Mutation and haplotype analysis for Duchenne muscular dystrophy by single cell multiple displacement amplification

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Duchenne muscular dystrophy (DMD) is an X-linked recessive genetic disorder with mutational heterogeneity. The scarcity of DNA from single cells in preimplantation genetic diagnosis (PGD) for DMD limits comprehensive genetic testing. Multiple displacement amplification (MDA) is reported to generate large amounts of template and give the most complete coverage and unbiased amplification to date. Here, we developed mutation and haplotype analysis in conjunction with gender determination on MDA products of single cells providing a generic approach that widens availability of PGD for female carriers with varied mutations. MDA amplified with 98.5% success for single lymphocytes and 94.2% success for single blastomeres, which was evaluated on 60 lymphocytes and 40 blastomeres. A total of six commonly mutant exons, eight short tandem repeat markers within dystrophin gene and amelogenin were incorporated into subsequent singleplex PCR assays. The mean allele dropout rate was 9.0% for single lymphocytes and 25.5% for single blastomeres. None of the blank controls gave a positive signal. Genotyping of each pedigree for three families provided 2–3 fully informative alleles per dystrophin haplotype besides specific mutant exons and amelogenin. We suggest that this approach is reliable to identify non-carrier female embryos other than unaffected male embryos and reduce the risk of misdiagnosis.

Keywords: Duchenne muscular dystrophy/multiple displacement amplification/haplotype analysis/preimplantation genetic diagnosis

Introduction

Duchenne muscular dystrophy (DMD, MIM#310200) is an X-linked recessive, progressive muscle-wasting disease affecting all world populations equally, with an incidence of one in every 3500 live male births (Emery, 1993). This lethal heritable disease is caused by mutations of dystrophin gene at Xp21 spanning ~2.6 million base pairs (bp) of genomic sequence and consisting of 79 exons (Coffey et al., 1992; Monaco et al., 1992; Van Deutekom and van Ommen, 2003). This extremely large size makes the gene prone to re-arrangement and recombination which may cause various mutations. The majority of mutations are deletions of one or more exons (~60%), although duplications (~6%), translocations and point mutations (~30%) have also been found (Leiden muscular dystrophy pages, www.dmd.nl). As DMD is inherited in an X-linked recessive manner, female carriers have a 50% chance of transmitting the mutation to their offspring. Males who inherit the mutation are affected, whereas females who inherit the mutation are carriers (Den Dunnen et al., 1989).

Preimplantation genetic diagnosis (PGD) for female carriers with DMD offers an alternative to prenatal diagnosis to avoid termination of affected pregnancies and provides an opportunity for bearing healthy children. But, it remains difficult due to the enormous size of the gene, mutational heterogeneity, high intragenic recombination rate and high frequency of de novo mutations. Thus, there is no universal PGD protocol for DMD as yet. Sexing of embryos for PGD has previously been described (Handyside et al., 1989; Munne et al., 1994; Staessen et al., 1996). However, with a ‘gender-only’ selection strategy, all male embryos will be discarded even though half of these are not affected, and female embryos are transferred regardless of carrier status. Subsequently, PGD protocols were designed for direct deletion test at the single cell level, which would allow the transfer of healthy male embryos. Liu et al. (1995) performed PGD for DMD in a carrier with a deletion of exons 3–18 by using a polymerase chain reaction (PCR) assay of exon 17, resulting in a normal female singleton pregnancy. However, female embryos and healthy male embryos could not be distinguished by this strategy. Furthermore, it could not be distinguished between non-carrier female embryos and female carrier embryos. Heterozygous carrier embryos would be transferred as non-carrier female embryos. Thus, these resulting females remain at risk of such inheritable disease and have to make similar decisions for their own reproduction in future.

Haplotype analysis identifies family-specific high-risk and low-risk disease regions of the genome in individuals from families with inherited single gene defects. For those families showing no deletions but duplications, haplotype analysis would be the only current option since these mutations cannot be identified easily at single cell level. In addition, if haplotype analysis is done simultaneously for families with deletion-type mutations, it could facilitate selection of real normal female embryos. However, a series of informative markers flanking or within the dystrophin gene is required for haplotype analysis to minimize errors due to high likelihood of recombination. Lee et al. (1998) analyzed two informative polymorphic sites in single blastomeres from embryos of a DMD non-deletion carrier, but no pregnancy was achieved. Obviously, the limited number of markers...
used in these single cell PCR tests was due to very small amounts of DNA. Moreover, the need to optimize PCR conditions for efficient co-amplification of different markers at the single cell level restricts such genetic testing. Hence, with whole genome amplification (WGA) as an initial step for PGD to provide a supply of starting template, and then having more loci amplified simultaneously for extensive genetic analysis are necessary to increase the accuracy and reliability of single cell analysis.

Several methods of WGA have been developed, such as primer extension preamplification (PEP, Zhang et al., 1992), degenerated oligonucleotide primed PCR (DOP-PCR, Telenius et al., 1992). The general applicability of these methods is limited by non-specific amplification artifacts (Cheung and Nelson, 1996), incomplete coverage of loci (Paunio et al., 1996) and the small size of the DNA products (Telenius et al., 1992). Multiple displacement amplification (MDA), first proposed by Lizardi, differs from other WGA methods in not being based on PCR (Lizardi et al., 1998). This isothermal method relies upon the strand displacement amplification approach (Dean et al., 2002). It utilizes bacteriophage Φ29 polymerase of high processivity and fidelity and generates large amounts of template from small DNA samples. Thus MDA gives the most complete coverage and unbiased amplification of any WGA method reported to date (Dean et al., 2002; Hosono et al., 2003).

Our aim was to develop mutation and haplotype analysis in conjunction with gender determination on MDA products of single cells providing a generic approach that widens the scope and availability of PGD for female carriers with varied mutations of dystrophin gene.

Materials and Methods

Patients

Three couples at risk of transmitting DMD to their offspring attended our clinic hoping to achieve healthy pregnancies.

Family 1: the patient had two sons affected with DMD due to a deletion of exons 48–52 in the dystrophin gene.

Family 2: the brother and son of the patient were diagnosed as DMD with a duplication of exons 3–11.

Family 3: the patient's son had DMD with a duplication of exons 53–63. Molecular analysis showed that both the mother and her sister were carriers.

Pedigree analysis

Genomic DNA was extracted from peripheral blood of DMD families using DNA Blood Midi Kit (Qiagen, Germany). Then eight short tandem repeat (STR) markers within the dystrophin gene were used to genotype reproductive partners, their affected children and the female carriers' relatives. The allele sizes obtained from the affected children identified high-risk DMD haplotype. Markers of the two reproductive partners had different size alleles, which were fully informative with MDA products served as positive controls.

Each 25 μl reaction consisted of 2.5 μl of PCR buffer, 3 μl of 1/50 dilution of MDA products or genomic DNA, 0.2 μM of each primer set, 200 μM dNTPs, 1.5 mM MgCl2, and 1 unit of AmpliTaq DNA polymerase (Perkin Elmer, USA).

There were five different sets of amplification condition used for analysis as described elsewhere. For markers exon 8, 12, 17, 44, 48, 50 and AMEL, denaturation was carried out at 94°C for 30 s, annealing at 56°C for 1 min and extension at 70°C for 2 min. For the marker 5’DYSII, the condition was at 94°C for 1 min, 55°C for 2 min and 72°C for 3 min (Hugnot et al., 1991). For markers STR44, 45, 49 and 50, 94°C for 30 s, then 62°C for 30 s followed by 65°C for 2 min (Clemens et al., 1991). For the marker 3’CA, denaturation at 94°C for 1 min, annealing and extension at 65°C for 4 min (Beggs and Kunkel, 1990). For markers STRMP and STR79GT2, 94°C for 30 s, 56°C for 1 min and 72°C for 1 min (Renwick et al., 2006). About 25–35 cycles of amplification were undertaken for each set of primers. PCR products were run on an ABI 3100-Avant genetic analyzer.

Ethics

The present study was approved by our hospital ethics committee. Informed consents to donate discarded embryos were obtained from the patients.

Results

Pedigree analysis

Pedigree analysis for three families revealed that each patient was heterozygous for 3–5 STRs. Genotyping of each pedigree provided 2–3 fully informative alleles per dystrophin haplotype besides specific mutant exons and amelogenin (Fig. 1).

Genomic DNA from Family 1 confirmed the previous molecular diagnosis of a deletion of exons 48–52 by showing the absence of STR49, EXON 50 and STR50 in two affected boys and the absence of one allele of STR49 and STR50 in their mother as a carrier. The mother was heterozygous for markers 5’DYSII, STRMP, STR44, STR45 and STR79GT2. For the female carrier in Family 2, markers STRMP, STR44, STR49, STR50 and STR79GT2 were heterozygous, among which markers STRMP, STR44 and STR49 were fully informative. Markers 5’DYSII, STRMP and STR49 were heterozygous for the carrier in Family 3. Two fully informative markers STRMP and STR49 located at the same side of the mutant locus (Fig. 1).
Table 1: Primer sequences used for analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRMP</td>
<td>ACTCAAGGTTCCAGATATCTAC</td>
<td>CTATCTTTTTGCATTCCAGTTGAG</td>
<td>Renwick et al. (1990)</td>
</tr>
<tr>
<td>AMEL</td>
<td>CCCTGGGCTCTGTAAAGAATAGTG</td>
<td>ATCAGAGCTTAAACTGGGAAGCTG</td>
<td>Chamberlain et al. (2005)</td>
</tr>
<tr>
<td>STR44</td>
<td>TCCAACATTGGAAATCACATTTCAA</td>
<td>TCATCACAAATAGATGTTTCACAG</td>
<td>Clemens et al. (1991)</td>
</tr>
<tr>
<td>EXON44</td>
<td>CTTGATCCATATGCTTTTACCTGCA</td>
<td>TCCATCACCCTTCAGAACCTGATCT</td>
<td>Chamberlain et al. (2005)</td>
</tr>
<tr>
<td>STR50</td>
<td>AAGGTTCCTCCAGTAACAGATTTGG</td>
<td>TATGCTACATAGTATGTCCTCAGAC</td>
<td>Clemens et al. (1991)</td>
</tr>
<tr>
<td>STR79G</td>
<td>GTTGTACCCTTCTTTAAGGCTTG</td>
<td>GTTTGAGCAGCCTAGCAGATGTCC</td>
<td>Renwick et al. (1990)</td>
</tr>
<tr>
<td>STR49</td>
<td>CGTTTACCAGCTCAAAATCTCAAC</td>
<td>CATATGATACGATTCGTGTTTTGC</td>
<td>Clemens et al. (1991)</td>
</tr>
<tr>
<td>EXON48</td>
<td>TTGAATACATTGGTTAAATCCCAACATG</td>
<td>CCTGAATAAAGTCTTCCTTACCACAC</td>
<td>Chamberlain et al. (2005)</td>
</tr>
<tr>
<td>EXON50</td>
<td>CACCAAAAGTTAAGATGTTTCATGAAT</td>
<td>TCTCTCTCACCAGTCTACTCATCTATAG</td>
<td>Beggs et al. (1990)</td>
</tr>
<tr>
<td>STR50</td>
<td>AAGGTCTCTCACCAGTACAGATTTGG</td>
<td>TATGCTACATAGTATGTCCTCAGAC</td>
<td>Clemens et al. (1991)</td>
</tr>
<tr>
<td>STR79G</td>
<td>CCAACATTGGAAATCACATTTCAA</td>
<td>TCATCACAAATAGATGTTTCACAG</td>
<td>Clemens et al. (1991)</td>
</tr>
<tr>
<td>3’CA</td>
<td>GAAAGATGTTAAGATCAGTGTTG</td>
<td>GGTAGCAAAACACACGGTACCC</td>
<td>Beggs and Kunkel (1990)</td>
</tr>
<tr>
<td>AMEL</td>
<td>CCCCTGGCCTCTGTAAGAATAGTG</td>
<td>ATCAGAGCTTAAACTGGGAAGCTG</td>
<td>Nakahori et al. (1991)</td>
</tr>
</tbody>
</table>

Amplification efficiency with MDA

A total of 60 single lymphocytes (20 from each patient in Family 1, 2 and 3) and 40 single blastomeres taken from 11 embryos were subjected to MDA. MDA amplified with 98.5% success for lymphocytes and 94.2% success for blastomeres.

Consistency

For each of 11 embryos, alleles observed for blastomeres from the same embryo were completely consistent. No recombination event, chromosomal mosaicism or aneuploidy for chromosome X was found (Fig. 2).

PCR efficiency and ADO

ADO was defined as one of two alleles at a heterozygous locus failing to amplify. Zero allele at a hemizygous, homozygous or heterozygous locus was counted as amplification failure. The PCR results obtained using MDA products from single lymphocytes of DMD carriers were compared with those from DNA extracted from their blood, from which a mean amplification failure rate of 1.5% (4/260, range 0.0–2.5%) and ADO rate of 9.0% (23/256, range 5.0–12.8%) were determined (Table 2). Allele sizes matched those amplified from genomic DNA. Single blastomeres were analyzed by using 15 markers. PCR results for six specific exons on MDA products of single blastomeres were all detected (Fig. 2). The mean amplification failure rate for single blastomeres was 5.8% (35/600, range 0–15%). Amplification results were also compared among blastomeres from the same embryo and within marker panels in order to identify heterozygous loci to calculate ADO. The mean ADO rate for single blastomeres amounted to 25.5% (27/106, range 0–37.5%) (Table 2). The occurrence of ADO events in each of 11 embryos was summarized. It was interesting to find that ADO was more frequently observed in some embryos such as embryo 5 and 6 than in others (Fig. 2).

Contamination

None of the blank controls gave a positive signal in subsequent PCR assays.

Discussion

MDA amplified with 98.5% success for single lymphocytes and 94.2% success for single blastomeres in our study due to its high efficiency. PCR results on MDA products showed significant consistency in the high sequence fidelity of MDA with previous description of MDA use in WGA (Paez et al., 2004). Kristjansson et al. (1994) used PEP followed by a heminested PCR-based assay for five DMD exons (4, 8, 12, 45 and 48) as well as ZFX/ZFY for gender assignment from single lymphocytes and blastomeres and reported 93.0% in amplification efficiency. MDA as a non-PCR-based isothermal WGA method has been introduced to provide microgram quantities of high-quality DNA from small samples (Dean et al., 2002). It is based on the usage of bacteriophage λ29 DNA polymerase. The high processivity accounts for its ability to generate long DNA products with an average length of 12 kb, compared with short DNA products of only 100–1000 bp by using other PCR-based WGA such as PEP and DOP. Accurate replication results from its high fidelity, which has an error rate of only 1 in 10^{5}–10^{6}. It makes only 6-fold difference in copy number between loci, compared with 10^{5}-fold for PEP and 10^{6} for DOP (Lasken and Egholm, 2003). Therefore, the higher efficient amplification with MDA is attributed to more complete and unbiased coverage of the genome than PEP and DOP.

Nevertheless, routine use of MDA on single cells is still being evaluated. Some recent works showed levels of ADO in subsequent PCR assays varied from 0.0–60.0% (Handyside et al., 2004; Hellani et al., 2004, 2005; Burlet et al., 2006; Lledo et al., 2006; Renwick et al., 2006; Spits et al., 2006). ADO events cause interpretation difficulties, leading to misdiagnosis. As yet, the origins of ADO remain elusive and no ideal protocol has been devised to eliminate it. Hellani et al. (2005) reported first clinical application of MGD of β-thalassaemia and cystic fibrosis. ADO rate was 10.3% for β-thalassaemia and 17.9% for cystic fibrosis, respectively. Two cycles of PGD were performed for these two diseases, resulting in two pregnancies. Spits et al. (2006) optimized a single-cell MDA protocol and evaluated it on two series of 30 single lymphocytes by 22 locus-specific PCR. ADO occurred on average in 25.8% of the samples, ranging between 0.0% and 60.0%. Burlet et al. (2006) assessed different markers on MDA products of single lymphocytes, getting ADO rates between 7.0% and 34.0%. Eight PGD cycles were optimized. Some recent works showed levels of ADO in subsequent PCR assays varied from 0.0–60.0% (Handyside et al., 2004; Hellani et al., 2004, 2005; Burlet et al., 2006; Lledo et al., 2006; Renwick et al., 2006; Spits et al., 2006). ADO events cause interpretation difficulties, leading to misdiagnosis. As yet, the origins of ADO remain elusive and no ideal protocol has been devised to eliminate it. Hellani et al. (2005) reported first clinical application of MGD of β-thalassaemia and cystic fibrosis. ADO rate was 10.3% for β-thalassaemia and 17.9% for cystic fibrosis, respectively. Two cycles of PGD were performed for these two diseases, resulting in two pregnancies. Spits et al. (2006) optimized a single-cell MDA protocol and evaluated it on two series of 30 single lymphocytes by 22 locus-specific PCR. ADO occurred on average in 25.8% of the samples, ranging between 0.0% and 60.0%. Burlet et al. (2006) assessed different markers on MDA products of single lymphocytes, getting ADO rates between 7.0% and 34.0%. Eight PGD cycles were carried out for DMD family with a deletion of exons 51–53. A non-carrier female embryo was transferred with no
resulting pregnancy. In our study, the mean ADO rate was 9.0% for single lymphocytes and 25.5% for single blastomeres. Having all loci amplified individually in singleplex PCR on MDA products rather than attempting all loci in multiplex PCR might account for lower ADO rates in our results than Renwick et al. (2006) reported. Much more extensive optimizations have to be performed in multiplex PCR. These technical difficulties would not be encountered in singleplex PCR, but on the other hand, singleplex PCR would be labor-intensive, time consuming and costly. Nonetheless, it could serve as an alternative approach for multiple loci assessment from single cells since large amounts of DNA obtained using MDA are absolutely sufficient for repeatable independent PCR assays in PGD.

Figure 1: Pedigree analysis for three families (A) Family 1 with a deletion of exons 48–52, (B) Family 2 with a duplication of exons 3–11 and (C) Family 3 with a duplication of exons 53–63. Npat and Nmat correspond to paternal and maternal normal dystrophin haplotype, respectively. Del and Dup represent dystrophin deletion and duplication haplotype, respectively. Arrows show where mutations are located in each family. Markers indicated by higher case letters are fully informative for each couple.
Our data showed that ADO rates varied from locus to locus. It was relatively high for markers STR45, STR49, STR50 and STR79GT2 compared with other markers. As there is no published literature available to be referred to, the underlying reason remains unclear. Access to the target genomic sequence by the primers and Taq DNA polymerase may be restricted by, for example, adjacent G/C rich regions or different degrees of folding perhaps related to the stage of the cell cycle (Ray and Handyside, 1996). If such markers are to be used for analysis in PGD cycles, interpretation of PCR results has to be cautious. Moreover, a combination of other markers should be kept in mind.

We also found that ADO events were observed more frequently in dissociated blastomeres from embryo 5 and 6 than in others. Since these two embryos arrested at the 4-cell stage, they may be undergoing apoptosis. Although each blastomere had a visible single nucleus under inverted stereomicroscope, the nuclei might be abnormal and these degenerating or degenerated cells suffered from different degrees of DNA degradation, which would be responsible for poor PCR amplification (Cui and Matthews, 1996). Consequently, selection of a nucleus with good characteristics (i.e. a clear and regular nuclear membrane, transparent nucleoplasm and prominent nucleoli), which indicates a living cell with integrity of the DNA structure, is extremely crucial for the precision of preimplantation diagnosis (Cui and Matthews, 1996). In addition, with only two target molecules present in a single diploid cell, ADO could arise from DNA damage or incomplete cell lysis during preparation. The reduction of ADO rate observed with an alkaline lysis buffer could partly reflect protection from, for example, endogenous nucleases (Ray and Handyside, 1996).

We applied direct deletion test and haplotype analysis to pedigree analysis for Family 1. The absence of one allele of STR49 and STR50 confirmed carrier status in their mother as the familial deletion involved in these two loci. Therefore, it is obvious that, for informative families, haplotype analysis is able to identify non-carrier female embryos for transfer in PGD for DMD carriers with deletion-type mutations. In addition, when direct deletion testing is undertaken simultaneously, it not only helps to avoid misdiagnosis by detection of the possible occurrence of two or more recombination events (though the incidence is low) but also allow confirmation of diagnosis. Here, we have to point out that female carrier embryos are regarded as healthy ones to be transferred routinely in most reproductive centers. In our clinic, it is the couple who will make decisions with regard to the transfer of carrier embryos after being informed extensively about its advantages and limitations even in the case if there are only carrier embryos among the embryos available after PGD. The couples’ agreements are confirmed by written informed consents.

Considering duplications could not be easily identified at single cell level, only haplotype analysis was done for Families 2 and 3. For Family 2, since three fully informative markers flanked the mutant locus, recombination events between mutant locus and linked

### Table 2: Results of PCR efficiency and ADO in single lymphocytes and blastomeres

<table>
<thead>
<tr>
<th>Locus</th>
<th>Lymphocytes (n = 60)</th>
<th>Blastomeres (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplification failure (%)</td>
<td>ADO&lt;sup&gt;a&lt;/sup&gt; (%)</td>
</tr>
<tr>
<td>5’DYSII</td>
<td>1/40 (2.5)</td>
<td>3/39 (7.7)</td>
</tr>
<tr>
<td>STRMP</td>
<td>1/60 (1.7)</td>
<td>5/59 (8.5)</td>
</tr>
<tr>
<td>STR44</td>
<td>1/40 (2.5)</td>
<td>4/39 (10.3)</td>
</tr>
<tr>
<td>STR45</td>
<td>0/20 (0.0)</td>
<td>1/20 (5.0)</td>
</tr>
<tr>
<td>STR49</td>
<td>0/40 (0.0)</td>
<td>4/40 (10.0)</td>
</tr>
<tr>
<td>STR50</td>
<td>0/20 (0.0)</td>
<td>1/20 (5.0)</td>
</tr>
<tr>
<td>STR79GT2</td>
<td>1/40 (2.5)</td>
<td>5/39 (12.8)</td>
</tr>
<tr>
<td>3’CA</td>
<td>5/40 (12.5)</td>
<td>1/40 (2.5)</td>
</tr>
<tr>
<td>AMEL</td>
<td>3/15 (20.0)</td>
<td>0/3 (0.0)</td>
</tr>
<tr>
<td>Total</td>
<td>4/260 (1.5)</td>
<td>23/256 (9.0)</td>
</tr>
</tbody>
</table>

<sup>a</sup>ADO rates were calculated on heterozygous cells.

<sup>b</sup>Missing data were due to unavailability of patients who were heterozygous for this marker.

<sup>c</sup>Marker AMEL was not amplified on MDA products of single lymphocytes.
markers could be detected, then the misdiagnosis rate would be low except for occurrence of more than one recombination events. For family 3, as only two markers located at the 5' and central region were fully informative for construction of haplotype, the chance of misdiagnosis was 5.0% due to recombination events with an estimated frequency of 10.0% between both ends of the dystrophin gene (Abbs et al., 1990). This family should be included in a PGD cycle only if more markers in the 3' region are analyzed, to avoid misdiagnosis.

In conclusion, with the protocol described here, PGD can be applied to the majority of female carriers with varied mutations of dystrophin gene to get accurate results. First, it is reliable to identify non-carrier female embryos other than unaffected male embryos. Secondly, this assay allows diagnosis for most couples (e.g. non-deletion carriers), as it is possible to follow the segregation of the mutant allele. Thirdly, this test reduces the risk of misdiagnosis by the use of linked markers and can also detect possible contaminations. Fourthly, additional markers (microsatellites, etc.) can easily be added to the PCR reaction in the case of 'non-informative' couples. In addition, the co-amplification of amelogenin sequences enables embryonic gender determination simultaneously with the direct and indirect analyses.

Acknowledgements

We thank Dr Tao Li and Minfang Zhang for providing the donated embryos for analysis, Prof. Guanglun Zhuang and Dr Zexu Jiao for experimental design advices and are grateful to all patients and their families for their collaboration.

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Submitted on February 16, 2007; resubmitted on March 1, 2007; accepted on March 8, 2007.