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Identification of embryonic chromosomal abnormality using FISH-based preimplantaion genetic diagnosis

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Abstract: Objective: Embryonic chromosomal abnormality is one of the main reasons for in vitro fertilization (IVF) failure. This study aimed at evaluating the value of Fluorescence in-situ Hybridization (FISH)-based Preimplantation Genetic Diagnosis (PGD) in screening for embryonic chromosomal abnormality to increase the successful rate of IVF. Method: Ten couples, four with high risk of chromosomal abnormality and six infertile couples, underwent FISH-based PGD during IVF procedure. At day 3, one or two blastomeres were aspirated from each embryo. Biopsied blastomeres were examined using FISH analysis to screen out embryos with chromosomal abnormalities. At day 4, embryos without detectable chromosomal abnormality were transferred to the mother bodies as in regular IVF. Results: Among 54 embryos screened using FISH-based PGD, 30 embryos were detected to have chromosomal abnormalities. The 24 healthy embryos were implanted, resulting in four clinical pregnancies, two of which led to successful normal birth of two healthy babies; one to ongoing pregnancy during the writing of this article; and one to ectopic pregnancy. Conclusion: FISH-based PGD is an effective method for detecting embryonic chromosomal abnormality, which is one of the common causes of spontaneous miscarriages and chromosomally unbalanced offsprings.

Key words: Preimplantation genetic diagnosis, Fluorescence in-situ Hybridization (FISH), Chromosome abnormality
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INTRODUCTION

The development of in vitro Fertilization (IVF) and related techniques has dramatically increased the possibility of obtaining healthy babies from infertile patients. However, IVF operation has certain limitations. It is well known that lower implantation rate and higher spontaneous abortion rate are closely correlated with an increase in maternal age. Even in the young patients, unexplained multiple IVF failures have been frequently reported.

Studies of Gianaroli *et al*.(1997) showed that infertile patients with a poor prognosis have increased risk of having embryos with chromosomal abnormality, which could be one of the main reasons of implantation failure. To solve this problem, Preimplantation Genetic Diagnosis (PGD) for chromosomal abnormality before embryonic transfer has been used in some laboratories in advanced countries. Generally, PGD is performed by biopsy of day 3 embryos to obtain one or two blastomeres. Isolated blastomeres are fixed and analyzed for chromosomal status using Fluorescence in situ Hybridization (FISH). Healthy embryos selected from PGD are transferred to the uterus. This technique has been demonstrated to efficiently increase the IVF implantation rate. However, the PGD technique has not been established in many IVF centers in China. Many patients are still suffering from the low successful rate of the expensive IVF

procedure. This study showed that we have successfully developed FISH-based PGD technique in our IVF laboratory and used PGD to identify embryonic chromosomal abnormality in high risk patients. Our study showed that FISH-based PGD is an efficient method for achieving a high success rate in IVF procedure for infertile patients.

MATERIALS AND METHODS

Clinical cases

From October 2001 to July 2003, a total of 10 couples were treated with PGD-combined IVF procedure in our IVF center. The research was approved by the Ethics Committee of our Institution. Each individual case is described below.

In case 1, the patient was a male carrier who had a balanced Robertsonian 45, XY, t (13q14q) translocation. His wife had undergone three spontaneous miscarriages. This couple had been infertile although they had tried to induce pregnancy in the recent three years.

In case 2, the patient was a mosaic 47, XXY/46, XY (with 5 cells in 100 showing a 47, XXY karyotype) infertile male. The semen analysis showed that sperm concentration was 2.2×10^6 ml^{-1}, with 35% of the sperms graded as C (motion with no progression) and 65% as D (no motion) according to their motility.

In case 3, the patient was a 43 years old woman who had delivered two babies both with trisomy 21 karyotype.

In case 4, the patient was a 38 years old woman, who had miscarried twice with massive fetal malformation. The first infant had low-set ear, micrognathia and overlapping fingers. The other infant showed hydrocephalus and visceral malformation. Detailed description was absent because autopsies were not performed. This couple has been infertile in the recent five years.

In the rest of the cases, the patients were all infertile women, aged from 37 to 42.

Oocyte retrieval, assessment of fertilization and embryo development

In each case, the female patients were treated to induce multiple follicular growths using a long desensitization protocol consisting of long-acting GnRH analogue and exogenous gonadotropins. Oocytes were retrieved transvaginally under ultrasound guidance, followed by insemination using Intracytoplasmic Sperm Injection (ICSI) (Jin *et al*., 1998). Fertilized oocytes were examined for pronuclei and polar bodies at 17 hours after insemination. Successfully fertilized oocytes were cultured in P-1 medium (Irvine Science, USA) and examined once again after 44 hours. For PGD, day 3 embryos with 5–10 blastomeres of equal size and fewer than 30% fragmentation were selected for embryo biopsy. Up to 6 embryos were biopsied for each patient (except patient 1) to obtain normal embryos. The rest of the embryos were cryopreserved for further use.

Embryonic biopsy and blastomere fixation

For biopsy, embryos were incubated in calcium magnesium-free EB-10 medium (IVF Science, Sweden) for 20 minutes. For each embryo, a 20–22 μm breach was opened on the Zona Pellucida (ZP). Zona drilling was done with 3 methods: using Tyrode's solution, mechanically and by laser beam. To do zona drilling mechanically, microtool consisting of one holding pipette and one microneedle was needed. The embryo was held loosely in position, and the microneedle was passed through the zona pellucida tangentially and on through the perivitelline space. Then the embryo was released from the holding pipette and held by the microneedle. The first cut was made by gently rubbing the microneedle with sawing motion against the holding pipette and then the emryo was released. The embryo was rotated again vertically and held firmly in position. A second cut was made by passing through the zona pellucida, under the first slit opening and out the other side, starting and ending in the same positions as in the first cut. Laser-assisted zona drilling was done simply with a noncontact 1.5 ms laser (RI, UK). Usually one blastomere was aspirated from each embryo. A second blastomere was aspirated if no cell nucleus was found in the first one. One or two blastomeres

were removed for biopsy using a polished glass needle, which was introduced into the perivitelline space through the hole opened on the ZP. After biopsy, embryos were transferred to the blastocyst medium (Irvine Science, USA) for further culture. Aspirated blastomere samples were lysed in hypotonic buffer for 1–2 minutes. The cell nuclei were separated and fixed on glass slides using 0.01 N HCl/0.1% Tween 20. The slides were air-dried, washed with PBS for 5 minutes, and dehydrated in a series of 70%, 85% and 100% ethanol solutions before FISH analysis.

FISH analysis

Probes used for hybridization were all purchased from Vysis Company. For patient 1, nuclei were hybridized with probes specific for chromosome 13 (LSI 13) and 14 (Telvysion 14q) labeled with Spectrum-Green and Spectrum-Orange, respectively. For Patient 2, nuclei were hybridized with probes specific for chromosome X (CEP X, DXZ1, alpha satellite in Spectrum-Green) and Y (CEP Y, DYZ1, satellite III in Spectrum-Orange). For patient 3–8, 3 probes were used for the detection of chromosome 13, 18 and 21. The probes were labeled as follows: LSI 13 Spectrum-Orange, CEP 18 (D18Z1, alpha satellite) Spectrum-Aqua, LSI 21 spectrum Green. For patient 9 and 10, probes used for hybridization were LSI 21 spectrum Green, CEP X alpha satellite in Spectrum-Green, and CEP Y, satellite III in Spectrum-Orange.

Slides with fixed blastomeres were denatured in 70% formamide at 73 °C. Hybridization solution heated to 73 °C were added to the fixed blastomeres. The slides were then sealed and placed in a moist chamber at 37 °C for overnight hybridization. After hybridization, the slides were washed once with 0.4×SSC/0.3% NP-40 for 90 seconds at 73 °C and once with 2×SSC/0.1% NP-40 for 1 minute at room temperature. The slides were counterstained with DAPI in an antifade buffer, and observed under a fluorescence microscope (Olympus AX) equipped with a triple-band-pass filter set for simultaneous observation of the spectrum-orange, spectrum-green and spectrum-aqua fluorescence.

RESULTS

Collecting normally-developed embryos for FISH-based PGD

A total of 158 oocytes were obtained from 10 patients. As shown in Table 1, 137 oocytes were mature and inseminated using ICSI, resulting in 97 successfully fertilized oocytes (70.8%). Ninety-three cleaved embryos were suitable for implantation. Among them, 54 embryos were biopsied and subjected to FISH-based PGD. The rest of the embryos were frozen for storage.

Results of embryonic biopsy and FISH analysis

Embryos were biopsied using the methods described in "MATERIAL AND METHODS" section. From the 54 embryos, 61 blastomeres were obtained for detection of chromosal abnormality using FISH-based PGD. As shown in Tables 2 and 3, 27 embryos were diagnosed as embryos with chromosomal abnormalities, 3 were not diagnosed because no fluorescence signals were observed. The rest of the 24 embryos were diagnosed as containing normal or balanced chromosomes and were transferred to the uterus.

Outcomes of pregnancies

In each case, except case 3, embryos confirmed by biopsy and FISH analysis to have normal chromosomes were implanted into the uterus. In case 3, both embryonic biopsies showed chromosomal abnormalities, so no embryo was transferred. Four gestations were resulted from a total nine cases who accepted embryonic transfer (cases 1, 2, 4 and 5). In case 1, one single euploid embryo was transferred to the wife, who is currently undergoing pregnancy. Ultrasonic examination showed normal development of the fetus at week 7 and 10. In case 2, the wife condition was diagnosed as tubal pregnancy 30 days after implantation of three normal embryos. The pregnancy was ended with surgery. Two and three normal embryos were transferred respectively to patients 4 and 5, who both successfully delivered healthy babies after full-term pregnancy. In cases 6–10, no patient was pregnant after embryonic transfer.

Table 1 A summary of collecting normally-developed embryos for FISH-based PGD

Case No.	Age	Oocyte retrieved	Mature oocytes	Fertilized eggs	Cleaved embryos	Biopsed embryos	Embryos for storage
1	33	33	23	14	14	10	0
2	35	6	5	4	4	4	0
3	43	3	3	2	2	2	0
4	38	18	17	10	9	6	7
5	38	17	13	13	13	6	4
6	39	18	17	12	12	6	6
7	38	22	21	14	14	6	8
8	37	18	16	10	10	6	7
9	42	15	15	14	13	6	3
10	38	8	7	4	2	2	0
Total		158	137	97	93	54	35

Table 2 Results of FISH-based PGD and outcomes of transferred embryos

Case	Method	Embryos biopsed	Blastomere obatained	Number of embryos			Embryo transferred	Gestation number
				Abnormal	Unknown	Normal/balanced		
1	Tyrode's	10	10	7	2	1	1	1
2	Laser	4	6	1	0	3	3	1
3	Tyrode's	2	4	2	0	0	0	0
4	Tyrode's	6	6	4	0	2	2	1
5	Tyrode's	6	6	3	0	3	3	1
6	Mechanical	6	8	2	1	3	3	0
7	Tyrode's	6	6	2	0	4	4	0
8	Mechanical	6	7	3	0	3	3	0
9	Tyrode's	6	6	2	0	3	3	0
10	Tyrode's	2	2	0	0	2	2	0
Total		54	61	27	3	24	24	4

DISCUSSION

Carriers of reciprocal and Robertsonian chromosomal translocation have high probability of multiple spontaneous abortions upon pregnancy, or producing offspring with chromosomal abnormalities. Application of preimplantation chromosomal diagnosis can significantly reduce the rate of abnormal pregnancies and help chromosomal-imbalanced couples to obtain healthy babies. The ideal method for PGD is FISH analysis on interphase blastomeres. Good FISH probes should be specifically designed for individual types of translocation, either spanning the breakpoints or flanking the breakpoints (Munné *et al*., 1998a). This method not only can detect aneuploid embryos, but also can differentiate balanced translocation from normal embryos. However, preparation of multiple specific probes is expensive and some times difficult, because translocation could occur atany locus on the chromosome. Currently, some laboratories simply use two pairs of subtelomeric probes and two pairs of centromeric probes simultaneously for PGD analysis. However, balanced translocation cannot be differentiated from normal embryos using this method (Knight and Flint, 2000).

In this study, we attempted to perform PGD using fewer but more specific probes to keep the low cost and high specificity. In case 1, the husband was a male carrier who had a balanced Robertsonian 45, XY, t (13q14q) translocation. Based on this genetic information, we used only two specific probes and successfully screened out multiple embryos with 13, 14 monosomy or trisomy. The only one euploid embryo obtained was transferred and

successfully implanted. Now the wife is undergoing pregnancy with normal fetal development.

IVF technology is very useful in increasing the pregnancy rate for patients with mosaic sex chromosomal abnormalities (Munné *et al*., 1998b). However, children of these patients also have high risk of sex chromosomal abnormalities (Bielanska *et al*., 2000). Clinical features of these patients can vary from germinal aplasia, sub-infertile, to nearly normal phenotypes. For these patients, IVF should be combined with FISH-based PGD to reduce the incidence of sex chromosomal abnormality in the next generation. In our study, the husband of case 2 was an oligoasthenozoospermic patient with 46, XY/47, XYY karyotype. Among four embryos biopsied, three embryos were normal karyotype and one embryo was 49, XXYYY karyotype. This result suggested that patients with mosaic sex chromosomal abnormality are not only at high risk of producing offspring with sex chromosome trisomy, but also tends to generate new types of aneuploidy (Staessen *et al*., 2003). Therefore, karyotypes of the adult patients should be analyzed to evaluate the risk of embryonic abnormality before treating with assisted reproductive technology.

Newly formed chromosomal abnormalities are frequently detected in preimplantation embryos created from stimulated cycles (Munné *et al*., 1998b). In addition, incidence of chromosomal trisomy is closely associated with increased maternal age (Verlinsky and Kuliev, 1996). It was reported that among all PGD cases currently performed worldwide, two-third of the cases are those using FISH to screen embryonic aneuploidy for women with advanced age (Simpson, 2001). Embryonic chromosomal aberrations are generated from two types of non-disjunction: non-disjunction occurring during gamete meiosis results in embryonic aneuploidy; non-disjunction occurring during zygote mitosis leads to mosaic chromosomal abnormality. Aneuploidy screening studies for oocytes and embryos from women aged over 35 showed that chromosome 13, 18 or 21 abnormality in oocytes was as high as 43%; whereas the abnormal rate of cleaved embryos could reach 58%~66% (Wilton, 2002). In our study, cases 3–10 were female patients with advanced age. Among these cases, more than half of the embryos were diagnosed as aneuploidy. The incidence of trisomy, monosomy and trisomy/monosomy were 30%, 12.5% and 5%, respectively.

Table 3 Chromosomal status of embryonic biopsy samples

Case	FISH probe	Chromosome status	Embryo number
1	LSI13 (13q14), TelVysion 14q	Normal/balanced	1
		13 monosomy/14 trisomy	3
		13 trisomy/14 monosomy	2
		14 trisomy	2
		No signal detected	2
2	CEP X, CEP Y	Normal	3
		XXYYY	1
3–8	LSI13, CEP18, LSI21	Normal	15
		13 trisomy	2
		21trisomy	3
		21 monosomy	2
		13 trisomy/21 trisomy	2
		18 trisomy/21 trisomy	2
		13 monosomy/18 monosomy	1
		18 monosomy/18 monosomy	2
		13 monosomy/18 monosomy/21 trisomy	1
		13 trisomy/18 monosomy/21 trisomy	1
		No signal detected	1
9–10	LSI21, CEP X, CEP Y	Normal	5
		21 trisomy	3

Our results were in good agreement with the aneuploidy rate reported in the literature.

In this study, ten cycles of FISH-based PGD were performed in IVF procedures for ten couples.Within nine cycles, 24 embryos confirmed without chromosomal abnormality were transferred resulting in 4 singletons. Our study suggested that FISH-based PGD should be performed to screen out aneuploid embryos and therefore decrease the rate of abnormal pregnancy in high-risk patients. This technique can efficiently increase the successful rate of IVF procedure and should be routinely used in high-risk populations.

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