Preimplantation diagnosis of non-deletion Duchenne muscular dystrophy (DMD) by linkage polymerase chain reaction analysis

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The use of preimplantation diagnosis for sex determination and detection of exon deletion means that unaffected babies can be born to parents suffering from Duchenne muscular dystrophy (DMD). However, those who do not have exon deletion should also be considered for further investigation. A new method, known as linkage analysis, has been developed to diagnose the presence of non-deletion DMD in preimplanted embryos. Linkage analysis uses informative intragenic and flanking markers to track the chromosome bearing the mutated gene. The present study reports the analysis of two polymorphic sites, in blastomeres biopsied from embryos from a female carrier of DMD. A single male embryo was obtained who had inherited alternate maternal alleles to the woman’s affected surviving son, and this embryo was transferred.

Key words: Duchenne muscular dystrophy/non-deletion/linkage analysis/preimplantation diagnosis

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive, lethal degenerative disease with an incidence of one in 3500 male births. DMD results from mutations in the human dystrophin gene, an enormous genetic locus that spans 2×10^6 bp of DNA on the short arm of the X chromosome (Hoffman et al., 1987; Koenig et al., 1987).

Consistent with an X-linked recessive disorder, males carrying a DMD-causing mutation express the disease and females generally are non-expressing, reproductive carriers. However, those females having one copy of DMD-causing mutation may express the disease (Chamberlain et al., 1988).

Analysis of dystrophin cDNA and genomic clones has revealed that 55–65% of all cases of DMD result from exon deletion of one of >70 exons; another 5–10% cases result from partial gene duplication. A further 5% of the cases have been reported as due to point mutations (Monaco et al., 1988).

Both polymerase chain reaction (PCR) and Southern blot assays have been used for the identification and characterization of the extent of deletion. Deletions detected by multiplex PCR are confirmed via Southern blot analysis using the appropriate cDNA probes for the deleted region (Koenig et al., 1988; Baumbach et al., 1989; Claustres et al., 1991).

Once a deletion has been identified in an affected male in a family, the probe that detected the mutation can be used to determine carrier/affected status in related families. If no deletions or duplications are found, linkage analysis using intragenic and flanking extragenic markers is suggested (Koenig et al., 1987; Claustres et al., 1991). The intragenic markers include several (CA)n repeats. The extragenic markers include several 5′ and 3′ (CA)n repeats (Beggs and Kunkel, 1990; Oudet et al., 1990; Clemens et al., 1991; Feener et al., 1991).

The first girls were born in 1990 after preimplantation genetic diagnosis (PGD) by gender determination in cases with a variety of X-linked recessive diseases affecting only boys (Handyside et al., 1990). Human embryos can undergo PGD, following either standard in-vitro fertilization (IVF), or intracytoplasmic sperm injection (ICSI) (Liu et al., 1996).

Preimplantation diagnosis of DMD may be an alternative to prenatal diagnosis for women carriers who have a one in four chance of having an affected child and a one in two chance if the child is a boy. The first child after PGD for a DMD gene deletion was born in 1994 (Liu et al., 1995). However, diagnostic methods have been limited to gender determination to obtain female embryos and/or the detection of exon deletion (Kontogianni et al., 1990; Chong et al., 1993; Verlinsky and Kuliev, 1993; Delhanty, 1994; Kristjansson et al., 1994).

We have developed linkage analysis to detect non-deletion DMD cases. By using linkage PCR, unaffected male embryos can be selected for embryo transfer. The present study represents the first report on the preimplantation diagnosis of non-deletion DMD patients.

Materials and methods

Patient

The patient was a 38 year old woman, who had two affected brothers and two affected sons. Both brothers and one of her sons have died of DMD (Figure 1).

Oocyte stimulation and oocyte collection

Ovarian stimulation was achieved by desensitization in the previous luteal phase using gonadotrophin-releasing hormone (GnRH) agonist (buserelin; Hoechst AG, Seoul, Korea) followed by ovarian stimula-
tion with human menopausal gonadotrophin (HMG, Pergonal; Serono, Seoul, Korea).

When at least two follicles measuring >18 mm in diameter were detected, ovulation was induced with 10 000 IU human chorionic gonadotrophin (HCG, Profasi; Serono). Oocytes were retrieved using transvaginal ultrasound-guided aspiration 34–36 h after HCG administration. The oocytes were cultured for 4–6 h in TC199 medium (Gibco/BRL, Gaithersburg, MD, USA) containing 15% inactivated human mature follicular fluid (hFF). All oocyte preparation steps were performed at 37°C in an atmosphere of 5% CO₂ in air.

**Intracytoplasmic sperm injection (ICSI)**

A sperm sample was obtained from the father (sperm count 110×10⁶ spermatids/ml, motility 80%, volume 3.7 ml) and ICSI was used for fertilization of the oocytes (Van Steirteghem et al., 1993). The oocyte–cumulus complexes (OCC) were placed in 0.1% hyaluronidase solution for 1 min. After removal of the cumulus cells and corona radiata, the oocytes were washed with fresh culture medium. Those oocytes with the first polar body extruded underwent ICSI. Following ICSI, the oocytes were cultured for 16–18 h and then examined for fertilization. The fertilized [two pronuclear (2PN)] oocytes were co-cultured with Vero cell monolayers until embryo transfer.

**Co-culture**

Vero cells (CRL 1587; ATCC, USA), stored in a liquid nitrogen (LN₂) tank, were thawed in a water bath at 37°C, placed in a tube containing 5 ml fresh culture medium and centrifuged at 90 g to remove cryoprotectant. After centrifugation, the supernatants were discarded and 2 ml fresh culture medium was added and mixed well. The cell concentration was adjusted to 3–5×10⁶ cells/ml and cultured in an organ culture dish (Falcon 3037; Falcon, Franklin Lakes, NJ, USA) for 24 h. Non-adhered cells were then removed and 2 ml of fresh culture medium was added. The cells were then cultured for an additional 24 h until the formation of 70–80% monolayers.

**Biopsy of cleavage-stage embryos**

Embryos were assessed daily for further development and those reaching the 8-cell stage on day 3 were biopsied. Two blastomeres with a distinct nucleus were biopsied using acidified Tyrode’s medium (Gibco, Gaithersburg, MD, USA) as described by Cohen et al. (1990). Briefly, micromanipulation (Handyside, 1991) was carried out in dishes containing 5 µl droplets of phosphate-buffered saline (PBS) overlaid with warmed mineral oil. Embryos were stabilized with a holding pipette at 9 o’clock and a 3 µm diameter pipette containing the acidic Tyrode’s medium was orientated at 3 o’clock next to an empty perivitelline space. A 30 µm defect in the zona was created by blowing the acidic Tyrode’s medium with a mouth-controlled delivery system. Suction was applied immediately after breaching the zona to avoid excess acid entering the perivitelline space. Embryos were rinsed several times and returned to co-culture. Each biopsied blastomere was checked for the presence of a nucleus by interference contrast microscope.

Each blastomere was then carefully washed twice in 1× PCR solution (Promega, Madison, WI, USA) and transferred to 0.5 ml tubes containing 10 µl tridistilled sterilized water. The blastomeres were then twice frozen in liquid nitrogen and thawed at room temperature, overlaid with mineral oil, and heat-denatured at 94°C for 35 min. The total time for this preparation was 55 min.

**Multiplex PCR for carrier mother and affected son**

Multiplex PCR was performed using DNA extracted from peripheral blood samples of the carrier mother and her affected son for deletion detection. A total of 16 different exons of the dystrophin gene, including Pm, were tested (Table I).

DNA (100–200 ng) was placed in PCR tubes, was overlaid with one drop of mineral oil, centrifuged and placed in a 72°C heating block. The buffer master mixture was prepared according to the sample size and consisted of 2.0 µl 10× Stock buffer (Perkin Elmer, Norwalk, CO, USA), 1.8 µl 100 mM MgCl₂ (final concentration 9 mM), 1.2 µl 25 mM dNTP stock (1.5 mM each dATP, dCTP, dGTP, dTTP), 0.6 µl each primer (final concentration 0.15 µM), 2 µl 100% DMSO, 0.4 µl Taq DNA polymerase Stock (10 IU/µl, Perkin Elmer), and sterilized water to a total volume of 8 µl for each sample.

The primer stock was divided into four parts: (A) exons 48, 51, 17 and 12; (B) exons 44, 8, 19, 45 and 4; (C) Pm, exons 50, 6, 60 and 3; (D) exons 43, 47 and 52. Aliquots of 2 µl of each primer stock was pipetted into labelled tubes. Then 8 µl of buffer master mix and 10 µl of master mix was placed in each tube, mixed well, and centrifuged for 3 s. The tubes then underwent 24 cycles of denaturation (94°C) for 1 min, annealing (52°C) for 30 s, and extension (65°C) for 4 min. Finally, samples were held at 65°C for 10 min and cooled to 4°C. The sizes of the PCR products were analysed by electrophoresis on a 3% Nusieve (FMC, Rockland, USA) agarose gel at 100 V for 2 h and stained with ethidium bromide.

**Linkage analysis for carrier mother, affected son, and biopsied blastomere**

To amplify DNA from a single blastomere, two sets of primer for the highly polymorphic sites at the beginning and the end of the gene were used. The primer for the beginning of the gene was used to amplify 88–108 bp fragments.

PCR was performed on each individual blastomere in 12.5 µl of master mixture containing 2.5 µl 10× Promega buffer, 1.5 µl of each primer (forward primer sequence: TGACTAAATGTATGAGTAGT, reverse primer sequence: AATAGTTGTTTTCTAAGG, 0.6 µl Taq polymerase (5 IU/µl, Promega) and 12.5 µl dH₂O. An aliquot of 25 µl of total reaction mixture was incubated at 94°C for 4 min followed by temperature cycling (35 rounds) at 92°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final incubation at 72°C for 7 min. The sizes of the PCR products were analysed by electrophoresis on a 12% polyacrylamide (17:1) gel at 58 V for 6 h and staining with ethidium bromide. pUC/MspI was used as a marker.

The primer of the end of the gene was used to amplify differently sized products: 143, 134, 121 and 119 bp fragments. PCR was performed on each individual blastomere in 12.5 µl of master mixture containing 2.5 µl 10× Promega buffer, 1.5 µl 25 mM MgCl₂, 0.625 µl 40× dNTP stock (0.2 mM each dATP, dCTP, dGTP, dTTP), 0.625 µl of each primer (forward primer sequence: GAAAGATTGTAAAC-TAAAGTGTGC, reverse primer sequence: GATAGTTGTITTTCCTAAGG), 0.6 µl Taq polymerase (5 IU/µl, Promega) and 12.5 µl dH₂O. Aliquots (25 µl) of total reaction were incubated at 94°C for 1 min, followed by temperature cycling (25 rounds) at
Table I. Sequence of oligonucleotide primers for multiplex polymerase chain reaction (PCR)

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer (5'→3')</th>
<th>Reverse primer (5'→3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>GTCCTTTACACACTTTACCTTTGAG</td>
<td>GGCCTCA TTCTCA TGTTCTTA TTAG</td>
<td>360</td>
</tr>
<tr>
<td>17</td>
<td>GACCTTGAGTGATGTTCACCTTCCC</td>
<td>AAGCTTGAGATGCTTCACCTTTCC</td>
<td>416</td>
</tr>
<tr>
<td>19</td>
<td>TTCTACACATCTTTCTTTCCTAAG</td>
<td>CATCCTCTGACCTGTGCCCCCTAC</td>
<td>457</td>
</tr>
<tr>
<td>44</td>
<td>CTTGGAGAATTCTCTCAGAAGCTGAGA</td>
<td>GGAGA TAAAGTGA TTGGTGGAAAAATC</td>
<td>506</td>
</tr>
<tr>
<td>45</td>
<td>AAACATGGGACAAACACTCTGTTGGGAC</td>
<td>CCGAGCAGCAGAATTGGAAGAAGT</td>
<td>547</td>
</tr>
<tr>
<td>47</td>
<td>CTGAAATGTAACCTCTCATCCATACGTTA</td>
<td>TCTTCCGAAGTAAATGGCCTCCAGAT</td>
<td>535</td>
</tr>
<tr>
<td>50</td>
<td>CCACATGTAAATGTTACTACCTTACCTTTCC</td>
<td>GTGCAGGATATGCTAAATGGAATGCA</td>
<td>410</td>
</tr>
<tr>
<td>43</td>
<td>GAACATGTCAAGTACCTGACATGG</td>
<td>AATATGTTTACCTACCTTCTGGTGTC</td>
<td>357</td>
</tr>
<tr>
<td>47</td>
<td>CTGTTGTGGTTGTTTCCCCGTTTCATATGG</td>
<td>GTCTAATCTTTATCCTACCTGAGATTG</td>
<td>181</td>
</tr>
<tr>
<td>50</td>
<td>CACAAATGATTAGATGTCATGACGAT</td>
<td>TCTCTCTTACCATCCTATCATCAT</td>
<td>271</td>
</tr>
<tr>
<td>52</td>
<td>AAATTTAGATTTGCAAGAACAGGGGCTCC</td>
<td>TTTGACGCAGATATGTTGGTTCAGCTCC</td>
<td>113</td>
</tr>
<tr>
<td>60</td>
<td>AGGAGAAATGCTTCCAGTGA</td>
<td>AGGAGAGAGC TTTGGAACAGAGGCGTCC</td>
<td>139</td>
</tr>
</tbody>
</table>

Multiplex PCR

Using multiplex DNA amplification kits, 17 exons were all amplified in the DNA sample from the carrier mother and her surviving affected son. No deletion was detected in the DNA sample from the father of the embryo; his phenotype was normal. Therefore, we were unable to confirm the disease diagnosis genetically. However, we performed linkage analysis to determine carrier/affected probabilities for the preimplanted embryo (Figure 2).

Linkage analysis

As shown in Figure 3, the carrier mother has 88 and 98 bp alleles at the beginning of the gene and 134 and 143 bp alleles at the end of the gene. The surviving affected son has a 88 and a 143 bp allele, while the biopsied embryo has a 98 and a 134 bp allele.

Discussion

The first child after PGD of a single gene defect was born to a couple at risk for the cystic fibrosis (CF) ΔF508 mutation (Handyside et al., 1992). DNA amplification of selected exons using multiplex PCR followed by separation of the DNA fragments with gel electrophoresis is now used routinely for deletion detection in DMD (Chamberlain et al., 1988). In carrier women with the DMD gene deletion, specific PGD using PCR can be performed which allows the transfer of unaffected male embryos as well as of normal and carrier female embryos (Kristjansson et al., 1994; Liu et al., 1995).

Fertilized oocytes were co-cultured with Vero cells since 93.9% of embryos have been found to cleave under these conditions (K.Cha et al., unpublished data). Co-culture of oocytes with somatic cells has been reported to be beneficial

Results

Oocyte retrieval and embryo biopsy

Three oocytes were obtained but only one was fertilized after ICSI. At the 8-cell stage on day 3, two blastomeres from the embryo were successfully biopsied.
Figure 3. Linkage polymerase chain reaction (PCR). (A) 12% polyacrylamide (17:1) gel showing linkage PCR products at the beginning of the dystrophin gene. Lanes 1–3: biopsied embryo; lanes 4–6: carrier mother; lanes 7–9: surviving affected son; lanes 10–12: father. (B) 8% polyacrylamide (17:1) gel showing linkage PCR products at the end of the dystrophin gene. Lane 1: surviving affected son; lane 2: biopsied embryo; lanes 3–4: carrier mother; lanes 5–6: marker pUC/MspI; lanes 7–8: father.

Table II. Polymerase chain reaction (PCR) analysis of single human cells of the end of the dystrophin gene

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Blastomeres analysed</th>
<th>Size of amplified product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>134</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>143, 119</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>134, 121</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>134</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>143</td>
</tr>
</tbody>
</table>

Table III. Reliability of single blastomere in linkage polymerase chain reaction (PCR) of dystrophin gene

<table>
<thead>
<tr>
<th>Blastomeres analysed</th>
<th>Total no. amplified</th>
<th>5‘UTR</th>
<th>3‘UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2</td>
<td>1/2</td>
<td>1/1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0/1</td>
<td>1/1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>9</td>
<td>4</td>
</tr>
</tbody>
</table>

for the in-vitro development and implantation of embryos (Wiemer et al., 1989; Bongso et al., 1990). Using multiplex DNA amplification, ~35% of the families affected by DMD were found to have no deletion in the DNA sample and, therefore, it is impossible to confirm the disease diagnosis genetically (Clemens and Caskey, 1992). However, linkage PCR can determine carrier/affected probabilities for family members using informative intragenic and flanking markers to track the chromosome bearing the mutated gene (Ward et al., 1989).

Recently, highly polymorphic short tandem repeat (STR) sequences have proved very useful in linkage analysis. Such STR loci can be easily amplified with PCR and analysed with polyacrylamide gel electrophoresis. Among the human genome, ~50 000–100 000 (dC-dA)n • (dC-dT)n loci are present and these STR sequences are very useful (Hamada and Kakunaga, 1984; Tautz and Renz, 1984). Several of these loci are found at the beginning, at the end, and in the middle of the dystrophin gene (Beggs and Kunkel, 1990; Oudet et al., 1990; Feener et al., 1991).

We performed linkage analysis to determine carrier/affected probabilities for a biopsied embryo whose family members have no deletion in the multiplex DNA amplification. DNA polymorphisms at the dystrophin gene locus were used to distinguish the two maternal alleles in this family. Analysis of two polymorphic sites, 5‘ untranslated region and 3‘ untranslated region, was carried out. The beginning of the gene, a polymorphic (CA)14 repeat, is located ~1.5 kb upstream of the first brain-type exon of the human dystrophin gene at Xp21 (Feener et al., 1991). The end of the gene, consisting of (CA)nTA(CA)n, was identified at nucleotides 11727–11758 of the human dystrophin cDNA. Oligonucleotide primers for PCR were constructed from sequences 70 bp upstream and 31 bp downstream. The end of the gene consists of (CA)nTA(CA)n, located in the 3‘ UTR region of the human dystrophin gene Xp21 (Oudet et al., 1990).

Table II reports the results of PCR analysis of single blastomeres from donated embryos; 25 cycles were found to be successful for analyses of the end of the dystrophin gene in all cases. Table III shows the efficiency of linkage PCR when 11 blastomeres from five donated embryos were analysed in a blind test.

A family study reveals the diagnostic power of using STR markers for preimplantation diagnosis. As in the pedigree shown, the surviving brother of the biopsied embryo was diagnosed as having DMD and we have used genetic analysis to determine the affected/carryer status of the embryo. DNA polymorphisms at the dystrophin gene loci were used to distinguish the two maternal alleles in this family. Analysis of two highly polymorphic sites, at the beginning of the gene and at the end of the gene have shown that the biopsied embryo inherited the alternate maternal allele to his affected
brother. These results are 95% informative; 5% uncertainty is due to a 5% frequency of recombination across the dystrophin gene. A recombination event could separate the mutation from the polymorphic site and give a falsely negative result. This unaffected embryo was transferred 4 days after oocyte retrieval, but no pregnancy was achieved (Abbs et al., 1990; Claustres et al., 1991).

In conclusion, preimplantation diagnosis of a case of non-deleted DMD can be performed by using a rapid linkage analysis.

References

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Preimplantation diagnosis of non-deletion Duchenne muscular dystrophy