Molecular genetics analyses of the muscular dystrophies in the Hungarian affected families

Ph.D. thesis

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Introduction

Muscular dystrophies are a genetically heterogeneous group of degenerative muscle disorders, characterized by the progressive loss of muscle strength and integrity. The muscular dystrophies are varying in age of onset, degree of severity, mode of inheritance and the muscle groups that are primarily affected. There is marked clinical similarity among patients with genetically different diseases, suggesting that many of the gene products may participate in a common functional structure or biochemical cascade. Duchenne/Becker muscular dystrophy (DMD/BMD) is a lethal/severe X-linked recessive disorder affecting 1 in 3500 (DMD) and 1 in 30 000 (BMD) male births. DMD is generally noticed between the ages of 2 and 5 years. Affected children do not walk until 18 months of age or later and often have a waddling gait and difficulty in climbing stairs with an early involvement of the proximal musculature. Confinement to a wheelchair is inevitable by the age of 12 years due to severe muscle atrophy and weakness. Progressive weakness of the diaphragm and intercostals muscles eventually leads to fatal respiratory insufficiency. The allelic BMD is similar to DMD, but with a later manifestation of symptoms related to muscle defects, sometimes referred as myalgia and

cramps. The clinical course is milder and patients are often able to walk into adulthood.

The DMD/BMD diseases are caused by mutations in the dystrophin gene located on Xp21.2, which encodes a protein of the membrane cytoskeleton in skeletal muscle (dystrophin). The gene consists of 79 exons that span 2.5 Mb, the genomic structure of which has been determined. Mutations responsible for the disease are deletions or duplications, which account for 60-65% and 5-8% of cases of DMD/BMD respectively and the remaining cases, about 30-35%, are caused by single point mutations. Approximately one-third of the DMD/BMD patients originate through new mutations while the rest are inherited through carrier mothers or arise from germinal mosaicism.

Aim of the study

My aims were to standardize the diagnostic procedure for Duchenne/Becker muscular dystrophy. The above techniques have been used successfully to give a proper genetic diagnosis for most of the patients and to quantify gene mutation dosage for DMD/BMD female relatives.

- Identification of mutation in the affected males and

determine the exact border of the mutation in order to give the phenotype forecast.

- Carrier status determine of the female dependent in the affected Hungarian families.
- Introduce the new method in order to determine the carrier status in those supposed female carriers in which we did not have any DNA sample or information about the mutation in the affected male relatives.
- Introduce the prenatal analysis in order to prevent supposed affected foetus.

Materials and Methods

Patients affected with DMD/BMD were assessed in our laboratory between 2001 and 2007. In all of them the diagnosis was established through clinical and neurological examinations in several clinical departments, family history, serum creatine kinase (CK) analysis and where available, through histopathology of the muscle biopsy.

Multiplex PCR

Two sets of PCR primers were used which cover the exons in the deletion hot spots. Set I covers exons 4, 8, 12, 17, 19, 44, 45, 48 and 51. Set II covers exons 1, 3, 6, 13, 43, 47, 50, 52 and 60. PCR

was performed by standard protocols. Amplification products were detected on 3 % agarose gels stained with ethidium-bromide.

Southern blot and cDNA probes

Genomic DNA was digested with HindIII and BglII restriction enzymes separately and were electrophoretized on a 0.7% agarose gel and transferred to Hybond N^+ membrane using the standard capillary transfer technique. After blotting, the membrane was hybridized with a 32P-dCTP radioactively labelled cDNA probe using the random priming procedure. The quantitative analysis was performed by Packard Instant Imager technique using the relative intensity of the signals. DNA samples of female relatives were tested together with their affected offspring's if available and fragment densities of the different exons were also compared to normal controls.

Multiple ligation-dependent probe amplification (MLPA)

The MLPA kit contains one probe for each of the 79 DMD exons of the dystrophin gene and in addition, a probe for the alternative exon 1 (Dp427c). These 80 probes have been divided in two probe mixes. For the individual analysis 100 ng DNA in 5 µl volume per sample was used. Further analysis was done according to the manufacturer's protocol. PCR products were analyzed on an ABI model 3130 capillary sequencer with the Genescan software, using Genescan 500 size standards. Individual peaks corresponding to

each exon were identified based on the difference in migration relative to the size standards. I determined the relative probe signals of each probe by dividing each measured peak area (A_s) by the sum of all peaks areas ($\sum A_s$) of the sample. This relative peak area ($A_s / \sum A_s$) was then divided by the relative peak area of the corresponding probe obtained from a control DNA sample ($A_c / \sum A_c$) as unknown samples were always run parallel to control samples. The relative peak areas of the amplified probes derived in this way were analyzed using a Microsoft Excel based script.

Results

Affected male patients analyses

A total of 149 DMD and BMD unrelated Hungarian patients were classified as non-ambulatory and ambulatory. Molecular analysis of the patient's dystrophin gene was performed by multiplex PCR reactions using multiplex amplifications of 2x9 exons and Southern blot with cDNA probe and MLPA analyses. Screening of deletions in dystrophin gene demonstrated a deletion in 93 among 149 analyzed cases (63 %) in accordance with other population studies. Deletions were located in the major hot spot (e43-e52) in 60 (64%) cases, while in 18 (20 %) cases deletions were detected in the minor hot spot region (e2-e10). In addition, I detected 15

(16%) atypical or rare mutations and in one case I confirmed a duplication event with DMD phenotype using the new MLPA technique. At one twins brother I confirmed point mutation in the ex30 which resulted DMD phenotype. Two patients had a deletion extending at least from the muscle promoter to exon 79 since no amplification products were obtained by the MLPA assay. In these two cases we made additional analyses of the neighbouring genes:

- ARX gene Xp22.13 presented
- IL1RAPL1 gene Xp22.1-p21.3 deleted
- NR0B1 gene Xp21.3-p21.2 deleted
- Glycerol kinase gene (GK) Xp21.3-p21.2 deleted
- Dystrophin gene Xp21.2 deleted
- PRRG1 gene Xp21.1 presented

In one of these two patients the first symptom was the glycerol kinase deficiency which is associated with severe developmental delay and also had adrenal insufficiency and episodic vomiting, metabolic acidosis. After he began a low-fat diet, no further GK deficiency symptoms. In the other patient glycerol kinase deficiency, congenital adrenal hypoplasia, and Duchenne muscular dystrophy symptoms were developed and the muscular dystrophy was prominent. In 46 cases we had DNA sample of female dependents so we offer the carrier analysis and in 42 affected male we had any DNA samples from the female dependents.

Carrier detection

For the deletion and duplication quantitative screening I could use Southern blotting and Multiplex Ligation-dependent Probe Amplification (MLPA). At the quantitative analysis I made the dosage identification, because the normal female sample contain two copy of each dystrophin exon (relative signal intensity between the female control versus index female sample 2 : 2 and carrying deletion dosage is 2 :1 and carrying duplication dosage is 2 : 3. From the 93 female relatives, we were able to assess carrier status in 42 (45%), using cDNA and MLPA analyses. Moreover, we detected a duplication event in two women, who had an affected male relative. Unfortunately, we did not have any information about the possible mutation and neither did we receive any DNA sample from the deceased affected male, so the only possibility was to use the MLPA analysis to determine the carrier status of the female relatives. Similar issues complicated the detection of 28 supposed female carriers; as we did not have any DNA sample or information about the mutation in the affected male relatives. Therefore, the whole dystrophin gene was screened by MLPA technique in order to determine the carrier status and out of this group; the DMD/BMD carrier status was confirmed in 13 cases. In three cases we confirmed the manifest carrier status one

with severe (wheelchair dependent at the age of 14) and two with mild (muscular cramp and muscular pain at the age of teens) symptoms and the CK levels of the manifest carrier were moderate elevated. Mothers of two in three manifest carriers were carrier without any symptoms and one in three proved as a de novo manifest status.

We confirmed the whole dystrophin deletion in two patients and we made mothers carrier analyses using the new MLPA technique and proved the carrier status in either mothers.

In those cases in which we not confirmed the carrier status we have to consider the germ line and somatic mosaicism so we have to offer the carrier analysis if the index female has one affected son and the other opportunity to make analyses toward the other muscular dystrophies.

Outcome of prenatal diagnoses

If the carrier status was confirmed, prenatal diagnosis was offered. In total, prenatal molecular analyses were performed in 15 cases and in three cases deletions in the dystrophin gene were confirmed. The parents decided for termination of the pathological pregnancies. In eight cases DNA samples of the affected patients were available, therefore the diagnostic procedure was straightforward due to previous determination of the exact

deletions in the index patients. In contrast, in six families the affected DMD/BMD patients were not available for study.

Protein analysis

We made the protein analyses with German collaboration (Munich, Ludwig Maximillians University (LMU) Friedrich Baur Institut). Muscle biopsies were performed and processed according to standard immunnohitochemistry and immunoblot techniques. We analysed eight male muscle biopsies and in five cases we confirmed the dystrophin protein defect. Four cases in five we determine the mutation with DNA analyses (one case 45. exon deletion, two cases with whole dystrophin gene deletion and one case with 45-49 exons duplication).

Discussion

The molecular strategies to confirm the most common dystrophin gene impairments use the following techniques: multiplex PCR, Southern blotting and hybridization with specific radioactive labelled DNA probes and MLPA.

- In my samples analyzed until now, 63% of the detected deletions were located in the major hot spot region and 20% of the detected deletions were in minor hot spots and 16 % of

the detected deletion were in atypical or rare mutation which were in good agreement with the results reported in other populations.

- In the atypical cases, the multiplex PCR analyses were not sufficient as the exact boundaries of the deletions were not detected or the deletions were out of the hot spot region of the Beggs and Chamberlain kit, so I used the Southern blotting and MLPA technique to define the exact deletion boundaries.
- In this study, deletions breakpoints were located mainly in introns 44 (39%) agreement with several authors have previously shown that intron 44 is the most frequent site for rearrangements in the dystrophin gene.
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- The phenotype prognosis is based on the alteration of the reading frame: out of frame mutations, leaving no dystrophin protein, produce the more severe Duchenne phenotype, whereas in-frame deletions result in the production of altered protein length and the milder Becker phenotype. The patients represented two phenotypes: 65 DMD (severe form) and 28 BMD (mild form) with elevated serum CK in all patients.
- In this work we analysed 93 female relatives and in 42 (45%) we confirmed the carrier status using quantitative Southern

blotting and MLPA analyses .

- We proved three manifest carriers and in two of them we confirmed the mother's carrier status as well. Although mothers of the manifest carriers have the same deletion in one of theirs X-chromosome as theirs daughters have, they are asymptomatic. In the manifest carrier samples could be a non random X-chromosome inactivation that means there is a deletion carrier X-chromosome inactivation preferences. This explain the differences between the asymptoms carrier and manifest carrier females but it is not the answer the differences between the manifest carrier and their mothers. Mothers of the manifest carrier females carry the same mutation in the X-chromosome all the same there is a phenotype distinction, in which some epigenetic modifying factors could be the background.
- We found two cases in which the carrier status were confirmed on the whole dystrophin gene according to the affected son mutation and in these two mothers we couldn't proved any symptoms.
- Using the MLPA technique I could make the carrier analyse for those cases in which I hadn't DNA sample of the index DMD/BMD patient.
- The carrier detection and prenatal analyses should be used as

a prevention strategy for families with DMD/BMD disease before family planning.

In my Ph.D. study I represented the routine diagnosis of DMD/BMD which relies on the detection of deletions in the mutation hot spots of the dystrophin gene; approximately 95% of the macrodeletions will be detected by the multiplex PCR. Direct diagnosis of female carriers is possible only when a deletion or duplication was detected in the index case and the carrier analysis was performed by quantitative Southern blot using specific cDNA probe. The MLPA assay, which I introduced newly, proved to be time saving and more precise compared to the Southern blot assay. Especially, it is the only solution for carrier detection when DNA sample of the index patient is not available.

This comprehensive study with a high number of patient and female relatives shows that Duchenne/Becker carrier detection and prenatal diagnosis have become both feasible and reliable using cDNA probes and MLPA technique. I am confident that the use of the MLPA assay described in this report represents a reliable alternative approach for a simpler and faster analysis of the Hungarian DMD/BMD patients. Applying these new techniques, a more reliable molecular genetic diagnosis of the specific muscular dystrophy became available. This development provides not only the opportunity for proper genetic counselling and prenatal

diagnosis for the affected families, but can help to choose the correct therapeutic approach, where available.

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