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BRIEF REPORT

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Exome Sequencing as a Diagnostic Tool for Pediatric-Onset Ataxia

Sarah L. Sawyer,[1] Jeremy Schwartzentruber,[2] Chandree L. Beaulieu,[1] David Dyment,[1] Amanda Smith,[1] Jodi Warman Chardon,[1] Grace Yoon,[3] Guy A. Rouleau,[4] Oksana Suchowersky,[5] Victoria Siu,[6] Lisa Murphy,[6] Robert A. Hegele,[7] Christian R. Marshall,[8] FORGE Canada Consortium, Dennis E. Bulman,[1] Jacek Majewski,[9] Mark Tarnopolsky,[10†] and Kym M. Boycott[1*†]

[1]Children's Hospital of Eastern Ontario Research Institute, University of Ottawa, Ottawa, Ontario, Canada; [2]McGill University and Genome Quebec Innovation Centre, Montréal, Quebec, Canada; [3]Divisions of Neurology and Clinical and Metabolic Genetics, Hospital for Sick Children, University of Toronto, Toronto, Ontario, Canada; [4]Montreal Neurological Institute and Hospital, McGill University Montreal, Quebec, Canada; [5]Departments of Medicine (Neurology) and Medical Genetics, University of Alberta, Edmonton, Alberta, Canada; [6]Department of Pediatrics, Division of Medical Genetics, Western University, London, Ontario, Canada; [7]Robarts Research Institute, University of Western Ontario, London, Canada; [8]Program in Genetics and Genome Biology, Hospital for Sick Children and McLaughlin Centre, University of Toronto, Toronto, Ontario, Canada; [9]Department of Human Genetics, McGill University, Montréal, Quebec, Canada; [10]Department of Pediatrics, McMaster Children's Hospital, Hamilton, Ontario, Canada

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ABSTRACT: Ataxia demonstrates substantial phenotypic and genetic heterogeneity. We set out to determine the diagnostic yield of exome sequencing in pediatric patients with ataxia without a molecular diagnosis after standard-of-care assessment in Canada. FORGE (Finding Of Rare disease GEnes) Canada is a nation-wide project focused on identifying novel disease genes for rare pediatric diseases using whole-exome sequencing. We retrospectively selected all FORGE Canada projects that included cerebellar ataxia as a feature. We identified 28 such families and a molecular diagnosis was made in 13; a success rate of 46%. In 11 families, we identified mutations in genes associated with known neurological syndromes and in two we identified novel disease genes. Exome analysis of sib pairs and/or patients born to consanguineous parents was more likely to be successful (9/13) than simplex cases (4/15). Our data suggest that exome sequencing is an effective first line test for pediatric patients with ataxia where a specific single gene is not immediately suspected to be causative.

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KEY WORDS: ataxia; whole-exome sequencing; clinical diagnosis

Whole-exome sequencing (WES) has been very successful for novel gene discovery for rare diseases [Bamshad et al., 2011; Boycott et al., 2013] as well as identifying de novo mutations in novel genes causing more common conditions such as intellectual disability [de Ligt et al., 2012; Rauch et al., 2012]. While several reviews and editorials suggest that WES is poised to facilitate a dramatic advancement in our ability to arrive at a genetic diagnosis for a particular patient with a rare genetic disease [Bick and Dimmock, 2011; Kingsmore and Saunders, 2011; Majewski et al., 2011; Singleton, 2011], studies demonstrating improvement over traditional testing based on diagnostic success rate, cost effectiveness, or time to diagnosis are still lacking. In addition, large-scale studies that focus on specific phenotypic presentations are needed to delineate the precise diagnostic utility of this technology for different clinical indications.

Ataxia is a clinically and genetically heterogeneous presentation that is a feature of more than 100 neurological disorders with onset in childhood (http://neuromuscular.wustl.edu/ataxia/aindex.html), thereby presenting a significant diagnostic challenge. Children with ataxia present with poor coordination and balance difficulties, which may either represent a static manifestation or be part of a degenerative disorder. In the latter instance, affected children may present with only a subset of features early in the course of the disease, further complicating the diagnostic process. As a result, a diagnostic odyssey often begins for these children. This process takes years, costing more than $10,000 [Kingsmore and Saunders, 2011] and ultimately may not yield a definitive molecular diagnosis. Receiving a genetic diagnosis has a significant and positive impact on patient care by ending the diagnostic odyssey, informing and optimizing patient management, facilitating accurate recurrence risk counseling and prenatal options for families, securing patient resources, and identifying other relatives who may be at risk [Kingsmore and Saunders, 2011].

There are few studies assessing the utility of WES as a diagnostic tool in ataxia. In one study, three consanguineous families were analyzed and three known genes were identified [Hammer et al., 2013], in another, a large autosomal dominant family with ataxia was found to harbor a mutation in *PRKCG*, known to cause spinocerebellar ataxia (SCA)14 [Sailer et al., 2012], and finally WES in a child

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Additional Supporting Information may be found in the online version of this article.

†These authors contributed equally to this work.

*Correspondence to: Kym M Boycott, Children's Hospital of Eastern Ontario Research Institute, 401 Smyth Road, Ottawa, ON, K1H 8L1, Canada. E-mail: kboycott@cheo.on.ca

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identified a homozygous *SACS* mutation [Liew et al., 2013], causing autosomal recessive spastic ataxia of Charlevoix–Saguenay. Broader assessment of the role of WES as a diagnostic tool has been undertaken in other neurological disorders. For example, WES in 25 patients with Charcot-Marie-Tooth (CMT) disease provided a diagnosis for eight patients, half of whom had a mutation in one of the four common CMT genes [Choi et al., 2012]. Mitochondrial disorders represent an active area of assessment. In a study that included 84 patients without a prior molecular diagnosis, five (6%) were diagnosed with a mitochondrial disorder after exome sequencing [Lieber et al., 2013]. In contrast, in an earlier study, 10/42 patients (24%) with a mitochondrial disorder were given a molecular diagnosis using MitoExome, which includes sequencing mt DNA and exons from ~1,000 nuclear genes encoding mitochondrial proteins [Calvo et al., 2012]. In one of the largest studies to date, 118 pediatric patients from consanguineous families with undiagnosed neurodevelopmental disorders underwent WES and 8% were diagnosed with a known disorder [Dixon-Salazar et al., 2012]. While all of these studies highlight the usefulness of WES as a diagnostic tool, additional data are needed to inform both clinicians and payers about the appropriate clinical indications and diagnostic yield for WES.

The FORGE (Finding Of Rare disease GEnes) Canada project is a nation-wide initiative to rapidly identify novel genes for rare childhood genetic disorders using WES. Patients undergo standard-of-care molecular investigations for known causes of their disease available to them in their province prior to inclusion in the study. Our experience from the completed analysis of the first 100 disorders in FORGE has demonstrated that a significant number of patients were found to have a mutation in a known disease gene, not tested in their standard-of-care diagnostic testing, that explained their clinical presentation. We report here the diagnostic yield of WES for the subset of FORGE patients with a phenotypic description of cerebellar ataxia.

A FORGE disorder/project consists of a patient, family or group of patients with a clinical diagnosis for which the genetic basis is unknown despite appropriate investigations. We retrospectively selected all FORGE Canada projects that included cerebellar ataxia as a clinical feature. In total 28 families, comprising 42 patients, were included: (A) six families (10 patients) born to consanguineous parents with one or more affected child; (B) seven families (17 patients) born to nonconsanguineous parents with more than one affected child (multiplex); and, (C) 15 patients born to nonconsanguineous parents with one affected child (simplex). Family histories did not suggest an autosomal dominant ataxia segregating in any of the families. All patients selected for WES had undergone previous molecular diagnostic testing. In Canada, physicians must apply for funding from the provincial health authority for genetic testing not available within the province and approval rates vary across the country. Thus, standard-of-care diagnostic testing for patients presenting with ataxia across the country varies by province but generally includes: SCA panel (SCA 1, 2, 3, 6, 7, 8, 17), Friedreich's ataxia, vitamin E, lactate, very long chain fatty acids, urine organic acids, plasma amino acids, and transferrin isoelectric focusing. In addition, diagnostic investigations usually include MRI, and may include lumbar puncture, muscle or skin biopsy, and other invasive investigations as indicated. For families in category A there were a variety of study designs including WES of one or two affected siblings with or without an unaffected parent. In category B, two affected siblings and an unaffected parent underwent WES in most instances. In category C only the affected patient underwent WES. In all cases, variant calls were confirmed by Sanger sequencing in patients and segregation was assessed using parent samples to confirm that the variants were in trans. Details regarding the number of affected individuals who had WES per family are outlined in Table 1.

Exome capture and high-throughput sequencing of DNA was performed at McGill University and Genome Québec Innovation Centre (Montréal, Canada) or The Centre for Applied Genomics (Toronto, Canada). Total genomic DNA was extracted from blood following standard procedures. Exome target enrichment was performed using the Agilent SureSelect 50Mb All Exon Kit (V3 or V4), followed by sequencing on Illumina Hiseq 2000 with three samples multiplexed per sequencing lane. Reads were processed by clipping adapter sequences and quality trimming from the 3′ end to the first base with phred-like quality 30, and then aligned to hg19 using BWA [Li and Durbin, 2009]. Duplicate reads were marked using Picard (http://picard.sourceforge.net) and were excluded. Average coverage of consensus coding sequence (CCDS) exons was calculated for each sample using GATK [DePristo et al., 2011]; mean coverage was 106x after duplicate read removal, with a range of 55× to 162×. Single nucleotide variants and short insertions and deletions (indels) were called using SAMtools mpileup [Li et al., 2009] and bcftools and quality-filtered to require a minimum 20% of reads supporting the variant call. Variants were annotated using Annovar [Wang et al., 2010] as well as custom scripts to select coding and splice-site variants, and to exclude common (≥1% minor allele frequency) polymorphisms represented in the 1000 genomes project or the NHLBI exome sequencing project, or with frequency > 4% in our FORGE control exomes, which increased in size from ~100 to 1,000 exomes as the FORGE project progressed over 2 years. To identify single or multi-exon copy number variants we used the tools FishingCNV [Shi and Majewski, 2013] and XHMM [Fromer et al., 2012].

We adopted a stepwise approach to identify the causal variants for each patient. First, we examined all homozygous, rare, nonsynonymous coding variants. There is usually a low number (1–40) of such variants, even in individuals from consanguineous families. We included homozygous deletion CNVs in this category. We manually compared the phenotypes of our patients with any known phenotypes reported in Online Mendelian Inheritance in Man (OMIM) associated with these genes to identify candidate gene(s). We next considered all genes broadly associated with a phenotype of ataxia with two rare variants, and evaluated their likelihood of pathogenicity. This is straightforward in the case of likely truncating variants—frameshift indels, stopgain SNVs, and canonical splice site changes—which are most always deleterious. For nonframeshift indels, we considered evolutionary conservation of the region and whether any other indels had been observed nearby in humans. For SNVs, we considered deleteriousness predictions from a set of tools: PolyPhen [Adzhubei et al., 2010], SIFT [Kumar et al., 2009], LRT [Chun and Fay, 2009], and MutationTaster [Schwarz et al., 2010], as well as conservation reported by GERP [Cooper et al., 2005] and PhastCons [Siepel et al., 2005]; however, no strict cutoff score were used as a primary filter. When the same rare variants had been previously associated with disease, this was considered strong evidence that they were likely to be involved in the disorder. In a number of cases, two nonsynonymous variants predicted to be deleterious were observed in a single known ataxia gene suggesting this gene as an excellent candidate for the phenotype. When only one such variant was seen, we also considered all variants in the same gene, including synonymous and intronic variants, to determine whether any were potentially deleterious or were inadvertently filtered out. If no likely causal variants were identified in known disease genes, the data was reanalyzed to consider novel candidate disease-causing genes and were subsequently validated in a research setting. The latter approach to novel gene discovery is

Table 1. Diagnosis by WES for FORGE Projects Presenting with Cerebellar Ataxia

FORGE ID	Original diagnosis/clinical features	Phenotype in addition to ataxia	# Exomes (Affected)	Category	Molecular Diagnosis	Final diagnosis-if revised
78	Congenital cerebellar atrophy	Hand tremor, dysarthria, dysphonia	2	B	*PMM2* (NM_000303.2) c.422G>A; p.R141H c.722G>C; p.C241S	Congenital disorders of glycosylation
108	Holmes syndrome	Hypogonadotropic hypogonadism	2	A	*RNF216* (NM_207111.3) c.2056C>T; p.R686X c.2056C>T; p.R686X	No change
109	Autosomal recessive spinocerebellar ataxia of childhood onset	Delayed motor milestones, cognitive delays, mild chorea	1	A	*SACS* (NM_014363.5) c.9278dupC; p. A3096Dfs∗4 c.9278dupC; p. A3096Dfs∗4	ARSACS
171	Perrault syndrome	SNHL, demyelinating polyneuropathy	2	B	*HSD17B4* (NM_00414.3) c.101C>T; p.A24V c.1547T>C; p.I516T	D-bifunctional protein deficiency (1)
254	Ataxia with cognitive impairment	Mild Intellectual disability, areflexic, progressive ataxia	2	A	*SETX* (NM_015046.5) c.6139G>T; p.G2047C c.6139G>T; p.G2047C	Autosomal recessive spinocerebellar ataxia
324	Hutterite cerebellar atrophy and short stature (CASS)	Hypotonia, strabismus, developmental delay, short stature, mild skeletal dysplasia, connective tissue abnormalities	1	A	*Gene X*	New disorder (2)
330	Cerebellar atrophy	Developmental delay, hearing loss, ophthalmoparesis, peripheral neuropathy	3	B	*HSD17B4* (NM_00414.3) c.1537C>A; p.P513T c.1628G>C; p.R543P	D-bifunctional protein deficiency
242	Marinesco-Sjögren syndrome	Severe intellectual disability, spasticity	1	A	*RAB3GAP1* (NM_001172435.1) c.363G>A; p.W121X c.363G>A; p.W121X	Warburg Micro syndrome
C1012	Ataxia, developmental delay, seizures	Intellectual disability, autism, seizures	1	C	*SYNGAP1* (NM_006772.2) c.2184delC; p.N729Tfs∗31	Intellectual disability (3)
C1026	Ataxia	Normal cognition, spasticity, motor, sensory neuropathy	1	C	*SACS* (NM_014363.5) c.5151dupA; p.S1718Ifs∗20 c.5151dupA; p.S1718Ifs∗20	ARSACS
212	Epilepsy with ataxia	Seizures, developmental delay	3	A	*KCTD7* (NM_153033.4) c.827A>G; p.T276C c.827A>G; p.T276C	Progressive myoclonic epilepsy type 3, with or without intracellular inclusions
C1010	Ataxia	Intellectual disability	1	C	*Gene X*	New disorder
C1002	Developmental delay and hypotonia	Regression, atypical neuropathy	1	C	*PLA2G6* (NM_001004426.1) c.1741C>T; p.R581X c.2208_2209delTG; p.Y736fs	Neurodegeneration with brain iron accumulation

(A) Patients born to consanguineous parents; (B) Patients with affected siblings born to nonconsanguineous parents; and (C) Patients without a family history (simplex).
ARSACS: autosomal recessive spastic ataxia of Charlevoix–Saguenay; SNHL: sensorineural hearing loss.
(1) [McMillan et al., 2012]; (2) Unpublished; (3) [Berryer et al., 2013].

essentially an ad-hoc process of selecting genes with variants that appear sufficiently rare and deleterious, and examining the literature and other gene-based resources to manually identify variants that seem likely to be causative for the patient's disease.

Twenty-eight families, including a total of 42 patients, with a phenotypic description of cerebellar ataxia, were retrospectively identified from >100 completed FORGE Canada projects. Analysis of all 28 families resulted in a molecular diagnosis for 11 (39%) in a known disease gene (Table 1). In 10 of the 11 families, we identified autosomal recessive disorders: autosomal recessive spastic ataxia of Charlevoix–Saguenay (MIM #270550), congenital disorder of glycoslylation (MIM #212067), D-bifunctional protein deficiency (MIM #261515) [McMillan et al., 2012], Holmes syndrome (MIM #212847), autosomal recessive spinocerebellar ataxia (MIM #606002), neurodegeneration with brain iron accumulation (MIM #256600), Warburg Micro syndrome (MIM #600118), and progressive myoclonic epilepsy 3 (MIM #611726). In the 11th family, we identified a de novo dominant mutation in *SYNGAP1* causing intellectual disability (MIM #612621) [Berryer et al., 2013]. Additionally, two new disease genes were identified, one in a consanguineous family and one in a nonconsanguineous family, and subsequently validated in a research setting (unpublished data) for a combined molecular diagnosis rate of 46%. The high success rate in this cohort may be influenced by the pediatric focus of this study as the presentation of ataxia in this patient population is more likely to be due to autosomal recessive disease, hence is more readily detected in the analysis of a single exome than a dominant disorder.

Table 2. Projects with a Revised Diagnosis of Ataxia Subsequent to WES

FORGE ID	Original diagnosis	Phenotype	Exome result	New diagnosis
84	Hereditary spastic paraplegia	Spasticity	*SIL1*	Marinesco–Sjögren syndrome
261	Early onset dystonia	Dystonia	*ATM*	Ataxia telangiectasia
381	Fitzsimmons–Guilbert syndrome	Brachydactyly, ataxia	*SACS*	Autosomal recessive ataxia of Charlevoix–Saguenay

For all families where a molecular diagnosis was achieved, arriving at the correct diagnosis without using WES would have been challenging due to variable access to funding for testing in international laboratories for Canadian patients, and the significant phenotypic and genetic heterogeneity of these disorders. Of the known genes that we identified, only *SACS* full gene sequencing is available on a clinical basis and only in the province of Ontario. For one patient with ARSACS, (C1026), clinical testing by Sanger sequencing had previously missed the homozgyous mutation. Sanger sequencing of the other eight genes identified by WES are only available in international laboratories, requiring funding approval for out-of-country testing, for access by Canadian patients.

We did not identify a molecular etiology for the clinical presentation of cerebellar ataxia in 54% of the families. Some of these may be because a nongenetic cause of ataxia is responsible for the clinical presentation. Others may be due, in part, to the limitations of WES which can include poor coverage of certain regions, and an inability to detect noncoding mutations, trinucleotide repeats in known [Sailer et al., 2012] and unknown genes, and large chromosomal rearrangements. However, a significant factor is that there remain a large number of variants in novel genes in the unsolved families that cannot be further validated at this time given the limited size of the current cohort and may represent rare and new causes of ataxia. To identify novel disease genes in these families will require large-scale collaborations to identify additional families with a similar phenotype and variants in the same gene. Detailed phenotypic data and variant datasets will need to be shared to facilitate the validation of such novel genes. The ability of WES to transition from clinical testing to research studies in such a fashion will increase the diagnostic success rate in the long-term and is a significant advantage of WES over current clinical methodologies [Ku et al., 2012].

In ten of the 11 families, the original clinical diagnosis/ description was clarified after WES analysis (Table 1). In all cases, the clinicians involved determined that, in retrospect, the gene identified did indeed explain the phenotype of the patient and the results were further supported by biochemical assays when possible. Interestingly, FORGE has also identified three patients who had been referred for WES with a clinical presentation that did not include ataxia who were found to have mutations in known ataxia genes, which in retrospect did explain their disorder (Table 2). These data highlight the challenges of clinical phenotyping and the unknown phenotypic spectrum of many genetic disorders, as has been reported in other studies [Dixon-Salazar et al., 2012]. This suggests that relatively unbiased approaches to genetic testing, such as WES, would be an efficient and successful approach to reach a molecular diagnosis in at least a subset of patients.

The diagnostic success rate of the three categories (A, B, and C) varied. The diagnostic success rate was highest for the consanguineous families (Category A) where it was successful in 6/6 (100%). For patients born to nonconsanguineous parents with more than one affected child (Category B) the diagnostic success rate was 3/7 (43%). In contrast, for simplex cases (Category C) the diagnostic success rate was 4/15 (27%). Although the success rates were better in the former two categories, a 27% rate of diagnosis after analysis of a single exome is anecdotally superior to testing that is currently available to most Canadian physicians for patients without a phenotype clearly consistent with a mutation in one gene. In addition, our data indicate that the diagnostic success rate in this study was influenced more by the presumed mode of inheritance (i.e., presence of an affected sib or consanguineous parents supporting autosomal recessive disease) than the WES study design (the number of individuals who underwent WES).

The success of WES in providing a molecular diagnosis to patients has broader implications with regard to the approach and thus cost of testing. The cost of the pre-exome genetic testing was calculated for six patients who were felt to have a typical evaluation from three different clinicians in two provinces. The average cost of in-province and international genetic testing alone, not including hospital stays and other invasive investigations, was $7,200 CAD and ranged from $1,800 to $16,000, with no diagnosis forthcoming (Supp. Table S1). The current cost of exome analysis offered by a clinical lab in the United States is in the range of $5,000 USD for a single patient and $9,000 USD for a trio. There is potential for cost savings for healthcare providers by ordering clinical WES early in the diagnostic assessment of patients presenting with cerebellar ataxia in childhood, particularly if the differential diagnosis is long.

In summary, our results illustrate the significant diagnostic yield of WES to identify mutations in genes causing pediatric-onset cerebellar ataxia in patients that had undergone standard-of-care investigations. If only a few genes are likely candidates, Sanger sequencing in the clinical laboratory should be ordered first to ensure complete interrogation of the gene(s). However, in instances where a long differential diagnosis exists then a relatively nonbiased approach to molecular diagnosis would appear to be an efficient option. Our findings suggest that when an autosomal recessive ataxia is suspected, a single exome will provide a genetic diagnosis at least 30% of the time in a known disease gene and that this diagnostic rate will increase to ∼50% if there is an affected sibling or a history of consanguinity. One approach to clinical testing could begin with WES of an affected individual and progress to analysis of a second affected sibling or both parents if an etiology is not identified. It remains to be seen if the diagnostic success rate of WES in ataxia is generalizable to other clinical presentations.

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