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REVIEW

DIAGNOSIS PARTICULARITIES IN DYSTROPHINOPATHIES IN WOMEN

Pop Anita[1], Cornițescu M.[1], Pleșca Doina Anca[1]

[1] *"Carol Davila" University of Medicine and Pharmacy - Bucharest*

Abstract. Dystrophinopathies are X- linked muscle diseases, more frequently expressed in males, but infrequently also the substrate of muscular pathology in females. Laboratory methods in the diagnosis of dystrophinopathies have been continuously improving, which permits a higher accuracy in the study of the involved genetic alterations and of their modes of transmission and expression.
Key words: dystrophin, Duchenne and Becker muscular dystrophies, X-linked pathology in women, X chromosome inactivation

General data

Dystrophinopathies are caused by a functional deficit of dystrophin, a protein with an essential role in the cell membrane binding of the actin-myosin complex. Clinically, dystrophinopathies cause impairment of the motor function, leading to respiratory deficit as well as skeletal deformities; they may be accompanied by cardiac and intellectual impairment. Initially described as muscle conditions affecting young boys, dystrophinopathies were linked to the dysfunction of the dystrophin protein, encoded by a gene located in the Xp21.2 region of the human X chromosome.

In the muscle fiber, dystrophin is located close to the internal side of the cell membrane as a component of a glycoprotein complex (the dystrophin-associated protein complex) [1] with a role in the anchoring of the actin-myosin myofibrils to the cell membrane. Dystrophin deficit leads to increased sarcolemmal deformability [2], one of the proposed explanation for the loss of muscle fibers in dystrophinopathies being that sarcolemmal lesions produced during muscle contractions favor the chronic necrosis of muscle fibers that becomes clinically manifest as muscle weakness.

Utrophin, a homologue of dystrophin, is overexpressed in dystrophinopathies [3]. It is not able to overcome the effects of the absence of dystrophin in human subjects, but its expression attenuates the effects of dystrophin deficiency in mdx mice [4].

The dystrophin gene is the largest identified in the human genome to date, comprising approximately 2.5 megabase in the Xp21.2 region of the human X chromosome. The gene consists of 79 exons; the presence of multiple alternative promoters that are differentialy expressed in tissues allows the tissue-specific messenger RNA synthesis. Several mechanisms enable the generation of alternative transcripts from the dystrophin gene: the existence of three full-length transcription promoters (brain B, muscle M, Purkinje cell P), of internal promoters (retinal R, brain-3 B3, Schwann cell S, general G) and of an alternative splicing mechanism at the 3' end of the gene [5]. The transcript generated in the striated and cardiac muscle, under the control of the M promoter, includes all exons and is translated in the muscular dystrophin of 427 kDa molecular weight [6].

Doina Anca Plesca
21 Basarabia Blvd, Bucharest, Romania
e-mail: doinaplesca@yahoo.com

Clinical aspects in dystrophinopathies

Clinical aspects vary in dystrophinopathies: from mild, asymptomatic forms, with increased creatine kinase serum levels or with muscle cramps and myoglobinuria to severe forms that impair the function of skeletal and cardiac muscles (Duchenne and Becker muscular dystrophy) or the cardiac muscle alone (X-linked dilated cardiomyopathy) [7-10]. In what concerns the muscle impairment, three dystrophinopathy phenotypes are most prominent: Duchenne and Becker muscular dystrophies and the intermediate phenotype [11]

Duchenne muscular dystrophy (DMD)

Initially described by the French physician Guillaume- Benjamine Armand Duchenne at the end of the 1800s [12], DMD is the most frequent dystrophinopathy phenotype. DMD affects 1 in 3500 male newborns [13] and is caused by the total absence of the dystrophin gene expression [5]. The onset in boys occurs between two and three years of age and manifests itself as a delay in acquiring motor skills, or as a global developmental delay [11].

Muscle weakness is more pronounced proximally and manifests itself as a difficulty in climbing and running. The child falls more frequently than other children of same age, develops lumbar hyperlordosis and a waddling gait. A characteristic sign of the disease is the Gowers sign: climbing from the floor is possible by walking up one's own body. The pseudohypertrophy

of calf muscles can be identified [14].

As the disease progresses, male patients lose their motor skills and develop permanent muscle contractures, generalized muscle atrophy, defective thorax and spine conformation (kyphoscoliosis). They become wheelchair dependent generally by the age of 12 [10].

The fatigue of the diaphragm muscle and the pathological conformation of the thorax favour the frequent occurrence of respiratory infections and impede the cough effort. The respiratory condition requires adequate treatment, including mechanical ventilation [15].

The cardiac impairment manifests itself as primary dilated cardiomyopathy (DCM), conduction abnormalities and a variety of arrythmias, primarily supraventricular. The cardiomyopathy is characterized by extensive fibrosis of the posterobasal and eventually of the lateral free wall of the left ventricle [11].

Children suffering from DMD have a global developmental delay that is manifest from the first years of life. Qualitatively, they have a more severe impairment of verbal than non-verbal performances. Deficits in working memory and executive function have been found [10].

Male DMD patients do not transmit the dystrophin mutation [10]. Their health state seriously deteriorates before reaching the reproductive age.

The main cause of death of DMD patients is cardiorespiratory failure. Only few of them survive beyond the third decade [10].

Becker muscular dystrophy

Becker muscular dystrophy (BMD) represents a less frequent phenotype, with a milder impairment of the striated muscle but with frequent cardiac involvement [16]. It is generated by the presence in the skeletal muscle of either qualitatively abnormal or decreased quantity dystrophin [5].

The onset of Becker muscular dystrophy occurs at an older age than that of DMD, with milder clinical signs. Most patients preserve their motor skills by at least the age of 15, some remaining mobile at even older age [11].

Clinical signs include muscle weakness in the limb girdles, often calf pseudohypertrophy and activity-induced cramping in some individuals [10]. Muscle contractures and mental retardation are not as frequent or severe as in DMD [11].

In contrast to DMD patients, BMD patients have unaffected reproductive function, thus transmitting the BMD genetic trait to their daughters who will have a carrier phenotype. By not inheriting the X chromosome from fathers, their sons do not carry the dystrophin mutation [10].

Death generally occurs in the fifth decade due to heart failure [10].

The intermediate phenotype (outliers)

The intermediate phenotype was described in patients with disease signs that resembled either mild DMD of severe BMD forms. These patients become wheelchair dependent between the ages 12 to 16 [11].

Dystrophinopathies in women

Unlike males with pathological alterations in the dystrophin gene who are always symptomatic, females can be asymptomatic carriers or symptomatic individuals.

Asymptomatic carrier females have this status due to the inheritance of the dystrophin mutation on a single X chromosome and to the random X inactivation that occurs within the general female population. Although generally asymptomatic, they may reveal the presence of minor myopathic signs such as muscle cramps and myalgia. High creatine kinase serum levels are generally present [17].

Symptomatic women represent approximately one third of women with a pathological mutation in the dystrophin gene. 5% of them show myalgia and cramps without muscle weakness; 17% show mild to moderate muscle weakness while 8% show dilated cardiomyopathy, with a mean onset age of 33 [18]. A small fraction even show a severe muscular dystrophy phenotype that causes invalidity [19,20]. The onset of disease generally occurs after the age of seven [21].

The disease shows a slower progression in females than in males due to their expression of dystrophin in skeletal muscles at closer to normal levels as they age (genetic and biochemical normalization) [22].

The motor deficit in DMD women is of medium intensity, proximally located and affects especially lower limbs. Calf pseudohypertrophy is present [23].

Changes in muscle tone are less pronounced than in males with DMD; girls are able to walk until advanced ages, but they fall frequently and are unable to run. In rare cases they are wheelchair-bound [17].

Cardiac abnormalities are often found in women with dystrophin mutations. The most frequently reported are left ventricular dilation for both DMD and BMD female patients and dilated cardiomyopathy for DMD women. Studies are not concordant regarding the frequency of ECG abnormalities in women with dystrophin mutations [24].

Symptomatic girls also experience behavioural and mild learning abnormalities [17].

Pathophysiology of dystrophinopathies in women

In the event of a pathological mutation in the dystrophin gene, the dystrophinopathy phenotype can occur in women through several mechanisms:

1. By a **translocation between the X chromosome and an autosome** that disrupts the dystrophin gene on the X chromosome [25].
2. By the **simultaneous presence of only an X chromosome** (Turner syndrome; 45, X0 karyotype) **and a dystrophin gene mutation** on the only X chromosome in the genome [26-28].
3. By **uniparental disomy of the X chromosome carrying the dystrophin gene mutation** [29].
4. By the occurrence of a **pseudohermafroditism in a person with female phenotype and male genotype** (46, XY) that has on the only X chromosome in the genome **simultaneous mutations in both the androgen receptor (AR)**

gene and the dystrophin gene [30].

5. By having **simultaneous mutations in the dystrophin gene alleles on both X chromosomes** [19,31].
6. By **preferential inactivation of the X chromosome without the dystrophin gene mutation (non-random X inactivation, skewed X-chromosome inactivation)**.

The mechanism of skewed X inactivation seems to be the most frequent cause of dystrophinopathies in women [20,32,33], the other mechanisms being detected only rarely.

X chromosome inactivation in women is definitive and usually random, taking place in the first mitoses in the human embryo [34].

Random X inactivation is a mechanism of balanced expression of genes on both X chromosomes, in the event of genetic mutations affecting the function of synthesized proteins. A dystrophin expression yield of 30% is sufficient to prevent the occurrence of muscle dystrophy [35].

Skewed X-chromosome inactivation is rare and generally due to some particularities of the regulating XIST center of inactivation on the X chromosome. The X chromosome inactivation is said to be skewed if more than 80% of a woman's blood lymphocytes have the same parental X chromosome inactivated [36]. Although the X chromosome inactivation is assessed on a regular basis in the peripheral blood lymphocytes, the result of this test does not always correlate with the inactivation pattern in the muscle nuclei; a more accurate assessment of the X chromosome inactivation in muscles uses samples obtained from muscle biopsy [37].

Mutations in the dystrophin gene responsible for the occurrence of dystrophinopathies are large intragenic rearrangements (exon deletions and duplications) in 65-75% of cases; a smaller fraction of cases involve point mutations or small gene rearrangements [38,39].

In most patients, the clinical evolution can be predicted by using the reading frame rule. Thus, the majority of DMD patients have truncating mutations that lead to the absence of dystrophin expression, while BMD patients have mutations that preserve the reading frame, permitting the expression of semi-functional dystrophins [9,40].

Diagnosis of dystrophinopathies

1. Diagnosis of dystrophinopathies in male patients

The diagnosis of muscle dystrophy is suspected based on clinical criteria: proximal distribution of muscle weakness; progressive decrease of force and generally a decrease of muscle volume; loss of osteotendinous reflexes; calf pseudohypertrophy; occurrence of the Gowers sign. A positive family history suggestive of X-linked inheritance strongly supports the suspicion of a dystrophinopathy.

Confirmation of the clinical diagnosis is based on biochemical, electrophysiological, histological, immunohistochemical and genetic assays.

The assessment of the muscular enzymes in serum reveals increased levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, aldolase, creatine kinase. The increase of the creatine kinase levels, particularly of the MM isoenzyme, is the most sensitive and specific biochemical marker suggesting muscle lesion. Creatine kinase levels increase even in the early stages of the disease, prior to clinical signs; in advanced stages of the disease the serum levels of the enzyme decrease along with the decrease of the muscle volume. Higher values of enzyme serum concentrations are also found in asymptomatic carriers of the dystrophin mutations [17].

Electromyography shows myopathic changes.

Muscle biopsy allows the identifying of the histological aspects characteristic to muscle dystrophies as well as the quantitative **assessment of muscle dystrophin synthesis, through immunohistochemical tests on the biopsy product or through Western Blot or ELISA assays on muscle tissue lysate**.

The histological aspect specific to Duchenne and Becker dystrophies is represented by necrotic and regenerating muscle fibers that appear frequently in clusters, with abnormal fiber size variations, increase in the number of fibers with internal nuclei and replacement of the muscle tissue by connective and fat tissue [41].

Immunohistochemical tests on muscle biopsy samples reveal the absence of dystrophin expression and the overexpression of utrophin in Duchenne muscular dystrophy, while in Becker muscular dystrophy one can detect the presence of low level or qualitatively altered dystrophin [5].

Genetic diagnosis of dystrophinopathies

Several methods are available for the detection of mutations in the dystrophin gene that lead to the DMD and BMD phenotypes. The array of available diagnostic tools expanded towards higher resolution, allowing the detection of single nucleotide polymorphisms in the gene sequence.

Testing for the presence of dystrophin gene deletions and duplications

Intragenic deletions and duplications represent the main types of mutations identified in the dystrophin gene in children with dystrophinopathies [38,39]. Both qualitative and quantitative tests are available for testing these mutations.

Qualitative methods

Multiplex PCR is a qualitative method for testing the existence of deletions in the dystrophin gene that relies on simultaneous amplification in a PCR reaction of a set of gene exons, known to be most frequently affected.

Multiplex PCR primer sets for the dystrophin gene, described starting with the works of Beggs and Chamberlain [42,43] and then continuously improved, allow the detection of the most common deletions in the dystrophin gene. The method presents several disadvantages: it can confirm the existence of tested mutations but does not specify the ends of these since it does not test all gene exons; it cannot be used for the detection of deletions in the dystrophin gene in carrier women as it is not quantitative (the presence of the normal sequence in a dystrophin gene masks the existence of the mutation in the other allele).

Quantitative methods

Quantitative methods can be used in the diagnosis of dystrophin mutations carrier women.

Many quantitative methods are able to detect all exon deletions and duplications, also specifying the ends of most rearrangements. Methods used in the past such as **quantitative multiplex PCR of selected exons [44] or Southern blot hybridization of cell DNA with synthetic DNA probes** [45] have been currently replaced by the optimized and commercially available method of **multiplex ligation-dependent probe amplification (MLPA)** [46].

A newer quantitative method for the high resolution testing of the dystrophin gene, including at the level of introns and splicing sites is the **array comparative genomic hybridization (array CGH)** [47-49]. This method employs sets of thousands of oligonucleotide sequences on solid support for hybridization with the corresponding sequences in the patient's DNA genome, thus delivering data on the number of copies of the gene sequence along the entire 2.5 megabase dystrophin region, including all exons and introns. It maps the gene rearrangement points with a precision that depends on the selection of test probes in the respective set. A major advantage of the method is that, depending on the position in the gene sequence of the chosen probes, deletions and duplications are detected by several probes in the set (redundance of the obtained results).

Testing for the existence of small size mutations in the dystrophin gene

A small proportion of mutations in the dystrophin gene that induce dystrophinopathies are small size, including point mutations [38,39]. These mutations can be detected through **gene sequencing** [50], a currently highly accessible method.

Gene sequencing allows the identification of the entire nucleotide sequence of the assessed genetic material. The DNA material used in sequencing can be either genomic DNA extracted from the patient's tissue or complementary DNA obtained through reverse transcription from the patient's tissular messenger RNA. The obtained nucleotide sequences present differences depending on the nature of the assessed DNA material: genomic DNA contains both gene exons and introns while complementary DNA obtained by reverse transcribing the messenger RNA contains only exons.

Mutations identified in the complementary DNA must be confirmed in the genomic DNA. In some cases this step is difficult to perform: the cause of occurrence of mutations in the exon splicing regions in the messenger RNA may be located in the intronic sequence between the respective exons in the dystrophin gene. In such cases, the description of the modified sequence in the complementary DNA represents the information identifying the mutation [51].

2. Diagnosis of dystrophinopathies in women

The diagnosis of dystrophinopathies in women benefits from the above-mentioned methods, also applicable in male patients.

The clinical suspicion of the presence of a dystrophin mutation in asymptomatic carrier women is generally raised by a family history of dystrophinopathy. In the case of symptomatic women the trigger for diagnostic thinking is represented by the very signs of myopathy of the patient.

If within the family there are blood relatives with dystrophinopathy, their DNA material can be used as a comparative probe in the genetic testing. If test results from assays such as gene sequencing are already available for other affected members of the family, they can become referential for the women in study. Still, the identification of an anticipated mutation does not rule out other mutations in the person's dystrophin genes.

In the absence of preliminary information on the dystrophin mutation of a female patient, previously mentioned tests can be applied successively, bearing in mind that the selected method should have the ability to detect the mutation in heterozygous state (in the presence of a corresponding normal allele in the genome).

Particularly in the case of women with dystrophinopathy it is useful to study **the intrafamilial pattern of mutation transmission**. Two methods are useful in this regard: ***the X chromosome segregation*** and ***the X chromosome inactivation analysis***.

The X chromosome segregation analysis employs testing, with the aid of specific markers, of polymorphic regions on the X chromosomes in blood related members of the family and the evaluation of the inheritance pattern of the X chromosomes [52].

The X chromosome inactivation analysis may be performed by evaluating the X chromosome polymorphic sequences methylation. One version of this method is based on the discovery that the methylation of a HpaII restriction site located in the proximity of a variable length repetitive (CAG)n region in the X chromosome androgen receptor (AR) gene correlates with the X chromosome inactivation [53]. For each studied member of a family, the HpaII enzyme digestion of genomic DNA followed by PCR amplification may be used to determine the methylation status of parental X chromosomes. The quantitative result expressing the ratio between the amplified androgen receptor alleles provides the information regarding the X chromosomes inactivation pattern. In dystrophinopathies the study can be performed on DNA extracted from peripheral blood lymphocytes, but it is more relevant if performed on DNA extracted from muscle biopsy samples [37].

If non-random X chromosome inactivation is identified, the possible cause of the phenomenon can be evaluated by studying **the non-coding XIST gene,** whose RNA product is involved in the mechanism that determines the X chromosome inactivation [54]. Mutations in the promoter area of the gene have been associated with the non-random inactivation of the X chromosome [55].

A possible link has been described between **skewed X chromosome inactivation and discordant X-related phenotypes in monozygotic twin girls**. In such twin pairs, one girl presents the severe phenotype, while the other is only mildly affected or is clinically healthy. The phenomenon has been described for several X-linked diseases: Duchenne muscular dystrophy [56-60], clotting factor VIII deficiency (hemophilia A) [61,62] or clotting factor IX deficiency (hemophilia B, Christmas disease) [63]. The

mechanism of production of the discordant X-related phenotype in monozygotic twin girls is not yet fully understood.

Conclusions

Dystrophinopathies are muscle diseases first described in males, caused by mutations in the dystrophin gene on the X chromosome. The clinical aspect of the disease in more variable and mild in women in comparison with that in males, mainly due to the high probability of inheriting another normal dystrophin allele in the genome and to the mechanism of random X chromosome inactivation in cell nuclei. Although the diagnosis benefits from the same methods as in men, supplementary tests for female patients are also available; these tests are useful for the study of the transmission patterns of the pathologic phenotype in families.

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