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Genetic Diagnosis of Sex and Trisomies 13, 18, 21 in Human Single Cell Embryo by Multiplex Fluorescent Polymerase Chain Reaction

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ABSTRACT

A multiplex fluorescent PCR was developed for preimplantation genetic diagnosis (PGD) to investigate aneuploidy screening of chromosomes 13, 18, 21, X and Y in a single human blastomere. The multiplex fluorescent PCR was able to give successful amplification in 30 cases. This technique was found to be sufficiently sensitive to reveal the peaks of trisomic alleles in 20 pg DNA of control samples. Multiplex fluorescent PCR products of short tandem repeats (STRs) located on chromosomes 13, 18, 21, X and Y from a single human blastomere in a single tube have recently been used for the detection of common chromosome aneuploidies in a single human blastomere.

Key word : aneuploidy, multiplex fluorescent PCR , PGD

INTRODUCTION

The first *in vitro* fertilization (IVF) baby is now more than 25 years old, and the number of patients using assisted reproductive technology (ART) have been increasing. However, the ART still faces a problem which mainly is chromosome abnormalities of embryos. Aneupliody results predominantly from non-dysjunction of the chromatids in the first meiotic division of the oocyte and increases with maternal age (Verlinsky and Kuliev, 1998). Of this abnormality, trisomies 13,18,21, X and Y can be commonly found in the cleavage and the blastocyst stage (Magli *et al*., 2000). These aneuploidies usually cause implantation failure of the blastocyst or spontaneous abortion of the fetus in the first trimester, although a small proportion of trisomy 13,18 and 21 fetuses can develop to term.

Preimplantation genetic diagnosis (PGD) is a technique used to identify genetic defects in embryos created through *in vitro* fertilization (IVF). PGD is now an interesting tool for patients carrying genetic defects(Menezo *et al*.,2000).Technically, embryos or oocytes are biopsied during culture *in vitro* and genetic analysis is carried out on material derived from a polar body or a blastomere. The only embryos without genetic abnormalities will be thereupon selectively transferred to the uterus.

PGD tests have largely focused on two methods, fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR). The FISH has been applied to PGD of common aneuploidies (at least chromosomes 13, 18, 21, X and Y).

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However, the cost of FISH is rather expensive. In the past, PCR-based methodology has only been used to diagnose single gene disorders. More recently, this technique has been developed to screen both the single gene disorders and the chromosomal abnormalities.

Fluorescent PCR, a new technique of PCR, has been shown to be a rapid and accurate technique to screen the genetic disorders of single cells. This method has many distinct advantages for clinical PGD (Findlay *et al*., 1995). There is less chance of non-specific amplification of contaminating DNA. The fluorescent-based systems are highly amenable to multiplex PCR. Fluorescent PCR detection technology, first developed for automated sequencing, has been adapted for PCR fragment analysis and applied for genetic screening (Sermon *et al*.,1998). Incorporation of fluorescent primers into PCR products enable a laser detection system to register the DNA fragments as excitation peaks that provides more sensitivity than standard gel electrophoresis (Hattori *et al*., 1992). In addition, the signal intensities of the fluorescent peaks can be compared and applied for quantitative PCR analysis. As a consequence of the increased sensitivity with fluorescent-PCR, a low allelic drop-out (ADO) rate has been reported due to an ability to distinguish true ADO from preferential amplification of one allele over the other (Findlay *et al*., 1995).

In this study, we have used multiplex fluorescent PCR techniques to investigate aneuploidies involving chromosomes 13, 18, 21, X and Y at short tandem repeats (STRs) of specific loci of these particular chromosomes. Quantitative analysis of the amplified products allowed the diagnosis of trisomies 13, 18, and 21 while sexing was performed simultaneously using PCR amplification of DNA sequences derived from the chromosomes X and Y. The aim of this study is to develop multiplex fluorescent PCR for screening of common aneuploidies (at chromosome 13, 18, 21, X and Y) of a single human blastomere.

MATERIALS AND METHODS

Embryo selection and collection

Blastomere cells were isolated from 'spare' embryos donated for research by patients undergoing routine treatment at the Reproductive Endrocrinology and Infertility (REI) Division, Department of OB&GYN, Ramathibodi Hospital, Faculty of Medicine, Mahidol University. This work was approved by the ethical clearance committee on human rights related to researches involving human subjects of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University.

A total of 30 blastomere samples received from Rama-IVF laboratory in a 7-month period for PGD were used in the present study. Since high-grade embryos are generally kept for *in vitro* fertilization (IVF) of the patients, only lower-grade (3 or 4) are available for this research used.

Blastomere biopsy and lysis

Zona pellucida was biopsied using a laser optical system (ZILOS-tk, Hamilton Thorne Biosciences, USA). Each blastomere was retrieved and checked for the presence of a nucleus. The blastomere was rinsed and collected in a 0.2 ml PCR tube. For each embryo,one blank of 3 µl of the last washing droplet was used and transferred to a PCR tube to monitor contamination. The single cell (blastomere) was lysed after 60 min incubation at 37°C in proteinase K and sodium dodecyl sulphate buffer and followed by inactivation of the enzyme at 95°C for 15 min (Holding *et al*., 1993).

Multiplex fluorescent PCR reactions and analysis

The multiplex fluorescent PCR amplification was performed in a total reaction volume of 20 µl containing 1.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 1x AmpliTaq Gold PCR Buffer

(Perkin Elmer; 500 mmol/l KCl, 100 mmol/l Tris HCl, pH 9.0), 1.5 mM $MgCl_2$, 200 µmol/l each of deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), 10-20 pmol of each primer (Applied Biosystems, USA), and 5.7 µl molecular biology grade H_2O. The PCR was started with an initial denaturation step of 10 min at 95°C, followed by 35 cycles of 95°C for 50 sec, 59°C for 50 sec, 72°C for 1 min and terminated with a final extension step of 10 min at 72°C. The PCR products (1.5 µl) were mixed with 12.0 µl of formamide buffer and 0.5 µl GeneScan LIZ-500 internal standard (Applied Biosystems). Samples were denatured at 95°C for 3 min and cooled immediately on ice. The amplified products were analysed and their relative fluorescent intensities were calculated using an ABI310 Genetic Analyzer (ABI PRISM Applied Biosystems). The results were analysed with GeneScan Analysis software version 377 provided by the manufacturer.

The blastomere cells were tested with chromosomes 13, 18, 21, X and Y derived STR D13S258 (Toth *et al*., 1998), D18S51 (Straub *et al*., 1993), D21S1413 (Findlay *et al*., 1998), and Amelogenin (Mansfield, 1993 ; Cirigliano *et al*., 1999), respectively. The fluorescent PCR products were identified by different sizes of fluorochromes labelled in the STR markers (Table 1). The multiplex fluorescent PCR patterns are considered to be normal diallelic if the peaks have similar activities in a 1:1 ratio. However, trisomic patterns usually reveal the presence of either three peaks (1:1:1 ratio), or two peaks of approximately 2:1 ratio. This rapid approach is particularly valuable when applied to preimplantation genetic diagnosis of aneuploidy.

RESULTS AND DISCUSSION

Aneuploidies can be rapidly diagnosed by the analysis of multiplex fluorescent PCR of chromosome specific short tandem repeat (STR) markers. The quantitative STR patterns showed 2 peaks of 1:1 ratio in a normal heterozygous sample using 20 pg genomic DNA (Figure 1A) . For aneuploidies involving sex chromosome, or trisomies of chromosomes 13, 18, and 21 the samples were tested with 20 pg control DNA extracted from aneuploid blood samples diagnosed by karyotype diagnosis (Figure 1B).

Table 2 illustrated condition of the 30 biopsied embryo cells. Five of them contained normal males and showed normal electropherogram (Figure 2 D) and nine of them

Table 1 STR markers and different fluorochromes for the detection of aneuploidy.

STR marker	Labeled fluorochrome
AMXY	VIC
D13S258	6-FAM
D18S51	PET
D21S1413	NED

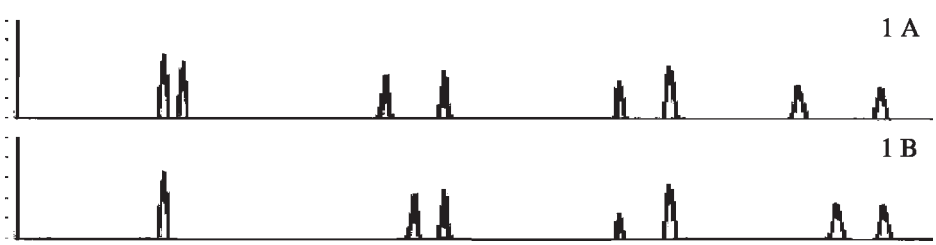

Figure 1 Amplification of chromosome 13, 18 and 21 short tandem repeats (STR), D13S258 (blue), D18S51 (red) and D21S1413 (black) from (A) 20 pg of a normal male DNA (B) 20 pg of a female DNA with trisomy 13.

contained normal females. The trisomic allele profiles are shown in Figures 2 A, B and C.

The satisfactory results using multiplex fluorescent PCR technique conducted under our clean environment laboratory showed no contamination of external DNA. Also, the ADO was low due to our suitable selection of blastomere. The high quality blastomere must contain nucleus. The nuclear DNA in the blastomere has positive effect on the amplification success of multiplex fluorescent PCR single cell analysis. For summary, multiplex fluorescent PCR products of short tandem repeats (STRs) located on chromosomes 13, 18, 21, X and Y provide possible opportunity for the detection of common chromosome aneuploidies in single human blastomere.

Table 2 Characteristics of male and female single cells.

	Male (n)	Female (n)
Normal	5	9
Trisomy 13	1	3
Trisomy 18	2	1
Trisomy 21	1	5
Trisomy 13 + 18	1	0
Trisomy 18 + 21	1	0
Trisomy 13+18 + 21	0	1
Total	11	19

Previously published multiplex fluorescent PCR developed for single cells (6 pg of DNA) has been plagued with various problems, including high rates of preferential allelic amplification and allelic drop out (Sherlock *et al*., 1998; Findlay

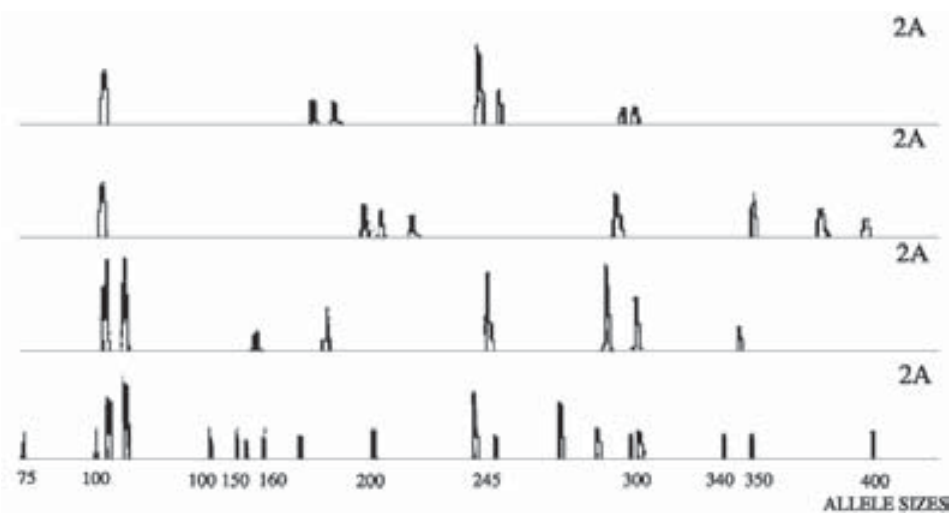

Figure 2 Single human blastomere.

(A) A single human female blastomere with Patau's syndrome: Heterozygous loci (D18S51 and D21S1413) and diallelic locus (D13S258) type 2:1.

(B) A single human female blastomere with Down's syndrome: Heterozygous loci (D13S258 and D18S51) and triallelic locus (D21S1413).

(C) A single human male blastomere with trisomy 18 and 21 : Heterozygous loci (D13S258) and diallelic locus D18S51 type 2:1 and D21S1413 type 1:2.

(D) A normal single human male blastomere: Heterozygous loci (D13S258, D18S51 and D21S1413). Fluorochromes are as follows : green (VIC) =AMXY, blue (6-FAM) =D13S258, black (NEC) =D21S1413, red (PET) =D18S51, and orange (LIZ) peaks indicate internal molecular standard size.

et al., 1998, 1999). In 2002, Katz *et al*. published multiplex fluorescent PCR developed for a single blastomere using specific short tandem repeats (STRs) markers, but the number of blastomeres were small.

CONCLUSION

Multiplex fluorescent PCR was developed for aneuploidy screening in preimplantation genetic diagnosis. It can increase accuracy by using multiple markers for each chromosome for diagnosis. The reason for this is that the second marker will generate a positive result if the first marker fails to perform accordingly. Additional study focussed on providing diagnosis and confirmation of the major trisomies 13, 18, 21, and sex as well as DNA in single cells. The success of MF-PCR in amplifying small quantities of DNA to a level at which they can be visualized and subjected to further genetic analysis has made the technique one of the most important diagnostic techniques in the modern molecular laboratory. MF-PCR protocol for single cell analysis has proved to be challenging, but remains to be explored for adding markers for specific chromosome of the major trisomies 13, 18, 21, and sex in human preimplantation embryos. The limited amount of template DNA (6 pg) available in a single diploid cell leads to a number of problems which are rarely, if ever, observed in routine diagnostic PCR (DNA template of at least 10 ng). Further evaluation using standardized MF-PCR and more STR loci also are suggested for trisomy diagnosis in single cell blastomeres. Moreover, the PGD scope for detection of genetic disorders in Thailand should be extended, e.g., Thalassemia.

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