Preimplantation genetic diagnosis of spinocerebellar ataxia 3 by \((CAG)_n\) repeat detection

M.Drüsedau\(^1,3\), J.C.F.M.Dreesen\(^1\), C.de Die-Smulders\(^1\), K.Hardy\(^1\), M.Bras\(^2\), J.C.M.Dumoulin\(^2\), J.L.H.Evers\(^2\), H.J.M.Smeets\(^1\), J.P.M.Geraedts\(^1\) and J.Herbergs\(^1\)

Research Institute Growth & Development (GROW), \(^1\)Department of Clinical Genetics and \(^2\)Department of Obstetrics & Gynaecology, Academic Hospital Maastricht, Maastricht, The Netherlands

\(^3\)To whom correspondence should be addressed at: Academic Hospital Maastricht, Department of Clinical Genetics, P.O.Box 5800, 6202 AZ Maastricht, The Netherlands. E-mail: Marion.Drusedau@gen.unimaas.nl

Spinocerebellar ataxia 3 (SCA3) is an autosomal dominant neurodegenerative disorder characterized by variable expression and a variable age of onset. SCA3/MJD (Machado–Joseph disease) is caused by an expansion of a \((CAG)_n\) repeat in the MJD1 gene on chromosome 14q32.1. A single cell PCR protocol has been developed for preimplantation genetic diagnosis (PGD) of SCA3 to select unaffected embryos on the basis of the CAG genotype. Single leukocytes and blastomeres served as a single cell amplification test system to determine the percentage of allelic drop-out (ADO) and PCR efficiency. Out of 105 tested heterozygous single leukocytes, 103 (98.1%) showed a positive amplification signal, while five cells (4.9%) showed ADO. Amplification in single blastomeres was obtained in 13 out of a total of 14, and ADO was observed in two out of the 13 single blastomeres. PGD of SCA3 was performed in a couple with paternal transmission of the SCA3 allele. Seven embryos were available for biopsy, all biopsied blastomeres showed amplification and no ADO occurred. One embryo was diagnosed as affected whereas six embryos were diagnosed as unaffected. Two unaffected embryos were transferred and resulted in a singleton pregnancy and the birth of a healthy girl.

Key words: allelic drop-out/preimplantation genetic diagnosis/spinocerebellar ataxia 3/triplet repeats

Introduction

Spinocerebellar ataxia type 3 (SCA3; OMIM accession number: 109150) is a progressive neurodegenerative disorder that belongs to the clinical group type I of the autosomal dominant late onset cerebellar ataxias (ADCA) (Harding, 1982). The neuropathology consists of neuronal loss and gliosis in the substantia nigra, motor cranial nuclei, dentate nucleus of the cerebellum, and variable neuronal loss with gliosis in the cerebellar cortex and neostriatum (Rosenberg, 1992). The clinical features of SCA3 are variable: progressive ataxia, hyperreflexia, nystagmus and often visual blurring and diplopia. All patients develop slow saccadic eye movements and an ophthalmoparesis evolves. The age of onset is also variable, but symptoms start usually in the third decade.

SCA3 is caused by an expanded and unstable \((CAG)_n\) repeat in the MJD1 (Machado–Joseph Disease) gene on chromosome 14q32.1 (Kawaguchi et al., 1994; Cancel et al., 1995). The \((CAG)_n\) repeat is highly polymorphic, and varies in normal individuals from 12 to 43 repeats (Cancel et al., 1995; Maciel et al., 1995; Matilla et al., 1995; Ranum et al., 1995; Sasaki et al., 1995; Takiyama et al., 1995; Limprasert et al., 1996; Matsumura et al., 1996), whereas SCA3 patients have a CAG expansion ranging from 62 to 84 CAG repeats (Maciel et al., 1995). A strong inverse correlation has been observed between the repeat expansion size and the age of onset (Ranum et al., 1995).

Until now, 14 neurological diseases are known to be caused by expansion of unstable trinucleotide repeats (Masino and Pastore, 2001). For three of them, Huntington’s disease, fragile X syndrome and myotonic dystrophy, a single cell PCR protocol has been developed for the respective polymorphic trinucleotide repeats, making preimplantation genetic diagnosis (PGD) possible (Piyamongkol et al., 2001; Sermon et al., 2001; Stern et al., 2002). This procedure, performed on embryos obtained via IVF with ICSI, allows for the selection of unaffected embryos for transfer, by the detection of two \((CAG)_n\) repeats in the normal range.

In this report we describe the development and the first clinical application of a single cell PCR protocol for the detection of the polymorph \((CAG)_n\) repeat in the MJD1 gene of single blastomeres to diagnose embryos for SCA3.

Materials and methods

Collection of human leukocytes and blastomeres

Single leukocytes and blastomeres were used to develop and test the PGD PCR protocol for SCA3. Leukocytes were isolated from fresh blood in 2 µl Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate-buffered saline (PBS) with 1% polyvinylpyrrolidone (PVP; Sigma–Aldrich Chemie BV, The Netherlands) and 0.1 mg/ml Phenol Red (Sigma) as described previously (Dreesen et al., 1998). After transferring the single cells to 0.2 ml reaction tubes, they were stored at −20°C until the PCR was performed.

Blastomeres were obtained from three different human embryos, donated by a couple after an IVF treatment (day 3 after ICSI). These embryos were left over after transfer and considered to be unsuitable for cryopreservation because of retarded embryonic development and poor morphology. The couple had given consent for their surplus embryos to be used in these experiments. The protocol of this study has been approved by the hospital Ethical Committee.
Single blastomeres were collected in PBS with 1% PVP and Phenol Red (0.1 mg/ml) (Dreesen et al., 1998) and transferred into 0.2 ml reaction tubes. After collection, the single blastomeres were lysed directly followed by PCR. From the non-transferred embryos remaining after the PGD cycle, blastomeres were collected and processed in the same way in order to confirm the results of the initial PGD analysis.

**Family history**

In a family (Figure 1), spinocerebellar ataxia 3 was clinically diagnosed in female member 2 (Figure 1) at 61 years of age and confirmed by DNA diagnosis. Initially her son (member 4) was asymptomatic and therefore he declined presymptomatic DNA testing. His wife (member 3) became pregnant and they applied for direct genetic testing of the fetus by means of a chorionic villus biopsy without prior testing of the future father. A repeat length of 70 CAG was found, indicating the fetus to be affected. The detection of this expansion in the fetus indicated indirectly that the father had to be carrier of SCA3. The couple decided to terminate the pregnancy. For their next pregnancy, they applied for PGD.

**Blastomere biopsy**

Oocytes were fertilized by ICSI to prevent contamination with sperm cells during the biopsy of the PGD. The ICSI procedure was performed as described previously (Dumoulin et al., 2000).

On the morning of day 3 after fertilization, blastomeres were biopsied from each cleavage stage embryo. A small hole was made in the zona pellucida with acid Tyrode’s solution (Handyside et al., 1990). From embryos containing ≥8 cells, two blastomeres were removed and from embryos with 4–7 cells, only one blastomere was biopsied and analysed. Blastomeres were rinsed and collected in a 0.2 ml PCR tube containing 2.5 μl lysis buffer. The lysis buffer contained 50 mmol/l DTT, 200 mmol/l NaOH (Merck, The Netherlands) and 200 mmol/l NaOH (Merck, The Netherlands). For each embryo, one blank of 2 μl was transferred to a PCR tube to monitor contamination.

**Cell lysis and PCR**

Cells (blastomeres and human leukocytes) were lysed by incubation at 65°C for 10 min in lysis buffer (50 mmol/l DTT, 200 mmol/l NaOH).

For PCR of the (CAG)n repeat, the Expand™ Long Template PCR System kit (Roche, The Netherlands), developed for PCR of long DNA templates and suitable for the amplification of triplet repeats at the single cell level was used (Sermon et al., 1998a,b, 1999).

The PCR reaction mixture consisted of 1×PCR buffer 2 provided by the manufacturer, 0.2 mmol/l dATP, dCTP, dGTP and dTTP (Pharmacia), 15 pmol/μl of each primer (PE Applied Biosystems, The Netherlands) (MJD52-HEX 5'-CCAGTGACTACTTTGATTCG-3' and MJD25 5'-TGG-

Table I. Allelic drop-out (ADO) rate and PCR efficiency of the PCR system

<table>
<thead>
<tr>
<th>Single cells</th>
<th>No. of cells</th>
<th>ADO n (%)</th>
<th>PCR efficiency n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unaffected</td>
<td>80</td>
<td>3 (3.8)</td>
<td>78 (97.5)</td>
</tr>
<tr>
<td>Affected</td>
<td>25</td>
<td>2 (8)</td>
<td>25 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>5 (4.9)</td>
<td>103 (98.1)</td>
</tr>
<tr>
<td>Blastomeres</td>
<td>14</td>
<td>2 (15.4)</td>
<td>13 (92.9)</td>
</tr>
</tbody>
</table>

Results

**Single cell testing**

Single leukocytes (with unaffected and affected CAG genotypes) and blastomeres served as a single cell test system to determine the percentage of allelic drop-out (ADO) and PCR efficiency. A total of 80 single leukocytes of an unaffected individual, heterozygous for CAG alleles in the normal range, were analysed as were 25 single leukocytes of a patient, containing a CAG expansion. Seventy-eight (97.5%) unaffected leukocytes gave a positive amplification signal, and three (3.8%) of these showed ADO (Table I). In two of the cells with ADO, the smaller allele had dropped out and the third cell showed ADO for the bigger allele. All the 25 affected cells showed amplification results (100%). Two (8%) of these demonstrated ADO, both for the expanded allele. The overall PCR efficiency and ADO rate demonstrated by the single leukocytes were 98.1 and 4.9% respectively. No case of contamination was detected in any of the negative controls.

The lysis and PCR method were tested on single blastomeres obtained from three donated human embryos at day 3 after fertilization. A total of 14 blastomeres in which a nucleus was visually confirmed was collected from a 3-cell embryo, a 6-cell embryo and an 8-cell embryo. In two blastomeres ADO was observed, and one blastomere showed no amplification, which resulted in a PCR efficiency of 92.9% (Table I).

**PGD results**

The male partner (35 years old; person 4, Figure 1) of the couple had an expanded allele of 67 CAG repeats in combination with a normal allele of 26 repeats. His wife (33 years old; person 3, Figure 1) had two normal alleles with 21 and 22 CAG repeats respectively.

After the first induced superovulation cycle, 10 cumulus-oocyte complexes were obtained. Nine oocytes were at the metaphase II stage and could be fertilized with ICSI. At day 1 after fertilization, eight of the nine oocytes showed two pronuclei (2PN), and on day 3 it was possible to biopsy one or two blastomeres from seven of the eight 2PN

![Figure 1. Pedigree of the first preimplantation genetic diagnosis (PGD) couple for spinocerebellar ataxia 3 (SCA3). The black/filled symbols represent the SCA3 carriers and the white/open symbols are unaffected family members. Circles are females and squares are males. Person 2 died at 64 years of age. TOP = termination of pregnancy.](image-url)
One embryo was not suitable for biopsy, because it consisted of <4 cells. In all the biopsied blastomeres a nucleus was visible. From all blastomeres subjected to PCR two alleles were obtained, so the PCR efficiency was 100% and no ADO was observed. All blank controls gave a negative signal.

Only one embryo contained the paternal allele with the expansion, but the expansion had decreased to 66 CAG repeats. All the other embryos displayed both paternal and maternal alleles in the normal range (Figure 2). On the morning of day 4, two of the embryos with a normal SCA3 genotype, both developed to the morula stage (Table II, embryo number 1 and 6), were transferred. The remaining five

![Figure 2. Electropherogram of the single cell CAG trinucleotide repeat analysis for preimplantation genetic diagnosis of spinocerebellar ataxia 3 (SCA3).](image-url)

**Table II.** Results of the first preimplantation genetic diagnosis of spinocerebellar ataxia 3

<table>
<thead>
<tr>
<th>Embryo number</th>
<th>Development stage/ morphological grade at day 3</th>
<th>No. of analysed blastomeres</th>
<th>Genotype (CAG)n repeat</th>
<th>Development stage/ morphological grade at day 4</th>
<th>Re-analysis genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12-cell/3</td>
<td>2</td>
<td>22–26</td>
<td>Morula</td>
<td>ET</td>
</tr>
<tr>
<td>2</td>
<td>10-cell/3</td>
<td>2</td>
<td>21–66</td>
<td>Morula</td>
<td>21–66</td>
</tr>
<tr>
<td>3</td>
<td>6-cell/2</td>
<td>1</td>
<td>22–26</td>
<td>6-cell/3</td>
<td>22–26</td>
</tr>
<tr>
<td>4</td>
<td>6-cell/2</td>
<td>1</td>
<td>22–26</td>
<td>Fragmented</td>
<td>22–26</td>
</tr>
<tr>
<td>5</td>
<td>8-cell/3</td>
<td>2</td>
<td>21–26</td>
<td>7-cell/3</td>
<td>21–26</td>
</tr>
<tr>
<td>6</td>
<td>10-cell/3</td>
<td>2</td>
<td>22–26</td>
<td>Morula</td>
<td>ET</td>
</tr>
<tr>
<td>7</td>
<td>4-cell/3</td>
<td>1</td>
<td>21–26</td>
<td>3-cell/2</td>
<td>21–26</td>
</tr>
</tbody>
</table>

*Values of 1 for poorest and 4 for best morphological grade (Bolton et al., 1989).

ET = embryo transferred.

embryos (Table II). One embryo was not suitable for biopsy, because it consisted of <4 cells. In all the biopsied blastomeres a nucleus was visible.

From all blastomeres subjected to PCR two alleles were obtained, so the PCR efficiency was 100% and no ADO was observed. All blank controls gave a negative signal.
embryos were collected for re-analysis. All embryos showed the same (CAG)n repeat genotype as established by PGD. The transfer of the two unaffected embryos resulted in a singleton pregnancy and the birth of a healthy girl. Postnatal testing of their daughter was discussed by the parents, but was not encouraged in accordance with international guidelines for presymptomatic testing of children for late onset diseases (McLean, 1998).

Discussion

SCA3 is one of the 14 neurological diseases caused by a trinucleotide repeat expansion (Masino and Pastore, 2001). Several disorders associated with a trinucleotide repeat expansion, such as Huntington’s disease, myotonic dystrophy and the fragile-X syndrome, have been proven suitable for PGD (Sermon et al., 1998a,b, 1999). Unlike other mutations associated with autosomal dominant genetic disorders, trinucleotide repeat expansions do not give a false negative PGD outcome due to ADO, provided that embryos are selected for transfer on the basis of the presence of two trinucleotide repeat alleles in the normal range. A prerequisite for PGD is that the normal allele of the affected parent differs from the normal alleles of the other parent. We developed and tested a single cell SCA3 CAG trinucleotide repeat PCR protocol and subsequently introduced it into the clinic to perform PGD.

The PCR efficiency for the CAG repeat single cell PCR system for SCA3 was 98.1% in single leukocytes. The overall ADO percentage of the tested leukocytes, 4.9%, is comparable with ADO percentages found and published for other trinucleotide repeats such as Huntington’s disease (heterozygous lymphoblasts: unaffected 4%, affected 9%; Sermon et al., 1998b), myotonic dystrophy (heterozygous unaffected lymphoblasts: 6.5%; Sermon et al., 1998a) and fragile X syndrome (heterozygous female lymphoblasts: 1.9%; Sermon et al., 1999). The higher ADO percentage and lower PCR efficiency found in our tested single blastomeres may be explained by the fact that we used embryos unsuitable for transfer and freezing, implying that these embryos are of a lower morphological quality.

An alternative for the first SCA3–PGD couple could be a prenatal exclusion test and an exclusion–PGD test but exclusion-testing PGD is not offered for trinucleotide repeat diseases by the Maastricht PGD team, because of the risks of assisted reproduction and biopsy for the prospective mother and child-to-be (Braude et al., 1998). In half the cases these risks could be avoided.

The CAG repeat expansion of the only affected embryo showed intergenerational instability. The father contained a 67 CAG repeat expansion and in the affected embryo the repeat number decreased to 66. Intergenerational instability has been described in other neurodegenerative diseases caused by a CAG expansion, such as Huntington’s disease, SCA1, SCA2, spinal and bulb muscular atrophy (SBMA) and dentatorubral pallidoluysian atrophy (DRPLA) (La Spada et al., 1991; Huntington’s Disease Collaborative Research Group, 1993; Orr et al., 1993; Koide et al., 1994; Nagafuchi et al., 1994; Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996). Expanded CAG repeats tend to be more unstable on paternal transmission.

The unaffected embryos were selected for transfer on the basis of morphology. In the case where two blastomeres were biopsied from one embryo they were required to show consistent results to be considered suitable for transfer. This was the case for all embryos.

The PGD embryos could be clearly genotyped for the (CAG)n repeat of SCA3 after the fluorescent PCR analyses of the biopsied blastomeres and detection on the Genetic Analyzer ABI3100. The single cell SCA3 CAG repeat PCR protocol is an efficient and accurate method for PGD of SCA3, as was shown by the single leukocyte test procedure and the re-analysis of the non-transferred PGD embryos. The results can be obtained in 6–7 h, depending on the number of embryos, after biopsy. The PGD procedure described in this paper led to the transfer of two morula embryos and resulted in a singleton pregnancy and the birth of a healthy girl.

References


Submitted on June 3, 2003; resubmitted on August 25, 2003; accepted on August 28, 2003