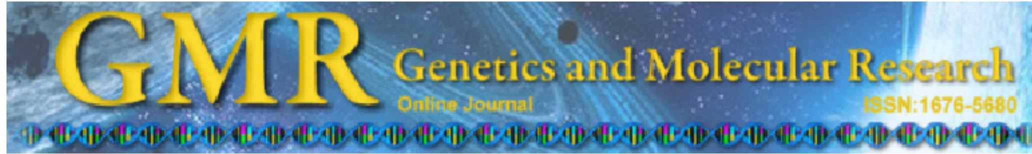

Methodology

Multiple displacement amplification for preimplantation genetic diagnosis of fragile X syndrome

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ABSTRACT. Preimplantation genetic diagnosis (PGD) has become an assisted reproductive technique for couples that have genetic risks. Despite the many advantages provided by PGD, there are several problems, including amplification failure, allele drop-out and amplification inefficiency. We evaluated multiple displacement amplification (MDA) for PGD of the fragile X syndrome. Whole genome amplification was performed using MDA. MDA products were subjected to fluorescent PCR of *fragile X mental retardation-1* (*FMR1*) CGG repeats, *amelogenin* and two polymorphic markers. In the pre-clinical tests, the amplification rates of the *FMR1* CGG repeat, DXS1215 and FRAXAC1 were 84.2, 87.5 and 75.0%, respectively, while the allele dropout rates were 31.3, 57.1 and 50.0%, respectively. In two PGD treatment cycles, 20 embryos among 30 embryos were

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successfully diagnosed as 10 normal embryos, four mutated embryos and six heterozygous carriers. Three healthy embryos were transferred to the uterus; however, no clinical pregnancy was achieved. Our data indicate that MDA and fluorescent PCR with four loci can be successfully applied to PGD for fragile X syndrome. Advanced methods for amplification of minuscule amounts of DNA could improve the sensitivity and reliability of PGD for complicated single gene disorders.

Key words: Fragile X syndrome; DNA amplification technique; Multiple displacement amplification; Trinucleotide repeat expansion; Preimplantation genetic diagnosis; Allele drop-out

INTRODUCTION

Preimplantation genetic diagnosis (PGD) for numerical and structural chromosomal abnormalities and single-gene disorders has been successfully applied as an alternative to prenatal diagnosis of inherited diseases. PGD can be used to differentiate between unaffected and affected embryos before embryo transfer in human *in vitro* fertilization-embryo transfer programs. Therefore, PGD can avoid the initiation of affected pregnancies (Grace et al., 2006; Lee et al., 2007).

Fragile X syndrome (FXS, MIM 309550) is the most common mental retardation disorder. FXS is caused by mutations in the *fragile X mental retardation-1* (*FMR1*) gene located at Xq27.3, more than 95% of which involve hyperexpansion (>200 CGG repeats) and abnormal hypermethylation of a polymorphic CGG trinucleotide repeat in the 5' untranslated region of the *FMR1* gene (Oostra and Chiurazzi, 2001). Phenotypical characteristics include macroorchidism in males, as well as mild facial features like a long face and large protruding ears (Hagerman, 1996). The incidence of this syndrome is estimated to be 1 in 4000 males and 1 in 6000 females based on molecular studies (Turner et al., 1996; de Vries et al., 1997).

Advances in molecular biological techniques allow the application of specific and highly sensitive strategies, such as nested polymerase chain reaction (PCR), multiplex PCR, fluorescent PCR, and whole genome amplification (WGA) for single-cell analysis for PGD. These techniques have been applied to select unaffected embryos for FXS (Sermon et al., 1999; Apessos et al., 2001). However, direct detection of a non-expanded CGG repeat allele or indirect detection using linked polymorphic markers was difficult because of the G+C-rich sequence and the heterozygosity of the repeats and surrounding sequence in the *FMR1* gene, as well as the non-informativity and allele drop-out (ADO) of the linked polymorphic markers. Nevertheless, these direct and indirect detection methods are usually used in the PGD for FXS.

Protocols for PGD of single cells are based on DNA amplification by PCR, which is sufficiently sensitive to detect genetic or genomic variations. Although PCR-based PGD cases are currently performed in all PGD centers, several difficulties associated with single-cell DNA amplification have become evident. These include total amplification failure, ADO, inefficiency of microsatellite amplification, and a limited number of markers for PCR from a single blastomere. Therefore, during the past decade, several WGA techniques, such as primer extension preamplification (Zhang et al., 1992) and degenerate oligonucleotide primed-PCR (Telenius et al., 1992), have been developed and successfully applied to the clinical field, es-

pecially in PGD, prenatal diagnosis, and forensic medicine. Recently, another WGA method called multiple displacement amplification (MDA) was developed (Dean et al., 2002). Bacteriophage Phi29 DNA polymerase and random exonuclease-resistant hexamers are used to yield large amounts of DNA and consistent DNA amplification regardless of the amount of starting DNA template. This MDA method is also more accurate than the PCR-based WGA methods previously used (Dean et al., 2002). Because of these advantages, MDA is also used in PGD (Hellani et al., 2005; Lledo et al., 2007).

We have developed a PGD strategy for FXS using MDA as a pre-diagnostic step for WGA. Using the MDA product as a template, four separate fluorescent PCRs to amplify the non-expanded CGG repeats and 2 polymorphic markers linked to *FMR1* and the *amelogenin* gene, which allows gender to be determined, were carried out in two consecutive clinical PGD programs for FXS.

MATERIAL AND METHODS

Patient description

The patient was a 27-year-old woman carrying a pre-mutation (28 repeats and over 100 repeats of CGG). Her husband showed a number of CGG repeats within the normal range (31 CGG repeats). This couple had experienced a termination of pregnancy for an FXS-affected fetus (31 CGG repeats and a full mutation) that was detected by amniocentesis. As a result of polymorphic marker screening, the DXS1215 (Gyapay et al., 1994) and FRAXAC1 (Richards et al., 1991) polymorphic markers were identified as semi-informative markers in this couple (Figure 1). This couple submitted informed consent before the PGD cycles were started. Ethical approval was obtained from the Cheil General Hospital Ethics Committee before initiating the study.

Isolation and preparation of single lymphocytes

Lymphocytes were isolated from peripheral blood collected from the heterozygous female partner, her normal male partner, and a normal female control who was heterozygous for the *FMR1* CGG repeat, DXS1215 and FRAXAC1 using Ficoll-Paque density gradient separation (Ficoll-Paque™ PLUS, Amersham Biosciences AB, Uppsala, Sweden) according to the manufacturer protocol. The cell layer containing lymphocytes was removed and diluted with sterile phosphate-buffered saline to a suitable cell density for single-cell isolation. Lymphocytes were then handled with a mouth-controlled fine heat-polished glass micropipette. The lymphocytes were selected and retrieved individually under visual control through an inverted microscope. Each single lymphocyte was loaded into 0.2-mL thin wall PCR tubes containing 1.5 μL lysis buffer (200 mM KOH and 50 mM dithiothreitol). The samples were stored at -70°C before analysis.

In vitro fertilization and embryo biopsy procedure

Ovarian stimulation was done as previously described (Kyu et al., 2004). Following ovarian stimulation, follicles were aspirated and fertilized by intracytoplasmic sperm injec-

tion. Fertilized zygotes were cultured separately in G-1 medium version 3 (Vitrolife Sweden AB, Kungsbacka, Sweden) for 3 days at 37°C in an atmosphere of 5% CO_2. On the third day of culture, embryos were biopsied in Ca^{2+}/Mg^{2+}-free medium (Biopsy medium; Medicult, Jyllinge, Denmark). Acid Tyrode's solution (Medicult) was applied to create a small hole in the zona pellucida. Biopsy was performed by gentle aspiration using a polished micropipette. One or 2 blastomeres among 6 to 9 cells were biopsied using a single pipette with an inner diameter of 30 µM. After the blastomere biopsy procedure, the embryos were then repeatedly washed, transferred to G-2 medium version 3 (Vitrolife Sweden AB), and cultured at 37°C with 5% CO_2 in air. Each blastomere was washed twice with 2 drops G-2 medium version 3 and transferred to sterile 0.2-mL PCR tubes containing 1.5 µL alkaline lysis buffer. For each embryo biopsied, blank negative controls were prepared from the wash drops. The embryos were cultured under standard culture conditions until the diagnosis was accomplished. The embryos with normal genotypes were selected and transferred on the fourth day of culture.

Alkaline lysis and DNA extraction procedure

The single lymphocytes and biopsied blastomeres stored with alkaline lysis buffer were incubated at 65°C for 10 min. Then, the alkaline lysis buffer was neutralized by the addition of 1.5 µL Stop Solution (in REPLI-g® UltraFast Mini Kit; QIAGEN GmbH, Hilden, Germany) before proceeding to MDA.

Multiple displacement amplification

Denatured cell lysates were used directly for MDA. The isothermal MDA method was performed using bacteriophage Phi29 DNA polymerase, exonuclease-resistant phosphorothioate-modified random hexamer primers and reaction buffer according to the manufacturer instruction (REPLI-g® UltraFast Mini Kit; QIAGEN GmbH) in a 20-µL reaction volume for at least 2 h at 30°C. The reaction was finished by incubation at 65°C for 3 min to inactivate the Phi29 polymerase, and DNA amplified using the MDA method was stored at -20°C until required.

Direct analysis of *FMR1* CGG repeats and indirect analysis of linked polymorphic markers

After inactivation of the Phi29 polymerase, the MDA product was diluted to 1:10 with distilled water. Fluorescent PCRs were prepared for 4 separate analyses. The first reaction for direct analysis of CGG repeats was carried out with the diluted MDA product, using *FMR1* CGG-specific primer pairs. The total reaction volume was 20 µL and contained 3 µL of the diluted MDA product, 2 µL 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 5% DMSO, 200 mM dATP, dTTP, dCTP, and 7-deaza-dGTP (Roche Diagnostics GmbH, Mannheim, Germany), 1 IU Neotherm DNA Polymerase (GeneCraft Co., Munster, Germany) and 2 pmol primer (Table 1). The thermal cycling conditions were a 96°C denaturation step for 5 min followed by 15 cycles consisting of 96°C for 30 s, 67°C for 1 min, and 72°C for 3 min, and then 25 cycles consisting of 95°C for 30 s with an auto-increment of 0.1°C per cycle, 67°C for 1 min and 72°C for 3 min, followed by a final extension step of 20 min at 72°C on a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA).

Table 1. Sequences of oligonucleotide primers used for PCR.

	Sequences of primers (5' → 3')
FMR1 CGG repeats	
FMR1-forward (outer)	ACGTGACGTGGTTTCAGTGTTTAC
FMR1-forward (inner)	FAM-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT
FMR1-reverse	AGCCCCGCACTTCCACCACCAGCTCCTCCA
DXS1215	
DXS1215-forward (outer)	CCTTCCTTTCCTTGACTCTACACCA
DXS1215-forward (inner)	NED-GCCCACTATGTGCCAGTAGGTATG
DXS1215-reverse	GGCAAAACATTAAACCTCTCTCATCC
FRAXAC1	
FRAXAC1-forward	FAM-TGGAGACTTCAACACCTCTCTG
FRAXAC1-reverse	TGATGAGAGTCACTTGAAGCTG
Amelogenin	
Amelogenin-forward (outer)	TGGGCACCCTGGTTATATCAACT
Amelogenin-forward (inner)	FAM-CCCTGGGCTCTGTAAAGAATAGTG
Amelogenin-reverse	AGGCCAACCATCAGAGCTTAAACT

FMR1 = *fragile X mental retardation-1*; outer = primers used for the first 'outer' PCRs without multiple displacement amplification.

The second reactions for linkage analysis and gender selection were performed by 3 separate PCRs with primers specific for DXS1215- and FRAXAC1-linked polymorphic markers and the *amelogenin* gene for gender selection. For each PCR, 1 µL of the diluted MDA product, 200 mM of each dNTP (Roche Diagnostics GmbH), 1 IU Neotherm DNA Polymerase (GeneCraft Co), 2 µL 10X PCR buffer with 25 mM $MgCl_2$, and 2 pmol of each specific primer pair (forward primers were FAM- or NED-labeled; Table 1) were added to each tube. The reaction was conducted in a total volume of 20 µL. The thermal cycling profile was as follows: 94°C for 2 min followed by 35 cycles of 95°C for 30 s, 64°C for 30 s and 72°C for 40 s, followed by a final extension step of 10 min at 72°C on a GeneAmp PCR System 2700. The sequences of the oligonucleotide primers used for PCR are shown in Table 1.

Fragment analysis of the amplified products

One microliter of the PCR products was added to 9 µL genetic analysis grade Hi-Di formamide (Applied Biosystems) and 0.2 µL GeneScan-ROX 1000 Size Standard (Applied Biosystems). After boiling for 5 min at 96°C, mixtures were capillary-electrophoresed in an ABI 3100 Avant Automatic Genetic Analyzer (Applied Biosystems). Results were analyzed by the GeneScan Analysis version 3.7 software (Applied Biosystems).

RESULTS

Pre-clinical test using single lymphocytes

As a result of linkage analysis for this family, the (CA)n microsatellite marker DXS1215 and the intragenic microsatellite marker FRAXAC1 were identified as semi-informative markers (Figure 1). The efficiency and accuracy of our MDA protocol were first evaluated in pre-clinical tests on 19 single lymphocytes collected from the control woman who was heterozygous for the *FMR1* CGG repeat, DXS1215 and FRAXAC1. After MDA, four separate reactions were carried out (DXS1215, FRAXAC1, *amelogenin*, and *FMR1* CGG repeats). The

amplification rate of the *FMR1* non-expanded CGG repeats was 84.2% (16/19), and the ADO rate was 31.3% (5/16). For the polymorphic markers DXS1215 and FRAXAC1, amplification rates were 87.5 and 75.0%, while the ADO rates were 57.1 and 50.0%, respectively (Table 2).

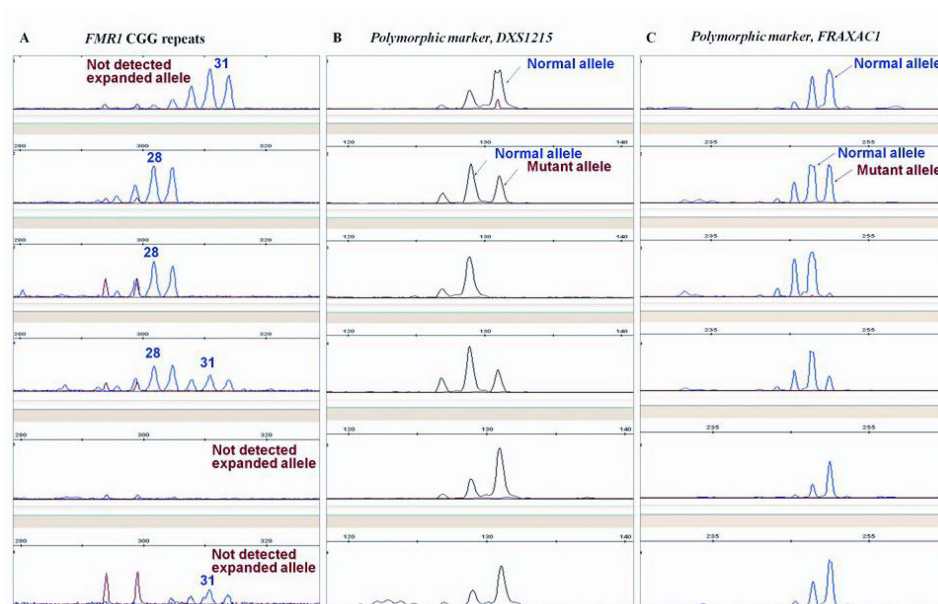

Figure 1. Electropherograms of the non-expanded *fragile X mental retardation-1* preimplantation genetic diagnosis (*FMR1* CGG) repeats (**A**) and polymorphic markers, DXS1215 (**B**) and FRAXAC1 (**C**) in the second clinical PGD for fragile X syndrome using multiple displacement amplification followed by fluorescent PCR and fragment analysis. *Line 1* = normal male partner; *line 2* = heterozygous carrier female partner; *line 3* = normal male embryo; *line 4* = normal female embryo; *line 5* = mutant male embryo; *line 6* = heterozygous carrier female embryo. The fully expanded mutant allele (more than 200 repeats) derived from the female partner was not detected (A-1, A-5, and A-6). Red and blue arrows indicate the peaks from the mutant allele and normal allele, respectively. Red peaks in *Panel A* indicate the GeneScan-ROX 1000 Size Standard.

Table 2. Amplification and allele drop-out rates of pre-clinical tests using single lymphocytes for fragile X syndrome with or without multiple displacement amplification.

Applied methods	*FMR1* CGG repeats		DXS1215	FRAXAC1
	Fluorescent-nested PCR without MDA	MDA and fluorescent PCR	MDA and fluorescent PCR	MDA and fluorescent PCR
No. of single lymphocytes	7	19	8	8
No. of amplified cells	0	16	7	6
Amplification rates	0.0%	84.2%	87.5%	75.0%
ADO rates	-	31.3%	57.1%	50.0%

FMR1 = *fragile X mental retardation-1*; MDA = multiple displacement amplification; ADO = allele drop-out.

Clinical PGD for fragile X syndrome

After the pre-clinical test, the clinical PGD program was applied to a couple who experienced a termination of pregnancy for a fragile X syndrome-affected fetus. In the first

clinical PGD program, a blastomere was biopsied on embryos with fewer than 6 cells and 2 blastomeres on 6 or more cells. A total of 23 blastomeres were biopsied and diagnosed from 15 embryos on day 3 after fertilization. Of 15 embryos analyzed after pre-amplification using MDA, no PCR product was amplified from 1 embryo (6.7%) and only *amelogenin* products were obtained from 3 embryos (20.0%). Three embryos were diagnosed as unaffected embryos, 1 embryo was identified as mutant, and 3 embryos were carriers. The genetic statuses of four embryos (26.7%) were not determined because their results were ambiguous (ADO or incomplete data). Unfortunately, half of the embryos analyzed gave no or incomplete results (8/15, 53.3%). Three healthy embryos were transferred to the mother's uterus on day 4, resulting in biochemical pregnancy. In the second PGD cycle, a total of 25 blastomeres were biopsied from 15 embryos and analyzed. Four separate reactions for each blastomere were carried out after MDA amplification. Unlike the first cycle, PCR products were amplified and diagnosed from all embryos analyzed. Seven embryos were diagnosed as unaffected embryos, 3 embryos were identified as mutant, and 3 embryos were carriers. Results were not determined in only 2 embryos (13.3%), because their results were inconclusive (different results for 2 different markers). Of 7 normal embryos, 3 well-developed embryos were transferred to the mother's uterus on day 4. Unfortunately, no pregnancy was achieved (Figure 1 and Table 3).

Table 3. Summary of the results for the preimplantation genetic diagnosis of fragile X syndrome with multiple displacement amplification followed by fluorescent PCR.

	1st cycle	2nd cycle	Total (mean)
No. of biopsied embryos	15	15	30 (15.0)
No. of diagnosed embryos	7	13	20 (10.0)
Successful diagnosis rate	46.7%	86.7%	66.7%
No. of unaffected embryos	3 (42.9%)	7 (53.9%)	10 (50.0%)
ADO rates (DXS1215/FRAXAC1)*	50.0/40.0%	50.0/40.0%	50.0/40.0%
No. of transferred embryos	3	3	6 (3.0)
Clinical pregnancy	Biochemical pregnancy	No	

*ADO (allele drop-out) rates were calculated only on female normal embryos for polymorphic markers, DXS1215 and FRAXAC1 because semi-informative markers alone were available in this family.

DISCUSSION

Multiplex (fluorescent) nested PCR methods followed by direct sequencing and/or fragment analysis provided reasonable reliability in single-cell analysis in our center. These protocols have been successfully applied to many clinical PGD cases for couples at high risk of having children with single-gene disorders (Lee et al., 2005, 2006, 2007).

Initially, as for other single-gene disorders, we developed a strategy for FXS using fluorescent nested PCR with genomic DNA followed by fragment analysis without whole genome amplification. However, when we applied this strategy to single lymphocytes in the pre-clinical tests, we did not obtain any results (Table 2). Direct detection of the normal non-expanded CGG repeat allele as well as the expanded CGG repeat allele is difficult because of the lack of PCR sensitivity and the high G+C content of the CGG repeat sequences and the surrounding sequences of the *FMR1* gene despite specific PCR protocols using the GC-RICH PCR System (Roche Diagnostics GmbH) and Expand Long Template PCR System (Roche Diagnostics GmbH). Therefore, we developed a new PGD strategy for FXS. We used

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MDA as a WGA method because large amounts of high-quality DNA could be obtained. MDA produces enough DNA from a single cell for multiple PCRs. Using the MDA products as a template, we performed only a single round of PCR, which decreases the time required for the PGD program. This strategy is a reliable protocol for obtaining material from a sample with a minuscule amount of DNA, such as a single cell, and several reports have demonstrated high amplification rates (88-100%) with an ADO rate of 7-31% when using MDA-amplified DNA as a template (Handyside et al., 2004; Hellani et al., 2004, 2005; Burlet et al., 2006; Lledo et al., 2007). In particular, Burlet et al. (2006) reported successful diagnosis, pregnancy and delivery in the PGD program using MDA for FXS. The amplification rate was increased by this method from 41 to 66% so that embryos with no results were rarer (14 *vs* 45% without MDA).

In our pre-clinical test for *FMR1* non-expanded CGG repeats and the DXS1215 and FRAXAC1 polymorphic markers, amplification rates were 75-84% and ADO rates were 31-57%. Although our data showed lower PCR amplification rates and higher ADO rates than those previously reported, the reliability and the accuracy of the diagnosis were improved by combining both direct detection of the CGG repeat and indirect detection of two polymorphic markers. The use of the non-expanded CGG repeats, two polymorphic markers, and the *amelogenin* gene for gender selection allowed us to decrease misdiagnosis due to ADO and to increase the successful diagnosis rate. Furthermore, in the case of the clinical PGD program, we have biopsied two blastomeres from embryos with more than 6 blastomeres. Amplification failure or misdiagnosis due to ADO at one blastomere or one locus was avoided by extrapolating the results obtained from other blastomeres or other loci. Inconclusive or ambiguous results led to no transfer of the embryos. In our cases, a total of 10 embryos (33.3%) were diagnosed as normal, and 6 of these embryos were transferred to the uterus. Unfortunately, no clinical pregnancy was achieved.

Our data demonstrate that MDA as a WGA method can produce enough DNA from a single cell for multiple PCR analyses. A PGD strategy using MDA followed by fluorescent PCR may be applied to the diagnosis of many kinds of single-gene disorders. Moreover, by using MDA methods, molecular karyotyping of a single cell such as array comparative genomic hybridization may be used to assess chromosomal abnormalities.

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