Genome Analysis for Genetic Diagnosis of Epilepsies and its Challenges in Clinical Practice

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Summary

High-throughput sequencing (HTS) has proven to be particularly useful for the causative molecular diagnosis of genetically heterogeneous Mendelian disorders, such as epilepsies. It presents clear benefits in the routine clinical setting, namely higher diagnostic yield, as well as improved time and cost effectiveness, compared to conventional sequencing technologies.

Different approaches are currently available, including targeted sequencing of predetermined panels of genes, or exome sequencing with targeted bioinformatics analysis. Daily practice of HTS has necessitated the development of specialized task forces, who work on selection of cases, variants' interpretation and classification, as well as on the establishment of good practice guidelines and adoption of reimbursement policies. Pre- and post-test genetic counselling remains a central aspect; moreover, multidisciplinary collaboration between health care specialists and clinical geneticists familiar with new sequencing technologies is required to warrant successful diagnostic application of HTS in the clinic. The current diagnostic yield of HTS for suspected Mendelian disorders in general, and neurologic disorders specifically varies between 23 and 31%. HTS is the method of choice for establishing the exact molecular defect in these disorders.

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Key words: High-throughput sequencing, exome sequencing, diagnostic, epilepsy

L'analyse génomique pour le diagnostic génétique des épilepsies et ses défis dans la pratique clinique

Le séquençage à haut-débit (SHD) a clairement montré son utilité dans le diagnostic des maladies mendéliennes avec hétérogénéité génétique, comme les épilepsies. Son utilisation en pratique clinique a permis d'augmenter de manière significative le rendement diagnostique, pour un coût et un temps d'analyse inférieurs à ceux des technologies de séquençage classique.

Différentes approches sont disponibles : il est possible de séquencer de manière ciblée un groupe de gènes prédéfinis ou d'effectuer un séquençage complet de l'exome, avec analyse bioinformatique ciblée sur les gènes d'intérêt. L'introduction du SHD en clinique a motivé la création de groupes de travail spécialisés, impliqués dans la sélection des cas, l'interprétation et la classification des variants, la production de recommandations de bonne pratique et la mise en place des modalités de remboursement. Le conseil génétique avant et après analyse reste un aspect très important de la prise en charge. Par ailleurs, le succès de l'implémentation de ces nouvelles technologies nécessite une collaboration pluridisciplinaire entre les différents spécialistes et les généticiens. Le rendement diagnostique actuel du SHD en cas de suspicion d'affection mendélienne en général, et de conditions neurologiques plus spécifiquement, est estimé à 23-31%. Le SHD est donc considéré comme la méthode de choix pour le diagnostic moléculaire de ces maladies.

Mots clés : Séquençage à haut-débit, séquençage de l'exome, diagnostic, épilepsie

Genomanalyse in der genetischen Diagnostik von Epilepsien und ihre Herausforderungen in der klinischen Praxis

Die Hochdurchsatzsequenzierung (HDS) hat sich in der Diagnostik mendelscher Erkrankungen mit heterogener Genetik (z. B. Epilepsie) als ausserordentlich nützlich erwiesen. Ihr Einsatz im Klinikalltag führte zu einer signifikanten Erhöhung der diagnostischen Ausbeute und dies mit geringeren Kosten und Analysezeiten als

bei herkömmlichen Sequenzierungsverfahren.

Dazu gibt es verschiedene Ansätze: Die gezielte Sequenzierung einer im Vorfeld definierten Gruppe von Genen oder die Sequenzierung eines vollständigen Exoms mit gezielt auf die gewünschten Gene gerichteter bioinformatischer Analyse. Die Einführung der HDS in den Klinikalltag bedingte die Schaffung spezieller Arbeitsgruppen, die mit der Fallauswahl, der Interpretation und Klassifizierung der Variablen, der Erstellung von Empfehlung zur Guten Klinischen Praxis und der Umsetzung der Kostenerstattungsmodalitäten befasst sind. Die genetische Beratung vor und nach der Analyse ist auch weiterhin ein extrem wichtiger Aspekt der Betreuung. Im Übrigen setzt die erfolgreiche Umsetzung dieser neuen Technologien eine pluridisziplinäre Kooperation der verschiedenen Spezialisten und Genetiker voraus. Gegenwärtig liegt die diagnostische Ausbeute der HDS bei generellem Verdacht auf eine mendelsche Erkrankung und speziell auf neurologische Erkrankungen, bei 23-31%. Daher gilt die HDS als bevorzugte Methode zur molekularen Diagnostik dieser Krankheiten.

Schlüsselwörter: Hochdurchsatzsequenzierung, Sequenzierung des Exoms, Diagnostik, Epilepsie

1. Introduction

High-throughput sequencing (HTS) has been introduced in clinical practice in order to simultaneously test hundreds of genes for diagnostic purposes in Mendelian disorders, resulting in increased diagnostic yield, reduced time to diagnosis, and improved cost effectiveness [1, 2]. It has been particularly useful in genetically heterogeneous diseases, such as epilepsy, in which a considerable number of genes have been implicated [2]. The implementation of HTS requires pluridisciplinary collaborations between health care specialists and clinical geneticists familiar with sequencing techniques and strategies, such as gene panel designs and management of the diverse implications of HTS results.

2. Technical aspects of high-throughput sequencing

The main technologies currently used for molecular diagnosis allow the simultaneous sequencing of hundreds of millions of small DNA fragments [1]; the subsequent bioinformatics analysis permits the detection of single-nucleotide substitutions and small (8-10 nucleotides or less) deletions or insertions [1]. Depending on the specific application, it is now possible to sequence the whole genome of an individual, the whole protein-coding region of the genome (exome) or selected number of genes at a lower cost and faster turnaround-time [3]. The exome sequence provides the possibility to detect variants in hundreds or thousands of genes of interest in one single test. This increases dramatically the analytical sensitivity of the test making it particularly useful in the clinic [4, 5].

The notion of coverage, or in other words how many times each nucleotide position is read, determines the accuracy of the test. While there are no specific guidelines, most laboratories tend to consider a coverage of 30 ("30x") acceptable for the detection of heterozygous Single Nucleotide Variants (SNV).

2.1. High-throughput sequencing approaches

In clinical practice, most centres select genes potentially causative of the patient's disease in order to minimize the risk of possible unsolicited findings. Different strategies are being offered [6], but in most cases, the different teams are selecting one of the following two common approaches:

1. Targeted gene capture and high-throughput sequencing
2. Exome sequencing and targeted bioinformatics analysis

2.1.1. Targeted gene capture and high-throughput sequencing

In this approach, there is a capture of sequences from a predetermined panel of genes related to the phenotype in question. The main advantage of this option is the extensive sequencing depth and coverage of the targeted genes, within reasonable cost and shortened turn-around-time. The principal limitation of this strategy, however, is linked to the non-dynamic nature of the established panels. If no variant is found within the tested panel, the sample of the patient may require further sequencing, either with a new panel, or with the whole exome/genome.

2.1.2. Exome sequencing and targeted bioinformatics analysis

This alternative strategy uses a whole-exome sequencing (WES), but the gene panels of interest are then bioinformatically selected and analysed. WES usually has a lesser coverage than the previous alternative however, it offers the great advantage of flexibility: the genes are targeted by means of bioinformatics, offering therefore the possibility to easily update the gene panel to the most recent discoveries. It is therefore conceivable to update the panels and to reanalyse the stored data periodically, without a need of further sequencing. Many diagnostic laboratories, including ours

at the Geneva University Hospitals, have decided to use this highly dynamic strategy [7].

2.2. Designing a gene panel

Panels of genes offer flexibility in the analysis, but are difficult to design: indeed, the update of gene panels requires a constant surveillance of the literature and consensus statements among clinicians and scientists. Our experience has shown that a gene panel design should be debated and the criteria for inclusion/exclusion of genes are not always widely accepted. Some argue that only genes with proven pathogenicity should be included, while others support a more inclusive attitude: obviously, panels obtained using more stringent criteria result in less false positive, and more false negative diagnoses. In order to offer high and uniform standards of care across Europe, the latest European guidelines introduce the concept of core genes, i.e. genes that should always be interrogated when a specific disorder is tested (EuroGentest guidelines, unpublished, http://www.eurogentest.org/index.php?id=958). Expert groups per phenotype should be created and updates of gene panels need to be maintained.

2.3. Limitations of the method

Coverage of sequenced regions is unevenly distributed across the exome (or genome). Some regions are less covered than others, which can lead to false negative results (absence of detection) if the causative variants lay in a poorly covered genomic area [8]. Precise knowledge of the coverage is crucial in the evaluation of HTS results; the gaps in coverage need to be completed either by improving the capture reagents, or by additional techniques (i.e. Sanger sequencing).

Additionally, long stretches of repetitive DNA (including trinucleotide repeats), chromosomal aneuploidies, Copy Number Variants (CNVs) or structural variations (i.e. translocations) can be either missed or very difficult to detect. If genetic disorders with such pathogenic variants are suspected, they must first be investigated by an appropriate technique such as specific tests for the detection of trinucleotide expansions or array comparative genomic hybridization (array-CGH) for CNVs bigger than 20-100 Kb. In the case of epilepsy specifically, array-CGH can detect causal genetic variants in 10-15% of the patients and it is recommended as a first tier analysis [9 - 11].

3. Challenges of HTS in diagnostic practice

High-throughput sequencing has brought new challenges in clinical practice. While the principal aim was traditionally the detection of variants, there is a shift in our concerns: the main difficulty is no longer the identification of variants (although improved algorithms are still needed for insertions and deletions), but their interpretation, as well as the correct and concise transmission of the genetic information to the patient.

3.1. Evaluation of variants' pathogenicity

Using a multitude of tools and available databases to annotate the variants (**Table 1**), the analytical team of every laboratory classifies each variant according to its appreciated level of pathogenicity (from pathogenic and likely pathogenic to variant of unknown clinical significance to likely benign and benign). Recently, specific guidelines have emerged in order to assist the analytical teams to this task, by using the recognized qualities of each variant and judging whether they support a pathogenic or benign function [12, 13].

Given the total number of attributes that must be evaluated before pronouncing each variant's role in disease [13], variant interpretation is becoming increasingly complex. Recent publications have shown that the analysis of the sequencing results is the more time consuming and costly aspect of HTS [14, 15]. To overcome this problem, many clinical teams, including ours, host regular multidisciplinary meetings with experts from different fields (clinical and molecular genetics, bioinformatics and ethics) in order to discuss the interpretation of the identified variants.

In this process of interpretation, detailed clinical description, and familial history, remains essential [16]. High-throughput sequencing does not replace clinical expertise: it renders it even more important. When several variants are identified, in addition to bioinformatics tools and scientific literature, the precision and accuracy of clinical information is crucial. The geneticists' competences and clinical expertise of neurologists and other medical specialists is required to ascertain the variants' pathogenicity and link the genotypic findings to the patients' phenotype [17].

3.2. Variants of Unknown clinical Significance (VUS)

In certain cases, the available data are currently insufficient to conclude to the pathogenicity of a variant, therefore called "Variants of Unknown clinical Significance" (VUS). Because pathogenicity's arguments are insufficient, inconclusive or conflicting, such variants cannot be used to explain the patient's phenotype, but can neither be completely discarded as non-pathogen-

Table 1: Databases and tools used for the variants' interpretation and annotation.

Population, disease-specific, and sequence databases	
Population databases	
Exome Aggregation Consortium	http://exac.broadinstitute.org/
1000 Genomes	http://browser.1000genomes.org
dbSNP	http://www.ncbi.nlm.nih.gov/snp
dbVar	http://www.ncbi.nlm.nih.gov/dbvar
Disease databases	
ClinVar	http://www.ncbi.nlm.nih.gov/clinvar
OMIM	http://www.omim.org
Human Gene Mutation Database	http://www.hgmd.org
Locus/disease/ethnic/other-specific	http://www.hgvs.org/dblist/dblist.html
Leiden Open Variation Database	http://www.lovd.nl
DECIPHER	http://decipher.sanger.ac.uk
Sequence databases	
NCBI Genome Source	http://www.ncbi.nlm.nih.gov/genome
RefSeqGene	http://www.ncbi.nlm.nih.gov/refseq/rsg
Locus Reference Genomic (LRG)	http://www.lrg-sequence.org
MitoMap	http://www.mitomap.org/MITOMAP/
In-silico predictive algorithms	
Missense prediction	
SIFT	http://sift.jcvi.org
MutationTaster	http://www.mutationtaster.org
PolyPhen-2	http://genetics.bwh.harvard.edu/pph2
CADD	http://cadd.gs.washington.edu
Splice site prediction	
GeneSplicer	http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml
Human Splicing Finder	http://www.umd.be/HSF/
MaxEntScan	http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html
NetGene2	http://www.cbs.dtu.dk/services/NetGene2
NNSplice	http://www.fruitfly.org/seq_tools/splice.html
FSPLICE	http://www.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind
Conservation scores	
GERP	http://mendel.stanford.edu/sidowlab/downloads/gerp/
PhastCons	http://compgen.bscb.cornell.edu/phast/
PhyloP	http://compgen.bscb.cornell.edu/phast/

ic. Concerning the reporting of such variants, opinions and practices differ, yet in most cases in current practice, they are not reported [18]. Nevertheless, all laboratories record the information since advances in the analyses and the scientific knowledge may elucidate their role in human disease. It is therefore desirable to introduce a re-analysis of the VUS when new knowledge become available, or when additional cases with the same VUS have been identified and reported in the literature or in databases. As follow-up strategies are thus so far not automatized, we suggest that patients contact the diagnostic services every 2-3 years. Finally, even with such a powerful analysis, it is common not to find the cause of the disease [16].

3.3. Incidental findings; informed consent

Incidental findings are universal in medicine. Indeed, each medical exam can lead to unexpected findings: for example, chest radiography for pulmonary infection can reveal the presence of a tumour. HTS can generate results unlinked to the indication of the sequencing. It is estimated that 1 to 3% of patients undergoing WES have such findings [19]. These incidental findings may be clinically useful, in different ways, but need to be anticipated and discussed with the patient.

Incidental findings can be of different kinds:

1. Actionable variants, such as cancer predisposition (i.e. *PTEN* responsible for Cowden Syndrome)
2. Non actionable variants of adult-onset diseases, such as late-onset neurodegenerative diseases (i.e. *NOTCH3*, implicated in CADASIL)
3. Carrier status, for variants in autosomal recessive or X-linked genes, implicating specific risk for the offspring (i.e. *SURF1* causing autosomal recessive Leigh syndrome)

Few attitudes concerning their testing and reporting are proposed from different national societies. In 2013, the American College of Medical Genetics and Genomics provided recommendations proposing to routinely search and report specific variants within a minimum set of 56 genes, responsible for 24 disorders that are highly actionable [18]. These recommendations were later changed to an opt-out policy. In Europe, the general attitude is more reserved concerning the seeking out of actionable variants, and the Swiss Society of Genetic Medicine has proposed an opt-in strategy that is being reflected in the consent form for the patients. Thus, the patient has the option to choose which types of variants he/she wishes to know and controls the flow of information [18].

3.4. Pre- and post-test counselling

Pre-test consultation generally resumes the principles of HTS, including its limitations with regard to sensitivity, and mentions the potential results. Families have to be prepared to the possibility of not finding the molecular cause, as the diagnostic yield of HTS reaches about 25-30% [5, 4], thus avoiding unrealistic hopes. Depending on the gene panel used, the issue of unexpected findings is explained, as well as the potential uncertainty due to VUS. The specific informed consent is explained and signed.

Post-test consultation is dedicated to the results of the analysis. When positive, explanations about the disease, its inheritance and family implications are given. In the cases of negative result (or VUS), the expert counsellor needs to explain that a negative result does not reliably exclude the presence of a causative variant (limitation of coverage and number of genes tested). We propose a regular follow-up (every 2-3 years) in order to update the scientific data when a VUS has been identified or to discuss a novel bioinformatics analysis if new genes are described.

4. Health insurance consideration and costs

In Switzerland, HTS was officially introduced as a reimbursed genetic test in January 2015 and entered the so-called « Liste des analyses » (LA) (http://www.bag.admin.ch/themen/krankenversicherung/00263/00264/04185/index.html?lang=fr).

The recommended reimbursable cost of the HTS test is based on three steps: the high-throughput sequencing, the bioinformatic analysis, and the additional confirmatory analyses such as Sanger sequencing and/or the detection of large rearrangements (e.g. by MLPA).

More specifically:

1. High-throughput sequencing has a fixed price of 2300.- CHF, irrespectively of the technology used for selection of the genes of interest and the sequencing technology.
2. The cost of the bioinformatics analysis varies accordingly to the number of analysed genes: 600.- CHF for 1-10 genes, 1000.- CHF for 11-100 genes and 1500.- CHF for 101 genes and more.
3. For the confirmatory tests, there are limitations according to the number of genes analysed: 2 Sanger confirmations for 1-10 genes, 4 for 11-100 and 6 for > 100 genes. In all the cases a maximum of 4 MLPA can also be added.

This modular aspect provides flexibility to the diagnostic laboratories and allows some steps to be performed separately and several times, i.e. consecutive

bioinformatic analyses.

Nevertheless, within Swiss law, the diseases for which genetic analysis are reimbursed are positively defined, i.e. a genetic condition has to be clearly mentioned in the "Liste des analyses" in order to be potentially "reimbursable". Unfortunately, currently "Epilepsy" as a general term does not figure in this list. Therefore, clinicians may have to formally request, by correspondance to Orphan disease procedure (http://www.sgmg.ch/view_page_professional.php?view=page&page_id=29), or to the insurance health company the acceptance of reimbursement of the analysis, before performing the test.

Because of the complex issues in the HTS analyses and interpretations the Swiss Federal Office of Public Health has limited the prescription of HTS for more than 10 genes to clinical geneticists with the Medical Genetics FMH title.

5. An example

Dizygotic 12-months-old male twins were referred to our service because of seizures since the age of 4 months associated with severe developmental delay, for which no clear cause could be identified after extensive laboratory and radiological exams. The twins were born to non-consanguineous parents and family history was unremarkable. An array-CGH analysis, using Agilent 180K, was performed and did not reveal any obvious abnormality. Whole-exome sequencing with targeted bioinformatics analysis of 395 epilepsy genes was subsequently performed. This analysis revealed a pathogenic variant in the PIGA gene on chromosome Xp22.2 (MIM 311770), thus establishing the diagnosis of Multiple Congenital Anomalies-Hypotonia-Seizures Syndrome 2 (MCAHS2, MIM 300868). The variant was transmitted to both twins from their unaffected carrier mother. In this case, HTS allowed us to make a precise diagnosis and to provide crucial genetic counselling to the couple for future pregnancies, given the X-linked inheritance with a 50% risk of each pregnancy for an affected male offspring.

6. Conclusions

HTS has changed the diagnostic possibilities of highly heterogeneous genetic disorders, such as epilepsies, raising the diagnostic rate to about 25 to 30% of unsolved cases. HTS is already widely used in the clinical practice, but it brings new challenges, regarding interpretation of variants and management of patients. The health professionals are now working to implement guidelines in order to define good practice procedures. Acknowledgment and anticipation of potential implications, as well as multidisciplinary participation, are required to warrant successful implementation.

Daily practice of HTS has encouraged the development of task forces, which work on clinical cases (selection of cases and genes, interpretation of variants, reporting aspects) as well as on the establishment of strategies and good practice guidelines.

Clinical expertise and detailed phenotyping of patients is critical in such approaches and emphasizes the importance of concerted efforts and active collaboration between health professionals. Moreover, genetic counselling remains a central aspect in the HTS practice.

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