GENETICS

Preimplantation Diagnosis by Fluorescence In Situ Hybridization Using 13-, 16-, 18-, 21-, 22-, X-, and Y-Chromosome Probes

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Submitted: June 24, 1998

Accepted: September 9, 1998

Purpose: Our purpose was to select the proper chromosomes for preimplantation diagnosis based on aneuploidy distribution in abortuses and to carry out a feasibility study of preimplantation diagnosis for embryos using multiple-probe fluorescence in situ hybridization (FISH) on the selected chromosomes of biopsied blastomeres.

Methods: After determining the frequency distribution of aneuploidy found in abortuses, seven chromosomes were selected for FISH probes. Blastomeres were obtained from 33 abnormal or excess embryos. The chromosome complements of both the biopsied blastomeres and the remaining sibling blastomeres in each embryo were determined by FISH and compared to evaluate their preimplantation diagnostic potential.

Results: Chromosomes (16, 22, X, Y) and (13, 18, 21) were selected on the basis of the high aneuploid prevalence in abortuses for the former group and the presence of trisomy in the newborn for the latter. Thirty-six (72%) of 50 blastomeres gave signals to permit a diagnosis. Diagnoses made from biopsied blastomeres were consistent with the diagnoses made from the remaining sibling blastomeres in 18 embryos. In only 2 of 20 cases did the biopsied blastomere diagnosis and the embryo diagnosis not match.

Conclusions: If FISH of biopsied blastomere was successful, a preimplantation diagnosis could be made with 10% error.

When a combination of chromosome-13, -16, -18, -21, -22, -X, and -Y probes was used, up to 65% of the embryos destined to be aborted could be detected.

KEY WORDS: karyotype analysis; chromosomal anomaly; preimplantation diagnosis; fluorescence in situ hybridization; spontaneous abortion.

INTRODUCTION

One of six clinical pregnancies achieved by in vitro fertilization (IVF) terminates in spontaneous abortion (1). Approximately half of these abortions are caused by chromosome errors (2). It is possible to remove a single blastomere from a four- to eight-cell stage embryo and study its genetic makeup prior to embryo transfer (3). If IVF programs could avoid transferring aneuploid embryos after IVF, then implantation rates might improve (4) and spontaneous abortion rates might decline (5).

Obtaining a metaphase plate for conventional cytogenetic analysis from a single blastomere is a formidable task. Fluorescence in situ hybridization (FISH), however, permits us to study the chromosome complement in the interphase nuclei. Although it is desirable to study all 23 autosomes and sex chromosomes in a given interphase blastomere nucleus, currently available technology permits us to study only a limited number of chromosomes.

The purpose of this study was to consider which chromosomes should be selected for FISH analysis in this circumstance and to carry out a feasibility study

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to determine whether we could examine those chromosomes efficiently with fluorescent probes.

MATERIALS AND METHODS

Selection of Chromosomes

To find out the frequency distribution of aneuploidy, 250 consecutive spontaneous abortions, 70 with IVF and 180 without IVF, were studied. The maternal age of these abortuses ranged from 19 to 43 years with a mean age of 33 ± 5 years. Tissues obtained by suction curettage were examined under a dissection microscope, and chorionic villi were dissected out for tissue culture. Karyotype analysis was successful in 92% of the cases.

Blastomere Biopsy, Fixation, and FISH Analysis

Thirty-three abnormal or excess embryos at the 4- to 12-cell stage from 17 patients at Toho University were used for blastomere biopsy and FISH analysis after obtaining informed consent from the patients and approval of the institutional review board.

One or two biopsied blastomeres from each embryo were handled as follows. A slit was made at the 12-o'clock position in the zona pellucida with a microneedle. The tip of a beveled biopsy pipette was then inserted under the zona pellucida at the 6-o'clock position, and one or two blastomeres were pushed out through the 12-o'clock slit by a gentle flow of medium through the biopsy needle (6) (Fig. 1).

The blastomeres were then fixed on the glass slide using Tarkowski's (7) air-drying method modified by Yoshizawa *et al.* (8). After the blastomere was exposed to a hypotonic solution (1% sodium citrate in water) for 5 min, it was prefixed with 2.5% fixative (methanol:acetic acid, 3:1) in hypotonic solution (1% sodium citrate in water) and then fixed on a regular slide with 4–8 drops of full-strength fixative (methanol:acetic acid, 3:1). All of the remaining blastomeres in each embryo were then fixed on a regular glass slide in a similar manner, after removing them from the zona pellucida with Tyrode's solution.

The probes for chromosomes 13, 18, 21, 22, and X, were labeled with fluorescence directly (Vysis, Naperville, IL), and the probe for chromosome 16 was labeled with digoxigenin (Oncor, Gaithersburg, MD) and required fluorescence detection and amplification

Fig. 1. Procedure for embryo biopsy. The first slit was made at the 12-o'clock position in the zona pellucida using a microneedle (1 and 2), then the tip of a beveled biopsy pipette was inserted under the zona pellucida at the 6-o'clock position (3) and a blastomere was pushed out through the first slit by gentle flow of medium through the biopsy pipette (4).

Fig. 2. Frequency distribution of aneuploidy in abortuses. Frequent aneuploidy was noted in chromosomes 16 and 22 and sex chromosomes.

procedures. The probes for chromosomes 16, 18, and X, were hybridized at the centromere regions, and the probes for chromosomes 13, 21, 22, and Y were hybridized at the long arms of chromosomes. The chromosome probes were divided into two sets, and the FISH procedure was carried out in two steps.

Two milliliters of probe mixture for the first probe set was applied to the fixed blastomere and covered with a small coverslip. A piece of parafilm was then placed over the small coverslip. Denaturation of probes and chromosomes of blastomere were performed simultaneously on a slide warmer at 75°C for 3 min, and the slides were placed in a humidified case at 37°C for 1.5 hr. After the parafilm was removed, the slide was immersed in 50% formamide in 2× sodium chloride/sodium citrate (SSC) and 0.1% Nonidet-40 (NP-40) in 2× SSC at 40°C for 10 min each. The coverslip was allowed to drop off from the slide in

Fig. 3. Chromosomal anomaly detection rates in abortuses with different chromosome combination sets for the FISH probe, showing increasing efficacy of detection with the addition of chromosomes 16 and 22.

these solutions. After counterstaining with 4'6-diamidino-2-phenylindole (DAPI), the slide was evaluated under the fluorescence microscope with proper filters. Before proceeding to the second FISH, the slide was washed with 2× SSC and dehydrated with 70%, 80%, and 99% ethanol at room temperature. The denaturation, hybridization, and post hybridization wash for the second FISH were the same as for the first FISH. The blastomeres remaining in each embryo after the biopsy were treated in the same manner, and the results were compared with those of the biopsied blastomeres.

RESULTS

Chromosome Selection by Abortus Karyotyping

Of 231 abortuses karyotyped successfully, 117 abortuses (51%) showed normal karyotypes and the remainder showed abnormal karyotypes. The prevalence of abnormal karyotypes, namely, aneuploid, polyploid and mosaic, were 73%, 16%, and 11%, respectively. The distribution of aneuploidy for each chromosome is shown in Fig 2. The distribution of abnormalities was similar in the abortuses from natural and IVF conceptions. Chromosomes 16 and 22 trisomies and sex chromosome monosomy were frequently found in abortuses. Although the aneuploid distribution of embryos might be different from that of the abortuses studied here, these chromosomes were

Table I. Correlation Between Diagnosis of Biopsied Blastomere and Embryo

Embryo No.	Chromosomes of biopsied blastomere ^a	Diagnosis of embryo
1	XX, +16	Chaotic mosaic
2	X	
3	XX, +(13 or 21)	Trisomy 13 or 21
4	XXY, +18, +2(13 or 21), +16, +22	Triploid
5	XXX	Diploid mosaic ^b
6	XX, +18	
7	XX	Diploid mosaic
8	XX, -(13 or 21)	
9	XY, +(13 or 21), +16	Mosaic (NDJ) ^c
10	XY, +(13 or 21), -16	
11	XY	Diploid
12	XXY, +16	Chaotic mosaic
13	XX, -18, -(13 or 21), -16, -22	Haploid mosaic
14	XY, -18, -(13 or 21), -16, -22	Chaotic mosaic
15	Y	Mosaic (NDJ)
16	XY	Diploid mosaic
17	XXY, +18, +(13 or 21)	
18	XX	Diploid mosaic
19	XY	Diploid
20	XY	Chaotic mosaic
21	XY	
22	X, -18, -2(13 or 21), -16, -22	Chaotic mosaic
23	XY	
24	XX	Chaotic mosaic
25	XY	
26	X, -18, -2(13 or 21), -16, -22	Chaotic mosaic
27	XY, -(13 or 21)	Monosomy 13 or 21

^a Sex chromosomes plus any abnormality in the chromosomes tested, i.e., normal chromosomes, not shown.

^b Main cell line being diploid with a few chromosomal abnormal cells.

^c Mosaic by nondisjunction.

Fig. 4. Two sequential FISH for the same biopsied blastomere showing chromosome 13/21 in orange, 18 in yellow, and X in aqua signals (top) and 16 in orange and 22 in green signals (bottom).

selected for FISH analysis. Chromosomes 13, 18, and 21 were also selected, because trisomies of these chromosomes are found in newborn infants.

Based on aneuploidy and polyploidy distribution of abortuses, it was conjectured based on a calculation that if we were to use a combination of chromosome-13, -16, -18, -21, -22, -X and -Y probes for detection of abnormal karyotypes, 65% of embryos destined to be abortus due to abnormal karyotypes could be detected prior to transfer into the uterus. If we limited the cases to those of maternal age over 35 (51 abortus), the detection rate with this combination could reach 71%. But in this study among IVF patients, the detection rate was up to 57% (Fig. 3).

FISH Analysis of Biopsied Blastomere

The first set of probes was made for chromosomes 13 (orange), 18 (yellow), 21 (orange), X (aqua), and Y (green), and the second set of probes was made for chromosomes 16 (orange), and 22 (green) for sequential FISH analysis in two steps (Fig. 4).

Fifty blastomeres were biopsied from 33 embryos. Due to technical difficulty, two blastomeres were dam-

aged during the biopsy procedure and two blastomeres were lost at fixation. Eight blastomeres were anuclear and two blastomeres showed no signals. Thirty-six (72%) of 50 blastomeres gave signals for diagnosis. The twenty embryos associated with these blastomeres were available for comparison. The other 13 embryos were not available for comparison because biopsied blastomeres from 8 embryos and 5 remaining embryos did not provide adequate signals for diagnosis. Except for two embryos (embryos 4 and 15), chromosome findings from the biopsied blastomere were consistent with those from the remaining blastomeres in the embryos (Table I).

DISCUSSION

Munné *et al.* (9) used a combination of 13, 18, 21, X and Y probes for preimplantation diagnosis by FISH. It was expected that 34% of the embryos destined to be aborted due to abnormal karyotypes might be detected by this method in embryos prior to transfer into the uterus. Benadiva *et al.* (10) added chromosome 16 probe to this combination. With this addition, up to 55% of the embryos destined to be aborted could be detected. By adding a chromosome 22 probe in our study, we projected that 65% of the embryos destined to be aborted could be detected.

Fourteen of 50 biopsied blastomeres did not give information concerning chromosomal complement due to technical difficulties. The most common difficulty was lack of nucleus in the biopsied blastomere. To minimize this technical difficulty, it may be desirable to remove two blastomeres from the embryos. It was, however, encouraging to note that only 2 of 20 embryos showed an inconsistent result between the biopsied blastomere and the embryo diagnosis. This suggested that if FISH diagnosis was obtained from a blastomere biopsy, it might be a 90% reliable diagnosis for the embryos, because the cause of 10% error was found to be mosaic in this study and a diagnosis made by one or two blastomeres biopsy would fail to assess mosaicism. The method presented here has dealt with only certain chromosomes and abnormality of the other chromosomes, mosaicism, and/or translocation could not be ascertained.

Although successful pregnancies after blastomere biopsies have been reported (4), these numbers are relatively small and the magnitude of damage sustained by the embryo in the process of blastomere biopsy

is unclear. When the safety of the biopsy process is established, this preimplantation diagnosis technique could be applied in cases where the chance of the embryo being aneuploid is thought to be very high.

CONCLUSIONS

Chromosomes (16, 22, X, and Y) and (13, 18, and 21) were selected on the basis of the high aneuploid prevalence in abortuses for the former and the presence of trisomy in the newborn for the latter. When a combination of chromosome 13, 16, 18, 21, 22, X, and Y probes was used, up to 65% of the embryos destined to be abortuses due to abnormal karyotypes could be detected. If FISH diagnosis was obtained from a blastomere biopsy, it might be a 90% reliable diagnosis for the embryos.

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