Article

Successful pregnancies after application of array-comparative genomic hybridization in PGS-aneuploidy screening



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Abstract

Recurrent IVF failure, implantation failure and early embryo demise can be attributed to the high frequency of chromosomal aneuploidy observed in human embryos. Preimplantation genetic screening (PGS) using multiple displacement amplifications (MDA) and array comparative genomic hybridization (aCGH) was successfully performed on eight patients with a minimum of seven recurrent IVF failures with the aim of detecting aneuploidy and ameliorating pregnancy rate. A total of 41 embryos with eight or more cells were biopsied by taking two blastomeres from each embryo. The DNA from these blastomeres were amplified and analysed by aCGH technology. The aCGH results showed a complex panel of chromosomal abnormalities in 60% of the diagnosed embryos. Some abnormalities could not be detected by the seven-probe panel (13, 16, 18, 21, 22, X and Y) used in fluorescence in-situ hybridization. Six out of eight patients had embryos for transfer with five out of those six showing positive pregnancy tests. As far as is known, this report is the first to show a pregnancy after PGS using the aCGH technology. The pregnancy rate obtained here is encouraging and will open the door for enrolment of more patients.

Keywords: *aneuploidy, array-CGH, comparative genomic hybridization, FISH, PGS, preimplantation genetic screening*

Introduction

Preimplantation genetic screening (PGS) is a technique used to identify chromosomal aneuploidies in embryos; only embryos that are euploid for the chromosomes tested are transferred. This technique has been suggested and used to improve pregnancy rates for the following indications: (i) advanced maternal age; (ii) repeated IVF failure; (iii) repeated miscarriage; and (iv) testicular sperm extraction (Caglar *et al.*, 2005; Gianaroli *et al.*, 2005; Donoso and Devroey, 2007; Kuliev and Verlinsky, 2008). Although improvements in IVF outcome after PGS have been observed in multiple case–control studies (Munné *et al.*, 2007a,b), its effectiveness in randomized controlled studies is still limited. One of the main reasons for this limitation is in the fluorescence in-situ hybridization (FISH) technique. Certainly, FISH has been used so far for aneuploidy assessment in embryos by using probes capable of enumerating up to 15 chromosomes (Baart *et al.*, 2007). However, two major concerns are still hampering the application of such a strategy. First, extending the FISH procedure beyond the usual two rounds of sequential hybridization is associated with a reduction in accuracy. Second, abnormalities affecting the remaining chromosomes and rearrangements involving deletions or duplications are usually missed. Therefore, assessment of the complete set of cell chromosomes using the technique of array comparative genome amplification (aCGH), which detects complete (aneuploidy) or incomplete gain or loss in genomic DNA, would be critically required. Consequently, PGS using aCGH on embryos could form the basis for most conclusions regarding the outcome of aneuploidy prevention in IVF pregnancies. As far as is known,

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there are currently no successful pregnancies reported as a result of applying aCGH in clinical PGS settings. In the current study, aCGH technology was applied for PGS-aneuploidy screening, in patients with recurrent IVF failures.

Materials and methods

Patient enrolment

Eight couples that had experienced a minimum of seven consecutive IVF failures (mean maternal age 36.25 years) were enrolled in the PGS programme and followed a routine stimulation protocol (Hellani *et al.*, 2008) after signing an informed consent.

FISH protocol

Two rounds of five-colour FISH were applied to single blastomeres or embryos. In the first round, FISH was performed for chromosomes 18 (aqua), X (blue), 13 (red), Y (gold) and 21 (green) using Multi-Vysion PGT Fluorescent Probe Kit (Abbott Molecular Inc., Des Plaines, IL) according to the manufacturer's protocol. Briefly, denaturation and hybridization were performed in the HYBrite using denaturation for 5 min at 73°C and 6 h at 37°C. After hybridization, cover slips were removed and slides were washed in 0.4× saline sodium citrate (SSC)/0.3% IGEPAL for 4 min at 73°C, then in 2× SSC/0.1% IGEPAL for 30 s at room temperature. The slides were mounted in anti-fade II medium (Abbott Molecular Inc.). After analysis of the fluorescent signals, slides were dipped in distilled water (73°C) until the cover slips were detached. A second round of FISH, as above, were applied for chromosomes 13 (red), 16 (aqua), 18 (blue), 21(green) and 22 (gold).

Multiple displacement amplification (MDA) protocol

The two blastomeres from each embryo were collected in 0.5 ml polymerase chain reaction (PCR) tubes containing 5 µl of 1× of phosphate-buffered saline (PBS). Blank samples were also run in parallel with the tested samples in order to detect any contamination (if present). As described previously (Sermon *et al.*, 1998), 3 µl of alkaline buffer (ALB; 200 mmol/l NaOH, 50 mmol/l dithiothreitol) were added to the samples and blanks. After 15 min incubation at 4°C, 3 µl of neutralization buffer (900 mmol/l Tris–HCl, 300 mmol/l KCl, 200 mmol/l HCl) were added to the solution. Cell lysates were used directly for whole genome amplification using MDA (GE Healthcare, USA) by adding 18 µl of the master mix in a total volume of 30 µl. The mix was then incubated at 31°C for 2 h and followed by heat inactivation at 65°C for 10 min. MDA yield was quantified on a fluorometer using picogreen quantification kit (Molecular Probes Inc., Eugene, USA). Blanks did not show any amplification.

Optimization of single-cell aCGH

Agilent human genome CGH 44B Oligo Microarray kit (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to detect chromosomal aneuploidies in two blastomeres of 20 non-transferred embryos due to known abnormalities previously diagnosed by FISH during routine PGS-aneuploidy screening. The blastomeres were amplified by MDA. In parallel, 0.5 ng of control DNA was amplified, using the same MDA technique, and used as control in the aCGH procedure. Equal quantities of DNA from amplified blastomeres and controls were purified using QIAprep Spin Miniprep columns (Qiagen, USA), collected in 50 µl of water and digested using *Rsa*1 and *Alu*1 restriction enzymes. Briefly, 50 µl of MDA product was digested using 50 units of *Alu*1 (Roche, Mannheim, Germany) and 50 units of *Rsa*1 (Roche) in a 100 µl volume with 10 µl 10 × Promega Buffer C. Digestions were carried out for 2 h at 37°C. The digested samples were purified using QIAprep Spin Miniprep columns (Qiagen) and eluted as per the manufacturer's instructions. The samples were then analysed using the Agilent 2100 bioanalyser with the DNA 7500 LabChip kit and DNA 7500 Software Script as per the manufacturer's instructions. The *Alu*1/*Rsa*1 digested DNA samples were labelled using the BioPrime aCGH Labeling kit (Invitrogen, USA) according to the manufacturer's protocol. Amplification samples (blastomeres and control DNA) were systematically labelled with Alexa Fluors 555 and 647, respectively. Labelled products of each sample and control DNA were purified using QIAprep Spin Miniprep columns (Qiagen), mixed together and checked on 2100 bioanalyser (Agilent) in order to evaluate the Alexa Fluors 555 integration into the DNA samples. To the purified Alexa Fluors 555 and 647 labelled samples, the following hybridization blocking reagents were added: 50 µg Cot-1 DNA (Invitrogen), and 50 µl 10 × control targets (Agilent). The volume was brought to 250 µl with ddH_2O and 250 µl 2 × hybridization buffer (Agilent) was added. The hybridization mixture was then denatured at 100°C for 3 min in a water bath. Samples were immediately transferred to a 37°C water bath for 30 min to allow pre-annealing of the blocking agents to the labelled sample. Samples were centrifuged for 5 min at 16,000 *g* and immediately applied to Agilent human genome CGH 44B Oligo Microarray Kit as per the manufacturer's recommendations. Hybridizations were performed at 65°C for 17 h. The microarrays were disassembled in the Agilent wash buffer-1 at room temperature, transferred to a slide holder and incubated for 5 min with stirring in the Agilent wash buffer-1 at room temperature. The second washing step was performed for 1 min in wash buffer-2 at 37°C. The third and fourth washing steps were done with acetonitrile (Fisher, USA) and stabilization solutions (Agilent) for 1 min and 30 s at room temperature, respectively. The microarray slides were immediately scanned in the Agilent DNA microarray scanner using the default settings. Data was extracted using the Agilent feature extraction software 8.1 using the default settings.

Quality control for aCGH

A method for gender determination of the blastomeres using the amelogenin gene was included in order to validate the quality of the aCGH-based PGS. 5 µl of the MDA product (1:10 diluted; 10 ng/µl) were amplified using primers designed for part of the amelogenin gene. Primer sequences were 5′-6FAM-CCCTGGGCTCTGTAAAGAATAGTG-3′ (forward) and 5′-AGGCCAACCATCAGAGCTTAAACT-3′ (reverse). PCR conditions were as follows: one cycle of denaturation at 97°C for 5 min followed by 30 cycles of denaturation at 97°C for 20 s, annealing at 60°C for 15 s and extension at 72°C for 45 s. Final extension was performed at 68°C for 10 min. PCR products were analysed on 3130xl Genetic analyser (Applied Biosystems, USA), two peaks at 107 bp and 112 bp indicated a male embryo, while one peak at 107 bp indicated a female embryo.

PGS using aCGH

A total of 41 embryos, at 8-cell stage or above, were generated by assisted reproduction technology and underwent two-cell embryo biopsy. Separated cells were amplified by MDA and assessed by aCGH as described in the optimization section. An additional step of sex determination on the MDA product using amelogenin gene primers was also included to confirm that equal quantities of samples and controls were used throughout the experiment (see above). Briefly, PCR was performed on the MDA product (diluted 1:10) and the PCR products were assessed on a genetic analyser 3130xl (Applied Biosystems).

Confirmation of aCGH result by FISH

Abnormal embryos, diagnosed by aCGH with at least one chromosome that could be detected by the FISH panel of probes, were fixed on slides and analysed by FISH for the following chromosomes (13, 16, 18, 21, 22, X and Y) as previously described (Staessen *et al.*, 2004).

Data analysis

Scanned images were quantified with Feature Extraction software (version 9.1; Agilent Technologies). The data was visualized with CGH Analytics software (version 3.4; Agilent Technologies) and evaluated the quality of each test with the quality-control metrics generated with CGH Analytics software. Copy-number aberration was indicated with the Aberration Detection Method 2 algorithm for the data that passed quality control testing. An aberration filter was set to indicate regions with at least 10 MB showing the same direction in copy-number change. Log_2 ratio values for all oligonucleotide probes are plotted as a function of their chromosomal position. Each point represents a single probe, with a 1 MB moving average (thin wavy coloured vertical lines) on the chromosome panels. Cut-off values for gain and loss were set +0.5 and –0.5 respectively.

Results

Optimization of single cell aCGH

Aneuploidies in the chromosomes 13, 16, 18, 21, 22 and sex chromosomes, previously diagnosed by FISH, were confirmed by aCGH on two single cells. Indeed, blastomeres being previously diagnosed each with aneuploidy (trisomy and monosomy) in one of the autosomes and the sex chromosomes using the FISH technique were confirmed by aCGH. Most abnormalities were detected by aCGH, except for five blastomeres, which were diagnosed abnormal by FISH and normal by aCGH. Such a discrepancy would be mainly due to mosaicism (**Table 1**).

PGS for aneuploidy detection using aCGH

Patients enrolled in the PGS programme had all experienced a minimum of seven recurrent IVF failures. Forty-one embryos were diagnosed using aCGH and the results obtained are shown in **Table 2**. Sex determination results of the blastomeres by amelogenin were used first as an internal control to confirm that an equal amount of control and sample DNA were used and then to confirm any noted abnormality that was seen by aCGH result. Two out of the 27 abnormal embryos had a sex chromosome abnormality.

Six out of the eight patients showed normal embryos for transfer and pregnancy test was positive in five out of the six patients. Non-transferred embryos (n = 11) with abnormalities in at least one of those chromosomes (13, 16, 18, 21, 22 and sex chromosomes) were retested by FISH. All FISH results were confirmed by aCGH (**Table 2**). Importantly, two of the 11 embryos (**Table 2**) analysed showed the presence of cells with normal chromosomes indicating the presence of mosaicism. **Figure 1** shows the panel of normal and abnormal aCGH profile, as detected by Agilent's high-resolution aCGH platform, for representative chromosomes. The moving average (thin wavy

Table 1. Comparative assessment of chromosomal aneuploidies using fluorescence in-situ hybridization (FISH) and array comparative genomic hybridization (aCGH) techniques.

Chromosome	*Aneuploidy*	*No. of embryos diagnosed by FISH*	*No. of embryos diagnosed by aCGH*
13	Trisomy	3	2[a]
	Monosomy	1	1
16	Trisomy	2	2
	Monosomy	1	1
18	Trisomy	2	2
	Monosomy	1	1
21	Trisomy	3	1[a]
	Monosomy	2	2
22	Trisomy	2	1[a]
	Monosomy	1	1
X/Y	XXY	2	1[a]

Due to differing protocol requirements, one blastomere was used in FISH, whereas two blastomeres were used in aCGH. Each embryo examined by aCGH had only one chromosome abnormality as detected by FISH.
[a]Discrepancy in the diagnosis observed between the two methods. A possible reason for discrepancy between the FISH and aCGH is embryo mosaicism due to the number of blastomeres diagnosed.

Table 2. Summary of blastomere screening by array comparative genomic hybridization (aCGH) technology.

Patient age (years)	*No. of recurrent IVF failures*	*No. of embryos assessed by aCGH*	*No. of normal embryos detected*	*No. of abnormal embryos detected and type of abnormalities*	*Pregnancy (heart beat)*	*Pregnancy duration*
38	10	6	3	**E1: Trisomy 18** **E2[a]: Trisomies 13, 16, 22** E3: Trisomy 20	Yes	Third trimester
41	11	4	2	**E1[a]: Trisomies 13, 21; monosomies 22, 14** E2: 8q deletion	Yes	Third trimester
44	9	3	0	E1: Trisomy 15 E2: Monosomy 10 E3: Trisomy 14	NA	Third trimester
42	10	4	0	E1: Monosomy 17 E2: Trisomy 15 E3: Trisomy 2 E4: Monosomy 10	NA	Third trimester
34	7	6	2	E1: Monosomy 1 **E2: Trisomy 21** E3: Trisomy 15 **E4: XXY**	Yes	Third trimester
28	9	6	2	E1: Trisomy 4 **E2: Trisomy 16** E3: Monosomy 12 **E4: X0**	Yes	Third trimester
31	7	6	3	**E1: Trisomy 16** **E2: Trisomy 21** **E3: Monosomy 18**	No	Third trimester
32	12	6	2	E1: Duplication 1p **E2: Trisomy 13** E3: Monosomy 5 E4: Trisomy 12	Yes	Third trimester

Systematically, two blastomeres from each embryo were pooled together and assessed.
E = embryo; NA = not applicable.
Embryos in bold were retested by FISH.
[a]Embryos found mosaic after aCGH result confirmed by FISH.

coloured vertical lines) was used as a reference to evaluate any type of abnormality. This method of analysis was used (instead of point by point) because of the quality of DNA amplified by MDA which most probably caused by allelic drop-out and/or preferential amplification. The presence of very dense array of 60-mer oligonucleotides made the reproducibility of the technique more pronounced.

Discussion

The last decade has witnessed an important focus on the role of chromosomal abnormalities in the success of assisted reproduction technology (Platteau *et al.*, 2006). Two prospective randomized trails (Staessen *et al.*, 2004; Mastenbroek *et al.*, 2007) reported the absence of a benefit from preimplantation genetic diagnosis (PGD) in couples with advanced maternal age. In spite of the interest given to these reports, one of the criticisms was the number of chromosomes assessed in these studies, which was limited to seven (Munné *et al.*, 2007b; Wilton, 2007).

The current report describes the first clinical application of aCGH in PGS. Routine PGS was based on FISH for the seven chromosomes. aCGH was first performed and validated on embryos previously diagnosed using FISH. Such a validation was the basis of the preclinical analysis. The discrepancies observed (25%) are attributed to mosaicism. As expected, many abnormalities detected by aCGH were missed using the FISH technique. The results presented in **Table 2** demonstrate abnormalities in chromosomes 1, 2, 4, 5, 8, 10, 12, 14, 15, 17 and 20 in many embryos tested. Such abnormalities could not be detected using the seven-probe FISH panel used in routine PGS. The percentage of embryos with those abnormal chromosomes was 60% when compared with the total number of abnormal embryos. Therefore, FISH panel for chromosomes 13, 16, 18, 21, 22 and sex chromosomes could only detect 40% of the chromosomal abnormalities, which is comparable to a recently published rate of 48% (Cohen *et al.*, 2007). On the other hand, it is important to mention that many embryos are aneuploid for several chromosomes, increasing the likelihood of detection even when a restricted set of chromosomes is screened. More interestingly and as previously reported (Wells and Delhanty,

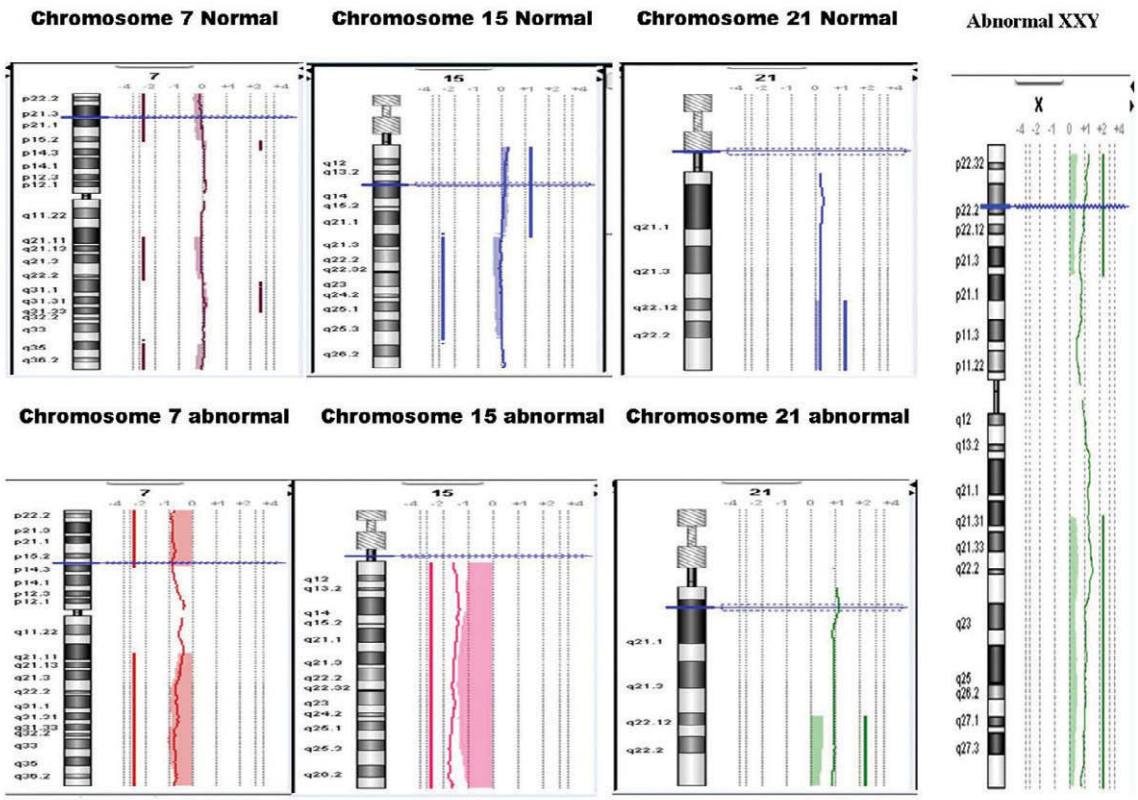


Figure 1. Result of a set of representative chromosomes with normal and abnormal aCGH status. Abnormality in the sex chromosomes is also shown where the embryo was diagnosed as male by amelogenin PCR and the microarray showed XX profile when hybridized with XY DNA.

2000), two embryos showed chromosomal structural changes (1p duplication and 8q deletion), an abnormality that could not be detected by FISH-aneuploidy screening.

Theoretically, cells can be removed for diagnosis at any stage between the 2-cell stage embryo and the blastocyst. In human embryos, the 8-cell stage is ideal because the cells are still totipotent (each cell can replace another cell). Although up to a quarter of cells from a human embryo can be removed without impairing its in-vitro development (Hardy *et al.*, 1990), the random removal of two cells could interfere with the embryo's early differentiation (Edwards and Beard, 1997; Cohen *et al.*, 2007). However, routine 2-cell biopsy (Van de Velde *et al.*, 2000, Burlet *et al.*, 2006; Goossens *et al.*, 2008) has resulted in pregnancies and live birth rates comparable to 1-cell biopsy. The routine in the study PGD protocol is to take one cell for FISH and two cells for aCGH and PCR analysis (Goossens *et al.*, 2008). DNA produced from two cells by MDA leads to more reproducible results on aCGH (Agilent 44K array) than one cell (unpublished data, A Hellani *et al.*). Two cells are systematically diagnosed for each embryo; consequently, mosaicism could not be easily detected which may explain the high percentage of normal embryos (40%) compared with the 25% previously reported (Voullaire *et al.*, 2000) (during the aCGH validation process, 25% of the embryos were mosaics).

Equally, the confirmation of the aCGH result by FISH on the non-transferred embryos showed the presence of two out of 11 embryos with mosaicism which involved more than one chromosome (2/11, 18%), resulting in discarding mosaic embryos. On the other hand, another unknown percentage (in theory close to 18%) would result in transferring mosaic embryos. Those two factors represent aCGH bias in detecting mosaicism. The 18% figure should be added to the percentage of biased factor due to missed mosaicism by aCGH (embryo diagnosed as normal and transferred). Such a bias would be most probably caused by mitotic non-disjunction with one chromosome involved. Indeed the chaotic type of mosaicism would be more likely detected by aCGH, since more than one chromosome is involved. Embryos with mitotic non-disjunction are capable of developing into good-quality blastocysts, but little is known about their implantation potential and their ability to develop into a healthy child. This will probably depend on the level of mosaicism, the type of chromosome aberration and the chromosomes involved. Embryos mosaic for trisomy 21 appear to be less viable than normal embryos (Katz-Jaffe *et al.*, 2005), but for other chromosomes this is unknown. More research is needed to determine whether some mosaic embryos can be considered suitable for transfer, depending on the type of aneuploidy and the chromosome involved.

It is debatable whether implantation failure in patients with recurrent IVF failure is due to chromosomal abnormalities (Caglar *et al.*, 2005). Munné *et al.* (2003) found that patients with recurrent IVF failure have similar rates of abnormal embryos when compared with patients with one or no previous

cycles. They suggested that in this group of patients, the poor implantation rate was not due to an excess of chromosomally abnormal embryos. Gianaroli *et al*. (2002) found that the most frequent chromosomal defects in patients with recurrent IVF failure (with more than three IVF failures) were mosaicism, haploidy and polyploidy. In contrast, others reported that these patients had a 1.9-fold higher rate of chromosomal anomalies, mostly aneuploidy, when compared with patients who underwent PGD for sex-linked diseases (Pehlivan *et al*., 2003). Moreover, it was found that the number of chromosomal anomalies increases with the number of failed IVF cycles. The rates are 40% and 50% respectively with two and three failed IVF cycles, and 67% with more than five failed IVF cycles (Gianaroli *et al*., 1997). Despite the small sample size in this study, it is in agreement with the observation of an increase in chromosomal abnormality due to the recurrent IVF failure. Interestingly, the patients were chosen based on long history of recurrent IVF failure (minimum of seven failures; **Table 2**), which may explain the high percentage of chromosomal abnormalities detected. In this study, the overall percentage of abnormal and mosaic embryos was higher than 60%).

The percentage of chromosomal abnormalities obtained utilizing the seven probes in FISH compared with the total number of abnormalities detected was 40%. If chromosome 15 is added to the seven-probe panel, as per Munné *et al*. (2003), an increase in the number of abnormal embryos of 11% would be obtained and the total number of chromosomal abnormalities will reach 51% of the abnormalities shown in this report. Further reports on aCGH with larger sample size will be of benefit to identify chromosomes implicated in the aneuploidy and, if added to the panel of FISH, will increase the likelihood of abnormality detection.

One inherent limit of aCGH is that it cannot detect whole ploidy errors: that is, it cannot distinguish for example haploid, triploid and tetraploid cells from diploid ones. However, embryologists at fertilization could detect many if not most, of these aberrations when they closely observe pronuclei and discard any zygotes that have other than two. In addition, it is possible that many of the tetraploid cells viewed in early embryos represent a normal developmental process, perhaps underlying the later formation of the syncytial trophoblast.

The diagnosis of the entire set of chromosomes on single cell has been successfully applied in PGD resulting in pregnancy and delivery of normal babies (Wilton *et al*., 2003). The greatest limitation of this approach is the long time (more than three days) required to obtain a result and so biopsied embryos had to be frozen and thawed and transferred in a subsequent cycle if they were normal. To avoid cryopreservation, other CGH strategies have been applied (Wells *et al*., 2002) that involve the conjunction of both polar body and blastomere biopsy. As a result, CGH could be performed on the polar body and FISH used to confirm the result on blastomeres. Consequently, any abnormality caused by a male partner and not included in the FISH panel would not be detected.

The aCGH protocol presented here would: (i) be less labour intensive (only one technique is used); (ii) not require double biopsy; and (iii) be capable, in theory, of identifying any abnormality if present. Regarding timing, the biopsy is performed on the morning of day 3, hybridization on the evening of day 3, wash and slide reading in the afternoon of day 4 afternoon (18 h hybridization) and transfer, if any, is done on day 5.

Sex determination of the blastomeres proved to be very useful as an internal control for the detection of the sex chromosome abnormalities. Sex mismatching control should always be run along with the tested embryo. If the result obtained is as expected regarding the X and Y chromosomes, then the embryo is normal and the aCGH experiment is valid. On the other hand, if the result is not matching then the two feasible scenarios will be: (i) the embryo is Turner (X0), then a male control needs to be used; and (ii) the embryo is Klinefelter (XXY) then a female control needs to be used. In the first scenario, the embryo is X0 and the controls is XY, therefore the XX ratio will be one and the Y chromosome will only be detected in the control sample. However, in the second scenario, the embryo is XXY and the control is XX, therefore the XX ratio is one and the Y is detected only in the embryo. These two scenarios are most frequently encountered in embryos.

The key to success of aCGH analysis on single cell is the quality of DNA amplified. MDA has been previously applied on single cell for aCGH application (Hellani *et al*., 2004; Hu *et al*., 2004, 2007; Wells *et al*., 2004; Le Caignec *et al*., 2006). The type of aCGH technique used is the main difference between those reports (bacterial artificial chromosome arrays) and the current one (60-mer oligonucleotides).

These results are in agreement with recent studies reporting an increase in the detection of abnormal embryos when additional chromosomes are screened (Wells *et al*., 2004; Hu *et al*., 2007; Lathi *et al*., 2008). The current aCGH outcome in terms of successful clinical pregnancies (positive fetal heart beat) is promising although firm conclusions may not be made due to a relatively small sample size. Nevertheless, the results obtained here are very encouraging and pave the way to test more couples with recurrent IVF failure, recurrent abortion and advanced maternal age.

Although aCGH has previously been applied with success on single cells (Le Caignec *et al*., 2006), the current report is the first to show a successful clinical application of this technique with an encouraging pregnancy rate. In conclusion, aCGH can be used routinely in PGS with a potentially high successful pregnancy rate and with superior efficiency over the seven-probe FISH panel. Agilent technology made it more convenient with the possibility of screening eight embryos using one slide (8 × 15 k slide format).

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