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Review

Molecular genetic testing and diagnosis strategies for dystrophinopathies in the era of next generation sequencing

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ABSTRACT

Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are X-linked recessive, inherited neuromuscular disorders, caused by pathogenic variants in the dystrophin gene that encodes the dystrophin protein. A number of mutations have been identified in the past years, producing dystrophin diversity and resulting in mild to severe phenotypes in patients. Mutations in the dystrophin gene can be characterized by laboratory testing to confirm a clinical diagnosis of DMD/BMD. Traditional genetic diagnostic strategy for DMD/BMD involves the initial detection of large mutations, followed by the detection of smaller mutations, where two or more analytical methods are employed. With the development of next generation sequencing (NGS) technology, comprehensive mutational screening for all variant types can be performed on a single platform in patients and carriers, although further optimization and validation are required. Furthermore, the discovery of cell-free fetal DNA (cffDNA) in maternal plasma provides basis for noninvasive prenatal diagnosis of DMD/BMD. Here, we discuss the correlation between genotype and phenotype, the current methods of molecular genetic testing and genetic diagnostic strategy for probands and female carriers of DMD/BMD, the diagnostic ability of a comprehensive targeted NGS strategy and the possibility of it replacing conventional methods.

1. Introduction

Pathogenic variants in the dystrophin gene (DMD, MIM #300377), encoding the dystrophin protein, lead to inherited neuromuscular diseases: dystrophinopathies [1–3]. These genetic disorders are progressive, and are classified as Duchenne muscular dystrophy (DMD) (MIM #310200), which is a severe form, characterized by early-onset (before age 5 years in childhood) and Becker muscular dystrophy (BMD) (MIM #300376), which is a slowly progressive and milder form. Both neuromuscular disorders are compatible with X-linked recessive inheritance and present primarily in male patients [1,3]. Recently, it was demonstrated that the average global DMD incidence is now closer to ~1:5000, with a drop in the reported average incidence, which was previously 1:3500 [4,5]. However, BMD is estimated to have an incidence between 6 and 20% of that of DMD [6].

The dystrophin gene, located on the short arm of the X chromosome (X-p21.2) [7], is the largest known gene in the human genome, spanning 2.5 Mb (10 times bigger than any other gene). Its coding sequence, with a length of 11.3 kb, contains 79 exons and 7 unique promoters and introns that make up 99.5% of the DMD gene [8]. Since the identification of the locus of the DMD gene in 1986 by independent experimental approaches [9,10], clinicians have focused largely on the molecular diagnosis of DMD/BMD, which forms the basis of treatment. Therapies under investigation, pregnancy management for female carriers, genetic counseling for risk to family members and family planning, prenatal testing and preimplantation genetic diagnosis (PGD) are all dependent on the correct diagnosis of the disorders. Numerous diagnostic testing methods are available for DMD mutation testing, including multiplex polymerase chain reaction (PCR), multiplex ligation-dependent probe amplification (MLPA), array comparative genome hybridization (array CGH), PCR-based Sanger sequencing, and others [11]. In the past few years, advances in next generation sequencing (NGS) technologies and improvements made in informatic techniques have improved sequencing accuracy, and greatly increased the speed of generating larger amounts of clean sequencing data. Massively parallel sequencing of multiple genes has successfully entered the clinical setting to identify pathogenic variants for rare Mendelian genetic diseases, though many of the genetic variations remain undetected till date [12,13].

In this review, we discuss the correlation between genotype and

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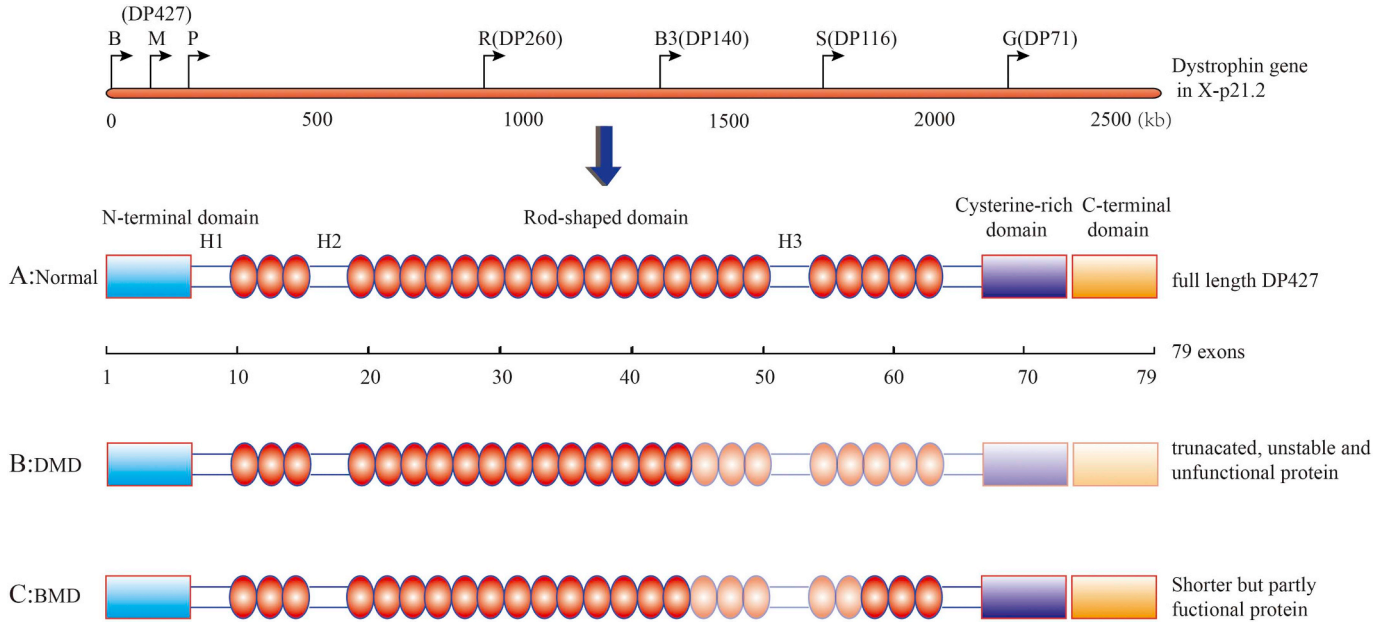

Fig. 1. Dystrophin gene and structural domains of dystrophin.
The dystrophin gene, containing 79 exons, is located at X-p21.2. Various promoters drive the expression of dystrophin in specific cells. The arrows of B, M, P represent the promoters in brain (B), muscle (M) and Purkinje cells (P), encoding full-length protein (DP427). The arrows of R, B_3,S, G represent the promoters of short isoforms of dystrophins: DP260, DP140, DP116 and DP71, respectively. (A) In normal condition, the full-length protein product (DP427), encoded by 79 exons, consists of four domains: N-terminal domain, internal rod-shaped domain, cysteine-rich domain and carboxy-terminal domain.
(B) In patients with DMD, out-of-frame large deletions and duplications, or small mutations result in the expression of truncated, unstable and unfunctional protein, which is rapidly degraded. (C) In mild BMD patients, shorter protein is expressed, with part function because of in-frame mutations.

phenotype of patients with DMD/BMD or female carriers of these disorders. Next, we outline the methods of molecular genetic testing, discuss the advantages and limitations of targeted NGS for genetic testing of DMD and the possibility that it replaces conventional dosage screening and Sanger-based sequencing. Finally, we outline the genetic diagnosis strategy for proband and female carriers of DMD/BMD and discuss the possible clinical indications of noninvasive prenatal tests and diagnosis based on NGS, for DMD/BMD.

2. Genotype and phenotype: an overview

Because of its large size, the mutation rate for DMD gene is relatively high among the human genes. Furthermore, a variety of mutations have been identified over the past years, producing dystrophin diversity and resulting in different phenotypes in the patients [14]. (Fig. 1) Large mutations (covering ≥1exon) are the most common type of mutations accounting for ~79% of total mutations, including deletions (~68%) and duplications (~11%). Small mutations (< 1exon, ~20%) are also observed, including small deletions and insertions, and point mutations [15,16]. Some examples for differences at gene level between DMD and BMD are shown in Fig. 2.

Large deletions and duplications of different sizes, occurring at different positions can be observed anywhere in the DMD gene [14–16]. The effects of these mutations on the phenotype are governed by whether the deletion or duplication disrupts the translational reading frame or not [8,17]. In patients with DMD, the truncated, unstable, and unfunctional protein is expressed and rapidly degraded. In patients with mild BMD, shorter protein is expressed with partial function because of in-frame mutations. Reading frame rules have been successful in explaining the phenotypic differences in ~90% cases [14–16]. However, this rule has failed to explain close to 10% cases [11]. No correlation was observed between the size of the deletion or duplication and the phenotype in these cases [8]. The size and location of in-frame mutations may influence the phenotype to some degree. [11] Moreover, it has been reported that an intact cysteine-rich domain is required for dystrophin function [18]; therefore, deletions in this domain, have always been found to cause DMD [11,14]. In addition, loss of large parts of the rod domain, comprising the N-terminal and C-terminal regions of dystrophin, often causes BMD [8], while in-frame deletions of all four actin-binding domains, with part of the central rod domain, are known to cause DMD [11,14,19]. Removing almost all of the central rod domain of dystrophin resulted in DMD, suggesting that threshold effect for deletion size should be noted [19]. Except for contiguous deletion or duplication events, complex genomic rearrangements, consisting of two or more breakpoint junctions, have been demonstrated in DMD. Though infrequent, these mutations are being increasingly characterized at the nucleotide level [19–21]. Small mutations, consisting of frameshift mutations, nonsense mutations, missense mutations, splice-site mutations and mid-intronic mutations, are distributed throughout the DMD gene [15,16]. Small frameshift lesions can lead to non-functional dystrophins and result in severe DMD [11]. However, patients with BMD are also known to harbor small frameshift lesions. The frequency of frameshift small lesions differs in DMD and BMD phenotype. Higher frequency is predicted to lead to severe DMD phenotypes, whereas lower frequency is associated with a mild Becker phenotype. Most of the small frameshift mutations involve about one to four nucleotides [15].

Close to 50% of small mutations and ~10% of all mutations are nonsense mutations [15,16], associated with both, the severe DMD and milder BMD phenotypes [22]. Nonsense mutations that occur in in-frame exons, resulting in BMD, are possibly due to a point mutation induced exon skipping mechanism, i.e. exclusion of the nonsense mutation-containing exon [11,22]. Therefore, nonsense stop codon read-through therapy is an alternative therapy for genetic disorders, caused by nonsense mutations [23]. Missense mutations can cause DMD, BMD, or X-linked cardiomyopathy in only a small percentage of patients. Nearly one-half of reported missense mutations, resulting in disease, are located in the actin-binding domain 1 [24].

The prevalence of splice-site mutations was estimated to be 3.1–6.3% of all DMD mutations and 7.3% of all BMD mutations. Most of

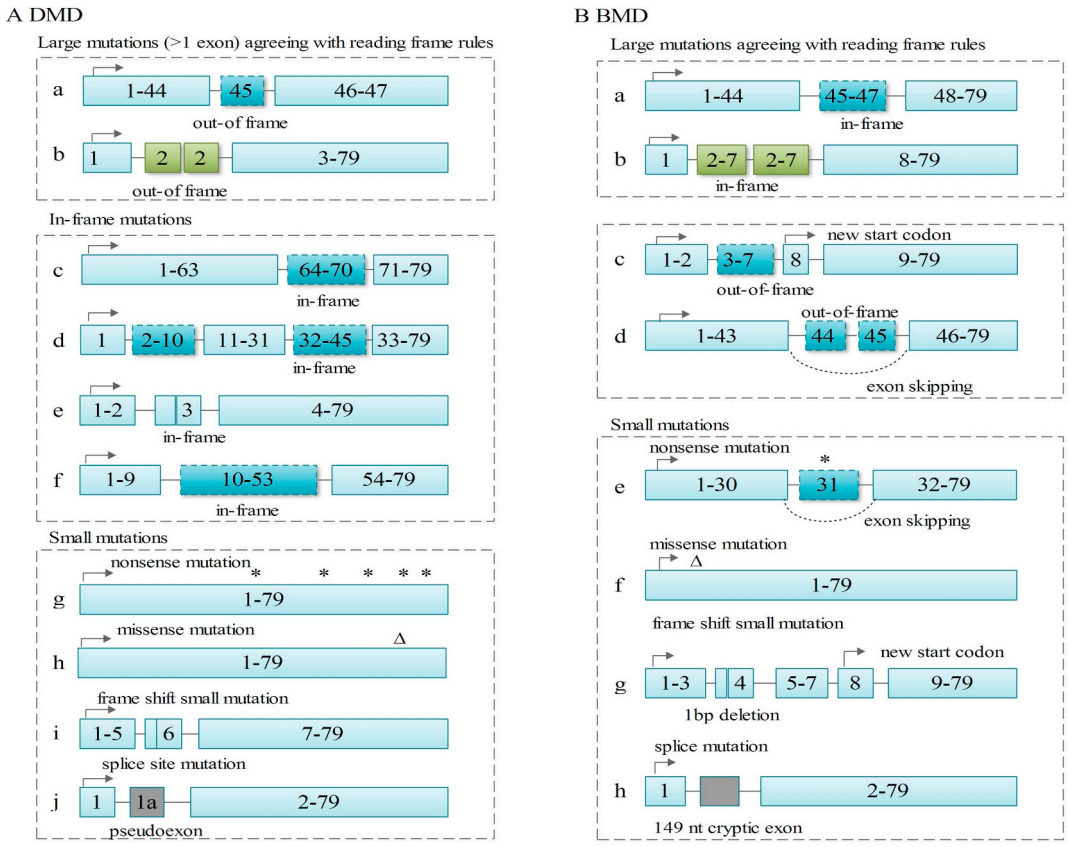

Fig. 2. Some examples for the differences at gene level of DMD and BMD.
Large variations in mutations of dystrophin gene were identified, resulting in different phenotypes in patients, including deletions, duplications, small deletion and insertion and point mutations. In most cases, the effect on phenotype depends on whether the mutation disrupts the reading frame or not. Cases not fulfilling the reading frame rule do exist, where different mechanisms function. Some examples are shown in fig. A) DMD: a) b) out-of-frame large deletions and duplications, e.g., deletion of exon 45 and duplication of exon 2; c) d) e) f) in-frame mutations, e.g., deletion of exons 64–70 coding cysteine-rich domain; deletion of exons coding all four actin-binding domain; small in-frame deletions disrupting 5′ region and deletions of exons removing almost all central rod domain; g)nonsense mutations widely distributed; h)missense mutation, e.g., missense mutation located in cysteine-rich domain;i) frame-shift small mutation (del/ins/dup), e.g., c.372delG in exon 6; j) splice-site mutation, e.g., deep intron mutation leading to inclusion of the preudoexon, containing premature termination codons. B) BMD. a) b) in-frame large deletions and duplications, e.g., deletion of exons 45–47 and duplication of exons 2–7; c) d) out-of-frame mutations, the examples are deletion of exons 3–7 leading to an alternative new start codon in exon 8 and frame shift deletion of exon 45, inducing exon skipping of exon 44;e) nonsense mutation, e.g., missense mutation of c.4250 T > A in exon 31, inducing exon skipping of exon 31; f) missense mutation, e.g., missense mutation located in N-terminal domain; g) frame-shift small mutation (del/ins/dup), e.g., 1 bp deletion of c.193delG in exon 4 leading to a new start codon in exon 8; h) splice-site mutation, e.g., deep intron mutation of c.31 + 36,947 G > A leading to two transcripts: full-length mRNA, out-of-frame mRNA, with an inserted 149-nt cryptic exon.

the reported splice-site mutations generally result in exclusion of single or multiple exons from the mRNA. Mutations at conserved AG and GT sites are known to occur more frequently than less conserved nucleotides or deep intronic mutations. The donor splice site is found to be more frequently mutated than the acceptor site. Deep intronic point mutations located at > 10 bp from the canonical splice site, often create novel splice sites, resulting in the inclusion of intronic sequences as pseudo exons or cryptic exons, within the mRNA [25]. The difference in phenotype may be due to variable splicing efficiency of the novel splicing signal. In patients with BMD, inefficient splicing at the intronic cryptic regions, often results in the presence of wild-type dystrophin transcript [15,26].

Most of the DMD female carriers have no clinical presentation, however, some clinical studies estimated that female carriers present with clinical features, including some degree of weakness and/or cardiomyopathy [27].Moreover, in a few cases, the affected females have severe clinical manifestations, similar to that seen in affected males [28]. A previous study has reported the incidence of skeletal muscle damage among female carriers to be 2.5%–19%, and the incidence of dilated cardiomyopathy to be 7.3%–16.7% for DMD and 0%–13.3% for BMD [27]. Skewed X-chromosome inactivation (XCI) may be one mechanism as skewed XCI patterns have been shown to determine the DMD phenotype in female carriers. DMD carriers with moderate/severe muscle damage, present a moderate or extremely skewed XCI, while DMD carriers with mild muscle damage exhibit a random XCI [27,29]. Some females who have X; autosome translocations, disrupting the X chromosome within band p21 and have been reported to develop DMD symptoms [30]. Moreover, females with Turner syndrome (45,X) have been observed to manifest DMD, if the remaining X chromosome carries a dystrophin mutation [31]. Uniparental isodisomy of the affected X chromosome is an additional mechanism in karyotypically normal females affected with DMD [32]. In addition, compound heterozygotes, such as contiguous exon deletions, exonic deletions and a splice-site mutation, may also cause DMD in females, indistinguishable from DMD in male patients [33,34]. It should be noted that a small number of women who are not carriers may still be able to transmit a faulty DMD gene to their offspring. The phenomenon is called germline mosaicism for mutation in the DMD gene, which might have occurred during mitosis, in early germline proliferation [35]. Germinal mosaicism deletions have been reported in at least 11.6% of families of DMD patients [36]. The question of germline mosaicism is important for genetic counseling and estimating the recurrence risk in DMD families [35].

Table 1
Published studies using NGS strategy for molecular diagnosis of dystrophinopathies.

Study and year	Strategy	Samples	Target-enrichment strategy and reagent	Sequencing platform	Data analysis	Results
Alame et al [55]	Targeted NGS for all coding regions and exon-intron boundaries of the DMD gene	DNA samples of 6 patients for SNV analysis and DNA samples of 50 patients for CNV analysis (24 males and 26 females)	Multiplex PCR, DMD MASTR Kit (Multiplicom)	GS-Junior instrument (Roche) and MiSeq instrument (Illumina).	MiSeq Reporter (Illumina) and SeqNext (JSI Medical Systems)	Testing of six patient DNA samples carrying 72 SNVs in the DMD gene showed an experimental sensitivity of > 99.9% with both Software. Analytical specificity was > 98%. Analysis of 50 DNA samples identified 28 CNVs, sensitivity and specificity of > 99.9%.
Lim et al [49]	Sequencing of whole dystrophin gene and other muscular-dystrophy-related genes	25 male patients: 16 patients for SNV analysis and 9 patients with a known large deletion/duplication.	Multiplexing strategy, SureSelect Target Enrichment System Kit (Agilent Technology)	Illumina Genome Analyser IIx	Genomic Short-read Nucleotide Alignment Program (GSNAP) alignment tool	Pathogenic small mutations were identified in 15 of the 16 patients. The deleted or duplicated exons in the 9 patients were accurately predicted with known mutations. The breakpoint junction was identified.
Wang et al [52]	Whole dystrophin gene (2.10 MB) sequencing using NGS	26 male patients: 21 had known large deletion/duplications and 5 did not have detectable large deletion/duplications	Target enriched, DMD whole gene enrichment kit (MyGenostics)	Illumina HiSeq 2000	Laboratory developed pipeline	Five small variants, including 4 novel mutations, were identified. The large deletion/duplications and breakpoints in the five randomly chosen subjects were precisely identified.
Wei et al [53]	Targeted NGS, all exons including the 100 bp flanking region	A total of 89 patients, 18 female carriers and 245 non-DMD patients	Target enriched, custom Sequence Capture 2.1 M Human Array from Roche NimbleGen	Illumina HiSeq2000	Illumina Pipeline software (version 1.3.4) and laboratory developed pipeline	99.99% specificity and 98.96% sensitivity for CNVs and 100% accuracy for the SNVs. Breakpoints located within exonic regions, were identified.
Okubo et al [54]	Targeted NGS, 99.6% of the exonic region, including 30 bp flanking region	67 cases: 37 with deletions/duplications and 30 with small mutations or indels	Multiplex primer pools, Ion AmpliSeq Designer software	Ion PGM using 318 chip	NA	Diagnosed 92% of patients. Small insertions in consecutive bases could not be detected.

3. Conventional methods of molecular genetic testing for DMD gene variants

Laboratory analysis of dystrophin gene can be used to characterize the types of mutations and confirm a clinical diagnosis of DMD/BMD. Multiplex PCR is used to identify common dystrophin deletions [37] and is still used in some laboratories as it is easy to perform, as well as for its cost-efficiency [11]. The ability to scan deletions at a hemizygous locus, but incompetence to fully characterize a deletion, due to its inability to cover the whole gene and failure to identify duplications or female carriers, are the main drawbacks of this technique [38]. Furthermore, with this technique, it is difficult to identify if the deletion detected is in-frame or out-of-frame, and hence exon skipping therapy cannot be applied. Moreover, boundaries of the deletion cannot be determined [11].

MLPA was first described by Schouten et al. [39], and it was subsequently applied for semi-quantitative detection of deletions and duplications in the DMD gene [40]. MLPA analysis can simultaneously screen all 79 DMD gene exons for deletions and duplications in DMD/BMD patients and carriers [40]. However, the technique does not detect most of the small mutations, inversions, translocations, and copy number variants (CNVs) outside the probe target sequence (www.mlpa.com). Moreover, it does not provide information regarding the intronic breakpoints [11]. On the other hand, a number of studies have revealed aberrant MLPA results, such as false-positives of duplicates, due to contamination [41], false-positive events, due to sequence variations at the probe-binding sites [42,43], false-positive results caused by the combination of a low melting temperature, due to MLPA probes and cytosine-adenine(CA) mismatch. Therefore, confirmatory testing of single-exon deletions, detected by MLPA, is required [43,44].

ArrayCGH is an alternative technique for detection of copy number losses and gains within the dystrophin gene. In this method, thousands of exonic and intronic oligonucleotide CGH probes, ranging from 45 to 60 bases, with isothermal Tm, are used. The vast number of probes permits all the unique sequences in this 2.2 Mb DMD gene to be interrogated, thereby enabling comprehensive detection of not only all deletions and duplications, but also reveals approximate intronic boundaries of the mutation event. High density arrayCGH may eliminate false-positives caused by sequence variants on probe- or primer-binding sites, since several oligos detect a single exon [45,46]. However, it cannot detect balanced translocations or inversions, and may miss CNVs < 30 kb with a resolution of ~50–100 kb [47].

PCR-based Sanger sequencing is still the “gold standard” for detecting sequence variants. Due to the absence of particular point mutation hotspots, and specific mutations carried in most/vast majority in each affected family, Sanger sequencing analysis of the dystrophin gene requires sequencing of all 79 exons, promoters and known intronic mutations. Therefore, direct sequencing is considered not only more labor intensive, but also expensive and time consuming [48].

In most laboratories, separate methods are employed for the detection of large and small mutations [49]; however, some laboratories do not conduct sequencing for detection of small mutations, due to the high costs involved [50]. Some variants, such as deep intronic mutations, or complex rearrangements, may not be straightforward to identify by traditional DNA-based mutation screening techniques [45].

4. Targeted NGS for DMD gene variants testing

Diagnostic tests based on NGS, used for diagnosis of rare genetic disorders, include sequencing of whole single genes, panels, clinical exomes and sequencing of all protein-coding genes by whole-exome sequencing (WES) and entire genomes by whole-genome sequencing (WGS) [47]. The application of NGS technology to the genetic diagnosis of dystrophinopathies has been reported. In a work aiming to identify novel intellectual disability genes using WES, an incidental finding of a missense mutation in the dystrophin gene in a patient prompted a

Table 2
Advantages and limitations of targeted NGS for molecular diagnosis of dystrophinopathies.

Advantages
Can simultaneously detect SNVs and CNVs
More cost effective than combination of two methods
Whole DMD gene sequencing can detect deep intronic SNVs and identify breakpoints
Technical and analysis time required is less compared to combination of two methods
Reduces the number of muscular biopsies as specific indication
Can simultaneously sequence multiple muscular-dystrophy-related genes
Limitations
Difficult to standardize the testing procedure and bioinformatics pipeline of different laboratories
Targeted NGS for only coding regions cannot detect variants in deep intronic regions and complex SVs
Targeted NGS for only coding regions cannot identify breakpoints in intronic regions, necessary for personalized treatments
Transcript analysis would still be required in the presence of a contradiction between phenotype and genotype

clinical reevaluation of the subject, and the clinical phenotype indeed was compatible with the diagnosis of DMD [51].

Targeted NGS for dystrophin gene variants is particularly useful for diagnosing dystrophinopathies and have been widely published [49,52–55]. The technology involves sequencing of the whole DMD gene [21,49,52,56], including its 79 exons and the flanking regions [52–55]. Some published studies using targeted NGS strategy for molecular diagnosis of dystrophinopathies are summarized in Table 1. Though NGS technology is often not reliable for detecting small CNVs, such as exon deletions or duplications [47], studies have demonstrated that targeted NGS strategy has the capacity to become a comprehensive mutational search tool for single nucleotide variants (SNVs) and CNVs on a single platform (the one-for-all test). In comparison, currently used diagnostic strategies make use of two or more analytical methods because of the complex mutational spectrum and the large size of the gene in single disease entities for dystrophinopathies. It provides a number of key advantages, compared to traditional genetic testing (Table 2). First, targeted NGS is able to identify CNVs, even for those single exon heterozygous duplications in female carriers, which are otherwise difficult to detect [55]. Therefore, it has the capacity to replace both MLPA and chromosomal microarrays in the clinical setting. Second, because ~20% dystrophinopathies might be caused by SNVs or small insertions and/or deletions (indels), the combination of MLPA and Sanger sequencing is necessary in patients without identified CNVs. However, this strategy is considered more labor intensive and expensive than the NGS strategy. Because sequencing companies are developing the NGS technology further, the cost of NGS may ultimately outweigh the current cost benefits of MLPA for CNVs. Third, although Sanger sequencing is still widely accepted as the gold standard for DNA sequencing, the sensitivity of NGS for SNVs and indels might prove to be higher, particularly for detecting female heterozygous variants [47,57]. Moreover, the technical and analysis time required for NGS is less compared to the combination strategy. These factors not only enable patients living with DMD/BMD to receive accurate genetic diagnosis and available therapy but also allows them and their families to seek prompt genetic advice and make informed reproductive choices as early as possible. Fourth, although DMD/BMD is the commonest and most serious neuromuscular disorder, muscular dystrophies are a group of inherited neuromuscular diseases sharing similar clinical features, characterized by variable degrees of progressive muscle weakness with or without mental retardation [58]. Therefore, clinicians can request simultaneous sequencing of muscular-dystrophy-related genes, rather than single dystrophin gene sequencing by NGS technology. For example, Lim et al. performed targeted sequencing on 26 genes involved in muscular dystrophies [49]. Fifth, NGS strategy can also reduce the need for an invasive and expensive muscle biopsy (MB). Even with targeted NGS only for coding regions and exons-introns boundaries of the DMD gene, MB is not necessary before performing an SNV screening [55]. Sixth, compared to other gene variants related to genetic disorders, inherited in an autosomal manner, hemizygosity of DMD gene variants and no reported pseudogenes, makes target enrichment and cut-off threshold setting more feasible [49].

Targeted NGS for DMD variants testing has its own limitations (Table 2). First and foremost, because of the use of various DNA target enrichment and library preparation methods, sequencing platforms and the bioinformatic pipelines, it is difficult to standardize the testing procedure of different laboratories [49]. In addition, short-read NGS platform does not reliably detect complex structural variants (SV) such as insertions, inversions, and translocations due to the average 100–300 bp fragment reads. Although research has identified two complex genomic rearrangements in the DMD gene by whole dystrophin gene sequencing, detection of SVs is currently still challenging. Targeted NGS for only coding regions, along with the flanking regions, also has its limitations in the detection of variants in deep intronic regions and identification of breakpoints located in introns. The deep intronic SNVs may create novel splice sites and lead to pseudo exons or cryptic exons in the transcript. The precise information on the location of breakpoints is important for personalized treatments, such as exon skipping treatment. Whole dystrophin gene sequencing technologies have been demonstrated for detection of deep intronic variants and determination of breakpoints [52,53].

In the near future, compared to the current traditional method, which involves a combination of MLPA and Sanger sequencing, targeted NGS assays will reduce the time and cost burden of the genetic diagnosis of DMD/BMD. Moreover, it can be expected to replace the existing detection method. Currently, further optimization and validation are required because of the cost involved, analytical challenges and standardization of procedures.

5. Genetic diagnosis strategies for proband and female carriers of DMD/BMD

When signs or symptoms are consistent with DMD/BMD, and creatine kinase concentrations are increased in a male patient, irrespective of family history, confirmation of diagnosis should be considered. For patients with positive biopsy diagnosis, DMD genetic mutation testing is still necessary, providing clinical information for genetic counseling, prenatal diagnosis, and gene therapy. Therefore, when a patient is suspected to have DMD/BMD, genetic testing for a DMD mutation is first done for dystrophinopathy diagnosis rather than invasive MB [38].

Currently, the widely adopted strategy for genetic diagnosis of DMD/BMD is to perform quantitative analysis of all exons in order to detect large deletions or duplications by MLPA or arrayCGH; in case the large mutations testing is negative, Sanger sequencing or alternatively NGS strategy is employed to detect small mutations [59]. With the development of NGS technology, detection of CNVs and SNVs using a single platform is performed in some laboratories. In most cases, if a genetic diagnosis is confirmed and the correlation of genotype and phenotype is determined, then no further protein testing is required. However, if a correlation between genotype and phenotype is not observed in a patient, but phenotype is clearly indicative of dystrophinopathies, then an MB is necessary and protein analysis often helps to identify the underlying abnormal dystrophin (quantity and/or quality) [28]. When MB is available, muscle RNA can be extracted from the muscular tissue and reverse transcription PCR for transcript analysis can be performed for identification of the genetic defect [11]. If normal dystrophin is detected, it may be concluded that the patient is negative for dystrophinopathies and diagnosis of other alternative muscular

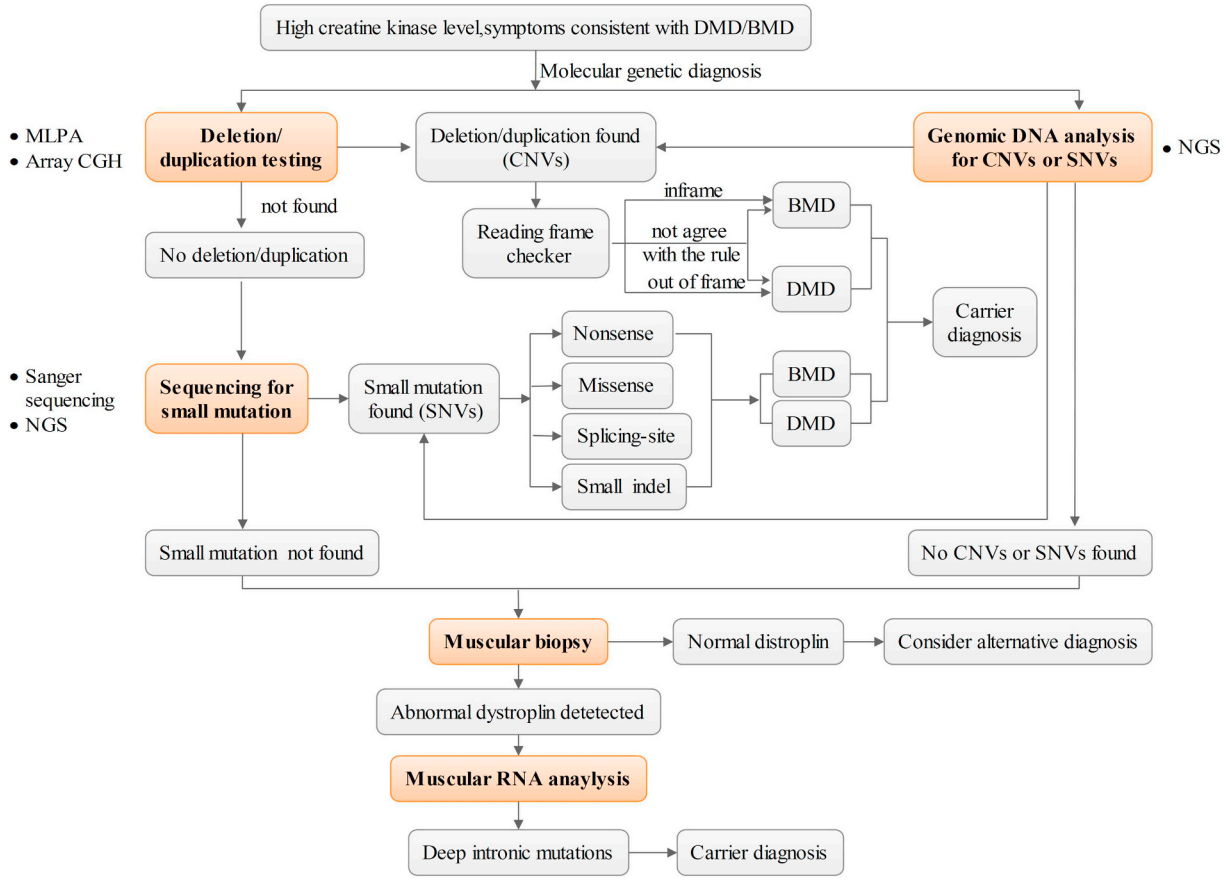

Fig. 3. Molecular diagnosis strategy in a patient with DMD/BMD.
When a patient is suspected to have DMD/BMD, genetic testing for DMD mutations is first performed at the DNA level. Quantitative analysis of all exons is conducted to detect large deletions or duplications, followed by sequencing, in order to detect small mutations. When NGS technology is used, CNVs and SNVs can be detected using a single platform. If no mutation is detected, a MB can provide information of dystrophin, and transcript analysis can be performed. Carrier diagnosis for female relatives at risk can be conducted when an affected male is detected with a DMD gene variant.

dystrophies should be considered. Genetic diagnosis for females manifesting DMD/BMD follows the same strategy described as above. Molecular diagnosis strategy in a patient and carrier with DMD/BMD is shown in Fig. 3.

When the DMD gene variant is known in an affected male, carrier diagnosis can be conducted for the mother and/or female relatives at risk, who are heterozygotes for the pathogenic gene variant. The method chosen depends on the pathogenic variant type in the proband. If the DMD gene variant is not detected in the mother of the proband, germline mosaicism should still be considered, conferring a recurrence risk for the next son [60]. When the DMD gene variant of an affected male is unknown and patient DNA is not available for analysis, molecular genetic testing of the at-risk females could be performed, usually starting with the proband's mother, as per the strategy in testing affected males. In families with more than one affected male with unequivocal diagnosis, if the DMD pathogenic variant in the affected male is not known and no DMD pathogenic variant is detected in at-risk females, linkage analysis can be offered to at-risk females to identify carrier status, depending on the availability and willingness of family members [61]. Asymptomatic heterozygous females require monitoring of cardiac abnormalities and should consider a cardiac evaluation, before conception or when she is pregnant [11].

6. Noninvasive prenatal genetic diagnosis

Prenatal diagnosis for DMD was first introduced in the Netherlands in 1984 [62]. Prenatal diagnosis for DMD/BMD is offered for a male fetus, albeit, no testing is performed for female fetuses [45]. Conventional prenatal diagnosis involves testing male fetal DNA for a known familial mutation, through chorionic villus sampling or amniocentesis [63]. The same method can be used to verify the mutation of the proband and female carriers [45]. If the familial mutation is unknown, haplotyping is an option for prenatal diagnosis [64]. However, chorionic villus sampling or amniocentesis are invasive methods, carrying associated risks. The discovery of cell-free fetal DNA (cffDNA) in maternal plasma has led to the development of noninvasive prenatal diagnostic tests, which are used for screening of aneuploidies, providing basis for prenatal diagnosis of monogenetic disorders [63]. The haplotype-based noninvasive prenatal testing for DMD, using cffDNA in maternal plasma, had been developed by targeted massively parallel sequencing, facilitating genetic diagnosis in female carriers, probands and fetuses efficiently, using a single platform [63,65]. However, as currently prenatal diagnosis is offered based on the presence of an affected boy, it will not be useful in detecting fetuses with de novo mutations (de novo mutations account for about one-third of DMD patients). This problem can be resolved by offering prenatal diagnostic testing for the dystrophin gene to every pregnant woman. The development of noninvasive prenatal diagnostic testing may change this scenario in the future, although many more hurdles are yet to be overcome [64]. On the other hand, though targeted sequencing has many advantages in proband diagnosis, carrier detection, and noninvasive prenatal diagnostic testing, there are still various limitations to the technique. PGD is another option for carrier women, and it was introduced in the Netherlands in 1995. With PGD, either unaffected male embryos or females can be selected and implanted into the uterus. Special requirements of PGD need to be considered, which are not within the scope of the present review.

7. Conclusion

Pathogenic variants in the dystrophin gene lead to DMD/BMD. It is important to correctly confirm the presence of genetic variants in affected patients and their families because this will affect disease diagnosis, prediction of disease risk, option of treatment and therapy, and family planning. The laboratories offering molecular genetic testing for DMD gene should maintain the highest possible quality standards. With the advances in molecular genetic technologies, especially the development of NGS technology, routine genetic diagnosis of DMD/BMD will need less laborious efforts and both, large duplications/deletions and small mutations, can be detected using a single platform. Certainly, before the NGS technology is widely used in routine genetic diagnosis of probands, carrier status confirmation and prenatal diagnosis, it will require further optimization and validation. The development of non-invasive prenatal diagnostic tests, using cffDNA in maternal plasma, has indeed increased the options for prenatal diagnostic testing of DMD. Personalized therapeutic strategies, such as exon skipping treatment and several antisense oligonucleotides, have entered clinical trials, and the genetic confirmation of variants can provide information that will aid in the selection of appropriate genetic therapies.

Contributors

KZ and JL discussed the outline of the review. KZ wrote the review. XY drafted the figures and tables. GL and YH provided suggestions and performed literature searches.

References

[1] E.P. Hoffman, R.H. Brown Jr., L.M. Kunkel, Dystrophin: the protein product of the Duchenne muscular dystrophy locus, Cell 51 (1987) 919–928.
[2] A.H. Ahn, L.M. Kunkel, The structural and functional diversity of dystrophin, Nat. Genet. 3 (1993) 83–291.
[3] A.E. Emery, The muscular dystrophies, Lancet 359 (2002) 687–695.
[4] J.A. Ellis, E. Vroom, F. Muntoni, 195th ENMC International Workshop: Newborn screening for Duchenne muscular dystrophy 14-16th December, 2012, Naarden, The Netherlands, Neuromuscul. Disord. 23 (2013) 682–689.
[5] J.R. Mendell, M. Lloyd-Puryear, Report of MDA muscle disease symposium on newborn screening for Duchenne muscular dystrophy, Muscle Nerve 48 (2013) 21–26.
[6] K.M. Bushby, M. Thambyayah, D. Gardner-Medwin, Prevalence and incidence of Becker muscular dystrophy, Lancet 337 (1991) 1022–1024.
[7] J.M. Murray, K.E. Davies, P.S. Harper, L. Meredith, C.R. Mueller, R. Williamson, Linkage relationship of a cloned DNA sequence on the short arm of the X chromosome to Duchenne muscular dystrophy, Nature 300 (1982) 69–71.
[8] F. Muntoni, S. Torelli, A. Ferlini, Dystrophin and mutations: one gene, several proteins, multiple phenotypes, Lancet. Neurol. 2 (2003) 731–740.
[9] A.P. Monaco, R.L. Neve, C. Colletti-Feener, C.J. Bertelson, D.M. Kurnit, L.M. Kunkel, Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene, Nature 323 (1986) 646–650.
[10] L.M. Kunkel, J.F. Hejtmancik, C.T. Caskey, A. Speer, A.P. Monaco, W. Middlesworth, et al., Analysis of deletions in DNA from patients with Becker and Duchenne muscular dystrophy, Nature 322 (1986) 73–77.
[11] A. Aartsma-Rus, I.B. Ginjaar, K. Bushby, The importance of genetic diagnosis for Duchenne muscular dystrophy, J. Med. Genet. 53 (2016) 145–151.
[12] H. Lee, J.L. Deignan, N. Dorrani, S.P. Strom, S. Kantarci, F. Quintero-Rivera, et al., Clinical exome sequencing for genetic identification of rare Mendelian disorders, JAMA 312 (2014) 1880–1887.
[13] Y. Yang, D.M. Muzny, J.G. Reid, M.N. Bainbridge, A. Willis, P.A. Ward, et al., Clinical whole-exome sequencing for the diagnosis of mendelian disorders, N. Engl. J. Med. 369 (2013) 1502–1511.
[14] A. Aartsma-Rus, J.C. Van Deutekom, I.F. Fokkema, G.J. Van Ommen, J.T. Den Dunnen, Entries in the Leiden Duchenne muscular dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule, Muscle Nerve 34 (2006) 135–144.
[15] S. Tuffery-Giraud, C. Béroud, F. Leturcq, R.B. Yaou, D. Hamroun, L. Michel-Calemard, et al., Genotype-phenotype analysis in 2,405 patients with a dystrophinopathy using the UMD-DMD database: a model of nationwide knowledgebase, Hum. Mutat. 30 (2009) 934–945.
[16] C.L. Bladen, D. Salgado, S. Monges, M.E. Foncuberta, K. Kekou, K. Kosma, et al., The TREAT-NMD DMD Global Database: analysis of more than 7,000 Duchenne muscular dystrophy mutations, Hum. Mutat. (36) (2015) 395–402.
[17] S.B. England, L.V. Nicholson, M.A. Johnson, S.M. Forrest, D.R. Love, E.E. Zubrzycka-Gaarn, Very mild muscular dystrophy associated with the deletion of 46% of dystrophin, Nature 343 (1990) 180–182.
[18] R.D. Bies, C.T. Caskey, R. Fenwick, An intact cysteine-rich domain is required for dystrophin function, J. Clin. Invest. 90 (1992) 666–672.
[19] M. Fanin, M.P. Freda, L. Vitiello, G.A. Danieli, E. Pegoraro, C. Angelini, Duchenne phenotype with in-frame deletion removing major portion of dystrophin rod: threshold effect for deletion size? Muscle Nerve 19 (1996) 1154–1160.
[20] A. Ishmukhametova, J.M. Chen, R. Bernard, B. de Massy, F. Baudat, A. Boyer, et al., Dissecting the structure and mechanism of a complex duplication–triplication rearrangement in the DMD gene, Hum. Mutat. 34 (2013) 1080–1084.
[21] Y. Xu, H. Wang, B. Xiao, W. Wei, Y. Liu, H. Ye, et al., Novel noncontiguous duplications identified with a comprehensive mutation analysis in the DMD gene by DMD gene-targeted sequencing, Gene 645 (2018) 113–118.
[22] K.M. Flanigan, D.M. Dunn, A. Niederhausern, P. Soltanzadeh, M.T. Howard, J.B. Sampson, et al., Nonsense mutation-associated Becker muscular dystrophy: interplay between exon definition and splicing regulatory elements within the DMD gene, Hum. Mutat. (32) (2011) 299–308.
[23] S.W. Peltz, M. Morsy, E.M. Welch, A, Jacobson. Ataluren as an agent for therapeutic nonsense suppression, Annu. Rev. Med. 64 (2013) 407–425.
[24] D.M. Henderson, A. Lee, J.M. Ervasti, Disease-causing missense mutations in actin binding domain 1 of dystrophin induce thermodynamic instability and protein aggregation, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 9632–9637.
[25] S. Tuffery-Giraud, J. Miro, M. Koenig, M. Claustres, Normal and altered pre-mRNA processing in the DMD gene, Hum. Genet. 136 (2017) 1155–1172.
[26] O.L. Gurvich, T.M. Tuohy, M.T. Howard, R.S. Finkel, L. Medne, C.B. Anderson, et al., DMD pseudoexon mutations: splicing efficiency, phenotype, and potential therapy, Ann. Neurol. 63 (2008) 81–89.
[27] M. Ishizaki, M. Kobayashi, K. Adachi, T. Matsumura, Kimura..Female dystrophinopathy: review of current literature, Neuromuscul. Disord. 28 (2018) 572–581.
[28] E. Viggiano, M. Ergoli, E. Picillo, L. Politano, Determining the role of skewed X-chromosome inactivation in developing muscle symptoms in carriers of Duchenne muscular dystrophy, Hum. Genet. 135 (2016) 685–698.
[29] E. Viggiano, M. Ergoli, E. Picillo, L. Politano, Determining the role of skewed X-chromosome inactivation in developing muscle symptoms in carriers of Duchenne muscular dystrophy, Hum. Genet. (135) (2016) 685–698.
[30] H. Trippe, S. Wieczorek, J. Kötting, W. Kress, U. Schara, Xp21/A translocation: a rarely considered genetic cause for manifesting carriers of duchenne muscular dystrophy, Neuropediatrics 45 (2014) 333–335.
[31] J. Chelly, F. Marlhens, B. Le Marec, M. Jeanpierre, M. Lambert, G. Hamard, et al., De novo DNA microdeletion in a girl with Turner syndrome and Duchenne muscular dystrophy, Hum. Genet. 74 (1986) 193–196.
[32] F. Quan, J. Janas, S. Toth-Fejel, D.B. Johnson, J.K. Wolford, B.W. Popovich, Uniparental disomy of the entire X chromosome in a female with Duchenne muscular dystrophy, Am. J. Hum. Genet. 60 (1997) 160–165.
[33] P. Soltanzadeh, M.J. Friez, D. Dunn, A. von Niederhausern, O.L. Gurvich, K.J. Swoboda, et al., Clinical and genetic characterization of manifesting carriers of DMD mutations, Neuromuscul. Disord. 20 (2010) 499–504.
[34] E. Takeshita, N. Minami, K. Minami, M. Suzuki, T. Awashima, A. Ishiyama, et al., Duchenne muscular dystrophy in a female with compound heterozygous contiguous exon deletions, Neuromuscul. Disord. 27 (2017) 569–573.
[35] E. Bakker, C. Van Broeckhoven, E.J. Bonten, M.J. van de Vooren, H. Veenema, W. Van Hul, et al., Germline mosaicism and Duchenne muscular dystrophy mutations, Nature 329 (1987) 554–556.
[36] C. Bermúdez-López, B. García-de Teresa, A. González-del Angel, M.A. Alcántara-Ortigoza, Germinal mosaicism in a sample of families with Duchenne/Becker muscular dystrophy with partial deletions in the DMD gene, Genet. Test. Mol. Biomarkers. 18 (2014) 93–97.
[37] J.S. Chamberlain, J.R. Chamberlain, R.G. Fenwick, P.A. Ward, C.T. Caskey, L.S. Dimnik, et al., Diagnosis of Duchenne and Becker muscular dystrophies by polymerase chain reaction. A multicenter study, JAMA 267 (1992) 2609–2615.
[38] K. Bushby, R. Finkel, D.J. Birnkrant, L.E. Case, P.R. Clemens, L. Cripe, et al., Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care, Lancet Neurol. 9 (2010) 177–189.
[39] J.P. Schouten, C.J. McElgunn, R. Waaijer, D. Zwijnenburg, F. Diepvens, G. Pals, Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification, Nucleic Acids Res. 30 (2002) e57.
[40] T. Lalic, R.H. Vossen, J. Coffa, J.P. Schouten, M. Guc-Scekic, D. Radivojevic, et al., Deletion and duplication screening in the DMD gene using MLPA, Eur. J. Hum. Genet. 13 (2005) 1231–1234.
[41] R. Santos, A. Gonçalves, J. Oliveira, E. Vieira, J.P. Vieira, T. Evangelista, et al., New variants, challenges and pitfalls in DMD genotyping: implications in diagnosis, prognosis and therapy, J. Hum. Genet. 59 (2014) 454–464.
[42] R.E. Varga, R. Mumtaz, A. Jahic, G.E. Rudenskaya, E. Sánchez-Ferrero, M. Auer-Grumbach, et al., MLPA-based evidence for sequence gain: pitfalls in confirmation and necessity for exclusion of false positives, Anal. Biochem. 421 (2012) 799–801.
[43] K. Koczok, G. Merő, G.P. Szabó, L. Madar, É. Gombos, É. Ajzner, et al., A novel point mutation affecting Asn76 of dystrophin protein leads to dystrophinopathy, Neuromuscul. Disord. 28 (2018) 129–136.
[44] M.J. Kim, S.I. Cho, J.H. Chae, B.C. Lim, J.S. Lee, S.J. Lee, et al., Pitfalls of multiple ligation-dependent probe amplifications in detecting DMD exon deletions or duplications, J. Mol. Diagn. 18 (2016) 253–259.
[45] S. Abbs, S. Tuffery-Giraud, E. Bakker, Best practice guidelines on molecular diagnostics in Duchenne/Becker muscular dystrophies, Neuromuscul. Disord. (20) (2010) 422–427.
[46] A. shmukhametova, P. Khau Van Kien, D. Méchin, D. Thorel, M.C. Vincent, F. Rivier, et al., Comprehensive oligonucleotide array-comparative genomic hybridization analysis: new insights into the molecular pathology of the DMD gene, Eur. J. Hum. Genet. 20 (2012) 1096–1100.

[47] C.F. Wright, D.R. FitzPatrick, H.V. Firth, Paediatric genomics: diagnosing rare disease in children, Nat. Rev. Genet. 19 (2018) 25.
[48] K.M. Flanigan, A. von Niederhausern, D.M. Dunn, J. Alder, J.R. Mendell, R.B. Weiss, Rapid direct sequence analysis of the dystrophin gene, Am. J. Hum. Genet. 72 (2003) 931–939.
[49] B.C. Lim, S. Lee, J.Y. Shin, J.I. Kim, H. Hwang, K.J. Kim, et al., Genetic diagnosis of Duchenne and Becker muscular dystrophy using next-generation sequencing technology: comprehensive mutational search in a single platform, J. Med. Genet. 48 (2011) 731–736.
[50] T.W. Prior, C. Bartolo, A.C. Papp, P.J. Snyder, M.S. Sedra, A.H. Burghes, et al., Dystrophin expression in a Duchenne muscular dystrophy patient with a frame shift deletion, Neurology 48 (1997) 486–488.
[51] E. Giorgio, A. Brussino, E. Biamino, E.F. Belligni, A. Bruselles, A. Ciolfi, et al., Exome sequencing in children of women with skewed X-inactivation identifies atypical cases and complex phenotypes, Eur. J. Paediatr. Neurol. 21 (2017) 475–484.
[52] Y. Wang, Y. Yang, J. Liu, X.C. Chen, X. Liu, C.Z. Wang, et al., Whole dystrophin gene analysis by next-generation sequencing: a comprehensive genetic diagnosis of Duchenne and Becker muscular dystrophy, Mol. Gen. Genomics. 289 (2014) 1013–1021.
[53] X. Wei, Y. Dai, P. Yu, N. Qu, Z. Lan, X. Hong, et al., Targeted next-generation sequencing as a comprehensive test for patients with and female carriers of DMD/BMD: a multi-population diagnostic study, Eur. J. Hum. Genet. 22 (2014) 110–118.
[54] M. Okubo, N. Minami, K. Goto, Y. Goto, S. Noguchi, S. Mitsuhashi, et al., Genetic diagnosis of Duchenne/Becker muscular dystrophy using next-generation sequencing: validation analysis of DMD mutations , , J. Hum. Genet. 61 (2016) 483–489.
[55] M. Alame, D. Lacourt, R. Zenagui, D. Mechin, F. Danton, M. Koenig, et al., Implementation of a reliable next-generation sequencing strategy for molecular diagnosis of dystrophinopathies, J. Mol. Diagn. 18 (2016) 731–740.
[56] R. Ebrahimzadeh-Vesal, A. Teymoori, M. Azimi-Nezhad, F.S. Hosseini, Next Generation Sequencing approach to molecular diagnosis of Duchenne muscular dystrophy; identification of a novel mutation, Gene 644 (2018) 1–3.
[57] T.F. Beck, J.C. Mullikin, NISC comparative sequencing program, L.G. Biesecker, Systematic evaluation of Sanger validation of NextGen sequencing variants, Clin. Chem. 62 (2016) 647–654.
[58] E. Mercuri, F. Muntoni, Muscular dystrophies, Lancet 381 (2013) 845–860.
[59] M.S. Falzarano, C. Scotton, C. Passarelli, A. Ferlini, Duchenne muscular dystrophy: from diagnosis to therapy, Molecules 20 (2015) 18168–18184.
[60] E. Bakker, C. Van Broeckhoven, E.J. Bonten, M.J. van de Vooren, H. Veenema, W. Van Hul, et al., Germline mosaicism and Duchenne muscular dystrophy mutations, Nature 329 (1987) 554–556.
[61] https://www.ncbi.nlm.nih.gov/books/NBK1119/#dbmd.Testing_Strategy.
[62] E. Bakker, M.H. Hofker, N. Goor, J.L. Mandel, K. Wrogemann, K.E. Davies, et al., Prenatal diagnosis and carrier detection of Duchenne muscular dystrophy with closely linked RFLPs, Lancet 1 (1985) 655–658.
[63] Y. Xu, X. Li, H.J. Ge, B. Xiao, Y.Y. Zhang, X.M. Ying, et al., Haplotype-based approach for noninvasive prenatal tests of Duchenne muscular dystrophy using cell-free fetal DNA in maternal plasma, Genet. Med. 17 (2015) 889–896.
[64] A.T. Helderman-van den Enden, K. Madan, M.H. Breuning, A.H. van der Hout, E. Bakker, C.E. de Die-Smulders, et al., An urgent need for a change in policy revealed by a study on prenatal testing for Duchenne muscular dystrophy, Eur. J. Hum. Genet. 21 (2013) 21–26.
[65] S.K. Yoo, B.C. Lim, J. Byeun, H. Hwang, K.J. Kim, Y.S. Hwang, et al., Noninvasive prenatal diagnosis of duchenne muscular dystrophy: comprehensive genetic diagnosis in carrier, proband, and fetus, Clin. Chem. 61 (2015) 829–837.