Article

Increased efficiency of preimplantation genetic diagnosis for aneuploidy by testing 12 chromosomes

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Abstract

One of the most important factors in increasing the screening potential of preimplantation genetic diagnosis (PGD) for aneuploidy is to increase the number of chromosomes analysed. Inclusion of chromosomes 8, 14 and 20 to the standard set of chromosomes X, Y, 13, 15, 16, 17, 18, 21 and 22 allows the analysis of 12 chromosomes in three rounds of fluorescent in-situ hybridization (FISH) without decreasing the efficiency of the technique. Pregnancy rate was significantly increased when only embryos that had been diagnosed as normal for the 12 chromosomes analysed were transferred compared with transfer of embryos with any abnormality for chromosomes 8, 14 or 20 ($P < 0.05$). This study proves that the high efficiency and practical feasibility of FISH analysis of 12 chromosomes in PGD for aneuploidy is a superior approach than the standard nine-chromosome analysis in order to screen for abnormalities.

Keywords: *aneuploidy, FISH, pregnancy rate, preimplantation genetic diagnosis*

Introduction

Since preimplantation genetic diagnosis (PGD), or preimplantation genetic screening, for infertility was first introduced in clinical diagnosis, several parameters, such as fixation technique (Velilla *et al.*, 2002), biopsy procedures (De Vos and Van Steirteghem, 2001), number of cells analysed (Cohen *et al.*, 2007; Goossens *et al.*, 2008), type of cell analysed (Munné *et al.*, 1995; Wells *et al.*, 2002; Magli *et al.*, 2004), type of probes (Agerholm *et al.*, 2005), scoring criteria (Munné *et al.*, 1998a) and reanalysis of unclear results (Colls *et al.*, 2007), have been established or optimized in order to maximize the efficiency of the technique. In addition to those improvements, the number of chromosomes analysed remains ultimately the most important factor in order to accomplish the main purpose of PGD, which is to increase the pregnancy rate and reduce the rate of spontaneous abortion by selecting chromosomally normal embryos. Therefore, the number of chromosomes analysed in a single interphase nucleus has increased from testing only chromosome Y (West *et al.*, 1987) to the test of 13 chromosomes (Abdelhadi *et al.*, 2003; Agerholm *et al.*, 2008). However considerable technical limitations of the fluorescence in-situ hybridization (FISH) technique, such as available fluorochromes, rounds of hybridization, fixation quality and timing for results, put some limits to the number of chromosomes that can be analysed in routine clinical PGD. Due to these limitations and in order to maximize the efficiency of the test, only those chromosomes more likely to allow implantation or to lead to fetal abnormalities in their aneuploid condition should be prioritized to be included in PGD analysis over chromosomes that, if aneuploid, would not allow the embryo to reach the blastocyst stage.

It is well known that embryos aneuploid for chromosomes X, Y, 13, 18 or 21 can develop into morphologically normal blastocysts (Sandalinas *et al.*, 2001), progress beyond

implantation and produce spontaneous abortions (Warburton *et al.*, 1986) or even lead to chromosomally abnormal offspring (Chandley, 1981). In addition to these five chromosomes, and based on incidence at cleavage stage, other chromosomes such as 16, 15, 17 and 22 have been included in PGD screening in order to increase the efficiency of the test with successful results (Munné *et al.*, 1998b, 2004). Thus, this nine-chromosome approach has become standard practice for the vast majority of PGD laboratories. Due to limited availability of fluorochromes, it is carried out via two rounds of FISH. Therefore, attempts to increase the number of chromosomes beyond nine or 10 would necessarily increase the number of FISH rounds, which can jeopardize the benefits of the approach by decreasing the efficiency of the FISH in the last round. However, recently described approaches such as 'no result rescue' (NRR; Colls *et al.*, 2007) can compensate this negative effect, thus allowing an increase in the number of chromosomes tested.

The choice of probes for this third panel is as follows. Chromosome 8 was chosen because it is the next most common in spontaneous abortions in infertile patients (Lathi *et al.*, 2008) after chromosomes 2 and 7. However, abnormalities for these two chromosomes were infrequently found to occur independently of abnormalities for the other nine chromosomes already tested (Abdelhadi *et al.*, 2003; and authors' unpublished results). Chromosome 20 was chosen because preliminary data in the laboratory showed high incidence of chromosome abnormalities at cleavage stage. As previously published (Munné *et al.*, 2004), some abnormalities are more frequent at cleavage stage than at first trimester. Additionally, inclusion of chromosome 14 to the regular nine-chromosome panel would cover all possible Robertsonian translocations. Therefore, addition of chromosomes 8, 14 and 20 to the X, Y, 13, 15, 16, 17, 18, 21, 22 standard test could be of great value to improve the efficiency of PGD for aneuploidy.

This report presents clinical experience with the use of an extended 12-chromosome PGD test versus the standard nine-chromosome test.

Materials and methods

Embryos analysed

Two different groups of patients, referred to the authors' unit for PGD of aneuploidy, were used in this study for different purposes. Indication for PGD in both groups, i.e. advanced maternal age, repeated IVF failure and recurrent pregnancy loss, were similar. No cases for which the indication was 'family balance' were included in this study.

Group 1 included embryos analysed with the standard nine-chromosome panel (X, Y, 13, 15, 16, 17, 18, 21, 22) and after transfer, the slides were reanalysed with probes for chromosomes 8, 14, and 20 for the purpose of comparing the pregnancy rate of cycles with only normal embryos transferred with those in which embryos abnormal for 8, 14 or 20 were transferred.

Group 2 included patients undergoing PGD with the 12-chromosome panel (X, Y, 8, 13, 14, 15, 16, 17, 18, 20, 21, 22), and those embryos classified as abnormal were reanalysed in all their cells with the same probes used in the original PGD analysis in order to determine the error rate of the 12-panel test, following the guidelines from the Preimplantation Genetic Diagnosis International Society (2007).

Embryos were biopsed on day 3 of development by removing a single blastomere, followed by fixation of the nucleus using the slightly modified Carnoid method (Velilla *et al.*, 2002). The fixed cells were sent to Reprogenetics laboratory in Livingston, NJ for FISH analysis, and results were provided on days 3–5 of development.

Fluorescence in-situ hybridization procedures

Embryos screened for chromosomes X, Y, 13, 15, 16, 17, 18, 21 and 22 were analysed by two rounds of FISH as previously described (Munné *et al.*, 1998b). The first round contained probes for chromosomes 13, 16, 18, 21 and 22 (MultiVision PB; Vysis, USA) (LSI 13q14.1–q14.3, RB1; CEP 16q11.2, D16Z3; CEP 18p11.1–q11.1, D18Z1; LSI 21q22.13–q22.2, D21S259, D21S341, D21S342; LSI 22q11.2, BCR), and the second round contained probes for chromosomes X, Y, 15 and 17 (MultiVision 4 CC; Vysis) (CEP Xp11.1–q11.1, DXZ1; CEP Yq12, DYZ1; CEP 15p11.1–q11.1, Alpha Satellite D15Z4; CEP 17p11.1–q11.1, D17Z1).

Embryos screened for chromosomes X, Y, 8, 13, 14, 15, 16, 17, 18, 20, 21, 22 were analysed as described above for the nine-chromosome test followed by an extra FISH round which included probes for chromosomes 8 (CEP 8p11.1-q11.1, D8Z2), 14 (Telomere 14q32.3, STS-X58399, SHGC-36156, STS-AA034492, Telomeric IGHV segments) and 20 (Telomere 20p13, 20PTEL 18A (D20S1157, GDB:624489)). FISH for this extra panel was performed by mixing the three probes and LSI/WCP hybridization buffer (Vysis) in a 1:1:1:7 proportion, respectively, followed by denaturation at 73°C for 5 min and hybridization for a minimum of 4 h at 37°C. Post-hybridization washes were performed in 0.7 × SSC/0.3% NP40 solution at 71°C for 2 min and DAPI was applied as counterstain. Each probe lot was evaluated on lymphocytes, giving an efficiency between 98% and 100%, depending on the lot.

In both groups, after the regular analysis, extra NNR FISH panels were performed in cases where doubtful results for one or more of the analysed chromosomes were obtained. In such cases, a new probe that binds to a different locus than the previous was used; these being a sub-telomeric probe binding to the p or q arm, or an LSI probe that targets a different locus of the chromosome in question. These extra hybridization panels were performed following protocols previously described (Colls *et al.*, 2007).

Thus, for chromosome 8, the probe (LSI 8q22, ETO) was used. For chromosome 13, the probes were (Telomere 13q34, VIJyRM2002) or (LSI 13q34, D13S25, D13S319,

RB1), for chromosome 14 (LSI 14q32.3, IGH), for chromosome 15 (Telomere 15, AFM A224XH1) or (LSI 15q22, PML), for chromosome 16 (Telomere 16p13.3, 16PTEL05) or (Telomere 16q24.3, 16QTEL013), for chromosome 17 (Telomere 17p13.3, 17PTEL80), (Telomere 17q25.3, AFMZ17yD10) or (LSI 17q12–q21, RARA), for chromosome 18 (Telomere 18p11.3, VIJyRM2102), (Telomere 18q23, VIJyRM2050, 18QTEL11) or (LSI 18q21.1–q21.3, BCL2), for chromosome 20 (LSI 20q12, D20S108) or (LSI 20q13, ZNF217, D20S183, 188T), for chromosome 21 (Telomere 21q22.3, VIJyRM2029), for chromosome 22 (Telomere 22q13.3, MS607) or (LSI 22q11.2, TUPLE1, 22q13.3, ARSA), and for chromosomes X and Y (Telomere Xp22.3/Yp11.3, DXYS129) or (Telomere Xq28/Yq12, EST Cdy 16c07) were used. All individual probes as well as multicolor probe hybridization mixtures were obtained from Abbott (Downers Grove, IL, USA).

In group 1, after the PGD report was sent to referring centre, the slides were reanalysed for chromosomes 8, 14 and 20 in an extra FISH panel as described above.

In group 2, for quality-control purposes and to assess the error rate, non-transferred embryos classified by PGD as abnormal and donated for research purposes were reanalysed with all or most of their cells fixed individually. The same sets of probes used in the original PGD analysis were used. The reanalysis and PGD results were compared in order to determine the error rate.

Embryos were classified after PGD as normal or abnormal based on previous published criteria (Munné *et al.*, 2004). Basically, if all chromosomes had two signals, the embryo was classified as normal, and any other combination as abnormal. Abnormal embryos that were reanalysed were fully disaggregated and the cells were fixed individually to assess PGD error rate. In this case, different scoring criteria were used that more finely classified the chromosome abnormality (Munné *et al.*, 1998c).

Embryo transfer groups

IVF cycles in group 1 were divided in two subgroups (A and B). Subgroup A included cycles where at least one embryo was normal for all chromosomes analysed (X, Y, 8, 13, 14, 15, 16, 17, 18, 20, 21 and 22). Subgroup B included cycles where all embryos transferred were normal for chromosomes X, Y, 13, 15, 16, 17, 18, 21 and 22 but were abnormal for any of the chromosomes analysed *a posteriori* (8, 14 and 20).

Statistical analysis

A chi-squared test using the algorithm GraphPad InStat3 was used to evaluate statistical differences between proportions.

Results

Chromosome abnormalities

In group 1, a total of 817 blastomeres from 806 embryos produced in 100 IVF cycles were analysed with PGD for aneuploidy for chromosomes X, Y, 13, 15, 16, 17, 18, 21 and 22. Of these, 275 (34.1%) were diagnosed as normal and 531 (65.9%) as abnormal. After reanalysis of the same nuclei with probes for chromosomes 8, 14 and 20, 240 (29.8%) were diagnosed as normal, and 566 (70.2%) as abnormal. Therefore 12.7% (35/275) of embryos diagnosed as normal with the standard test carried some abnormalities for chromosomes 8, 14 or 20 (**Figures 1** and **2**). Results are summarized in **Table 1**. In addition, of the 253 abnormal embryos classified as aneuploid by the first and second panel, 29 (11.5%) also had abnormalities for chromosomes 8, 14 or 20.

Of the 817 cells analysed, 85 (10.4%) showed inconclusive results for one or more chromosomes, with a total of 82

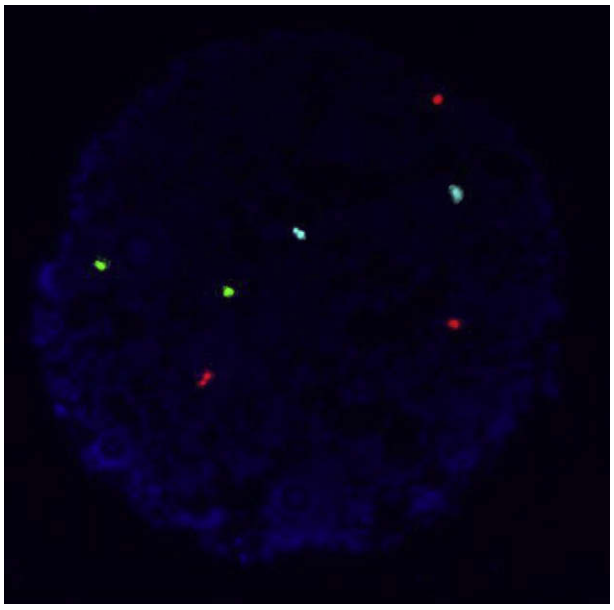

Figure 1. Blastomere from an embryo rediagnosed as having trisomy 14 (Telomeric 14q Spectrum Orange). This embryo was diagnosed as normal after standard PGD FISH analysis.

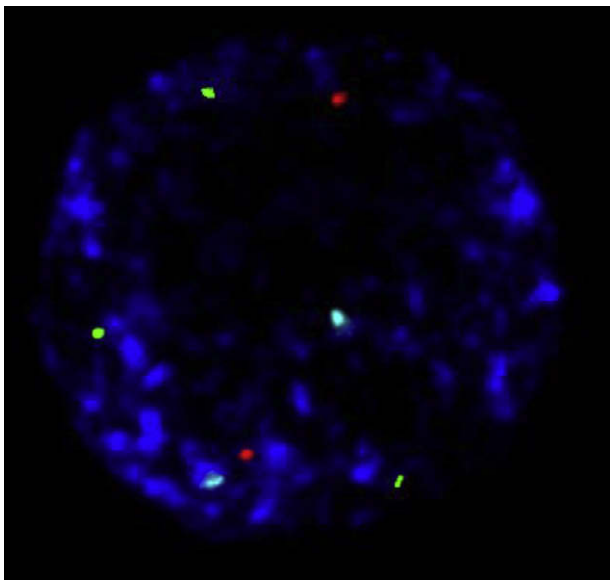

Figure 2. Blastomere from an embryo rediagnosed as having trisomy 20 (Telomeric 20p Spectrum Green). This embryo was diagnosed as normal after standard PGD FISH analysis.

Table 1. Comparison of results between subgroups A and B.

	Subgroup A	*Subgroup B*
Embryos	806	806[a]
Normal	275 (34.1)	240 (29.8)
Abnormal	531 (65.9)	566 (70.2)
Abnormal for chromosome 8, 14 or 20 only	–	35 (12.7)
Pregnancy rate	52/89 (58.4)[b]	1/8 (12.5)[b]

Values are *n* or *n* (%). For a detailed description of the subgroups, see Materials and methods.
[a]Embryos of subgroup A analysed *a posteriori* with probes for chromosomes 8, 14 and 20.
[b]$P < 0.05$.

no result (NR) events. Chromosomes 20, 16, 15, 13, 8, 14, 18, 21, 22 and 17 accounted respectively for 23.4%, 14.8%, 12.3%, 9.8%, 8.6%, 8.6%, 6.2%, 6.2%, 7.4% and 2.5% of the total NR events.

Based on several criteria, such as embryo quality, number of normal embryos in the cohort, fixation quality and probe availability, 93.7% of inconclusive results for chromosomes X, Y, 13, 15, 16, 17, 18, 21 and 22 were reanalysed with the NRR approach, obtaining conclusive results in 91% of them. Therefore, four (1.5%) of the 275 embryos diagnosed as normal had inconclusive results for one chromosome and were diagnosed as normal NR. For chromosomes 8, 14 and 20, NRR was applied in 94% of the dubious results, obtaining conclusive results in 100% of them. Therefore the efficiency of the NRR after the standard test is not significantly different than that observed after the extra 8, 14, 20 panel.

Error rate

In group 2, 143 embryos that were diagnosed as abnormal after being analysed by FISH for aneuploidy for chromosomes X, Y, 8, 13, 14, 15, 16, 17, 18, 20, 21 and 22 had all or most of their cells analysed with the same set of probes used in the original PGD. After reanalysis, 12 of them were rediagnosed as normal, giving an error rate of 8.4%.

Pregnancy outcome

In 97 of the 100 IVF-cycles embryos were transferred to the patient. For the remaining three cycles, embryo transfer was cancelled due to lack of embryos (two cycles) or elevated progesterone concentration. Pregnancy resulted in 53 of the 97 cycles, giving a total pregnancy rate of 54.6%. In subgroup A, the pregnancy rate was 58.4% (52/89) which was significantly higher ($P < 0.05$) than the 12.5% (1/8) observed in subgroup B. The average number of embryos transferred in subgroups A and B were 1.49 and 1.2, respectively. The small sample size of subgroup B is taken into account in the calculation of the *P* value, the difference in pregnancy rate being sufficiently large so as to be considered statistically significant. Age was not significantly different between subgroups A and B.

Discussion

Increasing the number of chromosomes analysed by FISH in order to increase the screening potential of PGD is a

challenging approach. Although the number of chromosomes analysed has been increased by the introduction of several protocols (Munné *et al.*, 1993, 1994, 1998a,b; Munné and Weier, 1996), fluorochrome availability, rounds of FISH and timing for results remain the main obstacle to that purpose. However, the protocol presented in this study allowed the analysis of three extra chromosomes without jeopardizing the efficiency of the technique and, thus, increasing the pregnancy rate in PGD for aneuploidy.

The highest number of available fluorochromes in the visible spectrum that can be combined in a single FISH panel is five (Spectrum Red, Spectrum Green, Spectrum Blue, Spectrum Aqua and Spectrum Gold; Vysis). However only a very limited number of multicolour probe hybridization mixtures include the five different fluorochomes, Spectrum Orange, Spectrum Green and Spectrum Aqua being the only fluorochromes currently commercially available for individual probes. Since it is known that there is a correlation between decrease in the efficiency of the FISH technique and increase in the number of FISH panels, the main purpose was to combine the 12 chromosomes analysed in a way that allows performance of the analysis in the lowest number of FISH rounds. Based on this, Telomeric 20p (Spectrum Green), Telomeric 14q (Spectrum Orange) and Centromeric 8 (Spectrum Aqua) were chosen as the probes for chromosomes 8, 14 and 20. This combination allows for the analysis of 12 chromosomes in three rounds of FISH, which would not jeopardize the efficiency of a potential extra panel for NNR. Regarding timing for results, although telomeric probes are included, the relatively large size of the probes 160 kb and 110 kb for Telomeric 20p and Telomeric 14q, respectively, allows acceptable signals to be obtained with a minimum of 4 h of hybridization. An assessment of proper performance of this extra panel can be made by looking at the NR rate observed for chromosomes 8, 14 and 20 compared with that observed for the standard nine-chromosome test. NR rate for chromosomes 8 and 14 fall within the range observed for the nine chromosomes included in the two previous panels, with only chromosome 20 being slightly above, probably due to the difficulty of obtaining proper signals with Spectrum Green in poorly fixed nuclei. Another parameter that assesses the effectiveness of this 12-chromosome analysis approach is the high efficiency of the NRR technique, which does not show any significant difference if applied before or after the 8, 14, 20 panel is applied, and which is higher than that previously described (Colls *et al.*, 2007).

Although all the parameters analysed are essential to the proper performance of FISH analysis in PGD, the ultimate purpose of the approach is to increase the efficiency of the PGD test itself by increasing the pregnancy rate. In order to accomplish this, it is paramount to transfer embryos normal for all chromosomes analysed. It is known that trisomies for chromosomes 8 and 20 are highly involved in spontaneous abortions (Lathi *et al.*, 2008) and that, in general, true monosomies do not reach the blastocyst stage, with the exception of those for chromosomes X and 21, but that they can lead to blastocyst formation in their mosaic form (Sandalinas *et al.*, 2001). Recent studies on comparative genomic hybridization indicate however that, using new culture systems, monosomies can indeed reach the blastocyst stage (Fragouli *et al.*, 2008).

This study has shown that, although only 4.3% of the total analysed embryos showed aneuploidy for chromosomes 8, 14 or 20 only, the extended panel allowed screening against 12.7% of embryos that were diagnosed as normal with the nine-chromosome test. The extended panel allowed re-diagnosis as aneuploid for chromosomes 8, 14 or 20, 12.7% of embryos that were diagnosed as normal with the nine-chromosome test. Since the extended test was performed *a posteriori*, some embryos abnormal for chromosomes 8, 14 or 20 were transferred to the patient, alone or along with some normal embryos for these chromosomes. Therefore, three different combinations of embryos resulted for transfer: all embryos being normal, all embryos being abnormal or a mixture of normal and abnormal embryos. As expected, when the pregnancy rate for each group was analysed, it was significantly higher ($P < 0.05$) when at least one normal embryo was transferred to the patient (58.4%, 52/89) versus when only abnormal embryos were transferred (12.5%, 1/8). Pregnancies when only abnormal embryos for chromosomes 8, 14 or 20 are transferred can be due to errors in diagnosis (8.4%) and can lead to spontaneous abortions. Indeed, the only pregnancy resulting in this group was a biochemical pregnancy.

Additionally, there was no significant difference in pregnancy rate between transferring only normal embryos (57.5%) compared with transferring a mixture of normal and abnormal embryos (62.5%) suggesting that either embryos abnormal for chromosomes 8, 14 or 20 do not have implantation potential, which is the most likely explanation for true monosomies, or that they do implant but they are eliminated at a later stage in the case of trisomies or mosaics.

In a previous study by Abdelhadi *et al.* (2003), a third panel consisting of chromosomes 2, 3, 4, and 11 was assessed and, although 15% of all embryos were abnormal, the majority of them were also abnormal for the two first panels and only 3% were abnormal only for these chromosomes; thus, this panel gave little extra value. Instead, in the present study, 35 (12.7%) of the embryos were abnormal only for chromosomes 8, 14 or 20.

Regarding accuracy of the expanded 12-chromosome panel plus NRR, the reanalysis results of group 2 embryos showed a 8.4% error rate, which is slightly above the 4.7% found for the standard nine-chromosome panel plus NRR (Colls *et al.*, 2007). The two main reasons that account for this increase are the analysis of chromosomes 8, 14 and 20 as a third-panel FISH and the use of telomeric probes, which due to their smaller size are more prone to produce erroneous signals especially when the fixation is suboptimal. Still, this is a significant improvement on previous studies showing 20% (Liu *et al.*, 1998; Vollmer *et al.*, 2000) or 12% (Abdelhadi *et al.*, 2003) error rate with a third panel. This difference could be due to the use of NRR and more attention to fixation technique. Also, the present error rate and the error rate obtained by Colls *et al.* (2007) by

analysing all the cells from abnormal embryos compare very favourably with other reported error rates for centres not using NRR and different fixation and scoring methods (Ziebe *et al.*, 2003; Baart *et al.*, 2004; Coulam *et al.*, 2007). Most probably, the error rate and NR rate of a PGD laboratory are the most objective measures of the quality of that laboratory and it is highly consequential in the clinical outcome of PGD, probably just as much as the damage produced by embryo biopsy. In that regard, an acceptable error rate and low no result (Staessen *et al.*, 2004) can be jeopardized by biopsing two cells (Staessen *et al.*, 2004; Cohen *et al.*, 2007; Goossens *et al.*, 2008) or by detrimental biopsing practices (Cohen and Grifo, 2007; Kuliev and Verlinsky, 2007; Mastenbroek *et al.*, 2007; Munné *et al.*, 2007; Simpson, 2008).

To conclude, this study proves that the high efficiency and practical feasibility of this FISH protocol for the analysis of 12 chromosomes plus 'no result rescue' in PGD for aneuploidy is a superior approach than the standard nine-chromosome analysis in order to screen for embryo chromosome abnormalities.

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