Preimplantation Genetic Diagnosis of Canavan Disease

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\textbf{Key Words} Canavan disease \textperiodcentered Autosomal recessive disorder \textperiodcentered Diagnosis, preimplantation genetic \textperiodcentered Ashkenazi Jews \textperiodcentered Aspartoacylase deficiency

\section*{Abstract}

\textbf{Objective:} Canavan disease is an autosomal recessive disorder which is relatively common in Ashkenazi Jews. It is characterized by developmental delay, severe hypotonia and early death, and is caused by a deficiency of aspartoacylase which is encoded by the \textit{ASPA} gene. In Ashkenazi Jews, one major mutation (E285A) and one minor mutation (Y231X) account for the majority of cases. The objective of this study was to develop a preimplantation genetic diagnosis (PGD) protocol for Canavan disease.

\textbf{Methods:} Two carrier couples requested PGD for Canavan disease. In 1 couple each was a carrier of a different \textit{ASPA} mutation (Y231X and E285A). In the other couple both were carriers of the minor mutation (Y231X). A single-cell duplex nested polymerase chain reaction (PCR) protocol was first developed on single leukocytes obtained from the known carrier parents. Following verification in single leukocytes, clinical PGD was offered to both couples.

\textbf{Results:} We evaluated 115 single leukocytes from known carriers and found an allele drop out rate of 1.7\% for the fragment harboring the Y231X mutation and 0\% for the fragment harboring the E285A mutation. One cycle of PGD was performed in each family. In the first, 11 embryos were successfully analyzed and 4 were found to be affected. Two unaffected embryos were transferred, but no pregnancy resulted. In the other family, 4 embryos were analyzed, 1 was affected, 2 were heterozygotes and 1 was homozygous normal. Following transfer of 2 unaffected embryos, a singleton pregnancy resulted, currently ongoing at 18 weeks gestational age. Amniocentesis performed at 16 weeks confirmed the diagnosis. \textbf{Conclusion:} Reliable PGD for Canavan disease is possible using a single-cell nested PCR approach.

\section*{Introduction}

Canavan disease is an autosomal recessive disorder characterized by developmental delay, beginning at 3–5 months of age, with severe hypotonia and failure to achieve motor milestones such as sitting, walking or speech. The disease is caused by a deficiency of aspartoacylase, resulting in accumulation of N-acetyl aspartic acid [1]. Aspartoacylase is encoded by the \textit{ASPA} gene on chromosome 17pter-p13 [2]. The disease is rare in most populations, but is relatively prevalent among Ashkenazi Jews, with a carrier frequency of 1:37–1:58 [3]. In Ashkenazi Jews, one major mutation (E285A) and one minor mutation (Y231X) account for 98\% of the cases [3, 4].

For a carrier couple at a 25\% risk of affected offspring, preimplantation genetic diagnosis (PGD) offers an alternative to prenatal diagnosis and termination of affected...
pregnancies. PGD requires in vitro fertilization (IVF) and analysis of single blastomeres obtained at the 6- to 10-cell stage. Only the unaffected embryos are transferred [5, 6]. To the best of our knowledge, PGD for Canavan disease has not been reported. The purpose of this study was to develop a single-cell duplex nested polymerase chain reaction (PCR) protocol for PGD of Canavan disease.

Materials and Methods

A single-cell duplex nested PCR protocol was first developed on single leukocytes obtained from known carriers of the Y231X and E285A mutations. Isolation of leukocytes from peripheral blood and cell lysis were performed as previously described [7]. Following establishment of the single-cell duplex nested PCR, clinical PGD was performed. Controlled ovarian stimulation, oocyte aspiration, intra-cytoplasmic sperm injection and embryo biopsy were also previously described in detail [7].

Duplex Nested Polymerase Chain Reaction in Single Cells

A duplex nested PCR protocol was designed for co-amplification of the two genomic fragments harboring the major (E285A) and the minor (Y231X) mutations of the ASPA gene, respectively (table 1). Following PCR, the resulting amplicons are subjected to digestion by restriction enzymes to distinguish between the wild-type and the mutated alleles. To evaluate for possible contamination, allele drop out (ADO), amplification failure or incomplete enzymatic digestion, 5 single-cell controls from known mutation carriers are simultaneously analyzed.

First Round – Outer PCR

For the first-round PCR, the following are added to the reaction tube containing the single cell in alkaline lysis buffer: 2 μl 10 x PCR buffer (OptiBuffer™, Bioline); 1 μl MgCl₂ (50 mM); 2 μl 5 x Specificity Enhancer (Bioline); 6 μl H₂O; 0.5 μl Tricine 1 M; 1 μl dNTP mixture stock (5 mM); 1 μl DMSO; 0.5 μl gelatin (1% w/v), and 0.7 μl of each outer primer (13 μM) for ASPA fragments harboring the Y231X and E285A mutations (table 1). The mixture is exposed to a prolonged denaturation at 96°C for 8 min and temperature is then decreased to 75°C. At this stage, 5 μl of enzyme mix containing 0.5 μl 10 x PCR buffer (OptiBuffer™, Bioline), 0.25 μl MgCl₂ (50 mM), 0.25 μl Taq polymerase (Bio-X-Act, 4 U/μl) and 4 μl H₂O, is added.

Amplification begins with a single denaturation step of 98°C for 2 min, followed by 10 cycles of denaturation at 96°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 3 min. This is followed by 8 more cycles of denaturation at 94°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 3 min. Final extension is performed at 72°C for 8 min.

Second Round – Inner PCR

Aliquots of the first-round PCR product (1 μl) are divided into 2 separate PCR tubes for each of the individual inner (nested) PCR. The following are added to each tube: 2 μl 10 x PCR buffer (OptiBuffer™, Bioline); 1 μl MgCl₂ (50 mM); 2 μl 5 x Specificity Enhancer (Bioline); 1 μl of dNTP mixture stock (5 mM); 1 μl DMSO, and 1.5 μl of each inner primer (13 μM) for Y231X- or E285A-containing fragments (table 1). H₂O is added for a final volume of 20 μl. The mixtures are exposed to prolonged denaturation at 96°C for 8 min and temperature is then decreased to 75°C. At this stage, 5 μl of enzyme mix containing 0.5 μl 10 x PCR buffer (OptiBuffer™, Bioline), 0.25 μl MgCl₂ (50 mM), 0.4 μl Taq polymerase (Bio-X-Act, 4 U/μl) and 3.85 μl H₂O, is added.

Amplification begins with a single denaturation step of 98°C for 2 min, followed by 14 cycles of denaturation at 96°C for 1 min, annealing at 60°C for 2 min and extension at 72°C for 2 min. This is followed by 20 more cycles of denaturation at 94°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 2 min. Final extension is performed at 72°C for 8 min.

Restriction Enzyme Digestion

Nested PCR is followed by digestion with specific restriction enzymes that distinguish between the wild-type and the mutant alleles. An aliquot of 10 μl of the second-round PCR product is digested with 20 units of either Eagl (for the E285A mutation) or Msel (for the Y231X mutation) and incubated at 37°C for 2 h. In the mutant allele responsible for the E285A mutation, the A at nucleotide position 584 is replaced by C, creating a novel Eagl restriction site. Enzymatic digestion of the mutant allele with Eagl results in cleavage of the 443-bp nested PCR amplicon into two fragments of 262 and 181 bp, while the non-mutated allele remains intact. In the mutant allele responsible for the Y231X mutation, a C at nucleotide position 693 is replaced by A, creating a novel Msel restriction site. Enzymatic digestion of the mutant allele with Msel results in cleavage of the 163-bp nested PCR amplicon into two fragments of 103 and 60 bp, while the non-mutated allele remains intact. The products of enzymatic restriction digestion are resolved by electrophoresis on a 3% agarose SFR™ (Amresco USA) stained with ethidium bromide. Products are visualized under UV light.

Results

Duplex Nested PCR for Canavan in Single Leukocytes

We first evaluated 115 single leukocytes from known carrier parents. ADO was defined as the absence of the normal or mutated alleles in single cells obtained from a

Table 1. Primer sets used for duplex nested PCR for PGD of Canavan disease

<table>
<thead>
<tr>
<th>E285A region</th>
<th>Y231X region</th>
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<tbody>
<tr>
<td>Outer forward</td>
<td>GGG TAT TTT TAG TGG AGA TGG GTG TC</td>
</tr>
<tr>
<td>Outer reverse</td>
<td>ATG TAC GCG AAG TGC TGT AGT AGC TA</td>
</tr>
<tr>
<td>Inner forward</td>
<td>CCA GAG ATG TTT TTA GTT GCC ATT GA</td>
</tr>
<tr>
<td>Inner reverse</td>
<td>TGT TAC CTG CAG ATT AGG ATG GAT GA</td>
</tr>
</tbody>
</table>

| Outer forward | TGC GCT TAG ATG CCT ACC GAA TAA GG |
| Outer reverse | GTG AGA TCA CTT GCC TGC ATC TCA AT |
| Inner forward | TGC AGT TGA ATA AGG CAC AAC CCT AC |
| Inner reverse |
known heterozygote carrier. There were 2 instances (1.7%) of ADO of the wild-type allele in the PCR for the fragment harboring the Y231X mutation. For the fragment harboring the E285A mutation, no ADO was encountered. In all negative controls no amplification was demonstrated. Figure 1 describes the duplex nested PCR in 3 single leukocytes from each of the carrier parents in case 1. Analysis of the E285A mutation in the mother’s sample (fig. 1A, lanes 1–3) demonstrates only the uncut 443-bp fragment, whereas the father’s sample (fig. 1A, lanes 4–6) has both the 443-bp uncut fragment and the digested fragments of 262 and 181 bp, representing the wild-type and E285A mutant alleles, respectively. Conversely, analysis of the Y231X mutation in the mother’s sample (fig. 1B, lanes 1–3) demonstrates both the 163 uncut fragment and the digested fragments of 103 and 60 bp, representing the wild-type and Y231X mutant alleles, respectively. In contrast, the father’s sample (fig. 1B, lanes 4–6) has only the uncut 163-bp fragment, corresponding to the wild-type mutation.

Clinical Cases of PGD for Canavan

Following establishment of the single-cell nested PCR protocol, PGD for Canavan disease was offered to 2 carrier couples. The first had given birth to an affected child who was found to be a compound heterozygote of the major (E285A) and the minor (Y231X) mutations. A subsequent pregnancy also resulted in an affected fetus, as diagnosed by chorion villus sampling (CVS) and the pregnancy was terminated. Following extensive counseling regarding reproductive options, including CVS or amniocentesis and termination of affected pregnancies versus the possibility of PGD, the couple opted for the latter.

The second couple was ascertained by population-based genetic screening for Canavan, offered in Israel by several genetic laboratories, according to guidelines specified by the Israeli Society of Medical Geneticists [8]. Interestingly, both parents were found to be carriers of the minor mutation (Y231X). They had previously conceived twice by IVF (performed for mechanical factors). However, in both pregnancies, prenatal diagnosis by CVS demonstrated an affected fetus and termination was performed.

One cycle of PGD for Canavan disease was performed in each family. Results are summarized in table 2. In the first, a total of 21 oocytes were aspirated, resulting in 12 2PN zygotes, which were all subjected to biopsy and single-cell molecular analysis. Of these, 4 embryos were found to be affected compound heterozygotes, 2 carried only the Y231X mutation and 3 carried only the E285A mutation, 2 embryos were homozygous normal and 1 was uninterpretable. The 2 homozygous normal embryos were transferred but no pregnancy resulted. Reanalysis of the embryos that were not transferred confirmed the initial analysis.

In the second case, 14 oocytes were aspirated and fertilized by intra-cytoplasmic sperm injection. This result-
ed in 4 embryos, all of which were successfully biopsied and analyzed. Two blastomeres were aspirated from each 8-cell embryo and molecular analysis was performed separately for each blastomere. One embryo was found to be homozygous affected, 2 were heterozygous carriers, and one was homozygous normal. A normal homozygous and a heterozygous embryo were transferred on day 4 post-fertilization. A singleton pregnancy resulted, currently ongoing at 18 weeks gestational age. Amniocentesis performed at 16 weeks demonstrated the implantation of the normal homozygous embryo. Reanalysis of the embryos that were not transferred confirmed the initial analysis.

Discussion

Canavan disease is relatively common among Ashkenazi Jews with a carrier frequency of 1:37–1:58. Thus, population screening in Ashkenazim is advocated by the American College of Medical Genetics in its position statement on carrier testing for Canavan disease [9]: ‘If both reproductive partners have an Ashkenazi Jewish background, we recommend that carrier testing for Canavan disease be offered before pregnancy...’ This position was echoed in a consensus statement on screening for Canavan Disease issued by the Society of Medical Geneticists in Israel [8], where the majority of the Ashkenazi Jews reside. Today, most carrier couples in Israel will be detected as a result of this screening strategy, rather than after the birth of an affected child. Such carrier couples have a 25% risk of having an affected child. Terminating an affected pregnancy following prenatal diagnosis is not always an option, particularly among Orthodox Jews. Other couples (like case 2) required IVF for other reasons. For such couples, PGD provides the ultimate solution.

In this article we describe a duplex nested PCR method for PGD of Canavan disease, based on the simultaneous amplification of the ASPA fragments harboring the 2 mutations most prevalent in the Ashkenazi Jewish population (E285A and Y231X).

Since duplex nested PCR on single blastomeres is technically difficult, and in order to avoid misdiagnoses, we attempt to base our analysis on two independent observations. In a previously published article on PGD for spinal muscular atrophy (SMA) we described a duplex nested PCR approach for each of the 2 deleted exons (7 and 8) of the SMN1 gene [7]. In patients with autosomal recessive disease caused by point mutations, we attempt to base our diagnosis on the analysis of 2 blastomeres from the same embryo, as this assures the accuracy of the diagnosis [10]. Indeed, all blastomeres from untransferred embryos that were re-analyzed, confirmed the initial diagnoses.

In conclusion, we describe a reliable protocol for PGD of Canavan disease that may be used clinically in carrier couples.

Acknowledgement

We humbly thank the Canavan carrier families for their courage and trust.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (Canavan disease; [MIM 271900]).

References


