PERSPECTIVE

Genetic testing in neurology exploiting next generation sequencing: state of art

Next generations sequencing (NGS) is definitely one of the most revolutionary technology of the last years in genetic and medical field (Kricka and Di Resta, 2013). It brought important changes in genetic testing of inherited human disorders, in particular in neurological Mendelian forms, such as inherited neuropathies, ataxias or monogenic form of epilepsy, where "diagnostic odyssey" is quite common. This term refers to the single-gene test approach where patients are evaluated by multiple providers, sometimes for years, without a genetic diagnosis (Di Resta et al., 2018). Indeed, in the Sanger sequencing era, neurologists were quite frustrated by the low diagnostic yield obtained by testing selected candidate genes, also due to the difficulties in differentiating genetic forms from acquired one, having the same clinical manifestations. In this context, clinicians had to pick a candidate gene to analyze and the gene-by-gene sequencing approach was not economical or efficient (Di Resta et al., 2018).

In recent years, NGS increased the diagnostic success rate, allowing a comprehensive genetic analysis. In particular NGS brought advantages for the testing of neurologic disorders characterized by a genetically heterogeneous picture, in which the same phenotype can be caused by different mutated genes (Di Resta and Becchetti, 2010; Di Resta and Ferrari, 2018). Here we will discuss the different NGS approaches that can be adopted.

Different genetic testing approaches and related open challenges: The importance of obtaining a positive genetic result has a good impact for i) the patient, avoiding improper therapies and additional invasive testing, such as biopsy; ii) for the disease management and prognosis; iii) for family genetic counselling; iv) for the opportunities to participate in drug clinical trials.

Three different approaches of analysis are available (whole-genome sequencing (WGS), whole-exome sequencing (WES) and targeted-panel sequencing) using next-generation sequencing (Di Resta and Ferrari, 2018).

WGS: WGS consists of the analysis of approximatively 3 billion base-pairs of DNA sequence. However, due to current technical limitations, the obtained coverage is not homogenous in several causative genes of inherited disorders and it is not adequate for a diagnostic test. Moreover, there is no a clinical utility for the analysis of variants in intronic or intergenic region, whose pathogenic role is unknown and interpretation is quite challenging (Di Resta and Ferrari, 2018), as it will be discussed below. Not at least, the sequencing cost of WGS is 5-fold higher than a WES analysis with a 100X coverage. Also the computational effort, including bioinformatic data analysis, data storage and interpretation, is remarkably greater than other NGS approaches (Peretto et al., 2018).

WES: WES covers all the protein-coding regions of the genome and recently it has been already adopted by several clinical laboratories for genetic testing. However, also in this case, the main issue is the need to have an average coverage of 150X, in order to assure that most analyzed regions have a robust read depth, with at least 20X coverage (Peretto et al., 2018).

A recent study exploited WES approach and analyzed the 56 genes recommended by the American College of Medical Genetics (ACMG) for their pathogenic relevance (Di Resta et al., 2018). Considering a coverage threshold of at least 70X, more than 7/56 genes had an insufficient read depth. These data suggested that the use of WES for diagnostic purpose may lead to sequencing gap in some critical genes or regions of interest, mainly due to intrinsic sequence composition or GC content (Di Resta et al., 2018).

The use of WGS or WES opens also a debate on the possible detection of "incidental findings", defined in 2013 by the ACMG as "genetic variations identified by genomic sequencing but not related to the disease being investigated". According to the European Society of Human Genetics guidelines, the targeted diagnostic testing should be performed minimizing the likelihood of detecting incidental findings, focusing only on genes clinically actionable. It means that genetic testing should analyze only the causative genes associated with the primary clinical questions, even if a broader panel of genes or the WES has been performed (Di Resta et al., 2018).

Recently, ACMG guidelines have been created for the proper handling of these results with important ethical implications but there is not a univocal consensus and the discussion is still open (Di Resta et al., 2018). Moreover, the analysis of WES can lead to the identification of several rare variants in novel genes, whose disease-association have not been previously validated and further studies are necessary for assessing their pathogenicity (Di Resta et al., 2014). So, reporting these variants is not useful for diagnostic purpose, since, as already explained, a definitive genetic diagnosis can be performed only for known causal genes (Mancini et al., 2015; Di Resta and Ferrari, 2018).

Despite the limitations above, WES approach may be useful in patients affected by multiple congenital anomalies or autism spectrum disorders, whose targeted analysis of known causative genes was negative (**Figure 1**). Furthermore, for research purpose, WES or WGS approaches are appropriate in order to identify new candidate genes or to analyze clinical cases without a specific phenotype and a clear indication for a definite panel of causative genes. In particular, for the identification of a novel gene, the trio analysis, the segregation analysis in a family with affected and unaffected persons or a large number of affected patients with similar phenotype are necessary. Mainly for this aim, a worldwide collaboration between research team is need in order to look for patients with the same disorders and to share and collect NGS results in an integrate large database (Peretto et al., 2018). The identification of new causative genes leads to the need of a continuous updating of each diagnostic gene panel content, that is one of the main challenges in the targeted sequencing approach.

Targeted panel sequencing: The NGS analysis of targeted panel is an effective approach in several disease categories, such as inherited neuropathy, myopathy, genetic epilepsy syndromes, neuromuscular or motor neuron disorders, characterized by genetic heterogeneity and in which targeted genetic testing achieved a high diagnostic yield (Tosetti et al., 2017; Peretto et al., 2018).

In contrast to WGS and WES, targeted analysis allows the enrichment and sequencing of approximately 50 to 300 genes, achieving a very high coverage depth with a drastic cost reduction. The high coverage is essential for a robust data in clinical diagnostics, where the accurate variant identification is crucial. So, the targeted panel approach presents the lowest number of false-negative than the other two NGS approaches (Di Resta et al., 2018) and it is the most efficacious approach in molecular diagnosis of neurologic disorders. Indeed, the diagnostic yield of disease gene panel analysis ranges from 10 to 70%, since more causative genes remain to be discovered. It is not surprising, and it is related to the well-established clinical and genetic heterogeneity of neurological disorders (Di Resta and Ferrari, 2019).

In targeted testing approach, the clinician involvement and collaboration with laboratory geneticists are essential in defining the disease category and in choosing the associated causative genes to be analysed (Di Resta et al., 2018). Moreover, also for the interpretation of variant pathogenicity, the straight collaboration is essential between neurologists, for their complete knowledge of clinical picture, and geneticists and bioinformaticians, who know the requirements for clinical testing and the data analysis process, respectively (Di Resta and Ferrari, 2018). Variant interpretation is one of the most critical steps in the NGS diagnostic process and it will be discussed further in the next section.

The classification and interpretation of a rare variant: the main issue of diagnostic testing: So far, in genetic analysis one challenging step is without doubt the pathogenicity interpretation of a rare detected variant.

Reliable interpretation of the multiple variants identified through NGS will require additional experience and validation before it reaches the clinical purpose on a large scale, particularly for diagnosis of complex traits (Di Resta et al., 2018).

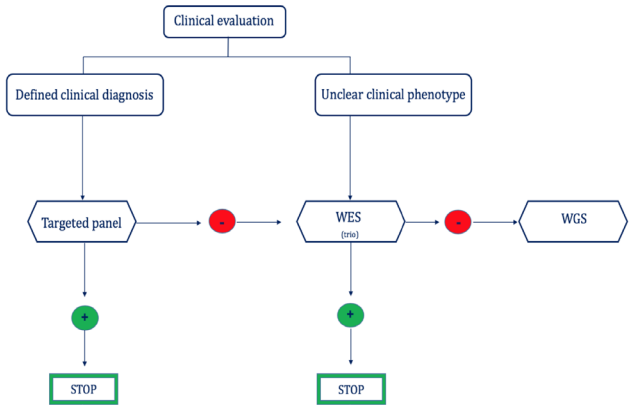

Figure 1 Schematic workflow for the identification of a causative mutation exploiting high-throughput sequencing approach.
At first, the clinical phenotype has to be evaluated and defined. If there is a clear clinical diagnosis, the targeted analysis of the known associated panel genes is recommended. If this first level of analysis is negative or if the clinical phenotype is undefined, WES or WGS can allow to identify new candidate mutations for research purpose. WES: Whole-exome sequencing; WGS: whole-genome sequencing.

Actually, the interpretation of genetic variants is based on criteria published by the American College of Medical Genetics and Genomics (ACMG). The ACMG recommends that the variants should be allocated to one of the five defined categories (Richards et al., 2015): a) pathogenic variant (class V): the sequence variation is previously reported and described as causative of the clinical suspicious; b) likely pathogenic variant (class IV): the sequence variation is not previously reported as expected to cause the disorder, frequently localized in a known disease gene; c) variant of unknown clinical significance or VUS (class III): the sequence variation is unknown or expected connected with a clinical presentation; d) likely benign (class II): the sequence variation is probably not causative of the pathology; e) benign (class I): the sequence variation is already reported and documented as neutral variant. The criteria for the classification of these classes of variants are based on literature data, population frequencies, clinical findings and mutation databases.

The clinical management of VUS represents an open issue for the diagnostic process. Sometimes the segregation analysis including affected members can be useful for the interpretation and prioritization of VUS. So far, clear guidelines for the VUS interpretation are lacking or incomplete. For example, in order to try to assign a pathological score to VUS, it is important to consider the allelic frequency in a control population (1000 Genomes or exome sequencing project consortium [ExAC]), the amino acidic conservation and the predicted effect on protein function obtained by in silico prediction tools. Unfortunately, the use of in silico prediction algorithms presents some intrinsic limitation, affecting their specificity and sensitivity, that can lead to possible false-positive and false-negative interpretations. Also the population frequency data have some caveat, related with the control populations sequenced in 1000 Genome project or ExAC. Indeed, they represent only a fraction of the worldwide populations, therefore the declared allelic frequency may not reflect the real population groups.

So far, the overcoming of this issue on the management of VUSs is crucial. The collection of detected VUSs in a database containing both genetic and clinical information is fundamental for a further re-evaluation when the same VUS is detected in other patients with a similar phenotype. Moreover, also the sharing of these variants and their associated phenotypes between different referee medical centers can allow a progressive and definitive classification of these variants, such as in LOVD (Leiden Open Variation Database). On the other hand, over time also several mutations at first classified as pathogenic can be re-classified as polymorphisms on the light of new reported clinical data or functional studies.

The correct interpretation of the possible pathogenic role of a rare variant is crucial to avoid the report of false-positive results to patients, in order to prevent emotional burden for patients and difficulties in the clinical management, especially in genes known to be highly polymorphic (Di Resta and Ferrari, 2018). Moreover, genetic testing may be recommended also for additional relatives of the proband, often still asymptomatic, for the disease risk assessment with important psychological issues.

Therefore, it is necessary to keep in mind that a clinical report is not a list of detected rare variants, but it has an impact on the patient management. Therefore, the clinical expertise is necessary for explaining the report at patient level and for requiring additional clinical testing for a further confirmation of clinical diagnosis related with genetic findings. Hence, only the strength collaboration of geneticists and clinicians can improve the quality of patient care (Kricka and Di Resta, 2013; Di Resta et al., 2018).

Conclusions: So far, there are still limitations to overcome in the use of NGS in clinical diagnostics. For example, there are some genetic abnormalities that cannot be reliably identified using NGS. Indeed, the short reads of 200–300 reads obtained in the NGS enrichment don't allow an accurate analysis for the copy number variation (CNV) or for nucleotide repeat expansions, which are a common genetic cause underlying neurologic disorders, such as fragile X syndrome ore Friedreich ataxia. So far, new algorithms and bioinformatic tools are developing for detecting CNV using NGS with an increased accuracy (Di Resta et al., 2018). They could be useful also for the detection of large deletions or duplications underlying some neuromuscular disorders, such as Duchenne or Becker dystrophy (Di Resta and Ferrari, 2019). Moreover, in the future the use of sequencers of third generation, such as PacBio instruments, can allow to analyze CNV or nucleotide expansion, sequencing up to 20-kb fragments of DNA (Di Resta and Ferrari, 2018).

To date, in our diagnostic laboratory experience we exploit the targeted approach. It is the best suitable for diagnostic purpose, increasing the diagnostic yield reached in neurology medicine compared with previous Sanger sequencing era (Di Resta and Ferrari, 2018). However, as sequencing technology and analysis algorithms are continuously improving, we think that in the future WES or WGS will be routinely used in clinical laboratories, bringing great advances in the discovery of novel candidate genes, mainly exploiting the trio analysis. For sure, the knowledge gained by NGS will be essential to meliorate the patient care and to carry on the application of personalized and precision genomic medicine, opening new opportunities for neurologic care in the near future (Malentacchi et al., 2015; Prodan Žitnik et al., 2018).

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