Assessment of preimplantation genetic diagnosis (PGD) for childhood-onset spinal muscular atrophy (SMA) using duplex fluorescent PCR

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Abstract

Preimplantation genetic diagnosis (PGD) is a very early form of prenatal diagnosis (PND) by which genetic diagnosis is performed on a single embryonic blastomer obtained by embryonic biopsy. Hence the genetic status of the embryo can be defined prior to embryo transfer; thus termination of pregnancy would not happen. The objective of the present paper was to establish preimplantation diagnosis for spinal muscular atrophy (SMA), a prevalent monogenic disorder in Iran, and to find the efficacy and sensitivity of the developed protocol. The fluorescent duplex single cell PCR technique for detection of SMN gene exon 7 deletion was developed first on single lymphocyte and then on a single blastomer. The protocol was duplexed with one of the three linked polymorphic markers namely, D5S112, D5S435 and D5S679. The PCR products of SMN exon 7 were restricted with DraI restriction endonuclease. Linkage analyses through polymorphic markers were also used for diagnosis. The amplification rates for SMN exon 7 deletion on a single lymphocytes and single blastomers were 96 and 88% respectively, using the aforementioned methods. The mean amplification rates of 3 polymorphic markers were 94% on single lymphocytes and 84% on single blastomers, respectively. Mean allele drop out (ADO) for the 3 markers on single lymphocytes was 4%. In conclusion the developed duplex single cell fluorescent PCR seems to be an accurate and feasible method at single cell level and could be easily applied in clinical preimplantation genetic diagnosis.

Keywords: *SMA, PGD, fluorescent PCR*

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INTRODUCTION

The spinal muscular atrophies (SMAs) are a clinically and genetically heterogeneous group of hereditary neuromuscular disorders caused by degeneration of anterior horn cells in the spinal cord. Childhood-onset proximal SMA is a frequent autosomal recessive neurodegenerative disorder with progressive weakness and atrophy of voluntary muscles. It is estimated to have an incidence of 1 in 10,000 individuals and a carrier frequency of 1/40 to 1/60 (Pearn, 1980; Melki *et al*., 1994). Thus, SMA represents one of the most common genetic causes of death in childhood. The gene responsible for this disease is survival motor neuron (*SMN*) present in two highly homologous copies (telomeric and centromeric) located on 5q13 (Lefebvre *et al*., 1995). Approximately 95% of patients lack a functionally critical sequence in exon 7 of both copies of the telomeric *SMN (SMNt)* gene. Interestingly however, the homologous deletion of exon 7 centromeric *SMN (SMNc)* gene does not cause any clinical symptom (Ogino *et al*., 2002).

There is no effective, routinely used, and well established therapy to diminish the devastating outcome of SMA. Due to religious, ethical and moral objections to induced abortion, prenatal diagnosis (PND) may not be a readily acceptable choice for many patients. Therefore preimplantation genetic diagnosis (PGD) would be a suitable alternative to be considered. In PGD, one or two blastomer biopsies will be obtained from embryos after in vitro fertilization (IVF) for the purpose of genetic diagnosis. As the diagnosis of the genetic status of the embryos takes place before

embryo transfer, pregnancy termination is avoided (Knavakis and Traeger-Synodinos, 2002).

There are several inherent difficulties associated with single-cell DNA amplification that renders PGD as a challenging strategy. These include potential sample contamination, total PCR failure and failure of one of the alleles to amplify at all, or at least up to a detectable level, named allele drop out (ADO). The incidence of each should be minimized for any PGD protocol before it is clinically applied (Egozcue *et al.*, 2000).

In this study a duplex single–cell fluorescent polymerase chain reaction (PCR) protocol for *SMN* exon 7 deletion and a linked polymorphic marker was developed. The protocol was evaluated and proved to be efficient for accurate diagnosis of SMA at a single cell level making it well suited for clinical applications of PGD.

MATERIALS AND METHODS

Lymphocyte preparation: Lymphocytes were separated by centrifugation through ficollpaque (Amersham Biosciences, UK) from 10 ml of blood with EDTA anticoagulant, as instructed by the manufacturer. The isolated lymphocytes were then washed in droplets of Ca^{++}/Mg^{++}-free medium, supplemented with 15 mg/ml bovine serum albumin (BSA) under a layer of mineral oil. Individual single cells were collected under an inverted microscope and transferred into a 0.5 ml thin-wall PCR tube, containing 2.5 µl alkaline lysis buffer (200 mM KOH, 50 mM DTT) (Cui *et al.*,1989). A few microliters of the medium from the last droplet were collected in a second PCR tube to serve as blank. Prior to PCR amplification, the tubes were heated at 65°C for 10 min, to lyse the cells.

The blastomers were obtained from spare embryos that were not suitable for transfer or cryopreservation. The embryos were sequentially transferred to two droplets of acidic Tyrode's under oil, with a finely drawn Pasteur-pipette and left until the zona pellucida disappeared. The embryos were then transferred to a Ca^{++}/Mg^{++} free medium and blastomers were separated by gentle up and down blowing. These single blastomers were then washed in fresh droplets of medium and transferred to PCR tubes containing 2.5 µl of alkaline lysis buffer.

PCR conditions: A two round duplex PCR protocol was used: In which the product of first round was used as the template for the second round (Table 1). The reaction mix contained 1x neutralization buffer (90 mM Tris–HCl pH 8.3, 30 mM KCl and 20 mM HCl) 50 mM KCl, 100 mM Tris–HCl pH 8.3, 0.1 mg/ml gelatin, 2 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmole of primers and 1.25 U Taq polymerase (Roche, Germany), and then covered with 30 µl of mineral oil. Three linked microsatellite polymorphic markers flanking the *SMNt* genes, namely D5S112, D5S435 and D5S679, were selected to be used in this study. These markers are highly informative; with a high polymorphism information content (PIC) score and a low recombination rate (Scheffer *et al.*, 2001).

Table 1: Primer sequences and PCR condition.

Primers	Duplex PCR Programs*
SMN exon 7	
5'-AGA CTA TCA ACT TAA TTT CTG ATCA-3' (FL)**	1st round 5min 95°C, 10× (30s 95°C, 30s 53°C, 30s 72°C)
5'-CCT TCC TTC TTT TTG ATT TTG TTT-3'	2nd round 5min 95°C, 40× (30s 95°C, 30s 55°C, 30s 72°C)
Polymorphic linked markers	
D5S112	1st round 5min 95°C, 10× (30s 95°C, 30s 55°C, 30s 72°C)
5'-TGT TCT TGG CAT CAC TGC-3' (FL)**	2nd round 5min 95°C, 35× (30s 95°C, 30s 57°C, 30s 72°C)
5'-TTT GAA GCC CTG GAA TAT-3'	
D5S435	1st round 5min 95°C, 10× (30s 95°C, 30s 54°C, 30s 72°C)
5'-CAA GAG CAC AGT TTG GAG TGA-3' (FL)**	2nd round 5min 95°C, 37× (30s 95°C, 30s 56°C, 30s 72°C)
5'-ACA CAC ATG CAC GCT CTCTC-3'	
D5S679	1st round 5min 95°C, 10× (30s 95°C, 30s 56°C, 30s 72°C)
5'-TTG CAT TTG GGA AGGAG-3' (FL)**	2nd round 5min 95°C, 35× (30s 45°C, 30s 57°C, 30s 72°C)
5'-GAC ACT TCT TTA CTCTC-3'	

*All second round PCR programs followed by 10 minutes final extension at 72°C.
(FL)** Fluorescently labeled

Initial optimization of PCR protocol and informativity test for selected markers was carried out on 100 ng genomic DNA from each individual. Subsequently, the method was applied to 50 single lymphocytes for each of the 3 polymorphic markers duplexed with *SMN* exon 7 and parameters such as contamination, ADO and amplification rate were calculated. The efficacy of the method was tested on 45 single blastomers in the same way as carried out for the lymphocytes. The primer sequences and PCR programs are summarized in table 1. Appropriate precautions were taken at all the stages to avoid contamination.

Detection of *SMN* telomeric copy: To distinguish between the two copies of *SMN* genes, restriction fragment length polymorphism (RFLP) was performed on PCR products using *DraI* restriction enzyme (Amersham, Biosciences, UK). Ten microliters of PCR aliquots were added to 2 µl of 10x enzyme buffer, 1 µl *DraI* enzyme and double–distilled water upto a final volume of 20 µl. Tubes were then incubated at 37°C for at least one hour.

Analysis of PCR products: Three microliters of the fluorescent labeled amplified DNA was mixed with an equal volume of loading dye (0.5% dextran blue in 100% formamide) and denatured by heating it at 90°C for 5 minutes before its loading on 6% denaturing polyacrylamide gel (Amersham Biosciences, UK). Electrophoresis was done on an Automated Laser Fluorescence Express DNA sequencer (ALF express) from Pharmacia Biotech, Sweden. Fragment analysis was carried out using AlleleLinks software provided by the ALF express DNA sequencer manufacturer.

RESULTS

Incorporation of fluorescent labeled primers into the PCR products makes the subsequent automated analysis of the fragments possible, through an excitation/detection system. The DNA fragments are recorded as peaks of variable amplitudes corresponding to their concentrations (Fig. 1). These peaks are the same as the bands observed in the conventional gel electrophoresis.

To detect the *SMN* exon 7 deletion in single cells, we used a PCR strategy enabling us to distinguish the differences between the centromeric and telomeric copies of *SMN* genes. Digestion of the PCR Products

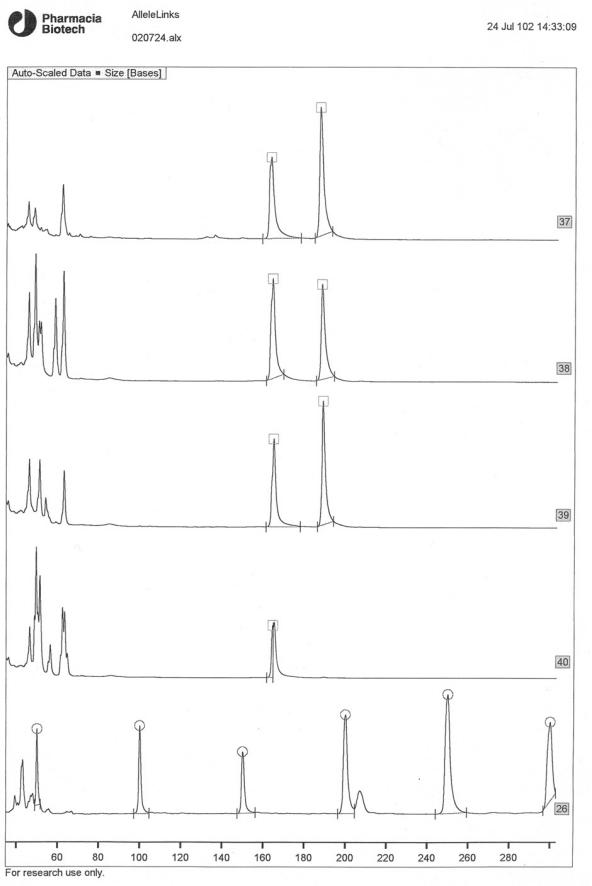

Figure 1*:* Single blastomer fluorescent PCR for SMN exon 7 (lanes 37 to 40) restricted with *DraI* restriction endonuclease enzyme; note an affected case (homozygous deletion of exon 7) in lane 40. Lane 26 is a 50 base pair ladder.

from exon 7 amplification of *SMNc* gene with *DraI* enzyme resulted in production of one fluorescent fragment of 165 bp. The *SMNt* PCR products did not possess the *DraI* restriction site and therefore remained as uncleaved fragments of 185 bp (Fig. 1). Using polymorphic linked markers amplification, we were able to further confirm the results obtained by amplification/*DraI* digestion of exon 7. Each duplex PCR was applied on 50 single lymphocytes to determine the rates of amplification, contamination and ADO as summarized in table 2. Efficiency and reproducibility of the results carried out on 45 blastomers were also comparable to the results obtained from studies single lymphocyte (a total of 150 single lymphocytes). Since, only a few of the blastomers were heterozygous for the given linked polymorphic markers, ADO rate could not be calculated (Table 2).

The amplification rate for *SMN* exon 7 was 96% on single lymphocyte. Polymorphic marker amplification

Table 2: Amplification, contamination and ADO rates for heterozygous amplified cell with duplex fluorescent PCR.

Polymorphic marker	Amplification (%)	Contamination (%)	ADO(%)
D5S112			
Lymphocytes	49/50 (98)	2/50 (4)	1/50(2)
Blastomers from research embryos	13/15(86)	0/15 (0)	
D5S435			
Lymphocytes	45/50(90)	1/50 (2)	3/50(6)
Blastomers from research embryos	12/15 (80)	0/15 (0)	
D5S679			
Lymphocytes	48/50 (96)	1/50(2)	2/50 (4)
Blastomers from research embryos	13/15 (86)	0/15 (0)	
Exon7			
Lymphocytes	145/150 (96)	4/150(3)	NA*
Blastomers from research embryos	40/45 (88)	0/45(0)	

*not applicable

rates were 98%, 90% and 96% on single lymphocytes, when duplexed with D5S112, D5S435 and D5S679 markers, respectively. The rate was slightly lower on single blastomers compared to that of single lymphocytes being 88% for *SMN* exon 7. The amplification rates were 86% for D5S112 and D5S679, and 80% for D5S435 polymorphic markers. ADO rates of 2% for D5S112, 6% for D5S435 and 4% for D5S679 were obtained on single lymphocytes duplex PCR.

DISCUSSION

This is the first attempt to assess the feasibility of PGD for SMA, a prevalent monogenic disorder in Iran. As a recently introduced procedure, PGD brings together several technically challenging areas like: *in vitro* fertilization, culture and biopsy of embryo, along with genetic diagnosis on single cells.

The methods of detecting monogenic disorders, using single cells, are mostly based on PCR (Li *et al.*, 1988). Since the performance of the first PCR-based PGD cases (Handyside *et al.*, 1989 and 1990), many inherent problems associated with single cell DNA amplification have become evident. The most important difficulties in employing these methods are those that may lead to misdiagnosis like sample contamination and ADO. Any single cell PCR should take appropriate measures to minimize these pitfalls. Multiplex PCR with linked informative polymorphic markers have shown to be quite helpful for the detection of both contamination and ADO (Wells and Sherlock, 1998; Sermon, 2002).

The causes of ADO are not well understood, but the most important issues receiving attention are: the cell lysis method, PCR conditions, the sequence of the template DNA, the size of the PCR products, and the degradation of target DNA molecule (Ray and Handyside, 1996; El-Hashemite and Delhanty, 1997; Thornhill, 2001; Verttou *et al.*, 1999; Findlay, 1995). Development of sensitive detection methods such as fluorescent PCR (Findlay *et al.*, 1996) followed by automated analysis using computer software and a higher denaturing temperature in PCR (Ray and Handyside, 1996) reduce the rate of ADO, thereby improve the diagnostic procedure.

De Ryke *et al.*, (2001) compared the results obtained by non-fluorescent and fluorescent PCR for sickle cell anemia and β-thalassemia. The amplification efficiency dropped from 94% to 78% in conventional PCR, while ADO increased from 8 to 33%. Findlay *et al.* (1995) obtained a low ADO rate of about 4% due to increased sensitivity using fluorescent PCR. Using duplex single-lymphocytes PCR a high mean amplification rate of about 96% was achieved for exon 7 of *SMN* gene and about 94% for markers. However, the mean amplification rate of exon 7 and markers were 88% and 84% on blastomers. These results were in favor of the results of preclinical and clinical PGD cycles performed by others (Vandervors *et al.*, 2000; Thornhill *et al.*, 2000).

Inclusion of informative polymorphic markers in

our duplex PCR assay provided two opportunities to detect chromosomes carrying disease–causing genes. Pickering *et al*. (2003) performed a PGD for *SMA* without incorporation of linked polymorphic marker that resulted in misdiagnosis. Therefore using these markers is of great importance in PGD. Moreover, PCR-RFLP alone just differentiate affected from unaffected individuals, and further differentiation of SMA carriers from homozygous normal individuals would not be possible. The easiest way for accurate classification of individuals into *SMA* carrier, healthy and affected would be possible through linkage analysis using informative polymorphic markers.

Fluorescent-PCR protocols so far have been successfully applied in clinical PGD for a number of genetic disorders including myotonic dystrophy (Sermon *et al.,* 1998a), Marfan's syndrome (Sermon *et al.,* 1999), Huntington's disease (Sermon *et al.,* 1998b), embryo sexing (Findlay *et al.,* 1996), β-thalassemia and sickle cell anemia (De Ryke *et al.,* 2001), trisomy 21 and SMA (Blake *et al.,* 1999). To our knowledge so far only one report on using duplex PCR for polymorphic linked markers and *SMN* exon 7 deletion is available (Moutou *et al.,* 2003). The results obtained in this study, suggest that the duplex fluorescent PCR assay developed in our laboratory is not only very efficient in diagnosis of suspected carriers of SMA, but also could be successfully applied in clinical PGD cycles.

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