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PGD for reciprocal and Robertsonian translocations using array comparative genomic hybridization

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BACKGROUND: Fluorescence *in situ* hybridization (FISH) is the most widely used method for detecting unbalanced chromosome rearrangements in preimplantation embryos but it is known to have several technical limitations. We describe the clinical application of a molecular-based assay, array comparative genomic hybridization (array-CGH), to simultaneously screen for unbalanced translocation derivatives and aneuploidy of all 24 chromosomes.

METHODS: Cell biopsy was carried out on cleavage-stage embryos (Day 3). Single cells were first lysed and DNA amplified by whole-genome amplification (WGA). WGA products were then processed by array-CGH using 24sure + arrays, BlueGnome. Balanced/normal euploid embryos were then selected for transfer on Day 5 of the same cycle.

RESULTS: Twenty-eight consecutive cycles of preimplantation genetic diagnosis were carried out for 24 couples carrying 18 different balanced translocations. Overall, 187/200 (93.5%) embryos were successfully diagnosed. Embryos suitable for transfer were identified in 17 cycles (60.7%), with transfer of 22 embryos (mean 1.3 $\pm$ 0.5). Twelve couples achieved a clinical pregnancy (70.6% per embryo transfer), with a total of 14 embryos implanted (63.6% per transferred embryo). Three patients delivered three healthy babies, during writing, the other pregnancies (two twins and seven singletons) are ongoing beyond 20 weeks of gestation.

CONCLUSIONS: The data obtained demonstrate that array-CGH can detect chromosome imbalances in embryos, also providing the added benefit of simultaneous aneuploidy screening of all 24 chromosomes. Array-CGH has the potential to overcome several inherent limitations of FISH-based tests, providing improvements in terms of test performance, automation, sensitivity and reliability.

Key words: PGD / preimplantation genetic screening / chromosomal translocation / array comparative genomic hybridization / 24 chromosomes screening

Introduction

Individuals who carry a balanced reciprocal or Robertsonian translocation are known to have high rates of unbalanced gametes following meiotic segregation and embryos with unbalanced chromosome complements (Munné *et al.*, 2000; Verlinsky *et al.*, 2005). These patients have a greater chance of being infertile and/or at high risk of conceiving chromosomally abnormal pregnancies that lead to recurrent spontaneous abortions or children with congenital anomalies and mental retardation (Scriven *et al.*, 1998).

Previous studies have shown that preimplantation genetic diagnosis (PGD) for translocations has the potential to improve live birth rates by reducing the risk of recurrent spontaneous abortions, minimizing the risk of conceiving a chromosomally abnormal baby or improving pregnancy rates in infertile couples (e.g. after failed IVF attempts) (Munné *et al.*, 1998a, 2000, 2002; Scriven *et al.*, 1998; Fischer *et al.*, 2010). Use of PGD for carriers of balanced translocations can decrease the risk of adverse outcomes by selecting for transfer only those embryos with a normal/balanced chromosomal complement (Munné *et al.*, 1998a; Verlinsky *et al.*, 2005; Fischer *et al.*, 2010).

Fluorescence *in situ* hybridization (FISH) is, to date, the most widely used method for detecting unbalanced chromosome rearrangements in embryos (Harper *et al.*, 2010). Historically, different strategies have been used in PGD for structural chromosome abnormalities. Early studies involved the use of chromosome painting probes

applied to polar bodies after metaphase conversion to diagnose translocations of maternal origin (Munnè et al., 1998b; Verlinsky and Evsikov, 1999). A major drawback of this method is that only translocations carried out by the mother can be analysed. A more widely used method involves analysis of interphase chromosomes in cleavage-stage embryos. FISH strategies for detecting reciprocal translocations on blastomeres initially involved probes that spanned (Munnè et al., 1998c) or flanked (Conn et al., 1998; Munnè et al., 1998b) the specific translocation breakpoints. Strategies using FISH probes that span the breakpoints have the advantage that normal embryos can be differentiated from those carrying a balanced translocation, but they were limited by the cost and the time required to develop case-specific probes for each translocation carrier (Munnè et al., 1998c; Pierce et al., 1998).

A commonly used FISH strategy for detection of the abnormal segregation in reciprocal and Robertsonian translocations involves the use of commercially available centromeric, locus-specific and subtelomere probes, allowing for a simplified approach that laboratories can apply routinely (Scriven et al., 1998, 2001; Munné et al., 2000).

Although relatively successful, the FISH procedure is technically demanding and harbours several technical limitations that are well-documented (Munnè, 2002; Velilla et al., 2002; De Ugarte et al., 2008) and include hybridization failure (lack of FISH signals), signal overlap, signal splitting and poor probe hybridization, as well as problems related to the fixation process, such as cell loss and variable cell fixation. Since FISH was first introduced in clinical diagnosis, improvements have been established to diminish the error rate of the technique (Cohen et al., 2007; Colls et al., 2007; Goossens et al., 2008). Technical skill and sound laboratory practices can minimize most of the limitations of FISH-based methods but certain shortcomings remain. Errors related to the technical issues described earlier can affect the accuracy of the interpretation of results, leading to misdiagnosis of embryos both in eliminating suitable (normal/balanced) embryos for transfer, or worse, including abnormal embryos in the transfer cohort (Wilton et al., 2009). Error rates of FISH protocols for translocation have been reported in some studies to range from 0 to 10%, with an average error rate of 6% (Munné et al., 2000; Velilla et al., 2002; Li et al., 2005).

Increasingly, new techniques for chromosome analysis in embryos are being sought in an attempt to improve on current FISH test method performance. Recently, a PCR-based PGD approach for the detection of chromosomal imbalances in embryos from both reciprocal and Robertsonian translocation carriers was proposed as a valuable alternative to the FISH-based PGD protocols (Fiorentino et al., 2010; Traversa et al., 2010). This approach aimed to overcome several of the limitations listed above related to the FISH technique, also providing significant improvements in terms of test performance, automation, turnaround time, cost effectiveness, sensitivity and reliability of the information obtained.

It is relatively common for PGD of chromosomal translocations to be combined with aneuploidy screening, to assess common aneuploidies for patients of advanced maternal age. This can be carried out using both FISH and PCR-based protocols but involving aneuploidy screening of only a limited number of chromosomes, focusing on the chromosomes most often found to be aneuploid in prenatal samples or from miscarriages (Kuliev et al., 2002; Jansen et al., 2008; Colls et al., 2009). This approach may result in the transfer of reproductively incompetent embryos with aneuploidy for chromosomes not analysed.

Comprehensive chromosome screening techniques, such as array comparative genomic hybridization (array-CGH) Le Caignec et al. (2006), Wells et al. (2008), Gutiérrez-Mateo et al. (2011) have been introduced recently into routine PGD laboratory practices. The array-CGH procedure involves screening of the entire chromosome complement, rather than the limited chromosome assessment typically used for the purpose of preimplantation genetic screening (PGS).

Some of the most promising progress toward developing a comprehensive 24 chromosome analysis method has been made possible through the combination of whole-genome amplification (WGA) and array-CGH (Hu et al., 2004; Le Caignec et al., 2006; Fiegler et al., 2007). Although this combined approach represents exciting and potentially important advances towards improved PGS, its possible clinical use in PGD for structural chromosomal abnormalities still remains unexplored. This technique, applied to embryos derived from translocation carriers, would offer a complete analysis of the embryo by providing information for not just the chromosomes involved in the translocation but for all 24 chromosomes.

This study describes the clinical application of array-CGH technology to simultaneously screen embryos from both reciprocal and Robertsonian translocation carriers for unbalanced translocation derivatives, as well as aneuploidy of all 24 chromosomes. The assay was clinically applied in 28 PGD cycles, resulting in the establishment of chromosomally balanced pregnancies in 12 couples.

Materials and Methods

Case referrals and patient counselling

All the couples involved in the study were first seen by a clinical geneticist, who assessed the feasibility of carrying out the diagnosis for the specific chromosomal translocation at single cell level. Genetic counselling consisted of reviewing the couple's genetic history and their reason(s) for requesting PGD, followed by an explanation of the PGD process for the specific translocation involved, and a discussion on the accuracy of the procedure and its limitations. A calculation of the possible genetic outcomes, the success rates and the risk of misdiagnosis were also discussed. The patients were then referred to the collaborating IVF clinics to arrange the clinical aspects of the treatment. Only couples undergoing fresh or cryopreserved cycles, where the female partner was younger than 43 years old, with normal ovarian reserve, no uterine malformation, no other genetic indication and with at least cumulatively four good quality Day 3 embryos, were finally included.

A written informed consent was obtained from the couples, as approved by the Institutional Review Board of both GENOMA and the collaborating IVF clinics, in which the possible risk of misdiagnosis was specified and confirmatory prenatal diagnosis for any ensuing pregnancy was recommended. All the centres participating in the study have obtained ethical approval.

Study design and clinical cases

Briefly, the couples included in this prospective longitudinal cohort study were 8 Robertsonian carriers and 16 reciprocal translocation carriers. Primary outcome measure was accuracy of the chromosomal analysis; secondary outcome measures were clinical outcomes. The karyotype of the 18 different translocations for which PGD has been performed is listed in Table I.

Table I The karyotype of the 18 different reciprocal and Robertsonian translocations for which PGD has been performed using array-CGH.

Translocation	Chromosome A[a]		Chromosome B[b]	
	Centric segment[c]	Translocated segment[c]	Centric segment[c]	Translocated segment[c]
45,XY,der(13;14)(q10;q10)	–	115.2	–	107.3
45,XY,der(14;21)(q10;q10)	–	107.3	–	48.1
46,XX,t(1;5)(q24;q13)	165.5	83.7	114.2	66.7
46,XX,t(1;8)(p34;p22)	202.3	46.9	127.6	18.8
46,XX,t(1;10)(p12;p11)	129.4	119.8	97.5	38.0
46,XY,t(2;16)(q12;p11)	102.7	140.5	55.8	34.6
46,XX,t(3;10)(q13;p13)	102.8	95.22	118.3	17.2
46,XY,t(3;18)(p26.3;q12)	195.5	2.5	53.2	24.9
46,XY,t(5;8)(q31.1;q22.1)	130.6	50.3	99.0	47.4
46,XY,t(5;19)(q31;p13.1)	130.6	50.3	39.2	19.9
46,XY,t(7;10)(q32;q26)	127.1	32.0	119.1	16.4
46,XX,t(7;17)(q33;p12)	132.5	26.6	65.4	15.8
46,XX,t(10;12)(q24;q24.2)	96.9	38.6	114.2	19.6
46,XX,t(10;18)(q25;q21.2)	105.7	29.8	48.2	29.9
46,XX,t(11;13)(p15;q22)	113.3	21.7	73.2	42.0
46,XY,t(15;20)(q26;q11)	89.0	13.5	29.2	33.8
46,XX,t(17;19)(q21;q13.1)	38.1	43.1	32.3	26.8
46,XX,t(18;19)(q12;q13.2)	53.2	24.9	38.6	20.5

[a]First chromosome of the karyotype formula.
[b]Second chromosome of the karyotype formula.
[c]Expected minimum size of imbalance in Mega base (Mb), according to Ensembl database (http://www.ensembl.org/Homo_sapiens/).

IVF and embryo biopsy procedure

Cleavage-stage embryos were obtained using a standard IVF procedure, as previously described (Kokkali *et al*., 2007). At 62–64 h post insemination, embryos with ≥6 cells and ≤50% fragmentation were placed into 20 μl of Ca^{2+}/Mg^{2+} free buffered media under mineral oil and subjected to biopsy following zona ablation using a non-contact laser. One blastomere from each embryo was removed with micromanipulation; biopsied cells were washed and placed into sterile 0.2 ml PCR tubes containing 2 μl of phosphate-buffered saline (Cell Signalling Technologies, Beverly, MA, USA). Several negative controls, consisting of 1 μl taken from the cell washing drops, were placed in separate tubes.

Cell lysis and WGA

Single blastomeres and negative controls were then lysed and genomic DNA amplified using the SurePlex DNA Amplification System (BlueGnome, Cambridge, UK), according to the manufacturer's instructions. SurePlex uses a WGA procedure based on random fragmentation of genomic DNA and subsequent amplification by PCR utilizing flanking universal priming sites. One nanogram of genomic DNA and one reagent negative control (amplification mixture only) were also subjected to WGA.

Array-CGH

WGA products were processed according to the BlueGnome 24sure + protocol (available at www.cytochip.com). These products are fluorescently labelled and competitively hybridized to specific arrays for translocations (24sure+, BlueGnome, Cambridge, UK) with a matched control in an array-CGH experiment format. The increased genomic coverage of 24sure+ arrays (approximate screening resolution 0.5 Mb across the genome and 0.25 Mb in peri-centromeric and sub-telomeric regions, human genome build NCBI36) enables a better detection and characterization of segmental chromosome imbalances in single cells.

A laser scanner InnoScan® 710 AL (INNOPSYS, Carbonne, France) was used to excite the hybridized fluorophores and read and store the resulting images of the hybridization. Scanned images were then analysed and quantified by algorithm fixed settings in BlueFuse Multi Software (BlueGnome, Cambridge, UK), a software package that performed the steps of grid placement, quantification, normalization and post-processing automatically. Once a specific amplification was observed (i.e. low autosomal noise), autosomal profiles were analysed for gain or loss of whole chromosomal ratios using a 3 × SD assessment, greater than ± 0.3 $\log_2$ ratio call, or both. To pass hybridization quality controls, female samples hybridized with a male reference DNA (sex mismatch) had to show a consistent gain on chromosome X and a consistent loss of chromosome Y (Gutiérrez-Mateo *et al*., 2011).

The whole procedure was completed within 24 h, and the results were obtained in time for an embryo transfer at Day 5 or Day 6, in a fresh cycle.

PCR-based detection of unbalanced chromosomal rearrangements

WGA products were also processed, in parallel, with a different molecular-based assay, previously clinically validated on embryos (Fiorentino *et al*., 2010). This approach consisted of a PCR-based PGD protocol for detection of chromosomal imbalances and was applied as described elsewhere (Fiorentino *et al*., 2010).

Classification of the results

For the array-CGH approach, abnormalities are detected as copy number imbalances, when the ratio of a test sample to reference deviates from 1:1 for a series of data points. Embryos were diagnosed as 'normal/balanced' if the array-CGH plot generated shows no gain or loss greater than 3 × SD of autosome. Embryos were diagnosed as 'unbalanced' if the array-CGH results showed a 3 × SD of autosome (Gutiérrez-Mateo *et al*., 2011). A trisomy (partial or full) is detected as a shift of the clones for the specific chromosome towards the green line (gain). On the contrary, a monosomy (partial or full) is identified as a shift towards the red line (loss) (Figs 1–4). An 'inconclusive' diagnosis was assigned for those embryos where the signal pattern was not a clear-cut normal result. Sex-mismatched female samples show a consistent gain on chromosome X and a consistent loss of chromosome Y. Sex-matched male samples, instead, show no change on either chromosome X or Y.

For the PCR-based protocol, results were classified as described earlier (Fiorentino *et al*., 2010).

Follow-up analysis

In order to confirm the PGD results obtained with the array-CGH strategy, embryos diagnosed as unbalanced, that developed to blastocyst stage were re-biopsied on Day 6. Briefly, several trophectoderm cells (5–10 cells) were removed by micromanipulation from the same hole in the zona pellucida used on Day 3, as described elsewhere (Kokkali *et al*., 2007). The collected cells were then placed in individual tubes for re-analysis by array-CGH, using the same approach as described previously. Follow-up analysis was also performed for embryos that had been previously diagnosed as aneuploid, with the aim of evaluating the incidence of the mosaicism and to ascertain whether the tested blastomeres were representative for the whole embryo, so confirming the diagnosis performed at Day 3. We considered a followed-up embryo as misdiagnosed when the embryo was initially classified as abnormal, but found to be normal after re-analysis, regardless of the actual abnormal genotype.

According to the Italian regulation that forbids embryo wastage, all abnormal embryos that developed into morphologically normal blastocysts were cryopreserved immediately after re-biopsy.

In cases in which pregnancies were achieved, patients were advised to undergo conventional prenatal diagnosis to confirm the karyotype of the fetus. Whenever possible, DNA extracted from buccal cells from newborns was analysed by array-CGH analysis to demonstrate balanced-normal karyotypes.

Clinical data and definitions

The number of fertilized (two pronuclei) oocytes and the number of biopsied embryos were calculated on the basis of the total number of mature injected oocytes. According to Farquharson *et al*. (2005), clinical pregnancy was determined by ultrasound demonstration of a gestational sac at 7 weeks. Miscarriage was classified as 'early' (before 12 weeks) or 'late' (after 12 weeks). Implantation rate and ongoing implantation rate were defined as the number of gestational sacs per transferred embryo, and number of fetuses with heart activity beyond 12 weeks of gestation per transferred embryo, respectively.

Results

Twenty-eight cycles of PGD were carried out for 24 couples carrying 16 different reciprocal translocations and two different Robertsonian translocations (Table I). Eight cycles involved embryo cryopreservation at the two pronuclei stage for collection owing to a low response to ovarian stimulation.

A total of 363 oocytes were collected (range 6–23), 310 (85.4%) of them were mature metaphase II stage, 249 (80.3%) fertilized normally (range 4–18), resulting in 200 embryos (mean number 7.1 ± 3.1 per cycle; range 4–16), which were biopsied on Day 3 (Table II).

WGA was successful in 187 of 200 blastomeres (93.5%). Amplification failed in 6.5% (13/200) of the cells. Amplification was not detected in any of the negative controls (washing media or reagent negative controls).

A clear and well-defined array-CGH profile was achieved in 187/187 (100%) of the blastomeres with positive WGA. Overall, 187 (93.5%) embryos were successfully diagnosed; no diagnosis was obtained for 13 embryos, because of an amplification failure.

Of the embryos diagnosed, 16.0% (30/187) were normal or balanced for every chromosome, 17.1% (32/187) were unbalanced for the translocation and normal for aneuploidy, 39.6% (74/187) had aneuploidy and were unbalanced, 27.3% (51/187) were normal or balanced but also showed aneuploidy of chromosomes not involved in the translocation.

Overall, unbalanced translocation products were observed in 56.7% [106/187; 95% confidence interval (CI) 49.6–63.8] of the embryos tested; aneuploidy for non-translocation chromosomes observed in 66.8% (125/187; 95% CI 60.1–73.5) of the embryos. Eighty-one (43.3%; 95% CI 43.3–50.4) of the embryos were normal-balanced for the two chromosomes involved in the translocation (Supplementary data, Table S1).

The diagnosis obtained by array-CGH was also confirmed by the PCR-based PGD approach. In fact, paired comparison between microarray and PCR-based testing from individual embryos showed concordant results for all the embryos.

Embryos suitable for transfer at the blastocyst stage were identified in 17 of the 28 cycles (60.7%). In two PGD cycles, embryo transfer was cancelled because the morphology of the embryos was not of sufficiently high quality for transfer; in nine cycles, only aneuploid and/or unbalanced embryos were produced.

Following transfer of 22 embryos (mean 1.3 ± 0.5, range 1–2), 13 women (mean age 37.0 ± 2.6 years, range 33–42) had positive hCG levels (76.5% pregnancy rate per embryo transfer): 12 pregnancies continued, confirmed by at least one fetal sac and heart beat (70.6% pregnancy rate per embryo transfer) and one was a biochemical pregnancy only. A total of 14 embryos implanted and led to the presence of a fetal sac (63.6% implantation rate), resulting in 14 fetuses with a heart beat detected (63.6% fetal heart beat/embryo transferred). All pregnancies (2 twins and 10 singleton) have completed at least 20 weeks of gestation. Three patients have delivered three healthy babies, while the others are currently ongoing. Microarray analyses of newborn buccal cells have shown balanced-normal results for the translocation and euploidy.

After the clinical cases, 57 non-transferred embryos were reanalysed under the same conditions and procedure as that used for clinical PGD samples. Twenty-five embryos were previously diagnosed as both unbalanced and aneuploid, 32 were normal or balanced but also showed aneuploidy. The follow-up analysis was successful in all embryos, confirming the diagnosis in all non-transferred embryos reanalysed. Aneuploidy mosaicism in at least one chromosome was found in 24/57 (42.1%) embryos, involving a total of 80 chromosomes. Despite the high level of mosaicism found, all embryos that were classified as aneuploid after Day-3 diagnosis were diagnosed again as

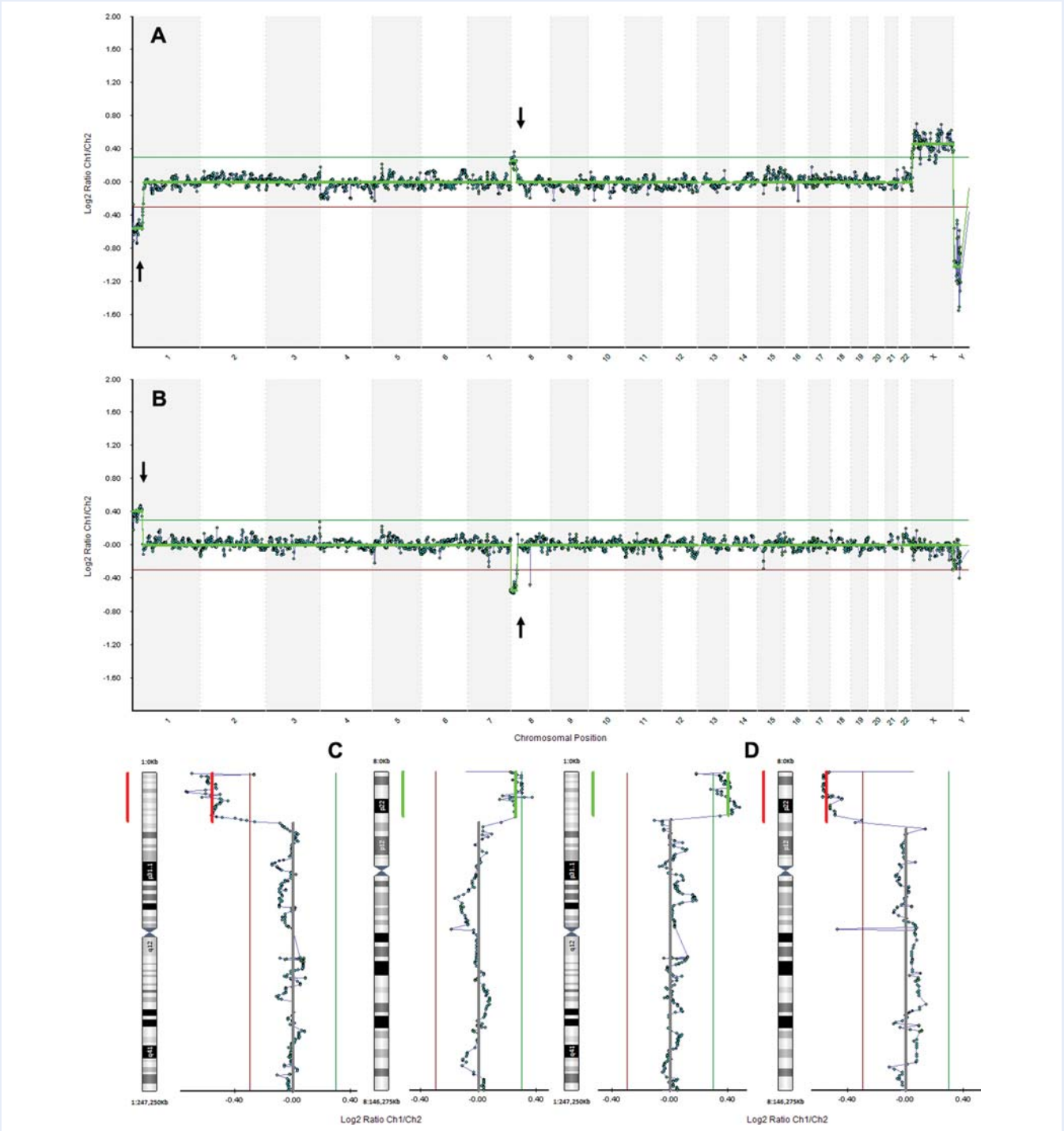

Figure 1 Examples of array-CGH-based PGD results from chromosomally unbalanced embryos derived from a patient carrying a balanced translocation 46,XX,t(1;8)(p34;p22), consistent with meiotic adjacent-1 segregation. Black arrows indicate chromosomal imbalances. (**A**) Embryo with a partial monosomy 1p34-pter, detected as a shift of the clones located in the above region towards the red line (loss), and a partial trisomy 8p22-pter identified with a shift of the specific clones towards the green line (gain). (**B**) Embryo with a partial trisomy 1p34-pter and a partial monosomy 8p22-pter. (**C**) Chromosomal details for segmental imbalances from (A), and (**D**) segmental imbalances from (B).

abnormal after re-analysis, confirming the previous results regardless of the actual abnormal genotype.

Examples of array-CGH results are shown in Figs 1–4. These results illustrate the utility of the approach for successfully identifying a variety of segmental imbalances associated with the inheritance of unbalanced translocation products (Figs 1–3), as well as aneuploidies of chromosomes unrelated to the translocation (Figs 2–4).

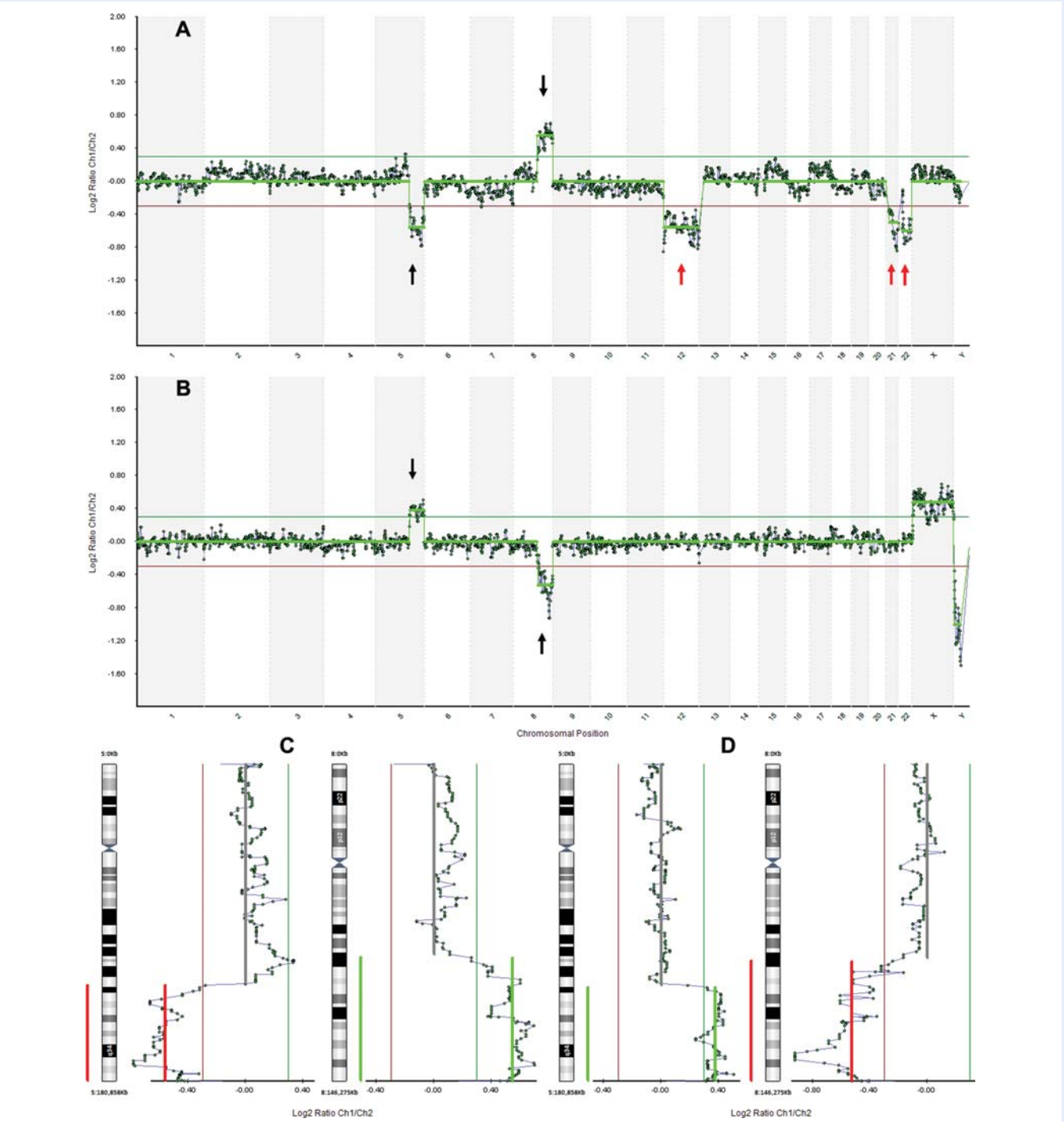

Figure 2 Examples of array-CGH-based PGD results from chromosomally unbalanced embryos derived from a patient carrying a balanced translocation 46,XY,t(5;8)(q31.1;q22.1), consistent with meiotic adjacent-1 segregation. Black arrows indicate chromosomal imbalances. (**A**) Unbalanced embryo with aneuploidy of chromosomes not involved in the translocation (loss of chromosomes 12, 21 and 22, indicated by the red arrows). The imbalances include a partial monosomy 5q31.1-qter, detected as a shift of the clones located in the above region towards the red line (loss) and a partial trisomy 8q22.1-qter identified with a shift of the specific clones towards the green line (gain). (**B**) Embryo with a partial trisomy 5q31.1-qter and a partial monosomy 8q22.1-qter. (**C**) Chromosomal details for segmental imbalances from (A), and (**D**) segmental imbalances from (B).

Discussion

Recently, we proposed a PCR-based PGD protocol for translocation testing as a valuable alternative to the FISH-based protocols (Fiorentino *et al.*, 2010) with the aim of overcoming several well-known technical limitations of the FISH technique.

This study has described the clinical application of a different molecular approach, known as array-CGH, for the detection of

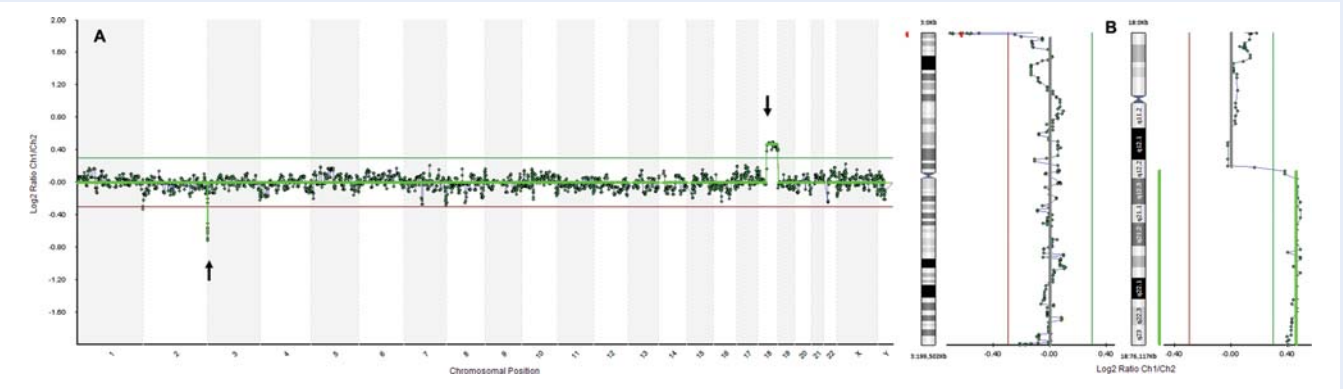

Figure 3 Estimation of the detection limits of array-CGH-based analysis of segmental imbalances. Examples of array-CGH results from an unbalanced embryo derived from a patient with karyotype 46,XY,t(3;18)(p26.3;q12). Black arrows indicate chromosomal imbalances. (**A**) Detection of a 2.5 Mb-sized segmental deletion in chromosome 3 (3p26.3-pter) and a 24.9 Mb-sized segmental duplication in chromosome 18 (18q12-qter). (**B**) Chromosomal details for the above segmental imbalances. These results demonstrate that array-CGH analysis can identify segmental aneusomy as small as 2.5 Mb with as few as 15 BAC clones in embryos from patients carrying a translocation.

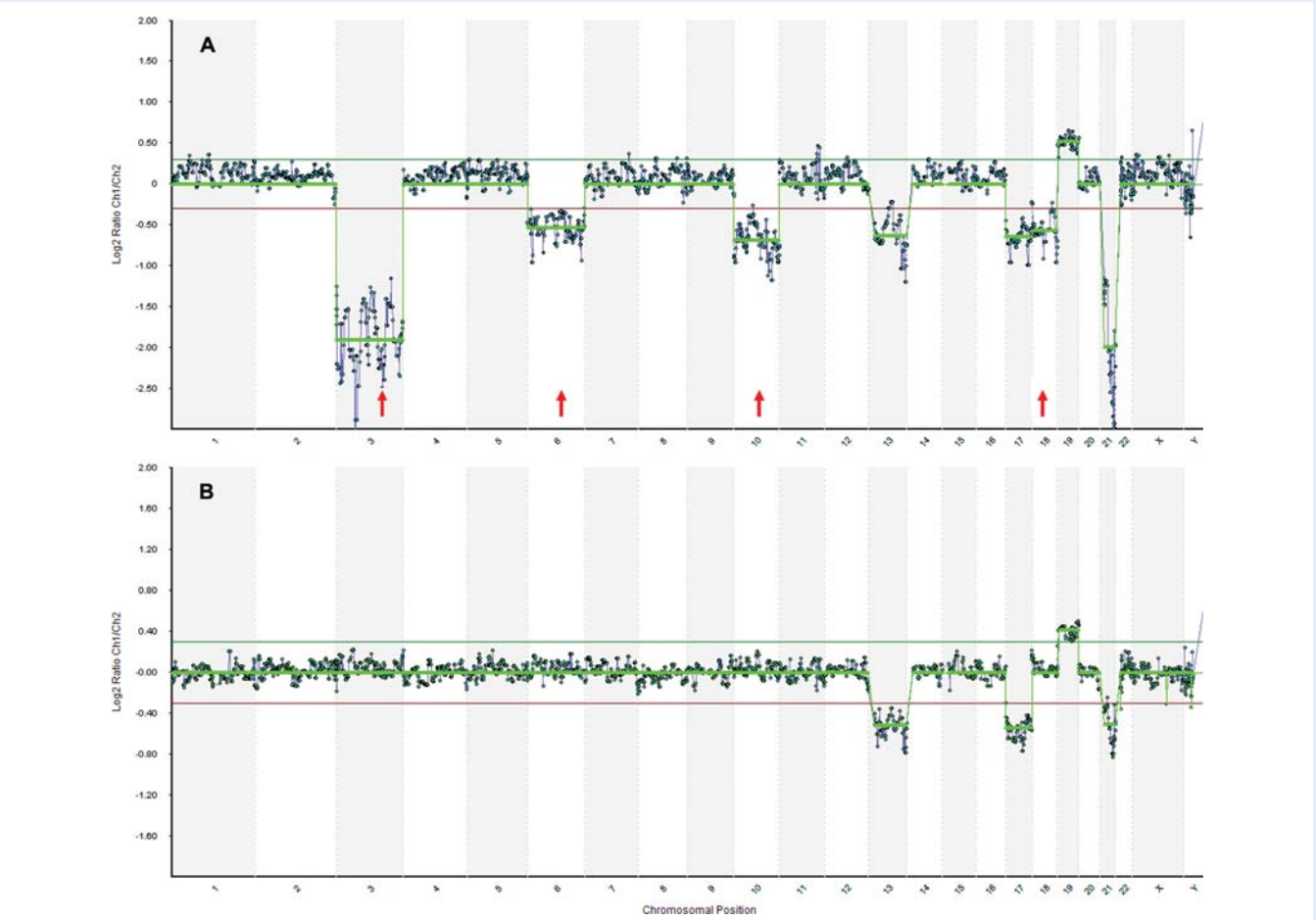

Figure 4 Example of mosaicism detected after re-analysis at blastocyst stage of an embryo that was normal-balanced for the two chromosomes involved in the translocation but presenting aneuploidy of chromosomes unrelated to the translocation. (**A**) Results after Day-3 diagnosis. (**B**) Results after re-biopsy at blastocyst stage. Follow-up analysis revealed a mosaicism for chromosomes 3, 6, 10 and 18 (red arrows). However, the embryo was still found to be abnormal, confirming the previous diagnosis at cleavage stage, regardless of the actual abnormal genotype.

Table II Clinical results from 28 PGD cycles for reciprocal and Robertsonian translocations.

Clinical data	Couples		Total
	Robertsonian translocation	Reciprocal translocation	
No. of couples treated	8	16	24
Maternal age (average, years)	38.0 ± 2.1	37.1 ± 3.7	37.4 ± 3.2
No. of cycles performed	10	18	28
No. of oocytes retrieved	121	242	363
No. of mature oocytes injected (%)[a]	97 (80.2)	213 (88.0)	310 (85.4)
No. of oocytes fertilized (%)[b]	73 (75.3)	176 (82.6)	249 (80.3)
No. of embryos thawed	2	15	17
No. of embryos surviving after thawing	2	15	17
No. of embryos biopsied	57	143	200
Mean	5.7 ± 1.8	7.9 ± 3.4	7.1 ± 3.1
No. of blastomeres analysed	57	143	200
No. of blastomeres with a WGA failure	6	7	13
No. of embryos diagnosed (%)	51 (89.5)	136 (95.1)	187 (93.5)
Balanced (%)	14 (27.5)	16 (11.8)	30 (16.0)
Unbalanced (%)	6 (11.8)	26 (19.1)	32 (17.1)
Balanced + aneuploid (%)	19 (37.3)	32 (23.5)	51 (27.3)
Unbalanced + aneuploid (%)	12 (23.5)	62 (45.6)	74 (39.6)
Total no. of balanced embryos (%)	33 (64.7)	48 (35.3)	81 (43.3)
Total no. of unbalanced embryos (%)	18 (35.3)	88 (64.7)	106 (56.7)
Total no. of aneuploid embryos (%)	31 (60.8)	94 (69.1)	125 (66.8)
No. of embryos transferable	14	16	30
No. of embryo transfers (%)	6 (60.0)	11 (61.1)	17 (60.7)
No. of embryos transferred	9	13	22
Mean	1.5 ± 0.5	1.2 ± 0.4	1.3 ± 0.5
Clinical pregnancy rate per OR	5/10 (50%)	7/18 (38.9%)	12/28 (42.9%)
Clinical pregnancy rate per embryo transfer	5/6 (83.3%)	7/11 (63.6%)	12/17 (70.6%)
Implantation rate	6/9 (66.7%)	8/13 (61.5%)	14/22 (63.6%)
Ongoing implantation rate	6/9 (66.7%)	8/13 (61.5%)	14/22 (63.6%)
Miscarriage rate (early and late)	0/5 (0%)	0/7 (0%)	0/12 (0%)
No. of pregnancies still ongoing	4	5	9
No. of pregnancies going to term	1	2	3
No. of babies born	1	2	3

OR, oocyte retrieval; ET, embryo transfer; WGA, whole-genome amplification.
[a]Calculated on no. of oocytes retrieved.
[b]Calculated on no. of mature oocytes injected.

chromosomal imbalances in embryos derived from translocation carriers. The analysis is fully automated and the whole procedure can be performed within 24 h. For Day 3 biopsy, it is possible to perform the embryo transfer on Day 5 of embryo development, in a fresh cycle.

A major advantage of the PCR-based and array-CGH PGD protocols over FISH is that they are not dependent on cell fixation onto a microscope slide, a critical step that requires skill and experience. Because the reliability of FISH analysis is linked to the quality of cell fixation, poorly fixed single cells will affect probe hybridization. The removal of a requirement for the more technically demanding cell fixation step simplifies the sample preparation procedure, with the potential to increase the percentage of embryos with a positive result. It may also make transport PGD easier, enabling broader use of PGD in IVF programmes because it is easier to train staff in, and monitor, the placing of single cells into PCR tubes than teaching any of the current spreading methods (Treff *et al.*, 2010a,b).

In addition, data analysis are performed by computational analysis of signal intensities and not by subjective signal scoring, as occurs with FISH analysis, allowing for easier and more reliable data interpretation. Finally, the molecular approaches are also amenable to automation for high throughput processing. The use of automated workstations for all manipulation greatly increases the number of samples that a laboratory can process and also reduces the risk of errors.

Beyond the technical advantages described for the molecular-based techniques lies another important advantage. The above approaches not only diagnose unbalanced inheritance of chromosomes in translocation carriers but also they allow the simultaneous screening of the embryos for single-gene disorders by PCR-based methods (such as for cases with combined indications for the couple, e.g. translocation + carrier status for a single-gene disorder), using the same DNA produced after the WGA reaction.

The array-CGH approach has several further advantages compared with the other techniques. In contrast to FISH and PCR, array-CGH allows screening for all aneuploidies in addition to the unbalanced derivatives associated with the specific translocation. It is relatively common for PGD of chromosomal translocations to be combined with aneuploidy screening to assess aneuploidies for patients of advanced maternal age (Gianaroli *et al*., 2002; Munnè *et al*., 2005). PGS aims to provide a means of identifying potentially viable euploid embryos for transfer, which may have higher chances of producing a pregnancy. With FISH, this is typically achieved by sequential rounds of hybridization followed by chemical stripping of bound FISH probes. However, it is well recognized that the accuracy of the FISH analysis decreases with each additional round of hybridization, as the DNA degenerates and hybridization efficiency is reduced (Harrison *et al*., 2000). Furthermore, the persistence of signals from the first round of FISH could result in incorrect interpretation of signals in the second round, thereby leading to a higher risk of misdiagnosis or loss of normal embryos to false-positive errors.

The PCR-based approach combines both tests in a single set of reactions, thereby minimizing loss of information. However, both FISH and PCR allow identification of aneuploidies only for a limited number of chromosomes (5, 9 or 12 chromosomes) (Kuliev *et al*., 2002; Jansen *et al*., 2008; Colls *et al*., 2009; Fiorentino *et al*., 2010). Several studies have demonstrated that aneuploidies may involve all 24 chromosomes (Fragouli *et al*., 2009; Vanneste *et al*., 2009; Treff *et al*., 2010a,b; Gutiérrez-Mateo *et al*., 2011) and this may result in the transfer of reproductively incompetent embryos with aneuploidy for chromosomes not analysed.

Recently, microarray-based approaches for 24-chromosome PGS have been proposed in order to overcome the technical difficulties that beset earlier PGS studies, allowing screening of the entire chromosome complement, rather than the limited chromosome assessment (Gutiérrez-Mateo *et al*., 2011; Treff *et al*., 2010a,b). Data from comprehensive aneuploidy screening showed that aneuploidies may occur in preimplantation embryos in any of the 24 chromosomes, indicating that aneuploidy screening of all chromosomes is necessary to determine whether an embryo is chromosomally normal (Gutiérrez-Mateo *et al*., 2011; Treff *et al*., 2010a,b).

The importance of performing aneuploidy screening in parallel with the diagnosis of chromosomal imbalances in embryos derived from translocation carriers was suggested in previous studies (Gianaroli *et al*., 2002; Pujol *et al*., 2006). This was also confirmed in this study by the prevalence of aneuploidy in embryos that were diagnosed normal or balanced for the chromosomes involved in the translocation. In fact, among 81 embryos diagnosed as normal-balanced for translocation, 24-chromosome aneuploidy screening identified as not suitable for transfer 51 embryos presenting aneuploidy of chromosomes unrelated to the translocation. These embryos would have been selected for transfer if only translocation screening had been performed: this represents 63% of the embryos that would have been diagnosed as balanced-normal by translocation screening alone, and 27.3% of the entire cohort of embryos tested.

It can be argued that PGS analysis performed on Day-3 embryos could have a negative impact on clinical outcome of PGD because the tested blastomeres may not representative for the whole embryo owing to mosaicism or the possibility of self-correction of aneuploidy within the embryo. In order to verify the exact cytogenetic constitution of the embryos, non-transferred aneuploid embryos were re-biopsied at blastocyst stage before cryopreservation. Although follow-up analysis revealed a high level of mosaicism in at least one chromosome (42.1%), all embryos that were diagnosed as aneuploid after Day-3 analysis were again identified as abnormal after re-analysis, confirming the previous diagnosis regardless of exact genetic conformation. A larger follow-up study, involving re-analysis of embryos from Day-3 24-chromosome PGS cycles, is ongoing in order to confirm the above results in a wider sample size. However, it is reasonable to assume that patients with balanced translocations will also benefit from aneuploidy screening even if embryo biopsy is performed at cleavage stage.

Another advantage of the use of array-CGH technology is that it does not require preclinical validation before each IVF cycle, which is required for FISH or PCR-based methods of translocation screening. This spares couples the cost of work-up testing and also avoids delays to the start of IVF treatment. In fact, a PGD cycle can be scheduled directly on the day of biopsy, based on the number of embryos available for biopsy.

Finally, array-CGH can also be used for more complex karyotypes, with multiple rearrangements, where FISH testing is generally very complicated, although the chances of getting euploid normal/balanced embryos from these cases are rare.

The array-CGH approach carried out for this series of patients has been shown to be efficient at the single cell level (Figs 1–4), producing profiles with a low amount of noise, that can often be introduced by the WGA reaction. An accurate array-CGH profile was achieved in 187/187 (100%) of the blastomeres with positive WGA and a successful diagnosis in 187 (93.5%) of the embryos analysed. Paired comparison between microarray and PCR-based testing confirmed the array-CGH results from 106 embryos diagnosed as unbalanced, as well as 81 normal-balanced embryos. These results demonstrate the ability of the array-CGH approach to detect segmental chromosomal deletions and duplications in embryos derived from translocation carriers, allowing the successful identification of a variety of 68 segmental imbalances derived from 18 different translocations.

The results obtained also allowed estimation of the detection limits of array CGH-based analysis of segmental imbalances from single cells. The probability of detecting an unbalanced translocation, and therefore the success of the array-CGH-based analysis, is dependent upon the location of the translocation breakpoints in the chromosomes and the size of the unbalanced region(s). Small imbalances can be difficult to detect when their breakpoints fall close to the telomeres. In the case of 46,XY, t(3;18)(p26;q12) translocation, loss of a 2.5 Mb chromosome 3p26.3-pter segment could be observed, with a 15 bacterial artificial chromosome (BAC) clone-set with coverage sufficient to observe a loss (Fig. 3). These results indicate that array-CGH analysis can reliably identify segmental aneusomy as small as 2.5 Mb, with as few as 15 BAC clones, in embryos from patients carrying a

translocation. Compared with another comprehensive approach, such as single nucleotide polymorphism (SNP) microarrays, recently proposed for PGD from trophectoderm cells (Treff et al., 2010a,b), array-CGH provided a similar high resolution and efficiency, even if performed from single blastomeres.

Although the above results indicate that the array-CGH procedure is reliable and suitable for routine clinical application, the limitations must be considered.

Array-CGH cannot detect haploidy and some polyploidies, such as 69,XXX, 92,XXXX or 92,XXYY, as well as balanced translocations, as there is no imbalance in the total DNA content.

Additionally, the occurrence of a contamination event with exogenous DNA may reduce the number of embryos with a conclusive diagnosis using this technique.

Another potential limitation for routine application of this approach is that DNA amplification protocols require a minimum level of experience in molecular biology as well as a laboratory environment where DNA contamination is avoided.

Unlike PCR and SNP microarrays, array-CGH does not allow for tracking of inheritance of each chromosome; so, it cannot detect uniparental disomy, a rare event where both chromosomes are inherited from one parent and no chromosomes are inherited from the other (Kuwano et al., 1992; Tomkins et al., 1996).

Finally, microarrays represent at this time an expensive option for embryo testing compared with the other conventional methods, although the cost of array-based testing will hopefully continue to drop, as often happens for most new technologies. The initial set-up costs as well as the expertise levels required may mean that FISH or PCR-based protocols are still appropriate for some PGD laboratories.

The clinical outcomes obtained in this study from PGD cycles performed with the array-CGH approach were very encouraging, resulting in a clinical pregnancy rate of 70.6% per embryo transfer (mean maternal age 37.0 $\pm$ 2.6 years) and an ongoing implantation rate of 63.6%, which is readily comparable with recent results from other molecular-based approaches, but with lower mean maternal age (Fiorentino et al., 2010; Treff et al., 2010a,b). In fact, Treff et al. (2010a,b) and Fiorentino et al. (2010) reported a clinical pregnancy rate per embryo transfer of 75%, with a mean maternal age of 29.6 and 36.1 $\pm$ 4.4 years, respectively. Other previous studies (Gianaroli et al., 2003; Verlinsky et al., 2005; Lim et al., 2008; Verpoest et al., 2009; Fischer et al., 2010) as well as the most recent European Society of Human Reproduction and Embryology PGD Consortium data collection (Harper et al., 2010) using FISH-based testing on Day-3 blastomeres reported clinical pregnancy rate and implantation rate values per embryo transfer significantly lower than those obtained with the molecular studies, ranging from 23.2 to 39.0% (mean maternal age range: 31.3–34.0 years).

In conclusion, we reported healthy births following PGD for chromosomal translocation on cleavage-stage embryos by array-CGH. The results achieved demonstrate the reliability and feasibility of the array-CGH protocol for detection of chromosomal imbalances in embryos. The method presented here provides a valuable alternative, and an improvement, on currently available FISH tests for translocations, especially in terms of test performance, automation, reliability of the information obtained, sensitivity and pregnancy outcome. Considering the encouraging clinical outcomes obtained, it is expected that simultaneous translocation and comprehensive aneuploidy screening will enhance embryo selection and improve IVF outcomes for patients carrying balanced reciprocal or Robertsonian translocations.

Authors' roles

F.F. conceived the study, validated array-CGH results, performed data collection and data analysis and prepared the manuscript. S.L. performed the array-CGH experiments and interpreted the results. B.S. performed the array-CGH experiments and interpreted the results. B.A. performed the array-CGH experiments, carried out PCR analysis and interpreted the results. K.G. performed embryology and was involved in sample collection. R.L. performed embryology and was involved in sample collection. U.F.M. was involved in patients' management. G.A. provided both guidance and support. P.K. was involved in patients' management.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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