Preimplantation genetic diagnosis of spinal muscular atrophy

Introduction
Preimplantation genetic diagnosis (PGD) is an alternative for prenatal diagnosis for couples who have a high risk of an inherited disease in their offspring. PGD is performed on one or two single blastomeres biopsied from 4–10-cell embryos on day 3 after fertilization (Handyside et al., 1990; Hardy and Handyside, 1992). The possibility of selecting and transferring only unaffected embryos to the uterus is an alternative to elective abortion following prenatal diagnosis of an affected fetus.

Spinal muscular atrophy (SMA) is characterized by degeneration of the anterior horn cells of the spinal cord resulting in progressive symmetrical limb and trunk paresis with proximal muscular atrophy. The disease can be subdivided into three clinical types (I, II and III), characterized by decreasing severity of the symptoms. Children with type I (Werdnig–Hoffman disease), show symptoms within the first 6 months after birth. These children usually die from respiratory failure before reaching the age of 2 years. Children with SMA type II express the first clinical signs before 18 months. They are able to sit, but they will never walk or stand unaided. They may, however, survive beyond 2 years. Patients with type III or mild SMA (Kugelberg–Welander disease) show evidence of muscle weakness after 18 months and a more benign clinical course. These children may reach adulthood (Dubowitz, 1995).

After Duchenne muscular dystrophy, spinal muscular atrophy (SMA) is the most common severe neuromuscular disease in childhood. Since 1995, homozygous deletions in exon 7 of the survival motor neuron (SMN) gene have been described in >90–95% of SMA patients. However, the presence of a highly homologous SMN copy gene complicates the detection of exon 7 deletions. This paper describes the adjustment and evaluation of an established SMN exon 7 polymerase chain reaction (PCR) protocol at the single cell level, and the first preimplantation genetic diagnosis (PGD) of SMA with this PCR protocol. To determine PCR efficiency and allelic loss, 200 leukocytes of normal individuals, SMA carriers and patients, and 25 blastomeres were tested. The PCR efficiency of the SMN exon 7 and the adjacent copy gene sequence, tested in the leukocytes, were 90% and 91% respectively. No allelic loss was detected. One out of 25 blastomeres tested revealed a negative PCR signal for the SMN exon 7 sequence. All 25 showed the copy gene sequence. PGD of SMA was offered to a couple with an affected child homozygous for the SMN exon 7 deletion. After intracytoplasmic sperm injection, four and five embryos could be genotyped for the SMN exon 7 in two cycles respectively. After embryo transfer in the second PGD cycle an ongoing gemelli pregnancy was achieved. This study demonstrates that PGD for SMA is feasible when a previous child is homozygous for the SMN exon 7 deletion.

Key words: preimplantation genetic diagnosis/single cell diagnosis/spinal muscular atrophy/survival motor neuron gene

After cystic fibrosis, SMA is the most frequent fatal autosomal recessive disorder. The birth prevalence of SMA types I, II and III together is ~1 in 10 000 newborns in the North West European population (Pearn, 1973, 1978; Spiegler et al., 1990). For all three clinical subtypes linkage was demonstrated with markers localized to the q13 region of chromosome 5 (Brzustowicz et al., 1990a,b). Different candidate genes in this region were identified (Lefebvre et al., 1995; Roy et al., 1995; Thompson et al., 1995). These genes frequently demonstrate homozygous deletions in SMA patients. For the survival motor neuron (SMN) gene, apart from a high frequency of homozygous deletions, point mutations were also described. The presence of these point mutations provided strong evidence that the SMN gene is an SMA determining gene. In the Dutch population 93% of all SMA patients show a homozygous deletion of at least SMN exon 7 (Cobben et al., 1995). This deletion was not found in a control group. Furthermore the SMN gene has a highly homologous copy gene which is present in 95.5% of the normal population (Lefebvre et al., 1995). This copy gene has a more centromeric location and its exon 7 sequence differs by only a single nucleotide from the SMN gene. Deletions of the SMN copy gene are not associated with SMA. Although the role of the SMN gene in the pathogenesis of SMA remains to be elucidated, deficiency
of the SMN proteins in tissue of SMA patients, especially the spinal cord, provides additional evidence that SMN is the major SMA-determining gene (Coovert et al., 1997; Lefebvre et al., 1997). On the basis of the one nucleotide substitution between the SMN gene and the copy gene and the use of a mismatch primer, discrimination between SMN exon 7 and the SMN copy gene is possible (van der Steege et al. 1995) (Figure 1). This allows the detection of SMN exon 7 deletions as a powerful diagnostic tool for SMA. Prenatal diagnosis of SMA by SMN-deletion analysis after chorionic villus biopsy is routinely performed in The Netherlands (Cobben et al., 1996).

Figure 1. The survival motor neuron (SMN) gene has a highly homologous copy gene with a more centromeric location as a result of an inverted duplication (Lefebvre et al., 1995). During polymerase chain reaction amplification of SMN exon 7, and hence of the SMN exon 7 copy sequence, mismatch primer X7-DraI generates a DraI restriction site in the SMN exon 7 copy sequence but not in SMN exon 7. Discrimination between the SMN exon 7 and SMN exon 7 copy sequence is accomplished by a DraI restriction enzyme digestion.

The aim of this study was to develop single cell analysis for the SMN exon 7 deletion, in order to establish a polymerase chain reaction (PCR) protocol for PGD of SMA. The first application of this PGD protocol for SMA will be described.

Materials and methods

Single cell testing for developing PGD method

Collection of leukocytes

Human leukocytes from normal individuals (presumed to be homozgyously ‘non-SMN deleted’, WT/WT), heterozygous carriers of the exon 7 deletion and SMA patients homozygous for the exon 7 deletion of the SMN gene, were used as single cells for testing the PCR method. Single leukocytes were collected in 2 µl Ca²⁺-/ and Mg²⁺/-free phosphate-buffered saline solution (PBS) with 1% polyvinylpyrrolidone (PVP; mol. wt 360 kDa; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and 0.1 mg/ml phenol red (Sigma), with the help of a micromanipulator (Narishige, ONO-121, Paes Nederland bv, Zoeterwoude, The Netherlands) mounted on an inverted microscope (Olympus, IX-70, Paes Nederland). After transferring the cells to a 0.5 ml reaction tube, cellular DNase heat inactivation was accomplished by a 10 min incubation at 65°C. Cells were stored at –20°C until PCR was performed.

Collection of blastomeres

Supernumerary human embryos considered to be unsuitable for freezing following in-vitro fertilization (IVF) treatment were collected. Each couple had given informed consent that surplus embryos could be used for these experiments. The protocol was approved by the local Ethical Committee. Blastomeres were collected from surplus embryos after IVF procedures. The zona pellucida was removed from the embryo by a 3–5 min incubation in a 1/1 mixture of 500 U/ml pronase (Sigma) and PBS. The blastomeres were separated from each other by gently flushing them in a small pipette. Subsequently, the blastomeres were rinsed in three droplets of PBS, with 1% PVP and phenol red (0.1 mg/ml), and transferred into a 0.5 ml reaction tube with the help of a dissection microscope. Cellular DNase inactivation and storage of the collected cells was performed as described previously.

Patient

A known couple with type 1 SMA was referred for PGD of SMA. The first child of this couple, a son, had suffered from SMA type I. Hypotonia was noted from the first month of life. He died at the age of 9 months. The woman, aged 29 years, had had one spontaneous abortion and one ectopic pregnancy. The couple had no healthy children. DNA analysis of the affected son revealed a homozygous SMN exon 7 deletion. This indicates that both parents are carriers of the SMN exon 7 deletion.

ICSI and blastomere biopsy of embryos for PGD

To obtain embryos from the couple described above for PGD of SMA, the female carrier was induced to ovulate using an established protocol as described previously (Land et al., 1996). Intracytoplasmatic sperm injection (ICSI) was performed ~5 h after oocyte retrieval using the protocol as described by Van Steirteghem et al. (1993). Cultured embryos were biopsied 64–68 h after ICSI and after incubation of the embryos in Ca²⁺-/Mg²⁺/-free HEPES-buffered medium for 5 min (Dumoulin et al., 1998). Only embryos developing from 2-pronuclear zygotes which were 4-cell or more were used. After the embryo was immobilized by a holding pipette, a small hole was made in the zona pellucida with acid Tyrode’s solution (pH 2.4) (Handyside et al., 1990). One or two blastomeres were removed from the embryo by aspiration into a micropipette. When the embryo had 4–7 cells one blastomere was removed, whereas two cells were biopsied from embryos consisting of ≥8 cells. Biopsied cells were rinsed and transferred into a 0.5 ml reaction tube with the help of a dissection microscope as described above. From the last washing droplet, a blank was taken to monitor contamination. Transfer of embryos in which at least one wild type allele could be demonstrated was performed at the fourth day after ovum retrieval.

PCR procedure

After topping the PCR target material with 40 µl mineral oil (Sigma–Aldrich Chemie BV, Zwijndrecht, The Netherlands), cells were lysed by adding 5.0 µl of alkaline lysis buffer followed by 10 min of incubation at 65°C. The alkaline lysis buffer contained 50 mM dithiothreitol (DTT) (Pharmacia Biotech B.V., Woerden, The Netherlands) and 200 mM KOH (Merck Nederland B.V., Amsterdam, The Netherlands). Prior to adding the PCR reaction mix of the first PCR round, 5 µl neutralization buffer (300 mM KCl (Merck), 200 mM HCl (Merck) and 900 mM Tris–HCl (Merck) pH 8.3) was added to the lysed cell (Cui et al., 1989). Subsequently 49 µl PCR first round reaction mixture was added followed by an initial denaturation step of 5 min at 96°C. The amplification was started by adding 1 µl of Ampli-Taq DNA polymerase (1.5 U/µl; Perkin Elmer Nederland, Nieuwekerk a/d IJssel, The Netherlands) at a sample temperature of 80°C.

SMN exon 7 of the SMN gene was amplified by two consecutive PCR rounds of 25 and 35 cycles in a DNA Thermal cycler 480 (Perkin Elmer Cetus, Norwalk, CT, USA) with the same set of primers in both rounds. The cycle steps for both rounds were: an initial denaturation step of 96°C for 5 min, followed by 1 min of 94°C denaturation, 1 min annealing at 55°C and 1 min 72°C elongation. The PCR was completed with a final elongation step of 5 min at 72°C. The first round reaction mixture contained 6.0 µl 10×buffer [30 mM MgCl₂ (Merck), 500 mM KCl (Merck), 100 mM Tris–HCl (Merck) pH 8.4, 0.1% Gelatin (Merck) and 1% Triton-X (Sigma)], 0.48 µl dNTP 25 mM from each of the four

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Figure 2. Polymerase chain reaction (PCR) products of the survival motor neuron (SMN) exon 7 PCR system, separated on a 10% polyacrylamide gel and stained with ethidium bromide. Lane 1: 100 bp base pair marker. Lanes 2 and 3: single cell PCR products of a normal individual homozygous wild type (WT/WT). Lanes 4 and 5: single cell PCR products from a spinal muscular atrophy (SMA) carrier heterozygous for the SMN exon 7 deletion (WTdel7). Lanes 6 and 7: single cell PCR products from an SMA patient homozygous for the SMN exon 7 deletion (del7del7).

Preimplantation genetic diagnosis of SMA

Table I. Results of survival motor neuron (SMN) exon 7 polymerase chain reaction (PCR) deletion detection in single leukocytes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Leukocytes</th>
<th>n</th>
<th>Positive PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SMN exon 7</td>
</tr>
<tr>
<td>WT/WT</td>
<td>70</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>WTdel7</td>
<td>50</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>del7del7</td>
<td>80</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>108/120 (90%)</td>
<td>181/200 (91%)</td>
</tr>
</tbody>
</table>

WT = SMN exon 7 wild type allele; del7 = SMN exon 7 deletion; n = number of leukocytes tested.

SMN copy sequence were visible. In the single cells obtained from the SMA patient, only the SMN copy sequence was demonstrated. The SMN exon 7 sequence was absent as expected.

In order to evaluate the efficiency of the PCR system, 200 leukocytes and 25 blastomeres were tested. Leukocytes were obtained from individuals with a normal (n = 2), carrier (n = 1) and patient (n = 1) genotype. The blastomeres were obtained from three surplus embryos after an IVF procedure, of which two were at the 8-cell stage and one was at the 9-cell stage. In 181 of 200 single leukocytes collected, a PCR product was generated (Table I). In 63 of 70 leukocytes with the homozygous WT/WT genotype, the expected PCR signal for the SMN exon 7 and SMN copy sequence was observed. And in 45 of 50 leukocytes heterozygous for the exon 7 deletion, a positive SMN exon 7 signal and SMN copy sequence were obtained (Table I). In the heterozygous group none of the single cell samples were genotyped as homozygous exon 7 deleted. This indicates that within this group no allelic dropout had occurred. In 73 of the 80 homozygously deleted SMN exon 7 leukocytes, only the SMN copy sequence was observed. In total, 108 from the 120 cells with one or two WT alleles gave a positive SMN exon 7 signal. Therefore the PCR efficiency was 90%. The SMN copy gene was amplified with the same efficiency. Of the three tested surplus embryos, all 25 blastomeres gave a positive SMN exon 7 signal and 24 gave a positive SMN exon 7 PCR signal. Thus in one blastomere the SMN exon 7 was absent although the SMN copy gene was visible. Moreover in the seven sister blastomeres the SMN exon 7 was present. In a total of 40 negative controls no false positive was observed.

PGD of SMA

From the couple with a 25% risk for SMA, eight metaphase II oocytes were retrieved under ultrasound guidance after the first stimulation to multifollicular development. After 18–20 h the ICSI procedure resulted in four zygotes with two pronuclei (PN), two 1PN oocytes and one oocyte in which no PN were observed. One oocyte was found to be degenerated. On the third day after ICSI four embryos were considered to be suitable for biopsy according to the criteria described above. Two blastomeres were biopsied from embryos 1 and 4, and one blastomere was biopsied from the 5-cell and 6-cell embryo. A second stimulation and ICSI cycle of the couple at risk yielded 13 metaphase II oocytes resulting in eight 2PN zygotes,
Table II. Intracytoplasmic sperm injection/in-vitro fertilization, embryo development and preimplantation genetic diagnosis results

<table>
<thead>
<tr>
<th>Embryo developmental stage</th>
<th>Blastomere biopsy</th>
<th>No. of blastomeres</th>
<th>Nucleus present</th>
<th>Determined alleles</th>
<th>Phenotype</th>
<th>Blanks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo no. Day 3 Day 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>First cycle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8-cell</td>
<td>morula 1</td>
<td>yes</td>
<td>WT</td>
<td>normal</td>
<td>neg</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9-cell</td>
<td>yes</td>
<td>WT</td>
<td>normal</td>
<td>neg</td>
</tr>
<tr>
<td>3</td>
<td>4-cell</td>
<td>10-cell 1</td>
<td>yes</td>
<td>WT</td>
<td>normal</td>
<td>neg</td>
</tr>
<tr>
<td>4</td>
<td>8-cell</td>
<td>morula 1</td>
<td>yes</td>
<td>WT</td>
<td>normal</td>
<td>neg</td>
</tr>
<tr>
<td><strong>Second cycle</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8-cell</td>
<td>morula 1</td>
<td>yes</td>
<td>WT</td>
<td>normal</td>
<td>neg</td>
</tr>
<tr>
<td>2</td>
<td>8-cell</td>
<td>9-cell 1</td>
<td>yes</td>
<td>WT</td>
<td>normal</td>
<td>neg</td>
</tr>
<tr>
<td>3</td>
<td>4-cell</td>
<td>6-cell 1</td>
<td>yes</td>
<td>WT</td>
<td>normal</td>
<td>neg</td>
</tr>
<tr>
<td>4</td>
<td>7-cell</td>
<td>8-cell 1</td>
<td>yes</td>
<td>NR</td>
<td>?</td>
<td>neg</td>
</tr>
<tr>
<td>5</td>
<td>8-cell</td>
<td>morula 1</td>
<td>yes</td>
<td>NR</td>
<td>?</td>
<td>neg</td>
</tr>
<tr>
<td>6</td>
<td>9-cell</td>
<td>9-cell 1</td>
<td>yes</td>
<td>del7/del7</td>
<td>affected</td>
<td>neg</td>
</tr>
<tr>
<td>7</td>
<td>5-cell</td>
<td>6-cell 1</td>
<td>yes</td>
<td>NR</td>
<td>?</td>
<td>neg</td>
</tr>
</tbody>
</table>

*WT = survival motor neuron (SMN) exon 7 wild type allele; del7 = SMN exon 7 deletion; NR = no polymerase chain reaction result.

Discussion

The recent finding and characterization of the SMN gene as the candidate gene for SMA (Lefebvre et al. 1995) has provided tools to perform mutation analysis in SMA. Van der Steege et al. (1995) published a PCR protocol for exon 7 of the SMN gene, in which restriction enzyme digestion is used to discriminate between the SMN exon and a highly homologous copy sequence. The presence or absence of the SMN exon 7 sequence on both chromosomes 5 can refute or prove clinical suspicion of SMA, and is therefore a powerful diagnostic test for SMA. Couples with SMA-affected children have a 25% risk of having another affected child, and can only opt for prenatal diagnosis to have a healthy child. We established a protocol for PGD as an alternative in cases where both parents are carriers of the SMN exon 7 deletion. In PGD, unaffected embryos are selected before implantation by genetic analysis of single blastomeres.

PCR efficiency for the SMN exon 7 detection in leukocytes with one or two wild type alleles or with a homozygous deletion for SMN exon 7 is 90 and 91% respectively. The presence of the SMN copy sequence, which is found in 95.5% of the normal population (Lefebvre et al., 1995), serves as a measure for the PCR efficiency in cells in which the SMN exon 7 is homozygously deleted. The presence of the copy sequence allows discrimination between a deletion and PCR failure and prevents misdiagnosis by serving as an internal control for the PCR reaction. None of the heterozygously deleted SMN exon 7 leukocytes was genotyped as homozygously deleted, indicating that no allelic loss occurred. None of the leukocytes with a positive PCR signal showed discrepancies regarding the determined genotype and the actual genotype.

In 25 blastomeres tested no amplification failure was found. However, in one blastomere only the copy sequence was
detected whereas in the other seven blastomeres from the same embryo, the SMN exon 7 sequence was clearly present. This difference in genotype can be explained by allelic loss of the wild type allele. However, since there was no allelic drop-out observed in leukocytes of the carrier tested, it is very unlikely that in case of a homozygous WT embryo both wild type alleles failed to amplify.

Therefore, a more plausible explanation may be that the embryo is heterozygous for the SMN exon 7 deletion. The chance of a normal individual being a carrier of the SMN exon 7 deletion is ~1 in 50 (Pearn, 1973, 1978; Spiegler et al., 1990). Discrimination between the homozygous wild type and heterozygous genotype is not possible with this PCR system.

Assuming that the amplification failure observed in the leukocytes tested is the same as in the biopsied blastomeres during PGD, implies that in 10% of the embryos no diagnosis can be made if only one blastomere should be available for analysis. However, if the embryo at the time of biopsy consists of eight cells or more, and two blastomeres can be biopsied for analysis, the rate of non-diagnosed embryos will be reduced to 1%. Furthermore, by analysing two blastomeres, contamination can be monitored more easily if inconclusive results are obtained. Thereby the chance of misdiagnosis is decreased.

Embryos from a couple requesting PGD could be genotyped for the SMN exon 7 deletion by analysing the blastomeres. The PCR analyses of the blastomeres obtained from the 2PN embryos were clearly interpretable and all but three blastomeres could be genotyped. No discordance was found when two blastomeres from the same embryo were biopsied. The PGD analysis led to the transfer of two embryos at the morula stage in both cycles. The transferred embryos contained at least one SMN exon 7 allele. After the first cycle no pregnancy was achieved, but after the second attempt an ongoing gemelli pregnancy was achieved. The results of this study demonstrate that SMA genotyping of single cells is possible within a time span of 11 h. Therefore, PGD for SMA is feasible for couples with a child homozygous for the SMN exon 7 deletion.

References


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