Different Probe Combinations for Assessment of Postzygotic Chromosomal Imbalances in Human Embryos

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Purpose: We compared three different probe combinations for detection of postzygotic mosaic imbalances in human preimplantation embryos.

Methods: Two hundred and two spare cleavage stage embryos were hybridized with fluorescently labelled DNA probe mixtures specific to chromosomes X, Y, 18 ($N = 67$), chromosomes 2, 7, 18 ($N = 71$), or chromosomes 13, 16, 18, 21, 22 ($N = 64$).

Results: An overall higher incidence of abnormalities was detected using probe mixture for five (69%) or three (72%) autosomes compared to one autosome and chromosomes X and Y (54%). The rate of aneuploidy detected increased with the number of autosomes hybridized from 4% (X, Y, 18) to 11% (2, 7, 18) to 19% (13, 16, 18, 21, 22). Postzygotic mosaicism comprised the most frequent abnormality detected by all probe combinations, and the percentage detected by each was similar, 48% (X, Y, 18), 56% (2, 7, 18), and 50% (13, 16, 18, 21, 22).

Conclusions: A probe combination of five autosomes, particularly those of clinical relevance, may be more beneficial for screening embryos from patients at risk of maternal-age-related aneuploidy. However, all three probe combinations are as efficient at identifying postzygotic mosaicism, and may be used for identifying embryos with less potential of developing to term.

KEY WORDS: Chromosome abnormalities; fluorescence in situ hybridization; mosaicism; preimplantation embryos; preimplantation genetic diagnosis.

INTRODUCTION

Multicolor fluorescence in situ hybridization (FISH) with chromosome specific DNA probes is the most common method used for analysis of chromosomes in human embryos. Application of FISH probes to one or two blastomeres biopsied from early cleavage in vitro fertilized embryos allows assessment of the embryonic chromosome status prior to its replacement to the patient. FISH has been successfully used to determine the sex of embryos from patients with

X-linked disease (1–3) and to identify specific chromosome imbalances in embryos from patients with translocations (4). This technique has also been performed to screen embryos from patients of advanced maternal age for specific aneuploidies observed in spontaneous abortions and live births (5–9).

Application of FISH to spare, nontransferred embryos from both, patients seeking assisted reproductive technology for infertility, as well as from fertile patients undergoing preimplantation genetic diagnosis (PGD) of inherited conditions has revealed that up to 50% of in vitro generated embryos are mosaic, comprising of blastomeres with varied, and often multichromosomal imbalances of postzygotic origins (5,10,11, reviewed in 12). It has been suggested that the high incidence of the observed postzygotic errors is induced by certain hormonal stimulation protocols or embryo culture conditions (13), and the resulting

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imbalances persist throughout cleavage divisions due to nonfunctional cell cycle checkpoint during preimplantation development (11,14,15). The rarity, or absence of these type of abnormalities in spontaneous abortions and live births (reviewed in 16), provides evidence that majority of the affected embryos do not survive to time of recognition of pregnancy.

Detection of postzygotic imbalances during current preimplantation diagnosis of inherited conditions and maternal-age-related aneuploidy may be providing the means of identifying those embryos destined for wastage and of increasing the likelihood of replacing embryos with the highest potential of developing to term. The purpose of this study was to consider the probe combinations most suitable for negative selection of embryos carrying different forms of postzygotic imbalances. To address this question, we compared the frequency and type of abnormalities detected by three different probe mixtures, specific to five autosomes (13, 16, 18, 21, 22), three autosomes (2, 7, 18), and one autosome plus sex chromosomes (X, Y, 18).

MATERIALS AND METHODS

Embryo Source

Surplus cleavage—stage normally fertilized (2PN) embryos were donated by 91 patients undergoing in vitro fertilization (IVF) at the McGill Reproductive Center. All patients gave written consent for the embryos to be used for research purposes. The mean maternal age of patients was 36 years (range 22–45 years). Embryos were considered arrested when no cleavage had occurred during the 24 h prior to embryo spreading. Alternatively, when the extent of cleavage previous to time of spreading was not known, embryos were classified as arrested when they consisted of ≤ 5 cells on the evening of Day 3 postfertilization, ≤ 8 cells on Day 4, ≤ 12 cells on Day 5. Embryos with > 5 cells on Day 3, > 8 cells on Day 4, and > 12 cells on Day 5 were considered as developing.

Embryos were subjected to one of three different FISH protocols, simultaneous hybridization of a 3-color probe combination specific to chromosomes X, Y, 18 ($N = 67$), simultaneous hybridization of a 3-color probe combination specific for chromosomes 2, 7, 18 ($N = 71$), or a simultaneous hybridization of a 5-color probe combination specific to chromosomes 13, 16, 18, 21, and 22 ($N = 64$). The distribution of developmental stages of the embryos, the proportion of

Table I. Distribution of Developmental Stages, Embryonic Arrest, and Maternal Age Among Embryos in Three Probe Categories

	X, Y, 18 ($N = 67$)	2, 7, 18 ($N = 71$)	13, 16, 18, 21, 22 ($N = 64$)
Developmental stages			
2–4 cells	23 (34.3)	23 (32.4)	21 (32.8)
5–8 cells	30 (44.8)	31 (43.7)	28 (43.7)
Morula	14 (20.9)	17 (23.9)	15 (23.4)
Developing	34 (50.7)	34 (47.9)	33 (51.6)
Arrested	33 (49.2)	37 (52.1)	31 (48.4)
Mean maternal age (years)	35.4	35.8	36.6

Note. Values in parentheses represent percentages.

arrested embryos, and the mean maternal age among the embryos in three probe categories is indicated in Table I.

FISH Procedure

All probes were commercially available from VYSIS (Downers Grove, IL, U.S.A.). Probes specific to chromosomes X (CEP X, alpha satellite in spectrum green), Y (CEP Y, alpha satellite DYZ3, spectrum orange), and 18 (CEP 18, alpha satellite, spectrum aqua) were obtained separately and combined in the ratio of 1:1:1 in 50% formamide, $2 \times$ saline sodium citrate (SSC) hybridization buffer (HB) (Oncor, Gaithersburg, MD, U.S.A.) to form a 3-color X, Y, 18 probe mixture. Probes specific to chromosomes 2 (CEP 2, alpha satellite, spectrum orange), 7 (CEP 7, alpha satellite, spectrum green), and 18 (CEP 18, alpha satellite, spectrum aqua) were obtained separately and combined in the ratio of 2:1:1 in HB to form a 3-color 2, 7, 18 probe mixture. Probes for chromosomes 13 (LSI 13 region 13q14, spectrum red), 16 (CEP 16, satellite II, D16Z3, spectrum aqua), 18 (CEP 18, alpha satellite, spectrum aqua), 21 (LSI 21, region 21q22.13–21q22.2, spectrum green), and 22 (LSI 22, region 22q.11.2, spectrum gold) were obtained as a 5-color probe mixture from the supplier.

Embryos were washed in phosphate buffered saline (PBS) and transferred onto poly-l-lysine coated slides. Embryos were spread and fixed on glass slides using 0.01 N HCl, 0.1% Tween 20 solution (17). The preparations were air-dried, washed in PBS for 5 min and dehydrated in 50, 70, and 100% ethanol. Embryonic nuclei were pretreated with pepsin (0.1 mg/mL in 0.01N HCl) for 10 min at 37°C, and again dehydrated. The target embryonic DNA was codenatured with the 3-colour or the 5-colour probe mixtures by heating

the slide to 74°C–78°C, for 4–6 min. Simultaneous hybridization was carried out in a moist chamber at 37°C, for 5–18 h. The unbound probe was washed off using $0.4 \times \text{SSC}/0.3\%$ Tween 20, or $0.7 \times \text{SSC}/0.1\%$ Tween 20 solution at 73°C, for 2–6 min, followed by rinsing in $2 \times \text{SSC}/0.1\%$ Tween 20 at room temperature. Preparations were mounted in antifade solution (*p*-phenylenediamide dihydrochloride in PBS, Vector) or in antifade with DAPI nuclear stain (Sigma, 0.25–0.5 ng/mL).

Analysis of FISH Results

Nuclei were viewed using a fluorescence Olympus DX 60 microscope equipped with appropriate filters. Images were captured using a CCD camera and Cytovision software (Applied Imaging, Santa Clara, U.S.A.).

Signals were scored as described in Bielanska *et al.* (18). Only those embryos yielding results in 75% of cells or more were included in the study. Chi-square test (χ^2) was used to compare statistically the chromosome patterns in embryos subjected to three different probe combinations.

RESULTS

The developmental stages, the frequency of arrest, and the mean maternal age of embryos subjected to probe combination X, Y, 18, combination 2, 7, 18, and combination 13, 16, 18, 21, 22 were not statistically different (Table I). Chromosome patterns detected in embryos in the three different probe categories are presented in Table II. An overall higher incidence of abnormalities was detected using probe mixture for five (69%) or three (72%) autosomes compared to one autosome and chromosomes X and Y (54%). A noticeable difference between the three probe mixtures was observed in the rate of aneuploidy (uniform monosomy or trisomy), which increased with the number of autosomes hybridized from 4% (X, Y, 18) to 11% (2, 7, 18) to 19% (13, 16, 18, 21, 22). Uniform haploidy or polyploidy were rare among all three probe categories.

Mosaicism (blastomeres with varied chromosome patterns) comprised the most frequent abnormality detected in the embryos and the incidence found with each probe combination was similar, 48% (X, Y, 18), 56% (2, 7, 18), and 50% (13, 16, 18, 21, 22). Mosaic embryos were classified based on the predominant type of abnormal chromosome complement detected

Table II. Chromosome Patterns in 202 Human Preimplantation Embryos as Detected by Three Different Probe Combinations

Chromosome pattern	X, Y, 18	2, 7, 18	13, 16, 18, 21, 22
Normal (2N)	31 (46.3) ^a	20 (28.2) ^a	20 (31.2)
Abnormal	36 (53.7) ^a	51 (71.8) ^a	44 (68.7)
Aneuploid	3 (4.5) ^{b,c}	8 (11.3) ^d	12 (18.7) ^{b,a,e}
Haploid	0 (0.0)	1 (1.4)	0 (0.0)
Polyploid	1 (1.5)	2 (2.8)	0 (0.0)
Mosaic	32 (47.8)	40 (56.3)	32 (50.0)
Chaotic	22 (32.8)	23 (32.4)	21 (32.8)
2N-aneuploid	6 (8.9)	11 (15.5)	7 (10.9)
2N-haploid	1 (1.5)	3 (4.2)	3 (4.7)
2N-polyploid	3 (4.5)	3 (4.2)	1 (1.6)

Note. Values in parentheses represent percentages. 2N: diploid for chromosomes tested. No. of embryos analyzed: 67 for X, Y, 18; 71 for 2, 7, 18; 64 for 13, 16, 18, 21, 22. No. of nuclei analyzed: 455 for X, Y, 18; 520 for 2, 7, 18; 485 for 13, 16, 18, 21, 22.

^a Values with this superscript in the same row are statistically different (χ^2 test, $P < 0.05$).

^b Values with this superscript in the same row are statistically different (χ^2 test, $P = 0.01$).

^c The aneuploidies detected were XXY, XXX, and XO.

^d Aneuploidies detected included two Ts 2, two Ts 7, three Ts 18, and a double aneuploidy of Ts 7 plus Mn 18.

^e Aneuploidies detected included trisomies 13, 21, 22, two Ts 16, two Mn 13, Mn 16, two Mn 22, double aneuploidy of Ts 13 plus Mn 21, triple aneuploidy of Ts 22 plus Mn 16 and Mn 13.

(Table II). The largest proportion of mosaic embryos in each of the three probe categories (~33%) showed blastomeres with chaotic chromosome patterns (imbalances for various, and often each of the chromosomes tested). The chaotic imbalances usually affected most, or all cells per embryo. This was followed by diploid (2N)-aneuploid mosaicism (a combination of diploid blastomeres and blastomeres with gain or loss for one of the chromosomes tested). The incidence of mosaic embryos with 2N and haploid or 2N and polyploid cells was low, and was similar among all three probe categories. Individual frequencies of 2N-aneuploid and/or polyploid, and/or haploid mosaicism are presented in Table II.

DISCUSSION

Till date, the various FISH probe combinations used for PGD in human embryos have been selected based on their relevance to particular inherited conditions, and for prevention of aneuploidies with severe clinical consequences. In this study we addressed probe combinations for a single hybridization detection of postzygotic mosaic imbalances.

Comparison of the results from FISH with probe combinations specific to chromosomes X, Y, 18,

chromosomes 2, 7, 18, and chromosomes 13, 16, 18, 21, 22 revealed that the detection of postzygotic chromosome gains and losses is not dependent on the number, nor the specificity of probes used. The similar frequency of mosaicism ascertained by testing for three or for five chromosomes is largely attributed to the large proportion of chaotic embryos, with imbalances for multiple, and often each of the chromosomes tested. The lack of disparity in rate of mosaic imbalances detected using probe for chromosomes X, Y, 18 versus chromosomes 2, 7, 18 could be explained by the mechanism of errors during cleavage divisions. Unlike errors from maternal meiosis, cleavage stage errors may not be dictated by chromosomal differences in length, recombination patterns, or gene density (reviewed in 19), but may result from general defects of the spindle (20).

Similar rates of mosaic imbalances detected by probe mixtures targeting chromosomes X, Y, 18, chromosomes 2, 7, 18, and chromosomes 13, 16, 18, 21, 22 suggest that screening for postzygotic errors could be performed using any of the above chromosome combinations. Negative selection of mosaic embryos in patients undergoing PGD for X-linked disease and translocations may be achieved by use of a single three probe mixture containing sex or break-point specific probes, without the additional time requirement and technical errors associated with rehybridization for a greater number of chromosomes (21,22). If the lack of differences in rate of postzygotic errors among chromosome combinations used in this study also applies to other chromosomes, as suggested by Wells *et al.* (23), the third chromosome incorporated into the combination used for PGD of inherited conditions could be any of the 21 remaining chromosomes. Certain probes, such as for chromosomes 2 and 7 provide the benefit of identifying trisomies commonly observed in cases of confined placental mosaicism (24). Other chromosome probes addressed in this study (chromosomes 13, 16, 18, 21, and 22) are effective for detecting maternal-age-related aneuploidies (5,8,25,26) and would therefore be more appropriate for detecting mosaicism in older patients. Where maternal age is not a factor, the choice of the probe may be dictated by the cost, the availability, and performance of the particular probe at the specific laboratory performing PGD.

Patients undergoing IVF at an advanced maternal age show high rates of aneuploidy in their embryos (27–31). The increase in the rate of uniform aneuploidies with the number of chromosomes targeted in this study clearly demonstrates that a

5-chromosome combination is the method of choice for screening of embryos from this population of patients. In clinically recognized pregnancies aneuploidy for sex chromosomes is much more common than aneuploidy for other chromosomes (reviewed in 16). The higher rate of aneuploidy obtained in this study by testing for chromosomes 2 and 7 compared to the sex chromosomes, confirms that distribution of aneuploidy in the embryo differs from that observed in clinically recognized pregnancies. Furthermore, higher rate of aneuploidy for chromosomes 2 and 7 compared to XY, suggests that the aneuploidies for these, and other chromosomes observed in preimplantation embryos, but rarely in clinical pregnancies (31,32), are eliminated at very early stages of development, possibly prior to, or at implantation. Because the increase in aneuploidy with advanced maternal age is not restricted to those chromosomes involved in trisomic offspring (31), negative selection of abnormal embryos by testing for any five chromosomes may decrease implantation failure in older patients. However, as shown by Munne *et al.* (8), the use of chromosomes in the 5-color combination in this study (chromosomes 13, 16, 18, 21, 22), would help avoid more severe clinical consequences such as spontaneous abortion or trisomic offspring with multiple systemic defects, malformations and/or mental retardation.

The number of chromosomes screened per embryo may be extended from 5 to 11 by subjecting the embryo to further rehybridization with different probes (7,21,22,29,31,33). Detection of all 23 chromosomes in blastomeres may also be using comparative genomic hybridization (CGH) (23,34). The increase in the rate of aneuploidy with number of chromosomes hybridized in this study suggests that rehybridization or CGH technology would increase the overall rate of abnormalities, and therefore benefit patients undergoing PGD for reasons of advanced maternal age. However, both techniques have drawbacks for clinical use. Analysis by CGH requires 3–4 days (23,34). Rehybridization also extends the time required for diagnosis, leads to loss of nuclei, as well as a lowers hybridization efficiency which may result in overestimation of monosomies or underestimation of trisomies detected by the second or third probe combination (21,22,29). Further to these technical disadvantages, our finding that the detection of postzygotic imbalances is not dependent on the number of chromosomes tested suggests that detection of higher number of chromosomes would not increase the number of mosaic embryos identified. Our study indicates the

current use of single-hybridization multicolor FISH for PGD of inherited disease or aneuploidy already provides the optimal method for the detection of mosaic embryos with postzygotic imbalances. The degree to which elimination of mosaic embryos from cohorts being transferred benefits pregnancy outcome remains to be investigated.

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