Am. J. Hum. Genet. 46:553–558, 1990

Determination of Y Chromosome Aneuploidy in Human Sperm Nuclei by Nonradioactive in Situ Hybridization

M. Guttenbach and M. Schmid

Department of Human Genetics, University of Würzburg, Würzburg

Summary

Sperm nuclei from eight normal, healthy donors were hybridized in situ with the biotin-labeled Y-specific pHY2.1 DNA probe to evaluate the X:Y ratio, the location of the Y chromosome, and the frequency of Y aneuploidy in human sperm. The streptavidine-horseradish-peroxidase and DAB detection system used permitted the unequivocal identification of sperm heads with zero, one, or two hybridization signals and proved superior to either quinacrine staining or radioactive in situ hybridization. The low incidence of 0.27% of sperm nuclei with two Y chromosomes that was found is similar to the frequency of XYY males among newborns. The average proportions of X- and Y-bearing sperm nuclei were 50.3% and 49.4%, respectively, corresponding to the expected 1:1 ratio. The Y heterochromatin was located in the central part of the nucleus in 58% of the Y-carrying sperm cells.

Introduction

Various efforts have been made to analyze the chromosome complements in mature human spermatozoa. The discovery of a specific, bright fluorescence of the Y chromosomal heterochromatin after quinacrine staining (Caspersson et al. 1970) and its identification in interphase nuclei (Pearson et al. 1970; Tishler et al. 1974) resulted in the estimation of the Y nondisjunction rate in human sperm nuclei (e.g., see Barlow and Vosa 1970; Sumner et al. 1971; Pawlowitzki and Pearson 1972; Klasen and Schmid 1981). However, data obtained with this technique were not very reliable, because other brightly fluorescing spots could be misinterpreted as being Y chromosomal material. In 1978, Rudak et al. (1978) described a method which for the first time permitted a direct analysis of human sperm chromosomes after the sperm had penetrated hamster eggs. With this technique, human sperm could be scored for both numerical and structural aberrations (Martin et al. 1982, 1983; Brandriff et al. 1984, 1988). Another approach, developed by Joseph et al. (1984) to determine numerical aberrations of the Y and 1 chromosomes in human sperm, consisted of in situ hybridization with ^{3}H-labeled repetitive DNA probes specific for these chromosomes to sperm nuclei. This method has limits, however, concerning the hybridization of sperm from ejaculates and the classification of hybridization signals. The present study attempts to overcome these problems by in situ hybridizations with a biotinylated Y-specific DNA probe to sperm from ejaculates. The results indicate that this technique, because of its simplicity and the distinct signals it produces, is well suited for the determination of chromosomal aneuploidy in human sperm.

Received August 4, 1989; revision received October 4, 1989.

Address for correspondence and reprints: M. Schmid, Department of Human Genetics, University of Würzburg, Koellikerstrasse 2, D-8700 Würzburg, Federal Republic of Germany.

0002-9297/90/4603-0019$02.00

Material and Methods

Cytogenetics

Chromosome preparations were obtained from peripheral blood lymphocyte cultures according to the method of Pfeiffer (1974). After hypotonic treatment, the cells were fixed in a fixative (methanol:glacial acetic acid 3:1) for 1 h at −20°C. After three rinses in fixative, the cell suspensions were dropped onto clean slides and air-dried. The slides were then frozen at −20°C. Quinacrine mustard (Q)-, distamycin A/DAPI (DA/ DAPI)-, and C-banding were performed using con-

ventional techniques (Caspersson et al. 1970; Sumner 1972; Schweizer et al. 1978).

Preparation of Sperm Nuclei

Sperm ejaculates from eight normal, healthy donors aged 21–40 years were examined. The samples were incubated for 30 min at 37°C, diluted in Hank's balanced salt solution, and centrifuged for 8 min at 2,000 rpm. The pellet was fixed for 20 min in fixative at −20°C. After two rinses with fixative, the sperm suspensions were dropped onto clean slides and air-dried. The preparations were either stored for up to 14 d at 4°C or immediately frozen at −20°C for prolonged storage.

Hybridization Probe

Probe pHY2.1 (Cooke et al. 1982) was used as a Y-specific DNA probe. Under stringent conditions, pHY2.1 exclusively detects sequences in the heterochromatic region in the long arm of the Y chromosome. Biotin-11-dUTP and (^{32}P)-dCTP were incorporated into the DNA according to the oligonucleotide technique (Feinberg and Vogelstein 1983, 1984).

Southern Blot Hybridization

DNA was extracted from peripheral blood lymphocytes of normal male and female probands according to the method of Kunkel et al. (1977). After digestion with *Hae*III, the DNA fragments were separated in 0.8% agarose gels and blotted with nitrocellulose filters (Southern 1975). The filters were hybridized for about 20 h with ^{32}P-labeled, heat denatured pHY2.1 DNA (specific activity 5×10^8 to 1×10^9 cpm/μg DNA). Hybridization was performed at 68°C in 3 × SSC, 5 × Denhardt's solution, 1% SDS, 200 μg tRNA/ml. After a washing in 0.1 × SSC/0.01% SDS at 65°C, the filters were exposed to X-ray films for 1–4 h.

In Situ Hybridization

Fifty microliters hybridization mix, containing 50% formamide, 2 × SSC, 5% dextran sulfate, and 20 ng biotinylated pHY2.1 DNA probe were dropped onto the sperm preparations or mitotic chromosome preparations and were distributed with a coverslip. The preparations were denatured with the DNA probe for 10 min (sperm nuclei) or 3 min (mitotic chromosomes) in a moisture chamber at 72°C in a water bath. The moisture chamber was transferred to a 40°C water bath for hybridization. The coverslip was removed after 16–20 h, and the slides were washed for 2 × 15 min in 50% formamide/1 × SSC and for 2 × 15 min in 0.1 × SSC and then were rinsed in 1 × PBS/0.05% Tween and in 1 × PBS for 3 min each (all solutions pH 7.0). The slides were covered with streptavidine-horseradish-peroxidase complex, diluted 1:200 with dilution buffer (ENZO), and incubated for 30 min. After a rinsing with 1 × PBS/0.05% Tween, the slides were stained with DAB (0.5 mg diaminobenzidine/5 μl 30% H_2O_2/1 ml PBS). The preparations were then stained for 5–10 s (sperm nuclei) or for 3 min (mitotic chromosomes) in 5% Giemsa solution, were air-dried, and were covered with Corbite for photography. All preparations were analyzed with phase-contrast optics, which easily detects the dark-brown hybridization signals.

Results

In contrast to radioactively labeled DNA, in situ hybridization with biotinylated pHY2.1 DNA (Cooke et al. 1982) produces no disturbing background on the preparations. Since the system used to elicit the reaction was streptavidine-horseradish-peroxidase and DAB staining, the dark-brown hybridization signals could easily be distinguished from the Giemsa-blue staining of other chromatin structures. Under stringent conditions, the repetitive pHY2.1 probe exclusively binds to the heterochromatin region in the long arm of the Y chromosome (figs. 1*d*, 1*e*). The comparison with C-banded (fig. 1*f*), DA/DAPI-stained (fig. 1*g*), Q-stained (fig. 1*h*), and berenil-treated (not shown) Y chromosomes indicates that the hybridization encompasses the entire Yq12 band. Since the binding sites of pHY2.1 are not restricted to the distal end of Yq12 (Schmid et al., in press) as previously assumed (Szabo et al. 1979; Schmidtke and Schmid 1980), this pHY2.1 probe can also be used as a chromosome marker in males with small Y chromosomes. A single brown hybridization signal is recognized in diploid interphase nuclei of normal (46,XY) male probands (figs. 1*b*, 1*c*). As expected, the Southern blots of the *Hae*III-digested male DNAs always showed a clear 2.1-kb band after hybridization with pHY2.1, regardless of the length of the Yq12 heterochromatin of the various probands. As expected, in female *Hae*III-digested DNA, no hybridization of pHY2.1 was found under stringent conditions (fig. 1*a*).

Its specificity for Y chromosomes and the simple, nonradioactive in situ hybridization procedure made the pHY2.1 probe appear ideal for the evaluation of Y chromosome aneuploidy in mature human sperm. Hypotonic treatment during preparation of sperm suspensions turned out to be unnecessary, because no difference was observed in untreated cells. Similarly, no effort was

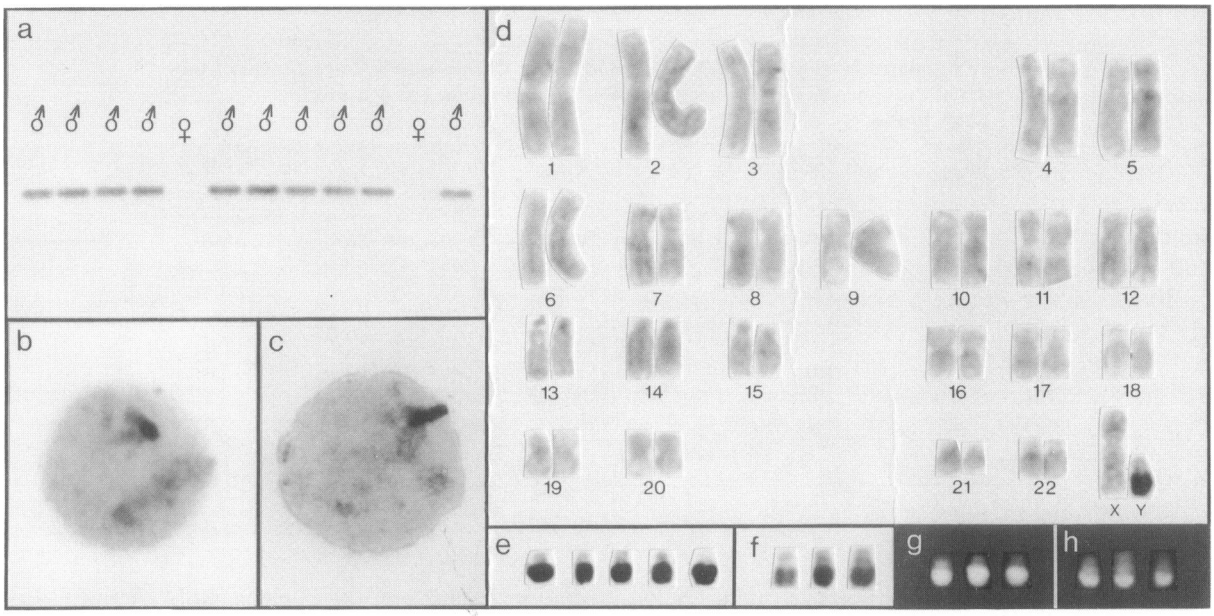

Figure 1 *a*, Southern blot hybridization of male and female *Hae*III-digested DNA with ^{32}P-labeled pHY2.1. The pHY2.1 probe only detects a 2.1-kb fragment in male DNA and does not hybridize to female DNA. *b–d*, In situ hybridization of biotinylated pHY2.1 probe to lymphocyte nuclei of a male, showing one compact hybridization signal in each nucleus (*b* and *c*) and a normal male karyotype (*d*). *e–h*, Selected Y chromosomes from the same proband demonstrating specific hybridization of the pHY2.1 DNA probe to band Yq12 (*e*), C-bands (*f*), DA/DAPI-bands (*g*), and Q bands (*h*).

made to decondense the sperm heads, because the long denaturation time prior to hybridization already had caused all sperm nuclei to swell. The presence of zero, one, or even two hybridization signals could be unequivocally identified in the sperm cells (figs. 2*a*–2*n*). A total of 8,061 mature sperm nuclei from eight normal, healthy men were analyzed. The results are shown in table 1. The mean age of the probands was 28.3 years, with a range of 21–40 years; three probands had fathered normal children. A minimum of 1,000 sperm nuclei from each proband were investigated. The proportion of YY sperm nuclei ranged between 0.1% and 0.5%

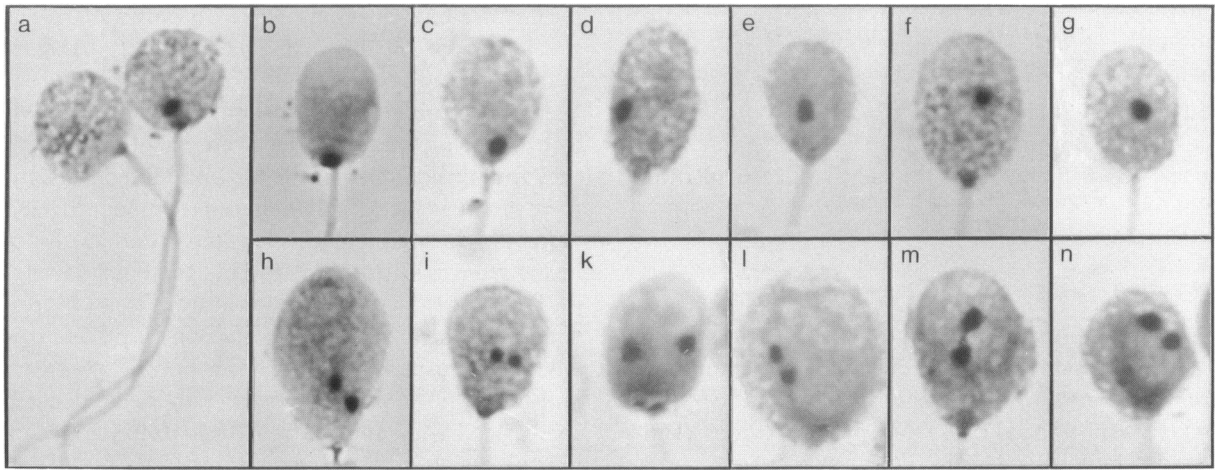

Figure 2 In situ hybridization of biotinylated pHY2.1 DNA probe to mature sperm nuclei. *a*, X- and Y-bearing sperm cells. *b–g*, Y-bearing sperm with one compact hybridization signal in each nucleus, located in the distal (*b* and *c*) or central (*d–g*) region of the nucleus. *h–n*, Sperm nuclei with two distinct hybridization signals.

Table 1

Frequency of Sperm Nuclei with 23,X, 23,Y, and 24,YY Chromosome Complements from Eight Normal Probands as determined by in Situ Hybridization Using a Biotinylated pHY2.1 DNA Probe

Proband (age in years)	No. of Sperm Nuclei Analyzed	No. (%) of 23,X Sperm Nuclei	No. (%) of 23,Y Sperm Nuclei	No. (%) of 24,YY Sperm Nuclei
1 (40)	1,010	506 (50.1)	502 (49.7)	2 (.2)
2 (32)	1,007	511 (50.7)	493 (49.0)	3 (.3)
3 (23)	1,000	503 (50.3)	495 (49.5)	2 (.2)
4 (28)	1,007	494 (49.1)	512 (50.8)	1 (.1)
5 (30)	1,007	510 (50.6)	495 (49.2)	2 (.2)
6 (30)	1,018	523 (51.4)	491 (48.2)	4 (.4)
7 (22)	1,000	503 (50.3)	492 (49.2)	5 (.5)
8 (21)	1,012	508 (50.2)	501 (49.5)	3 (.3)
Total	8,061	4,058 (50.34)	3,981 (49.39)	22 (.27)

in the different probands, the average frequency being 0.27%. The incidence of 24,YY sperm nuclei did not correlate with the age of the donors. The two hybridization signals were always clearly separated from each other, and each signal was about the size of the single signal in normal 23,Y sperm nuclei (figs. 2*h*–2*n*). The ratio of X-bearing sperm to Y-bearing sperm varied between 49.1%:50.8% and 51.4%:48.2% in the eight probands. An average of 50.34% of the sperm nuclei contained an X chromosome, and 49.39% contained a Y chromosome. This closely approximates the expected 50%:50% ratio.

The position of the Y chromosome was also determined in 500 sperm nuclei from one donor. The Y chromosome was located in the vicinity of the acrosome in 103 sperm heads (20.6%), in the central region of the nucleus in 289 sperm heads (57.8%) (figs. 2*d*–2*g*), and in the distal region near the neck of the spermatozoon in 108 sperm heads (21.6%) (figs. 2*a*–2*c*). A preference of the Y chromosome for the central region of the nucleus was also obvious in the sperm nuclei of all other probands.

Discussion

For the evaluation of the ratio of X-bearing sperm to Y-bearing sperm and the frequency of Y aneuploidy in mature sperm, in situ hybridization with the biotin-labeled, Y-specific pHY2.1 DNA probe provides a simple and reliable alternative to radioactive in situ hybridization or quinacrine staining. It is often impossible to discern Y chromosomal heterochromatin with the fluorescence Q-banding method, probably because of the differing staining properties of the Y chromosome and its location in the nucleus (Roberts and Goodall 1976). On the other hand, some other chromosome regions might fluoresce so brightly as to mimic a second Y chromosome. The aneuploidy rates determined for the Y chromosome in sperm with Q-banding are 0.18%–5% (Sumner et al. 1971; Pawlowitzki and Pearson 1972; Schwinger et al. 1976; Klasen and Schmid 1981). Although radioactive in situ hybridization with chromosome-specific DNA probes is far superior to the Q-banding technique, it is difficult to classify sperm nuclei with this method when background is high and when grains are scattered over wide areas (Joseph et al. 1984). After nonradioactive in situ hybridization, on the other hand, the dark-brown hybridization signals of the DAB staining are clearly distinguishable from the Giemsa-blue chromatin. There is only a sharp, compact signal with no distracting background, so that even two closely adjacent signals are discernible.

The main problem encountered by Joseph et al. (1984) in hybridizing sperm nuclei from ejaculates was that they found the sperm heads too condensed for signals to be properly identified. A special treatment to decondense the chromatin in turn resulted in damaged sperm heads after hybridization, forcing the investigators to perform hybridizations on sperm from testes biopsies. This phenomenon was not observed in the present study. The sperm suspensions were not hypotonically treated, and it was not necessary to specially decondense the sperm heads. The sperm swelled and decondensed during the denaturation process itself.

The average frequency of Y aneuploidy in equally decondensed sperm nuclei was 0.27% in our study. This frequency somewhat exceeds that of 1/1,000 (0.1%) for the number of XYY males found in newborns. The

excess may be due to the fact that only eight persons were examined or else may indicate that not all 24,YY sperm are capable of fertilizing an egg. Martin et al. (1983, 1987) analyzed sperm chromosome complements of normal men after in vitro fertilization of zona-free golden hamster eggs and failed to demonstrate any 24,YY condition in a total of 2,582 sperm investigated. Brandriff et al. (1984) demonstrated one 24,YY sperm complement in 909 human sperm. Another possible reason for the slightly increased frequency of 24,YY sperm nuclei reported in the present study might be that individual Y chromosomes give a two-dot signal. These signals appear to be comparatively smaller than those in other sperm nuclei and could be attributed to a splitting of the heterochromatin of a single Y chromosome (figs. 2*h*, and 2*l*).

While Martin and Rademaker (1987) found a negative correlation between donor age and the frequency of hyperhaploid sperm chromosome complements, no correlation between the frequency of YY sperm nuclei and the age of the probands was observed in the present study.

The ratio of 50.3% X-bearing sperm heads to 49.4% Y-bearing sperm very closely approximates the expected 1:1 distribution of X and Y chromosomes during meiosis. The position of the Y chromosome within the longitudinal structure of human spermatozoa studied in 500 sperm nuclei allocated it to either the acrosome region, the central region, or the distal region (close to the neck). The preferential location of the Y chromosome is the central region (58%), which confirms the findings of Barlow and Vosa (1970) and indicates that the arrangement of the Y chromosome in sperm nuclei might not be random.

In situ hybridization with nonradioactively labeled, chromosome-specific DNA probes appears to be a good alternative technique for the estimation of aneuploidy rates of specific chromosomes in human sperm. This method is especially suited for the assessment of numerical—and not of structural chromosomal—aberrations. Used for this purpose, it is much less time-consuming than either (*a*) the in vitro fertilization of golden hamster eggs with human sperm and the subsequent analysis of chromosome complements or (*b*) the radioactive in situ hybridization to sperm heads. Hybridization experiments with biotinylated DNA probes to chromosomes X and 1 are in preparation.

Acknowledgments

This study was supported by Deutsche Forschungsgemeinschaft grant Schm 484/4-1.

References

Barlow P, Vosa CG (1970) The Y chromosome in human spermatozoa. Nature 226:961–962

Brandriff B, Gordon L, Ashworth L, Watchmaker G, Carrano A, Wyrobek A (1984) Chromosomal abnormalities in human sperm: comparison among four healthy men. Hum Genet 66:193–201

Brandriff B, Gordon L, Moore D II, Carrano AV (1988) An analysis of structural aberrations in human sperm chromosomes. Cytogenet Cell Genet 47:29–36

Caspersson T, Zech L, Johannson C, Modest EJ (1970) Identification of human chromosomes by DNA-binding fluorescent agents. Chromosoma 30:215–277

Cooke HJ, Schmidtke J, Gosden JR (1982) Characterization of a human Y chromosome repeated sequence and related sequences in higher primates. Chromosoma 87:491–502

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

——— (1984) Addendum: a technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137:266–267

Joseph AM, Gosden RJ, Chandley AC (1984) Estimation of aneuploidy levels in human spermatozoa using chromosome specific probes and in situ hybridization. Hum Genet 66:234–238

Klasen M, Schmid M (1981) An improved method for Y-body identification and confirmation of a high incidence of YY sperm nuclei. Hum Genet 58:156–161

Kunkel LM, Smith KD, Boyer SH, Borgaonkar DS, Wachtel SS, Miller OJ, Breg WR, et al (1977) Analysis of human Y chromosome specific reiterated DNA in chromosome variants. Proc Natl Acad Sci USA 74:1245–1249

Martin RH, Balkan W, Burns K, Rademaker AW, Lin CC, Rudd NL (1983) The chromosome constitution of 1000 human spermatozoa. Hum Genet 63:305–309

Martin RH, Lin CC, Balkan W, Burns K (1982) Direct chromosomal analysis of human spermatozoa: preliminary results from 18 normal men. Am J Hum Genet 34:459–468

Martin RH, Rademaker AW (1987) The effect of age on the frequency of sperm chromosomal abnormalities in normal men. Am J Hum Genet 41:484–492

Martin RH, Rademaker AW, Hildebrand K, Long-Simpson L, Peterson D, Yamamoto J (1987) Variation in the frequency and type of sperm chromosomal abnormalities among men. Hum Genet 77:108–114

Pawlowitzki IH, Pearson PL (1972) Chromosomal aneuploidy in human spermatozoa. Humangenetik 16:119–122

Pearson PL, Bobrow M, Vosa CG (1970) Technique for identifying Y chromosomes in human interphase nuclei. Nature 226:78–80

Pfeiffer RA (1974) Cell cultures from blood and bone marrow. In: Schwarzacher HG, Wolf U (eds) Methods in human cytogenetics. Springer, Berlin, Heidelberg, and New York, pp 1–37

Roberts AM, Goodall H (1976) Y chromosome visibility in

quinacrine-stained human spermatozoa. Nature 262: 493–494

Rudak E, Jacobs PA, Yanagimachi R (1978) Direct analysis of the chromosome constitution of human spermatozoa. Nature 274:911–913

Schmid M, Guttenbach M, Nanda I, Studer R, Epplen JT. Organization of DYZ2 repetitive DNA on the human Y chromosome. Genomics (in press)

Schmidtke J, Schmid M (1980) Regional assignment of a 2.1-kb repetitive sequence to the distal part of the human Y chromosome. Hum Genet 55:255–257

Schweizer D, Ambros B, Anderle M (1978) Modification of DAPI banding on human chromosomes by prestaining with a DNA-binding oligopeptide antibiotic, distamycin A. Exp Cell Res 111:327–332

Schwinger E, Ites J, Korte B (1976) Studies on frequency of Y chromatin in human sperm. Hum Genet 34:265–270

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304–306

Sumner AT, Robinson JA, Evans HJ (1971) Distinguishing between X-, Y- and YY-bearing human spermatozoa by fluorescence and DNA content. Nature 229:231–233

Szabo P, Kunkel L, Yu LC, George D, Smith KK (1979) Chromosomal distribution of DNA sequences derived from the human Y chromosome in human higher primates. Cytogenet Cell Genet 25:212–213

Tishler PV, Rosner B, Lamborot-Manzur M, Atkins L (1974) Studies on the location of the Y fluorescent body in human interphase nuclei. Humangenetik 22:275–286