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Genome-wide sequencing technologies: A primer for paediatricians

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Abstract

Genetic testing has been a routine part of paediatic medicine for decades. Over time, the number of genetic tests available for children presenting with features thought to be explained by an underlying genetic aetiology has expanded considerably. Genome-wide sequencing approaches (e.g., whole-exome sequencing, whole-genome sequencing) are now emerging as the most comprehensive approaches to genetic diagnosis that we have seen to date; multiple serial tests that were once required for a child under diagnostic investigation can now be accomplished in a single assay. Moreover, the performance of this single assay appears to be superior to the sum of its parts. Despite this promise, technical, ethical and access-related complexities require considerable attention prior to the implementation of these tools in mainstream paediatrics. To ready paediatricians for the eventual transition to genome-based diagnostics, herein we review both the elements and delivery considerations of this emerging technology.

Keywords: *Diagnostic testing; Genome-wide sequencing; Paediatrics; Rare disease.*

WHAT'S NEW?

- Genome-wide sequencing approaches are emerging as powerful diagnostic tools in paediatrics. Compared to 10% to 15% detection rates associated with conventional genetic testing, genome-wide sequencing yields a diagnosis in 30% to 40% of the patients.
- Despite its diagnostic potential, analytic challenges and ethical controversies remain.
- Paediatricians will be called upon to initiate appropriate referrals to genetics for children with complex clinical presentations of suspected genetic aetiology and understand the types of results that can emerge genome-wide sequencing.

WHY THE PRIMER?

Genetic diseases are individually rare, but common in aggregate, particularly in paediatrics (1). Up to 34% of paediatric hospitalizations are for children with a genetic disorder and genetic

disease is a common cause of disability and death in children (2). The diagnosis of genetic disease is an essential part of clinical care—while often not leading directly to a specific treatment for the child, a diagnosis is important to exclude treatable disorders, provide accurate recurrence risks for family members, inform prognosis and end the diagnostic odyssey (3,4). Genome-wide sequencing approaches are emerging as a powerful diagnostic tool. For example, compared to chromosome microarray, the currently recommended first-tier test for children with developmental delay/intellectual disability (5), genome-wide sequencing achieves a threefold increase in diagnostic yield (6). Unlike the traditional approach of ordering multiple serial genetic tests, this improved diagnostic yield is achieved in a single assay. As such, genome-wide sequencing will change the way the diagnostic process for a suspected rare genetic disease is delivered in Canada. While these changes are beginning to take place in tertiary care genetics centres, it will be some time before test ordering capabilities will be available to community-based paediatricians. Overcoming complexities related to timing of and indication for testing, interpretation, ethics and access is warranted. However, an understanding of the current landscape of genome-wide diagnostics is necessary to prepare for the receipt of increasingly sophisticated genomic sequencing reports on patients referred to tertiary genetics centres. Here, we provide a brief overview of the basic and more complex features of genome-wide sequencing.

CASE EXAMPLE

Patient A is a 7-year-old nondysmorphic boy with medically intractable epilepsy with onset at age 4 months. He presented with nonfebrile status epilepticus repeatedly between ages of 4 and 8 months and failed five antiepilepsy medications. He stopped seizing altogether after starting the ketogenic diet, but remained significantly developmentally delayed. He has autistic features with a nonverbal, nonambulatory phenotype. Over a 6-year period, extensive genetic and metabolic testing using traditional methods was performed (e.g., chromosome microarray, Prader-Willi/Angelman testing, epilepsy gene panel, Rett syndrome sequencing, SCN1A, CDKL5, ATP7A sequencing, plasma amino acids, urine organic acids). Given the high probability of a genetic cause, genome-wide sequencing was performed and a single mutation in the phosphatidylinositol glycosylation protein A (PIGA) gene was identified in the patient and his mother. These findings are consistent with the diagnosis of X-linked recessive phosphatidylinositol glycosylation protein A deficiency (PIGA deficiency) (7).

GENOME-WIDE SEQUENCING: GENERATING THE SEQUENCE

Next-generation sequencing is a contemporary technology that performs sequencing of millions of small fragments of DNA in parallel. Of the entire genome, only 1% codes for proteins; this 1% is called the exome. The exome is organized into ~22,000 genes and is thought to contain 85% of known or potential disease-causing variants. In contrast, an individual's entire DNA content (protein coding and noncoding regions) is called the genome. Genome sequencing refers to sequencing the entire genetic code of a person and exome sequencing refers to sequencing only the parts of the genome that contain protein-coding genes; both are forms of genome-wide sequencing. Neither type of sequencing is readily available, clinically, in Canada yet. However, efforts are currently underway to deliver publically funded exome sequencing for specific indications in various provinces (8). Genome sequencing is only available on a research basis in Canada and most other countries.

For both approaches, the laboratory process begins with extracting DNA from cells. After extraction, the DNA is broken into short fragments (i.e., 100 to 150 base pairs) and the fragments are put through a process called library preparation. For exome sequencing, an additional enrichment procedure is needed to 'capture' only the protein-coding information contained within the exons. The sequencing instrument 'reads' the genetic code of these short sequences multiple times in parallel. Using bio-informatics tools, these short sequence reads are aligned and matched to specific positions in the human genome reference sequence. A computerized annotation of the patient's genotype (consisting of nucleic acids labelled A, T, C, G) at each position in the exome or genome is then created and compared to the reference genome. Similarities and differences between the patient's sequence and the reference sequence can be identified (4,9,10).

GENOME-WIDE SEQUENCING: INTERPRETING THE VARIANTS

Once analytic accuracy is verified, an interpretation team needs to determine which variants in the patient's genome may be clinically significant. While generating the raw sequence data is 'hypothesis free', understanding its meaning requires clinically-derived hypotheses that reflect possible associations between the patient's phenotype and potentially relevant variants in genes that are identified in the individual's sequence data. For this reason and at this time, variant interpretation is best conducted by a multidisciplinary team that includes a bio-informatician (i.e., develops variant pipeline), a genomicist (i.e., analyzes detected variants using relevant software) and a medical geneticist or other subspecialist (i.e., expertise in rare diseases and direct knowledge of the patient being analyzed) (11). First, the raw sequence data are filtered for rare variants (i.e., variants seen in <1% of the population). An exome sequence typically generates ~500 variants whereas a

Table 1. Types of results that can be generated by genome-wide sequencing

Variant type	Clinical example
Primary diagnosis	Detection of sequence-level variant in the NSD1 gene that is diagnostic of Sotos syndrome, in the presence of Marfan-like features (6)
Possible diagnosis	Detection of one sequence level variant in the ZFYVE26 gene in a child with spastic paraplegia. Homozygous or compound heterozygous mutations in this gene are associated with autosomal recessive spastic paraplegia type 15. However, with only one variant detected, the molecular diagnosis cannot be confirmed. Further investigations could identify a second variant (e.g., a deletion) which would confirm a diagnosis (12).
Uninformative test result	Detection of biallelic variants in TRIT1 gene in a child with microcephaly, profound developmental delay, hypotonia, epilepsy, and brain anomalies. This gene has never been reported to be a disease-causing gene in humans. The identification of genotypically and phenotypically similar children combined with more extensive analyses identified that mutations in this gene explain the phenotype among these children (14).
Dual diagnosis	Detection of a variant in the ITPR1 gene responsible for spinocerebellar ataxia type 29 in a 2-year-old female presenting with ataxia, motor, and language delay. Pathogenic mutation was inherited from her father. At age 5 she presented with seizures which had never been reported to be associated with SCA 29. Her mother had a childhood diagnosis of Landau-Kleffner syndrome with seizures. Analysis revealed a de novo pathogenic mutation in the GRIN2A gene in the proband and her mother, known to be associated with Landau- Kleffner syndrome. The family was counseled regarding the 2 separate autosomal dominant diseases that were identified in the proband, each inherited from a different affected parent (21).
Predictive risk result	Detection of sequence-level variant in the KCNH2 gene which is associated with risk for Long QT syndrome. Potential for medical actionability would typically prompt a laboratory to report this variant (22,23).
Pharmacogenomic result	Detection of polymorphisms in cytocrome P450 (CYP) enzymes (CYP2C9, CYP2C19) that can lead to differences in serum concentrations and anti-epileptic drug clearance with a greater risk of concentration-dependent adverse effects (30).

genome sequence can generate up to 20,000 (6). Based upon the patient's clinical features and the suspected mode of inheritance (i.e., or determined mode of inheritance via parental testing), the variant interpretation team then searches the published literature and various databases of genomic variation (e.g., Human Gene Mutation Database, ClinVar, Leiden Open-source Variation Database) for evidence of an association between suspected genes and the clinical presentation of the patient (4,9–11).

Based upon the current American College of Medical Genetics (ACMG) variant classification system (12), genome-wide sequencing can generate the following categories of results: (i) primary diagnosis, (ii) possible diagnosis, (iii) uninformative test, (iv) dual diagnosis, (v) predictive secondary variant and (vi) pharmacogenomic variant (Table 1).

A primary diagnosis refers to a variant that is identified in a disease-causing gene that is the likely cause of the child's health problem(s). The detection rate for primary diagnoses using genome-wide sequencing approaches 30% to 40% for children with developmental delay and congenital anomalies (6,7).

A *possible diagnosis* occurs when variants of uncertain significance (VUS) are identified; variants that neither confirm nor disconfirm a genetic aetiology for a set of presenting clinical features (12). VUS have posed longstanding challenges in molecular genetics but with the emergence of genome-wide sequencing, these variants are identified at greater frequencies (9–11). The ACMG advises that certain variant characteristics be used to guide decision making about the pathogenicity of a VUS. These include the mutation type, the frequency of the variant in control and patient databases, its predicted pathogenicity based upon in silico computer programs and its inheritance pattern (13). For example, if a variant is not inherited from a parent (so is de novo) and is associated with a dominant condition, pathogenicity is favoured. Large-scale data repositories of variants as well as novel research tools are emerging as additional strategies for characterizing these variants, the latter

including RNA expression, enzyme analysis, protein localization and animal modeling (9,10).

An *uninformative test result* occurs when no variants are detected, when a variant is detected that is not relevant to the child's presenting features, or when a variant is detected that has not been reported to be associated with human disease (14). Children who receive uninformative results might benefit from re-analysis of their sequence data at a later point in time or may benefit from access to research initiatives that aim to 'solve the unsolved' (e.g., Care4Rare Canada, Matchmaker Exchange, Undiagnosed Disease Network) (15–17). By sharing genotypic and phenotypic data through international data platforms, the likelihood of finding a patient 'match', leading to a shared and understandable diagnosis increases substantially. As a result of such international collaboration, gene discovery for rare disease has occurred at a rapid pace over the past 5 years (18,19). As such, re-interrogating an individual's 'null' sequence may generate new information, even after only 1 year. For example, upon re-evaluating the exomes of 40 'unsolved' cases, a genetic diagnosis was identified in 10%, due to new gene discoveries over the course of one preceding year (20). Children for whom a *possible diagnosis* is received may also be good candidates for sequence re-analysis.

A *dual diagnosis* occurs when mutations in more than one disease-causing genes are identified and each variant is thought to provide a partial explanation of the individual's composite presentation. Dual diagnoses have been reported in 4% to 15% of the patients (6,21).

A *predictive secondary variant*, also known as an incidental finding, refers to variants that are medically actionable but unrelated to the primary indication for testing. While the rate at which these are detected depends upon the bioinformatics filter used, it is estimated that secondary variants will be identified in up to 3.5% of children when the ACMG's recommended 59-gene list is interrogated (22,23). Mixed views on whether these variants *should* be actively sought and reported has resulted in conflicting professional guidance on this issue (4,24,25). In part, the controversy stems from varied views on the ethics of proactively searching for unsolicited information (26,27) and in part it stems from the absence of robust evidence on the actual positive predictive value of the secondary variants themselves (e.g., mutation of equivocal association with longQT syndrome detected in the absence of relevant family history) (11,28). This controversy is particularly charged with respect to children, given that traditional guidance in genetics recommends against predispositional testing in children (29). Some experts in the field retain the traditional view of preserving the child's autonomy related to knowing adult-relevant health information while others advocate that it is in the best interest of the child to generate this information so that parents and other implicated relatives can make preventive health care decisions (26). Against this backdrop, the ACMG recommends that laboratories actively search for and report variants in 59 genes (i.e., associated with hereditary cancer syndromes and cardiac dieases) but that parents and children should be given a choice about whether to receive results on variants deteced in these dynamics (24). In contrast, the European Society of Human Genetics (ESHG) recommends that laboratories analyze only sequence data relevant to the primary indication for testing to reduce the likelihood of generating secondary variants. Where inadvertently identified, however, ESHG advises that medically actionable variants should be reported to the family (25). Similar to the ESHG, the Canadian College of Medical Genetics (CCMG) does not endorse the intentional interrogation of a list of secondary genes, but recommends that if identified childhood-onset medically actionable mutations be reported and that competent adults be offered a choice about receiving secondary variants related to themselves (4).

A *pharmacogenomic result* occurs when a variant associated with a known drug response is identified (30,31). Genome-wide pharmacogenomics testing has been proposed as a tool for pre-emptive screening to provide anticipatory guidance to families. In one cohort, 95 of 98 children had at least one clinically actionable pharmacogenomic variant, suggesting that pre-emptive screening may in fact act as a patient safety measure embedded within diagnostic genome-wide sequencing for children (31).

GENOME-WIDE SEQUENCING: ANALYTIC LIMITATIONS

Certain analytic limitations of genome-wide sequencing warrant elaboration. First, coverage of the exome/genome using next-generation sequencing technology is estimated to be 85% to 92% (9,10,32). As well, variability in depth of coverage across the exome and genome leads to the possibility of gaps in sequencing and missing or uninterpretable data (32). While the diagnostic yield estimates for exome sequencing are robust (~30%), expansions, structural rearrangement and mutations in regulatory or intergenic regions of the genome cannot be detected (10). For genome sequencing, diagnostic yield estimates suggest it is slightly higher (6,33), but certain regions remain uninterpretable and expansions greater than sequence read lengths remain undetectable (e.g., expansions associated with Fragile X syndrome) (9,10,32). In addition, disease-causing variants reported in the literature and in large-scale databases can be incorrect; error rates have been reported to range from 4% to 23% (34–36). Current software tools for predicting variant pathogenicity should not be used in clinical decision making (37), and functional studies that characterize the effect of the variant on gene expression or protein–protein interaction, for example, are generally not available in a clinical setting (38).

GENOME-WIDE SEQUENCING: IMPLICATIONS FOR PRACTICE

Guidance is emerging to assist clinicians with determining appropriate clinical indications and timing for genome-wide sequencing. With respect to clinical indications, Table 2 presents broad factors that increase the likelihood of identifying a molecular cause for a given condition using genome-wide sequencing (4). Currently, the diagnostic yield using genome-wide sequencing is highest for specific conditions that are known to be genetically heterogeneous (e.g., ataxia) or for cases of nonspecific and unexplained clinical presentation (e.g., moderate-to-severe intellectual disability) (4,32). At the time of CCMG's position statement on the clinical use of these tools, there was insufficient evidence to recommend their use for children with nonsyndromic autism, learning disabilities and neuropsychiatric disease without additional features, or in the prenatal setting (Figure 1) (4). With respect to timing, a recent prospective study investigated the diagnostic yield and cost of exome sequencing in 44 Australian children at various timepoints in their diagnostic trajectory. The total health care expenditure on all diagnostic testing conducted on this cohort was A$568141 (US$430873). The cost per patient of the standard diagnostic pathway (i.e., without exome sequencing) was A$9901 (US$7509). The cost per patient of the standard diagnostic pathway *plus* exome sequencing was A$12 912 (US$9792). However, exome sequencing performed at initial tertiary presentation had the lowest cost per patient (A$5186 [US$3933]), followed by exome sequencing performed at the first genetics appointment (A$7047[US$5347]) (39).

Currently, professional guidelines stipulate that genome-wide sequencing can only be ordered by clinicians with specific training in genetics (4,32). Table 2 and Figure 1 can guide paediatricians' thinking about appropriate indications for referral. Prior to ordering genome-wide sequencing tests themselves,

Table 2. Factors that increase the likelihood of monogenic disease and/or facilitate the interpretation of genome-wide data

Family history	Similarly affected individuals
	Recognizable pattern of inheritance
	Consanguinity
Phenotype	Severity of phenotype
	Specificity of clinical presentation (e.g., neuropathy, metabolic disease)
Clinical interpretation	Careful patient phenotyping (e.g., detailed physical exam, imaging, chemistry)
	Normal chromosomal microarray analysis and other relevant laboratory testing
	Exclusion of acquired causes (e.g., infection)

Data taken from ref. (4).

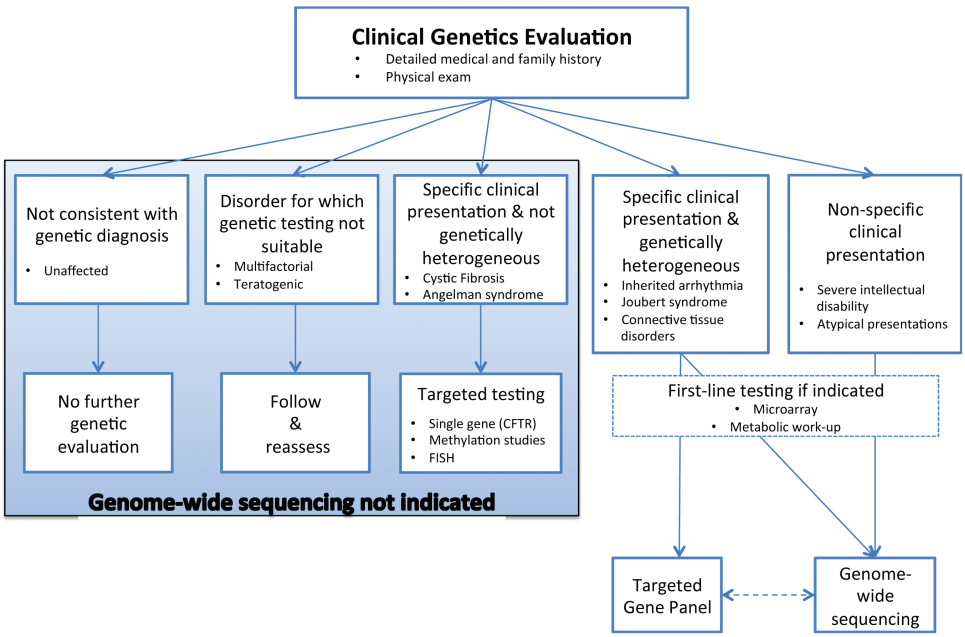

Figure 1. Decision aid to facilitate the diagnostic evaluation of patients with rare disease of suspected monogenic aetiology. This decision aid highlights where genome-wide sequencing may prove useful in the evaluation process. The conditions listed in each box are representative examples only. For specific clinical presentations associated with genetic heterogeneity, the decision regarding the use of a targeted panel versus genome-wide sequencing is dependent on a number of factors, including the availability of the testing options and the yield of such panels. Patients with negative targeted gene panels may benefit from subsequent clinical genome-wide sequencing. Conversely, consideration of a targeted panel subsequent to uninformative clinical genome-wide sequencing would be dependent on the depth of coverage achieved in the latter instance. NB: Fluorescence in situ Hybridization has now been replaced by multiplex ligation-dependent probe amplification in many centres. Reproduced with permission from ref. (4).

academic and nonacademic paediatricians will require proficiencies in conducting clinical genetics evaluations, understanding the indications for and strengths and limitations of genome-wide sequencing approaches, result interpretation and strategies for cascade testing in family members (4,32). In the not too distant future, efforts to enhance paediatricians' capacity for ordering genome-wide sequencing themselves will be warranted. When this time comes, we anticipate that providing diagnostic evaluation for phenotypically and genotypically complex cases, disease and disease-modifier gene discovery and the application of these data to tailor patient care will remain the core business of medical genetics experts.

While genome-wide sequencing promises to be a powerful tool for identifying the causes of genetic diseases in children and Canadian nondiscrimination legislation is favourable (40), interpretation, availability and ethics remain core challenges. However, a robust understanding of the aforementioned components of genome-wide sequencing approaches will equip paediatricians to play an active role in genome diagnostics when current analytic and ethical challenges resolve and accessibility improves, guiding patients and their families into the era of genomic medicine.

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