

PREIMPLANTATION DIAGNOSIS OF THE ANEUPLOIDIES MOST COMMONLY FOUND IN SPONTANEOUS ABORTIONS AND LIVE BIRTHS: XY, 13, 14, 15, 16, 18, 21, 22

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SUMMARY

The present preimplantation diagnosis test is able to screen for the most common aneuploidies from single blastomeres in about five hours with a 15 per cent misdiagnosis. This means that the risk of spontaneous abortion and trisomic offspring for women of advanced maternal age could be reduced to the same level as younger women for whom prenatal diagnosis is usually not necessary. Better probes and more fluorochromes could improve the success rate of the test. Copyright © 1998 John Wiley & Sons, Ltd.

INTRODUCTION

Numerical chromosome abnormalities are the most common genetic defects, and with the exception of Robertsonian translocations, they occur *de novo*, with maternal age being the only known risk factor (Hassold and Chiu, 1985). Ploidy assessment of single blastomeres has been postulated to have the potential to reduce the chances of delivering a trisomic baby, reduce embryo loss during pregnancy, and perhaps increase implantation rates in women of advanced maternal age (Munné *et al.*, 1995a). The causes of the decline in implantation observed with increasing maternal age are still under debate, and the only clear link between maternal age and embryo competence is aneuploidy. The increase in aneuploidy with maternal age in spontaneous abortuses and live offspring was also found both in cleavage-stage embryos (Munné *et al.*, 1995a) and

unfertilized oocytes (Dailey *et al.*, 1996). The rate of chromosomal abnormalities in embryos was higher than the one reported for spontaneous abortion, suggesting that a sizeable part of chromosomally abnormal embryos are eliminated before prenatal diagnosis is performed. Such loss of embryos could account for the decline in implantation with maternal age. For instance, the rate of embryonic monosomy is similar to the one for trisomy (Munné *et al.*, 1995a), while most autosomal monosomies are normally undetected in clinically recognized pregnancies, probably because, as in mice, they die before implantation (Magnuson *et al.*, 1985). Furthermore, even trisomies that can arrive at term (13, 18, 21) are probably selected against prior to, or during, implantation, since even recognized trisomy 21 pregnancies spontaneously abort in over 50 per cent of cases (Warburton *et al.*, 1986).

Because of the correlation between aneuploidy and declining implantation rates with maternal age, it was hypothesized that negative selection of chromosomally abnormal embryos could reverse this trend (Munné *et al.*, 1993a). Currently, negative selection of aneuploid embryos can only

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be achieved through preimplantation genetic diagnosis (PGD), either by polar body or blastomere analysis. Low metaphase yield and less than 30 per cent of karyotypable metaphases, together with the requirement of overnight culture in antimitotics (Santaló *et al.*, 1995), make karyotype analysis unsuitable for PGD. Fluorescence *in situ* hybridization (FISH) allows chromosome enumeration to be performed on interphase cell nuclei. FISH has been used for PGD of X-linked disease (Griffin *et al.*, 1992; Munné *et al.*, 1993b; Harper *et al.*, 1994), common aneuploidies (X, Y, 13, 18, 21) (Munné *et al.*, 1993a, 1995a,b, 1998c; Munné and Weier, 1996; Verlinsky *et al.*, 1996a,b), and recently translocations (Munné *et al.*, 1998a,b; Conn *et al.*, 1998). More powerful techniques, such as the use of spectral imaging, already allow the simultaneous enumeration of all 23 chromosome pairs and the detection of some structural abnormalities in metaphase-stage first polar bodies and oocytes (Márquez *et al.*, 1998). However, this technique cannot be used in blastomeres because it requires metaphase analysis.

The objective of our study was to obtain as much information as possible from a single cell using FISH. Specifically, we attempted to enumerate simultaneously the chromosomes X, Y, 13, 14, 15, 16, 18, 21 and 22 from a single blastomere, which would cover 70 per cent of the aneuploidies detected in spontaneous abortions. For that purpose, the cell was analysed in two rounds of hybridization and analysis over a total period of less than six hours, permitting embryo transfer to take place on the same day as the initial blastomere biopsy. Combination coding (Fox *et al.*, 1995) of the *in situ* hybridization probes with three spectrally-distinct fluorescent labels was used to provide visibly distinguishable colour staining of each chromosome target. The occurrence of false-positive and false-negative FISH results was evaluated in a PGD setting by analysing all or most of the cells of the non-transferred embryos.

MATERIALS AND METHODS

Embryo source

PGD cases were performed in two centres: The Institute of Reproductive Medicine and Science of Saint Barnabas Medical Center (Livingston, NJ, U.S.A.) and S.I.S.Me.R (Bologna, Italy). Written consent was obtained from all PGD patients in

accordance with the corresponding Internal Review Board protocols. Because PGD of aneuploidy has an estimated ≥ 10 per cent error rate (Munné *et al.*, 1998c), our consent forms recommended that patients undergo prenatal diagnosis. During day three of development, one or two cells per embryo were biopsied, and the embryos returned to culture as described elsewhere (Grifo, 1992). All of the embryos were at the 4 to 12-cell stage of development at the time of biopsy. Most embryos classified as normal after PGD were transferred to the uterus on the same day of analysis or on the next morning. Only those embryos classified as abnormal or developmentally arrested were fully biopsied and had most or all of their cells analysed. All blastomeres were fixed individually following our protocol for blastomeres (Munné *et al.*, 1996) with one modification. In previous protocols, one or two drops of fixative were applied to the blastomeres until the cytoplasm broke; then another drop of fixative was added to eliminate cytoplasm debris. In the present study the last drop was avoided. With appropriate humidity conditions (40–50 per cent) and blowing after the cytoplasmic breakage, it was observed that little debris remains on top of the nucleus and micronuclei do not get lost, as they can do if a drop is added after cytoplasmic rupture (Munné *et al.*, 1998c).

FISH procedure

Fixed cells were analysed by two rounds of FISH, the first hybridization with a previously described protocol for the simultaneous analysis of chromosomes X, Y, 13, 16, 18 and 21 (Munné *et al.*, 1998c). For the second round of hybridization a new protocol was applied using 14, 15 and 22 chromosome-specific probes. The first hybridization involved probes for chromosomes 13 (RB-1 locus, 13q14, expanding 440 Kb, Vysis, Inc., Downers Grove, IL), 21 (region 21q22.13-q22.2, Vysis), 16 (alpha satellite, Vysis), 18 (alpha satellite, D18Z1, Vysis), X (alpha satellite, DXZ1, Vysis) and Y (satellite III, DYZ1, Vysis). These probes were labelled with Spectrum-Orange[™], Spectrum-Green[™], and/or Spectrum-Aqua[™] (Vysis). They were combined in the following quantities (volumes refer to commercial stock solutions except where indicated with specific concentration values): 16 μ l Spectrum-Aqua CEP Y, 12 μ l Spectrum-Green CEP Y, 36 μ l Spectrum-Aqua CEP X, 22 μ l Spectrum-Aqua CEP 18, 14 μ l

Spectrum-Orange CEP 18, 30 μ l Spectrum-Orange LSI 13 (200 ng/ μ l), 24 μ l Spectrum-Green LSI 13 (200 ng/ μ l), 30 μ l Spectrum-Orange LSI 21 (200 ng/ μ l), and 30 μ l Spectrum-Green LSI 16 (125 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 90 μ l, and added to 210 μ l of WCP hybridization buffer (Vysis). 10 μ l of the resulting hybridization solution were applied to the glass slide containing fixed blastomeres and covered with an 18 mm \times 18 mm coverslip. The slide was then placed for three minutes on a slide warmer preheated to 78°C, sealed with rubber cement, and placed in a dark moist chamber at 37°C for at least two hours. After the hybridization, the slides were washed individually at 71°C in 0.4 \times SSC for two minutes. The slides were then mounted with 10 μ l of DAPI counterstain in antifade solution (4',6-diamino-2-phenyl indole, Vysis) and observed with a fluorescent microscope (Olympus BX60) equipped with the triple-band pass filter set for simultaneous observation of Spectrum-Orange, Spectrum-Green and Spectrum-Aqua fluorescence. When viewed through the triple-band pass filter set, the Y-chromosome specific signal appeared white to yellow, the X as blue, the 18 as magenta, the 13 as orange, the 21 as red and the 16 as a green domain. A DAPI filter (Olympus) was applied to find the nuclei.

As previously demonstrated by Benadiva *et al.* (1996), hybridization signals disappear after a second round of denaturation with a different set of probes, therefore allowing FISH re-analysis. For the re-analysis of cells with probes specific for chromosomes 14, 15 and 22 we followed Benadiva's protocol with few modifications. 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, and then dehydrated (70 per cent, 85 per cent, 100 per cent ethanol, two minutes each). 10 μ l of the second hybridization solution were applied per slide. This second hybridization solution was prepared by mixing 2.5 μ l of a probe for chromosome 14 (binding to the 14q11.2 region), labelled with Spectrum-Aqua, 1 μ l of a probe for chromosome 15 (binding to the alpha satellite region) labelled with Spectrum-Orange, and 1 μ l of a probe for chromosome 22 (binding to the 22q13.3) labelled with Spectrum-Green. This mixture was concentrated to 3 μ l with a Speed-Vac centrifugal evaporator (DyNA VAP) and mixed with 7 μ l of WCP

hybridization buffer (Vysis). Slides and probe were simultaneously denatured in a hot plate of 78°C for three minutes, followed by two hours of hybridization at 37°C and finally washed for 90 seconds in 0.4 \times SSC at 71°C. The washed slides were mounted with DAPI in antifade (Vysis) and analysed.

The scoring criteria described in Munné and Weier (1996) was used without modification. The criteria to classify embryos as normal, aneuploid, mosaic, polyploid or haploid based on FISH results has been previously described by us and was followed here without modification (Munné *et al.*, 1998d). However, only embryos with three or more of their cells analysed were included. Generally, most or all of their cells were analysed.

RESULTS

Patient information

The average maternal age of the 25 patients included in this study was 37.4 \pm 4.2. Of those, 14 patients were selected for PGD because of advanced maternal age (average maternal age of 39.6 \pm 2.1). The rest of the patients had suffered habitual losses (three patients, average maternal age 38.0 \pm 2.4), had poor embryonic development or failed more than three previous IVF attempts (four patients, average maternal age 34.0 \pm 4.1), or had a precondition such gonosomal mosaicism (four patients, average maternal age 33.5 \pm 5.1).

Abnormalities found by PGD

A total of 25 cycles of PGD resulted in 247 embryos having a single cell biopsied, of which 103 (42 per cent) were classified after PGD as normal, 56 as aneuploid (22.6 per cent; 31 monosomies, 25 trisomies) (Fig. 1), 55 as complex abnormal, 15 as polyploid and 8 as haploid. Embryos were classified as complex abnormalities when two or more chromosomes had an abnormal count but were not completely polyploid or haploid. The remaining 10 embryos could not be analysed by PGD either because of damage to the biopsied cell or anuclear biopsied cells.

Pregnancy outcome

Of the 25 clinical cases (13 from S.I.S.Me.R. and 12 from Saint Barnabas), 7 had no replacement for

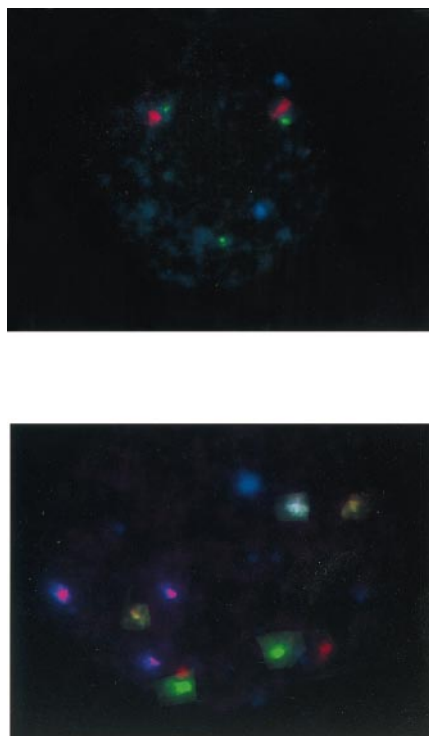


Fig. 1—Top panel: *in situ* hybridization with 14, 15 and 22 chromosome-specific DNA probes. The chromosome-14-specific probe is shown labelled in blue, chromosome 15 in red and chromosome 22 in green. The cell is from an embryo with trisomy 22 (14, 14, 15, 15, 22, 22, 22). Bottom panel: *in situ* hybridization with X, Y, 13, 16, 18 and 21 chromosome-specific DNA probes. The chromosome-X-specific probe is shown labelled in blue, chromosome Y in white, chromosome 13 in orange, chromosome 16 in green, chromosome 18 in magenta and chromosome 21 in red. The cell is from a male embryo with trisomy 18 (X, Y, 13, 13, 16, 16, 18, 18, 18, 21, 21)

lack of normal embryos, 10 became pregnant and 8 did not. Of these pregnancies, 9 are ongoing with 12 conceptuses (3 pregnancies from S.I.S.Me.R. and 6 from Saint Barnabas) and a singleton

pregnancy spontaneously aborted and was diagnosed as having trisomy 21 (from Saint Barnabas). The cells belonging to the transferred embryos of the case resulting in trisomy 21 were rehybridized with a probe for chromosome 21. The same results (normal) were obtained, therefore excluding an error produced by the overlap of a chromosome 21 (labelled in Spectrum-Orange) and another chromosome containing Spectrum-Orange (Y, 18, 13).

Error rate

Of the non-transferred embryos, 88 were re-biopsied and all or most cells analysed by FISH using the same protocol used for PGD. Two types of error were found. The most serious error classified an embryo as normal by PGD, when in fact it was abnormal, or classified the embryo as abnormal by PGD when in fact it was normal. We called these events type-1 errors (false positive and false negative, respectively). Of 88 re-analysed embryos, 9 previously classified as normal by PGD, were all confirmed as such after re-analysis, while 75/79 previously classified as abnormal were confirmed as such. The embryo that resulted in a spontaneous abortion with trisomy 21 should also be counted as an error of type 1. Therefore, the type-1 error rate was 6 per cent (5/88+1/13). A less serious error was when an embryo was classified as having a specific abnormality by PGD but was found to have a different abnormality after re-analysis (type-2 error). A total of 8 (9 per cent) embryos were found to be type-2 errors. Therefore, the total FISH error was 15 per cent although only the 6 per cent of type-1 errors could have consequences with pregnancy outcome. The frequency of errors is shown in Table I in relation to the PGD classification. Of the type-1 errors, two were

Table I—PGD and re-analysis results

Classified as:	PGD analysis	Re-analysed	Type-1 error	Type-2 error
Normal	103	9	0	0
Aneuploid	56	25	3	2
Complex abnormal	55	31	1	6
Polyploid	15	7	0	0
Haploid	8	4	0	0
No result	10			
Total	247	76 (87%)	4 (5%)	8 (9%)

Table II—Specific PGD errors found in the present study and Munné *et al.* (1998c)

	PGD analysis	Re-analysis or SPA	Number of events	
Type-1 errors	Normal	Monosomy 16	1	
	Normal	SPA with trisomy 21	1	
	Complex abnormal	Normal	3	
	Monosomy Y	Normal	1*	
	Monosomy 13	Normal	3†	
	Monosomy 16	Normal	3†	
	Monosomy 18	Normal	1*	
	Monosomy 21	Normal	2	
	Monosomy 22	Normal	1	
	Trisomy 13	Normal	1	
	Trisomy 18	Normal	1	
	Trisomy XYY	Normal	2	
	Type-2 errors	Trisomy 21	Trisomy 21 and 16	1
		Monosomy 22	Monosomy 22 and 13	1
Complex abnormal		Monosomy 21	3	
Complex abnormal		Monosomy 22	1	
Complex abnormal		Trisomy 14	1	
Complex abnormal		Trisomy 21	1	
Haploid		Trisomy 16/monosomy 16	1	
Trisomy XXY		Monosomy 18	1	
Trisomy 15		Trisomy 22	1	

*One embryo classified as monosomy Y and 18 simultaneously.

†One embryo classified as monosomy 13 and 16 simultaneously.

SPA: spontaneous abortion.

embryos classified as trisomy XYY, one as complex abnormal (the cell analysed by PGD was multinucleated), and one as monosomy 22, which after re-analysis were found to be normal. In addition, the other type-1 error was the embryo classified as normal, which subsequently spontaneously aborted as trisomy 21. Type-2 errors were mostly found among embryos previously classified as complex abnormal. Of the eight type-2 errors, six were classified as complex abnormal, when in fact four were single monosomies and two were single trisomies. The other two type-2 errors were: an embryo classified as monosomy 22, which in fact was double monosomic for chromosomes 13 and 22, and an embryo classified as trisomy 15, when in fact it was a trisomy 22. The last case was most probably an error in data recording.

Putting the errors found in this study together with those found in a previous one that also used probes for X, Y, 13, 16, 18 and 21 (Munné *et al.*, 1998c), the overall type-1 error rate was 18 per cent. Specifically, 4 per cent (1/20 re-analysed

embryos+1/26 conceptus) of the embryos classified by PGD as normal were in fact abnormal, 14 per cent (28/140) of the embryos classified as abnormal were in fact normal (type-1 error) and 8 per cent (11/140) were type-2 errors. The pooled errors are shown in Table II.

CONCLUSIONS

Based on the analysis of a single blastomere, the present protocol allows the detection of about 70 per cent of the numerical chromosome abnormalities found in spontaneous abortions. However, this technique is not free of error. We detected a frequency of type-1 errors ranging from 6 per cent in the present study, to 18 per cent when including all the cases in which probes for chromosomes X, Y, 13, 16, 18 and 21 were used (Munné *et al.*, 1998c and present study). The unchanged, or even improved error rate with extra probes is because they were applied during the second hybridization, each one having a single pure fluorochrome. In this

way, the complexity of the system was not increased. Furthermore, in the second hybridization stage, the use of one fluorochrome per probe, instead of combination coding, avoided errors produced by overlaps of different chromosomes sharing common fluorochromes.

We have now identified 20 kinds of type-1 errors and 11 kinds of type-2 errors, which allows us to propose changes to the protocol that may reduce the error rate. For instance, 11 type-1 errors and 1 type-2 error were false monosomies. Previously, we have reported a reduction in this type of error when fixing the blastomeres as described in the present study, which minimizes the loss of micronuclei during fixation. In previous protocols, one or two drops of fixative were applied to the blastomere and once the cytoplasm broke, another drop of fixative was added to eliminate cytoplasm debris (Munné *et al.*, 1998c). By eliminating the drop added after cytoplasm breakage we found very few false monosomies in the present study.

Not all false monosomies are due to the loss of micronuclei. Chromosomes in degenerating blastomeres can easily be lost among fragments of cytoplasm. Also, overlaps between the same chromosomes or with chromosomes sharing one of the fluorochromes may also produce false monosomies. As we reported previously, the bigger the fixed nuclear diameter, the less frequently overlaps occurred (Munné *et al.*, 1996). In future, the use of more fluorochromes, such as Spectrum-Gold and Spectrum-Blue, recently commercialized by Vysis, will ultimately allow the use of one fluorochrome per chromosome, perhaps completely eliminating errors produced by overlaps between different chromosomes.

Another common error is to classify a cell as complex abnormal (two or more chromosomes with an abnormal count) when in fact the rest of the embryo is normal (three events, Table II), or have a single abnormality (six events, Table II). Some of those are also due to false monosomies. Others were related to multinucleation. Multinucleated cells, as reported previously (Kligman *et al.*, 1996), are seldom representative of the rest of the embryo as they can be chromosomally abnormal; while the rest of the embryo is normal.

Mosaicism, which is very common in cleavage-stage embryos (Munné *et al.*, 1995a; Delhanty *et al.*, 1997), was not found to be a significant source of error, because most mosaic embryos were classified as complex abnormal, probably because an abnormal cell was biopsied. Neverthe-

less, only 20 embryos classified as normal were re-analysed in this and a previous study (Munné *et al.*, 1998c), which is not enough to know how many mosaics had a normal cell biopsied.

Finally, another common source of error is false trisomies in disomic embryos or false disomies in monosomic ones. This happens when the target splits into two signals and produces false-positive results. This can be caused by the fixation procedure (stretched nuclei), the type of probe (when some probes split more than others), or the cellular stage, where double-dotted signals can also represent two sister chromatids after reduplication (Wyrobek *et al.*, 1994; Munné *et al.*, 1998c). We have previously attempted to produce scoring criteria to deal with this problem (Munné and Weier, 1996; Munné *et al.*, 1998c) but errors still occur. A solution would be to substitute the probes that split more often (alpha satellite probe for chromosome 18, satellite III probe for chromosome Y) by non-repetitive probes.

Other errors could result from too short times of hybridization. Recent observations extending the time of hybridization to four hours per hybridization combined with late embryo transfer or transfer on day four of development produced clearer and brighter signals (Magli *et al.*, personal communication). The only obvious disadvantage is very long hours for the staff when embryo transfer takes place on the same day.

Even though the error rate is lower in this study than in previous ones (Munné and Weier, 1996; Munné *et al.*, 1998c), one PGD case resulted in the conception of a trisomy 21 fetus, which later spontaneously aborted. This error was not due to an overlap between a chromosome 21 and another chromosome, since a third round of hybridization with only a probe for chromosome 21 showed the same results. This false disomy was probably caused by mosaicism or an overlap between two chromosome 21 targets. PGD patients from the centres involved in this study were strongly recommended to undergo prenatal diagnosis. However, classifying 6 per cent of abnormal embryos as normal means that 94 per cent of trisomies would be detected, therefore decreasing the probability that women aged 40 or older would have a trisomic pregnancy to the levels found in women aged 34 or younger. Young women are usually not recommended to undergo prenatal diagnosis, therefore it is not completely clear if prenatal diagnosis should be offered to PGD patients.

In summary, the present protocol reduces the error rate over previous protocols while increasing the number of chromosomes analysed. It does so by reducing false monosomies generated during fixation, and by using cell recycling (Benadiva *et al.*, 1996), which allows the analysis of the same cell with a different set of probes without increasing the complexity of the system. Further improvements may be achieved by using more fluorochromes to eliminate the need of combination coding, and by changing some probes for others that would split less. However, errors due to mosaicism will not be eliminated unless more cells can be analysed, for example, by blastocyst analysis (Mugleton-Harris *et al.*, 1995; Veiga *et al.*, 1997).

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