Human Reproduction Vol.17, No.3 pp. 576–583, 2002

Sperm aneuploidy in fathers of children with paternally and maternally inherited Klinefelter syndrome

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BACKGROUND: It is unclear whether frequency of sperm aneuploidy is associated with risk of fathering children with trisomy. METHODS: We recruited 36 families with a boy with Klinefelter syndrome (KS), interviewed the fathers about their exposures and medical history, received a semen sample from each father, and collected blood samples from the mother, father and child. We applied a multicolour fluorescent in-situ hybridization assay to compare the frequencies of sperm carrying XY aneuploidy and disomies X, Y and 21 in fathers of maternally and paternally inherited KS cases. RESULTS: Inheritance of the extra X chromosome was paternal in 10 and maternal in 26 families. Fathers of paternal KS cases produced higher frequencies of XY sperm ($P = 0.02$) than fathers of maternal KS cases. After controlling for age, the major confounding variable, the difference between the two groups was no longer significant ($P \leq 0.2$). Also, there were no significant differences between the parental origin groups for disomy X, Y or 21. CONCLUSIONS: Men who fathered a child with a Klinefelter syndrome produced higher frequencies of XY sperm aneuploidy, which is explained, in part, by both paternal age and parent of origin.

Key words: aneuploidy/fluorescence in-situ hybridization human/Klinefelter syndrome/sperm

Introduction

Exposures of fathers to certain environmental or occupational agents have been associated with their offsprings' risk for cancers (Sorahan and Roberts, 1993), birth defects (Brender and Suarez, 1990; Hakim *et al*., 1991; Olshan *et al*., 1991), and spontaneous abortions (Savitz *et al*., 1994). The biological mechanisms linking paternal exposure to abnormal reproductive outcome remain uncertain, although certain exposures may induce transmissible genetic damage in sperm. In studies with male rodents, it has been conclusively demonstrated that exposure to chemical mutagens can increase the fraction of sperm with DNA damage and chromosomal defects (Wyrobek, 1993). Furthermore, exposures of male rodents prior to mating can result in a broad spectrum of abnormal reproductive outcomes including dominant lethality during development, morphological defects, heritable chromosomal translocations, and cancer in the offspring (Wyrobek, 1993).

Multicolour fluorescent in-situ hybridization (FISH) methods have been developed for the study of sperm aneuploidy (Downie *et al*., 1997; Egozcue *et al*., 1997; Guttenbach *et al*., 1997; Hassold and Hunt, 2001). Studies in humans using FISH have demonstrated that aneuploid sperm can be induced after paternal exposures to certain chemical agents, such as chemotherapeutics (Robbins *et al*., 1997a; De Mas, 2001), cigarette smoking (Robbins *et al*., 1997b; Rubes *et al*., 1998), organophosphate pesticides (Padungtod *et al*., 1999), and diazepam (Baumgartner *et al*., 1996). However, one of the major uncertainties is whether the fraction of chromosomally abnormal sperm is associated with the risk of having an abnormal reproductive outcome, such as an offspring with trisomy.

The objective of the current investigation was to determine whether men who had recently fathered a child with trisomy produced higher frequencies of aneuploid sperm. We limited this analysis to families with a boy with Klinefelter syndrome (KS; 47,XXY), because KS has a relatively high incidence at birth (~1/600 liveborn males), affected males generally live well through their reproductive years, and unlike autosomal trisomies where the majority are maternal in origin, a substantial fraction of sex chromosomal aneuploidies at birth are paternal in origin (Hassold *et al*., 1993). We conducted a study of families with a child with KS to determine whether the frequencies of aneuploid sperm involving chromosomes X, Y and 21 (Baumgartner *et al*., 1999) were higher in fathers who contributed the extra X to their sons (paternal KS group) compared to fathers where the mother contributed the extra X (maternal KS group).

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Materials and methods

Participants

We recruited families nationwide who had a child with Klinefelter syndrome. The children were primarily contacted from the membership list of Klinefelter Syndrome and Associates, a lay nationwide educational and advocacy group located in Roseville, California, and through additional referrals by genetic counsellors. A family was included in the study if the child was no more than 6 years old, had a non-mosaic karyotype, and both parents consented to participate. We identified 38 families, of which 36 were included in these analyses.

Procedure

This study was approved by the Institutional Review Boards of the participating institutions. A trained interviewer conducted a 30 min telephone interview separately with the mother and with the father about medical and reproductive history, habits, occupational and environmental exposure, and a genetic pedigree. The interviewer asked the father for detailed exposure and medical information for the prior 3 month period. Participation also included a single semen sample from the father and blood samples from the mother, father and child. The family was asked to contact a local branch of a nationwide service laboratory to schedule the blood draw and semen specimen collection. If this was not convenient or if they preferred, the family could choose to have the laboratory work done at a local medical facility or provider. In either case, the clinical person responsible for the blood draw and semen sample handling was given verbal and written instructions from the study office to standardize the protocols for sample collection, handling, and shipping. Instructions for the collection of semen samples and a sterile container were sent to each father, and the instructions were reviewed with him by phone.

Parental origin of the X chromosome

Blood was refrigerated immediately and sent within 2 h on cold packs to Lawrence Livermore National Laboratory (LLNL) in Livermore, California, by overnight carrier, where it was aliquoted and stored at –20°C. Parental origin of the extra X chromosome was determined using microsatellite polymorphisms (one trinucleotide repeat, AR, and three dinucleotide repeats DXS981, DXS106 and DXS573). DNA was extracted using QIAamp blood and tissue kit (Qiagen, Palo Alto, CA, USA) according to the manufacturer's protocol using ~200 µl of stored blood. Marker-specific primers that flanked the repeats were tagged with fluorescence labels, either HEX-green or FAM-blue. For every 50 µl of PCR amplification, the following reagents were used at the final concentration of ~200 ng of DNA template: 1×PCR buffer (Perkin–Elmer), 0.2 mmol/l dNTP, 20 pmol of each primer, and 25 U of *Taq* DNA polymerase (Perkin–Elmer). A template negative control was run simultaneously for every PCR. 10 µl of every PCR product was initially examined in 3% metaphor agarose gel to determine the quality, quantity and length of product with 4–6 bp resolution. 30% polyacrylamide sequencing gel was used to obtain 1 bp resolution with an automated sequencer (ABI 373). PCR products obtained by using four different marker-specific primers tagged with blue or green fluorescence labels were diluted at 1:10 to 1:50 based on the results of metaphor gel analysis. A desired concentration of PCR products was achieved to avoid overloading samples in the sequencing gel resulting in cross-contamination between the neighbouring lanes. These samples were then mixed and loaded onto acrylamide sequencing gel lanes along with the standard molecular size GS 2500 tagged with red fluorescence dye, one lane per subject. The fluorescence tags were designed for each primer so that multiple PCR products can be loaded and analysed within the same lane of the polyacrylamide gel. For example, one pair of markers, DXS981 and AR, produces PCR products of 182–199 and 195–22 bp in length, respectively. Because the length of PCR products is very similar, marker DXS981 was tagged with blue and marker AR was tagged with green fluorochrome and both were viewed in the green and blue channels. The other pair of markers, DXS106 and DXS573, have PCR products of 97–107 and 137–145 bp and were tagged with blue and green, respectively. The PCR products from these four markers for each subject were mixed and loaded into the same lane. Samples from members of the same family were always loaded in the adjoining lane for electrophoresis (2500 V, 40 A, 32 W and run time of 6–8 h). Genescan 672 software (Perkin–Elmer) was used to collect and analyse the data obtained from the automated sequencer ABI 373. The data were accepted only if they met the following criteria: (i) expected molecular size and pattern; (ii) there was no cross-contamination among adjoining lanes; (iii) data from father, mother and the KS boy of the same family were collected at the same time; and (iv) at least two markers provided informative and consistent results. Using these criteria, 36 of the 38 families provided informative data.

Semen preparation and multicolour sperm FISH analysis

Men abstained for 2–7 days prior to providing the semen specimen by masturbation into a provided plastic sterile container. The container was delivered to a clinical laboratory within 2 h of collection and exact time and specimen condition were noted. Specimens were then sent on dry ice to LLNL by overnight carrier. Upon receipt at LLNL, the specimen was thawed at room temperature, and mixed gently with a Pasteur pipette. A volume of 5–7 µl was smeared onto an ethanol-cleaned microscope slide, and air-dried overnight at room temperature. Slides were stored under nitrogen at –20°C until hybridized. The remaining specimen was stored at –80°C as aliquots.

Sperm decondensation was performed according to a published method (Wyrobek *et al*., 1990). Briefly, the disulphide bridges of protamines were broken by incubating the slide for 30 min in 10 mmol/l dithiothreitol in 0.1 mol/l Tris–HCl solution on ice. To provide efficient probe penetration, the nuclei were swollen in 4 mmol/l 3,5-diiodosalicylic acid in 0.1 mol/l Tris–HCl at room temperature for 30 min, and air-dried for at least 1 h prior to hybridization. This decondensation procedure results in swelling of sperm cells to 1.5–1.8 times their original longitudinal cell axis.

Each semen specimen was analysed by the XY21 sperm FISH assay (Baumgartner *et al*., 1999). This assay uses alpha centromeric DNA probes for chromosomes X and Y in addition to a locus-specific probe that hybridizes to the 21q22.13–q22.2 region on the chromosome. Specifically, a mixture of CEP X (Vysis) probes labelled with both SpectrumGreen and SpectrumOrange fluorophores was used in combination with centromeric alpha satellite DNA probe for chromosome Y (LLNL) labelled with a SpectrumGreen fluorophore and LSI probe for the q-arm of chromosome 21 (Vysis) labelled with a SpectrumOrange fluorophore. The hybridization efficiencies were ~99.5%.

Scoring was done under a Zeiss Axiophot fluorescence microscope using the spectral filters and scoring criteria as previously published (Baumgartner *et al*., 1999). Briefly, all slides were coded by a third party and a minimum of 10 000 sperm per semen sample was scored with the exception of one sample with ~6800 cells scored. All slides were scored by a single scorer using Cytoscore©, a computer program developed at LLNL for entry and management of sperm aneuploidy data. First, 5000 sperm were scored from each of a group of five slides. All slides were then recoded by the third party and a second set of 5000 sperm were scored in another area on the same slide while blind to the results of the

Table I. Frequency of XY, disomy 21 and disomy X or Y sperm in fathers of maternal and paternal Klinefelter syndrome cases

Donor			Frequency/10 000 sperm		
Maternal (M)/ paternal (P)	Paternal age	Sperm scored	XY	Disomy 21	Disomy X or Y
M	24	10 019	8.0	8.0	6.0
M	27	10 073	7.9	8.9	13.9
M	27	10 160	6.9	10.8	9.8
M	29	10 014	7.0	8.0	6.0
M	32	10 030	7.0	3.0	5.0
M	33	10 036	8.0	4.0	5.0
M	34	10 086	8.9	5.0	9.9
M	35	10 065	6.0	3.0	7.0
M	35	10 103	5.9	6.9	5.9
M	36	10 053	10.9	10.9	7.0
M	37	10 067	7.0	7.0	7.9
M	38	10 028	7.0	4.0	12.0
M	38	10 031	6.0	4.0	8.0
M	40	10 014	15.0	3.0	10.0
M	41	10 070	7.9	4.0	5.0
M	41	10 031	5.0	4.0	11.0
M	41	10 027	14.0	6.0	12.0
M	41	10 043	28.9	17.9	19.9
M	42	10 036	9.0	7.0	12.0
M	42	10 039	8.0	6.0	4.0
M	43	10 044	13.9	3.0	10.0
M	44	10 040	29.9	6.0	19.9
M	44	10 040	6.0	4.0	7.0
M	45	10 025	10.0	10.0	13.0
M	49	10 043	6.0	11.9	11.0
M	51	10 032	21.9	5.0	8.0
P	32	10 029	8.0	4.0	11.0
P	34	6893	24.7	5.8	24.7
P	34	10 029	9.0	4.0	8.0
P	36	10 037	14.9	17.9	9.0
P	39	10 022	11.0	17.0	10.0
P	41	20 071	7.0	3.0	11.0
P	49	10 050	17.9	6.0	10.9
P	51	10 041	9.0	6.0	6.0
P	51	10 026	13.0	4.0	7.0
P	57	20 090	26.4	8.0	6.5
Median (interquartile range)			8.4 (7.0, 14.0)	6.0 (4.0, 8.0)	9.4 (6.7, 11.0)

first scoring. To be considered valid results, we required that there was no significant difference between the two scorings by χ^2-analysis. Normal sperm consisted of cells containing one red and one yellow signal (21X) or one red and one green signal (21Y). For a nucleus to be considered as having an abnormal number of fluorescent domains, the characteristics of the affected domains (intensities and shapes) were required to be similar to the domains of nearby normal cells. In the case of two domains of the same colour within a cell, they were required to be separated by a distance of at least half of their average diameter and to be of similar sizes and intensities. All cells with abnormal fluorescent phenotypes were inspected under single and dual band-pass filters to determine whether one signal was located below or above a larger one. All abnormal cells were also evaluated under simultaneous fluorescence (double band-pass) and phase contrast imaging to determine how many flagella were attached to the nucleus.

Statistical analysis

We examined the distributions for three categories of sperm hyperhaploidy: XY sperm (which might produce KS upon fertilization); disomy 21 (which might produce Down's syndrome upon fertilization); and disomy X or disomy Y (meiosis II aneuploidy errors). Using the Mann–Whitney *U*-test, we determined whether the frequencies of sperm aneuploidy differed by parent of origin, i.e. higher frequency of sperm aneuploidy for fathers of paternal KS cases than for maternal KS cases. The maternal KS group served as the reference group, since there is no reason to believe that the sperm of these fathers differed from the general population. Because of the a-priori hypothesis, sperm aneuploidy frequencies for fathers of paternal versus maternal KS cases were compared using one-tailed *P*-values.

We determined whether the paternal and maternal KS groups differed in the father's sociodemographic characteristics such as paternal age at semen collection, race, and education; habits in the previous 3 months (e.g. smoking, alcohol, coffee, tea); any fevers of greater than 102°F reported in the previous 6 months; and maternal report of infertility, i.e. ever having tried for ⩾12 months to become pregnant. Two-tailed *P* values were presented for these comparisons. We also examined whether the differences between paternal and maternal KS groups in sperm aneuploidy frequencies were higher or lower in certain subgroups.

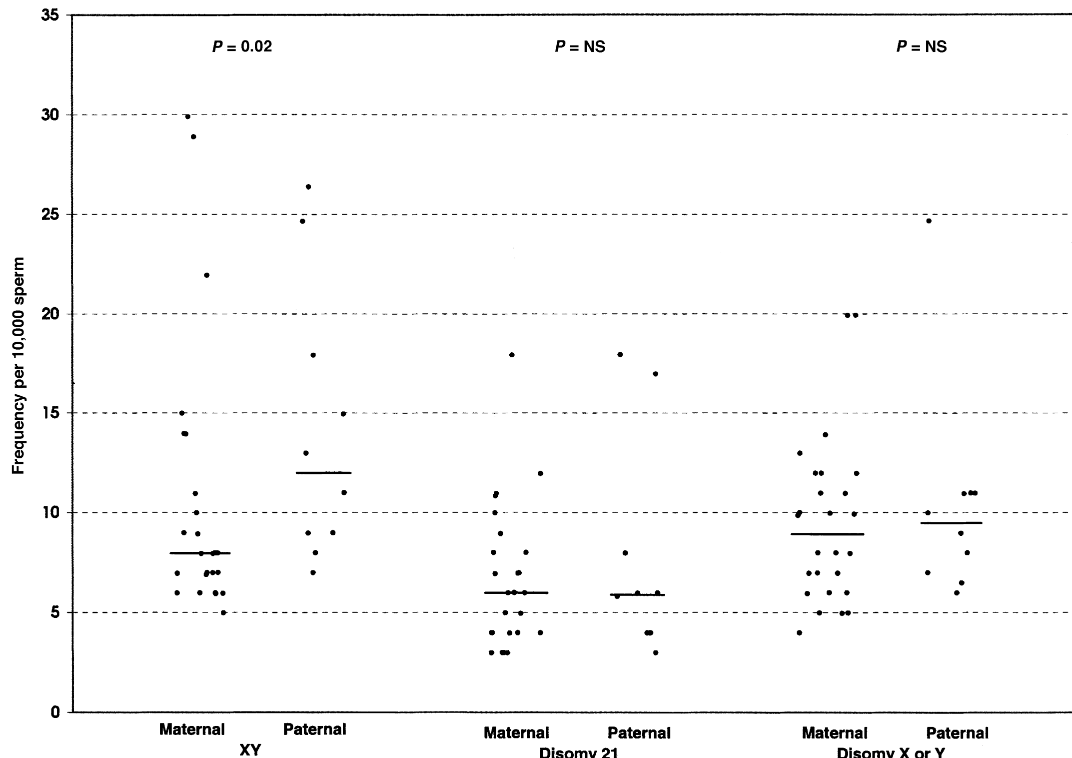

Figure 1. Frequencies of aneuploid sperm, and median frequency, by parent of origin in fathers of boys with Klinefelter syndrome ($n = 36$). Frequencies of aneuploid sperm were significantly (Mann–Whitney *U*-test) higher for paternal parent of origin groups for XY sperm. No other comparisons between paternal and maternal parent of origin groups were statistically significant.

We adjusted for co-variates in a multiple linear regression model with negative binomial regression (Stata, 1999). We also used an offset option that adjusts for differences across men in the number of sperm scored.

Results

Among the 36 informative families, the inheritance of the extra X chromosome was paternal in 10 and maternal in 26 families. The ratio of Y- to X-bearing sperm did not differ from unity in either group (overall ratio = 0.995). Table I presents the frequencies of XY, disomy 21, and disomy X or Y aneuploid sperm for each father by parent of origin and by age. The fathers of the paternal and maternal KS groups did not differ in their sociodemographic characteristics, habits, or reproductive histories, although numbers in some categories were small (data not shown). The fathers of the paternal KS group were on average slightly older than the fathers of the maternal KS group (mean ± SD: 42.4 ± 8.9 versus 38.0 ± 6.7 years), although the difference was not significant. The median ages were similar (40 versus 39 years).

The fathers of the paternal KS group had elevated frequencies of XY sperm compared with fathers of the maternal KS group [see Figure 1; median and interquartile ranges (IQR): 12.0 (9.0, 17.9) for paternal and 8.0 (6.9, 10.9) for maternal; $P = 0.02$, one-tailed]. There were three cases who had frequencies of XY sperm ⩾4 SD higher than the mean for the group. Frequencies of disomy 21 sperm [median (IQR): paternal, 5.9 (4.0, 8.0); maternal, 6.0 (4.0, 8.0); P = not significant (0.43), one-tailed] and the sum of disomy X and disomy Y sperm [median (IQR): paternal, 9.5 (7.0, 11.0); maternal, 8.9 (6.0, 12.0); P = not significant (0.31), one-tailed] did not differ for the paternal and maternal KS groups.

Table II suggests that men who were older had a tendency towards higher frequencies of XY sperm ($P = 0.07$, two-tailed). Disomy 21 was apparently higher in non-smokers and frequency of disomy X or Y was lower in men who had had a fever in the past three months; however, the number of smokers ($n = 10$) and men with fever ($n = 4$) were small. There were no other associations between sperm aneuploidy and sociodemographic characteristics, habits, or reproductive history.

In order to examine the interrelationship of age and parent of origin, the maternal and paternal KS groups were then divided into two age groups at the median age, ⩽40 years old and >40 years old, and examined by frequency of XY sperm. The older men of the paternal KS group had the highest frequency of XY sperm [median (IQR): 13.0 (9.0, 18.0)], followed by the younger fathers of the paternal KS group [11.0 (9.0, 14.9)], followed by the older fathers of the maternal KS group [10.0 (8.0, 15.0)], and last by the younger fathers of the maternal KS group [7.0 (6.9, 8.0)]. These four groups were statistically heterogeneous (Kruskal–Wallis $\chi^2 = 9.8$, $P = 0.02$). However, regression analyses revealed that only the group of younger fathers of the maternal KS cases was significantly different from each of the other three groups ($P < 0.02$), which were not different from each other, suggesting a contribution of both age and parent of origin.

Table II. Characteristics of fathers of boys with Klinefelter syndrome by frequency (per 10 000 cells) of XY, disomy 21, and disomy X or Y sperm aneuploidy

Characteristics	Maternal *n*	Paternal *n*	Frequency/10 000 sperm [median (IQR)]		
			XY	Disomy 21	Disomy X or Y
Paternal age at semen analysis (years)					
<40	13	5	8.0 (7.0, 9.0)*	6.4 (4.0, 8.9)	8.0 (6.0, 10.0)
⩾40	13	5	11.5 (7.9, 17.9)	6.0 (4.0, 7.0)	10.5 (7.0, 12.0)
Race					
Caucasian	22	8	8.0 (7.0, 14.0)	6.0 (4.0, 8.0)	9.4 (6.0, 11.0)
Other	4	2	10.5 (9.0, 13.0)	6.0 (4.0, 10.0)	9.0 (7.0, 12.0)
Education (years)					
⩽2	10	3	8.0 (7.0, 15.0)	6.0 (4.0, 8.0)	9.0 (7.0, 11.0)
>12	16	7	9.0 (7.0,13.0)	5.8 (4.0, 8.0)	9.8 (6.5, 11.0)
Current smoking					
Yes	7	3	8.0 (7.0, 15.0)	4.0 (3.0, 8.0)*	10.0 (6.5, 11.0)
No	19	7	9.0 (7.0, 13.0)	6.0 (4.0, 10.0)	8.5 (7.0, 11.0)
Alcohol (drinks per week)					
<1	14	6	9.0 (7.0, 14.0)	6.0 (4.0, 10.4)	10.0 (6.5, 11.0)
⩾1	10	4	8.0 (6.0, 22.0)	5.4 (4.0, 7.0)	8.0 (7.0, 12.0)
Caffeinated coffee (cups per day)					
<1	8	4	9.0 (7.0, 11.0)	6.0 (4.0, 8.0)	7.5 (6.2, 9.9)
⩾1	16	6	9.0 (7.0, 15.0)	5.9 (4.0, 8.0)	10.0 (7.0, 12.0)
Fevers/flu in past 6 months					
Yes	4	0	8.0 (7.0, 11.0)	6.4 (4.5, 7.4)	6.0 (5.0, 8.0)**
No	20	10	9.0 (7.0, 15.0)	5.9 (4.0, 8.0)	9.9 (7.0, 11.0)
History of infertility[a]					
Yes	7	5	14.4 (6.5, 21.3)	4.9 (3.5, 10.0)	10.5 (8.0, 11.0)
No	17	5	8.4 (7.0, 10.9)	6.0 (4.0, 8.0)	8.0 (6.0, 11.0)
No. of children					
1	9	1	7.5 (6.9, 8.0)	7.5 (4.0, 10.8)	8.9 (6.0, 11.0)
>1	17	9	9.0 (7.0, 14.9)	5.9 (4.0, 7.0)	9.4 (7.0, 11.0)

*$P < 0.10$, **$P < 0.05$; P values two-sided based on Mann–Whitney U-tests.
[a]Maternal report of trying to become pregnant for >12 months.
IQR = interquartile range.

A multiple regression model consisting of parent of origin and paternal age as categorical variables (maternal, paternal: <40 years, ⩾40 years) indicated that the adjusted increase in XY frequencies between maternal and paternal KS groups was 37.1% ($z = 1.81$, $P = 0.04$, one-tailed) and between men <40 years and ⩾40 years was 56.6% ($z = 2.79$, $P = 0.005$). The P-value for the interaction between age categories and parent of origin was not significant ($P = 0.16$), although the numbers in some of the subcategories were small. However, when age was considered as a continuous variable, the adjusted increase in XY frequencies between parental KS groups fell to 16.5% [$z = 0.86$, P = not significant (0.20), one-tailed]. Based on the coefficient for age in this model, we calculate that there was a 3.0% increase in XY sperm with each year of age, i.e. a man who is 50 years old would have more than double the frequency of XY sperm compared with a 20 year old man [risk = exp (30 years×0.0298816), $z = 2.8$, $P < 0.01$]. There was no interaction between parent of origin and age (P = 0.41). Figure 2 displays the adjusted negative binomial regression curves for the frequencies of XY sperm by paternal age for the maternal and paternal KS groups. The frequency of XY increases with age for both paternal and maternal KS groups.

We found a non-significant 0.4% increase in disomy X and Y for fathers of the paternal KS group compared to fathers of the maternal KS group [$z = 0.03$, P = not significant (0.49), one-tailed] controlling for history of fever and coffee consumption. We also found a non-significant 14.5% increase in disomy 21 [$z = 0.75$, P = not significant (0.23), one-tailed], controlling for smoking.

Discussion

Among 36 families who had a child with Klinefelter syndrome, we found that fathers of the paternal KS group had a 50% higher frequency of XY sperm than fathers of maternal KS cases ($P = 0.02$). However, paternal age was a significant confounding factor. After adjusting for age, the difference between the two parental groups in XY frequency was substantially reduced [P = not significant ⩽0.20)]. The paternal KS group did not produce significantly higher frequency of disomies X, Y or 21.

This is the first study investigating the relationship between sperm aneuploidy and the risk of paternally transmitted KS. The strength of our study is that it comprises a relatively large number of aneuploid children of paternal origin, the use of an

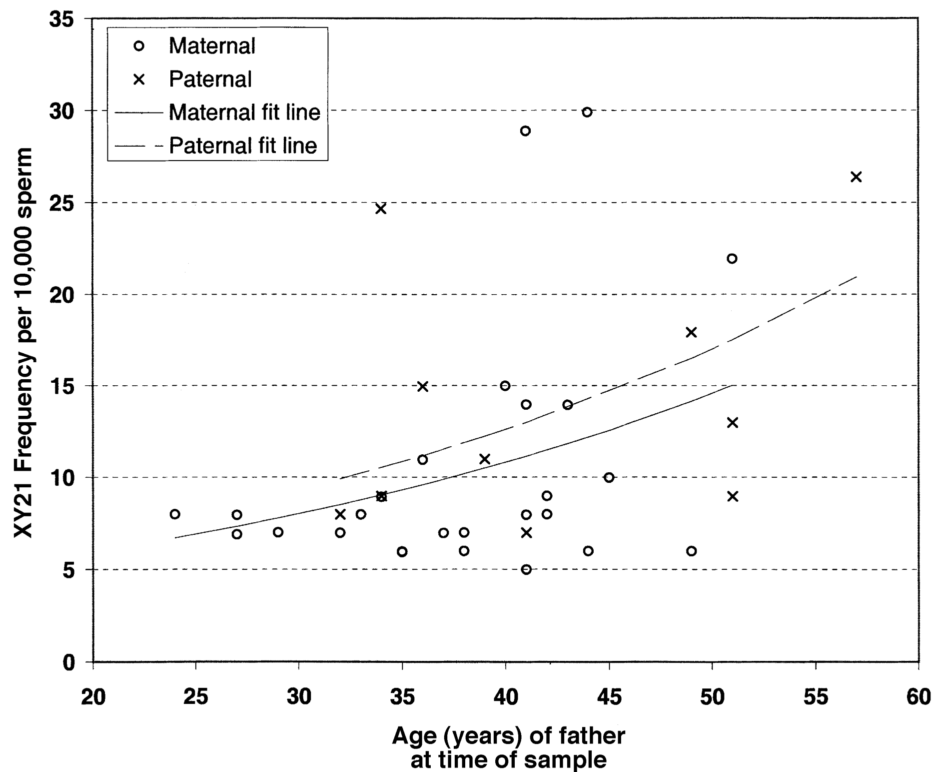

Figure 2. Negative binomial regression curves for the XY aberration frequency of XY sperm by paternal age for 26 maternal and 10 paternal Klinefelter syndrome (KS) cases. The XY sperm aneuploidy frequency for the paternal KS cases is higher than for the maternal KS cases. The frequency of XY sperm aneuploidy increases with age.

internal disease-positive reference group (maternal KS group), and detailed donor information to control for co-variates and to examine susceptible subgroups. Our findings are consistent with previous studies of sperm aneuploidy in fathers of children with Turner syndrome (Martinez-Pasarell *et al.*, 1999) and Down's syndrome (Blanco *et al.*, 1998; Hixon *et al.*, 1998). One study (Martinez-Pasarell *et al.*, 1999) reported increased XY and sex-null sperm (but not disomy X or Y) in fathers of four girls with Turner syndrome who had no paternal sex chromosome, compared with a reference group of healthy males. Blanco *et al.* reported higher rates of disomy 21 in two fathers of children with Down's syndrome of paternal origin compared with 10 fathers of children with Down's syndrome of maternal origin, and to healthy controls (Blanco *et al.*, 1998). However, compared with the general male population, another study (Hixon *et al.*, 1998) did not find a higher frequency of disomy 21 sperm in 10 men with a trisomy 21 offspring of paternal origin.

Only 10 of 36 KS cases (28%) were of paternal origin, which is lower than the 41–49% found in previous studies (Jacobs *et al.*, 1988; Harvey *et al.*, 1991; Lorda-Sanchez *et al.*, 1992; MacDonald *et al.*, 1994). Since our sample was comprised of volunteers, the observed proportion may be within the range expected due to random fluctuation. An estimate of the proportion of paternal origin in the population was ascertained by examination of consecutive cases in a birth cohort (Harvey *et al.*, 1991). Their proportion of 44% was not statistically different from ours [$\chi^2 = 2.61$, P = not significant (0.11)].

We assumed in our study design that the frequency of aneuploid sperm in a given man would remain stable over time. Two studies (Robbins *et al.*, 1995; Rubes *et al.*, 2001) have shown that certain men are stable variants over a period of ~2 years. However, little is known of the stability of aneuploidy frequencies over longer periods of time. For a few KS children in our study, there was as much as 6 years between conception and collection of the semen specimen. Although genetic factors may influence baseline levels of sperm aneuploidy, there is little evidence for this in the literature. It has been shown, however, that environmental or lifestyle factors can produce stable or transient changes. Indeed, chemotherapy and high doses of diazepam have been shown to induce transient increases in sperm (Baumgartner *et al.*, 1996; Robbins *et al.*, 1997a; Frias *et al.*, 1998). Chronic cigarette smoking and ageing may result in stable increases over time (Robbins *et al.*, 1997b; Rubes *et al.*, 1998).

We estimated a 3.0% increase in XY sperm per year of age. Some previous studies have reported an association of age and XY sperm (Griffin *et al.*, 1995; Guttenbach *et al.*, 2000), whereas others have not (Robbins *et al.*, 1995; Kinakin *et al.*, 1997); however, all previous studies had a considerably smaller sample size. Further analyses of the relationship of sperm aneuploidy and paternal age in this study are presented elsewhere (Lowe *et al.*, 2001).

The frequencies of sperm aneuploidy in our reference group (maternal KS) were within the range reported in previous investigations of normal healthy men using multicolour sperm FISH. Our maternal KS group averaged 0.27% of sperm with one of the four aneuploidies (disomy X, Y and 21; and XY), 0.20% with the sex chromosome aneuploidies alone, and

0.10% with XY alone. The frequency of sex chromosome aneuploidies is lower than the 0.43% reported for only five healthy men (Spriggs *et al.*, 1995), but considerably higher than reported for 13 controls in Belgium (Vegetti *et al.*, 2000). The frequency of XY sperm is very similar to the 0.11% observed by another group (Martinez-Pasarell *et al.*, 1999) and previously reported in our laboratory (Robbins *et al.*, 1993; Robbins *et al.*, 1995; Van Hummelen *et al.*, 1997; Baumgartner *et al.*, 1999). Slight differences in frequencies across studies may be due to different hybridization protocols, microscope scoring criteria, hybridization quality and efficiencies, and differences in other characteristics of the population such as age, smoking habits, diet, and health. A recent study in mice has shown that subtle differences in microscope scoring criteria for sperm aneuploidy are very important in understanding differences among laboratories and studies (Schmid *et al.*, 2002). Our study employed rigorous scoring criteria to minimize technical variation within our study.

Because the semen of healthy men usually contains a small baseline fraction of aneuploid sperm, it is plausible that a certain fraction of aneuploid children of paternal origin in the general population may be solely due to random fertilizations by abnormal sperm. However, our results suggest that a second and smaller fraction of fathers have inherently elevated sperm aneuploidies. Three donors (two maternal and one paternal KS fathers) were identified who had frequencies >4 SD above the cohort mean of 11.4 per 10^4 sperm. Although these donors were not unusual by their exposure or medical histories, the father of the paternal KS child reported an unusual reproductive history. All four of the pregnancies he fathered had been aneuploid, with at least two confirmed to be of paternal origin (Tomascik-Cheeseman *et al.*, 1999). Thus, our study provides a crude estimate of ~8% (i.e. three out of 36 men) for the fraction of men who produce higher rates of XY sperm. Further studies will be needed to examine the prevalence of such subgroups.

The results of this study help us to understand the reproductive consequences of aneuploid sperm. Although the proportion of XY sperm in the typical semen sample is very low, our data lend support to the hypothesis that all men in the general population are at some risk of producing an aneuploid child due to the baseline levels of aneuploid sperm they produce. Our data also suggest that some men in the population produce higher frequencies of aneuploid sperm, and consequently may be at greater risk of fathering an aneuploid offspring. Lifestyle factors and exposure to medical and environmental agents may increase the fraction of abnormal sperm. The examination of sperm aneuploidy in a population may be an efficient means for identifying the paternally mediated risk factors for trisomy.

Acknowledgements

We would like to thank Yue Jen Chuu, PhD and Angela Alme for their laboratory work, and Ed Sloter, Elana Wallenstein, and Lee Moore, PhD for their helpful comments. The research was funded by a grant from the NIEHS/EPA Superfund program P4ZES04705, and under auspices of the US DOE by LLNL under contract W-7405-ENG-48.

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Submitted on March 26, 2001; resubmitted on August 13, 2001; accepted on November 6, 2001