—Mini Review—

Preimplantation Diagnosis of Duchenne Muscular Dystrophy

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Abstract: *Duchenne muscular dystrophy (DMD), caused by mutations of the dystrophin gene, is a severe X-linked recessive neuromuscular disorder. Preimplantation diagnosis of DMD includes three approaches. The first approach is gender determination of embryos by either polymerase chain reaction (PCR) or the fluorescence in situ hybridization (FISH)-based method. While each method is well established, the FISH method has some advantages over PCR in gender determination. The second approach is diagnosis of specific gene mutation. The partial deletions are diagnosed by the PCR with primers constructed to amplify the deletion exons. The partial duplication cannot be detected by now available strategies. The small mutations can be diagnosed by the specific PCR based assay. The third approach is linkage analysis by means of linked markers. CA repeats have been shown to be highly polymorphic and to be of great diagnostic utility because they can be easily assayed by PCR.*

Key words: *Preimplantation genetic diagnosis, Duchenne muscular dystrophy, Polymerase chain reaction, Dystrophin, Linkage analysis*

Preimplantation genetic diagnosis (PGD) is a new technique that allows the identification of unaffected embryos of high risk couples prior to implantation. Embryos are obtained through *in vitro* fertilization (IVF), and their disease status is determined by DNA analysis of one or two blastomeres biopsied from embryos. Only the unaffected embryos are replaced into the uterus, therefore avoiding the risks associated with affected pregnancies and with artificial abortion. This technique offers a reproductive choice to families who find termination of pregnancy unacceptable, or who have already had several terminations after prenatal diagnosis [1–5]. The first PGD was polymerase chain reaction (PCR) based-gender determination for X-linked disorders and this led to the birth of several normal girls [6]. The first PGD for a single gene disorder was done for couples at risk of transmitting cystic fibrosis (cystic fibrosis ΔF-508 mutation) [7]. A similar approach is possible for Tay-Sacks disease, Lesch-Nyhan syndrome, hemophilia A, α-1-antitrypsin deficiency, and retinitis pigmentosa and an unaffected child has been born [1–3].

Duchenne muscular dystrophy (DMD) is a severe X-linked recessive neuromuscular disorder that affects about 1 in 3,500 live born males [8]. The number of DMD patients in Japan is estimated to be about 7,000 and the number of newborn patients is estimated to be about 150 per year [9]. It usually appears in childhood about the middle of the first decade of life. The onset of symptoms is gradual. A child which has previously been normal begins to walk clumsily, tends to fall, and has difficulty in getting up unaided. During the second decade of life, ambulation is lost. The patients die of respiratory failure or cardiac failure at about the age of twenty [8].

DMD is caused by mutations of the dystrophin gene [10–12]. Muscle dystrophin is absent or noticeably deficient in DMD muscle [13]. In 1987, dystrophin cDNA was cloned from muscle mRNA [10] and the complete coding sequence of the dystrophin gene made mutation analysis possible. Mutations of the dystrophin gene include partial deletions, partial duplications, and small mutations [8]. In the first PGD for identifying the dystrophin gene mutation, six embryos were diagnosed at the cleavage stage after intracytoplasmic sperm

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injection (ICSI). Four of these embryos appeared to be unaffected, 3 of them were transferred to the uterus, and an unaffected non-carrier female was born [14]. Since this assay is only available for those families carrying exon 13–18 deletions, assays to detect the full range of mutations are needed. While there are several diagnostic techniques in routine prenatal diagnoses, this paper will concentrate on more specialized techniques that are applied to preimplantation diagnosis of DMD.

The Dystrophin Gene and Its Mutations

DMD is caused by mutations of the dystrophin gene at Xp21. The dystrophin gene is about 2,300 kilobases (kb) in size, or about 1% of the entire X chromosome, and consists of more than 79 exons [15]. The dystrophin mRNA encodes a 3,685 amino acid protein of 427 kilodaltons (kDa) with overall similarity to the cytoskeletal proteins β-spectrin and α-actinin [10–12, 16]. Muscle dystrophin is present on the surface plasma membrane in normal and absent or markedly deficient in DMD muscle [13].

Large gene rearrangements

The majority of mutations are partial deletions (50–60%) or partial duplications (0–14%) [10, 15, 17, 18]. These mutations that shift the mRNA translational reading frame soon result in leading a stop codon. The frameshifting leads to the synthesis of a truncated dystrophin molecule whose carboxy-terminus is missing, resulting in impaired membrane attachment of dystrophin, severe dystrophin deficiency, and the Duchenne phenotype (Reading frame theory) [19]. The partial deletions (absence of a DNA segment) and partial duplications (addition of a duplicated DNA segment) are distributed unevenly, with hot spots occurring where the introns are particularly long [10, 11, 15]. Moreover, the distribution of deletion breakpoints is similar to the distribution of recombination events in the gene in healthy individuals, suggesting that the two phenomena share a common mechanism [8]. The stretch between the hot spots is a cold spot with relatively few deletions, and mutations beyond exon 55 of the gene are rarely detected [8]. The location and extent of the deletions in Japanese DMD patients are similar to those of other races [18].

The partial deletions are detected either by Southern blot analysis with dystrophin cDNA probes [10, 17, 18] or by the PCR with primers constructed to amplify the deletion-prone exons of the gene [20, 21]. Beggs *et al.* described oligonucleotide primer sequences that can be used to amplify 8 exons plus the muscle promotor of the dystrophin gene in a single multiplex PCR, which are able to detect 98% of the partial deletions diagnosed by the Southern blotting [21].

The partial duplications can be detected by quantitative Southern blot analysis with dystrophin cDNA probes [18]. The PCR strategy can be exploited by performing the assay quantitatively to enable the detection of duplication in males by observing a 2:1 ratio in the amount of PCR product generated from a duplicated locus compared with that from a normal single copy locus. The PCR should be restricted in the number of cycles performed so that assay remains the linear phase of amplification. The products of PCR are then assayed with a system that is able to quantitate the amount of products [22].

Small mutations

About 30% of DMD patients have no large gene rearrangements. Presumably, the deficiency is caused by small mutations (single base changes, microdeletions, or microinsertions). Because of the enormously large size of the gene, the small mutations are more difficult to identify, require special approaches, and must be distinguished from harmless polymorphisms [8, 23–26]. At least 16 small mutations in the dystrophin gene have been uncovered. Ten cases of nonsense mutation [23, 24, 26–29], 3 cases of single base deletion [25, 28, 30], one case of single base insertion [25], one case of donor site mutation [28], and one case of missense mutation [27] were reported.

Linkage Analysis and Polymorphic Markers

Linkage analysis

Most of the inherited diseases are caused by unknown mutations. In some instances, these disorders can be diagnosed indirectly by using markers for the mutant gene. These tags are variations in DNA that do not code for protein but have been demonstrated to be close to or linked to the gene of interest. This type of analysis can succeed only if family members are heterozygous for the markers employed, if markers associated with the disease allele can be identified, and if a sufficient number of markers within and flanking the gene are available to obviate the effects of possible intragenic recombinations. Numerous family members are needed to determine the form of the marker that segregates with the mutant gene in that family. The distance between the marker and the

mutant gene limits the accuracy of indirect DNA diagnosis. For each distance of 1 million bp between the marker and gene, there is a 1% chance of recombination during each meiosis, which can render the results incorrect.

Dinucleotide repeats

Simple dinucleotide sequence repeats, such as CA repeats, have been shown to be highly polymorphic and useful in linkage analysis. These repeats may occur adjacent to the coding regions of genes, in introns within genes, or within untranslated regions. There are approximately 50,000–100,000 CA repeats in the human genome. In addition, these CA repeats are of great diagnostic utility because they can be easily assayed by PCR [8].

There are over thirty CA repeat type polymorphic markers available that lie in or flanking the dystrophin gene [22]. CA repeat has been described in the 3' untranslated region and in the 5' terminus of the dystrophin gene. Beggs *et al.* determined allele frequencies and measures of variation for four CA repeats identified within a deletion-prone region of the dystrophin gene (these four being located in introns 44, 45, 49 and 50 of the dystrophin gene: STR-44, STR-45, STR-49, STR-50) [31]. Feener *et al.* have demonstrated new polymorphic markers for the 5' terminus of the dystrophin gene. The two most polymorphic markers, 5'DYS-I and 5'DYS-II, are located just upstream of the transcriptional start site for the brain promoter, making them true 5' flanking markers. 5'DYS-III and 5'DYS-IV map within the first intron, located 3.5 and 4.2 kb 3' to the transcriptional start site, respectively [32].

Restriction fragment length polymorphisms

Restriction fragment length polymorphisms (RFLPs) are inherited variations in the DNA sequence that result in the gain or loss of a site recognized by a restriction endonuclease or in alteration of the number of nucleotides between such sites. These RFLPs, usually detected by Southern blotting, are widely used in linkage analysis.

Roberts *et al.* described a *Mae* III polymorphism within 30 kb of the promoter region of the dystrophin gene, by means of amplification and mismatch detection analysis followed by direct sequencing [33]. Walker *et al.* characterized the normal cDNA hybridization pattern for *Taq* I and arranged the exon containing fragments into a partial *Taq* I map. The cDNA probes 2b-3, 5b-7-a, 8, 10, and 11-14-a each identified a *Taq* I RFLP, making a total of five cDNA RFLPs which are detected with this enzyme, spanning the length of the dystrophin gene [34].

Approach to Preimplantation Diagnosis of DMD

Preimplantation diagnosis of DMD includes three approaches. The first approach is gender determination of embryos by either PCR or a fluorescence *in situ* hybridization (FISH)-based method [1, 3–5]. The second approach is diagnosis of specific gene mutation, which is attempted by a PCR-based method [14, 35, 36]. The third approach is linkage analysis by means of linked markers [35].

Gender determination

For couples at risk of transmitting DMD, of which the underlying causes and gene mutations are not yet known, the only available option for avoiding affected fetuses prior to pregnancy is the identification of female embryos. But gender determination and the transfer of only female embryos is not optimal, as the unaffected male embryos cannot be considered for transfer. Furthermore, the disease may manifest in females who have Turner syndrome, a structurally abnormal X chromosome, or failure of inactivation of the mutation-bearing X chromosome. At present, two methods for gender determination use the FISH or PCR method.

The FISH method is well established and uses directly labeled probes for sex chromosomes and autosomes [42]. The FISH method has some advantages over the PCR method in gender determination. Firstly, the FISH method can detect aneuploidies since the sex chromosomes and autosomes are visualized. Secondly, FISH techniques are not ridden with problems such as contamination and allele dropout (ADO).

PCR-based gender determination has been used to identify both the gene mutation and gender simultaneously. PCR methods include the co-amplification of X- and Y-specific repeat sequences [37, 38], as well as the co-amplification of related genes on both chromosomes with the same primers, including those for amelogenin and amelogenin-like-sequences [39], the steroid sulphatase gene [40], and ZFX/ZFY sequences [41]. The advantage of strategies that use the same primers to amplify sequences on both chromosomes is that erroneous gender determination caused by the inability of certain primer pairs to amplify existing sequences (primer specific amplification failure) may be prevented. Since the target sequences of X/Y

co-amplification are derived from single copy genes, however, nicking of target sequences or priming failure at the beginning of the PCR may easily lead to amplification failure.

Mutation analysis

The majority of mutations are large gene rearrangements (partial deletions or partial duplications) and about 30% of mutations are presumably caused by small mutations (single base changes, microdeletions, or microinsertions). For single cell analysis, a highly efficient and accurate PCR assay is required. The PCR-based method should utilize a single cell as template DNA, therefore easily leading to misdiagnosis or an inconclusive result caused by amplification failure or DNA contamination. It is possible that the amplification success rate may be dependent on primer design, PCR conditions, sequences of target locus, or cell type.

The partial deletions are diagnosed by the PCR with primers constructed to amplify the deletion exons of the gene [20, 21]. A specific PCR assay for identifying embryos carrying a deletion of exon 13 to 18 was developed by Liu *et al*. ICSI was performed in metaphase oocyte from the carrier, and the resulting embryos were sampled at the cleavage stage. Of six embryos tested, four appeared to be unaffected, and three were transferred, resulting in a singleton pregnancy and an unaffected non-carrier female was born. This assay is available only for families whose mutation is exon 13-18 deletion. Therefore, several different assays to detect a full range of deletion mutations that would be present in different families are required. To make a diagnostic test widely available for PGD candidates, we have developed a PCR-based assay amplifying sequences derived from exons 8, 44, 45, 50 and 51, which was found to detect about 78% of c-DNA detectable deletions in Japanese patients with DMD. While we were able to amplify sequences derived from exons 8, 44 and 50 from as little as 0.1 ng of genomic DNA at an annealing temperature of 52°C and a MgCl2 concentration of 1.5 mM, the same sensitivity was achieved in an assay for exons 45 and 51 at an annealing temperature of 56°C. In our single cell analysis, we found the amplification success rate to be 80%. Our results are in agreement with those of Holding *et al.*, who described amplification success rates of 67–89% in a single cell deletion analysis of the dystrophin gene, and 97–100% in two cell analysis, and suggested two blastomeres would be necessary and sufficient for reliability PGD [35]. High amplification efficiency of exon 17 was reported by Liu *et al.* to be almost 100% in 50 blastomeres [14].

The partial duplications can be detected by quantitative PCR analysis by observing a 2:1 ratio in the amount of PCR product generated from a duplicated locus compared with that from a normal single copy locus. The PCR should be restricted in the number of cycles performed so that the assay remains the linear phase of amplification. The PCR products are then assayed with a system that is able to quantitate the amount of products [22]. Since DNA analysis should be done using only one or two blastomeres biopsied from embryos, this quantitative PCR strategy cannot be applicable to PGD, or it is impossible to detect embryos with partial duplication by now available strategies.

At least 16 small mutations in the dystrophin gene have been uncovered. Ten cases of nonsense mutation, 3 cases of single base deletion, one case of single base insertion, one case of donor site mutation, and one case of missense mutation were reported [23–30]. Once a mutation is identified in a family, a specific PCR based assay can be designed and the products can be analyzed by (1) PCR-RFLP; (2) PCR-single strand conformation polymorphism (PCR-SSCP); (3) PCR-Allele specific oligonucleotide (PCR-ASO); or (4) PCR-direct DNA sequencing. These molecular genetic techniques may be applicable to PGD.

Linkage analysis

The genetic condition of embryos can be diagnosed indirectly with polymorphic markers such as CA repeats or RFLPs. CA repeats have been shown to be highly polymorphic and to be of great diagnostic utility because they can be easily assayed by PCR [8]. There are over thirty CA repeat type polymorphic markers available that lie in or flanking the dystrophin gene [22]. STR50 is a CA repeat in intron 50 with a heterozygosity of 71.6%, and is thus of value as a marker for PCR contamination as well as for linkage analysis. Amplification success rate of STR50 in a single buccal cell was 67% [35].

Conclusions

We have presented three approaches to preimplantation diagnosis of DMD. The first approach is gender determination by either PCR or the FISH-based method. The second approach is diagnosis of specific gene mutation, which is attempted by the PCR-based method. The third approach is linkage analysis by means of linked markers. Further technical

development is required to raise the sensitivity and accuracy of the deletion detection assay. Deletion detection assays that cover the full length of the dystrophin gene, other mutation detection assays and linkage analysis in a single cell are also required.

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