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NEXT GENERATION SEQUENCING APPLICATION IN DUCHENNE MUSCULAR DYSTROPHY (DMD): DIAGNOSTIC EVOLUTION, MUTATION ANALYSIS AND CHALLENGES

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ABSTRACT

Duchenne and Becker muscular dystrophy (DMD) is a chronic debilitating and progressive muscle-wasting disease that leads to difficulties with movement and, eventually, to the need for assisted ventilation and premature death. As a result of mutations in the DMD gene (encoding dystrophin), muscle dystrophin production is abolished. Dystrophic muscles are more susceptible to damage, resulting in progressive weakness and cardiomyopathy. Detailed understanding of the mutational spectrum of the DMD gene is fundamental to genetic counseling, prenatal diagnosis, and selection of suitable patients for mutation-specific treatments in the future. However, a molecular diagnosis with accuracy and convenience is difficult, due to the immense size of the dystrophin gene and the diversity of causative mutations. Traditional methods of diagnosing DMD, including multiplex ligation-dependent probe amplification and Sanger sequencing, need multiple steps and have many flaws. A stop codon read-through approach and exon-skipping are the most promising therapeutic options to date for the treatment of DMD. To use either of these approaches, a very precise identification of the mutational status of the DMD gene must be made in patients with DMD. Identifying the causal variation in DMD within this difficult-to-diagnose group necessitated using novel contemporary methods. This Primer provides a comprehensive introduction to the practice of next-generation sequencing technologies for a more detailed characterization of the mutational spectrum within the human dystrophin gene.

***Keywords*:** Muscular Dystrophy; Therapeutic Treatment; Sequencing; Molecular Diagnosis, ventilation, heart failure, alanine aminotransferase

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1. INTRODUCTION

Muscular dystrophy is a group of hereditary disorders that cause skeletal muscle deterioration and weakness (Bushby et al. 2010). In muscular dystrophy, the damage of strong muscle fibers (2) and reinforcement with fat and fibrosis occurs with time (Aartsma-Rus et al. 2019; Alfano et al. 2019), causing muscle tissues to be less capable of generating force for ordinary activities (Bello and Pegoraro, 2019; Birnkrant et al. 2018). During the process of muscle wasting, patients suffer from weakness, although specific muscular dystrophies can affect different muscle groups (Bylo et al. 2020). If mechanical support is not provided (Doorenweerd et al. 2014), respiratory failure may limit the lifespan of muscular dystrophy (Hugnot et al. 1992). The heart can also be affected by some forms of muscular dystrophy, leading to cardiac complications, including heart failure (Duan 2018).

Duchenne is caused by a mutation on the dystrophin X gene chromosome is a main form of Dystrophy and characterized by continuous weakness and damage of muscles (Duboc et al. 2005; Hoffman et al. 1987). Boys with DMD often fail their capability to walk around the time of puberty and exhibit indications of muscular weakness as early as infancy, usually between the ages of 2 and 7 (Caskey et al. 1980). In most cases, wheelchair dependency occurs between 10 and 12 years old and supported ventilation begins at the age of 20 (Aartsma-Rus et al. 2016). It is estimated that a maximum of patients with DMD will die between the ages of 20 and 40 due to respiratory and cardiac failure, even with optimal care (Brison et al. 2019).

1.1. Dystrophin: Gene and Protein

In August 1987, positional cloning yielded the first successful DMD gene identification on the X chromosome, which opened the door to new directions in the study. Investigations of exceptional female DMD patients with

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stable X and autosomal translocations with the translocation division at Xp21 allowed researchers to pinpoint the gene's location to Xp21 (14). A DNA marker confirms the localization of the disease, revealing that it shares an allelic relationship with milder, similar-clinical disease, Becker muscular dystrophy (BMD) (Monaco et al. 1988). However, the gene was discovered by utilising a patient with a significant deletion who also had DM and four other X-linked disorders. The dystrophin gene comprising 79 exons spans about 2,200kb, unevenly 0.1% of the human genome (Birnkrant et al. 2018). There is a wide distribution of the DMD locus transcribed (14-kb mRNA), mostly in cardiac and skeletal muscles, and a smaller amount in the brain (Birnkrant et al. 2018; Helderman-van Den Enden et al. 2009). Since dystrophy results from its absence were given the name dystrophin produced by this protein product.

After the discovery of the DMD gene, many different mutations arise (Hugnot et al. 1992). In the present study of 7149 DMD patients, 79% had huge mutations, 68% of which were large deletions, and 11% had large duplications. Only 21% of the patients had minor changes, and 50% of them were nonsense mutations. 5%, 2%, and 3% of the population had minor insertions, deletions, and splice site alterations, respectively (Doorenweerd et al. 2014). Stimulatingly, the removed exons are often bunched in sections 43-55. Only a small percentage of patients have these hot spot sites. Mutation rates for exon 53 (10.1%), 51 (14%), exon 45 (9%), exon 44 (7.1%) and exon 43 (7.5%) are the highest, according to a large-scale study (Moser 1984; McNally et al. 2015). De-novo mutations are frequent in DMD and BMDB; de-novo germline alterations account for one-third of individuals' cases of DMD and BMDB (Iyombe-Engembe et al. 2016). In addition, due to germline mosaicism, mothers with children with DMD or BMDB who do not have DMD mutations are at risk of producing another child with the same condition, which is a percentage of her oocytes, is present). Sperm or oocyte germline mosaicism occurs in various individuals, but it can be as much as 14% (Moura et al. 2015; Bettica et al. 2016).

1.2. Diagnosis

A prompt and precise diagnosis of DMD is important for treatment. Since 2010, there has been no significant change in the method for diagnosing DMD (Brenman et al. 1995). When indicative symptoms and indications are seen, such as clumsiness, toe waliking, difficulty ascending stairs, weakness, and a gowers sign often start in early infancy (Bylo et al. 2020). It is possible to avoid diagnostic delays by promptly referring a patient to a neuromuscular specialist, assisted by a geneticist or genetic counsellor. Occasionally, the judgment is completed due to progressive elevated and delayed serum enzymes, like lactate dehydrogenase, alanine aminotransferase, creatine kinase, or aspartate aminotransferase (Birnkrant et al. 2018). It can be difficult to tell the difference between increased liver enzymes and DMD. However, an elevated ALT, AST, or concentration of lactate dehydrogenase, sometimes causes an incorrect emphasis on hepatic dysfunction, and diagnosis of DMD is delayed (Capogrosso et al. 2018; Duchêne et al. 2018). This is especially true when these increases are accompanied by increased bilirubin levels. The most common form of DMD is a gene deletion or duplication, in which a single or multi-exon region of the dystrophin gene is missing (Skuk et al. 2006). About 70% of people with DMD have this loss or duplication, thus it is often the first confirming test. The deletions were detected by multiplex PCR, the optimum testing methods are comparative genomic hybridization array or multiplex ligation-dependent probe amplification (MLPA) (De Palma et al. 2014). Using MLPA or a comparative genomic hybridization array may determine if a deletion or duplication mutation has boundaries that cause the reservation or dislocate the reading frame. If testing for duplication or deletion is negative, genetic sequencing should be carried out to check for the outstanding mutation types that are thought to account for 25–30% of DMD cases. These abnormalities, which comprise point mutations (missense or nonsense), little duplication, tiny deletions, small insertion, and duplication, may be found using next-generation sequencing (Aartsma-Rus and Goemans 2019). When everything else fails, genetic testing may be supplemented by western blotting or immunohistochemistry of tissue cryosections to confirm the clinical diagnosis of DMD (Wood et al. 2014).

1.3. Female Carriers

Genetic counselling should be provided to family members of a person with DMD to determine who is likely to be a carrier (Chamberlain et al. 1992). Female relative should be advised for carrier testing who has been diagnosed with DMD genetically. The American Medical Association's ethical rules for genetic testing of children should be followed if the relative is a minor (Eagle et al. 2007). Female carriers who are found have various options for conception, such as prenatal genetic testing or preimplantation genetic diagnosis via amniotic fluid or chorionic villus collection (Tuffery-Giraud et al. 2009).

1.4. Newborn Screening

The measurements of the creatine kinase level from dried blood spots were initially demonstrated in the middle of the 1970s (34). In a recently described two-tier newborn screening diagnostic approach, samples that showed a

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creatine kinase concentration elevation were subsequently examined for mutations in the dystrophin gene (Mendell et al. 2012). The Recommended Uniform Screening Panel, which is mostly restricted to neonatal-onset illnesses for whom early treatment indicates a better prognosis, does not contain DMD (Wood et al. 2014). However, some countries have carried out neonatal DMD screening trials (Wood et al. 2014). However, the majority of these studies have been stopped (Mendell et al. 2012; Gatheridge et al. 2016). Though support from stakeholders and the possibility that newly developed DMD medicines would be most successful if they are started before symptoms have rekindled interest in newborn screening (Mendell et al. 2012; Moat et al. 2017).

1.5. Evolution of Various DMD Diagnostic Methods

1.5.1. Multiplex PCR: Multiplex PCR is one of the patients' most promising tools for detecting DMD. It has been used to diagnose various genetic disorders, including DMD, cystic fibrosis, sickle cell anaemia and haemophilia (Frank et al. 2020). Multiplex PCR is frequently used in clinical studies to aid in discovering genetic variations. Multiplex PCR is currently used in targeted massively parallel sequencing (MPS) as an intrinsic part (Janssen et al. 2005). MPS is utilized in clinical laboratories as a genetic variation detection tool because of its speed and accuracy (36). There are various advantages of using multiplex PCR, including systematic protocol development, fast experimentation, and an accurate and efficient variant detection process (Satre et al. 2004). Multiplex PCR is thus extensively employed in medical and biomedical studies, despite its drawbacks, which include poor amplification efficiency for longer fragments, and poor specificity and sensitivity of certain specific targets dependent on the case. Multiple studies have used multiplex PCR to detect deletions in DMD patients (Chelly et al. 1990). For example, Murugan et al. found deletions in 103 DMD patients out of 150 that they believed to be affected (Crescimanno et al. 2019). In addition to identifying nine mutations via MLPA, Kumar et al. recently discovered deletion mutations in DMD affecting 30 exons. This study was conducted on 996 patients who were considered to be DMD patients. Only 623 of these patients had deletions identified via multiplex PCR. Additional deletions were detected through MLPA in 92 patients (De Palma et al. 2012; McDonald et al. 2017).

1.5.2. Multiplex Ligation-dependent Probe Amplification: In 2002, Schouten developed multiplex ligation-dependent probe amplification (MLPA) (Brison et al. 2019). It is currently employed to detect Duchenne muscular dystrophy (DMD) and is used as an initial screening test in clinical settings (Aartsma-Rus and Goemans 2019). It is a simple, economical, and sensitive technique that is available as an in vitro diagnostic assay (MRC Holland SALSA MLPA Probe mixes P034 and P035) for a reduced price (Bylo et al. 2020). A variety of pioneering studies have examined all 79 DMD gene exons using MLPA, a well-established technique, to detect genetic variations (deletions and duplications) (Garcia et al. 2014). These studies have shown that MLPA can detect additional genetic mutations in DMD patients, including intragenic deletions and carrier status detection using multiplex PCR as the first line of diagnostic testing (Gatheridge et al. 2016). MLPA can only detect large deletions/duplications (Satre et al. 2004). However, it is incapable of detecting small insertions/deletions and point mutations (Thangarajh et al. 2019). As a result, NGS technologies were developed to detect small variants.

***1.5.3. Next-Generation Sequencing*:** Small pathogenic variants, including missense, nonsense, small insertions and deletions, indel, and splicing, can be identified by NGS (Barlow and Ellard 2006) (Khalid 2022). The DMD gene (which includes 79 exons and intronic flanking sequences) is composed of a large number of indels. NGS can detect variants that MLPA cannot, therefore serving as the next or second line of DMD testing (Bradley et al. 1972; Eagle et al. 2007). The first-generation sequencing method was frequently used to sequence all DMD exons individually (Wasala et al. 2018). Although it is costly and time-consuming, it has high accuracy. Later, Sanger sequencing was replaced by new NGS approaches to decrease experiment costs and time. Sanger sequencing can sequence only one exon per experiment, whereas NGS can accomplish a large number of targets in parallel in different patients at once (Frank et al. 2020). It has been described that the whole DMD gene has been sequenced in addition to exons, introns, and promoters. Several NGS techniques, such as amplicon-based, whole genome, whole-exome sequencing, and DMD gene-targeted analysis, have been used thus far. Long-Read sequencing technologies, such as Pacific Biosciences and Oxford Nanopore Technologies, can detect Structural Variants, Single Nucleotide Variants (SNVs), and Single-Molecule Barcoding (SMB) (Wasala et al. 2018). Researchers recently used a 10x Linked-Read sequencing strategy to study the DMD gene, which combines single-molecule barcoding with short-read WGS (Goemans et al. 2018). In this instance, MLPA was used to analyse a female muscular dystrophy carrier with an undetermined genetic status, which could not detect exon deletions (Dumont et al. 2015). However, linked-read WGS could distinguish the two X chromosomes (Haenggi et al. 2005). The linked-read WGS method should be considered a valuable tool for understanding unresolved genetic conditions (Mendell et al. 2020). NGS might be used as a single comprehensive platform to detect mutations and diagnose DMD. 16-29 deletions were identified in the first allele, whereas the second allele had a 1-34 duplication, indicating that linked-read WGS should be

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considered a valuable method to detect unresolved genetic conditions (56). Recognition of cryptic splice sites or pseudo-exon insertion in mature transcripts, which leads to complex rearrangements, can evade level 1 and 2 DNA-based testings (40). RNA-based sequencing methods may be used to detect these escape variants. Micro-fluidic exome array can be sequencing DMD RNA and assess multiple splicing events (Holloway et al. 2008). It also provides the complete DMD mRNA sequence, including all exon-exon junctions. Technical difficulties in research have been identified in DMD variants from various tissues (blood, muscles, stem cells, and extracellular RNA from urine (Johnson et al. 2012). The presence of low amounts of illegitimate transcripts characterizes technical difficulties in the study. along with NGS-based RNA-seq, may encourage therapeutic approaches for DMD (Frank et al. 2020).

***1.5.4. NGS Data Analysis*:** The amount of data produced by NGS platforms is vast. Bioinformaticians with expertise must handle and analyse massive amounts of data. The reads are often provided in raw format by sequencing via NGS, including FASTQ or other raw sequence file formats (such as BCL, SFF, HDF5, bam, SOLID, etc. Pre-processing is an initial and crucial stage in bioinformatic analysis. A quality check (QC) is carried out on these pre-processed reads to obtain accurate and error-free reads (Kodippili et al. 2018). The downstream analysis can vary depending on the objectives of projects and scientific inquiries (Pinto Leite et al. 2018). The reference DMD gene is matched against DMD sequencing reads produced using the Sanger technique to identify causal mutations (Kieny et al. 2013). Only high-quality bases are mapped on DMD after the base calls are examined to determine the base-quality value before alignment (Doorenweerd 2020).

Additionally, methods like WGS and RNA-seq have been employed to find harmful DMD mutations (McNally et al. 2015). As previously noted, high-quality readings are obtained by filtering reads produced by WGS/WES methods. The GRCh38 reference genome is used to map high-quality reads to find SNVs, CNVs, and tiny indels. These discovered variations are contrasted with variants found in databases such as the Genome Aggregation Database (gnomAD) (MacArthur Lab, n.d.), dbSNP, ClinVar (Yu et al. 2017). Other tools, such as the Manta tool, may also be utilised depending on the requirements to find split read irregularities in the DMD. To verify the structural rearrangements, the probable DMD variants are ultimately manually examined using Integrative Genome Browser (IGV) (Uttley et al. 2018). RNA-seq reads are processed through quality checks similarly to WGS reads (Patel et al. 2018). These reads are matched against the reference genome using a two-step alignment method using a spliced aligner, such as STAR 2-Pass, and GENCODE is used for annotation. It removes unannotated junctions in the first step and identifies novel junctions (Wasala et al. 2020).

***1.5.5. NGS Data Analysis Challenges*:** The American College of Medical Genetics and Genomic (ACMG) divides the variations into four categories: harmful, probably pathogenic, and presumably benign (Nance et al. 2019). A significant amount of data is produced by NGS. Therefore, managing and storing data properly presents the first challenge. NGS data offers tens of thousands of variations (Wasala et al. 2018). The significance of the majority of the identified variants is assessed using data from mutation databases like the ClinVar database, Genome Aggregation Database (gnomAD), dbSNP, and Human Gene Mutation Database (HGMD), as well as information from the literature and clinical observations (Nance et al. 2019). Numerous losses of function variants in the human genome with unclear relevance complicate the interpreting process (Voit et al. 2017). Therefore, one of the key difficulties in diagnosing complex features is identifying the pathogenic and clinically important variants.

Additionally, interpreting expected markers necessitates software, tools, and algorithms, all of which need a significant amount of computer power (Uttley et al. 2018). To put it briefly, it is an expensive and difficult task to turn pathogenic genetic data into a diagnostic tool. It also requires the confirmation of variations, the expertise of professional bioinformaticians, a bioinformatics infrastructure, genetic counseling, and integration of all of them. By overcoming these obstacles, NGS technology can eventually become a single, all-inclusive tool for DMD diagnosis and mutation identification.

2. Conclusion

Currently, the most popular and successful method for the routine diagnosis of DMD is the targeted resequencing of genes of interest. The first-tier diagnostic for genetically varied illnesses such as skeletal muscle disorders will soon be exome and genome sequencing due to the declining cost of NGS and technological advancements. Genetic information will probably be available before other test results and during the patient's first clinical assessment because of the quick turnaround times of such procedures. The NGS industry is developing quickly, and new strategies are being developed to obtain long reads for sequencing longer contiguous chunks of DNA. Long-read sequencing approaches enhance the identification of longer repetitive components, copy number alterations, and structural variants by circumventing the length constraint of earlier NGS techniques. Using single-molecule real-time sequencing technology, provided the first sequence data for extended CGG-repeat FMR1 alleles,

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proving the practicality of such a long-read strategy for the diagnosis of triplet repeat disorders. For viral and malignant illnesses, RNA sequencing technologies are also becoming powerful diagnostic and prognostic tools. Similar techniques are already being used to evaluate the differentially expressed genes of known disease-causing transcripts, and they will probably soon be used to diagnose hereditary illnesses. The vast amount of data produced will aid in understanding the cellular and molecular mechanisms causing diseases of the skeletal muscle as well as the association between genotype and phenotype. These are the obligatory first steps toward potential therapeutic strategies.

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