Prenatal Diagnosis of Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused by mutations in the dystrophin gene, resulting in progressive muscle wasting. DMD is the most common lethal disease, affecting one in 3500 newborn males. One third of DMD cases are caused by a new mutation. Prenatal diagnosis is important in the detection and the prevention of DMD.

The isolation of the entire dystrophin gene and the identification of its protein product, dystrophin have led to the development of molecular genetic diagnosis. Prenatal diagnostic approach applied to DMD families consists of (I) DNA analysis of the dystrophin gene using a combination of multiplex polymerase chain reaction (PCR), Southern blotting with cDNA probes, restriction fragment length polymorphism (RFLP) and dinucleotide repeat length polymorphism-linkage analysis and (II) Analysis of the dystrophin protein in a fetal muscle biopsy sample by immunoblot and immunofluorescence. Each diagnostic method has its advantages, limitations and pitfalls and is best used under specific circumstances.

We present some exemplary patients in which prenatal diagnosis has been accomplished by these techniques and a diagnostic approach is outlined in the management of DMD families.

Key Words: Prenatal diagnosis, Duchenne muscular dystrophy, Multiplex polymerase chain reaction, Restriction fragment length polymorphisms, Dystrophin, Fetal muscle biopsy

When a pregnant woman with a family history of "muscular dystrophy", makes enquiry about the feasibility of early prenatal diagnosis, it is essential to know what type of dystrophy it is and what is its mode of inheritance before genetic risks or prenatal tests are discussed in detail. Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused by mutations in the dystrophin gene, which is located on the short arm of the X chromosome at Xp21. Becker muscular dystrophy (BMD) is a clinically milder form of DMD, resulting from mutations of the same gene.

DMD is the most common, affecting one in 3500 newborn males, and the most severe, resulting in progressive muscular wasting, which becomes clinically evident before the age of 5 yrs, and leads to a wheelchair confinement around the age of 12 yrs. The affected children die from respiratory failure in early twenties. DMD carries high recurrence risks within a family. It is maintained in the population by a high mutation rate of one in 104 because one third of DMD cases are caused by a new mutation. Coupled with the limitations of therapy at the present time, prenatal diagnosis is important in the detection and the prevention of DMD.
The isolation of the entire dystrophin gene\(^6\) and the identification of its protein product, dystrophin\(^7\) have led to the development of molecular genetic diagnosis. The dystrophin gene is 2.4 Mb in size, the largest gene and comprises 79 exons encoding a 14kb mRNA,\(^{(6,9)}\) which is then translated into the dystrophin protein, containing about 3685 amino acids.\(^8\)

Mutations in the dystrophin gene are due to either intragenic deletions,\(^6\) insertions\(^10\) or point mutations of nucleotides.\(^11\) About 65% of Duchenne patients have deletions of one or more exons in the dystrophin gene with preferential distribution around the middle of gene (exons 45-52), or near the 5' end (exons 3-19).\(^{6,12-14}\) Less than 5% have duplications.\(^{6,14}\) The remainder are due to point mutations of nucleotides.

Prenatal diagnostic approach applied to DMD families consists of (I) DNA analysis of the dystrophin gene by direct demonstration of the gene mutation, and indirect gene tracking, and (II) Analysis of the dystrophin protein in a fetal muscle biopsy sample by demonstration of a reduced or absent protein product using immunoblot and immunofluorescence technique.

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**Fig. 1.** Multiplex PCR based deletion screening for prenatal diagnosis of DMD. Multiplex PCR analysis of 19 exons of the dystrophin gene shows no deletion in patient 1 and missing band (□) corresponding to deletions in lane 3 and 5 in patient 2.

**Fig. 2.** Linkage analysis for prenatal diagnosis of DMD. RFLP with alleles 1 and 2 on X chromosome was detected by intragenic DNA probe with Mae III restriction endonuclease. The grandmother (Ib) is a carrier and allele 1 (236 bp fragment) indicates the mutant gene as it was inherited by the affected sons (IIb and IIc). Grandmaternal allele 1 segregates with dystrophy gene, indicating that the male fetus is predicted to be affected with 95% accuracy.
Fig. 3. Linkage analysis for prenatal diagnosis of DMD. The consultand is Ile for prenatal diagnosis. Of 5 linked markers, two DNA probes (pERT87-15/Bam HI and pERT84/MaeIII) detected polymorphic restriction sites in Ila and Ile. Ila is a carrier and alleles 2 indicates mutant genes. This male fetus (IIlc) is predicted to be unaffected as its gene is different from mutant genes of cousins (IIIa and IIIb). His normal status was confirmed postnatally.

Fig. 4. Flow chart for the prenatal diagnosis of DMD.

Direct detection of the gene mutation.
Direct demonstration of the gene mutations can be made from CVS or amniocytes\(^{(15)}\) by polymerase chain reaction (PCR)-based deletion screening\(^{(16,17)}\) and/or Southern blot-based deletion / duplication screening.\(^{(6)}\) In affected males, PCR can identify up to 98% of all deletions by amplifying 18 exons,\(^{(17)}\) which were chosen to include the two deletion "hot spots".

The primary advantages of PCR over Southern blotting are speed (1-2 days compared to 1-2 weeks to provide the results) and sensitivity (as little as one tenth of the amount required for Southern blot).

Many laboratories do not screen routinely for duplications. But the duplications can be detected by quantitative Southern blot or quantitative PCR. For accurate carrier detection of deletions and duplications, gene dosage analysis with automated fluorescence sequencing machines may be necessary\(^{(18-20)}\) because the normal X chromosome masks any deletion present on the other X. Given the size of the dystrophin gene, point mutation screening for single base changes is more difficult and not routinely performed.

Multiplex PCR analysis of 19 exons of the dystrophin gene shows no deletion in patient 1 and missing bands corresponding to deletions in lane 3 and 5 in patient 2. (Fig. 1)

Indirect mutant gene tracking (Linkage analysis)
If a deletion or duplication mutation can not be found in the patient, then prenatal diagnosis must be carried out by indirect gene tracking (gene linkage) through a family using DNA polymorphisms (genetic variations in non-coding DNA sequences) as linked markers (DNA probes) within or flanking the dystrophin gene on a Southern blot\(^{(21,22)}\) or PCR-based assays.\(^{(23)}\) By comparing the polymorphic pattern of a patient with the patterns of both of his parent, one can identify whether a patient has inherited a chromosome carrying a mutant gene or normal gene.

There are over 20 intragenic polymorphisms in non-coding DNA sequences of the dystrophine gene, ranging from restriction fragment length polymorphisms (RFLPs) with two alleles\(^{(21-24)}\) to microsatellite,
dinaucletide, CA repeat polymorphisms.\textsuperscript{(25-29)}

The results of indirect analysis are conditional on no recombination between the DNA polymorphism (marker) and the gene mutation site. Recombination between the two sites during meiosis may separate the loci, so that absolute prediction about transmission of the mutation from parent to child can not be made from linkage analysis. In family studies recombination frequency across the dystrophin gene is about 12%. 5% recombination is observed even with the intragenic markers and prediction of genetic state can therefore be made with 95% accuracy.\textsuperscript{(30,31)}

5 linked markers (pERT87-8/TaqI, pERT87-15/BamHI, pERT87-15/XmnI, pERT84/MaeIII, exon48/Msel) are used for RFLP for prenatal diagnosis of DMD in our lab.\textsuperscript{(32)} Of which these 3, pERT87-15/BamHI, pERT87-15/XmnI, pERT84/MaeIII showed high heterozygosity in a normal Korean population.\textsuperscript{(33, 34)}

Fig. 2 shows the way linkage analysis is used for prenatal diagnosis. RFLP with alleles 1 and 2 on X chromosome was detected by intragenic DNA probe with MaeIII restriction endonuclease. The grandmother (Ib) is a carrier and allele 1 (236 bp fragment) indicates the mutant gene as it was inherited by the affected sons (IIb and IIc). Grandmaternal allele 1 segregates with dystrophy gene, indicating that the male fetus is predicted to be affected with 95% accuracy.

This emphasizes the need to bank DNA samples from affected persons even if a prenatal diagnosis is not imminent as the DNA can be stored for prolonged periods prior to analysis.

Fig. 3 is another example of linkage analysis with a rather complicated family pedigree. The consultand is IIe for prenatal diagnosis. Of 5 linked markers, two DNA probes (pERT87-15/BamHI and pERT84/MaeIII) detected polymorphic restriction sites in IIa and IIe. IIa is a carrier and alleles 2 indicates mutant genes. This male fetus (IIlc) is predicted to be unaffected as its gene is different from mutant genes of cousins (IIIa and IIIb). His normal status was confirmed postnatally.

The need for typing multiple family members, and the possibilities of recombination and uninformative genotypes are disadvantages of linkage analysis.

**New mutations and mosaicism in DMD**

New mutations and mosaicism raise special problems in the DNA based diagnosis of DMD. 1/3 of DMD cases are new mutations. Therefore the mother of an isolated DMD boy has only a 2/3 chance of being a carrier. Moreover, the first mutation carrier in a DMD pedigree is very often a mosaic (male or female). It has been demonstrated that where the mother of an affected male has been shown not to be a carrier by any one of the direct detection methods available using somatic material, she still has a 5% chance of having another affected child.\textsuperscript{(35,36)} Therefore, the mother of an affected male can never be told she is definitely not a carrier and still offered prenatal diagnosis to guard against the possibility that she may be germline mosaic. New mutations, mosaicism, recombination and uninformative result are the indications of fetal muscle biopsy for the analysis of the dystrophin protein.

**Table 1. Dystrophin Assay**

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<th>Western Immunoblot % of dystrophin quantity</th>
<th>Immunohistochemistry Immunofluorescence staining</th>
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Detection of a reduced or absent dystrophin protein.

When DNA analysis fails to detect mutations of the dystrophin gene or yield an uninformative genotype, analysis of the dystrophin protein, which requires a fetal muscle biopsy, can be performed by immunoblot (IB)\(^{(37)}\) or immunofluorescence (IF)\(^{(38)}\) using antibodies directed against different regions of the protein product. (Table 1)

Dystrophin is a 427 KD, muscle-specific protein product\(^{(7)}\) of the DMD/BMD gene, consisting of 4 domains: amino-terminal, mid rod, cysteine-rich and carboxy terminal domain.\(^{(8)}\) Dystrophin is located at the internal face of the muscle fiber plasma membrane\(^{(38-41)}\) and acts as a structural cytoskeletal protein.\(^{(8,42,43)}\)

Dystrophin Immunoblotting

Almost complete absence (less than 3%) of dystrophin is predictive of DMD (>99% accuracy). Regardless of the protein size, dystrophin levels between 3% and to 10% of normal correlate with an intermedate phenotype (mild DMD or severe BMD), whereas levels greater than 20% correlate with a mild or moderate BMD phenotype (>95% accuracy).\(^{(37)}\) IB appears superior for the prognostic diagnosis of BMD\(^{(44)}\) and for the identification of many BMD carriers by their double-band pattern because their muscle often contains both normal and abnormally sized dystrophin.\(^{(45)}\) The test does not appear suitable for prenatal diagnosis or for the detection of symptom-free DMD carriers because it is not capable of detecting the small reductions of dystrophin levels in most DMD carrier females.

Dystrophin Immunohistochemistry

IF appears suitable for prenatal diagnosis because IB requires a relatively large amount (5 mg) of muscle tissue, whereas IF is accurate with as few as a half dozen muscle cells and most useful for the detection of DMD carriers although quite useful for DMD and BMD in males.

Continuous strong immunostaining of the plasma membrane in normal muscle\(^{(38-40)}\) and other muscular dystrophies,\(^{(46)}\) no staining in DMD, partial (patchy) faint staining in BMD\(^{(38,40,44)}\) and mosaic pattern-patches of negative fibers among positive fibers-owing to X chromosome inactivation in female DMD carriers\(^{(46)}\) were observed in muscle biopsy samples.

Since the first report of a fetal muscle biopsy for the diagnosis of DMD by Evans et al in 1991,\(^{(47)}\) less than 20 cases have been reported in the literature.\(^{(48,49)}\) In 1994, Evans reported the collaborative experience of US-guided fetal muscle biopsy between 18 and 23 gestational week.\(^{(50)}\) The muscle sample was always taken from the gluteal region in 11 of 12 (92%) cases, using a Klear Kut biopsy gun. Spontaneous abortion after the procedure occurred in 2 of 12 (17%) cases. Laboratory diagnoses using one IB and 10 IF were possible on small samples and 4 of 12 fetuses (33%) were affected with TOP. Repeat biopsies were performed in 2 cases.
Further experience is needed, not only to determine the best instrument for fetal muscle biopsy, but also to determine the most appropriate site and gestation for its performance. Preliminary postmortem examinations have shown that dystrophin analysis might also be possible by 15 weeks, but not much earlier.(51)

**Recommended protocol for molecular diagnosis of DMD**

For the prenatal diagnosis of DMD, DNA analysis is performed from CVS or amniocytes, first by PCR, and then by Southern blot if PCR did not detect a deletion. In cases without detectable mutations, linkage analysis must be done. When linkage analysis is uninformative or unavailable especially with sporadic case, fetal muscle biopsy will need to be considered for dystrophin immunohistochemistry. It should be remembered that this flow chart is based on the assumption of the firm diagnosis of DMD in families.(Fig. 4)

In conclusion, this review summaries current understanding of the genetics and biochemistry of DMD and gives an overview of the molecular diagnostic tools and their applications. Close collaboration between obstetricians and clinical geneticists should help to minimize the very real problems in genetic counselling and in the management of pregnancies at risk for this group of disorder.

**References**

Duchenne muscular dystrophy gene product localized in sarcrolemma


