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Disease-targeted sequencing: a cornerstone in the clinic

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Abstract

With the declining cost of sequencing and the ongoing discovery of disease genes, it is now possible to examine hundreds of genes in a single disease-targeted test. Although exome-and genome-sequencing approaches are beginning to compete, disease-targeted testing retains certain advantages and still holds a firm place in the diagnostic evaluation. Here I examine the current state of clinical disease-targeted sequencing and evaluate the benefits and challenges of incorporating sequencing tests into patient care.

Introduction

For many years, molecular diagnostics laboratories have been expanding the tests that they offer in the area of disease-targeted clinical sequencing. Early on, sequencing tests were available only for disorders for which a single causative gene was wholly or mostly responsible. Initial tests focused on genes for which a phenotype could quickly direct a clinician to a particular test, and most tests were ordered for the purpose of confirming a suspected diagnosis and for offering an assessment of recurrence risk. For example, cystic fibrosis has a reasonably well-defined phenotype, and a physician can direct testing towards one gene (namely, CFTR) and have a high likelihood of identifying the molecular aetiology of the patient's disorder. By contrast, tests for disorders with enormous genetic heterogeneity, such as retinitis pigmentosa, have been slower to develop, given the low clinical sensitivity of any individual gene.

Sanger sequencing (also known as dideoxy or capillary sequencing) is the gold standard in molecular diagnostics and has been the chosen clinical testing method for disorders in which rare and private mutations make up a large percentage of causative variants. Although the basic technique has remained unchanged for ~30 years, incremental improvements in instrumentation, methodologies and throughput have steadily reduced its cost, allowing laboratories to add content gradually to their tests. A few novel testing approaches have also gained some traction, such as pre-screening DNA fragments with mutation-scanning technologies that detect mutations on the basis of changes in the properties of the fragment[1] or array-based oligo-hybridization sequencing[2, 3].

However, there was little shift from Sanger sequencing until the recent introduction of high-throughput sequencing methods, which are often collectively referred to as 'next-generation' sequencing (NGS) and which have facilitated substantial increases in sequencing content while dramatically decreasing the cost per base. With NGS technologies, the amount of

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DNA to be sequenced is no longer a barrier to launching a new or expanded clinical test. Thus, the limiting factor in deciding the content of the test is no longer the size of the gene or its relative contribution but is instead the pace of the discovery of the genes relevant to a given phenotype.

There is much discussion of the potential of genome or exome sequencing in clinical contexts, but the major current application of NGS in diagnostics is through disease targeted tests. Here I discuss the existing practical applications of such tests, how they are already being integrated into patient care, why such tests remain important in an era of genomic sequencing and the challenges that remain.

Tests being applied

Moving to multi-gene tests

Next-generation sequencing (NGS) is gradually making its way into clinical laboratories. Although there is some use in infectious disease testing, most applications have been in diagnostic testing for hereditary disorders and, more recently, therapeutic decision-making for somatic cancers. The use of NGS technologies to move from testing single genes or small panels of genes to large multi-gene disease targeted panels was a logical first step for the clinical application of these technologies. This approach has given geneticists the ability to increase clinical sensitivity for many existing tests and to continue to investigate the substantial contribution of unique and rare variation to these diseases, which can be assayed only through sequencing.

Although the cost of sequencing can be as low as fractions of a penny per base, this benefit is realized only when a test involves a large amount of sequencing. This is primarily because there is a baseline cost to run an NGS test. Thus, for gene tests with a small amount of content, Sanger sequencing is still more cost-effective. Indeed, few tests that involve <10 genes are currently available using NGS, and to date this technology has been applied only to disorders for which both allelic and locus heterogeneity are substantial. However, as workflows improve, as costs continue to drop and as laboratories work to make the transition towards a common sequencing platform for all tests, fewer tests will be maintained on Sanger platforms.

Current tests

To assess the current implementation of NGS tests that focus on panels of genes, I reviewed panel-based tests listed in the GeneTests database and then queried laboratory websites to investigate methodologies for each test and to identify additional tests that are available. It should be noted that although this was the best resource at the time of writing, not all clinically available genetic tests are represented, and this resource will soon be replaced with the Genetic Testing Registry. As of September 2012, approximately 15 clinical laboratories in the United States had launched a total of ~50 disease-targeted clinical NGS tests: about one-third in the commercial sector and two-thirds in academically affiliated clinical laboratories. Disease-targeted tests are also available from a number of laboratories internationally. Although fewer clinical laboratories have launched somatic cancer NGS tests, many laboratories have tests under development, and this area is expected to expand quickly. In addition, NGS technologies are being used for noninvasive prenatal testing to detect trisomies using circulating fetal DNA[4].

Table 1 summarizes disorders for which multi-gene NGS panels have been launched. Each test typically includes ten to several hundred genes; some panels contain many genes that can give rise to indistinguishable presentations, and other panels represent several phenotypes with overlapping presentations. For example, mutations in many genes for

retinitis pigmentosa have nearly identical presentations[5]. By contrast, several tests for cardiomyopathy encompass several different presentations, such as dilated cardiomyopathy, hypertrophic cardiomyopathy and arrhythmogenic right ventricular cardiomyopathy[6]. Although most cardiomyopathy presentations can be distinguished by an experienced cardiologist, other cases may be more difficult, and having access to a broad panel can make test-ordering decisions much easier. In general, the variation in the numbers of genes included for a given test is largely dependent on the breadth of the spectrum of clinical presentations. For example, for muscular dystrophy, a small panel (of 12 genes) is used for addressing congenital presentations, but panels including more genes that encompass adult-onset forms are also available[7]. As the capacity to launch large gene panels has grown, the tightness of the connections among genes on a panel has begun to lessen, so broader ranges of phenotypes are being represented.

Integration into care

For a long time, genetic testing was only of marginal use in the diagnostic evaluation of a patient. A clinician would exhaust a battery of medical tests (such as chemical laboratory tests and imaging) and turn to genetic testing only if these tests did not yield a definitive diagnosis or if there was a need to assess recurrence risk for future family planning. Even positive genetic test results often did not change management of the patient, as the physician often already had in mind the most common aetiology for which genetic testing might be ordered. However, with the introduction of large multi-panel testing, many more genes that make rare contributions to a phenotype and genes involved in a broader range of phenotypes can be included in testing. For example, case 1 in BOX 1 discusses the inclusion of alpha-galactosidase (GLA) in a test for hypertrophic cardiomyopathy. This is a rarely considered gene for left ventricular hypertrophy, but sequencing it enabled a subset of patients to receive treatments that can slow or even cause a regression of their disease[8, 9].

With the continued increase in the rates of positive results for disease-targeted testing, clinicians have also begun altering the placement of genetic testing in the evaluation of their patients, as they are recognizing that a positive genetic test can save much time and cost in identifying an aetiology. This practice has also been argued for by the cytogenetics community, as the use of cytogenomic arrays has often resulted in a diagnosis before other work-ups[10, 11]. For a child with hearing loss, the traditional approach was to run a battery of low-yield tests (for example, infectious disease testing, radiology of the inner ear, electrocardiogram, renal ultrasound and thyroid hormone tests) to attempt to identify an aetiology, which could include a syndrome that has a risk for additional clinical symptoms. Today, many physicians begin their evaluation with a genetic test, given that a comprehensive genetic testing panel can identify an aetiology in over half of cases of hearing loss[12]. Similarly, the evaluation of a patient with left ventricular hypertrophy often begins with genetic testing, given that a genetic diagnosis of hypertrophic cardiomyopathy can now be achieved in roughly half of all cases[6]. Most targeted genetic tests return a result in 2–8 weeks, which is fast compared to the many years some patients wait trying to understand the cause of a rare disorder.

Targeted versus genomic approaches

The challenges that clinicians are beginning to face today involve the choice between starting their analysis with a disease-targeted test versus jumping immediately to exome or genome approaches. The cost for a large disease-targeted multi-gene sequencing test typically ranges from US$2,000–$10,000. Clinical exome- and genome-sequencing tests range from $5,000–$15,000. Not surprisingly, clinicians are beginning to wonder whether they should move directly to exome or genome sequencing. Although the question seems to

be a relevant one, there are still arguments for why targeted testing will remain a cornerstone of the diagnostic evaluation for at least a few more years.

Coverage

First, although exome and genome sequencing are often referred to as 'whole' exome or genome sequencing, these services might better be called 'hole' exome and genome sequencing, as no approach today is comprehensive in its coverage of even those genic regions that are included in typical disease-targeted testing. In exome sequencing, typical coverage of exons is approximately 90–95%[13], but when analysing small sets of genes that are implicated in a known disorder, coverage can be much lower. For example, more than 73 genes are known to be causative for nonsyndromic hearing loss or hearing loss that can present as nonsyndromic. Examining the publically available coverage data for the University of California, Los Angeles exome-sequencing service shows that these genes have an average coverage of 92%. Four poorly sequenced genes — stereocilin (STRC), protein tyrosine phosphatase, receptor type, Q(PTPRQ), mir-138 and mir-96 — have 0% to 44% coverage. Poor coverage can result from various factors, including probes that are not tiled for certain genes either because the genes were not chosen for inclusion during assay development or because repetitive sequences prevented inclusion, and poorly performing probes owing to GC-richness or low mapping quality.

By contrast, disease-targeted tests can have a much higher or often complete coverage of these genes by filling in missing NGS content with supplemental Sanger sequencing and other complementary technologies. For example, testing using long-range PCR can be carried out in parallel to compensate for inadequacy in NGS of genes with pseudogenes, such as STRC[14]. In addition, laboratories often supplement disease-targeted sequencing tests with copy number detection approaches to detect heterozygous and homozygous large deletions and other copy number changes, a category of mutations that is currently missed by exome-sequencing services[15]. This example highlights the basis for recent recommendations from the American College of Medical Genetics and Genomics that suggest that exome or genome sequencing approaches should be reserved for those cases in which disease-targeted testing is negative or unlikely to return a positive result in a timely and cost-effective manner[16].

Sanger sequencing

Sanger sequencing has been considered the gold standard in molecular diagnostics for many years, yet there is ongoing debate about its future in diagnostic testing. As noted above, laboratories that offer disease-targeted NGS panels often rely on Sanger to fill in the gaps missed by NGS[2, 7], whereas exome- and genome-sequencing services rarely fill in gaps. However, laboratories that offer targeted panels and those that offer exome- or genome-sequencing tests are both reliant on Sanger sequencing to confirm NGS-detected variants before returning the results to patients[17]. Although all applications of NGS can suffer from artefacts, confirmation is particularly important for exome and genome sequencing, given increased error rates as a consequence of lower coverage of many areas of the exome or genome. There is a commonly voiced concern in the community that Sanger sequencing is labour intensive and that its continued use will hinder the efficiency improvements and cost reductions that are required for the broader use of genetics in medicine. As such, it follows that efforts in NGS technology development need to focus on improvements in the sequencing technology and bioinformatics analyses to cover fully and to analyse accurately all clinically relevant areas of the genome, allowing minimization of the reliance on Sanger sequencing and other orthogonal approaches to supplement NGS.

Variants of uncertain significance

Faced with results containing thousands to millions of variants, exome- and genome-sequencing analyses rely on data-filtering approaches that must make automated assumptions about variants and their potential role in disease. For example, variants that are found in control populations might be filtered out, sometimes with an erroneous assumption of benign impact. Case 2 in BOX 1 demonstrates this problem. By contrast, for laboratories that use disease-targeted tests, the standard approach to result analysis is to interpret and to report fully the importance of every variant identified. This ensures that no obvious aetiologies are missed and better enables follow-up analysis to be carried out for variants of uncertain significance (VUSs). For a large multi-gene panel for an inherited disorder, typically tens to hundreds of variants are identified in each test. Most variants can be readily classified as benign or likely benign on the basis of population frequency; however, each test may yield several variants that are novel. For each novel variant, the laboratory typically searches published literature and online databases to look for any data that may have been published on the variant or to see whether it has been identified in large population cohorts, such as those that are available from the 1000 Genomes Project[18] and the Exome Variant Server[19]. However, most searches yield no data or insufficient data to determine the clinical significance of a rare variant. Next, the laboratory might carry out computational analyses to predict the likelihood that a variant may affect gene splicing or protein function. Unfortunately, these computational approaches have a low sensitivity and specificity and are rarely valuable in predicting the pathogenicity of a variant. The best evidence may come later through follow-up studies. For example, segregation studies can be done in large families that are affected by a dominant disease, or a research laboratory can demonstrate gene or protein disruption through functional studies. Such a follow-up is more likely when a laboratory reports only one or a few VUSs from a disease-targeted test than it is in the case of exome or genome sequencing, in which VUSs may simply be filtered out and not reported.

Expert knowledge and detailed phenotyping

Disease-specific expertise typically resides in laboratories that have long carried out disease-targeted testing; a non-speciality laboratory is likely to have less knowledge of the genes that are known for a given phenotypic presentation and less understanding of the clinical significance of variants identified in those genes. For this reason, physicians will often choose to order testing from laboratories with well-established experience in a disease area. This will remain an issue for some time to come until laboratories begin to structure and to share their knowledge more broadly with the larger community, allowing knowledge-enabled analytical pipelines to support more readily the broad community of testing laboratories.

Furthermore, if a laboratory has extensive clinical experience with a particular disease, they may be better able to prioritize variants. For example, in a test for hearing loss, if VUSs were identified in certain hearing loss genes, a laboratory with hearing-loss expertise may be more likely to recommend specific clinical evaluations — such as a temporal bone evaluation for SLC26A4 [20] or otoacoustic emission testing for OTOF[21] — that can help to determine which variant is the most relevant. By contrast, a laboratory using exome- or genome-sequencing approaches without specific expertise in the relevant clinical condition might be left with a long list of VUSs and might be unable to differentiate appropriately the variants and recommended follow-up studies.

This example also highlights the crucial need for accurate and detailed phenotyping and for the sharing of that information between the clinician and the laboratory. Effective phenotyping is a crucial component of the diagnostic testing process both in determining the

most appropriate testing strategy and in interpreting the results of testing to allow the highest yield of information. As diagnostics moves towards genomic approaches, accurate and detailed phenotyping will require more software-supported approaches that can draw on large data sets of curated clinical associations to aid the laboratory and physician in identifying less obvious candidate genes for aetiologic consideration[22, 23].

Return of results

An added challenge that faces laboratories offering genetic testing is the prospect of identifying variants that are unrelated to the indication for testing. This is largely not an issue for disease-targeted testing, as only those genes that are relevant to the phenotype are included. By contrast, this issue represents a major challenge for laboratories offering exome- and genome sequencing services, given the opportunity to analyse proactively or to stumble accidentally on disease-implicated variants that are unrelated to the reason for testing. Although there is some consensus developing around which variants to return, this topic is likely to remain hotly debated for some time[24].

Evaluation of disease-targeted tests

Although the expansion of gene content for disease-targeted tests is no doubt leading to increases in the likelihood of a diagnosis, the question remains whether these tests are really making a difference in patient care and at what cost. Although NGS tests have more content than do previous Sanger-based sequencing tests, the prices have largely remained the same. To date, there have been only limited evaluations of the cost-effectiveness of molecular tests. The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) working group, which is funded by the US Centers for Disease Control and Prevention, has begun the important task of evaluating the clinical validity and utility of genetic tests[25]. The first example of a multi-gene sequencing panel evaluation was the EGAPP analysis of genetic testing strategies for Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer). In this case, sufficient clinical validity and utility were identified to recommend that genetic testing should be included in the workup of patients with newly diagnosed colorectal cancer[26]. However, additional evaluation will be needed to examine the exact costs of testing using different strategies. Analyses of different testing strategies have not yet been extended to rare diseases, given the limited data available and the daunting task of examining thousands of disease genes that could be included in clinical tests. However, more efficient strategies have recently been proposed to increase the efficiency of genetic test evaluation in an era in which two or three new genetic tests are launched weekly[27].

A step in NGS test evolution

Although disease-targeted testing is likely to remain useful for the short term, laboratories are faced with the never-ending incremental costs to develop and to validate each new disease-targeted panel, as well as to update constantly the content of existing panels as new genes are identified. This burden is causing laboratories to consider more efficient approaches than targeted testing, including the development of a ‘disease-associated exome’ test, which includes all genes associated with disease: that is, 2,100–3,500 genes, depending on the source of data (for example, GeneTests, Online Mendelian Inheritance in Man(OMIM), and so forth). Indeed, commercial products have already been developed to support this approach (for example, from Illumina). Although all gene content would be on one physical test, analysis of the data could be limited to only those genes that are relevant to the patient’s phenotype. This approach would satisfy the simplicity of disease-targeted testing and would avoid interrogation of genes of unknown clinical relevance that laboratories are often not in a position to follow-up on. However, it would enable the

laboratory to minimize test development and validation efforts. Although such tests would still require updates as new gene associations are made, the updates would be limited to only one test. Similar but smaller-scale approaches are already used by many laboratories that offer disease-targeted tests as full panels or subpanels. For example, some laboratories combine sets of related disease genes into one large test but allow physicians to order a smaller set of genes in a subpanel; however, if the subpanel is negative, they can request analysis using the larger panel for a much smaller fee than that incurred by running a new test[6]. The same approach can be applied to the disease-associated exome: analysis can initially focus on a primary set of genes with a strong contribution to the patient's clinical presentation but then extend to a much broader gene set or even to the entire disease-associated exome, if initial testing is negative.

Future directions

There is little doubt that the introduction of massively parallel sequencing is having a dramatic effect on the diagnosis of genetic conditions. Although most testing today uses disease-targeted approaches, as genomic technologies improve and become increasingly able to detect all types of mutations across all genetic loci for an ever decreasing cost, it may not be long before every baby has its genome sequenced at birth to allow the most informative approach to health management. However, there are several key challenges that impede the widespread use of genetics in everyday medicine. The most important challenge is the lack of understanding of the impact of most genetic variants on human health and disease. Most variants are either extremely rare or have a weak effect.

As such, understanding these variants will require massive sources of genomic and phenotypic data and shared efforts in studying variants. Although there has been a recent movement towards laboratories embracing the desire to share data[28] — as supported by a recent position statement from the American College of Genetics and Genomics — systems to support data sharing and standards to allow compatibility of the data and annotations are only just beginning to be developed. Progress is being made with the recent launch of the ClinVar database at the US National Center for Biotechnology Information (NCBI)[29]. The International Collaboration for Clinical Genomics is working closely with NCBI to develop standards, to assist clinical laboratories in sharing their data and to develop approaches to curate the shared data. In addition, a recent workshop sponsored by the US National Human Genome Research Institute entitled 'Establishing a central resource of data from genome sequencing projects' provided hope for developing approaches to large-scale data sharing. In addition to our limited knowledge about genetic variation, another key challenge is the lack of physician and patient understanding of how to use genetic information for health benefits. A combination of better education in genetics as well as better tools for clinical decision support will be needed to integrate genomic data effectively into the practice of medicine. That said, there are many examples of the beneficial impact of genetic information on the health of individuals, and it is only a matter of time before the promise of genomic medicine begins to penetrate the many facets of clinical care.

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Box 1

Case examples demonstrating the application of targeted sequencing tests

Case example 1

The alpha-galactosidase (*GLA*) gene is primarily associated with Fabry's disease, which has a cardiac, renal and neurosensory phenotype. Testing for this gene was rarely ordered in the genetic evaluation of hypertrophic cardiomyopathy (HCM)—a cardiac disorder that is responsible for the most common cause of sudden cardiac death in individuals under age the age of 25 (REF. 30)— despite published evidence that patients with Fabry's disease can present with cardiac wall thickening that mimics HCM[31]. However, inclusion of *GLA* as a component of large multi-gene panel tests for HCM has led to the finding that nearly 2% of patients with an assumed HCM diagnosis are positive for pathogenic variants in this gene[32]. This is an important diagnosis as it is the only gene in the HCM test panels offered clinically that can currently be used to direct a substantive change in management: *GLA*-positive patients are able to receive enzyme replacement therapy[8, 9].

Case example 2

In the first case examined with genome sequencing in our laboratory, the cause of hereditary hearing loss was eventually found to be deletions in the stereocilin (*STRC*) gene. This aetiology was overlooked during the initial analysis of the genome sequencing data because this defect has a reasonably high occurrence (1.6%) in the general population. Therefore, this variant was filtered out in a data analysis pipeline of copy number variants. The variant was later identified when the sample was included in the validation of a new disease-targeted panel test for hearing loss, for which more stringent data analysis methods specific to hearing loss causes were used (H.L.R., unpublished observations).

Table 1

Clinically available disease-targeted tests

Disease area	Disease type	Ceres
Cancer	Hereditary cancers (for example, breast, colon and ovarian)	10–50
Cardiac diseases	Cardiomyopathies	50–70
	Arrhythmias (for example, long QT syndrome)	10–30
	Aortopathies (for example, Marfan's syndrome)	10
Immune disorders	Severe combined immunodeficiency syndrome	18
	Periodic fever	7
Neurological neuromuscular and metabolic disorders	Ataxia	40
	Cellular energetics, metabolism	656
	Congenital disonders of glycosylation	23–23
	Dementia (for example. Parkinson's disease and Alzheimer's disease)	32
	Developmental delay, autism, intellectual disability	30–150
	Epilepsy	53–130
	Hereditary neuropathy	34
	Microcephaly	11
	Mitochondrial disorders	37–450
	Muscular dystrophy	12–45
Sensory disorders	Eye disease (for example, retinitis pigmentosa)	66–140
	Hearing loss and related syndromes	23–72
Other	Rasopathies (for exemple, Noonan's syndrome)	10
	Pulmonary disorders (for example, cystic fibrosis)	12–40
	Short stature	12