DIAGNOSIS MANAGEMENT STRATEGY OF DUCHENNE MUSCULAR DYSTROPHY

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Abstract

Duchenne Muscular Dystrophy (DMD) – an X-linked recessive inherited disease affecting around 1 in 3600 - 6000 male newborns – is considered the most severe human neuromuscular disease with no cure. It's determined by a mutation of the dystrophin gene DMD (Xp21), which codes for the dystrophin protein. The aim of gene therapy is to targeting the underlying genetic abnormalities, as the only chance for DMD patients to improve the quality of life and to increase the lifetime.

Generally, the diagnosis of DMD might be easily established on the basis of the family history, specific muscular symptomatology, elevated CK level, immunohistochemical examination and molecular testing. Though, lacking of family history, mild clinical features and laboratory technique limitations could delay the diagnosis of DMD.

A fast and definite diagnosis (molecular testing included) could offer the possibility to include the patients in international clinical trials focused on specific mutation, changing thus the evolutiv prognostic of disease.

The present work is trying to validate the minimum standards for a fast and complete diagnosis of Duchenne Muscle Dystrophy which would allow the same prognostic perspective for the Romanian patients.

The study was performed on 8 patients, using clinical evaluation, creatine phosphokinase dosing, immunohistochemical examination and molecular analysis.

The results of our study prove that some clinical features, such as phenotypic combination of Gower`s sign and calf hypertrophy might be high significant for DMD clinical diagnosis. Diagnostic certainty needs further explorations: the immunohistological examination (essential for the differential diagnosis), as well as the molecular analysis which confirms the diagnosis and allows, according to the mutation type, the inclusion in a targeted therapeutic study.

Key words: Duchenne Muscular Dystrophy, diagnosis management, therapy

Introduction

Duchenne Muscular Dystrophy (DMD), an X-linked disease that affect 1 in 3600-6000 live male newborns, is the most common muscular dystrophy present in early childhood, for which a curative treatment is not yet developed.(1).

Affected individuals present proximal muscle weakness and calf hypertrophy, usually manifested between 3 and 5 years of age. The disease is rapid progressive and most of the patients become wheelchair-bound by the age of 12. Respiratory, orthopedic and cardiac complications emerge and the boys die in their late teen to early twenties (2).

DMD occurs as a result of mutations in the dystrophin gene (DMD, locus Xp 21), which lead to an absence of dystrophin in the muscles. A milder form of DMD, Becker Muscular Dystrophy (BMD) is caused by allelic variants of DMD gene, which exhibit a less severe phenotype and evolution than DMD (BMD individuals can survive till their 7th decade (3).

A diagnosis of DMD can be made based on familial history, clinical symptomatology and creatine kinase level (4).

Males suspected to have a DMD based on these explorations need to be referred for molecular confirmation, which is achieved by demonstrating the presence of a pathogenic variant in the DMD gene. Sometimes, due to technical limitations, the mutation cannot be identified; in these cases, dystrophin analysis by immunohistochemistry from a muscle biopsy might be needed in order to establish a definite diagnosis (5).

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Material and methods

Our study shows the diagnosis pathway for 8 male patients between 4 months and 8 years of age, coming from Pediatrics and Neurology clinics and addressed to the Medical Genetics Department of University of Medicine and Pharmacy of Craiova for DMD suspicion. For all patients a thorough genetic examination was performed, consisting in achieving the family tree, detailed disease history, clinical and paraclinical examination.

Results

Of all 8 patients, only 2 have presented a family history for clinically and histopathologically confirmed muscular dystrophy highly suggestive for DMD.

Due to the way of suspecting the DMD diagnosis, the patients were divided in two groups:

- 2 patients with very elevated plasma levels of CK (20 – 100 times normal)
- 6 patients with clinical features characteristic for DMD. (Tab.1).

Apart the clinical features mentioned in Tab. 1, physical examination has revealed for all patients, except P1 – too young to offer a relevant clinical findings – a progression of the disease with a symmetric increase of muscle weakness and atrophy (more evident proximal than distal), lumbar hyperlordosis, waddling gait – Trendelenburg, Achilles contractures.

At that moment, no patient presents any sign of impairement of upper limb muscles, or cardiac and respiratory involvement. For 7 patients (P2 –P8) supportive investigation that molecular analysis of the dystrophin gene were performed; muscle biopsy with immunohistochemical study for dystrophin was performed in for P2 - P8 patients; patient P1 was already having this analysis at the time of presentation.

All patients (including P1) exhibited a negative immunohistochemistry stain for dystrophin (Dys -1, Dys – 2, Dys-3) (Tab. 1). The samples were analyzed for identifying the mutations for the dystrophine gene through various techniques, such as: multiplex PCR, MLPA analyse of genomic DNA by direct sequencing or by next generation sequencing. The results are shown in Table 2, allowing to classify all our patients from the study as DMD.

Disscutions

DMD clinical diagnosis should be easily made in the context of characteristic clinical presentation. The mean age of diagnosis of boys with DMD, without a family history of DMD, is around 5 years, but the diagnosis can be suspected earlier because of the delay in attainment of developmental milestones (delayed walking or language achievement).

Initial symptoms might include frequent falls, difficulty in running, jumping, standing up from standing on the floor (Gowers' sign – "climbing up their legs"), and climbing stairs. The atrophy of pelvic muscles leads to a development of a lumbar lordosis. Toe – walking is a common feature of the disease, as well as the hypertrophy of calf (1, 6, 7).

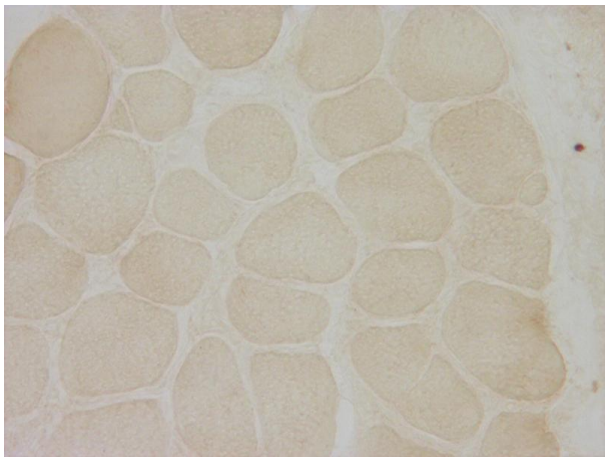

Fig. 1. Complete deficiency of dystrophin immunostaining on sarcolemm of the fibers from a patient of our study, x10.

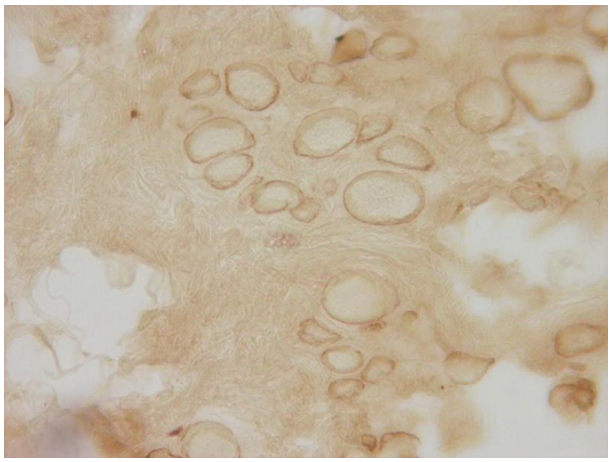

Fig. 2. Reduced dystrophin staining on sarcolem of the fibres from a control patient with BMD, x10.

The distinction between DMD and BMD is based on the symptomatology onset age (later-onset skeletal muscle weakness for BMD).

In keeping with its X-linked recessive pattern of inheritance, all the 8 patients of our study were males, and, except the P1, P2 and P8 cases, the age of initial presentation not different from those in other reports.

Table 1. Summary of clinical and paraclinical data for studied cases.

Patients	Family history	CK (U/l) serum level	First medical presentation age	First symptoms noticed	Age of first DMD diagnosis
P1	No	8500	4 months	- no clinical symptoms	4 months
P2	No	4320	2 years	- no muscular symptoms	2 years
P3	Brother	9300	3 years	- delayed walk - calf hypertrophy - difficulty to walk (slower than peers), to running, climbing stairs - frequent falls - Trendelenburg gait - hepatosplenomegaly	3 years
P4	Cousin of maternal grand mother	23451	4 years	- language delay, behaviour and cognitive problems - calf hypertrohy - difficulty to climbing stairs, to jump, and to run - muscular fatigability - Gower`s sign present	7 years
P5	No	6615	4 years	- delayed walk - calf hypertrophy - difficulty to climbing stairs, to jump, and to run - muscular fatigability	5 years
P6	Brother	7540	5 years	- delayed walk - calf hypertrophy -difficulty to walk (slower than peers), to running, climbing stairs - Gower`s sign present - Trendelenburg gait - hepatosplenomegaly	5 years
P7	No	18619	6 years	- delayed walk - calf hypertrophy - Gower`s sign present - Trendelenburg gait - toe walking	6 years
P8	Maternal oncle Maternal cousin	7385	6 years	- delayed walk - difficulty with climbing stairs, running - frequent falls - Gower`s sign present - calf hypertrophy - toe walking	8 years

CK – creatine kinase, DMD – Duchenne Muscular Dystrophy

Table 2. Results of IHC examination and genetics tests performed for including study patients.

Patients	Dystrophin evaluation by IHC	Molecular techniques used for DNA analysis	Mutations	Diagnosis
P1	Absent	Multiplex PCR	Del 45	DMD
P2	Absent	MLPA	Del 48-50	DMD
P3	Absent	Sequencing	ND	DMD
P4	Absent	Multiplex PCR	Del 46 – 47	DMD
P5	Absent	MLPA	Del 46 – 48	DMD
P6	Absent	Sequencing	ND	DMD
P7	Absent	MLPA	Del 51	DMD
P8	Absent	NGS	Nonsense mutation of exon 41	DMD

IHC – immunohistochemistry, PCR- polymerase chain reaction, MLPA – multiplex ligation-dependent probe amplification, ND – not detected, DMD – Duchenne Muscular Dystrophy

For 6 of the patients, symptomatology onset was between 3 and 6 years of age, with: difficulties to run or jump properly, frequent falls down, muscle fatigability, proximal muscle weakness (Gower`s manoeuvre present), calf hypertrophy. For all these patients we could notice a middle delay of walk (16 - 19 months old). They all had a normal neuropsychological and motor development in early childhood; the patient P1 had a normal evolution for his age (4 months) and the P4 patient had a language delay as well as behavior and cognitive problems. For 2 of the patients there was not an obvious clinical debut, the suspicion for a DMD diagnosis being based on random elevated levels of transaminases and further of the CK. Surprisingly, cases P4 and P8, with presumed DMD family history, are the latest patients evaluated in terms of age (seven and, respectively, eight years old), although both have presented specific clinical features and high levels of CK from early childhood. Thus, we can discuss about lacking of pathology approach in family, even without a diagnostic certainty in ancestry. For case P3, the diagnosis of his brother, case P6, was a trigger to early initiate the diagnosis protocol, part of exploration being assessed simultaneously for both, with a lower psychological impact on family.

Laboratory investigations were performed by several methods: biochemical analysis, DNA analysis, and histological examination.

Creatin kinase (CK) serum level

The characteristic finding in DMD is a noticeable increase of CK level. The normal serum level of CK varies with age, sex, and physical activity and may be elevated in several type of neuromuscular disorders (spinal muscular atrophy, myositis or other muscular dystrophies such Limb Girdle Muscular Dystrophies). In DMD occurs the most spectacular elevation of serum CK (50-100 times normal), even in newborns and prior to any symptoms (2, 6). Very elevated CK serum levels detected in our patients (4000 - 23000 UI/l) are at the highest point for literature existing serum levels and even if this is considered a non-specific DMD element, because of its association with other neuromuscular diseases, for two of our patients this was the first sign for a DMD diagnosis, due to their young age and lack of clinical features.

Genetic testing

Dystrophin gene is one of the biggest human genes (approximately 2.5 million base pairs, encoding 79 exons), so the molecular diagnosis for DMD can be complicated by the size of the gene and the multiple different mutation types (9). The majority of mutations in the DMD gene, accounting for 65% of cases of DMD and 85% of cases of BMD, is demonstrated to be deletions of one or more exons, especially in two "hot-spot" (mutation rich area) areas in the central genomic region (exons 2-20 and exons 45–53). The most frequent deletions, associated with DMD phenotype, in the literature, are those involving exons 45 (5, 3%), and 48-50 (5,1%) (10,11). Duplication of one or more exons accounts for 6-10% of cases of both DMD and BMD, and the remaining cases are due to point mutations, small insertions/ deletions or splice site changes. 2% of DMD cases occur by rearrangements and deep intronic changes.

The route to establish the molecular diagnosis depends on local availability of rapid and reliable testing and may include multiplex polymerase chain reaction (PCR), multiplex ligation-dependent probe amplification (MLPA), direct sequencing of all exons at the genomic level, or from cDNA of dystrophin gene. The two multiplex sets of Chamberlain et al. and Beggs et al. enable the detections of 98% of all DMD deletions. Even if the multiplex PCR is available, do not test all deletions, and do not characterize all deletion breakpoints (5, 10). The more recently developed MLPA technique is now the most widely used, since it will detect all whole exon deletions and duplications, and also characterize the end point of rearrangements at the exon level resolution (5). If MLPA does not reveal a deletion or duplication in DMD gene, the dystrophin gene sequencing should be done to search for point mutations or small deletions/insertions. Sequencing can be performed on either genomic DNA or on derived cDNA from muscle RNA. Complex rearrangements or deep intronic changes (approximately 2% of DMD mutations) will be not detecting using standards methods of genomic DNA analysis, and for these mutations the analysis of muscle RNA is required.

The next generation sequencing technique, recently developed, allows to sequence the whole gene, including introns, improved the probability of being able to detect the full spectrum of DMD mutations (5, 10). According to the lab possibilities were the tests were performed, 3 of the patients had the MLPA test, 2 of them had the multiplex PCR test, 2 patients benefited of sequencing analysis of DMD gene and 1 patient of next generation sequencing. In our study, two of patients had the most frequent deletions, associated with DMD phenotype (deletion of exon 45, and deletion 48-50), and the rest of them had deletions located in the two hot spot regions, of the dystrophin gene, described in the literature (Tab. 2). These findings are consistent with literature data, supporting the use of MLPA and multiplex PCR as first intention techniques for identifying dystrophin gene mutations. For P3 and P6 patients, even if the gene analysis was early carried, the mutation could not be identified, so additional RNA-based studies may be required in order to detect possible complex rearrangements, or variants located deep into the large introns of the gene. By chance, the only point mutation found in our cases was rapidly identified because the patient P8 was the only one who benefited by the NGS analysis as first approach.

Muscle biopsy

If a DNA mutation has been found, generally, a muscle biopsy is not needed any more for diagnosing DMD. But, when the genetic testing is not available in the centre where the patient is seen, if no mutation was identified, or if it is the case of a family history of DMD with unknown mutation, muscle biopsy can remain a routine investigation in DMD, and could be the only method able to establish a certain diagnosis (1, 2). Absent or markedly reduced dystrophin in muscles biopsies can be demonstrated by

immunostaining, using antibodies directed against different epitopes of dystrophin. As mentioned above, the immunohistochemistry continue to be the most specific method that can sustain a DMD diagnosis, and differentiate DMD from BMD (immunoreactivity is absent in DMD and significantly reduced in BMD) (2, 8, 12). For all our 8 patients immunostaing on muscle biopsy samples was performed. Sadly, since P1 and P2 patients had muscle biopsy previous to our study, we considered it as an impetuous act, due to their young age (4 months and 2 years, respectively). The others had immunohistochemistry for dystrophin on biopsy samples because of the impossibility of an initial genetic analysis or for exact setting of dystrophin deficit/absence, especially for the patients with a late presentation (6-8 years), for whom a differential diagnosis with BMD was to be considered. Dystrophin was entirely absent for all our studied patients. For 6 of them this was consistent with molecular analysis, while for P3 and P6 patients, with no identified mutation, this represented the main support for DMD diagnosis.

Even the size and distribution of expression of the dystrophin gene is challenging for the development of DMD therapies (10). Several experimental gene therapies are currently under investigation (8). Some of the most promising approaches to therapy for DMD, capable to convert DMD into a milder BMD phenotype: antisense oligonucleotide induced exon skipping, and non-sense mutation red-through, are targeted applied according to the mutation type (deletions, duplications, nonsens mutations (10). Because till now only corticosteroids offer a valid therapeutic benefit, we can say that no curative pharmacological treatment is available yet in DMD (6). Therefore, an earlier and complete diagnosis with genetic confirmation of the DMD mutation is crucial to identify patients eligible for experimental treatments (12).

Conclusions

A DMD diagnosis is one of the main diagnosis to be considered when we have a male patient with a delayed or abnormal global developmental (delayed walking and/or speech), early muscle impairment (calf hypertrophy, Gower`s sign, frequent falls), even doesn't exist a family history of DMD. A suggestive family history for DMD must be considered even in the absence of a certainty ascending diagnosis.

Significant family history for DMD should be considered, even without a diagnostic certainty in ancestry.

Finding an increase CK level (20 - 100 times normal), even if the patient is very young, represents a reason to immediately refer the patient to a specialist for confirmation of the diagnosis.

Actually, in the molecular diagnosis era, the muscle biopsy is no longer necessary, but it may be a very useful tool to establish the diagnosis in case when the genetic test could not identify any mutation. On the other hand, even if a molecular diagnosis is available, the immunohistochemical examination could differentiate DMD from BMD when the clinical phenotype is atypical.

If the experimental therapies in clinical trials based on targeted mutation proved their efficacy, it will be absolutely necessary to start this kind of treatments early, before significant muscle loss occurs, in order to obtain optimum benefits for the patients.

Thus, molecular diagnosis identification of the DMD responsible mutation is mandatory, impediments determined by technical or financial limitations must be overtopped by using a protocol for various genetic diagnosis techniques: (1) multiplex PCR for identification of deletions in deletional "hot-spots" of DMD gene, (2) MLPA, enable to detect all whole exon deletions and duplication, (3) sequencing of genomic DNA and cDNA or next generation sequencing for the other mutations (point mutations, small insertions/deletions, deep intronic changes, complex rearrangements)

An efficient testing strategy, whit a minimum rate of error, optimal for determining a quick and complete diagnosis could change the life prognosis for boys affected by DMD.

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