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Duplex PCR for preimplantation genetic diagnosis (PGD) of spinal muscular atrophy

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The main difficulty in developing a molecular diagnosis of spinal muscular atrophy (SMA) resides in the specific genomic structure of the locus. Indeed, two highly homologous survival motor neurone genes, *SMN1* and *SMN2*, are present at the locus. The detection of the homozygous deletion of exons 7 and 8 of the *SMN1* gene, which is present in 90 to 98% of the patients, is based on methods highlighting 1 of the 8 nucleotidic mismatches existing between these 2 genes. In order to offer preimplantation genetic diagnosis (PGD) for SMA, we developed a new allele-specific amplification method. The main disadvantage of our previously described strategy resided in the possibility of diagnosing, in case of amplification failure, an unaffected embryo as affected. We present here a new PGD-SMA method. We established the conditions for three different duplex PCRs, allowing the specific detection of the *SMN1* gene and one polymorphic marker, either D5S629, D5S1977, or D5S641. Of the 60 to 90 single cells tested, the PCR efficiency varied from 98 to 100% with a complete genotype obtained in a range between 81 and 87% with a global allele drop-out rate of 9%. Such a test was used to perform 1 PGD cycle for which 7 embryos could be analysed. All the embryos were fully diagnosed, six as unaffected and one as affected. Four embryos were transferred, but no pregnancy ensued. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS: allele-specific amplification; preimplantation genetic diagnosis (PGD); single-cell multiplex PCR; spinal muscular atrophy

INTRODUCTION

Spinal muscular atrophy (SMA) is the most common fatal muscular disease (prevalence of ∼1/10 000 and a carrier frequency of ∼1/50) in childhood after Duchenne muscular dystrophy (Melki *et al*., 1994; Pearn, 1980). It is characterised by degeneration of the anterior horn cells of the spinal cord resulting in symmetric proximal muscle weakness. Patients with SMA are classified into 3 groups according to the clinical course: type I SMA (Werdnig–Hoffmann disease; MIM 253300), the most severe form, with early onset before 6 months of age and death usually before the age of 2; type II SMA (MIM 253550), the intermediate form, with onset before 18 months of age and a significant reduced life expectancy, and type III SMA (Kugelberg–Welander disease, MIM 253400), the mildest form of the disease, with later onset and a more benign clinical course.

All three types of SMA are autosomal recessive diseases and linked to 5q13 (Brzustowicz *et al*., 1990; Gilliam *et al*., 1990; Melki *et al*., 1990a; Melki *et al*., 1990b), a complex region that includes a 500-kb duplication and inversion (Lefebvre *et al*., 1995). Several candidate genes have been identified in this region (for a review, see (Biros and Forrest, 1999). Of these, the survival motor neurone (*SMN*) gene shows homozygous deletions of exon 7 (most of them also affecting exon 8) in 90 to 98% of the SMA patients (Cobben *et al*., 1995; Lefebvre *et al*., 1995; Rodrigues *et al*., 1995; Velasco *et al*., 1996) or point mutations in SMA patients lacking the homozygous deletion (Burglen *et al*., 1996), review (Wirth, 2000). Two highly homologous *SMN* genes are present in the 5q13 region: a telomeric one (*SMN1*) and a centromeric one (*SMN2*). While *SMN1* and *SMN2* differ only in five intronic and three exonic nucleotides (Burglen *et al*., 1996; Lefebvre *et al*., 1995), SMA is caused only by deletions or mutations in the telomeric copy. This provides strong evidence that *SMN1* is the major SMA-determining gene.

Most molecular diagnoses of SMA is based on the detection of the homozygous deletion of exons 7 and 8 of the *SMN1* gene in patients suspected to be affected or in prenatal diagnosis in families with a history of SMA. Two kinds of DNA tests are usually performed, both based on nucleotidic mismatches between the *SMN* gene (*SMN1*) and its copy (*SMN2*): a single-strand conformational polymorphism method (SSCP), (Lefebvre *et al*., 1995), or a polymerase chain reaction (PCR) followed by a restriction enzyme digestion method (van der Steege *et al*., 1995). SMA preimplantation genetic diagnosis (PGD) has been available since 1998 (Blake *et al*., 1999; Dreesen *et al*., 1998; Fallon *et al*., 1999). The protocol involves the detection of the homozygous deletion of exon 7 or 8 of the *SMN1* gene by two consecutive rounds of PCR followed by enzymatic digestion.

We recently proposed a more rapid method for single-cell molecular diagnosis of SMA, based on allele-specific amplification, (ARMS for amplification refractory mutation system) of the SMN1 allele (Moutou *et al*., 2001). The strategy takes advantage of a mismatch present in exon 7 between the telomeric and the centromeric *SMN* genes. The main disadvantages of this method were that in case of amplification failure

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(AOF), embryos were diagnosed as affected, and therefore rejected, and that contamination by exogenous DNA could lead to transfer of an affected embryo. In order to detect both amplification failure and contamination, we have developed an improved method. Indeed, we present here three duplex fluorescent-PCR protocols allowing the allele-specific amplification of the *SMN1* gene concomitant to the amplification of one extragenic polymorphic marker, either D5S629, D5S1977 or D5S641. We also describe one SMA-PGD cycle performed in our centre using this new improved method.

MATERIAL AND METHODS

Single-lymphoblast testing and embryo biopsy

Three Epstein–Barr-transformed lymphoblast cell lines (kindly provided by Dr Judith Melki, Evry, France and Dr Karen Sermon, Brussels, Belgium) were used to perform single-cell analysis: one homozygous for a deletion of exon 7 of the *SMN1* gene (delSMN1: affected patient), one homozygous for a deletion of exon 7 of the *SMN2* gene (delSMN2: normal control1) and one normal at both loci (SMN1/SMN2: normal control2). Cell tubing and manipulation were performed as previously described (Moutou *et al*., 2002).

D5S629, D5S1977 and D5S641 are extragenic markers with heterozygous frequencies of 83, 78 and 77% and a distance to the *SMN1* gene of 582 kb, 5870 kb, and 11 569 kb, respectively. Primers are described in the Genome Database (www.gdb.org). One primer of each pair was fluorescently labelled (see Table 1) with 6-fam, tet or hex fluorochrome (Applera, Warrington, UK).

Aliquots of 22.5-μL PCR master mix containing neutralizing buffer (NB: 900 mM TrisHCl, 300 mM KCl and 200 mM HCl), 2 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM of each primer for the SMN1 locus and 0.4 μM of each primer for the D5S629, D5S1977 or D5S641 loci and one unit Amplitaq DNA polymerase (Applera, Courtaboeuf, France) was added to each reaction tube. This polymerase exhibits no 3′–5′ exonuclease activity and therefore no mismatch repair at the 3′-end of the specific primer, which is the basis of the ARM tests. PCR reactions were performed using a Mastercycler gradient PCR machine (Eppendorf, Hamburg, Germany). Forty-five cycles were performed. PCR products were then analysed on an ABI3100 automated sequencer (Applied Biosystems, Warrington, UK).

For the three cell lines, the marker profiles were first determined on genomic DNA. For all markers, DelSMN2 and SMN1/SMN2 cell lines were heterozygous, whereas delSMN1 was not. Tubing procedures and embryo biopsies were performed as previously described (Moutou *et al*., 2002). PCR efficiency was calculated from samples for which a PCR signal for at least one tested locus was obtained.

Clinical PGDs

One couple requested PGD for SMA

A 38-year-old woman and her 48-year-old husband had had an affected boy deceased at 2 months of age. Genomic DNA from the couple and from their affected child was extracted from whole blood samples using the standard saline protocol (Rousseau *et al*., 1992). Ovarian stimulation, oocyte recovery and intracytoplasmic sperm injection (ICSI) procedures were carried out using standard protocols as previously described (Van Steirteghem *et al*., 1993; Wittemer *et al*., 2000). Three days after ICSI, embryos were biopsied as previously described (Moutou *et al*., 2002) and two blastomeres per embryo were collected and analysed separately. For each biopsied cell, a blank control was prepared from the final wash drop. PCR controls containing single lymphoblast from control cell lines (delSMN1 and delSMN2) were added. After PGD, at least three blastomeres from

Table 1—Duplex PCR conditions and genotype of the cell lines

Locus (distance to SMN1)	Fragment size range (bp)	Primer sequence (name—fluorochrome)	Genotype (fragment size in bp)		
			DelSMN1	DelSMN2	SMN1/SMN2
SMN1 exon7	185	-AGACTATCAACTTAATTTCTGATCA (R111—6-fam) -TCCTTCTTTTTGATTTTGTCTG (specific SMN1)	Homozygous deletion	Normal	Normal
D5S629 (582-kb proximal)	230–255	-ACTCGGGAGGCTGAGA (AFM265wf5a—hex) -CCGGTTTGTTCCTGTGA (AFM265wf5m)	247/247	239/253	235/247
D5S1977 (5870-kb distal)	180–190	-TGACAAAGCAAGGCTCTC (AFMa204zf1m—ned) -GATGTTCATCAGACTCAGAACC (AFMa204zf1a)	185/185	181/187	185/187
D5S641 (11 569-kb distal)	250–280	-AGGGACAGTCCACTTCCAGT (AFM284cd1m—ned) -AGTTGTGTATTGGAGAATGTTATCA (AFM284cd1m)	268/268	264/268	264/268

untransferred affected embryos were individually reanalysed following the same procedure to confirm PGD results.

Prevention of contamination

To prevent contamination, reagent preparation, lymphoblast tubing and single-cell PCR were set up in a dedicated pre-PCR lab in clean laminar flow cabinets. For clinical application, the biopsy area was physically different from tubing as well as from pre-PCR areas. All pre-PCR steps were carried out wearing sterile gowns, gloves, overshoes, caps and masks and using dedicated tubes, racks and pipettes. Finally, post-PCR analysis was completely separated from pre-PCR steps.

RESULTS

Lymphoblast testing

Table 2 shows the results of the duplex PCRs performed on single lymphoblasts. We tested three duplex PCRs (SMN1-D5S629; SMN1-D5S1977 and SMN1-D5S641). According to the assay, we performed 88 to 90 single-cell analyses for which a PCR efficiency between 98 and 100% was obtained. A correct diagnosis for SMA was obtained in 97 to 99% of amplified lymphoblasts. A complete genotype was obtained for 86% of the tested cells for the duplex PCR SMN1-D5S629, for 87% for the duplex PCR SMN1-D5S1977 and for 81% for the duplex PCR SMN1-D5S641. The PCR efficiency varied from 82 to 90% for the markers. A global allele drop-out (ADO)allele rate of 9% was observed irrespective of the locus and the heterozygous cell line considered.

Clinical PGDs

Figure 1 represents the pedigree and the genotype analysis of both parents and their affected son. Haplotype analysis for all three microsatellites and segregation analysis with regard to the *SMN1* gene were performed. Of the three markers tested, only marker D5S629 was semi-informative, the other two were not informative. Therefore, it was decided to use the SMN-D5S629 duplex PCR for the PGD. The results of the PGD cycle are summarised in Table 3 and profile examples are shown in Figure 2. Twenty cumulus–oocyte complexes were retrieved and 13 inseminated by ICSI; 3 days later, 7 embryos with 6 or more cells were biopsied and 2 blastomeres were collected and analysed. A full diagnosis, using the SMN-D5S629 test, was obtained for all the embryos. Six embryos were diagnosed as unaffected

Table 2—Results of lymphoblast testing

Duplex PCR	No. of cells analysed (delSMN1, delSMN2, SMN1/SMN2)	No. of PCRs with a signal (%)	No. of cells with amplification failure at a single locus		No. of cells correctly genotyped (%)		Complete genotype (%)	ADO (%)[a]
			SMN1 locus[b]	Marker	SMN locus	Marker		
SMN1 + D5S629	90	90 (100)	2	2	87 (97)	77 (86)	77 (86)	9 (15)
SMN1 + D5S1977	88	87 (99)	0	9	86 (99)	78 (90)	76 (87)	2 (3)
SMN1 + D5S641	90	88 (98)	0	1	87 (99)	72 (82)	71 (81)	7 (10)

[a] ADO rate based on heterozygous cells (delSMN2 and SMN1/SMN2).
[b] AOF in cells from normal controls (i.e. delSMN2 and SMN1/SMN2).

Table 3—Summary of PGD performed

Embryo	Embryo grade at day 3	No. of cells taken	SMN1 exon7 results	D5S629 results	Diagnosis	Transfer	Embryo grade at day 4	Confirmation
1	7	2	SMN1 SMN1	1/2 −/2 (ADO)	Unaffected	No	5	Unaffected
2	7	2	SMN1 SMN1	1/2 1/2	Unaffected	Yes	8	—
3	8	2	SMN1 SMN1	2/2 2/2	Unaffected	Yes	8	—
4	6	2	SMN1 SMN1	2/2 2/2	Unaffected	No	4	Unaffected
5	7	2	SMN1 SMN1	1/2 1/2	Unaffected	Yes	>6 compacted	—
6	7	2	SMN1 SMN1	1/2 1/2	Unaffected	Yes	>6 compacted	—
7	8	2	Del Del	2/2 2/2	Affected	No	8	Affected

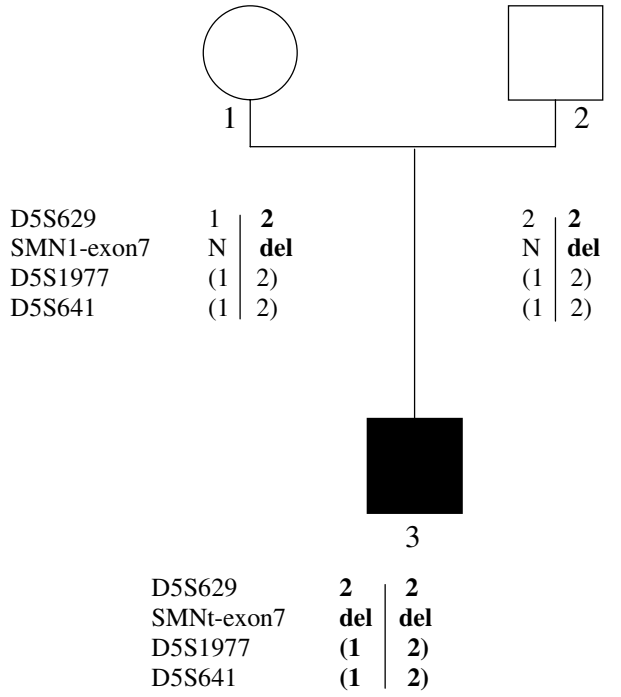

Figure 1—Pedigree of the couple asking for PGD for SMA with genotype for exon 7 of SMN1 and for markers D5S629, D5S641 and D5S1977. In bold are affected haplotypes and in brackets, genotypes with an unknown phase

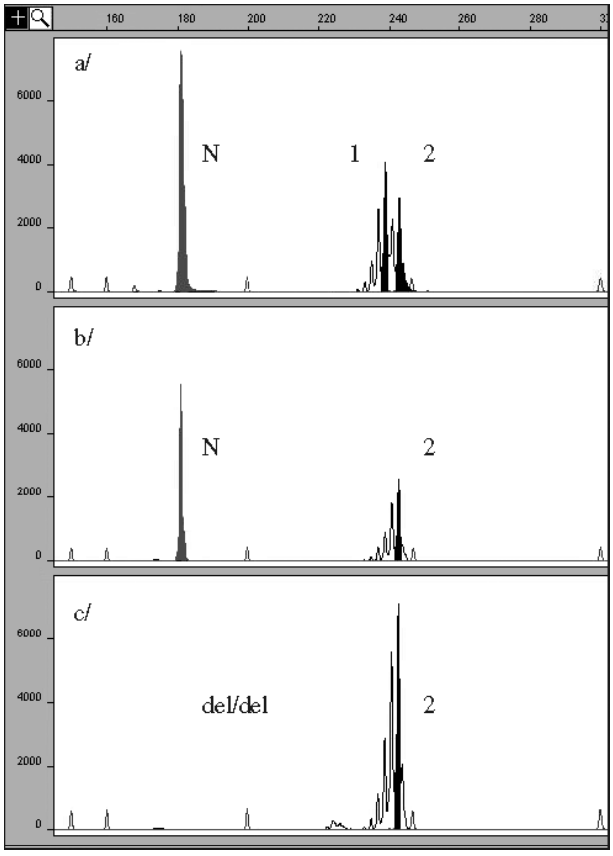

Figure 2—Example of PGD results. Alleles are shown in plain peaks: in grey, exon 7 of *SMN1* gene and in black, microsatellite D5S629. Empty peaks represent internal size standard Genescan Rox 500 (in grey) or stutters from the microsatellite marker (in black). Lane a/: result for a blastomere of embryo 1 with presence of *SMN1* gene and heterozygous (1/2) for D5S629 and diagnosed as unaffected. Lane b/: result for a blastomere of embryo 4 with presence of *SMN1* gene and homozygous (2/2) for D5S629 and diagnosed as unaffected. Lane c/: result for a blastomere of embryo 8 with homozygous deletion of *SMN1* gene and homozygous (2/2) for D5S629 and diagnosed as affected

(for four of them, the diagnosis was based on both loci) and one as affected. Four embryos were transferred; no pregnancy ensued. The reanalysis of the remaining three embryos confirmed the initial diagnosis.

In all cases, single-cell PCR controls were reliably diagnosed and no contamination was detected in blank controls. In total, we obtained a diagnosis for 13/14 of the blastomeres analysed (92.9%) and 1 detectable ADO. The ADO rate could not be ascertained since the marker used is semi-informative. We therefore could not distinguish between ADO and homozygosity.

DISCUSSION

Molecular diagnosis of SMA presents a specific difficulty due to the duplication and inversion of 500 kb in the region, resulting in the presence of two homologous *SMN* genes that differ only in 5 intronic and 3 exonic nucleotides. The detection of the homozygous deletion of exons 7 and 8 of the *SMN1* gene, which is present in 90 to 98% of the patients, is based on methods highlighting the nucleotidic mismatches between the *SMN1* and *SMN2* genes. In addition to this difficulty, PGD procedures were requested to be performed during daytime since most laboratories have a day 3 or 4 embryo-transfer strategy. Four protocols are available for SMA-PGD. Three of them involve two rounds of PCR followed by enzymatic digestion (Blake *et al*., 1999; Dreesen *et al*., 1998; Fallon *et al*., 1999). We developed a fourth one on the basis of allele-specific amplification of the *SMN1* gene using a single-round PCR strategy. The latter procedure is much faster than the other ones since it can be performed within about 7 h after biopsy (Moutou *et al*., 2001), as compared to 11 h (Dreesen *et al*., 1998) or more (Fallon *et al*., 1999).

Four causes of error can be encountered with the three previously described methods for SMA-PGD that require restriction digestion after SMN exon 7 and/or exon 8 amplification: (1) amplification failure, (2) PCR contamination that is possible with single-cell PCR irrespective of the method used, (3) digestion failure or partial digest that could lead either to transfer of an affected embryo or to rejection of an unaffected one, according to the restriction enzyme used, and (4) preferential amplification of SMN2 with absence of amplification of SMN1, since both genes are amplified using the same set of primers.

With our initial strategy of selectively amplifying the *SMN1* gene, an amplification failure could result in the misdiagnosis of unaffected embryos as affected. This would not lead to the transfer of an affected embryo

but would only reduce the number of unaffected transferable embryos. Since the rate of pregnancy is highly dependent on the number of transferable embryos, we have improved this initial test by including a polymorphic marker in addition to the specific detection of the *SMN1* gene. We established the conditions for three different duplex PCRs, allowing the specific detection of the *SMN1* gene concomitant to one marker, either D5S629, D5S1977 or D5S641. Of the 88 to 90 single cells tested per assay, the PCR efficiency varied from 98 to 100%. A correct diagnosis for SMA was obtained in 97 to 99% of amplified lymphoblasts, which is better than the success rate obtained with our initial test (Moutou *et al*., 2001). A complete genotype was obtained in a range between 81 and 87%. These results were obtained after trying numerous PCR protocols using different primer and $MgCl_2$ conditions, PCR enhancers, as always done in our lab to optimise PCR conditions when setting up a new PGD test.

This new test was used to perform 1 PGD cycle for which 7 embryos could be analysed. All embryos were fully diagnosed, six as unaffected and one as affected. Four embryos were transferred, but no pregnancy ensued.

Compared to our previous test, this one combines the advantages of detecting possible amplification problems (ADO or amplification failure), detecting exogenous DNA contaminations and, finally, considerably improving the reliability of the tests since it can give multiple information from a single cell. The D5S629 marker being very close to the SMN locus (582 kb) can also be used for linkage analysis when informative. In that case, duplex PCR can be considered as a double diagnosis test. The recombination rate between the SMN locus and D5S1977 (5.9%) or D5S641 (11.6%) is much higher than that between the SMN locus and D5S629 (0.6%). Therefore, duplex PCRs combining ARMS and D5S1977 or D5S641 should be used when no informativity is obtained for D5S629, and should only be considered as amplification, ploidy and contamination controls, which is the first aim of this duplex approach. Introduction of other makers closer to SMN1 could be suggested in order to have a better double diagnosis method combining detection of the deletion and linkage analysis. However, considering the extreme efficiency of the ARMS test (close to 100%), we do not feel that this would be essential.

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