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REVIEW

Impacts of massively parallel sequencing for genetic diagnosis of neuromuscular disorders

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Abstract Neuromuscular disorders (NMD) such as neuropathy or myopathy are rare and often severe inherited disorders, affecting muscle and/or nerves with neonatal, childhood or adulthood onset, with considerable burden for the patients, their families and public health systems. Genetic and clinical heterogeneity, unspecific clinical features, unidentified genes and the implication of large and/or several genes requiring complementary methods are the main drawbacks in routine molecular diagnosis, leading to increased turnaround time and delay in the molecular validation of the diagnosis. The application of massively parallel sequencing, also called next generation sequencing, as a routine diagnostic strategy could lead to a rapid screening and fast identification of mutations in rare genetic disorders like NMD. This review aims to summarize and to discuss recent advances in the genetic diagnosis of neuromuscular disorders, and more generally monogenic diseases, fostered by massively parallel sequencing. We remind the challenges and benefit of obtaining an accurate genetic diagnosis, introduce the massively parallel sequencing technology and its novel applications in diagnosis of patients, prenatal diagnosis and carrier detection, and discuss the limitations and necessary improvements. Massively parallel sequencing synergizes with clinical and pathological investigations into an integrated diagnosis approach. Clinicians and pathologists are crucial in patient selection and interpretation of data, and persons trained in data management and analysis need to be integrated to the diagnosis pipeline. Massively parallel sequencing for mutation identification is expected to greatly improve diagnosis, genetic counseling and patient management.

Keywords Neuromuscular disorders · Diagnosis · Mutation · Next generation sequencing · Neuropathy · Myopathy

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Neuromuscular disorders

Inherited neuromuscular disorder (NMD) is a wide term covering different genetic disorders affecting muscles (different types of myopathies and dystrophies, ion channel muscle diseases and malignant hyperthermia), nerves (Charcot–Marie–Tooth neuropathies also called hereditary motor and sensory neuropathies, amyotrophic lateral sclerosis, hereditary ataxias and spinal muscular atrophies) and neuromuscular junctions (myasthenic syndromes [41]) (http://musclegenetable.fr/) [44]. Muscle weakness, twitching, cramps and numbness are common features in several NMDs. These disorders are rare and often severe, affecting children and adults with considerable burden to the patients, their families and public health systems [25].

Provisions in genetic diagnosis laboratories

The most important criterion for using a test in standard health care is whether the test will lead to a better outcome for the patient [97]. The clinical test should address the specific problem. The aim of a clinical genetic service is to make genetic diagnosis and estimation of transmission risk for an affected patient and to provide genetic counseling and preventive and therapeutic guidance for family members [31]. Anonymity and confidentiality are important standards in genetic diagnosis. Data should be reliable and diagnosis should be cost-effective. The turnaround time for test results is another important issue in genetics diagnosis.

Challenges in molecular diagnosis

Genetic heterogeneity

NMDs are one of the most genetically heterogeneous disorders class with more than 300 implicated genes. This genetic heterogeneity can be seen, for instance in Charcot–Marie–Tooth (CMT) neuropathies with more than 30 causative genes [83] and more than 40 loci (http://www.molgen.ua.ac.be/CMTMutations) or in autosomal recessive limb girdle muscular dystrophy with 15 genes implicated to date [49]. This high degree of genetic heterogeneity is problematic for molecular genetics diagnosis as it could be time-consuming and costly to test the different implicated genes in diagnostic laboratories. The rarity of mutations in some genes explains the fact that samples should be sent to several specialized laboratories to cover all candidate genes.

Implication of large genes

Several of the largest human genes are mutated in neuromuscular disorders such as *DMD* (MIM #300377) spanning more than 2.3 Mb with 79 exons [86], *TTN* (MIM #188840) with 363 exons with an open reading frame spanning more than 100 Kb, *NEB* (MIM #161650) with 183 exons [22] and *RYR1* (MIM #180901) with 106 exons [73]. These genes are also mainly expressed in muscle, precluding cDNA analysis if such tissue is not available. While these genes are known to be implicated in diseases, they might not be fully tested or only the mutation hot spot regions may be analyzed at first [4].

Clinical heterogeneity

Clinical heterogeneity is another parameter in diagnosis of NMDs. For example, mutations in the Caveolin 3 gene (MIM #601253) have been detected in four different skeletal muscle disease phenotypes: rippling muscle disease, limb-girdle muscular dystrophy, hyperCKemia and distal myopathy [28]. Patients can have an overlap of these symptoms, or the same mutation can cause diverse clinical phenotypes with different severities. Thus, it is sometimes difficult to suggest the best candidate genes to direct molecular diagnosis.

Unspecific clinical features

Another reason precluding the suggestion of the best molecular diagnosis approach is the fact that a large number of patients display unspecific clinical and histopathological features. For instance, reviews of cases with congenital myopathies that are usually sub-classified into nemaline, core or centronuclear myopathies reveal no specific signs in almost half of them, whereas other patients have diverse but overlapping clinical and histopathological manifestations [64]. Moreover, when a sequence change is found in one of the prioritized genes in diagnostic laboratories, further investigation might be stopped, which may lead in some cases to missing the real disease-causing mutation. Conversely, when no change is found, patients undergo additional time-consuming, costly and sometimes painful tests to precise the clinical diagnosis and prioritize other genes [75].

Requirements for several approaches

Different types of mutations are detected in patients with NMDs. For example, 60–65 % of patients with Duchenne and Becker muscular dystrophies (DMD and BMD) have deletions in the dystrophin gene, *DMD* (MIM #300377), 5–15 % have duplications, and the rest have point mutations or small insertions–deletions [1, 63]. Thus, diagnostic laboratories should apply different techniques to detect disease-causing mutations in patients, which is laborious, expensive, necessitates different platforms and increases turnaround time [76].

Unidentified genes

The last obstacle is the unidentified genes in different NMDs. For instance, mutations in several genes, including *SOD1* (MIM #147450) [81], *SETX* (MIM #608465) [14], *ALS2* (MIM #606352) [35, 96], *TARDBP* (MIM #605078) [30], *FUS* (MIM #137070) [48], *ANG* (MIM #105850) [33, 39] and *C9orf72* (MIM #614260) [19, 79] can cause amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder characterized by the loss of motor neurons in brain and spinal cord. However, for a significant number of ALS patients, the causative mutation remains unknown, suggesting the involvement of other genes. More generally,

about 40 % of patients with NMDs do not have a genetic diagnosis.

Benefits of definitive molecular diagnosis

Identification of related disease-causing mutations helps to confirm the clinical findings and provide an accurate diagnosis. The knowledge of the mutation and mutated gene usually improves disease management, and allows for inclusion into therapeutic trials. Genetic counseling becomes possible, as carrier status determination and prenatal diagnosis can decrease the risk of recurrence in affected families. In addition, finding the disease-causing mutation permits potential phenotype–genotype correlations and a better understanding of the underlying pathophysiological mechanisms, a pre-requisite for the development of specific therapeutic approaches.

Routine molecular techniques in genetics diagnostic laboratories

Today, several techniques are used in routine diagnostic to find causative mutations. The most common method is PCR amplification of coding/exonic sequences of candidate genes from genomic DNA, followed by Sanger sequencing of PCR products. This approach is the gold standard for detection of point mutations and small insertions–deletions. If a gene is large and mutations are spread throughout the entire gene, using this technique is extremely time-consuming, expensive and laborious. An alternative approach is reverse transcription PCR (RT-PCR) followed by Sanger sequencing of the entire cDNA [34, 59] which needs the access to specific tissues such as muscle for the muscle-specific genes. Such tissues may not be available all the times. Multiplex PCR and long-range PCR (LR-PCR) are other PCR-based techniques. Using multiplex PCR of only 19 exons of the *DMD* gene, about 98 % of deletions could be detected in patients with DMD/BMD [6, 13, 70]. With the MLPA technique in patients with sporadic amyotrophic lateral sclerosis, it was shown that SMN1 duplications are associated with ALS susceptibility whereas SMN1 deletions and SMN2 copy number status are not associated with ALS [10]. In patients with spinal muscular atrophy (SMA), a neuromuscular disorder characterized by degeneration and loss of alpha-motor neurons in the anterior horn of the spinal cord, LR-PCR was used to detect deletions of the *SMN1* gene (MIM #600354) [3].

For detection of repeat expansions, a common type of mutation in NMDs, several methods are used, such as repeat primed PCR (RP-PCR) and Southern blot [32, 95]. In 56 out of 76 patients with myotonic dystrophy type 2 (DM2), a neuromuscular disorder characterized by myotonia and muscle dysfunction, and 25 out of 378 patients with spinocerebellar ataxia type 8, a slowly progressive neurodegenerative disorder, repeat expansions were detected using RP-PCR [46].

Comparative genomic hybridization (CGH) array is a method of choice for detection of large rearrangements and copy number variations. Using this technique, genomic rearrangements were detected in dysferlin (*DYSF*, MIM #603009) and calpain-3 (*CAPN3*, MIM # 114240) genes, implicated in two forms of limb-girdle muscular dystrophy types 2B and 2A, respectively [5].

Indirect diagnostic techniques such as monitoring the presence of a protein by Western blot [88] or enzymatic activity are other available approaches; however, they do not provide a specific knowledge of the mutation.

Massively parallel sequencing technology

As mentioned above, genetic and clinical heterogeneity, unspecific clinical features, implication of large genes and the necessity to apply multiple techniques are the main drawbacks in routine molecular diagnostic laboratories. Massively parallel sequencing (MPS), also called next generation (NGS) or high-throughput sequencing (HTS), allows to sequence target genes and regions, exome or whole genome, and is revolutionizing the molecular diagnosis as it now permits large-scale parallel sequencing and can be used to detect several types of mutations. The exome represents about 1–2 % of the genome but harbor 85 % of disease-causing mutations [15].

Template preparation and barcoding

The massively parallel sequencing technology is based on a combination of template preparation, sequencing, imaging/recording and data analysis [61]. DNA and library preparations are the first steps. Two to twenty microgram of high quality genomic DNA is randomly sheared into smaller size molecules by sonication or nebulization. Several DNA can also be pooled and sequenced together. The barcoding and pooling step can be done either before or after capture of specific targeted sequences, using a unique DNA tag sequence per sample [18]. In this way, it is possible to sequence a few genes in many patients or many genes from a few patients.

Targeted sequence enrichment

There is no enrichment step for whole genome sequencing [94]; however, if the aim is to sequence a portion of

genomic DNA which can be the protein-coding part of the genome (exome) or regions of interest such as selected genes or a genomic region linked to a disease, such targeted sequencing includes the enrichment of the target sequences.

This enrichment or capture can be done on microarray or in solution [60]. In solid-phase enrichment, high-density primers targeting the sequences of interest are covalently attached to the slide array [25], while in solution-phase enrichment, primers are generated on beads [20]. Several solutions such as Illumina, Agilent and Nimblegen capture kits can be used; they have technical differences such as using RNA baits (Agilent) or DNA baits (Illumina and Nimblegen) with almost similar performance [17]. Other enrichment methods include RainStorm microdroplet-based technology from Raindance technologies which is a multiplex PCR method using microdroplets containing PCR components loaded on microfluidic chip to compartmentalize the PCRs by single primer pairs [87].

Sequencing

For some of the sequencing platforms such as Helicos BioSciences, single molecule template is used, whereas in others such as Illumina, SOLiD and Roche/454, clonally amplified DNA is required to detect the signal produced by the incorporation of nucleotides [57]. Thus, for sequencing platforms using single molecule templates, the amount of starting DNA is lower and there is no PCR amplification step that could create artificial mutations and AT or GC-rich amplification bias.

In all the MPS platforms, either templates, primers or polymerase enzyme are immobilized on a solid support before the sequencing reaction [61]. Sequencing and recording steps are different [54]. Illumina technology is based on clonally amplified templates coupled with cyclic reversible termination method with four fluorescent colors. First, one fluorescently modified nucleotide complementary to the template sequence is incorporated. After washing and imaging for detection of the incorporated nucleotide, a cleavage step removes the fluorescent dye and a novel incorporation step is performed. These steps are done in a cyclic manner, 72 or 100 times or more [9]. It is possible to sequence from both extremities of the DNA template (paired-end sequencing). HeliScope single-molecule sequencer of Helicos BioSciences works with one fluorescent color (Cy5) using a cyclic reversible termination approach [12]. SOLiD (Sequencing by Oligonucleotide Ligation and Detection) sequencer from Applied Biosystems is another MPS technology based on sequencing by ligation using DNA ligase and a cleavable two-base-encoded probe consisting of two nucleotides combined with a particular dye [91]. Roche/454 sequencing platform is based on pyrosequencing. In this sequencing by synthesis method, a pyrophosphate (PPi) is released after incorporation of one dNTP. This released pyrophosphate is converted into detectable light through a series of enzymatic reaction [80]. Ion Torrent commercializes semiconductor sequencing machines where the release of a proton after incorporation of a nucleotide leading to change in pH is detected by voltage change. If two nucleotides are incorporated, the voltage will be double [82]. For human whole genome sequencing, apart from the above mentioned sequencing platforms, Complete Genomics offers a non-commercialized solution. In that case, DNA nanoballs containing hundreds of copies of a short DNA fragment are sequenced using combinatorial probe anchor-ligation (cPAL) where fluorescent molecules are attached to each nucleotide by a ligase enzyme [23] (Table 1).

Data analysis

The final steps in massively parallel sequencing are genome alignment, variant calling and data analysis. The generated sequence reads are aligned to a human reference genome such as UCSC assembly hg18/NCBI 36 or hg19/GRCH 37, and the variations are detected using different programs including BWA [50, 89] for alignment and Samtools [51] for variant calling. For filtering and scoring the variants and detection of disease-causing mutations, there are several strategies depending on the mode of inheritance and number of affected/non-affected sequenced cases [29]. Sequencing several affected and eventually non-affected individuals from the same family improves the filtering; variations common in affected and absent in non-affected persons will be prioritized. Several public websites display polymorphisms and allele frequencies such as dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), 1000 genomes (http://www.1000genomes.org/) or the Exome Variant Server, NHLBI Exome Sequencing (http://evs.gs.washington.edu/EVS/) which covers SNPs but no indels. Many softwares are available to filter sequence variants, such as ALAMUT software (Interactive Biosoftware, Rouen, France), Cartagenia benchlab (http://www.cartagenia.com/); SIMPLEX (http://simplex.i-med.ac.at) [27] or ANNOVAR (http://www.openbioinformatics.org/annovar/) [93], and most integrate tools to predict the pathogenicity of the variations on RNA splicing and protein function such as SIFT [65], Polyphen [2, 77] or NNSPLICE (Fig. 1).

Massively parallel sequencing for NMD diagnosis

In 2010, Lupski et al. [58] published the first paper using massively parallel sequencing technology in neuromuscular diseases, where they showed the result of whole genome sequencing of a patient with a recessive form of Charcot–

Table 1 Different massively parallel sequencing platforms and their principles

Sequencing machine	Company	How does it work	Read length	Website
HiSeq2000	Illumina	Cyclic reversible termination method with four fluorescent colors	2 × 100 bp	http://www.illumina.com/systems/hiseq_systems.ilmn
HeliScope Single Molecule Sequencer	Helicos BioSciences Corporation	Cyclic reversible termination with one fluorescent color	25–55 bp	http://www.helicosbio.com/
SOLiD v4	Applied Biosystems	Sequencing by ligation	50 + 35 bp/ 50 + 50 bp	http://www.appliedbiosystems.com/
454 GS FLX	Roche	Pyrosequencing	700 bp	http://www.454.com/
Ion Proton Sequencer	Ion Torrent/Life Technologies	Ion semiconductor sequencing	200 bp	http://www.iontorrent.com/
Complete Genomics' sequencing instrument	Complete Genomics	Combinatorial probe anchor-ligation (cPAL) technology	35-base mate pair reads	http://www.completegenomics.com/

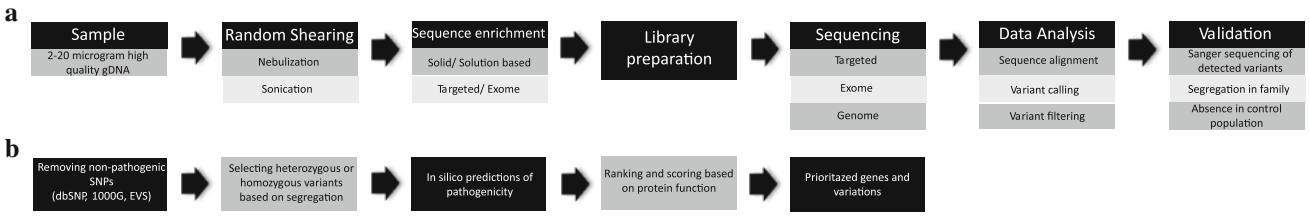

Fig. 1 Workflow of massively parallel sequencing and analysis. **a** 2–20 μg of genomic DNA is sheared randomly and targeted sequences are enriched following capture protocols in solid or liquid phases. Targeted genes/regions or exome can be enriched, or the whole genome can be sequenced. Amplified or single molecules are prepared for sequencing according to each platform. Output sequences are used for mapping to a reference genome, variation calling and filtering, using different algorithms and softwares. The last step is validation, where the interesting variations are confirmed by Sanger sequencing from the starting DNA, segregation of the variation in the family investigated, and a healthy control population checked. Sequencing of additional patients with similar phenotypes to identify more mutated patients with variations in the same gene, and functional studies could be done to confirm the implication of the gene or the pathogenicity of the variation if not previously known. **b** Different steps of filtering are shown. From the list of called variations, the known non-pathogenic variations are removed by comparison with dbSNP, 1,000 genomes and Exome Variant Server databases. Based on disease inheritance mode, homozygous or heterozygous changes are selected. Next, in silico prediction of pathogenecity and effect on splicing can be tested using different programs such as PolyPhen, SIFT or NNSPLICE. Scoring and ranking of the different variations/genes based on expression profile and function using different databases such as GENATLAS or Genecards provide a list of prioritized genes and variations

Marie–Tooth neuropathy. They prioritized sequence variants in genes known to be implicated in Charcot–Marie–Tooth neuropathies and found the compound heterozygous mutations p.Arg954X and p.Tyr169His in the *SH3TC2* gene as the causes of the neuropathy. Since then, several studies have been published using massively parallel sequencing for detection of causative mutations in different NMDs. Through exome sequencing, Montenegro et al. [62] found a missense variation, p.Val95Met, in the *GJB1* gene, a known gene in CMT neuropathies, in a family with CMT. Sanger sequencing confirmed this change and validated the complete co-segregation within the family.

In our recent publication, we showed the efficacy of massively parallel sequencing in molecular diagnosis of patients with different NMDs by capturing and sequencing 267 genes implicated in NMDs [92]. We could retrieve successfully all known mutations, detect and precise a large deletion linked to DMD, and identify novel disease-causing mutations in patients awaiting molecular diagnosis since more than 15 years. Other targeted sequencing focused on subsets of NMD genes. 25 patients with Duchenne or Becker muscular dystrophies (DMD/BMD) with known or unknown mutations were sequenced with the Illumina genome analyzer using a capture kit targeting the whole genomic sequence of muscular dystrophy-related genes after DNA barcoding [53]. The authors could detect disease-causing mutations in 24 patients out of 25 and concluded that this technology is useful for diagnosis of patients with DMD/BMD. Hoischen et al. [42] reported the validation of an array based sequence capture of seven genes and two loci related to the autosomal recessive form of ataxias, by sequencing these genes in five patients with known mutations and two unaffected persons using a one-quarter Roche GS FLX Titanium sequencing run. Other examples were dedicated to other specific disease classes [36, 45] (Table 2).

Such targeted parallel sequencing of all candidate genes is especially appropriate for disorders with high genetic heterogeneity like NMDs, and should ease the identification of allelic diseases, i.e. different diseases caused by mutations of the same gene. In addition, for large genes, such as *TTN*, which are difficult to fully test by conventional Sanger sequencing routinely even if known to be implicated in disorders, different studies have shown the effectiveness of massively parallel sequencing in variant detection of such a large gene [68, 72, 92]. Although there are some limitations in massively parallel sequencing as described below, important issues in genetic diagnosis laboratories such as cost and turnaround time can be resolved using massively parallel sequencing. Transfer of these technologies to diagnosis laboratories will benefit from the constantly dropping costs and increasing output. Reliable data can be produced if the depth of sequence coverage is high enough. In Fig. 2, an example of MPS achieved data is shown.

Possible applications of massively parallel sequencing in diagnosis

Diagnosis of affected individuals

Ideal strategies for diagnostic laboratories are methods that are simple with high accuracy and low error rate in a short run time; they should also be cost-effective with easy data analysis and interpretation [20]. Massively parallel sequencing is a useful alternative or complementary technique for molecular diagnosis. Routine use of massively parallel sequencing leads to a rapid screening and fast identification of mutations in rare genetic disorders through

Table 2 First reports using high-throughput sequencing for mutation and gene identification (rows 1–3), for neuromuscular diseases (row 4) and other recent examples using this strategy (rows 5–15)

Disease	Sequenced region	Sequencing machine	What was found	Publication year	Reference
Different disorders	Genome	Roche 454	Several putative mutations	2008	[94]
Freeman-sheldon syndrome (distal arthrogryposis type 2A)	Exome	Genome Analyzer IIx	Different mutations in *MYH3*	2009	[67]
Miller syndrome	Exome	Genome Analyzer IIx	Different mutations in *DHODH*	2010	[66]
Charcot–Marie–Tooth neuropathy	Genome	SOLiD	Compound heterozygous in *SH3TC2*	2010	[58]
Limb-girdle congenital myasthenic syndrome with tubular aggregates	Exome	Illumina HiSeq 2000 and Genome Analyzer IIx	Compound heterozygous in *DPAGT1*	2012	[7]
Hereditary myopathy with early respiratory failure	Exome	Illumina HiSeq 2000	Missense in *TTN*	2012	[69, 72]
Heterogeneous neuromuscular diseases	Targeted sequencing of 267 genes	Illumina Genome Analyzer IIx	Mutations in different genes detected in 12 patients	2012	[92]
Hereditary sensory autonomic neuropathy	Exome	Illumina HiSeq 2000	A deleterious mutation in *DST*	2012	[24]
Spinal muscular atrophy	Targeted sequencing of 73 genes in the 14q32 linkage interval	Illumina Genome Analyzer IIx	Missense in *DYNC1H1*	2012	[36]
Emery–Dreifuss muscular dystrophy	Exome	Illumina HiSeq 2000	Missense (homozygous) in *LMNA*	2012	[43]
Duchenne muscular dystrophy	Targeted sequencing of 79 exons of *DMD*	Illumina HiSeq 2000	Nonsense in *DMD*	2012	[55]
Nemaline myopathy	Targeted sequencing of 15 genes	SOLiD 4	Two missenses in *RYR1*	2012	[45]
Limb-girdle muscular dystrophy	Exome	Illumina HiSeq 2000	Missense in *DNAJB6*	2012	[37]
Genes associated with muscle disease and spastic paraplegia	Exome	Illumina Genome Analyzer IIx	Deleterious *CAPN3* mutation in myopathy	2012	[21]
Benign Samaritan myopathy	Exome	Illumina Genome Analyzer IIx	Allelic disease to RYR1 myopathies	2012	[11]

a

VariantID	Gene	TranscriptID	TranscriptLength	Chr	Start	End	Ref	Mut	HomHet	Coverage	ReadWithVar
X_149767058_DEL_4_1	MTM1	NM_000252.2	3421	X	149767058	149767061	AAAG	-	het	212.0	87.0
1_156848947_SNV_G_1	NTRK1	NM_002529.3	2638	1	156848947	156848947	T	G	het	52.0	16.0
12_863505_INS_3_1	WNK1	NM_001184985.1	11204	12	863505	863506	-	CTT	het	187.0	73.0

VariantID	VarType	CodingEffect	VarLocation	Exon	Intron	gNomen	cNomen	pNomen	rsID	rsValidation	hgmdID
X_149767058_DEL_4_1	DEL	Frameshift	exon	4	NA	g.149767058_149767061del	c.141_144del	p.Glu48Leufs*24	NA	NA	NA
1_156848947_SNV_G_1	SNV	Synonymous	exon	15	NA	g.156848947T>G	c.1839T>G	p.=	NA	NA	NA
12_863505_INS_3_1	INS	NA	intron	1	1	g.863506_863508dup	c.759+16_759+18dup	p.?	NA	NA	NA

b

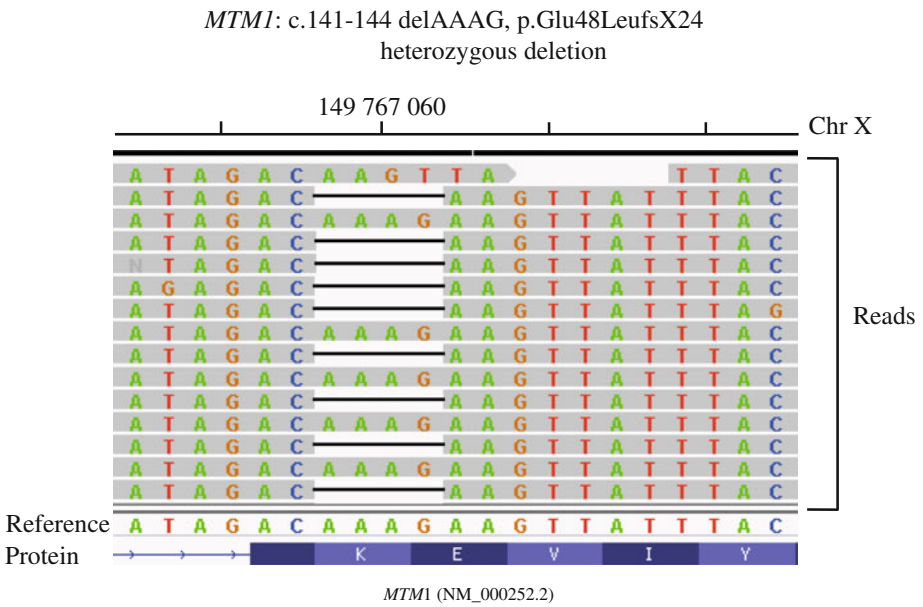

Fig. 2 An example of massively parallel sequencing data for a patient with myotubular myopathy. **a** The table shows three different variations: the *first row* is the disease-causing mutation which is a deletion of four nucleotides in the *MTM1* gene. The *second row* is a synonymous change and the *third* is an intronic insertion. **b** Deletion of four nucleotides at the beginning of exon 4 in the *MTM1* gene displayed with the integrative genomics viewer (IGV)

sequencing of either the exons or the genomic sequence of all genes or a subset of genes. Nowadays, with benchtop sequencers such as Ion Torrent PGM (Ion Torrent, Guilford, CT), MiSeq (Illumina Inc., San Diego, CA) and 454 GS Junior (454 Life Sciences, Roche, Branford, CT), these new technologies can be transferred easily to clinics for mutation identification in patients, carrier status determination and prenatal diagnosis. The cost of whole genome sequencing of a DNA without interpretation is about 6,500 USD and exome sequencing is offered for about 1,000 USD (an example: http://www.edgebio.com). High throughput screening at low cost and low complexity can be addressed by targeted sequencing rather than complete sequencing of exomes or genomes, combined with multiplexing of barcoded samples. The cost for this strategy depends on the number of mixed samples and the size of targeted regions and is generally less than 1,000 USD per sample. Moreover, targeted and exome sequencing are starting to be proposed on a routine diagnosis basis (for examples: http://www.bcm.edu/geneticlabs/ or http://genetics.emory.edu/egl/). Financing this cost depends on specific countries regulation and insurance policies.

Massively parallel sequencing will probably replace most of the genetic screening methods but will not substitute for clinical and histopathological investigations. While until now, clinical and pathological diagnoses were used to orient genetic screening, massively parallel sequencing may now be used on a first intention to better orient clinical tests that could be invasive, costly and necessitate patient travel. Although biopsies might be less needed for establishing the molecular diagnosis, they will be necessary for understanding the pathogenesis of a disease. Thus, massively parallel sequencing will promote a more efficient integrated diagnosis encompassing clinic, histopathology and molecular analyses.

A rapid and accurate molecular diagnosis will have important impacts on patients as it will improve disease management, may lead to inclusion into therapeutic trials, will help genetic counseling and will reduce further unhelpful investigations.

Prenatal diagnosis

This technology is also applicable to noninvasive prenatal diagnostic by massively parallel sequencing of fetal DNA present in the maternal plasma. The circulating cell-free fetal DNA is assessed using a set of differentially methylated markers [71] or by exome or genome sequencing compared to parents DNAs [26]. Currently, this approach is used for detection of severe monogenic disorders, fetal chromosomal aneuploidies and determination of blood groups [56]. Such approach will avoid the need for invasive and risky procedures such as chorionic villus sampling. Comprehensive diagnosis of any Mendelian disorders such as NMDs early in pregnancy might permit early termination of pregnancy in an ethically acceptable way. It can have a significant impact on the reproductive decision making and pregnancy management as it has been shown that families with increased risk of having a child with a severe disorder tend to have fewer children [78].

Carrier detection

The massively parallel sequencing approach might also apply to families without previous cases of severe genetic disorders. It would be technically possible to determine the carrier risks of individuals for any Mendelian disorders and predict for example, for which recessive diseases both parents are carriers of a heterozygous mutation [8]. Based on this knowledge, parents would have the possibility to test the fetus by sequencing the gene(s) with heterozygous mutations, or performing in vitro fertilization (IVF) and selecting non-affected zygotes. This approach will not predict de novo mutation, another source of disease-causing mutations.

Massively parallel sequencing limitations and improvements for clinical use

Although massively parallel sequencing is an appropriate alternative technique to use in diagnostic laboratories, some issues need to be addressed.

False negatives

One challenge in the use of massively parallel sequencing for diagnosis is the detection of different types of mutations, especially repeat expansions or structural variations. Some of the most common NMDs are due to repeat expansions; these mutations may be missed leading to false negative results. Increasing sequence coverage [40], improving bioinformatics algorithms and softwares and novel sequencing technologies may solve this problem in order to propose an exclusion diagnosis. Alternatively, a combination of different methods can be proposed. Detection of structural variations will benefit from whole genome sequencing. Another obstacle is the incomplete coverage of commercial exome capture libraries. Although new versions of capture kits are released consistently, none of them captures all coding parts of all genes [47]. This issue can be addressed by targeted sequencing of genes and regions of interest and improving the capturing process.

False positives

Another drawback is the high error rates in massively parallel sequencing compared to Sanger sequencing. Artificial mutations can be produced during templates amplification or sequencing, leading to false positive results. Thus, Sanger sequencing of interesting variants detected by massively parallel sequencing is an essential validation step which is increasing the cost and turnaround time. This issue can be addressed by improving capturing and sequencing approaches to increase variant coverage, and thus leads to achieve reliable data. In addition, better data filtering protocols can reduce the pool of false positives.

Volume of data

Massively parallel sequencing generates a high volume of data which becomes problematic for data management, analysis and storage in diagnostic laboratories [74]. As the genetic test results should be kept at least 5 years or even 10–20 years [85], substantial investment in infrastructure and informatics is needed. Cloud computing can be a solution for reducing the cost of expensive computing infrastructure [20, 84]. Importantly, decreasing costs in massively parallel sequencing outpaces the increase in calculation power and storage capacity of computers. As sequencing becomes cheaper than data storage of the corresponding sequence output, re-sequencing of a patient DNA might be more cost-effective than saving original data [38]. In other words, DNA is becoming the cheapest storage support.

Variants filtering and mutation identification

As a high number of variations are detected by massively parallel sequencing, it is difficult to distinguish between

individual, rare and non-pathogenic variations without clinical significance versus disease-causing mutations. Defining the complete list of polymorphisms in different populations will require sequencing a large part of the world populations [52]. A recent study hypothesized that 27 % of published mutations appear to be sequencing errors, common polymorphisms, or have a lack of pathogenicity evidence [8]. This issue can be addressed by further analysis and validation such as in silico predictions of pathogenicity, detection of mutations in the same gene in unrelated individuals with the same disorder and absence in a control population, co-segregation in affected families and finally functional studies [90]. Multiple criteria should be combined in order to prove the pathogenicity of the variation.

Incidental findings

With massively parallel sequencing, incidental findings are an issue and unsolicited/unexpected information can be problematic, especially for unreported diseases. Diagnostic laboratories should prepare informative, complete and precise consent and result reports to use massively parallel sequencing for diagnosis application. Guidelines should be established and implemented by national committees in order to determine whether the analysis and reports should be selective or complete and what is the best way to cope with the ethical issues [16, 31].

Trained people

Having enough trained people for data analysis and information transfer to patients will be a key point for the future. Specific trainings are needed for interpreting genetic data for rare and common diseases and eventually genetic predispositions, and how to transfer this information to patients. Ideally a team of bioinformaticians, scientists, geneticists and clinicians will collaborate together in order to provide an accurate and accessible results to patients. Development of robust, easy to use and practical softwares in the clinical setting and comprehensive mutations and polymorphisms databases are necessary. For a full understanding of genetic variations, considerable amount of genetic data from various populations should be gathered and interpreted. Recurrent re-analysis of the sequencing data will be needed while our understanding of genetic variations improves. Well-trained clinicians and pathologists in neuromuscular disorders and bioinformaticians will have crucial roles in the selection of patients and interpretation of the obtained data.

Conclusion

Although there are some limitations and challenges using massively parallel sequencing for diagnosis, this technology appears mature enough for a routine approach in clinics. As the cost of massively parallel sequencing declines consistently and the technology improves continually leading to obtaining faster and more accurate data, it is strongly anticipated that this recent technology will complement clinical and pathological investigations and will greatly improve diagnosis and management of Mendelian disorders.

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