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Reliability of comparative genomic hybridization to detect chromosome abnormalities in first polar bodies and metaphase II oocytes

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BACKGROUND: Preimplantation Genetic Diagnosis (PGD) using FISH to analyze up to nine chromosomes to discard chromosomally abnormal embryos has resulted in an increase of pregnancy rates in certain groups of patients. However, the number of chromosomes that can be analyzed is a clear limitation. We evaluate the reliability of using comparative genomic hybridization (CGH) to detect the whole set of chromosomes, as an alternative to PGD using FISH. METHODS and RESULTS: We have analysed by CGH both, first polar bodies (1PBs) and metaphase II (MII) oocytes from 30 oocytes donated by 24 women. The aneuploidy rate was 48%. Considering two maternal age groups, a higher number of chromosome abnormalities were detected in the older group of oocytes (23% versus 75%, $P < 0.02$). About 33% of the 1PB-MII oocyte doublets diagnosed as aneuploid by CGH would have been misdiagnosed as normal if FISH with nine chromosome probes had been used. CONCLUSION: We demonstrate the reliability of 1PB analysis by CGH, to detect almost any chromosome abnormality in oocytes as well as unbalanced segregations of maternal translocations in a time frame compatible with regular *in vitro* fertilization (IVF). The selection of euploid oocytes could help to increase implantation and pregnancy rates of patients undergoing IVF treatment.

Key words: aneuploidy/comparative genomic hybridization/first polar body/oocyte/preimplantation genetic diagnosis

Introduction

Implantation and pregnancy rates decrease with maternal age. Some authors have suggested that uterine factors could explain this decline in fertility (Meldrum, 1993) but high pregnancy and low miscarriage rates have been found in post-menopausal women after IVF cycles with donated oocytes (Abdalla *et al.*, 1993). The only clear link observed between maternal age and embryo competence is chromosome abnormality (Navot *et al.*, 1991; Munné *et al.*, 1995a; Dailey *et al.*, 1996; Nicolaidis and Petersen, 1998; Márquez *et al.*, 2000; Sandalinas *et al.*, 2001; Pellestor *et al.*, 2003). The increase in aneuploidy with maternal age leads to an increased risk of producing aneuploid offspring (involving mainly chromosomes 13, 18, 21, X and Y), an increased risk of spontaneous abortion (frequently involving chromosomes 15, 16, 22 and X) and a decrease in implantation rates. Some evidence suggests that there is a negative selection against chromosomal abnormalities during the first stages of embryonic development (Boué *et al.*, 1985). This early embryonic wastage is thought to be one of the main factors which contribute to the low fertility rate in humans (Bahçe *et al.*, 1999; Sandalinas *et al.*, 2001).

Several strategies have been used to discard chromosomally abnormal embryos, such as selection of embryos based on their ability to grow to the blastocyst stage (Menezo *et al.*, 1992), as well as morphological criteria (Plachot *et al.*, 1990). However, about 37% of trisomic embryos reach the blastocyst stage and 70% of morphologically normal embryos are, in fact, aneuploid (Iwarsson *et al.*, 1999; Sandalinas *et al.*, 2001).

Currently, a reliable identification of chromosomally abnormal embryos can only be achieved by preimplantation genetic diagnosis (PGD) using either polar body or blastomere analysis, in biopsies performed on day 0 or day + 3 after fertilization, respectively (Verlinsky *et al.*, 1990; Munné *et al.*, 1993; Munné *et al.*, 1995b; Durban *et al.*, 2001).

The technique most widely used for this purpose has been fluorescent *in situ* hybridization (FISH). Using FISH to allow identification and preferential transfer of embryos with normal numbers of the chromosomes assessed, has led to a reduction in spontaneous abortions and an increase in implantation and pregnancy rates for several groups of IVF patients: advanced maternal age and women with a history of recurrent miscarriages. (Gianaroli *et al.*, 1999; Munné *et al.*, 1999, 2003). However, PGD using FISH has several limitations; the most important of which is the number of chromosomes that can be analyzed simultaneously. Although the current panel of nine probes used in our laboratories covers the most frequent abnormalities detected in cleavage-stage embryos and oocytes (Pujol *et al.*, 2003; Munné *et al.*, 2004), some studies indicated that 25–30% of chromosomal abnormalities would remain undetected using FISH with nine chromosome-specific probes (13, 15, 16, 17, 18, 21, 22, X and Y), leading to the transfer of aneuploid embryos incorrectly diagnosed as normal (Boué *et al.*, 1985; Voullaire *et al.*, 2002). Current FISH protocols have used probes for up to 13 chromosomes (Abdelhadi *et al.*, 2003) but this represents only half of the whole karyotype and accuracy per probe is reduced when large numbers of probes are combined.

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique that allows the analysis of the full set of chromosomes (Kallionemi *et al.*, 1992) and it has been applied to detect aneuploidy in single cells (Voullaire *et al.*, 1999; Wells *et al.*, 1999). The more extensive analysis of the karyotype provided by CGH allows replacement of only chromosomally normal embryos, which are those most likely to establish a successful pregnancy. This also could lead to the transfer of fewer embryos and consequently reduce multiple pregnancies, which is one concern derived from assisted reproductive technology. As CGH is a labour intensive technique that requires as many as 4 days to obtain results, two different strategies have been proposed to apply CGH to PGD. The first one was the use of CGH in PGD by blastomere analysis (Voullaire *et al.*, 2002). In this case, embryo freezing was required to provide time enough to perform the CGH analysis. Although this approach has recently shown higher implantation and pregnancy rates than FISH, it produced considerable controversy, because 46% of the embryos did not survive the freezing–thawing process (Hill, 2003; Munné and Wells, 2003; Verlinsky and Kuliev, 2003; Wilton *et al.*, 2003a,b). The second strategy was to perform CGH for PGD by first polar body analysis (Wells *et al.*, 2002). Since polar body biopsy is performed on the same day as fertilization by intracytoplasmic sperm injection, CGH analysis was compatible with embryo replacement on day + 4, without embryo freezing.

In female meiosis I, a set of chromosomes, with two chromatids each, segregate to the first polar body (1PB) while the oocyte in metaphase II (MII) retains the reciprocal chromosome complement. Since the 1PB is thought to have no biological role once it has been extruded, the analysis of 1PBs allows the indirect characterization of the chromosome constitution of the MII oocyte (Gitlin, 2003). This means that if a segregation error occurs during this first meiotic division, and for instance, an extra chromosome is present in the MII oocyte, then the 1PB will show the complementary loss. Most embryo aneuploidies as well as most first trimester aneuploidies were classified as originating in female meiosis I (Nicolaidis and Petersen, 1998; Hassold and Hunt, 2001). However, FISH analysis results of first and second PBs has indicated that a sizable part of aneuploidy occurs in meiosis II, or at least, at the chromosome level, is expressed in meiosis II (Kuliev *et al.*, 2003). Therefore, the detection of abnormal oocytes through PGD using CGH should be performed in both, first and second PBs, but even biopsing on day 1, there is still enough time for CGH results prior to transfer, and no cryopreservation is needed (Wells *et al.*, 2002).

The aim of this study is to evaluate the limitations, error rate and reliability of CGH prior to its clinical application. To achieve this, a series of 1PB and MII oocyte doublets have been analyzed separately in a blind study and the results have been compared.

Materials and methods

Oocyte and polar body recovery

Immature oocytes discarded from IVF cycles were maturated *in vitro* (aged 12–24 h) before being processed. Only those that were considered to be at the metaphase II stage (having extruded the 1PB) were included in this study. The oocytes used were donated by 21 women with normal karyotype aged from 21 to 41 years (mean 33.2) and three translocation carriers (two Robertsonian translocation carriers aged 29 and 35 years and one balanced reciprocal translocation carrier aged 35 years). The material was obtained from the IVF programme of the Institut Universitari Dexeus (Barcelona, Spain) and the Institute for Reproductive Medicine and Science at Saint Barnabas Medical Center (West Orange, New Jersey, NJ) in accordance with guidelines set by the internal review board of these centres. Written informed consent was obtained from all patients.

1PB and MII oocyte isolation and lysis

The zona pellucida was removed using acid Tyrode's. After that, MII-oocytes and their 1PBs were isolated and washed in three PBS/0.1% polyvinyl alcohol (PVA) droplets. The single cells were transferred to individual PCR tubes and the presence of the single cell inside the tube was ascertained, although this was not always possible with polar bodies. The tubes were coded and randomized so that the CGH analysis was conducted blindly. Finally, 1 μl of sodium dodecyl sulphate (SDS, 17 μM) and 2 μl of proteinase K (125 μg/ml) were added and the sample was overlaid with light mineral oil. The lysis was performed by incubating at 37°C for 1 h followed by 10 min at 95°C to inactivate proteinase K.

Whole genome amplification

Single cell DNA was amplified using degenerate oligonucleotide primed PCR (DOP–PCR) as previously described (Wells *et al.*, 2002) with some modifications. In brief, each PCR tube contained 1 × buffer, 2 μM DOP primer (CCGACTCGAGNNNNNNATGTGG), 0.2 mM dNTPs and 2.5 U of SuperTaq Plus polymerase (Ambion, Austin, TX) in a final volume of 50 μl. The sample was spun and heated to 94°C for 4.5 min; 8 cycles of 95°C for 30 s, 30°C for 1.5 min and 72°C for 3 min; 40 cycles of 95°C for 30 sec, 56°C for 1 min and 72°C for 3 min with a final extension step of 72°C for 8 min. The PCR program was carried out in a Tgradient thermocycler

(Biometra, Goettingen, Germany) or alternatively in a 9700 PE thermocycler (Applied Biosystems, Norwalk, USA).

Stringent precautions against contamination were taken. Negative controls were included in each experiment to test the reaction solutions and the phosphate-buffered saline used for washing the single cells in the isolation step. The negative controls were subjected to the entire procedure. No DNA and no hybridization signal should be present after the DOP–PCR and the CGH experiment, respectively.

Genomic DNA extracted from peripheral blood diluted to 100 pg/μl or isolated and lysed single buccal cells, both from a normal female were also amplified and used as a reference sample in the CGH experiment.

Nick translation and probe preparation

Whole-genome amplification products were fluorescently labelled by Nick Translation (Vysis, Downers-Grove, USA) according to the manufacturer's instructions. 1PB and MII oocyte DNAs (test) were labelled with Spectrum Red-dUTP (Vysis), whereas reference DNA was labelled with Spectrum Green-dUTP (Vysis). The reaction time was adjusted to obtain a probe of a suitable size, and assessed by electrophoresis of 9 μl of product in a 2% agarose gel. Labelled reference and test DNA were mixed and ethanol precipitated with 10 μg of Cot-1-DNA. The pellet was dried and redissolved in 10 μl of hybridization mixture (50% formamide, 2 × SSC, 10% dextran sulphate, pH 7).

Comparative genomic hybridization

Normal male (46, XY) metaphase spreads (Vysis) were dehydrated through an alcohol series (70%, 85%, and 100% for 2 min each) and air dried. The slides were then denatured in 70% formamide, 2 × SSC at 73°C for 5 min and taken through a cold alcohol series and air dried. The probes were denatured at 73°C for 10 min and were applied to the slide; a coverslip was placed on top and sealed with rubber cement. Hybridization was performed in a moist chamber at 37°C for 36–72 h to evaluate the minimal hybridization time to ensure reliable results. After hybridization, the slides were washed at high stringency in 0.4 × SSC/0.3% NP-40 at 73°C for 2 min, 2 × SSC/0.1% NP-40 for 2 min and dipped in distilled water before being dehydrated through an alcohol series and air dried. Finally, the slides were mounted in Vectashield (Vector Labs, Peterborough, UK) containing DAPI to counterstain the chromosomes and nuclei.

Microscopy and image analysis

Metaphase preparations were examined using an Olympus BX 60 epifluorescence microscope equipped with a high-sensitivity camera and filters for the fluorochromes used. An average of 10 metaphases per hybridization were captured and analyzed using SmartCapture software and Vysis Quips CGH software, both supplied by Vysis. The average red/green fluorescent ratio for each chromosome was determined by the CGH software. In regions where the DNA sequence copy number of the test is identical to the reference DNA, the CGH profile shows no fluctuation and the ratio is expected to be close to 1.0. Deviations of the ratio below 0.8 (the test DNA is under-represented) or above 1.2 (the test DNA is over-represented) were scored as loss or gain of material in the test sample, respectively. Deviations of the ratio but within the threshold cut-off of 0.8 or 1.2 were also annotated to evaluate the sensitivity of the technique.

Results

In this work a total of 86 single cells, corresponding to 43 1PB-MII oocyte doublets were isolated. A total of 80 out of 86 single cells were successfully amplified by DOP–PCR (93%) showing a smear between 200 and 4000 bp. Only six

Table I. Summary of CGH data from 25 doubles 1PB-MII oocyte from 46. XX women

Doublet 1PB-MII	Age	CGH interpretation 1PB	Artifacts**	CGH interpretation MII	Artifacts**	Reciprocity
1	38	24, X, +21	22	22, X, −21	19	Yes
2	38	23, X	19	23, X	19,22	Yes
3	24	24, XX*	–	22, −X	–	No
4	39	24, X, +3*, −6, +8*	–	21, X, −3*, +6, −8*, −15*	–	No
5	32	23, X	–	23, X	19	Yes
6	25	23, X	–	23, X	–	Yes
7	41	22, X, −7	17, 22	24, X, +7*	19	No
8	41	21, X, −13, −21*	19	25, X, +13*, +21*	–	No
9	37	23, X, +2, −15	19	23, X, −2, +15	–	Yes
10	39	22, X, −2	19	24, X, +2	–	Yes
11	21	23, X	–	23, X	–	Yes
12	21	25, XX, +1	–	21, −X, −1	–	Yes
13	31	23, X	–	23, X	17, 19, 22	Yes
14	30	23, X	19	23, X	–	Yes
15	31	23, X	–	23, X	–	Yes
16	22	23, X	–	23, −X, −20*	–	No
17	34	23, X	17, 22	23, X	17	Yes
18	34	23, X	22	23, X	19, 22	Yes
19	37	24, X, +19*	–	22, X, −19	–	No
20	33	23, X	22	23, X	17, 19, 22	Yes
21	23	23, X	17	23, X	19, 22	Yes
22	39	24, X, +15	–	22, X, −15	22	Yes
23	41	23, X	–	23, X	–	Yes
24	40	23, X	22	23, X	17, 19	Yes
25	40	22, X, −9	–	24, X, +9	–	Yes

The 1PBs and MIIs karyotypes were based on the interpretation of the CGH profile determined by the CGH software (see materials and methods). IPB = first polar body; MII = metaphase II oocyte

*Chromosomes showing deviation but within the threshold cut-off of 0.8 or 1.2, such that it would be difficult to conclude loss or gain of these chromosomes, respectively.

**Chromosomes which show enhancement of the test signal, but they are regarded as artifactual and consequently they are excluded from the analysis.

cells failed to give any smear after amplification, suggesting that the cells had been lost during the isolation step or cell lysis had failed. All the MII oocytes amplified gave a smear with a band pattern at 600, 1250 and 1650 bp, whereas these bands were not visible in any 1PB amplification.

CGH results were obtained in 67 single cells (83.8%), 30 1PB-MII oocyte doublets and seven single 1PBs (data not show). Thirteen cells, three 1PB and 10 metaphase II oocytes, failed to give any result because the hybridization intensity of the test DNA was too weak. Out of the 30 matched pairs with adequate hybridization, 25 were donated by 21 normal females (46, XX) while five were donated by two Robertsonian translocation carriers [45, XX, der(13;14)(q10;q10) and 45, XX, der(13;15)(q10;q10)] and one balanced reciprocal translocation carrier [46, XX, t(1;5)(q21.1;p13.1)].

The results of the CGH analysis are given in Table I. Out of 25 1PB-MII oocyte doublets derived from normal females, 12 presented results consistent with aneuploidy in either the MII oocyte, the 1PB, or both (48%). Although most aneuploid doublets had one (seven), or two (four) chromosomes implicated in aneuploidy (Figure 1), we also found one doublet with extensive aneuploidy involving four different chromosomes.

Heterochromatic, telomeric and centromeric regions were not informative and therefore were excluded from the analysis because they usually show a deviation in the CGH pattern. In this study we have also observed artifactual gains of chromosomes 17 (12%), 19 (28%) and 22 (24% of the analyzed cells). Some studies have already reported the difficulty of interpreting the CGH profile of these chromosomes and commonly, when the test signal is enhanced, they are also excluded from the analysis (Moore *et al.*, 1997; Voullaire *et al.*, 2002).

Age-related aneuploidy has also been analyzed. Thirteen 1PB-MII oocyte pairs from women <37 years old (mean 27.8, range 21–34) were examined, three (23%) giving results consistent with aneuploidy. Additionally, 12 1PB-MII oocyte pairs from women ≥ 37 years old (mean 39.2, range 37–41) were investigated, with nine (75%) found to be aneuploid. The difference between the aneuploidy rate in these two age-related groups was statistically significant ($P < 0.02$, Fisher's Exact Test).

The highest rate of aneuploidy was found for chromosome 15, followed by chromosome 21, chromosome X and, interestingly, chromosome 2. It has been previously suggested that chromosome 2 may play a more significant role in human reproductive failure than is typical for such a large chromosome (Wells *et al.*, 2002).

The CGH analysis of the doublet 1PB-MII oocyte from the balanced reciprocal translocation carrier 46, XX, t(1;5)(q21.1;p13.1), revealed an adjacent two segregation and consequently, both cells, 1PB and MII oocyte were unbalanced (Figure 2). The four 1PB-MII oocyte doublets from the two Robertsonian translocation carriers [Rob(13;14) and Rob(13;15)] were found to be normal or balanced. No aneuploidy affecting chromosomes not involved in these rearrangements (interchromosome effect, ICE) was found (Table II).

In this study 24/30 (80%) of the CGH results of the 1PBs were confirmed by MII oocyte results. Six 1PB-MII oocyte doublets analyzed by CGH were found to give results that were not perfectly complementary between the CGH interpretations of the 1PB and the MII oocyte. Although in some cases results were in partial agreement (e.g. doublet four reveals imbalance affecting several chromosomes, but not all of them are detected in both 1PB and MII oocyte). In all six doublets where data was not entirely complementary, a loss was seen in one cell while the other did not display the reciprocal gain, but showed a normal or doubtful CGH profile for the chromosome in question (labeled by an asterisk in Table I).

Discussion

In this work we have studied chromosome abnormalities in 1PB and MII oocytes; a 48% aneuploidy rate was found. The incidence of chromosomal abnormalities varies from 9.4% to 47.5% (average 27.7%) across the studies which have used various approaches such as R-banding techniques (Pellestor, 1991, 2003), fluorescent *in situ* hybridization (FISH) for up to nine chromosomes (Dailey *et al.*, 1996; Mahmood *et al.*, 2000; Cupisti *et al.*, 2003; Pujol *et al.*, 2003), spectral karyotyping (SKY; Márquez *et al.*, 1998; Sandalinas *et al.*, 2002) or Multicolor fluorescence *in situ* hybridization (m-FISH; Clyde *et al.*, 2003). Our high rate of aneuploidy could be attributed to the fact that all the material used was retrieved at MI stage and matured *in vitro*, and this long *in vitro* maturation could produce morphological alterations of the oocyte spindle, increasing the aneuploidy rate of these oocytes (Pickering *et al.*, 1988). In addition, the reported incidence of abnormalities in embryos resulting from fertilization of *in vitro* matured oocytes was significantly higher than that found for *in vitro* matured oocytes (DeScisciolo *et al.*, 2000). However, it has also been described that unbalanced predivision and non-disjunction did not increase significantly with time in culture (Munné *et al.*, 1995b; Boiso *et al.*, 1997). Therefore, the rate of abnormalities presently found could be overestimated but the higher number of chromosomes being analyzed could also explain this high rate of aneuploidy. It is worth emphasizing that if these cells had been analyzed by FISH for nine chromosomes this rate would decrease to 32% because only eight out of 12 aneuploid doublets would have been detected.

The chromosome most involved in aneuploidy was chromosome 15, as suggested by other researchers (Clyde *et al.*, 2003). We also found aneuploidy for chromosomes 1, 2, 3, 6, 7, 8, 9, 13, 19, 20 and 21, in contrast with other studies where aneuploidy for chromosomes 1, 2 and 9 were not found (Mahmood *et al.*, 2000; Sandalinas *et al.*, 2002; Cupisti *et al.*, 2003). Unlike the only previous report of CGH conducted on 1PBs (Wells *et al.*, 2002), no relation was found between chromosome size and aneuploidy frequency in this cohort.

Current strategies for the detection of chromosomal abnormalities in oocytes by 1PB analysis are mostly performed using FISH for five (13, 16, 18, 21 and 22) to nine chromosomes (1, 13, 15, 16, 17, 18, 21, 22 and X), which are the most commonly involved in aneuploidy in spontaneous abortions and live births (Munné *et al.*, 2000; Kuliev

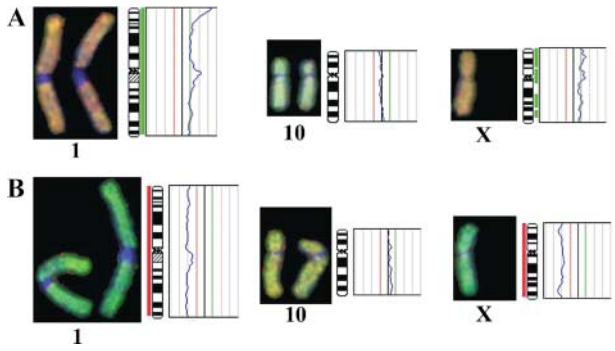

Figure 1. CGH results from doublet 12 (Table I) indicating a double aneuploidy for chromosomes 1 and X. Interestingly, this doublet came from a 21 year old donor. (A) CGH results from the 1PB showing chromosomes 1 and X more red than other chromosomes (e.g. chromosome 10) and deviation of the CGH ratio >1.2 indicating gain. (B) CGH results from the MII oocyte showing the same chromosomes more green and deviation of the CGH ratio to the left (<0.8) indicating the reciprocal loss.

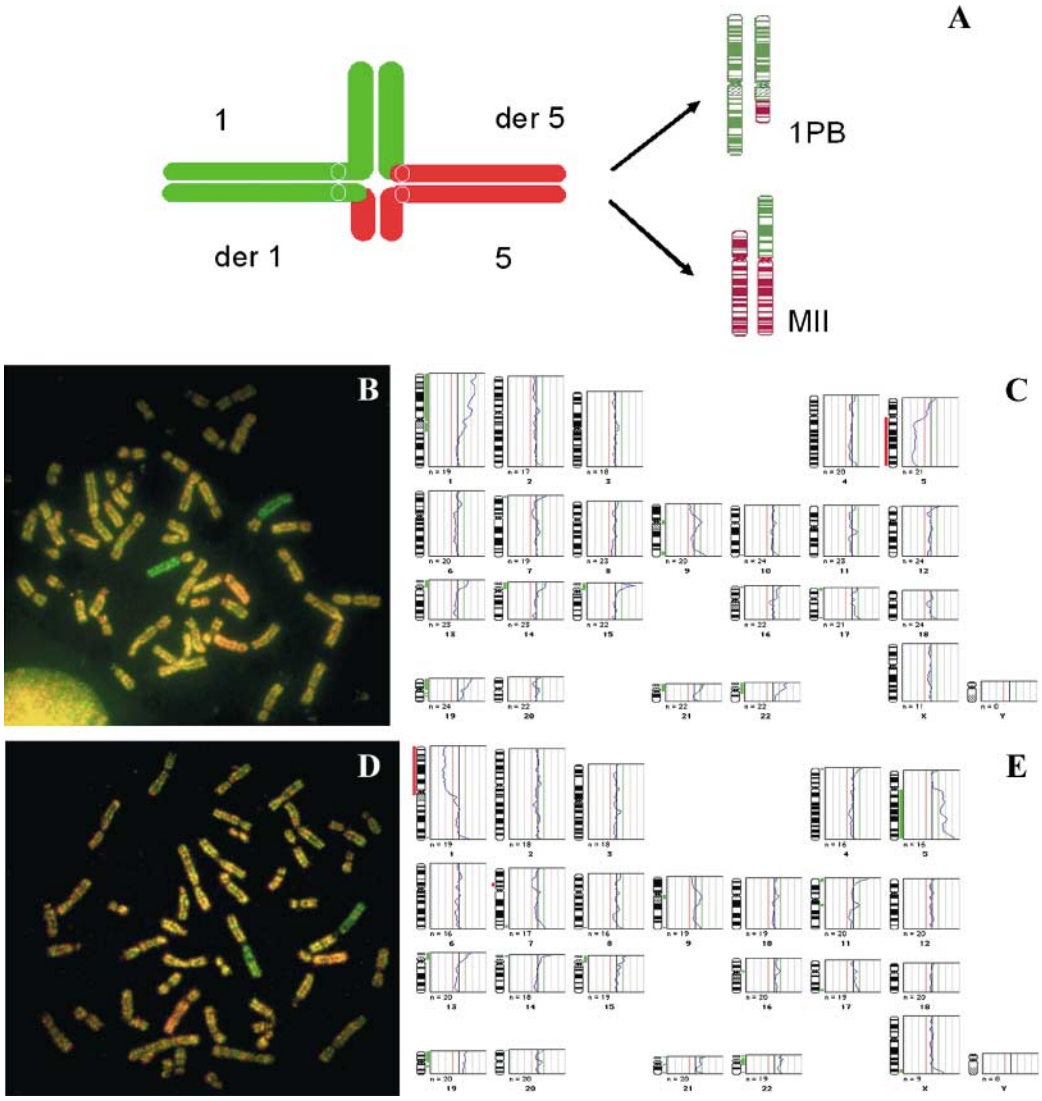

Figure 2. Results of CGH on a doublet 1PB-MII oocyte from a reciprocal translocation carrier 46, XX, t(1;5)(q21.1;p13.1). (A) Quadrivalent formed during prophase I. An adjacent two segregation occurred so that chromosome 1 and the derivative 1 (der 1) segregated to the 1PB while chromosome 5 and the derivative 5 segregated to the MII oocyte. (B) Results from a normal metaphase spread hybridized with amplified normal female DNA labelled with Spectrum Green and amplified DNA from the 1PB labelled with Spectrum Red. The short arm of chromosome 1 (1pter-1q21.1) appears relatively more red, while the long arm of chromosome 5 (5p13.1-5qter) appears relatively more green. (C) CGH profile showing the deviation of the region 1pter-1q21.1 to the right (gain) and the region 5p13.1-5qter to the left (loss). (D) Normal metaphase template hybridized with normal female DNA (green) and MII oocyte DNA (red). The region 1pter-1q21.1 appears more green and the region 5p13.1-5qter appears more red. (E) CGH pattern showing an extra region of chromosome 5, reciprocal to that missing in the 1PB, and the short arm of chromosome 1 deviated to the left indicating loss.

Table II. CGH analysis of doublets 1PB-MII oocyte from Robertsonian or reciprocal translocation carriers

Translocation	Doublet 1PB-MII	Age	CGH interpretation 1PB	CGH interpretation MII	Segregation	Reciprocity
t(13;14)(q10;q10)	1	29	23, X or 22, X, +der13;14	22, X, +der13;14 or 23, X	Normal or balanced	Yes
	2	29	23, X or 22, X, +der13;14	22, X, +der13;14 or 23, X	Normal or balanced	Yes
t(13;15)(q10;q10)	3	35	23, X or 22, X, +der13;15	22, X, +der13;15 or 23, X	Normal or balanced	Yes
	4	35	23, X or 22, X, +der13;15	22, X, +der13;15 or 23, X	Normal or balanced	Yes
t(1;5)(q21.1;p13.1)	5	35	23, X, +der1, −5	23, X, −1, +der 5	Adjacent two	Yes

et al., 2003; Pujol *et al.*, 2003). Consequently, only about one third of the chromosomes in each cell are analyzed.

PGD using CGH to detect aneuploidy for almost all the chromosomes might increase IVF pregnancy rates by detecting abnormalities not currently detected by the nine FISH probe set, thus assisting IVF laboratories in selecting viable embryos for transfer and avoiding transfer of aneuploid embryos with low implantation potential.

If we had used FISH for nine chromosomes instead of CGH, 57.1% (4/7) of the 1PB-MII oocyte doublets showing single aneuploidy and 47.3% (9/19) of individual chromosome errors (involving chromosomes 2, 3, 6, 7, 8, 9, 19 and 20) would have been missed. As it has been suggested (Abdelhadi *et al.*, 2003), some pairs (i.e. doublets 4, 9 and 16) showed double or extensive aneuploidy involving not only chromosomes that are routinely analyzed with the nine chromosome panel but also other chromosomes not included in the panel. Consequently the doublet would have been scored as abnormal even if FISH screening only had detected one chromosome error. Despite this fact, it is important to note that about 83% (10/12) or 33% (4/12) of the doublets classified with CGH as aneuploid would have been misdiagnosed as normal using FISH with probes for five or nine chromosomes, respectively. Our results are consistent with a recent study where 25% of the blastomeres diagnosed as aneuploid with CGH, would have been incorrectly diagnosed as normal using FISH for nine chromosomes (Wilton *et al.*, 2003a). However, the higher rate of no reciprocity (20%) with CGH compared to FISH's misdiagnosis (12%) (Abdelhadi *et al.*, 2003) would result also in either normal embryos not being replaced or replacement of some abnormal embryos.

Although the 25 1PB-MII oocyte doublets studied here represented a small sample, it was still possible to recognize a relation between maternal age and chromosomal abnormalities. Consideration of two maternal age groups (21–36 and $\geq$37 years old) revealed a significantly higher aneuploidy rate in the older group (75% verses 23%, $P < 0.02$). Since a recent study has found a 52.1% aneuploidy rate in women of advanced maternal age (average 38.5 years old) using FISH for the analysis of only five chromosomes (Kuliev *et al.*, 2003), our aneuploidy rate in older women is not unexpected. These results support previous reports where age-related aneuploidy is demonstrated analysing a more sizeable dataset (Dailey *et al.*, 1996; Pellestor *et al.*, 2003).

A total of 30 1PB-MII oocyte doublets have been successfully analyzed using CGH. The presence of bands after amplification by DOP–PCR, has been reported previously and they have been identified as mitochondrial DNA, which is selectively amplified by DOP–PCR (Voullaire *et al.*, 2000). Despite these high levels of mitochondrial DNA in MII oocytes, there is no interference with CGH profiles, as mitochondrial DNA does not hybridize to the template chromosomes (Voullaire *et al.*, 2000). However, mitochondrial DNA may compete with genomic DNA in the amplification and nick translation procedure. This could explain why MIIs, which contain many mitochondria, usually give a weaker hybridization than the 1PBs.

A 20% of non-reciprocity between 1PB and MII oocyte results was found, as six out of 30 1PB-MII oocyte pairs presented one or two missing chromosomes, while the sibling cell (1PB or MII oocyte) did not display a clear gain of material. There are two possible reasons that may explain these results.

First, in standard CGH, hypohaploidy affecting whole chromosome or single chromatid (DNA test: DNA reference ratio 0:2 or 1:2, respectively) is easier to detect than hyperhaploidy (ratio 4:2 or 3:2, respectively), as in the hypohaploidy there is a loss of 50–100% of the chromosomal material, while in the hyperhaploidy there is only a gain of 33–50%. Four out of six doublets which show no reciprocal results between the 1PB and MII oocyte (doublets 3, 7, 8 and 19; Table I) showed a missing chromosome in one of the cells, while the other cell displayed a deviation of red:green ratio that was suggestive of a gain of chromosomal material, but fell within the threshold cut-off of 1.2. Our data combined with other observations suggest that some hyperhaploidies, mainly the ones which could involve single extra chromatids could show a doubtful profile in the CGH analysis (Voullaire *et al.*, 2002).

Second, recent 1PB-MII oocyte FISH studies found evidence of oocytes from karyotypically normal women that appeared to have originated from trisomic germ cell lines (e.g. gonadal mosaicism). Some doublets had an extra chromatid in both the 1PB and the MII oocyte, while others had an extra chromosome with no reciprocal loss of material in the complementary cell (Mahmood *et al.*, 2000; Cupisti *et al.*, 2003; Pujol *et al.*, 2003). Considering that artifactual loss of chromosomes is not expected with CGH, our data indicate the possible existence of a gonadal mosaicism with a monosomic germ line in some of these patients, as one of the cells (MII oocyte or 1PB) has a missing chromosome while the other cell shows a normal karyotype.

One of the main limitations of CGH is that it is unable to detect alterations such as balanced predivision of chromatids, which predisposes to aneuploidy, but does not result in an immediate gain or loss of chromosomal material. Additionally, CGH is incapable of detecting changes in ploidy (e.g. diploid oocytes). Heterochromatic, telomeric and centromeric regions have to be excluded from the analysis because

they usually show a deviation in the CGH pattern. Some studies have already reported the difficulty of interpreting the CGH profile of chromosomes 17, 19 and 22 (CG-rich areas) in either classical CGH or CGH applied to the analysis of single cells. Therefore, when the test signal is enhanced, these chromosomes are also excluded from the analysis (Moore *et al.*, 1997; Voullaire *et al.*, 2002). It has been reported that CG-rich areas of the genome yield CGH artefacts but the specific mechanisms that create this artifact are still unknown. We have found artifactual gains of these chromosomes, despite the use of "reverse labelling" method, that may reduce hybridization artefacts in some of these problematic regions (Larramendy *et al.*, 1998). In addition, the use of 1PBs has inherent limitations in itself, since second meiotic, paternally derived and post-zygotic chromosome errors (i.e. embryonic mosaicism, which has been detected in 30% of cleavage-stage embryos; Munné *et al.*, 1995a) cannot be detected.

On the other hand, 90% of embryo aneuploidy is the result of errors in maternal meiosis I (Nicolaidis and Petersen, 1998), consequently, in non male-factor IVF patients, CGH analysis of 1PBs may allow the identification of most chromosomal abnormalities. In a clinical case, the biopsy would be carried out on day 0, after ICSI procedure (Durban *et al.*, 2001). Considering that hybridization times of 36 and 72 h gave comparable results, the total time required to perform the CGH would be about 60 h (counting as: 13 h for the DOP–PCR–CGH experiment, plus 45–48 h of hybridization). This timetable is compatible with regular *in vitro* fertilization and it would allow embryo replacement on day + 3 or + 4 (depending on the number of 1PBs being analysed). Clinical cases have been undertaken via this approach (Wells *et al.*, 2002).

In addition, other studies have shown the ability of CGH to detect chromosome breakage in human embryos (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). FISH probes only reveal information about the small area of each chromosome to which they hybridize and consequently most rearrangements that affect chromosomal regions, rather than the whole chromosomes, are not detected. CGH will be a more appropriate tool for the detection of *de novo* structural abnormalities that results in loss/gain of chromosomal material.

Further investigation involving other techniques such as FISH, SKY or m-FISH is needed to test the reliability of CGH to detect not only extra or missing full chromosomes, but also single chromatid abnormalities (precocious sister chromatid segregation; predivision), which is one of the most common mechanisms of aneuploidy in human oocytes (Angell, 1997).

A recent study showed the ability of CGH to detect unbalanced segregations of translocations, as long as the unbalanced region is larger than 10–20 Mb, which is the resolution of CGH applied to single cells (Malmgren *et al.*, 2002). On the other hand, interchromosomal effects (ICE) in spermatozoa, embryos or oocytes from translocation carriers have been found by some authors (Blanco *et al.*, 2000; Pellestor *et al.*, 2001; Gianaroli *et al.*, 2002; Pujol *et al.*, 2003). These studies have been performed using FISH for the analysis of up to 10 selected chromosomes; consequently, some ICE involving other chromosomes could remain undetected. In this study the complementary products of adjacent two segregation were detected in a 1PB-MII oocyte pair donated from a t(1;5) carrier. This indicates that CGH could be used for PGD of maternal translocations, revealing whether specific rearrangements do indeed induce an ICE during female gametogenesis.

In conclusion, in the present study we have demonstrated the reliability of CGH not only to detect single copy number changes involving whole chromosomes in 1PB and MII oocytes, but also to detect unbalanced segregations of a maternal translocation. Our results indicate that CGH analysis of the 1PB may be used for the indirect characterization of the chromosome constitution of the oocyte. Due to the limited number of oocytes being analysed, further investigation would be necessary to give a better estimation of the error rate of this methodology prior to its standard clinical application. The clinical application of this method for the purposes of PGD could increase success rates for couples undergoing IVF treatment, not only advanced maternal age patients but also female carriers of chromosome rearrangements and women with repeated implantation failure.

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