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What Has the Undiagnosed Diseases Network Taught Us About the Clinical Applications of Genomic Testing?

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Abstract

Genetic testing has undergone a revolution in the last decade, particularly with the advent of next-generation sequencing and its associated reductions in costs and increases in efficiencies. The Undiagnosed Diseases Network (UDN) has been a leader in the application of such genomic testing for rare disease diagnosis. This review discusses the current state of genomic testing performed within the UDN, with a focus on the strengths and limitations of whole-exome and whole-genome sequencing in clinical diagnostics and the importance of ongoing data reanalysis. The role of emerging technologies such as RNA and long-read sequencing to further improve diagnostic rates in the UDN is also described. This review concludes with a discussion of the challenges faced in insurance coverage of comprehensive genomic testing as well as the opportunities for a larger role of testing in clinical medicine.

INTRODUCTION

Established in 2014, the Undiagnosed Diseases Network (UDN), funded by the National Institutes of Health (NIH) Common Fund, is a multicenter collaborative effort that takes a multi-omic approach to inform deep clinical phenotyping to solve complex medical cases (1). The UDN was an extension of the Undiagnosed Diseases Program established in 2008 within the Intramural Research Program of the NIH (2, 3). The UDN incorporates state-of-the-art testing, including whole-exome sequencing (WES), chromosomal microarray (CMA), metabolomics, and model organism screening, as part of clinical research evaluations to arrive at a diagnosis (**Figure 1**). The overall diagnostic rate within the UDN is ~35% (4), which is notable since many patients have

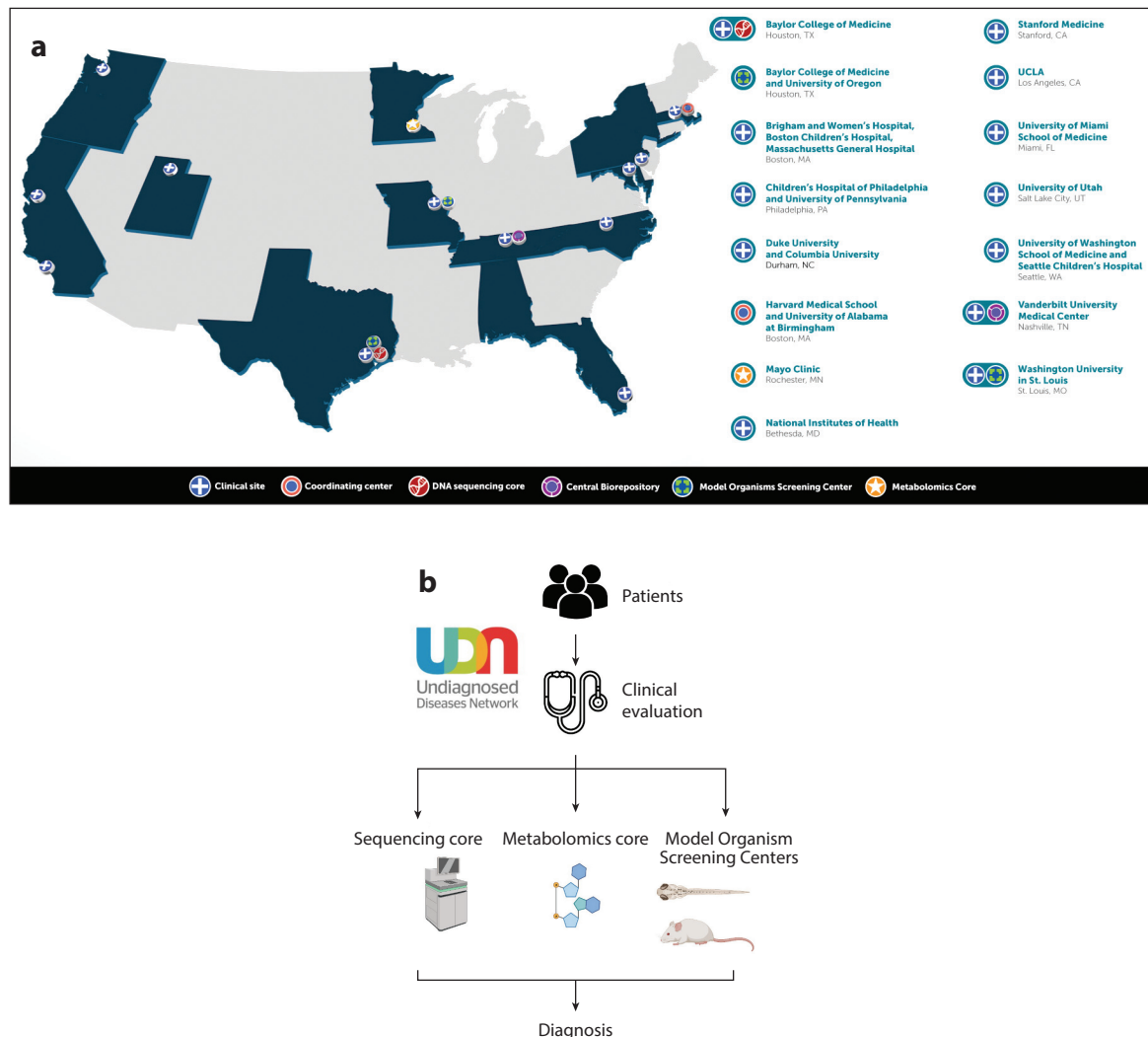


Figure 1

(a) Undiagnosed Diseases Network (UDN) composition of clinical sites and cores performing sequencing, metabolomics, and model organism screening. (b) Overall UDN workflow from patient enrollment to clinical evaluation through analysis of sequencing and other data to arrive at a diagnosis. Panel a taken as a screenshot from <https://undiagnosed.hms.harvard.edu/udn-sites/>.

already undergone extensive negative diagnostic workups, including with WES and CMA. The UDN has also led the way in implementing emerging technologies such as whole-genome sequencing (WGS), RNA sequencing (RNA-seq), and long-read sequencing to improve diagnostic rates of unsolved cases.

Clinical genetic testing has evolved rapidly from Sanger-based single-gene tests to multiple-gene panels to WGS. A major revolution in genetic testing occurred around 2008 with the advent of next-generation sequencing (NGS) that dramatically decreased the cost of such testing and revolutionized the field of molecular genetics (5). The primary provider of this technology is currently Illumina, and the approach of NGS is to generate millions of short reads of DNA, typically around 100–200 base pairs in length, that are then aligned to the human reference sequence. Before NGS, most genetic testing was based on Sanger sequencing, which was more expensive and slower than NGS and not suited for high-throughput applications (6). The Human Genome Project, for example, used Sanger sequencing to generate the human reference genome at a cost of \$3 billion over 13 years (7). In contrast, the cost (excluding interpretation and computational processing) of today's sequencing per human genome is less than \$1,000 and can be achieved in less than a day (8). However, it is important to note that NGS would not be possible today without the efforts of the Human Genome Project that generated the original human reference sequence.

The application and utility of genetic testing are well established in pediatrics and continue to expand in adults as precision medicine efforts aim to shift care from a reactive to a preventative model (9). For example, the American College of Medical Genetics and Genomics (ACMG) has published evidence of improved outcomes from genomic testing in pediatric patients with congenital anomalies or intellectual disability (10). Similarly, in 2018, the Heart Failure Society of America and ACMG published guidelines recommending genetic testing for adult patients with cardiomyopathies to assist in management (11). Large-scale NIH-funded programs such as Electronic Medical Records and Genomics (eMERGE) and All of Us aim in part to return actionable clinical genetic findings to improve health outcomes (12, 13). Our experience in the UDN has provided important lessons about the roles and applications of genomic testing in clinical medicine in both children and adults.

LIMITATIONS OF WHOLE-EXOME SEQUENCING AND ADVANTAGES OF WHOLE-GENOME SEQUENCING

Widespread adoption of WES in clinical practice has improved diagnosis for rare Mendelian conditions; however, studies generally report diagnostic rates of 25–30% using WES alone (14–16). The UDN has been a leader in implementing WGS for rare disease diagnosis. WGS is becoming a first-tier diagnostic test for individuals with suspected genetic conditions because it can detect most types of variants in a single test (**Table 1**), enabling more rapid and less costly diagnoses (17–20). It can detect single-nucleotide variants, small insertion/deletions, copy-number variants, balanced and complex structural variation, many repeat expansions, and mitochondrial variants (17, 21, 22). Also, WGS is a genome-wide screen covering all coding and noncoding regions, whereas WES targets only the 1% coding region of the genome and NGS gene panels test specific genes. As such, WGS probes not only exonic regions but also intronic, intergenic, and regulatory regions that have been shown to cause disease and are frequently missed with other testing modalities (23). One limitation is of course our ability to interpret the pathogenicity of this abundance of data.

One UDN clinical site recently reported that 28% of its diagnoses required testing beyond WES. The vast majority had a causative noncoding variant or copy number variant that had been missed on prior testing (24). In fact, of new diagnoses from July 2015 to September 2019 across four clinical UDN sites, WGS alone was responsible for 45% of the diagnoses in cases with prior

Table 1 Comparison of reportable variant types detected by different genetic testing assays

Genetic test	SNVs/Indels	CNVs	Mosaic variants	Repeat expansions	Balanced and complex SVs	mtDNA	Genes tested	VUS potential ^a	Cost ^a
Sanger	Yes	No	Limited	No	No	If included	Single–few	+	\$\$
CMA	No	Yes	Yes (CNV only)	No	No	No	Up to ~20,000	++	\$\$
NGS panel	Yes	Yes	Yes	No	No	If included	Few–hundreds	++	\$
WES	Yes	Limited	Limited (depends on coverage)	No	No	If included	~20,000 (coding regions only)	+++	\$\$\$
WGS	Yes	Yes	Limited (depends on coverage)	Yes	Yes	Yes	~20,000 (coding and noncoding regions)	++++	\$\$\$\$

^aVUS potential and cost are compared on a scale from one symbol (lowest) to four (highest).
Abbreviations: CMA, chromosomal microarray; CNV, copy-number variation; Indel, small (<150 base pair) insertion/deletion; mtDNA, mitochondrial DNA; NGS, next-generation sequencing; SNV, single-nucleotide variant; SV, structural variant; VUS, variant of uncertain significance; WES, whole-exome sequencing; WGS, whole-genome sequencing.

nondiagnostic WES (25). One downside of WGS is its increased cost compared to multigene panels and WES, although this is already rapidly decreasing with new technologies. However, because WGS can effectively replace serial genetic testing with a single assay, it has been shown to lead to faster diagnoses, improved outcomes, and decreased cost of care (19, 26). Another drawback of WGS is the increased number of variants of uncertain significance it identifies. This is because of our limited ability to interpret noncoding variation with current ACMG guidelines that focus primarily on coding changes (23, 27). However, techniques such as RNA-seq and high-throughput functional assays may help overcome this issue and bring WGS into mainstream practice. With the continued fall of sequencing costs, clinical WGS will likely replace WES and negate the need for multiple testing modalities such as CMA and mitochondrial DNA sequencing in the coming years.

ROLE OF RNA SEQUENCING IN RARE DISEASE DIAGNOSIS

RNA or transcriptome sequencing (RNA-seq) is emerging as another tool in the genetic diagnostic toolbox. Though RNA-seq is available in a limited manner for targeted clinical applications (28) and in the analysis of somatic fusion events in diagnosing cancer genomic changes, the UDN has pioneered its use for rare disease diagnosis across varied phenotypes and indications (23, 29, 30). In contrast to DNA sequencing, RNA-seq can provide evidence of the functional impact of noncoding variants on splicing, decreased gene expression via nonsense-mediated decay, allele-specific expression, and other findings (31). In the case of splice-altering variants, for example, bioinformatic predictions have mixed effectiveness (32), but RNA-seq can be used to support or refute the effects of a variant on gene splicing (23, 33). Overall, RNA-seq has led to a 7.5–36% improvement in diagnostic rates depending on the sampled tissue and clinical phenotype (23, 29–31, 34, 35) (**Table 2**) and aids in the resolution of variants of uncertain significance (28, 33). Among four clinical UDN sites specifically, RNA-seq contributed to a diagnosis in 16% of newly diagnosed cases (25).

A critical issue to consider with RNA-seq is what tissue to use, since gene expression varies widely with tissue type. While whole blood RNA is the easiest to collect, the expression of genes associated with most phenotypes is poor (23, 34), limiting the utility of RNA-seq to contribute to a diagnosis. At the Baylor College of Medicine (BCM) UDN clinical site, we perform RNA-seq on cultured fibroblasts obtained with a skin biopsy because studies have shown much higher expression of a broader number of genes in skin than in whole blood (23, 36).

Table 2 Comparison of published RNA-seq studies and their contrasting approaches and results

Reference	UDN study	Phenotypes	Tissue	Events detected	Analysis method ^a	RNA-seq diagnostic rate
35	No	Neuromuscular	Muscle	Splicing	Candidate + outlier	35%
34	No	Neuromuscular	Muscle, fibroblasts, fibroblast-derived myotubes	Expression, splicing, ASE	Outlier	36%
29	Yes	Multiple (neurologic most common)	Whole blood	Expression, splicing, ASE	Outlier	7.5%
30	Yes	Multiple (neurologic most common)	Whole blood, fibroblasts, muscle, bone marrow	Expression, splicing, ASE	Candidate	18%
23	Yes	Multiple (neurologic most common)	Whole blood, fibroblasts	Expression, splicing, ASE	Outlier	17%

^aThe candidate method relies on variants first identified on primary WES/WGS to direct RNA-seq analysis. In contrast, the outlier technique identifies novel abnormalities in expression and splicing in a large cohort to direct genomic analysis.

Abbreviation: ASE, allele-specific expression.

Using fibroblast-derived RNA-seq, we have increased diagnostic rates by 17%, in contrast to the 7.5% reported at another UDN site using blood transcriptome sequencing alone (29). Many genes are still not well expressed in clinically accessible tissues (36); however, techniques such as fibroblast trans-differentiation and/or use of induced pluripotent stem cell-derived target cells may overcome this limitation (34).

The method of RNA-seq analysis is another issue, specifically whether to pursue a traditional candidate variant approach or a transcriptome-directed strategy. The former is an extension of standard practice where candidate variants first identified on WES, for example, are manually reviewed in the transcriptome to determine any functional consequences. In contrast, a transcriptome-directed strategy uses outliers of expression or splicing in the RNA-seq itself to guide subsequent analysis of sequencing data and thereby find the underlying cause. At BCM, the RNA-seq directed method has proven to be more effective than a traditional candidate approach, revealing disease-causing variants commonly missed on WES and CMA and being a powerful tool for novel gene discovery (23). A comparison of the previously published studies, including tissues, analysis method, and diagnostic rates, is shown in **Table 2**.

RNA-seq shows promise to further improve diagnostic rates for rare diseases, in particular as a complement to WGS in WES/CMA-negative cases. Before RNA-seq becomes a widely accepted clinical test, standardization of techniques and interpretation criteria are needed, in the same way that the ACMG and the Association for Molecular Pathology (AMP) developed guidelines for DNA variants (27).

NEED FOR ONGOING REANALYSIS

Of the roughly 20,000 genes in the human genome, only ~4,400 are currently associated with a Mendelian phenotype in the Online Mendelian Inheritance in Man (OMIM) database (37). However, gene discovery continues rapidly, with ~250 new gene–disease associations entered annually in OMIM (38, 39). One major WES laboratory achieved a 22% improvement in diagnostic rate by reanalysis of existing sequencing data that was overwhelmingly driven by newly implicated disease genes (40). Bioinformatic tools continue to evolve, allowing for improved identification of pathogenic copy-number changes from NGS data (41). Adaptations to the 2015 ACMG and AMP

guidelines for variant interpretation (27) can also yield improvement in variant classification over time. For example, recent gene-specific interpretation guidelines permit upgraded variant interpretation for genes such as *MYH7* (42). Reanalysis of data with consideration of alternative transcripts has also been shown to increase diagnostic rates and resolve uncertain results (43). For these reasons, most diagnostic laboratories now offer periodic WES reanalysis, a practice endorsed by the ACMG (44). One UDN site recently reported 23% of individuals receiving a diagnosis after reanalysis of nondiagnostic WES done before UDN enrollment (45). At our own BCM clinical UDN site, we perform such reevaluation of pre-UDN WES data on a routine basis for these reasons before considering alternative testing, such as WGS or RNA-seq, with similar yields.

EMERGING TECHNOLOGIES: LONG-READ SEQUENCING

Sequencing technology continues to evolve, offering hope for further improvement in diagnostic rates and our understanding of genetic diseases. As impactful as NGS has been, it has several limitations resulting from the short (100–200 base pair) reads upon which it is based. For example, NGS has difficulty in genomic regions with high homology (e.g., pseudogenes), repetitive sequences, and high GC content (46, 47). Complex structural rearrangements are also challenging to detect and characterize with NGS; it has been shown to have low sensitivity (30–70%) and a high false discovery rate (as high as 85%) for these variant types (48). Particularly important for autosomal recessive conditions, phasing of heterozygous variants (cis or trans) is generally not possible with NGS without parental information (49).

In contrast, long-read sequencing from Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (Nanopore) generates reads from 10 kb to >1Mb in length (48), overcoming many of the shortcomings inherent to NGS. Long-read sequencing has already been applied to rare disease research, where it has been particularly adept with complex structural rearrangements (46, 48). For example, long reads were recently used to discover a pathogenic GGC repeat expansion missed by NGS in the *NOTCH2NLC* gene associated with neuronal intranuclear inclusion disease (50). Unlike short reads, long reads can span the entire expanded repeat, making detection more straightforward (46). We have begun using Nanopore sequencing at our UDN clinical site, in one case diagnosing a patient with deleted exon 5 autoinflammatory syndrome (NEMO-NDAS) (51) by identifying a pathogenic *IKBK*G (inhibitor of nuclear factor κ B kinase, regulatory subunit γ) variant missed on WES due to a nearby pseudogene. Despite the advantages of long-read sequencing compared to NGS, its higher cost and base calling error rates (46, 52) will need to improve before it is routinely used in the clinical space.

INSURANCE COVERAGE FOR COMPREHENSIVE GENETIC TESTING

Despite the well-documented utility and cost-effectiveness of genetic testing in the diagnosis and management of patients with rare diseases (53–55), insurance coverage for comprehensive molecular tests remains an obstacle. A retrospective analysis of patients enrolled in the UDN revealed that 40% of patients undergoing WES through the UDN had previously faced insurance coverage barriers to testing (56). Among those previously denied, WES yielded a diagnosis in 35% of these patients, resulting in management changes in a majority of them (61%) (56). Nevertheless, a frequent reason for insurance denial is that such testing is considered experimental or investigational and thus is not accepted practice (56, 57). Coverage for WES in adults is particularly low, despite the high diagnostic yield in patients with moderate–severe intellectual disability (58).

Even fewer insurers cover WGS at this time, with many considering it investigational. Payer opinions may evolve as studies demonstrate not only improved diagnostic rates with WGS but

also reduced healthcare costs (59). In a pilot study carried out in California from 2018 to 2020, rapid WGS in 178 critically ill babies led to a diagnosis in 43%, management changes in 31%, and \$2.5 million in healthcare savings (26). Cost savings were driven by fewer days hospitalized, fewer major surgeries and invasive procedures, and avoided diagnostic tests such as CMA (26). As a result of these findings, Blue Shield of California now covers the cost of rapid WGS in critically ill children (60). Similar pilot studies in other states are currently under way or are in development.

Nevertheless, a more rigorous economic analysis of genomic sequencing is needed to direct reimbursement policy and measure the cost-effectiveness of such testing. Such economic studies have been lacking and are challenging to do (61, 62). In a recent review of 171 articles describing the use of comprehensive genomic sequencing in infants and children, only 4 had a primary economic evaluation aim (55). One report on infants in neonatal intensive care units who were suspected to have a genetic diagnosis surprisingly found higher costs for patients who underwent WES than for those who did not when a detailed economic analysis was done (63). In addition, the perceived value of these comprehensive assays for clinicians and families will be important to measure (63–65). Collectively, such analyses will establish a framework from which to demonstrate clinical utility and cost-effectiveness of genomic sequencing. With the development of much less costly sequencing platforms, the fundamental determinants of cost-effectiveness will further help to overcome payer resistance for covering WGS.

CONTINUED CLINICAL PHENOTYPING

Detailed clinical phenotyping remains an integral component of the diagnostic process. In some cases, a molecular diagnosis is not needed if a patient is found to meet clinical diagnostic criteria for a specific disorder after deep phenotyping. Within the UDN, Shashi et al. recently diagnosed individuals with oral-facial-digital syndrome and multiple pterygium syndrome based on clinical features in the absence of a molecular diagnosis (45). Deep phenotyping may also suggest performing an alternative test if no causative variants are found on the initial assay. One UDN clinical site recently reported three such patients who were negative on WES but were diagnosed after targeted Sanger sequencing and CNV analysis revealed the causative variants (66). In those cases, detailed phenotyping prompted the sequencing of specific genes that were felt to be a strong fit for the clinical features. The disease-causing variants were missed on WES due to the inherent limitations of the technology. Phenotyping can also guide diagnosis by leading to the reinterpretation of variants of uncertain significance (45). Automated filtering algorithms may exclude or deprioritize certain variants from further analysis if they miss specific thresholds (e.g., in silico predictions, population frequency). Detailed phenotyping and the subsequent generation of an accurate differential diagnosis may rescue such uncertain variants and trigger additional investigations. Ultimately, phenotyping informs the generation of candidate gene lists that may lead to downstream functional studies, gene matching, and other studies that may enable the conversion of a variant of uncertain significance to a pathogenic variant. We have also previously demonstrated that nearly 5% of cases carry multiple molecular diagnoses (67). Broad phenotyping in such cases is critical in identifying features of each diagnosis that may erroneously be attributed to a single cause and lead to an incomplete molecular diagnosis.

CONCLUSION

Genetic testing technology continues to evolve with the goal of improving diagnostic rates for rare diseases. As sequencing costs fall and our understanding of the entire genome increases, the role of such testing in clinical medicine will continue to grow. The UDN has led the field in its application of current and emerging technologies for diagnostic purposes and a better

understanding of disease mechanisms. Increasing access to comprehensive genomic testing outside a research setting such as the UDN will be an important step in integrating genomics into routine health care. While this will increasingly drive a genotype-first approach to clinical medicine, the continued need for deep and broad clinical phenotyping will remain, especially as increasingly numerous rare variants are correlated with unique presentations or combinations of presentations that require old-fashioned hypotheses testing with downstream studies.

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