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Sperm aneuploidy and meiotic sex chromosome configurations in an infertile XYY male

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BACKGROUND: There is little information regarding the behaviour of the extra Y chromosome during meiosis I in men with 47,XYY karyotypes and the segregation of the sex chromosomes in sperm. We applied immunofluorescent and FISH techniques to study the relationship between the sex chromosome configuration in meiotic germ cells and the segregation pattern in sperm, both isolated from semen samples of a 47,XYY infertile man. METHODS: The sex chromosome configuration of pachytene germ cells was determined by immunostaining pachytene nuclei for synaptonemal complex protein 3 (SCP3) and SCP1. FISH was subsequently performed to identify the sex chromosomes and chromosome 18 in pachytene cells. Dual- and triple-color FISH was performed on sperm to analyse aneuploidy for chromosomes 13, 18, 21, X, and Y. RESULTS: 46,XY/47,XYY mosaic pachytene cells were observed (22.2% vs. 77.8%, respectively). The XYY trivalent, and X+YY configurations were most common. While the majority of sperm were of normal chromosomal constitution, an increase in sex and autosome disomy was observed. CONCLUSIONS: The level of germ cell moscaicism and their meiotic sex chromosome configurations may determine sperm aneuploidy rate and fertility status in 47,XYY men. Our approach of immunostaining meiotic cells in the ejaculate is a novel method for investigating spermatogenesis in infertile men.

Keywords: sperm; aneuploidy; sex chromosomes; meiotic configurations; 47,XYY

Introduction

The 47,XYY syndrome is found in 0.1% of the general male population (Jacobs *et al*., 1974), with the extra Y chromosome resulting from paternal non-disjunction at meiosis II. The presence of an extra unsynapsed chromosome is thought to activate checkpoint mechanisms to prevent the progression of meiosis (Roeder and Bailis, 2000). Nevertheless, the majority of XYY men are fertile (Linden *et al*., 1996), and it has been suggested that the extra Y chromosome is lost prior to meiosis in these men (Chandley *et al*., 1976; Faed *et al*., 1976; Speed *et al*., 1991). This hypothesis has been supported by studies on sperm aneuploidy in fertile and infertile XYY men, in which a majority of sperm were of normal karyotype (Mercier *et al*., 1996; Blanco *et al*., 1997; Chevret *et al*., 1997; Mennicke *et al*., 1997; Martin *et al*., 1999; Morel *et al*., 1999; Rives *et al*., 2003). However, the level of sex chromosome aneuploidy has been found to be significantly increased when compared to concurrent controls in the same studies. Thus, XYY men may have mosaic XY/XYY germ cell lines, and the presence of XYY germ cells may be indicative of their infertility status and the genetic makeup of their sperm.

Meiotic studies on XYY men have shown that the extra Y chromosome is present in varying proportions of testicular germ cells (Tettenborn *et al*., 1970; Hultén and Pearson, 1971; Luciani *et al*., 1973). Analyses of XYY germ cells at the pachytene stage from testicular samples have revealed different sex chromosome pairing configurations. The most prevalent configuration is the pairing of two Y chromosomes as a bivalent while the X chromosome remains unpaired (X + YY) (Hultén and Pearson, 1971; Speed *et al*., 1991; Solari and Rey Valzacchi, 1997). Other configurations include XY + Y, X + Y + Y, and XYY trivalents (Luciani *et al*.,1973; Speed *et al*.,1991; Gabriel-Robez *et al*., 1996; Solari and Rey Valzacchi, 1997). The XYY trivalent configuration is suggested to be capable of escaping the pachytene checkpoint, leading to the production of sex chromosome aneuploid sperm (Miklos, 1974; Rodriguez and Burgoyne, 2000; Roeder and Bailis, 2000; Milazzo *et al*., 2006). However, testicular tissues are not always available for study, and few studies have concurrently studied meiotic germ cells and sperm in XYY men. Recently, Milazzo *et al*. (2006) were able to identify pachytene cells from the ejaculate of two XYY men based on nuclei morphology, and examined the meiotic configurations of the sex chromosomes. Using morphology to identify particular meiotic cells can be difficult, as zygotene cells, which display incomplete pairing, can

often be confused with the pachytene nuclei, in which synapsis is complete. Previously published studies, including our own, have employed immunofluorescent techniques to study synapsis and recombination in pachytene cells from testicular tissue (Ma *et al*., 2006). These techniques allow for the direct analysis of synapsis and recombination, allowing confident identification of pachytene nuclei. In this study, we applied a similar technique to study pachytene cells present in the ejaculate of an XYY patient. We combined FISH on chromosomes 18, X and Y with immunostaining of synaptonemal complex protein 3 (SCP3) and SCP1 to study the sex chromosome configurations in pachytene cells from the ejaculate of an XYY infertile patient. We also studied aneuploidy rates of the sex chromosomes and autosomes in this patient's sperm to determine the relationship between XYY germ cells and the chromosomal constitution of the resulting sperm. To our knowledge, this is the first application of immunofluorescent techniques to study synapsis in pachytene nuclei from the ejaculate.

Materials and Methods

Clinical information

The 47,XYY patient was ascertained at 29 years old. At that time, hormone levels were normal. Semen analyses revealed oligoasthenoteratozoospermia with a sperm count of 2.6×10^6/ml, 54% normal motility and 5% normal morphology. Cytogenetic analysis of peripheral lymphocytes revealed a non-mosaic 47,XYY karyotype. Ejaculate samples from five proven fertile males were used as controls. Ethical approval was obtained from the University of British Columbia Ethics Committee before initiation of this study.

Combined immunofluorescence and fish on pachytene germ cells

To identify and analyse pachytene nuclei in the ejaculate, we modified a previous protocol (Ma *et al*., 2006). Ejaculate samples were washed in modified human tubal fluid (Irvine Scientific, Santa Ana, CA, USA), centrifuged to pellet cells and then resuspended and incubated in a hypo-osmotic buffer [30 mM Tris, 50 mM sucrose, 17 mM citric acid, 5 mM EDTA, 0.5 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulphonyl flouride] for 1 h. Cells were fixed on a slide using 1% paraformaldehyde and incubated at 37°C overnight in a humid chamber. The slide was air-dried and washed in 0.4% PhotoFlo (Kodak 200 solution) to minimize water marks. A second wash in 1× ADB (1% donkey serum, 0.3% bovine serum albumin, 0.005% Triton X, PBS; pH 7.2) followed. Primary antibodies of mouse anti-SCP3 and mouse anti-SCP1 (provided by P. Moens, York University) 1:300 and 1× ADB were applied to the slide and incubated for 24 h. The slide was then soaked in 1× ADB for 48 h at 4°C. The secondary antibody, rhodamine (TRITC) goat anti-mouse IgG (Jackson ImmunoResearch) 1:100, was applied to the slide and incubated at 37°C. The slide was washed in PBS and air-dried. Antifade and coverslip were applied.

The immunostained slide was analysed on a Zeiss Axioplan epifluorescent microscope. Images of SCs were captured using Cytovision V2.81 Image Analysis software (Applied Imaging International, San Jose, CA, USA). Pachytene cells were captured if they were appropriately spread and SCP-immunostained. Cell coordinates were recorded for subsequent analyses of the sex chromosomes identified by FISH.

FISH was performed to identify chromosomes 18, X and Y. The coverslip was removed and the slide was soaked in 4× saline sodium citrate (SSC)/0.05% Tween 20 solution. A standard ethanol series followed, and the slide was air-dried. Alpha-satellite DNA probes for chromosomes X (SpectrumGreen; Vysis Inc., USA), Y (SpectrumOrange; Vysis) and 18 [CEP 18 (D18Z1) SpectrumAqua; Vysis] were applied to the slide, followed by a coverslip and sealed with rubber cement. The slide was co-denatured at 75°C and incubated overnight. With the coverslip removed, the slide was washed in 0.4× SSC/0.3% NP-40 at 75°C, then in 2× SSC/0.1% NP-40 solution and air-dried. Antifade and a new coverslip were applied.

Pachytene cells captured beforehand were relocated and chromosomes 18, X and Y were identified. The meiotic configuration in pachytene nuclei were classified as: (i) XY bivalent (XY) when the signals for X and Y are in close proximity; (ii) X-univalent and Y-univalent (X + Y) when X and Y are distant from each other; (iii) XYY trivalent (XYY) when the three sex chromosomes are in close proximity; (iv) XY-bivalent and Y-univalent (XY + Y) when one X and one Y are in close proximity and the other Y is distant; (v) X-univalent and YY-bivalent (X + YY) when two Y's are in close proximity and the X is distant; and (vi) X-,Y-,Y-univalents (X + Y + Y) when all three sex chromosomes are not in close proximity to each other.

Sperm aneuploidy analysis by fish

Sperm preparation and FISH were performed according to our previously described protocol (Tang *et al*., 2004). Briefly, sperm fixed in methanol/acetic acid (3:1) were dropped on glass slides, washed in 2× SSC and incubated in DTT until sperm heads were adequately decondensed. Triple- and dual-color FISH were both performed on patient and control samples. Sperm nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vysis). The same DNA probes for chromosomes 18, X and Y were used for triple-color FISH. DNA probes for chromosomes 13 (LSI 13 SpectrumGreen; Vysis) and 21 (LSI 21 SpectrumOrange; Vysis) were used for dual-color FISH. Analyses were done using a Nikon Ellipse E600W epifluorescent microscope equipped with the appropriate filters. We followed previous criteria for scoring sperm nuclei (Tang *et al*., 2004). Only nuclei with intact morphology and sperm tails were scored.

Results

Combined immunofluorescent and fish analysis of pachytene nuclei

A total of 45 pachytene nuclei were identified by SCP3/SCP1 immunostaining from the ejaculate of our XYY subject and analysed by FISH (Table I). Of these cells, 78% were XYY constitution and 22% were XY. The most common meiotic configuration observed was X + YY (37.8%) (Fig. 1A and B). However, XYY trivalent cells were observed at a similar rate (35.6%) (Fig. 1C and D). Of the XY pachytene cells, half

Table I. Sex chromosome configuration at pachytene stage assessed by combined fluorescent immunostaining and fluorescent *in situ* hybridization with antibodies for SCP3/1 and probes for chromosomes 18, X and Y.

Pairing configuration of XYY pachytene cells *n* (% of total no. of cells)			
XYY	X + YY	XY + Y	Total
16 (35.6)	17 (37.8)	2 (4.4)	35 (77.8)
Pairing configuration of XY pachytene cells *n* (% of total no. of cells)			
XY	X + Y		Total
5 (11.1)	5 (11.1)		10 (22.2)
		Total no. of cells 45	

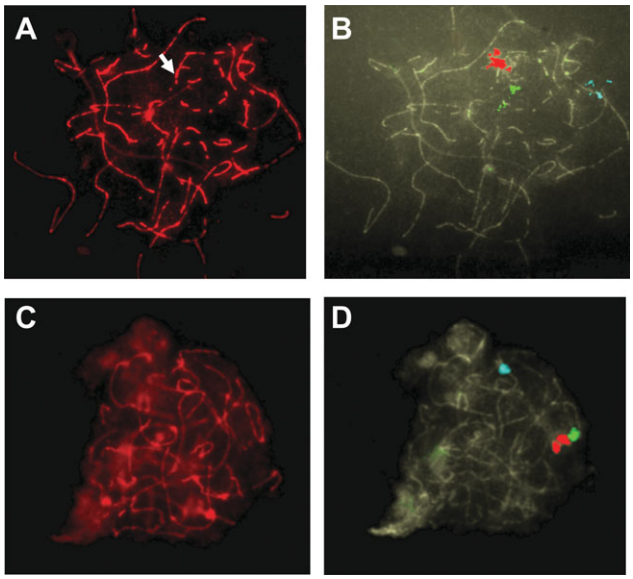

Figure 1: Immunofluorescent and FISH analysis of pachytene cells from the ejaculate of an XYY male
(**A, C**) Cells were immunostained with antibodies against SCP3/1 (red) to identify pachytene cells and observe meiotic chromosome pairing. Subsequent FISH for chromosomes 18 (blue), X (green) and Y (red) was performed to identify chromosomes. (**B**) Cells were classified as the X + YY configuration if a SCP3/1 positive Y bivalent was identified (**A**, white arrow) and the X chromosome was close to the Y bivalent, but not directly paired. (**D**) Cells were classified as the XYY configuration if the X and two Y FISH signals were in close proximity

were XY bivalents and half were X + Y. We also observed that all pachytene cells analysed had at least a small degree of SC fragmentation.

Fish analysis of spermatozoa

For the XYY patient, a total of 10 092 and 10 009 sperm were scored for FISH on chromosomes 18, X, Y and 13, 21, respectively. For the controls, a total of 50 752 and 51 234 sperm were scored using the same two sets of probes. The results are displayed in Table II. The ratio of X- to Y-bearing sperm was not significantly different in the patient. A significant increase in the frequency of each type of sex disomy was observed. The 24,YY sperm accounted for over 60% of sperm with sex disomy. The rates of autosomal disomy were significantly increased for chromosomes 13, 18 and 21. Sex chromosome nullisomy and nullisomy 13, 18 and 21 were also significantly increased. However, the rate of nullisomy and the rate of disomy for each chromosome investigated were different from each other.

Table II. Aneuploidy levels in sperm assessed by dual- and triple-FISH with probes for chromosomes 13 and 21, and 18, X and Y.

	47,XYY patient (%)	Controls (%)	*P*-value
Three-color FISH			
No. of sperm scored	10 092	51 234	
X:Y ratio	1.04	1.01	n.s
Overall aneuploidy	3.05	0.58	<0.001
Total sex aneuploidy	1.97	0.36	<0.001
XY disomy	0.33	0.20	<0.01
XX disomy	0.37	0.08	<0.001
YY disomy	1.27	0.11	<0.001
Sex nullisomy	0.45	0.00	<0.001
Disomy 18	0.16	0.07	<0.05
Nullisomy 18	0.33	0.06	<0.01
Total nullisomy	0.77	0.06	<0.001
Two-color FISH			
No. of sperm scored	10 009	50 752	
Total aneuploidy	1.89	0.61	<0.001
Disomy 13	0.35	0.10	<0.001
Nullisomy 13	0.52	0.24	<0.001
Disomy 21	0.63	0.15	<0.001
Nullisomy 21	0.39	0.11	<0.001
Diploidy	0.01	0.10	n.s

n.s., not significant. Statistical analyses were performed using a 2 × 2 Chi-square test.

Discussion

Using immunocytogenetic and FISH techniques, we observed that a majority of pachytene cells (77.8%) in the ejaculate of our patient contained the extra Y chromosome. Other studies have reported 58–100% frequencies of XYY pachytene nuclei in four patients (Solari and Rey Valzacchi, 1997; Blanco *et al*., 2001; Rives *et al*., 2005; Milazzo *et al*., 2006). Using silver-staining techniques, Solari and Rey Valzacchi (1997) found a complete absence of normal XY cells in pachytene nuclei of an XXY male with extremely low sperm count (625/ml). However, Gabriel-Robez *et al*. (1996) identified an XYY patient with normal sperm count in which all pachytene nuclei were normal 46,XY cells. It has been suggested that the level of XY/XYY mosaicism in pachytenes is negatively correlated with sperm count (Milazzo *et al*., 2006). As well, severe oligozoospermia in XYY men is often associated with the persistence of the extra Y chromosome in pachytene cells, at frequencies of 30–100% (Speed *et al*., 1991; Solari and Rey Valzacchi, 1997). Similarly, our patient also had low sperm count in addition to abnormal sperm morphology and low motility. Our findings are consistent with previous suggestions that sperm count may be indicative of the genetic makeup of meiotic cells in XYY men and their fertility status.

A high frequency of XYY pachytene cells in our patient contained two Y-chromosomes in close proximity in an X + YY configuration. Other studies on testicular pachytene cells showed that the two Y-chromosomes preferentially pair together (Solari and Rey Valzacchi, 1997; Blanco *et al*., 2001; Rives *et al*., 2005). This pairing occurs along at least 43% of their length and includes the p-arms (Solari and Rey Valzacchi, 1997). The preferential pairing of the Y-chromosomes may be due to greater homology compared with the X chromosome. However, X + YY cells are likely to be caught by the pachytene checkpoint, resulting in a low sperm count (Solari and Rey Valzacchi, 1997; Roeder and Bailis, 2000; Blanco *et al*. 2001; Rives *et al*. 2005; Milazzo *et al*. 2006). X + YY cells are likely to be lethal due to the escape of Y genes from meiotic sex chromosome inactivation, which normally silences the unsynapsed sex chromosomes (Turner, 2007). XY + Y and XYY trivalent cells may not cause cell lethality and proceed through spermatogenesis, resulting in aneuploid sperm. X + Y + Y, XY + Y and XYY trivalent cells were also observed in other XYY men at lower frequencies (4–30%, 8% and 8–35%, respectively) (Luciani

et al., 1973; Speed *et al*., 1991; Gabriel-Robez *et al*., 1996; Solari and Rey Valzacchi, 1997). XY + Y pachytene nuclei represented 2 of 45 cells (4.4%) in our patient. However, we did not observe any X + Y + Y cells. XYY trivalents were present in 16 of 45 cells (35.6%) in our patient. This is contrary to the findings by Milazzo *et al*. (2006), who observed that all XYY pachytene nuclei were in a probable trivalent configuration. The XYY trivalent is suggested to be the only configuration that can escape the pachytene checkpoint, due to saturation of pairing sites (Miklos, 1974). The escape of these cells from degradation may result in abnormal 24,XY or 24,YY sperm. It is important to note that all pachytene cells that we analysed displayed fragmentation of the SC. SC fragmentation may be a result of DNA breakage associated with apoptosis or meiotic arrest. Thus, pachytenes in the ejaculate may be representative of the germ cells that did not complete spermatogenesis, as a majority of sperm in our patient had normal chromosome constitution (97.0%).

We also determined the rate of sperm aneuploidy in our patient. Our patient had significantly higher rates of sex chromosome disomy when compared with normal 46,XY men. The higher incidence of 24,XY and 24,YY sperm is likely due to the presence of XYY germ cells. Significant increases in XY and YY disomy in the sperm have been observed in other XYY infertile men (Blanco *et al*., 1997; Rives *et al*., 2003; Milazzo *et al*., 2006). Similar to two other patients, we also observed a significantly increased rate of XX disomic sperm (Martin *et al*., 1999; Rives *et al*., 2003). All infertile XYY patients with significantly increased XX disomy sperm also display significantly increased XY, YY and chromosome 18 disomy, and diploid sperm (Rives *et al*., 2003). Our results are in accordance with this association. Additionally, increased chromosomes 13 and 21 disomic and nullisomic sperm were observed. Two other studies have investigated aneuploidy for chromosomes 13 and 21 (Martin *et al*., 1999; Shi and Martin, 2000), with only one patient displaying an increase in disomy 13 in the sperm (Martin *et al*., 1999). Similar to most studies that investigated chromosome 18, we also observed a significant increase in disomy 18 sperm (Rives *et al*., 2003; Milazzo *et al*., 2006).

In 50% of XY pachytenes of the patient, the sex chromosomes were not synapsed. The failure of XY synapsis may impede sex chromosome recombination, which is important for the proper segregation of the sex chromosomes (Hassold *et al*., 1991). We have previously found that reduced recombination is also associated with the production of aneuploid sperm in chromosomally normal infertile men (Ma *et al*., 2006; Ferguson *et al*., 2007). Absent XY recombination in pachytene nuclei may have resulted in non-disjunction, leading to sex aneuploid sperm in the patient. Surprisingly, aneuploidy for autosomal chromosomes also appeared to be increased in this XYY patient. It is possible that the unsynapsed sex chromosomes may undergo heterosynapsis with autosomal chromosomes in order to avoid detection by the pachytene checkpoint. However, this association may disrupt the proper segregation of the autosomal chromosome, leading to increased aneuploidy in the sperm. Heterosynapsis in order to avoid the pachytene checkpoint has been observed in carriers of structural chromosomal abnormalities (Gabriel-Robez *et al*., 1986; Johannisson *et al*., 1987), as well as in chromosomally normal males (Codina-Pascual *et al*., 2006). However, this process has not been reported in males with an XYY chromosomal constitution.

It is also possible that the general increase in sperm aneuploidy in this patient may be related to the abnormal testicular environment and impaired semen parameters in this man. Studies on infertile men who are chromosomally normal have shown that, in general, the presence of abnormal semen parameters is an indicator for an increase in sperm aneuploidy, DNA fragmentation and mitochondrial dysfunction (Liu *et al*., 2004; Calogero *et al*., 2001). Based on our results and that in the literature, infertility in XYY men appears to be dependent on the chromosome constitution of their meiotic germ cells. The proportion of XYY germ cells and the sex chromosome configuration during meiosis may be indicative of impaired sperm counts and elevated sperm aneuploidy. Furthermore, XYY infertile men with low sperm count may be at a greater risk of passing on a chromosomal abnormality to their offspring when undergoing infertility treatments such as intracytoplasmic sperm injection. Aneuploidy screening of these men may provide better risk assessments for infertility treatments.

To our knowledge, we are the first to apply fluorescent immunostaining for SCP3/1 on pachytene cells from the ejaculate. We observed fragmentation of the SC in the pachytene nuclei, indicating the apoptotic death of cells, which may have accounted for the high frequency of XYY pachytene nuclei. Thus, further immunofluorescent studies on immature germ cells will provide information on the differences in the chromosomal constitution and meiotic pairing between pachytene nuclei found in the ejaculate and those found in the testes. If differences are found between the ejaculate and testicular cells, it may provide insight into the meiotic configurations that are preferentially eliminated during meiosis. However, if immature germ cells in the ejaculate are a reflection of what is occurring in the testes, it will provide an alternative source for meiotic studies when testicular samples are unavailable.

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