testis post-meiotic aneusomy rate.

The discrepancies of the different studies carried out on XXY patients may be due to the number of cells analysed, which depends upon the severity of hypospermatogenesis, and may be related to the risk of aneuploidy. In cases of mild hypospermatogenesis, numerous spermatozoa may be saved for diagnosis and research purposes whereas in cases of severe hypospermatogenesis, and perhaps of high hyperaneuploidy risk, the number of spermatozoa saved is very restricted. For

this reason, we selected as controls two men with a normal katyotype and azoospermia due to spermiogenesis failure. We observed a spermatid aneuploidy rate of 1.5% (2/133). This 1.5% rate, though lower that the one observed for XXY patients' post-meiotic cells (i.e. 6.75%), is not significantly different (Fisher's test, $P = 0.132$). Due to the low number of cases, this data should be checked on a higher number of patients and a higher number of hybridized cells.

For the three XXY patients for whom testicular sperm was retrieved, the results and details of the aneuploidy rate were given to the couples who received genetic counselling. Patients were advised of the possibility, but also of the limits and risks of ICSI and all opted for this assisted reproduction treatment (although alternative solutions were also put forward). The possibility of preimplantation diagnosis or chromosomal prenatal diagnosis if ICSI was successful was also discussed. No couple opted for preimplantation diagnosis. The couple with an ongoing pregnancy refused amniocentesis. They underwent careful ultrasound follow-up and maternal serum markers (free βHCG) were determined.

Testicular biopsy and ICSI can be successful in Klinefelter patients. We believe that all azoospermic Klinefelter patients should be informed of this and that testis biopsy should be widely offered. The genetic risks resulting from injection of such spermatozoa should be discussed with each couple and the limited data yet obtained should be mentioned with caution. If ICSI is possible and successful, genetic counselling and pregnancy follow-up should be discussed in each case with the patients, taking into account the fact that the diagnosis of a fetus with a sex chromosome abnormality may be more acceptable to subfertile patients (Meschede *et al.*, 1997), mostly those who carry a sex chromosome anomaly (Ron-El *et al.*, 2000). After preimplantation diagnosis, prenatal chromosomal diagnosis and ultrasound scan have been discussed, the parents themselves should decide on which techniques should be used.

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