GENETICS

Fluorescence In Situ Hybridization with Chromosome Paint Probes: A Novel Approach to Assess Aneuploidy in Human Sperm Nuclei

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Purpose: Fluorescence in situ hybridization (FISH) using whole-chromosome paint probes was performed to evaluate disomy and diploidy frequency for chromosomes 1, 18, 19, and 22 in human sperm nuclei.

Methods: Ten subjects of proven fertility and normal spermatogenic parameters were included in the study. A dual-color FISH method was carried out.

Results: A total of 157,896 spermatozoa was scored. The mean frequencies of disomic sperm for chromosomes 1, 18, 19, and 22 were 0.22% (range, 0.19 to 0.28%), 0.24% (range, 0.14 to 0.37%), 0.22% (range, 0.17 to 0.30%), and 0.25% (range, 0.21 to 0.29%), respectively. The mean frequency of diploidy was 0.14% (range, 0.09 to 0.18%). No interindividual and interchromosomal variations in the aneuploidy frequency were observed between the different subjects.

Conclusions: FISH with whole-chromosome paint probes provides a novel and efficient approach for disomy assessment in human sperm nuclei.

KEY WORDS: aneuploidy; chromosome paint probes; fluorescence in situ hybridization; human spermatozoa.

INTRODUCTION

The analysis of spermatozoa chromosome constitution offers the possibility to determine the parental origin

of the additional chromosome in trisomy and to elucidate the mechanisms of aneuploidy. The incidence of aneuploidy in human sperm nuclei could be estimated by the cytogenetics analysis of human spermatozoa pronuclei after fusion with hamster eggs. This technique is, however, labor intensive and time-consuming, and interpretation of the karyotypes is sometimes not easy (1, 2). The advent of fluorescence *in situ* hybridization (FISH) technology in interphase nuclei offers new approaches for aneuploidy screening directly in human spermatozoa. Several studies have been performed, using one-, two-, or three-color FISH on human sperm nuclei of fertile men (3–15). Alpha-satellite centromeric probes were usually used. This type of probe has a good sensitivity, but the presence of high homology in the alpha-satellite DNA sequences of several chromosomes, (for example, chromosomes 13 and 21 and chromosomes 14 and 22) reduces their specificity (16, 17).

The primed *in situ* labeling technique provides another approach for chromosomal identification in spermatozoa and is more efficient than the FISH procedure for recognition of alpha-satellite DNA sequences. However, this procedure cannot be applied to all chromosomes because of the unavailability of specific primers and is not practicable in all laboratories.

We developed a FISH with chromosome-paint probes in human sperm interphase nuclei. Whole metaphasic and interphasic chromosomes can be directly identified in somatic cells by chromosome painting (18). This procedure has a high specificity and efficiency, and the ready availability of paint probes offers the possibility to assess the disomy rate of each chro-

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mosome individually (19). The present study describes this novel technical approach of disomy estimation in human sperm nuclei and reports the assessment of disomy rates for autosomes 1, 18, 19, and 22 in dual-color paint FISH. Disomy frequencies for chromosomes 19 and 22 have, to our knowledge and according to the recent review by Guttenbach *et al.* (20), not yet been evaluated.

MATERIALS AND METHODS

Semen Donors

The semen samples were obtained from 10 healthy and fertile males, aged between 25 and 42 years. All men gave their informed consent to participate in this study. Their spermiatic parameters were normal according to World Health Organization (WHO) criteria. The study was performed on the same ejaculate for each subject. Their constitutional karyotype was normal.

Slide Preparation

The samples were liquefied for 20 min at 37°C. A complete semen analysis was performed for each sample using WHO guidelines (1993). Then the sperm was diluted (v/v) in phosphate-buffered saline and washed three times by centrifugation for 10 min at 2000 rpm. The pellets were resuspended in fresh fixative (3:1, v/v, methanol:glacial acetic acid precooled to -20°C) and fixed for 60 min at 4°C. The spermatozoa suspensions were spread onto clean microscopic glass slides (SuperFrost/Plus, Menzel-Gläser, Germany). The slides were air-dried and stored at -20°C until use.

Decondensation of Sperm Nuclei

The slides were thawed at room temperature for 5 min and washed in 2 × SSC for 5 min. Sperm nuclear partial decondensation was performed by incubation for 5 min in 25 mM dithiothreitol (DTT) diluted in 1 M Tris-HCl, pH 9.5, under a phase-contrast microscope. The decondensation was stopped by washing in 2 × SSC, pH 7, for 5 min at room temperature, then in phosphate-buffered saline, pH 7.4, for 5 min at room temperature before dehydration in ethanol series.

DNA Probes

The probes used in this study were chromosome-specific DNA library or whole-chromosome paint

probes (STAR*FISH) purchased by Cambio Biosys (Compiègne, France). They were biotinylated for chromosomes 18 and 22 or directly labeled with fluorescein isothiocyanate (FITC) for chromosomes 1 and 19. The specificity of the different probes were verified by hybridization on human lymphocyte metaphase chromosomes. The labeled probes were diluted in a hybridization buffer (v:v) consisting of 50% deionized formamide/2 × SSC, pH 7, with 10% sulfate dextran.

Fluorescence *In Situ* Hybridization Procedure

A dual-fluorescence *in situ* hybridization was used. Two chromosomes were studied simultaneously using probes with different labels and signal colors (red or green). The slides were incubated for 2 hr at 37°C. Cytoplasmic substances were removed by incubation for 10 min at 37°C with a 100 µg/ml RNase A solution (Sigma Chemical Co., St. Louis, MO), then for 5 min at 37°C with a 0.25 µg/ml proteinase K solution (Sigma Chemical Co). Spermatozoa DNA was denatured in 70% formamide/2 × SSC, pH 7, for 3 min at 73°C and then immediately put into a 70% iced ethanol bath before dehydration. The probes were denatured for 10 min at 65°C and incubated for at least 30 min at 37°C during the preannealing stage. Aliquots of 14 µl of the hybridization mixture containing two differently labeled probes were applied to each slide under a 21 × 26-mm coverslip and sealed with cow gum. Hybridization was performed at 37°C overnight in a moist chamber. After hybridization, coverslips were carefully removed and the slides were washed twice in 60% formamide/0.05 × SSC/0.05% Tween 20 for 5 min at 42°C, twice in 2 × SSC for 5 min at 42°C, and in 4 × SSC/0.05% Tween 20 for 5 min at room temperature.

Detection

Detection of the labeling sites was performed by simultaneous incubations with avidine Texas Red/biotinylated antiavidine (Cambio Biosys) for the biotinylated probe and anti-FITC antibody (Cambio Biosys) for the FITC-labeled probe. After incubations, the slides were washed three times in 4 × SSC/0.05% Tween 20 for 5 min at 42°C. The slides were dehydrated in ethanol series and counterstained with DAPI (Cambio Biosys) (0.5 µg/ml) diluted in a mixture of glycerol and antifade reagent (Vectashield; Vector, Compiègne, France).

Scoring Criteria, Data Collection, and Statistical Analysis

The slides were examined at $\times 1000$ magnification using an epifluorescence microscope (DMRB; LEICA, Germany). An FITC/Texas Red double-band pass filter set was used to count and a DAPI single-band pass filter set to differentiate a spermatozoon from somatic cell. Images were captured at $\times 1000$ magnification with a digital imaging system (Mac Probe Version 3.3; Perceptive Scientific International Ltd. Chester, England). For each pair of chromosomes studied, a minimum of 5000 sperm nuclei was scored. Slides were accepted for scoring if the efficiency of hybridization was at least 98%.

Chi-square tests were performed to check for homogeneity in the rates of disomy for the different chromosomes in every subject and between all subjects (StatView for Windows 3.1, Abacus Concepts, Inc., Berkeley, CA, 1996).

RESULTS

Sperm nuclei with distinct and scorable signals were analyzed (Fig. 1). A minimum of 5000 sperm nuclei per chromosome per subject was scored, with a total of 157,896 spermatozoa for the different autosomes. The mean efficiency of hybridization was 99.34%, with a range from 98.77 to 99.99%. The frequency of disomy obtained for the different autosomes is represented in Table I. The mean disomy rates ranged from 0.22% for chromosomes 1 and 19 to 0.25% for chromosome 22. No significant differences were observed in the frequencies of disomy among the different autosomes in each subject ($0.07 \leq \chi^2 \leq 4.74$, $ddl = 3$). We did not find any significant differences in the

10 subjects studied ($\chi^2 = 24.057$, $df = 39$, $P = 0.97$) and for each chromosome between the 10 subjects ($1.35 \leq \chi^2 \leq 12.59$, $ddl = 9$, $P > 0.05$). The rate of diploid sperm nuclei ranged from 0.09 to 0.18%. No significant difference was observed among the 10 subjects ($\chi^2 = 4.35$, $ddl = 9$, $P > 0.05$).

DISCUSSION

Fluorescence *in situ* hybridization has been proven to be an alternative method for aneuploidy detection in human spermatozoa, to karyotypes performed by the human sperm/hamster oocyte fusion assay. This technique permits us to analyze a large number of sperm nuclei. Most of the studies published used alpha-satellite centromeric probes and specific detection for all chromosomes was not possible (17). The present study demonstrates the possibility of assessing aneuploidy in interphasic spermatozoa by chromosome painting. The availability of ready-labeled and highly purified paint probes gives a very powerful approach to obtain a specific and efficient detection of each chromosome (19).

Previous studies with alpha-satellite probes show an extreme variation in the frequency of autosomes disomy (Table II). The heterogeneity observed could reflect inter- or intraindividual and interchromosomal variability, as well as methodological variations. Some studies were performed with simple color FISH and direct estimation of the disomy rate was not possible (4,5,21–23). The hybridization efficiency and disomy estimate are both affected by the quality of the sperm nuclei decondensation (14). The structural organization of the spermatozoa DNA, with the presence of interprotamine disulfite bridges, reduces the target of

Fig. 1. Sperm nuclei hybridized with whole-chromosome paint probes specific for chromosomes 19 and 22. (A) A haploid spermatozoon (one red spot and one green spot). (B) A disomic spermatozoon for chromosome 22 showing two red spots. (C) A disomic spermatozoon for chromosome 19 showing two green spots.

Table I. Frequency of Disomy for Chromosomes 19 and 22 in Human Sperm Nuclei Estimated in Dual-Color Paint FISH

Subjects	Age (yr)	Disomy frequency (%) for chromosome				Hybridization efficiency (%)	Diploidy frequency (%)	No. of nuclei scored
		1	18	19	22			
A	28	0.20	0.23	0.18	0.27	98.77	0.09	10 933
B	27	0.21	0.37	0.17	0.22	98.84	0.09	10 323
C	37	0.24	0.37	0.18	0.26	98.88	0.18	10 756
D	27	0.22	0.25	0.22	0.24	98.32	0.12	11 061
E	28	0.19	0.22	0.29	0.22	99.16	0.12	10 484
F	35	0.21	0.23	0.20	0.21	99.52	0.17	10 526
G	25	0.24	0.14	0.20	0.23	99.98	0.17	10 416
H	42	0.28	0.14	0.19	0.27	99.97	0.16	10 965
I	32	0.26	0.25	0.25	0.27	99.98	0.15	11 059
J	26	0.19	0.16	0.30	0.29	99.99	0.14	10 509
Mean (%)		0.22	0.24	0.22	0.25	99.34	0.14	107 032

DNA hybridization (34). This particular compaction of the spermatozoa DNA presents one potential advantage, the limitation of the hybridization domain to a discrete territory in sperm nuclei, and suggested to us the use of paint probes in assessment of sperm nuclei aneuploidy. The decondensation of sperm nuclei with DTT at a high pH (35) improves the accessibility of

the probe to the target and the hybridization efficiency, and did not modify the exposure time between different samples. A short exposure time was sufficient, in our study, to effect a high hybridization efficiency, without overswelling the sperm nuclei and impairing the morphology of the spermatozoa. Only sperm nuclei with bright and nonextended spots were scored. The number and relative size of chromosome-specific domains can be accurately assessed in the majority of nuclei; all domains are not always clearly separable in these two-dimensional preparations. These domains of hybridization were excluded from the count. FISH by chromosome painting seems to be a novel way to study aneuploidy in human sperm nuclei.

The average disomy rates for autosomes 1, 18, 19, and 22 were evaluated as 0.22, 0.24, 0.22, and 0.25%, respectively. The incidence of disomy did not vary significantly among the various chromosomes analyzed. These values agree with some previous estimates by FISH (11,23,32) but differ significantly from others (4,6,30,34). However, our results are completely consistent with those reported by primed in situ labeling (25,29,36). These divergent results are more probably related to various methodological approaches than to interindividual variations (Table II). Our data support the hypothesis of equiprobable repartition of non-disjunction among chromosomes in the male gamete. Martin *et al.* (2) also observed the same result in an analysis of sperm chromosome complements performed by human sperm/hamster oocyte fusion assay. Nevertheless, the frequencies reported were lower because only a few sperm nuclei were studied, which probably underestimated these values.

The estimation of aneuploidy for chromosomes 13 and 21 and chromosomes 14 and 22 is not possible

Table II. Summary of Disomy Frequencies (%) Reported for Different Autosomes by Previous FISH Studies and by the Present Study in Simple or Dual-Color FISH and Primed In Situ Labeling

Study	Chromosome			
	1	18	19	22
Pieters <i>et al.</i> (1990) ^a [21]	0.80			
Coonen <i>et al.</i> (1991) ^a [22]	0.67			
Guttenbach <i>et al.</i> (1991) ^a [4]	0.41			
Holmes <i>et al.</i> (1993) ^a [5]	0.06			
Robbins <i>et al.</i> (1993) ^b [8]	0.14	0.08		
Bischoff <i>et al.</i> (1994) ^b [11]		0.39		
Guttenbach <i>et al.</i> (1994) ^a [23]		0.36		
Miharu <i>et al.</i> (1994) ^a [3]	0.14			
Chevret <i>et al.</i> (1995) ^b [5]	0.20			
Martin <i>et al.</i> (1995) ^b [24]	0.08			
Quennesson <i>et al.</i> (1995) ^c [25]				
Spriggset <i>et al.</i> (1995) ^b [13]	0.10	0.11		
Girardet <i>et al.</i> (1996) ^c [26]				
Martin <i>et al.</i> (1996) ^b [27]	0.11			
Pellestor <i>et al.</i> (1996) ^c [28]		0.33		
Pellestor <i>et al.</i> (1996) ^c [29]				
Spriggs <i>et al.</i> (1996) ^b [13]	0.09	0.11		
Van Hummelen (1996) ^b [31]	0.017			
Morel <i>et al.</i> (1997) ^{a, b} [32]		0.20		
Pellestor <i>et al.</i> (1996) ^c [33]		0.31		
Pellestor <i>et al.</i> (1997) [34]	0.19			
Present study	0.22	0.24	0.22	0.25

^a Simple FISH.

^b Dual-Color FISH.

^c Primed in situ labeling.

with alphoid or centromeric probes, because these chromosomes share a high homology in their alpha-satellite sequence (17). FISH with chromosome paint probes offers the possibility of studying chromosomes 13, 14, 21, and 22 individually without cross-hybridization in the FISH procedure. The disomy frequency for chromosome 19 was estimated, in our study, to be 0.22%, and the disomy frequency for chromosome 22 to be 0.25%. All chromosomes are predisposed to nondisjunction but chromosome size has been suggested to be a factor of nondisjunction (30, 37). No significant difference was observed, in our study, between the chromosomes according to their size, more precisely between the smallest chromosomes (Nos. 19 and 22) and the largest chromosomes (Nos. 1 and 18). This indicates that the susceptibility to malsegregation may be equal in human male germ cells, whereas the aneuploidy distribution varies in human oocytes according to chromosome group (38). Estimation of the disomy rate for the other chromosomes will be performed to confirm these findings. The disomy frequency for chromosome 19 (0.24%), as well as for chromosome 22 (0.25%), is reported, in the literature, for the first time. These two values agree with the average value of 0.23% observed in our data (Table I).

The diploid sperm frequency did not significantly vary among the 10 men. The mean value of 0.14% is very close to values reported by Rademaker *et al.* (39) (0.13%) and Pellestor *et al.* (29) (0.15%). We did not find major variation in the frequency of diploid spermatozoa among the 10 subjects, as reported by Williams *et al.* (9) and Blanco *et al.* (10), but the sample studied should be extended.

In conclusion, FISH by chromosome painting represents a powerful approach to screen a large number of human sperm nuclei with specific probes for each chromosome. Aneuploidy for all chromosomes will be evaluated to support the hypothesis of equal occurrence of nondisjunction in male gametes of fertile men.

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