Preimplantation Genetic Diagnosis of Aneuploidy: Were We Looking at the Wrong Chromosomes?

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Purpose: Our purpose was to study aneuploidy frequencies of chromosomes 1, 4, 6, 7, 14, 15, 17, 18, and 22 in cleavage-stage embryos. These frequencies were compared to spontaneous abortion data to determine differences in survival rate of their aneuploidies.

Methods: One hundred ninety-four embryos were analyzed with multicolor fluorescence in situ hybridization. Embryos were divided into three maternal age groups: 20 to 34.9 years, (2) 35 to 39.9 years, and (3) 40 years and older. Embryos were also divided into two developmental and morphological groups: arrested and nonarrested embryos.

Results: The rate of aneuploidy was 14.51%, 14.10%, and 31.48% for age groups 1, 2, and 3, respectively ($P < 0.005$). The chromosomes most frequently involved in aneuploidy events were 22, 15, 1, and 17.

Conclusions: The chromosomes most involved in spontaneous abortions are not necessarily the ones causing a decrease in implantation rates with maternal age. Other aneuploidies, such as for chromosomes 1 and 17, may seldom implant or die shortly after implantation.

KEY WORDS: FISH; monosomy; preimplantation genetic diagnosis; trisomy 1; trisomy 17.

INTRODUCTION

The causes of the decline in implantation observed with increasing maternal age are still being debated, but to date the only clear link between maternal age and embryo competence is aneuploidy. The increase in aneuploidy with maternal age in spontaneous abortions

and live offspring (1) was also found in both cleavage-stage embryos (2) and unfertilized oocytes (3). The rate of chromosomal abnormalities in embryos was higher than that reported for spontaneous abortion, suggesting that a sizable portion of chromosomally abnormal embryos is eliminated before prenatal diagnosis is performed. This embryo loss could account for the decline in implantation with maternal age. Because of the correlation between aneuploidy and declining implantation rates with maternal age, it was hypothesized that negative selection of chromosomally abnormal embryos could help reverse this trend (4). Currently, negative selection of aneuploid embryos can be achieved only through preimplantation genetic diagnosis (PGD), by either polar body or blastomere analysis, using fluorescence in situ hybridization (FISH). FISH has been used for PGD of X-linked disease (5–7) and common aneuploidies (X, Y, 13, 18, 21) (4, 8–12).

Most studies have been limited to analyzing the chromosomes involved in trisomies at risk of arriving to term (X, Y, 13, 18, 21) or involved in spontaneous abortions (Nos. 15, 16, 22). It is possible that the selection against aneuploid embryos does not work equally for each aneuploidy, and some trisomies survive less often than others to implantation and/or to the first trimester. If so, the chromosomes involved in implantation failure may be different from those involved in spontaneous abortions and trisomic offspring. To study this hypothesis, chromosomes 1, 4, 6, 7, 14, 15, 17, 18, and 22 were studied simultaneously. These chromosomes were chosen because, to our knowledge, there is only one previous report on chromosomes 14, 15, and 22 (9), scant data on chromosomes 1, 7, and 17 (13–18), and no data for chromosomes 4 and 6. We used chromosome 18 as an internal control to be able to compare the present data to previous data. Similarly, because the simultaneous study of nine chromosomes involved cell recycling, chromo-

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some 18 was added for both rounds of hybridization as an internal control of the efficiency of the second rehybridization.

MATERIALS AND METHODS

Classification of Embryos

Embryos were donated by patients undergoing in vitro fertilization (IVF) at the Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center, and in accordance with guidelines approved by the internal review board of Saint Barnabas Medical Center, including written consent from the patients in each case. Embryos donated for research were disaggregated or biopsied on day 3 of development, and their blastomeres were fixed individually following the protocol described by Munné *et al.* (19).

The embryos were divided into three groups according to maternal age: (1) between 20 and 34.9 years, (2) between 35 and 39.9 years, and (3) 40 years and older (Table I). The embryos were also divided into two groups according to developmental and morphological characteristics: arrested and nonarrested embryos. Arrested embryos were those that had not cleaved during the preceding 24-hr period. This classification was required because previous FISH studies found lower rates of aneuploidy in arrested embryos than nonarrested ones (2), which could bias the analysis of maternal age differences. There were no statistical differences in the frequency of arrested versus nonarrested embryos in these three maternal age groups, so comparison between these groups was valid (Table I).

FISH Procedure

Fixed cells were analyzed by two rounds of FISH; the first hybridization was performed with probes for chromosomes 4, 7, 14, 17, 18, and 22 and the second

round of hybridization used 1, 6, 14, 17, and 18 chromosome-specific probes according to the protocol described by Munné *et al.* (9). The first hybridization involved probes for chromosomes 4 (alpha satellite; Vysis Inc., Downers Grove, IL), 7 (alpha satellite; Vysis), 15 (alpha satellite; Vysis), and 18 (alpha satellite; D18Z1, Vysis). These probes were labeled with Spectrum-Orange, Spectrum-Green, and/or Spectrum-Aqua (Vysis). They were combined in the following quantities: 7.2 μ l Spectrum-Orange CEP 4, 4 μ l Spectrum-Green CEP 4, 18 μ l Spectrum-Aqua CEP 7, 4 μ l Spectrum-Orange CEP 7, 10 μ l Spectrum-Aqua CEP 18, and 16 μ l Spectrum-Green LSI 22 (200 ng/ μ l). The above probe mixture was concentrated with a Speed-Vac centrifugal evaporator (DyNA VAP; National Labnet Company) to a final volume of 40 μ l and added to 93.2 μ l of WCP hybridization buffer (Vysis). Ten microliters of the resulting hybridization solution was applied to a glass slide containing fixed blastomeres, and covered with an 18 $\times$ 18-mm coverslip. The slide was then placed for 5 min on a slide warmer preheated to 73°C, sealed with rubber cement, and placed in a dark moist chamber at 37°C for at least 2 hr. After the hybridization, the slides were washed individually at 71°C in 0.4 $\times$ standard saline citrate (SSC) for 2 min. The slides were then mounted with 10 μ l of DAPI (4',6-diamino-2-phenyl indole; Vysis) counterstain in antifade solution and observed with a fluorescent microscope (Olympus BX60) equipped with a triple-band pass filter set for simultaneous observation of the Spectrum-Orange, Spectrum-Green, and Spectrum-Aqua fluorescence. When viewed through the triple-band pass filter set, the domain colors were yellow for the 4 chromosome 4-specific signal, magenta for the 7, red for the 15, blue for the 18, and green for the 22. The chromosome 18-probe was used as an internal control to check the reliability of reanalysis. A DAPI filter (Olympus) was applied to find the nuclei.

After the analysis of the first set of probes, the slides were washed in 1 $\times$ PBD (Oncor, Gaithersburg, MD) until the coverslips fell off, then dehydrated (70%, 85%, and 100% ethanol, 2 min each). Ten microliters of the second hybridization solution was applied per slide. This second hybridization solution was prepared by mixing 28 μ l of a probe for chromosome 1 (alpha satellite region) labeled with Spectrum-Aqua, 10 μ l of a probe for chromosome 6 (alpha satellite region) labeled with Spectrum-Green, 8 μ l of the same probe for chromosome 6 labeled with Spectrum-Orange, 8 μ l of a probe for chromosome 14 (14q 11.2 region),

Table I. Classification of Embryos According to Age Group

Maternal age group (years)	No. arrested embryos (%)	No. nonarrested embryos (%)
(1) 20.00–34.99	9 (14.51)	53 (85.48)
(2) 35.00–39.99	15 (19.23)	63 (80.76)
(3) $\geq$ 40.00	8 (14.81)	46 (85.18)

8 μ l of the same probe for chromosome 17 labeled with Spectrum-Orange, 8 μ l of the same probe for chromosome 17 labeled with Spectrum-Green, 10 μ l of a probe for chromosome 18 (alpha satellite region) labeled with Spectrum-Aqua, and 10 μ l of the same probe for chromosome 18 labeled with Spectrum-Orange. This mixture was concentrated to 24 μ l with a Speed-Vac centrifugal evaporator (DyNA VAP) and mixed with 56 μ l of WCP hybridization buffer (Vysis). Slides and probes were simultaneously denatured on a hot plate at 73°C for 5 min, followed by overnight hybridization at 37°C, and finally, washed for 60 sec in 0.4 $\times$ SSC at 71°C. The washed slides were then mounted with DAPI in antifade (Oncor) and analyzed.

The scoring criteria to differentiate false positives and negatives from mosaicism were described previously (10). Similarly, the criteria to classify by FISH the different chromosomal abnormalities previously described by Munné *et al.* (8) were followed, but with one exception: mosaics with less than 25% abnormal cells were considered normal, since it was difficult to differentiate them from FISH errors in embryos with six or fewer cells.

The χ^2 and F tests were used to compare statistically the chromosomal abnormalities among the three maternal age groups.

RESULTS

A total of 194 embryos from 60 patients were analyzed and FISH results were obtained. Of those, 872 blastomeres had nuclei and provided FISH results, 150 had no nuclei, and 35 were lost during biopsy or fixation, with an average of 5.44 blastomeres per embryo. The chromosome 18 probe was used as an internal control to demonstrate that a second round of hybridization provided reliable results (Fig. 1). As such, 851 (97.59%) blastomeres of 872 had the same number of 18 chromosomes in both hybridization procedures. In the other cases, 14 showed a missing chromosome 18 signal and 7 an extra signal after the second hybridization.

The average age of the patients was 37.13 years. The percentage of normal embryos for the chromosomes analyzed in this study was 27.4%, 42.3%, and 27.8% for age groups 1, 2, and 3, respectively. These differences were not statistically significant. The rate of aneuploidy, however, was 14.51%, 14.10%, and 31.48% for age groups 1, 2, and 3, respectively. The aneuploidy rates of the first age group versus the third age group, and the second age group versus the third age group, were significant ($P < 0.05$ and $P < 0.025$,

Fig. 1. Top: In situ hybridization with DNA probes specific for chromosomes 1, 6, 14, 17, and 18. The chromosome 1-specific probe is labeled in blue, chromosome 6 in yellow, chromosome 14 in green, chromosome 17 in red, and chromosome 18 in magenta. The cell is from an embryo with trisomy 1 (1,1,1, 6,6, 14,14, 17,17, 18,18). Bottom: In situ hybridization with DNA probes specific for chromosomes 4, 7, 15, 18, and 22. The chromosome 4-specific probe is labeled in yellow, chromosome 7 in magenta, chromosome 15 in orange, chromosome 18 in blue, and chromosome 22 in green. The cell is from an embryo with trisomy 22 and monosomy 15 (4,4, 7,7, 15,0, 18,18, 22,22,22).

respectively). The aneuploidy rate of the first and second age groups (20 to 39.9 years old) versus the third group was also significant ($P < 0.005$). The other differences among the three maternal age groups were observed in their respective frequencies of polyploidy, haploidy, or mosaicism. Table II presents the incidence of numerical chromosome abnormalities for the chromosomes studied according to maternal age.

Regardless of maternal age, the embryos were also classified according to developmental potential into

Table II. Numerical Chromosomal Abnormalities

Chromosomal abnormality	Maternal age group (years)*		
	20–34.9	35–39.9	≥40
No. of embryos analyzed	62	78	54
Normal	17 (27.4%)	33 (42.3%)	15 (27.8%)
Aneuploid	9 (14.5%) ^a	11 (14.1%) ^b	17 (31.5%) ^c
Haploid (mosaic or not)	1 (1.6%)	4 (5.1%)	2 (3.7%)
Polyploid (mosaic or not)	15 (24.2%)	10 (12.8%)	8 (14.8%)
Extensive 2N mosaicism	20 (32.2%)	20 (25.6%)	12 (22.2%)

* a vs c, $P < 0.05$; a vs b, $P < 0.025$; (a + b) vs c, $P < 0.005$.

two groups, arrested and not arrested. Only 32 embryos were arrested, of which 10 (31%) were mosaics, 9 (28%) were polyploid, 8 (25%) were aneuploid, 4 (13%) were normal, and 1 (3%) was haploid. The other embryos ($n = 162$) were not arrested. Of those 51 (31%) were mosaics, 50 (31%) were normal, 31 (19%) were aneuploid, 24 (15%) were polyploid, and 6 (4%) were haploid. Although there was a tendency for arrested embryos to have more chromosome abnormalities, in particular polyploidy, the differences were statistically not significant.

There were 37 aneuploid embryos detected. However, of those, seven were double aneuploidies, two were triple aneuploidies, and one was a quadruple aneuploidy. When the aneuploidy rate per chromosome was based on the number of aneuploidy events, instead of aneuploid embryos, it was found that chromosome 22 was the most commonly involved in aneuploidy (12 events; 6.2%), followed by chromosomes 1 and 15 (8 events each; 4.1%) and chromosome 17 (6 events; 12.2%). The other chromosomes were less involved in aneuploidy, with 2.1% for chromosome 4 (four events), 1.5% for chromosomes 6 and 7 (three events each), and 1% for chromosomes 14 and 18 (two events each).

DISCUSSION

In the present study we have used cell recycling to obtain information about the status of nine chromosomes. FISH–FISH Cell recycling has been used previously (9, 20) but no internal controls regarding its efficiency were included. By using a probe for chromosome 18 in both rounds of hybridization, we were able to determine that the rehybridization error rate was very low (2.4%); it was attributed either to DNA loss during the second hybridization or scoring discrepancies in borderline samples.

Chromosome 18 was also used to compare the present group of embryos to previous studies in which the

chromosome 18 probe was used. The largest previous study (2) showed a 4.6% (24/524) aneuploidy rate for chromosome 18, which is significantly higher ($P < 0.05$) than that in the present study (1%, 2/194). There could be several differences between both studies because, although they were performed by the same group of embryologists, they took place at different centers. In the study by Munné *et al.* (2), the number of embryos from patients 40 years of age and older was higher (34%) than in the present study (28%). Another difference could be the differing stimulation protocols between the centers. As reported previously, different stimulation protocols may produce different rates of chromosome abnormalities (21).

As the study by Munné *et al.* (2), the present study indicates an increase in aneuploidy with maternal age. However, the present data indicate that this increase is not restricted to those chromosomes closely associated with trisomic offspring (Nos. 13, 18, 21) and spontaneous abortions (Nos. 15, 16, 22), but also occurs in others, seldom, if ever, observed in clinically recognized pregnancies, such as chromosomes 1 and 17. To the best of our knowledge, only one other large series of embryos has been analyzed for chromosome 1 (17, 18), which found an incidence of only 2% (3/157). The differences between that study and ours could be related to maternal age differences between the two patient groups, as well as embryo quality, as low-quality embryos are usually chaotic or polyploid mosaics in which aneuploidy is difficult to detect (2).

If the incidence of aneuploidy in cleavage-stage embryos for the chromosomes studied here is compared to the incidence in spontaneous abortions, there seem to be different degrees of selection against aneuploid embryos, depending on the type of chromosome aneuploidy that they carry. For instance, aneuploidy for chromosome 22 has an 11-fold reduction in incidence, from 24.5% at the cleavage stage (this study) to 2.26% in spontaneous abortions (22). However, chromosome 17 shows a 68-fold reduction, from 12.2% at the cleav-

age stage (this study) to 0.18% in spontaneous abortions (22). In addition, trisomy 1 is never detected in spontaneous abortions, while in our study, it occurred in cleavage-stage embryos at a frequency of 16%. The present finding is in contradiction with previous studies, in which karyotype analysis of one or few cells was used to compare aneuploidy rates in cleavage-stage embryos and spontaneous abortions (23). Perhaps their results were biased by the analysis of too few cells, as Almeida and Bolton (24) demonstrated, in which an underestimation of mosaicism and an overestimation of aneuploidy occurred when only one cell per embryo was analyzed.

The results of the present study do have implications for PGD of aneuploidy. We have hypothesized that selecting embryos based on their chromosomal content will help increase implantation rates in women of advanced maternal age (4). However, most centers applying PGD of aneuploidy, including ours, have concentrated on the chromosomes involved in producing live trisomic offspring (Nos. 6, 10, 11, 12). Recently probes for chromosomes involved in spontaneous abortions (Nos. 14, 15, 16, 22) have been included (8, 9). However, the present results indicate that other chromosomes are also involved in the decrease in implantation with maternal age, such as chromosomes 1 and 17. Based on our present knowledge, for PGD of aneuploidy using current FISH technology, the chromosomes analyzed should be X, Y, 13, 18, and 21, to eliminate the risk of trisomic offspring, with chromosomes 1, 15, 16, 17, and 22 analyzed to reduce spontaneous abortions and implantation failure. Other chromosomes, for which there are no data at the embryo level, may also be important.

Ideally, all chromosomes would be analyzed for the best chance of eliminating the decrease in implantation rates with maternal age. FISH with only three fluorochromes, even using cell recycling, seems to be at an upper limit for the numbers of chromosomes per cell than can be analyzed. Ultimately, other techniques such as spectral imaging could be used to obtain full karyotypes of single blastomeres or polar bodies in a rapid, reliable and efficient way. So far, full karyotypes of polar bodies have been achieved using spectral imaging (25), paving the way for a more complete selection process of gametes and embryos in the near-future.

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