Duchenne Muscular Dystrophy (DMD) Diagnosis: Past and Present Perspectives

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Abstract

Duchenne muscular dystrophy (DMD) is a fatal X-linked disorder, characterized by progressive skeletal muscle wasting. The disease is caused by various types of mutations in the dystrophin gene (DMD). The disease occurs at a frequency of about 1 in 5000 male births, making it the most common severe neuro-muscular disease. In addition to clinical examinations of muscle strength and function, diagnosis of DMD usually involves a combination of immunological assays using muscle biopsies, typically immunohistochemistry and western blotting, and molecular techniques such as DMD gene sequencing or Multiplex Ligation Dependent Probe Amplification (MLPA) using blood samples. In fact, precise molecular diagnosis is a prerequisite for determining the appropriate personalized therapeutic approach such as exon-skipping, gene therapy or stem cell-based therapies in conjunction with gene editing techniques like CRISPR-Cas9. However, the quest for reliable biomarkers with high sensitivity and specificity for DMD from liquid biopsy is still a hotspot of research, as such non-invasive biomarker(s) would not only facilitate disease diagnosis but would also help in carrier detection, which will eventually result in better disease management. In this chapter, we will illustrate the detailed current and prospect strategies for disease.

Keywords: DMD, diagnosis, biomarkers

1. Introduction

Dystrophin protein is present in myocytes in skeletal, cardiac, and smooth muscles, acting to connect the actin microfilaments, via N-terminus of the protein, to the extracellular matrix by binding membrane—bound (sarcolemma) glycoprotein complex (dystrophin associated glycoprotein complex; DGC) to the C-terminal end of the protein, and thus, plays an important role in normal muscle function [1]. Inactivating mutations occurring in DMD gene causes immature termination of protein translation, giving rise to C-terminally truncated protein product that fails to transmit muscle impulses, which causes increasing intracellular Ca^{2+} influx and thus, activating apoptotic machineries and eventually causes cell death and muscle atrophy/necrosis [2]. Death usually occurs in the third decade of life as a result of respiratory or heart failure [3].
2. Methods for DMD diagnosis

2.1 Clinical picture

Affected DMD boys are usually normal at birth but in early childhood they suffer from inability to get up from floor or climb stairs or run and they fell very often. Also, enlarged calf muscles (pseudo hypertrophy) are always noticed [4]. From the age of 7–12, the cases become more deteriorated, and the patients start to suffer from scoliosis [5], and joint contracture [6]. Also, patients will have an apparent reduction in bone-mineral density and will have hypocalciuria and osteoporosis [7].

Because the disease affects proximal as well as distal muscles, thus, in early teenage, DMD boys usually get respiratory infections and sleep apnea [8], and later, the patient will develop cardiomyopathy and eventually heart failure [9].

2.2 Circulating blood biomarkers

2.2.1 CK levels and other proteins/enzymes

One of the dystrophin protein main functions is to stabilize the muscle tissue, since it exists and binds to sarcolemma. The absence of dystrophin will eventually lead to the increased permeability of the muscular tissue and consequently the release of the muscle proteins [10], of which the creatine kinase (CK) enzyme that is responsible for the production of phosphocreatine and ADP from creatine and ATP as part of energy homeostasis. In normal condition, normal myocytes turnover, serum levels of CK ranges from 20 to 200 U/L, however, it can be slightly increased in some neurological disorders. On the other hand, in case of DMD boys, due to the accelerated muscular destruction, it may reach higher levels reaching several thousands of units/L, and in severe muscle damage it can reach 200,000 U/L [11–13]. However, CK levels sometimes can be misleading because in advanced stages of DMD, CK levels may come within normal range due to progressive muscular atrophy [14].

CK is considered one of the most used serum biomarkers in DMD diagnosis, however, many studies were performed to detect alterations in other muscle related proteins using immunoassay and MS-based detection to screen for other potential diagnostic biomarkers (Table 1).

2.2.2 MicroRNA

MicroRNAs (miRNAs) are a tissue—specific class of small, non-coding RNA molecules that function as gene regulators/silencers and consequently they are considered sensitive indicators for different cellular contexts. MiRNAs act through binding to a specific region in the 3’-UTR in the target mRNA molecules, thus, inducing mRNA degradation and inhibiting the translation process [42]. The circulating levels of miRNAs in serum reflect the intracellular status and hence, they are excellent biomarkers for many pathological conditions as they can be detected from liquid biopsies and/or tissue specimens [43]. Many studies attempted to study the modulation in the levels of different miRNAs (Table 2).

2.2.3 Lipids, metabolites, amino acid, and organic acid

In addition to the previously mentioned biomarkers, lipid profile and metabolites in the blood or urine are also very important parameters that reflect the status
| Tested marker | Levels (high or low) in DMD patients and/or other MDs | Location (serum/muscle) | Detection method | Ref. |
|---------------|-----------------------------------------------------|--------------------------|------------------|-----|
| Alkaline phosphatase (AP)-A | Elevated in Grade 1 and Grade 2 patients | Serum | Measuring enzyme activities | [15] |
| AP-B | No change | | | |
| Gly-AP | Elevated in Grade 1 and Grade 2 | | | |
| Ala-AP | Elevated in Grade 1 | | | |
| Ser-AP | Elevated in Grade 1,2,3 | | | |
| Leu-AP | Elevated in Grade 1 | | | |
| Met-AP | No Change | | | |
| Phe-AP | Elevated in Grade 1,2,3 | | | |
| Trp-AP | Elevated in Grade 1,2,3 | | | |
| Gly-pro-AP | Elevated in Grade 1 Reduced in Grade 3 | | | |
| Gly-Pro-Leu-AP | Reduced in Grade 1 and Grade 2 | | | |
| Trypsin | Reduced in Grade 1 | | | |
| Cathepsin C | Reduced in Grade 1 and Grade 2 | | | |
| Sulphatase | No change | | | |
| Phosphatase | No change | | | |
| Acetyl-choline esterase | Reduced in Grade 2 | | | |
| Esterase | Elevated in Grade 1,2,3 | | | |
| RNase | Reduced in Grade 1 and Grade 2 | | | |
| Angiotensin Converting enzyme | Reduced in Grade 3 | | | |
| Myostatin (Growth and differentiation factor 8; GDF8) | Elevated in DMD patients | Serum | ELISA | [16] |
| Interleukin 17 | Elevated in Emery-Dreifuss MD and Limb-Girdle MD 1B | Serum | ELISA | [17, 18] |
| TGF-β2 | Elevated in Emery-Dreifuss MD and Limb-Girdle MD 1B | Serum | ELISA | |
| IL6 | Variable | | | |
| Skeletal troponin I (sTnI), | Elevated in DMD, BMD, LGMD2B | Serum | ELISA | |
| Myosin light chain 3 (My3), | Elevated in DMD, BMD, LGMD2B | | | |
| Fatty acid binding protein 3 (FABP3) | Elevated in DMD, BMD, LGMD2B | | | |
| Muscle-type creatine kinase (CKM) | Elevated in DMD, BMD, LGMD2B | | | |
| Tested marker                                      | Levels (high or low) in DMD patients and/or other MDs | Location (serum/muscle) | Detection method                      | Ref.     |
|---------------------------------------------------|--------------------------------------------------------|--------------------------|---------------------------------------|----------|
| N-terminal α Dystroglycan (αDG-N)                  | Reduced in DMD patients                                 | Serum                    | ELISA                                 | [19]     |
| Fibronectin                                        | Elevated in DMD patients                               | Serum                    | ELISA                                 | [20]     |
| Basic fibroblast growth factor                    | Elevated in DMD patients                               | Serum                    | ELISA                                 | [21]     |
| Cardiac myosin light chain I                       | Elevated in DMD patients (correlated with CK levels)  | Serum                    | Immunoradiometric assay               | [22]     |
| Troponin I, fast skeletal muscle                   | Elevated in DMD                                        | Serum                    | SOMAscan assay “Aptamer-based proteomic technology” | [23]     |
| Carbonic anhydrase 3                               | Elevated in DMD                                        |                          |                                       |          |
| Fatty acid-binding protein, heart                  | Elevated in DMD                                        |                          |                                       |          |
| Troponin I, cardiac muscle                         | Elevated in DMD                                        |                          |                                       |          |
| Creatine kinase M-type                             | Elevated in DMD                                        |                          |                                       |          |
| Mitogen-activated protein kinase 12                | Elevated in DMD                                        |                          |                                       |          |
| Alanine aminotransferase 1                         | Elevated in DMD                                        |                          |                                       |          |
| Myoglobin                                          | Elevated in DMD                                        |                          |                                       |          |
| Fibrinogen                                         | Elevated in DMD                                        |                          |                                       |          |
| Phospholipase A2, membrane associated              | Elevated in DMD                                        |                          |                                       |          |
| Acidic leucine-rich nuclear phosphoprotein 32 family member B | Elevated in DMD                                                   |                          |                                       |          |
| Hepatoma-derived growth factor-related protein 2   | Elevated in DMD                                        |                          |                                       |          |
| 40S Glucose-6-phosphate isomerase ribosomal protein S7 | Elevated in DMD                                                  |                          |                                       |          |
| Heparin cofactor 2                                 | Elevated in DMD                                        |                          |                                       |          |
| Persephin                                          | Elevated in DMD                                        |                          |                                       |          |
| Calcium/calmodulin-dependent protein kinase II α   | Elevated in DMD                                        |                          |                                       |          |
| Malate dehydrogenase, cytoplasmic                  | Elevated in DMD                                        |                          |                                       |          |
| l-lactate dehydrogenase B chain                    | Elevated in DMD                                        |                          |                                       |          |
| Aminoacylase-1                                     | Elevated in DMD                                        |                          |                                       |          |
| Proteosome subunit α type-2                        | Elevated in DMD                                        |                          |                                       |          |
| Tested marker | Levels (high or low) in DMD patients and/or other MDs | Location (serum/muscle) | Detection method | Ref. |
|---------------|----------------------------------------------------|-------------------------|-----------------|------|
| C-X-C motif chemokine 10 | Elevated in DMD | | | |
| cAMP-dependent protein kinase catalytic subunit α | Elevated in DMD | | | |
| Heat-shock 70 kDa protein 1A/1B | Elevated in DMD | | | |
| Proto-oncogene tyrosine-protein kinase receptor Ret | Reduced in DMD | | | |
| Growth/differentiation factor 11 | Reduced in DMD | | | |
| Complement decay-accelerating factor | Reduced in DMD | | | |
| Cadherin-5 | Reduced in DMD | | | |
| Tumor necrosis factor receptor superfamily member 19 L | Reduced in DMD | | | |
| Gelsolin | Reduced in DMD | | | |
| Wnt inhibitory factor 1 | Reduced in DMD | | | |
| Contactin-5 | Reduced in DMD | | | |
| Prolyl endopeptidase FAP | Reduced in DMD | | | |
| Jagged-1 | Reduced in DMD | | | |
| Netrin receptor UNC5C | Reduced in DMD | | | |
| Kunitz-type protease inhibitor 1 | Reduced in DMD | | | |
| Protein SET | Reduced in DMD | | | |
| Disintegrin metalloproteinase domain-containing protein 9 | Reduced in DMD | | | |
| Cell adhesion molecule LI-like | Reduced in DMD | | | |
| Osteomodulin | Reduced in DMD | | | |
| WAP, Kazal, Ig, Kunitz and NTR domain-containing protein 1 | Reduced in DMD | | | |
| Bone sialoprotein 2 | Reduced in DMD | | | |
| Interleukin-34 | Reduced in DMD | | | |
| Neurogenic locus notch homolog protein 3 | Reduced in DMD | | | |
| Cytoplasmic aspartate aminotransferase | Elevated in DMD | Serum | Measuring enzyme activity | [24] |
| mitochondrial aspartate aminotransferase | Elevated in DMD | | | |
| Tested marker                          | Levels (high or low) in DMD patients and/or other MDs | Location (serum/muscle) | Detection method                        | Ref. |
|---------------------------------------|------------------------------------------------------|-------------------------|-----------------------------------------|------|
| Alanine transaminase (ALT)            | Elevated in DMD                                       | Serum                   | ELISA                                   | [25] |
| Aspartate transaminase (AST)          | Elevated in DMD                                       | Serum                   |                                         |      |
| Muscle-specific enolase (MSE, beta beta and alpha beta enolases) | Elevated in DMD and another progressive muscular dystrophies | Serum                   | Enzyme immunoassay                      | [26] |
| Serum carbonic anhydrase III (CA-III) | Elevated in DMD, limb-girdle dystrophy, facioscapulohumeral dystrophy and congenital dystrophy | Serum                   | Enzyme immunoassay                      | [27] |
| Creatine kinase (CK) isoenzymes (MM, MB, and BB) | Elevated in DMD                                       | Serum                   | Sensitive enzyme immunoassay            | [28] |
| Matrix metalloproteinase-9 (MMP-9)    | Elevated in DMD                                       | Serum                   | ELISA                                   | [29] |
| Tissue inhibitors of metalloproteinase-1 (TIMP-1) | Elevated in DMD                                       |                         |                                         |      |
| Osteopontin (OPN)                     | Normal                                               |                         |                                         |      |
| MT-1-MMP                              | Elevated in autosomal dominant EDMD                  | Serum                   | ELISA and zymography                    | [30] |
| MMP2                                  | Elevated in autosomal dominant EDMD and in X-linked EDMD | Serum                   |                                         |      |
| MMP9                                  | Non-significant elevation                            |                         |                                         |      |
| TIMP-1                                | Normal in AD-EDMD                                     | Serum                   | ELISA sandwich immunoassay              | [31] |
| TIMP-2                                | Non-significant decrease AD-EDMD/X-EDMD cases         |                         |                                         |      |
| TIMP-3                                | Reduced in AD-EDMD/X-EDMD                             |                         |                                         |      |
| Carbonic anhydrase III (CA-III, EC 4.2.1.1) | Elevated in DMD, congenital (Fukuyama-type), limb-girdle, also elevated in: polymyositis myotonic dystrophy amyotrophic lateral sclerosis spinal progressive muscular atrophy or Kugelberg-Welander disease and in carriers of DMD | Serum                   | Radioimmunoassay                        | [32] |
| Vitamin D binding protein (GC)        | Reduced in DMD                                        | Serum                   | 2D-HPLC off-line coupled to LC-MALDI-TOF-MS verified with ELISA | [33] |
| Fibulin-1 (FBLN1)                     | Elevated in DMD                                       |                         |                                         |      |
| Tested marker | Levels (high or low) in DMD patients and/or other MDs | Location (serum/muscle) | Detection method | Ref. |
|---------------|--------------------------------------------------|------------------------|-----------------|-----|
| Gelsolin (GSN) | Reduced in DMD | | | |
| Carbonic anhydrase 1 (CA1) | Elevated in DMD | | | |
| Apolipoprotein B100 | Reduced in DMD | | | |
| ALT, AST, LDH, and ALP | Elevated in DMD | Serum | Enzymatic assay | [34] |
| ALT, AST, and LDH | Elevated in BMD and LGMD | | | |
| FSHD and EDMD | lack of abnormal serum enzyme levels | | | |
| ALP | Highly elevated in LGMD2B, Elevated in non-LGMD2B | | | |
| Vascular endothelial growth factor | Highly elevated in BMD, Elevated in Bedridden DMD, spinal muscular atrophy, myotonic dystrophy | Serum | ELISA | [35] |
| Creatine kinase MB fraction | Elevated in DMD | Serum | Multiplex, microsphere-based immune-fluorescent assay | [36] |
| Tissue-type plasminogen activator PLAT | Slightly elevated in DMD | | | |
| Myoglobin | Slightly elevated in DMD | | | |
| Epidermal growth factor | Slightly elevated in DMD | | | |
| Chemokine (C-C motif) ligand 2 | Slightly elevated in DMD | | | |
| CD 40 ligand | Slightly elevated in DMD | | | |
| Vitronectin | Slightly elevated in DMD | | | |
| Carboxyterminal propeptide of type I procollagen | No significant alteration | Serum | Radioimmunoassay | [37] |
| Aminoterminal propeptide of type III procollagen | No significant alteration | | | |
| Laminin P1 | No significant alteration | | | |
| Creatine kinase | Elevated in DMD and BMD | Serum | Measuring enzyme activity | [38] |
| Pyruvate kinase | Elevated in DMD and BMD | | | |
| Myosin light chain—3 | Elevated in DMD | Serum | affinity proteomics-based screening approach using an antibody suspension bead array | [39] |
| Carbonic anhydrase III | Elevated in DMD | | | |
| Electron transfer flavoprotein A | Elevated in DMD | | | |
of the muscles and thus, they could be measured to indicate the extent of muscular dystrophy and can serve as good candidates for diagnostic purposes (Table 3).

2.3 Muscle imaging

Magnetic resonance imaging (MRI) is now used to visualize the composition of skeletal muscles and detect structural abnormalities in DMD patients [61]. The produced images can reveal the presence of fat infiltration of muscle tissue, a characteristic consequence of DMD, and thus, can be used for monitoring disease progression and response to treatment [62].

2.4 Genetic diagnosis

2.4.1 RFLP

Detecting the mutation, especially non-sense point mutations, in the 2.4 Mb gene represents a challenging task. In this context, restriction fragment length polymorphism (RFLP) analysis could be used by digesting the genomic DNA using specific restriction endonucleases followed by Southern blotting using DMD-specific DNA probes (genomic or cDNA probes). At 1985, Bamkan et al. developed 11 RELP markers that are present in the X chromosome and can be used for diagnosis. However, RFLP can detect only small percentage of the mutation and hence it cannot be used as gold standard technique in the diagnosis process [63–65].

2.4.2 Multiplex PCR

Multiplex PCR is one of the modified PCR protocols that allows the co-amplification of multiple products using different primer pairs that specially bind complementary regions in the target segment. This method showed a great potential
| microRNA      | Status   | Disease                  | Location                        | Ref.       |
|--------------|----------|--------------------------|---------------------------------|------------|
| miR-133a     | Upregulated | DMD, BMD, LGMD, FSHD     | Serum and skeletal muscles      | [44–47]   |
| miR-206      | Upregulated | DMD, BMD, LGMD, FSHD     | Serum and skeletal muscles      |            |
| miR-1        | Upregulated | DMD, BMD, LGMD, FSHD     | Serum and skeletal muscles      |            |
| miR-499      | Upregulated | DMD                       | Serum                           | [45]       |
| miR-208a     | Upregulated | DMD                       | Serum                           |            |
| miR-208b     | Upregulated | DMD                       | Serum                           |            |
| miR-95       | Upregulated | DMD                       | Serum                           | [48]       |
| miR-539      | Downregulated | DMD                    | Serum                           |            |
| miR-30c      | Upregulated | DMD                       | Serum                           | [49]       |
| miR-181a     | Upregulated | DMD                       | Serum                           | [50]       |
| miR-21       | Downregulated | DMD                    | Urine                           |            |
| miR-29       | Downregulated | DMD                    | Urine                           |            |
| miR-23       | Downregulated | DMD                    | Urine                           |            |
| miR-181a     | Upregulated | DMD                       | Serum                           | [51]       |
| miR-4538     | Upregulated | DMD                       | Serum                           |            |
| miR-4539     | Upregulated | DMD                       | Serum                           |            |
| miR-606      | Upregulated | DMD                       | Serum                           |            |
| miR-454      | Downregulated | DMD                    | Serum                           |            |
| miR-483      | Upregulated | DMD                       | Serum                           | [52]       |
| hsa_miR_146b, hsa_miR_368, hsa_miR_381, hsa_miR_487b, hsa_miR_495, hsa_miR_376a, hsa_miR_299.5p, hsa_miR_155, hsa_miR_382, hsa_miR_199a, hsa_miR_379, hsa_miR_335, ambi_miR_5021, hsa_miR_432, hsa_miR_199b, hsa_miR_369.5p, hsa_miR_21, hsa_miR_34a, hsa_miR_199a*, hsa_miR_154, hsa_miR_221, hsa_miR_214, hsa_miR_518a.2*, hsa_miR_409.3p, hsa_miR_452, ambi_miR_2537, hsa_miR_127, hsa_miR_493.3p, hsa_miR_130a, ambi_miR_4983, ambi_miR_13145, hsa_miR_148a, hsa_miR_210, hsa_miR_485.5p, hsa_miR_299.3p, hsa_miR_134, hsa_miR_222, hsa_miR_181d, ambi_miR_13258 | Upregulated | DMD                       | Serum       | [53]       |
| hsa_miR_423, hsa_miR_361, hsa_miR_197, hsa_miR_92, hsa_miR_26a, ambi_miR_7075, hsa_miR_30b, hsa_miR_30e.5p, hsa_miR_29a, ambi_miR_13156, hsa_miR_30a.5p, hsa_miR_193b, hsa_miR_331, hsa_miR_486, hsa_miR_30d, hsa_miR_29b, hsa_miR_101, hsa_miR_30c, hsa_miR_22 | Downregulated | DMD                       | Serum       |            |

Table 2.
List of different microRNAs that could be used as potential biomarkers in the diagnosis of DMD.
| Tested marker | Levels (high or low) | Location (serum/muscle) | Ref. |
|---------------|---------------------|-------------------------|-----|
| 24,25(OH)2D3 | Reduced in DMD      | Serum                   | [54]|
| 1,25(OH)2D3 | No change           |                         |     |
| 25(OH)D3    | No change           |                         |     |
| Creatinine   | Reduced in DMD, BMD, LGMD2A and LGMD2B | Serum | [55]|
| Imidazole acetic acid | Reduced in DMD and LGMD2B |     |     |
| 5α Dihydrotestosterone glucuronide // androsterone glucuronide // Etiocholan-3alpha-ol-17-one 3-glucuronide | Reduced in DMD |     |     |
| DL-p-Hydroxyphenyllactic acid // Isohomovanillic acid | Reduced in DMD |     |     |
| Creatine     | Elevated in DMD, DM1, LGMD2Aand LGMD2B |     |     |
| Guanidinoacetic acid | Reduced in DMD, BMD, DM1 and LGMD2A |     |     |
| p-Coumaric acid | Reduced in DMD |     |     |
| Citrulline   | Reduced in DMD      |                         |     |
| 5-Methoxyindoleacete // Indoleacetic acid | Reduced in DMD |     |     |
| L-Aspartic acid | Reduced in DMD |     |     |
| Ornithine    | Reduced in DMD      |                         |     |
| 2-Hydroxycaproic acid | Reduced in DMD |     |     |
| L-Serine     | Reduced in DMD      |                         |     |
| Dehydroisoandrosterone 3-sulfate | Reduced in DMD |     |     |
| Erythrose    | Reduced in DMD, BMD, FSHD |     |     |
| Glutamine    | Reduced in DMD, BMD, LGMD-2B, FSHD and elevated in DM-1 | Serum | [56]|
| Acetate      | Elevated in DMD, BMD, FSHD, LGMD-2B and DM-1 |     |     |
| Tyrosine     | Elevated in BMD     |                         |     |
| Lysine       | Reduced in FSHD, LGMD-2B and DM-1 |     |     |
| Citrate      | Reduced in FSHD     | Elevated in LGMD-2B    |     |
| Lactate      | Reduced in LGMD-2B  |                         |     |
| Histidine    | Reduced in FSHD     |                         |     |
| Serum creatinine | Elevated in BMD | Decreased in DMD | Serum | [57]|
| 3-Methylhistidine | Reduced in DMD and LGMD |     |     |
| N epsilon,N epsilon-dimethyllysine | No alteration |     |     |
| N epsilon, N epsilon, N epsilon-trimethyllysine | No alteration |     |     |
| NG,NG-dimethylarginine | Elevated in DMD and LGMD |     |     |
| NG,N’G-dimethylarginine | No alteration |     |     |
to diagnose DMD since the multiple primers covered commonly mutated locations across the entire DMD gene, hotspot regions [66–68]. This technique was first developed by Chamberlain et al. [69] through utilization of 6 primer sets that were modified to 9 sets and later to 10 by Beggs et al. [70] (to amplify exons 45, 48, 19, 17, 51, 8, 12, 44, 4). If no amplification take place, this will confirm deletion of this exon. The developed primer sets were successfully able to detect deletion mutations in the hot spot regions. One of the limitations of such technique was its inability to diagnose all cases with other deletion mutation in other regions, or patients with SNPs or deep intronic mutation.

2.4.3 Multiplex ligation dependent probe amplification (MLPA)

In order to simultaneously investigate the status of the 79 exons of the DMD gene, a PCR-based technique was developed to diagnose DMD in a multiplex PCR reaction. The assay uses multiple probes to target different exons in the DMD gene. Each probe consists of two oligonucleotides; one consists of a 5′-adapter and a 3′-exon-specific region, and vice versa for the second oligonucleotide, where the 3′-end of the first primer and the 5′-end of the second hybridize to two adjacent nucleotides in the target exon. Hybridized probes are subjected to ligation reaction, thus, only hybridized probes get ligated, amplified by PCR using adapter-specific primers and separated by capillary electrophoresis. Positive PCR product indicates the presence of the target exon, while deleted exon(s) will not produce corresponding product(s). In this assay, it is also possible to detect exon duplication, which will be detected as larger peak [71, 72]. However, this assay cannot detect non-sense nor in/del point mutations.

2.4.4 Microarray

High-throughput methods such as DNA microarrays were adopted using specific oligonucleotide probes that cover the entire 2.4 mbp DMD gene (targeted high density comparative genomic hybridization (CGH) microarray). Such method could effectively be used to detect known as well as novel intronic mutations [73–75].

2.4.5 Next generation sequencing (NGS)

The development of NGS and the massively parallel sequencing allowed the sequencing of 100 s of millions of independent short reads (100–300 bp) at the same time. Such approaches generate huge amount of data that uses bioinformatic analysis for annotations and alignments of the generated sequences to produce sequence information for large genes such as DMD and titin and delineate the exact locations of mutations [76]. One major advantage of resorting to NGS for DMD diagnosis is that it could be used for the analysis of MLPA-negative samples that could have small deletions/duplications or single nucleotides variants [77].

| Tested marker | Levels (high or low) | Location (serum/muscle) | Ref. |
|---------------|----------------------|-------------------------|------|
| Tetranor PGDM (PGD2 metabolite) | Elevated in DMD | Urine | [59] |
| Nitric oxide | Reduced in DMD | Serum | [60] |

Table 3. List of metabolites that can be used as potential biomarkers in DMD diagnosis.
Also, RNA sequencing by NGS (RNA-seq) is very useful in detecting the splicing pattern that occur in the DMD transcripts in the muscles through different developmental stages, muscle breakdown or muscle regeneration [78–80].

2.4.6 Muscle biopsy

In some cases, muscle biopsy is required to fully characterize the phenotypic effect of the mutation. The muscle tissue is used in immunoassays, using different antibodies targeting different regions of dystrophin protein (C-terminal, Rod and N-terminal domains), such as western blotting [81–83] or immunohistochemistry [83]. Uchino et al. [83] developed a multiplex western blotting assay to analyze the expression of other muscle proteins like dysferlin, merosin, different forms of sarcoglycan (alpha, beta, gamma, delta), and calpain in addition to dystrophin protein, due to the frequent epigenetic changes incited in these proteins as a consequence to the alteration in dystrophin expression.

3. Conclusion

In this chapter, we have presented a comprehensive review for the methods that have been used in the diagnosis of DMD. Because of the nature of the disease, an X-linked disorder, DMD symptoms of the first affected male births of asymptomatic carrier mothers are usually go unnoticed until the age of 5, where the progressive muscle weakness becomes obvious and fibrotic fatty tissue infiltration is prominent. However, it is well known that early diagnosis and treatment results in better disease management and improve the clinical outcomes. In fact, some studies have pointed out to the fact that initiating corticosteroids therapy early enough has delayed the loss of ambulation in most cases by about 2 years [84]. In addition, with the fast-paced progress in molecular/personalized therapies such as exon-skipping and gene-editing based approaches, precise diagnosis and mutation detection becomes a necessity. Moreover, the genetic testing has been extensively used in prenatal diagnosis and has assisted in decreasing disease burden by aborting affected male pregnancies. In a retrospective study conducted in the Netherlands, the authors reported 145 abortions of male fetuses over 26 years that had been found to carry inactivating mutations of the DMD gene [85]. Furthermore, identifying female carriers, is gaining momentum to decrease the possibility of giving birth to affected males and consequently contributes to the overall disease management.
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