

## Next generation sequencing for molecular diagnosis of neurological disorders using ataxias as a model

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Many neurological conditions are caused by immensely heterogeneous gene mutations. The diagnostic process is often long and complex with most patients undergoing multiple invasive and costly investigations without ever reaching a conclusive molecular diagnosis. The advent of massively parallel, next-generation sequencing promises to revolutionize genetic testing and shorten the 'diagnostic odyssey' for many of these patients. We performed a pilot study using heterogeneous ataxias as a model neurogenetic disorder to assess the introduction of next-generation sequencing into clinical practice. We captured 58 known

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human ataxia genes followed by Illumina Next-Generation Sequencing in 50 highly heterogeneous patients with ataxia who had been extensively investigated and were refractory to diagnosis. All cases had been tested for spinocerebellar ataxia 1–3, 6, 7 and Friedrich's ataxia and had multiple other biochemical, genetic and invasive tests. In those cases where we identified the genetic mutation, we determined the time to diagnosis. Pathogenicity was assessed using a bioinformatics pipeline and novel variants were validated using functional experiments. The overall detection rate in our heterogeneous cohort was 18% and varied from 8.3% in those with an adult onset progressive disorder to 40% in those with a childhood or adolescent onset progressive disorder. The highest detection rate was in those with an adolescent onset and a family history (75%). The majority of cases with detectable mutations had a childhood onset but most are now adults, reflecting the long delay in diagnosis. The delays were primarily related to lack of easily available clinical testing, but other factors included the presence of atypical phenotypes and the use of indirect testing. In the cases where we made an eventual diagnosis, the delay was 3–35 years (mean 18.1 years). Alignment and coverage metrics indicated that the capture and sequencing was highly efficient and the consumable cost was ~£400 (€460 or US\$620). Our pathogenicity interpretation pathway predicted 13 different mutations in eight different genes: *PRKCG*, *TTBK2*, *SETX*, *SPTBN2*, *SACS*, *MRE11*, *KCNC3* and *DARS2* of which nine were novel including one causing a newly described recessive ataxia syndrome. Genetic testing using targeted capture followed by next-generation sequencing was efficient, cost-effective, and enabled a molecular diagnosis in many refractory cases. A specific challenge of next-generation sequencing data is pathogenicity interpretation, but functional analysis confirmed the pathogenicity of novel variants showing that the pipeline was robust. Our results have broad implications for clinical neurology practice and the approach to diagnostic testing.

**Keywords:** ataxia; genetics; autosomal dominant cerebellar ataxia; autosomal recessive cerebellar ataxia; diagnosis

**Abbreviation:** NGS = next-generation sequencing

## Introduction

Ataxias are a highly heterogeneous group of neurological disorders affecting individuals of all age groups. The most common ataxias are those caused by trinucleotide repeat expansions and include spinocerebellar ataxias 1, 2, 3, 6, 7 and 17, and Friedreich's ataxia, but causal point mutations have been described in a wide variety of other genes. The repeat expansions can be easily and cheaply tested using standard PCR methods and most patients with ataxia have this testing done even when the clinical phenotype makes the diagnosis unlikely. Genetic testing is available for few other genes because development of Sanger-based (dideoxy) DNA sequencing, the gold standard for detecting mutations at the base pair level, is expensive on an individual gene basis. Thus a large proportion of patients with ataxia never have a molecular diagnosis made (Muzaimi *et al.*, 2004; Kraft *et al.*, 2005; Anheim *et al.*, 2010). This may be due to limited test availability, but it is also possible that many novel genes associated with ataxia remain to be discovered. In the past few years, next-generation sequencing (NGS; high-throughput massively parallel sequencing) has allowed an unprecedented increase in our ability to sequence large numbers of genes at an equivalent cost to traditional Sanger sequencing. Although there have been several reports using NGS in a diagnostic setting, including one pilot study of five patients with ataxia (Hoischen *et al.*, 2010), most studies include patients with very homogeneous clinical features (Shanks *et al.*, 2012) or a modest number of genes requiring analysis (Fokstuen *et al.*, 2011; De Keulenaer *et al.*, 2012; Schrauwen *et al.*, 2013) and there are no reports examining the realities of introducing NGS for highly varied disorders into clinical practice. As obtaining a specific molecular diagnosis has significant

implications for patient management including genetic counselling, prognosis, long-term investigations and development of therapeutic strategies, we decided to investigate a heterogeneous group of patients with ataxia who were a diagnostic challenge and are representative of the range of cases referred for serial genetic testing.

We recruited 50 patients from independent UK families with ataxia who did not have a molecular diagnosis. All cases had standard testing for spinocerebellar ataxias 1–3, 6, 7 and Friedreich's ataxia. In addition, all the patients had a vast array of other investigations as appropriate, including multiple standard biochemical tests (e.g. plasma amino acids, urine organic acids), specialist biochemical tests (e.g. ubiquinone, phytanic acid, hexosaminidase), invasive investigations (e.g. muscle biopsy, lumbar puncture) and other genetic tests (e.g. *FRAXA*, mitochondrial genes). Patients were only included if ataxia was considered to be the main clinical feature and, to enrich for genetic aetiologies, we only included patients with a family history or an age of onset below 50 years. We performed targeted capture on a total of 58 genes known to be associated with human ataxia and an additional 59 genes considered to be good candidates from functional data or animal models. NGS was then performed on the Illumina GAII platform and where necessary functional experimental analysis was performed to confirm the pathogenicity of novel mutations. In addition, we noted the time to diagnosis and the investigations that were performed prior to successful diagnosis using NGS. Our findings have significant implications for diagnostics and research into ataxia but can also be applied to other inherited neurological disorders and can be used to develop models for introduction of NGS services into diagnostic laboratories.

## Materials and methods

### Patients

Patients were recruited from throughout the UK and consent for participation in the study was obtained according to the Declaration of Helsinki and approved by the Central Oxford Research Ethics Committee and the Research and Development Department of the Oxford Radcliffe Hospitals NHS Trust, Oxford. Inclusion criteria were ataxia as the main clinical feature and either presence of a family history of ataxia or age of onset below 50 years. All cases had been tested for spinocerebellar ataxia 1–3, 6, 7 and Friedrich's ataxia and had multiple other biochemical, genetic and invasive tests. In any cases where a mitochondrial disorder was suspected (e.g. when retinitis pigmentosa was present), then all available mitochondrial genetic testing and muscle biopsies had also been performed. All patients or their parents provided written consent for the study.

### Targeted capture using SureSelect enrichment

The exonic and 25 bp intronic flanking sequences of 118 genes were selected for targeted capture and sequencing (Supplementary Table 1). These included genes known to be associated with ataxia in humans as the predominant phenotype (42 genes, 786 exons), genes causing ataxia in rodent models (18 genes, 514 exons), or good candidate genes based on function (35 additional transfer RNA synthetase genes, 580 exons). We included all the transfer RNA synthetase genes because at the time of the capture design there was emerging evidence that mutations in these were particularly involved in neurological phenotypes including ataxia, including mitochondrial transfer RNA synthetase genes (e.g. *RARS2* causing infantile encephalopathy with particular effect on the cerebellum, and *DARS2* causing leukoencephalopathy with brainstem and spinal cord involvement, LBSL; Scheper *et al.*, 2007), cytoplasmic transfer RNA synthetase genes (e.g. *Aars* causing cerebellar ataxia in a mouse model) and bifunctional genes (*GARS* and *YARS* causing autosomal dominant Charcot–Marie–Tooth disease; Antonellis and Green 2008). Indeed since the original capture design at least six further mitochondrial transfer RNA synthetases have been associated with neurological disorders (*YARS2*, *HARS2*, *EARS2*, *FARS2*; Riley *et al.*, 2010; Pierce *et al.*, 2011; Elo *et al.*, 2012; Steenweg *et al.*, 2012), including two associated with ataxia (*MARS2* and *SARS2*) (Belostotsky *et al.*, 2011; Bayat *et al.*, 2012). Several other genes associated with ataxia as part of the phenotype (six genes, 79 exons) and several speculative genes based on function (seven genes, 167 exons) were also included. Ten genes (243 exons) associated with Joubert syndrome, a specific ataxia disorder associated with the 'molar tooth sign' on MRI were included and results will be presented in a separate paper. The gene accession numbers and coordinates, were downloaded from the UCSC database, and 120-mer baits with 2 × tiling were designed using the Agilent eArray web-based design tool.

Libraries were prepared according to Agilent's protocol for SureSelect Target Enrichment for Illumina Paired-End Multiplexed Sequencing Version 1.0 (May 2010). One of 12 unique barcodes (Illumina Multiplexing Sample Preparation Oligonucleotides) was added to each sample. Groups of 12 sample libraries were sequenced per lane of a flow cell. Library quality was assessed using the Agilent DNA High-Sensitivity Bioanalyzer kit, and library quantity was assessed using the High-Sensitivity Qubit Fluorometer (Invitrogen).

### Illumina sequencing

Samples were sequenced on lanes producing 51 bp paired-end reads. The amount (in pM) of library sample run was determined by quantitative PCR so that the same cluster density was present in each lane.

### Sequence read mapping and variant calling

Sequence reads were aligned to the human reference genome (GRCh37/hg19) using STAMPY (Lunter and Goodson, 2011) with the option of using BWA (Li and Durbin, 2009) as a pre-mapper to speed up calculations. Single nucleotide variants were identified using the SAMTOOLS software package (Li *et al.*, 2009b) with custom modifications. Small insertion/deletions were identified using DINDEL (Albers *et al.*, 2011).

### Variant annotation

Variants were annotated with respect to gene and transcripts using the Ensembl Variant Effect Predictor (VEP) tool (release 62, April 2011) (Flicek *et al.*, 2011). For each variant, the VEP reports its functional consequences (non-synonymous coding, splice site, intronic, etc.) and checks if the mutation is already present in dbSNP (Build 132) (Sherry *et al.*, 2001).

### Variant filtering

Regions of interest were defined as exons plus 25 bp of intronic flanking sequences. All variants within the region of interest were filtered using an algorithm which ensured that all essential splice variants were retained in the analysis while common (minor allele frequency >5%) single nucleotide variants were removed, before analysis with standard pathogenicity prediction programs including PolyPhen2 (Adzhubei *et al.*, 2010), SIFT (Sim *et al.*, 2012), MutPred (Li *et al.*, 2009a) and the mutation interpretation software Alamut which incorporates five splice prediction programs (<http://www.interactive-biosoftware.com/>). Relatively stringent criteria were used such that pathogenicity had to be predicted by all three Bioinformatics programs (Shanks *et al.*, 2012). This strategy was designed to maximize true positives and minimize false positives. Potential pathogenic variants were compared with the clinical data provided to determine the likely relationship between the variants and the phenotype.

### Coverage analysis

Sequence coverage was calculated with custom scripts and the BEDTOOLS package (Quinlan and Hall, 2010). We calculated the average fold coverage over the target regions and the percentage of nucleotide positions covered by at least 5, 10, 20, 30, 50 and 100 reads. In order to evaluate the success of the capture experiment, we also calculated the percentage of reads that mapped to the target regions out of the total number of reads ('reads on target').

### DNA extraction and Sanger sequencing validation

All potential mutations were validated by Sanger sequencing using standard protocols. Primer sequences for the validations are found in Supplementary Table 2. PCR products were used as templates for sequencing with BigDye<sup>®</sup> Terminator reagents (Life Technologies) on

a 3730xl DNA Sequencing Analyzer (Life Technologies). The sequence traces were aligned to the gene-specific reference sequence (NCBI build 37) with Sequencher 4.10.1 (Gene Codes).

## Pathogenicity analysis of *PRKCG*

Both cysteine domains were aligned using the European Bioinformatics Institute ClustalW online alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), and the TEXshade alignment shading software (Beitz, 2000) was used to create the graph. The cysteine domains were overlaid with the published 3D structure Cys domain (PDB 1TBN; Xu, 1997) and the residues correlating to the known mutations were marked in MacPyMol. HEK293T cells were transfected with the indicated enhanced GFP-tagged PKCg constructs using Fugene (Roche) according to manufacturer's instructions. Thirty-eight hours post-transfection, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.4% Triton<sup>TM</sup> X-100/PBS and mounted with 4',6-diamidino-2-phenylindole (DAPI) (Vectashield<sup>®</sup> mounting medium, Vectorlabs).

## Results

### Clinical characteristics of patients

The clinical and genetic characteristics of the 50 patients can be found in Supplementary Table 3. Twenty-two patients (44%) had a clear family history, of which 12 showed autosomal dominant inheritance (mostly ADCA type III; Harding and Deufel, 1993). Childhood-onset ( $\leq 18$  years of age) or congenital cases represented 54% of cases (27/50) and 40% (20/50) had additional neurological features that included spasticity, epilepsy, cognitive impairment/decline, neuropathy or retinitis pigmentosa. The majority of cases (38/50) displayed a progressive phenotype, including eight childhood-onset/congenital cases and all adult ( $n = 24$ ) and adolescent ( $n = 6$ ) onset cases.

The majority of patients were not under review by paediatric neurologists, reflecting the long delay in diagnosis in many cases. In those patients in whom we were able to make a molecular diagnosis, the number of years from disease onset to diagnosis ranged from 3–35 years, with a mean of 18.1 years and a total of 163 years (Supplementary Table 3).

### Sequencing metrics

The regions of interest represented 2369 exons  $\pm 25$  bp flanking sequencing, totalling 603 248 bases. The designed capture bait

pool covered 95.2% of region of interest bases (Supplementary Table 4).

Sequencing metrics including alignment and coverage are shown in Table 1. Fifty-eight per cent of reads were on target with a mean coverage of 216 reads per base. Ninety-four per cent of regions of interest had  $>5\times$  coverage, 91% had  $\geq 20\times$  coverage and 73% had coverage of  $\geq 100$ -fold. Two samples had lower coverage, suggesting that the capture for these samples was less efficient (Table 1 and Supplementary Fig. 1).

### Identification of pathogenic variants and characteristics of patients

Over 5000 variants were identified in the 50 patients. After filtering,  $\sim 150$  variants remained, which we examined using our Bioinformatics pipeline in addition to validation by Sanger sequencing, and literature searches. Thirteen were considered to be clearly pathogenic and 9/13 were previously undescribed. We validated the majority of these using a variety of functional assays and in one case an animal model (Lise *et al.*, 2012) (Figs 2–4). The pathogenic mutations were found in nine cases (18%) (Table 2). In eight of these nine cases the age of onset was  $\leq 18$  years (Fig. 1A, B and Table 2) but most of them are now adults reflecting the long delay in diagnosis. Every patient had numerous outpatient investigations and two were admitted to specialist centres to determine the cause of the condition. These investigations routinely included multiple biochemical tests, with lumbar puncture and skin biopsy having been performed in some cases. It is not possible to obtain a formal costing for these investigations, but it is likely to far exceed the  $\sim £400$  for the consumables of the targeted capture and NGS of this study.

Figure 1A and B show the patients classified by clinical features and mutation status. We found that the most likely predictors of detecting a mutation were: an adolescent age of onset, a complex phenotype, a family history and a progressive disorder. The highest detection rate was therefore in the progressive adolescent-onset cases with a family history where three of four (75%) had a molecular diagnosis made. However, one notable exception were those cases of ataxia plus retinitis pigmentosa where no mutations were identified.

A more detailed examination of the gene-positive cases revealed the value of using the ataxia NGS panel (Table 2). In three cases (Cases 14, 14 and 37) the clinical phenotype included an ataxia with a prominent eye movement disorder (sometimes known as ataxia-telangiectasia-like disorder, because of the neurological

**Table 1** Sequencing metrics

Alignment %		% ROI coverage $> 5\times$		% ROI coverage $> 10\times$		% ROI coverage $> 20\times$		% ROI coverage $> 30\times$		% ROI coverage $> 50\times$		% ROI coverage $> 100\times$		Mean base coverage per sample	
Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
57.4	28.5–62.7	93.6	92.7–94.1	92.8	91.0–93.6	91.0	86.8–92.9	89.5	83.7–92.	84.6	75.7–90.7	73.2	45.2–87	215.3	88.7–471.3

Percentage and range of alignment as an average for all 50 samples in region of interest (ROI). Also shown is the mean percentage of exons covered at greater than  $5\times$ ,  $10\times$ ,  $20\times$ ,  $30\times$ ,  $50\times$  and  $100\times$  coverage, and the range in values. Finally, the mean base coverage in all samples is shown with the range in values across the patient samples.



Table 2 Clinical details and evidence for pathogenicity of mutation positive cases

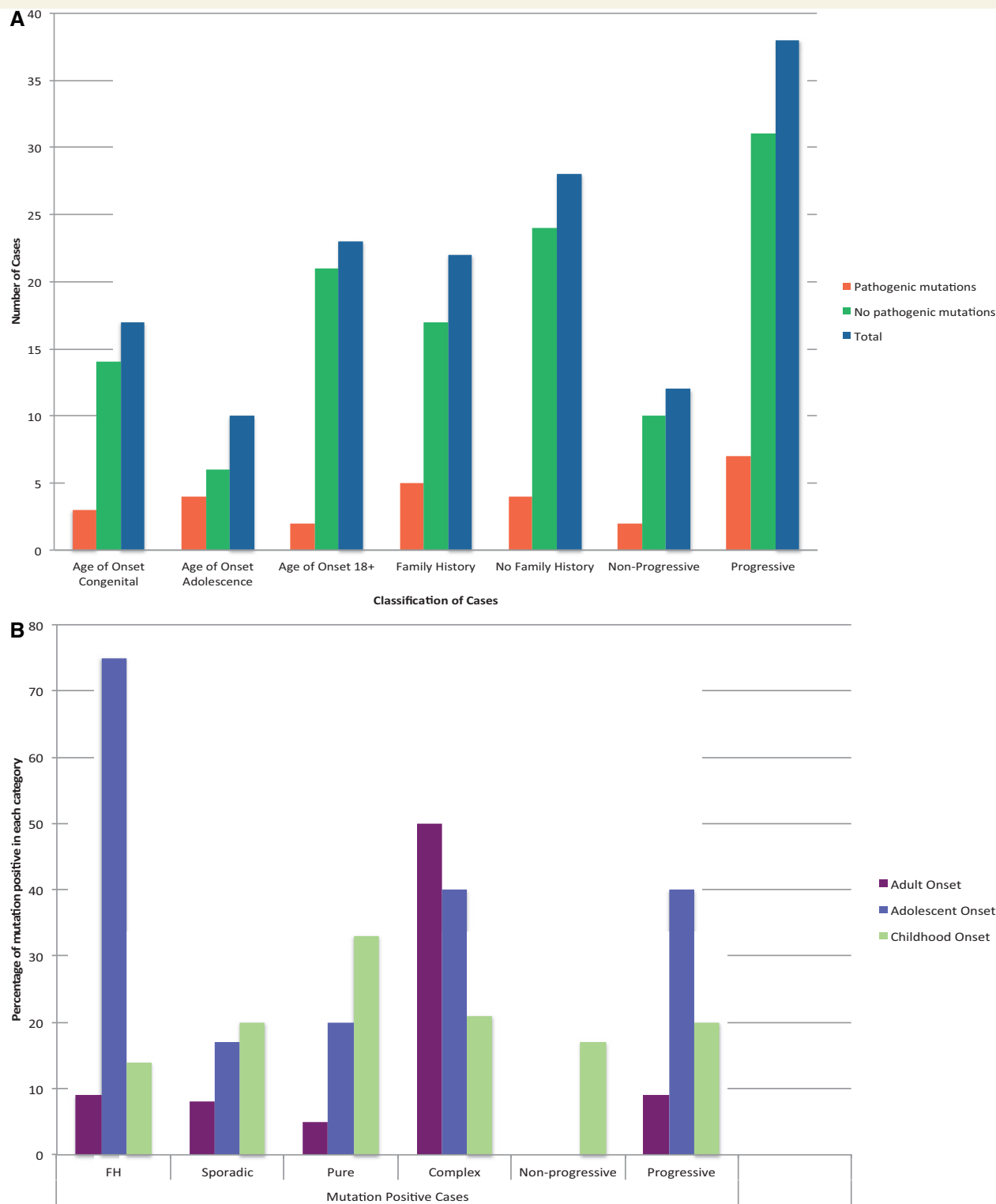
Case	Clinical features	Imaging	Previous investigations	Mutation	Mutations known or novel	Evidence for pathogenicity	Comment
3	Age of onset 17 years. No other neurological features. No FH initially reported, advised possible recessive inheritance with low recurrence risk. FH subsequently found and 50% recurrence risk advised.	Cerebellar atrophy on MRI	Four out-patient appointments	<i>TTRK2</i> (SCA11), heterozygote: R444TrsX7	known	<b>Previously reported mutation:</b> identified in a family from Devon, England (Houlden <i>et al.</i> , 2007).	Patient not known to be connected to previous family.
5	Age of onset 40s. Affected mother reported to have a rapidly progressive disorder. Dominant FH.	Cerebellar atrophy on MRI	Multiple out-patient investigations.	<i>PRKCG</i> (SCA14) (Chen <i>et al.</i> , 2003; Yabe <i>et al.</i> , 2003; Alonso <i>et al.</i> , 2005; Seki <i>et al.</i> , 2005): heterozygote: H36R	Novel	<b>Bioinformatics:</b> PolyPhen2 Probably damaging, MutPred Probably damaging <b>In vitro analysis:</b> see Fig. 2	No other family members available for segregation analysis. Extensive bioinformatics and functional assays required to confirm pathogenicity.
14	Age of onset 12 years. Slowly progressive ataxia and a sensorimotor neuropathy. Similarly affected sibling.	Cerebellar atrophy on MRI	AFP elevated	<i>SETX</i> (AOA2) (Izatt <i>et al.</i> , 2004), compound heterozygote: H2243Qfs*27 and V2390L	Two novel	<b>Bioinformatics:</b> one frameshift and other in crucial Helicase domain. <b>Western blotting:</b> reported as normal (Fig. 3)	Western blot normal, but mutations detected on sequencing.
15	Age of onset 17 years. Progressive cerebellar syndrome, marked amyotrophy, sensorimotor neuropathy. Similarly affected sibling.	Cerebellar atrophy on MRI	AFP elevated, normal Senataxin expression on western blotting (Fig. 4). Two hospital admissions to specialists units.	<i>SETX</i> (AOA2), compound heterozygote: F1756S and 7100+2 T>C (splice mutation).	One known, one novel	<b>Previously reported mutation</b> (F1756S) (Izatt <i>et al.</i> , 2004; Moreira <i>et al.</i> , 2004). <b>Bioinformatics:</b> on splice prediction, see Supplementary Fig. 2.	Clinical diagnosis of AOA2 was suspected but no sequencing available to confirm.
16	Congenital ataxia, squint and learning difficulties. Parents consanguineous, two identically affected cousins.	Normal MRI	Investigations of cousins for past 20 years. Patient had multiple out-patient appointments.	<i>SPTBN2</i> (SPARCA1), homozygote: C627X (Lise <i>et al.</i> , 2012)	Novel	<b>Previously reported dominant mutations in SCA5</b> (Ikeda <i>et al.</i> , 2006). <b>Whole-genome sequencing and analysis of mouse model.</b> Conclusion that the stop codon causes a novel syndrome, now known as SPARCA1 (Spectrin-associated Autosomal Recessive Cerebellar Ataxia, type 1, Lise <i>et al.</i> , 2012).	The homozygous stop codon strongly suspected to be the cause of the ataxia, but the recessive syndrome not reported before; significant downstream analysis required
29	Progressive cerebellar ataxia with spasticity from age 37. Mild cognitive decline.	Cerebellar atrophy and non-specific white matter abnormalities	Multiple out-patient assessments, muscle biopsy	<i>DARS2</i> : 536G>A, heterozygote: R179H	Known	<b>Previously reported mutation</b> (Scheper <i>et al.</i> , 2007). Very low carrier rate of the mutation in the population. Clinical phenotype consistent with Leukoencephalopathy with brainstem and spinal cord involvement (LBSL)	No second mutation found, but may be due to intronic mutations (Scheper <i>et al.</i> , 2007). Further work required to search for an intronic splice mutation.

(continued)

Table 2 Continued

Case	Clinical features	Imaging	Previous investigations	Mutation	Mutations known or novel	Evidence for pathogenicity	Comment
37	Normal early development but by age 2 years had progressive balance disorder with dystonia and myoclonic jerks. Now aged 25.	Marked atrophy of cerebellar vermis by age 12.	Two in-patient assessments at specialist centres including complex biochemistry, ubiquinone, lumbar puncture, skin biopsy, chromosome and DNA analysis (including AOA1 and AOA2 on a research basis).	<i>MRE11A</i> (Ataxia-Telangiectasia-Like Disorder (Stewart <i>et al.</i> , 1999): compound heterozygote: N117S and splice mutation, 314 + 4, 314 + 7del.	One known, one novel	<b>Previously reported mutation:</b> N117S (Stewart <i>et al.</i> , 1999) <b>Bioinformatics:</b> four programs predict effect on splicing (Fig. 4A) <b>In vitro analysis:</b> Western blotting shows abnormal Nbs1, which is an effect of MRE11 mutations (Fig. 4B)	ATLD very rare, easily detectable using NGS
45	Congenital cerebellar ataxia associated with mild learning difficulties, working diagnosis of 'ataxic cerebral palsy'.	Normal MRI brain aged 3	Multiple out-patient investigations including blood and urine biochemistry and for DNA repair disorders.	KCNC3 (SCA13): heterozygote: T428I	Novel	<b>Previously reported dominant mutations in the same domain of KCNC3 cause SCA13</b> (Waters <i>et al.</i> , 2006; Figueroa <i>et al.</i> , 2010; Minassian <i>et al.</i> , 2012) <b>Bioinformatics:</b> highly conserved nucleotide, highly conserved amino acid, Grantham score: 89 [0-215], Align GVGD: C65 (GV: 0.00 - GD: 89.28) SIFT: Deleterious (score: 0.00); PolyPhen2: probably damaging (0.982); <b>MutPred</b> T428 in protein domain involved in ion transport through voltage-dependent potassium channel.	Significant implications for offspring recurrence risk which is 50%.
50	Progressive ataxia and spasticity from the age of 3 years, now aged 38. Further deterioration in 20s.	Atrophy of cerebellar vermis and upper cervical cord (Supplementary Fig. 2)	In-patient assessment at specialist centre including complex biochemistry, muscle biopsy, skin biopsy, other genetic tests e.g. FRAXA, Hexosaminidase A, LHON mitochondrial mutations	SACS (Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay) (Engert <i>et al.</i> , 2000), compound heterozygote: Q4010X, P4539S	Two novel	<b>Bioinformatics:</b> Q4010X a stop codon, P4539S located in highly conserved HEPN domain and noted to be a critical residue (Kozlov <i>et al.</i> , 2011). Clinical review of the patient confirmed phenotype consistent with ARSACS although optic atrophy unusual.	Genetic testing for ARSACS is not available in the UK despite prevalence estimates suggesting that it is one of the commoner autosomal recessive ataxias (Figueroa <i>et al.</i> , 2010).

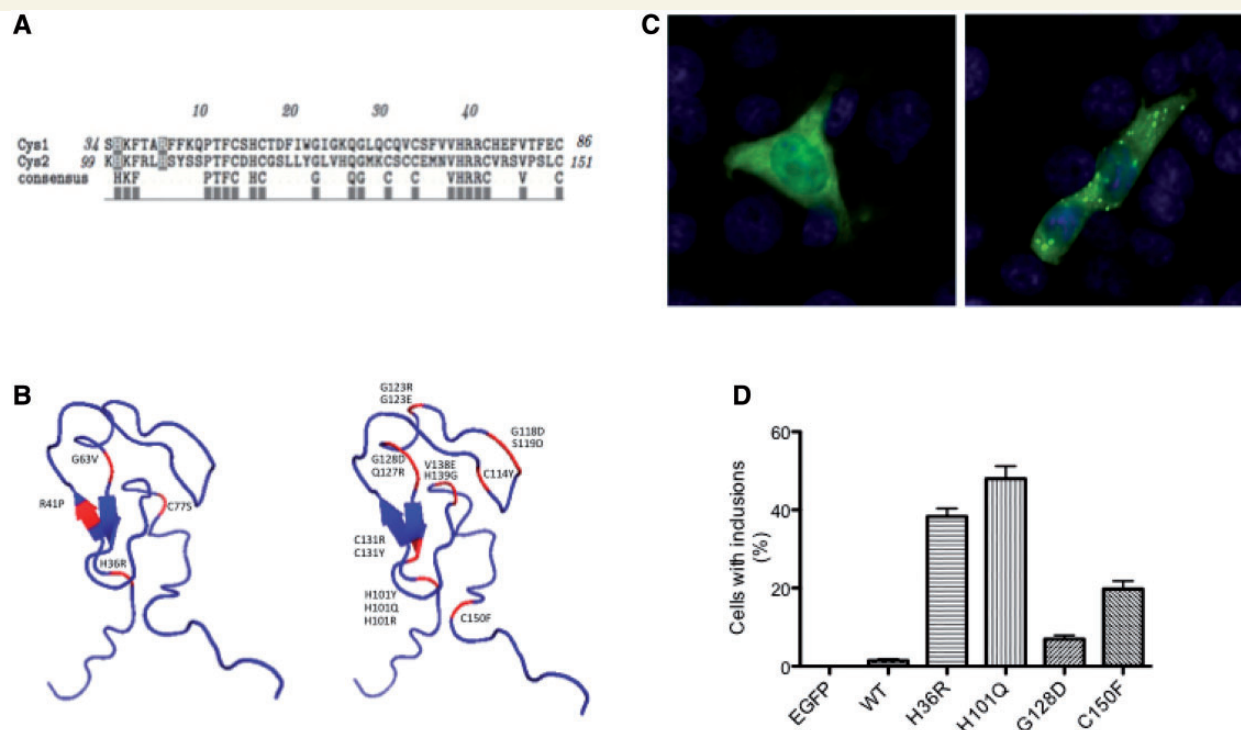
AFP = Alpha fetoprotein; FH = Family history.



**Figure 1** Clinical features of cases. (A) Total number of cases analysis with or without pathogenic mutations categorized by age of onset, family history and progression. (B) Percentage of mutation-positive cases analysed by age of onset, family history and progression.

similarities with ataxia-telangiectasia). The most common gene mutations associated with ataxia-telangiectasia-like disorder are in aprataxin (*APTX*; Moreira *et al.*, 2001) and senataxin (*SETX*; causing ataxia with oculomotor apraxia type 2; Németh *et al.*,

2000; Izatt *et al.*, 2004; Moreira *et al.*, 2004). In Case 14 the diagnosis of ataxia with oculomotor apraxia type 2 had not previously been made because of lack of availability of genetic testing, however Case 15 was more complex. The diagnosis of

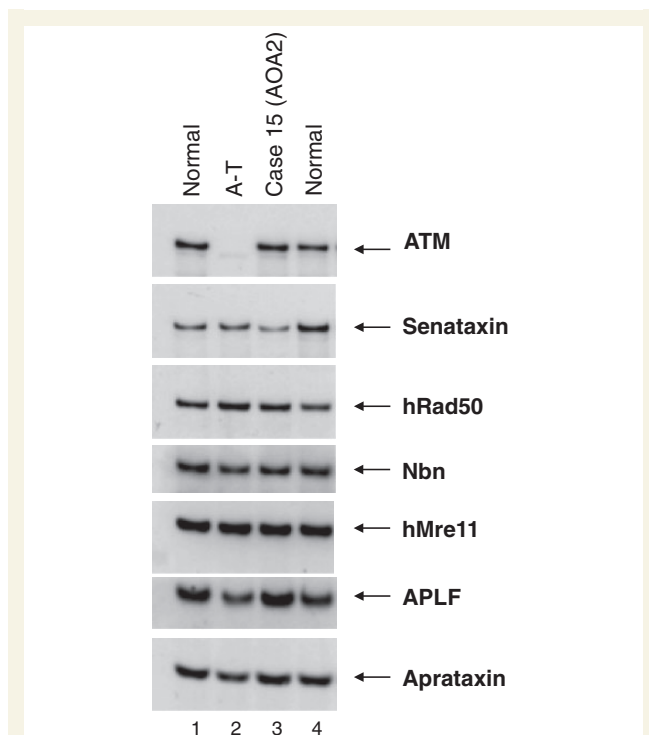


**Figure 2** Pathogenicity analysis of H36R in *PRKCG*. (A) The Histidine at position 36 is located in the first cysteine-rich domain. The equivalent Histidine H101, located in the second cysteine rich-domain is associated with several known spinocerebellar ataxia 14 (SCA14) mutations (Alonso *et al.*, 2005). (B) Comparison of the 3D structure of the cysteine-rich domains in *PRKCG* shows the located of H36R and other known mutations including H101Y, H101Q and H101R. (C) Comparison of HEK cells expressing wild-type (left) and H36R (right) with visible inclusions in the cells with the mutated SCA14 protein. (D) Susceptibility to aggregation in the H36R mutant compared with wild-type, reported in several SCA14 mutations (Seki *et al.*, 2005). At least 200 cells per transfection were counted in three independent experiments. ANOVA and Bonferroni's multiple comparison test: wild-type versus H36R  $P < 0.005$ ; wild-type versus H101Q  $P < 0.005$ ; wild-type versus G128D not significant; wild-type versus C150F  $P < 0.005$ .

ataxia with oculomotor apraxia type 2 was suspected on clinical grounds (adolescent onset ataxia, an eye movement disorder, raised alpha fetoprotein) but a western blot to screen for mutations in *SETX* was found to be normal (Fig. 3). However, on NGS testing two mutations were identified in *SETX*; a known missense mutation, F1756S, and a clear splice variant in intron 23 (Supplementary Fig. 2). The western blot probably appeared normal because the missense allele would produce a normal sized protein product whereas the splice variant either produced an abnormal protein size that was missed, or induced nonsense-mediated decay resulting in no protein produced from that allele, thus leading to a false negative result. In Case 37, the phenotype was also an ataxia-telangiectasia-like disorder with ataxia and a prominent eye movement disorder. The diagnosis of ataxia with oculomotor apraxia type 2 was considered but excluded by sequencing on a research basis. Mutations in *MRE11* also cause an ataxia-telangiectasia-like disorder phenotype (Stewart *et al.*, 1999) but sequencing of the *MRE11* gene was not available. Our analysis identified one known missense mutation, N117S [shown to be pathogenic by Stewart *et al.* (1999)] and one novel splice mutation, 314+4\_314+7del in *MRE11*. Bioinformatics predicted a damaging splice mutation and retrospectively a western blot confirmed the pathogenicity of these mutations (Fig. 4).

There were three cases with additional spasticity and in two we found mutations. Case 29 had an adult onset ataxia with spasticity and subtle cognitive decline, but an MRI with non-specific white matter abnormalities. A single known pathogenic mutation was found in the mitochondrial transfer RNA synthetase *DARS2*. Mutations in *DARS2* are associated with leukoencephalopathy with brainstem and spinal cord involvement (Scheper *et al.*, 2007). The clinical phenotype includes a slowly progressive cerebellar ataxia and spasticity with dorsal column dysfunction associated with MRI signal abnormalities in the white matter. Deterioration of motor skills usually starts in childhood or adolescence, but occasionally not until adulthood. The phenotype in our patient is unusual but nevertheless non-specific and mutations in this specific gene would not have been considered likely in this patient. Case 50 had a childhood onset gait disorder that deteriorated in adulthood. The key features included ataxia and spasticity. Optic atrophy was also noted. Mutations in *SACSIN* were identified, one a clearly pathogenic stop codon, Q4010X, and a missense mutation, P4539S, in the crucial HEPN functional domain (Kozlov *et al.*, 2011). Mutations in *SACSIN* cause ARSACS (autosomal recessive spastic ataxia of Charlevoix-Saguenay) which was originally reported in Quebec and Tunisia (Engert *et al.*, 2000), and although recognized elsewhere genetic testing is not available in the UK. The phenotype is usually an early-onset spastic





**Figure 3** Western blot of Case 15. Patient had adolescent onset ataxia with an eye movement disorder, sensorimotor neuropathy, *pes cavus*, amyotrophy and raised alpha fetoprotein. Lane 3 shows Case 15, with a normal-sized senataxin band. The blot was reported to be normal and the patient highly unlikely to have ataxia with oculomotor apraxia type 2, although in retrospect the band may be of reduced intensity. Two mutations in *SETX* were detected using the ataxia NGS panel: F1756S and 7100+2 T>C (splice mutation). Also see Supplementary Fig. 2.

ataxia with a sensorimotor neuropathy, although some cases present later.

Results of analysis in two of our cases with ataxia plus mental retardation were particularly surprising. Case 45, described as having ataxic cerebral palsy, had mild learning difficulties, a non-progressive ataxia and no family history. A novel *de novo* variant, T428I, in *KCNC3* was identified. Mutations in *KCNC3* cause spinocerebellar ataxia 13, an autosomal dominant ataxic disorder (Waters *et al.*, 2006; Figueroa *et al.*, 2010; Minassian *et al.*, 2012). To our knowledge *de novo* variants have not been associated with spinocerebellar ataxia 13 or indeed any ataxic disorders and illustrates the value of high throughput screening as this gene would not have been analysed in any other way. In Case 16 a homozygous stop codon was found in *SPTBN2*. Heterozygous mutations in *SPTBN2* are known to cause spinocerebellar ataxia 5 (Ikeda *et al.*, 2006), but recessive mutations in this gene had not previously been described. Our discovery prompted extensive research analysis that resulted in the description of a novel recessive ataxia disorder (spectrin associated autosomal recessive ataxia type 1, SPARCA1) associated with mental retardation (Lise *et al.*, 2012).

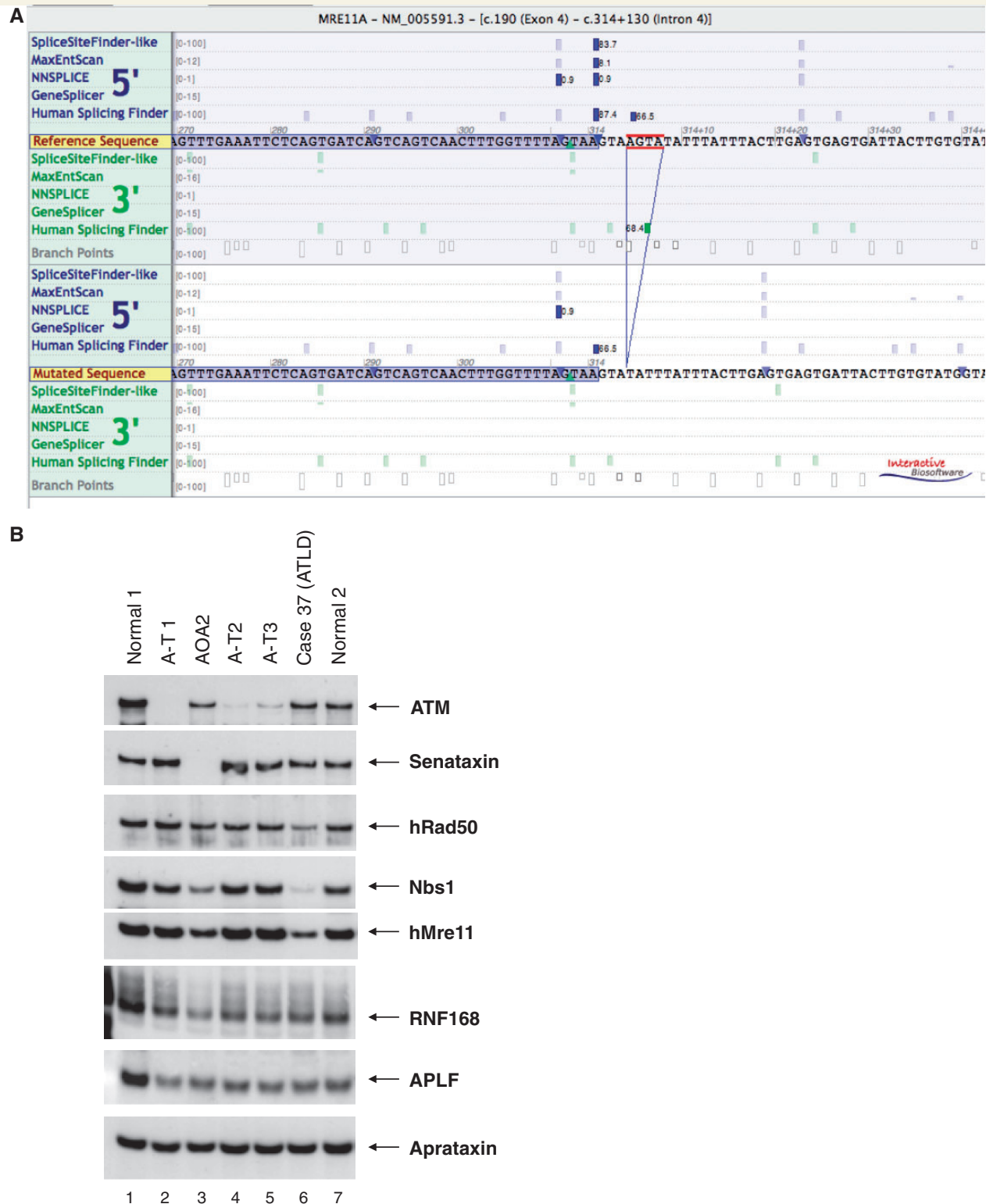
The cohort also included cases of non-specific late adolescent or adult onset ataxia without other neurological features and in two

cases we identified mutations. In Case 3 a mutation was found in *TTBK2* associated with spinocerebellar ataxia 11. The mutation is the same as that found in a family from South West England, suggesting that our case may link with this family (Houlden *et al.*, 2007). In Case 5 a novel mutation, H36R, in *PRKCG*, causing SCA14 (Chen *et al.*, 2003; Yabe *et al.*, 2003) was found. Bioinformatics analysis predicted that this missense mutation was pathogenic, but as it had not previously been described and family members were not initially available for segregation analysis, we performed functional studies which confirmed its pathogenicity (Fig. 2).

## Discussion

We used next-generation sequencing as a tool to investigate heterogeneous patients with ataxia without a molecular diagnosis in order to determine the feasibility of implementing this technology for diagnostic services and research. Our results have shown that even in a highly heterogeneous cohort of challenging patients likely to be representative of cases referred to Molecular Diagnostics laboratories, we were able to make a diagnosis in a significant number of patients. The most significant obstacle we identified was interpretation of pathogenic variants. In seven of nine cases we found at least one novel variant, which was predicted to be pathogenic based on our bioinformatics analysis. To confirm that these variants were pathogenic we performed extensive functional analysis in three cases and demonstrated that they had a pathogenic effect as expected, providing confidence in the use of bioinformatics programs and our pathogenicity assessment pipeline and suggesting that a diagnostic service laboratory would be able to interpret many variants without access to complex experimental procedures. These results have led us to set up genetic testing for ataxias within our own Molecular Diagnostic Laboratory and the experience we have obtained can be applied to many other neurological disorders in which there is phenotypic and genotypic heterogeneity. The total costs including analysis and reporting times will compare very favourably with the costs of single gene tests: currently a single test using Sanger sequencing is ~£700 and the Ataxia NGS panel of >50 genes is ~£1000; recommended reporting times for Sanger sequencing are currently 8 weeks compared with 16 weeks for NGS panels but this is likely to decrease as additional experience is gained with the new technology (document from the Clinical Molecular Genetics Society Professional Guidelines for Reporting Times, 26 October 2012).

Although the numbers are too small to draw major statistical conclusions our results represent a notable increase in diagnostic success rate and illustrate the potential for NGS to make a more rapid diagnosis, which has implications for health service economics and patient satisfaction. In the vast majority of the cases, the diagnosis was not made because of lack of available testing combined with unusual phenotypes (SACS), mechanisms (i.e. *de novo* mutation in *KCNC3*) or knowledge of the syndrome (*SPTBN2*). In some cases the phenotypes were too non-specific to select genes for testing (e.g. pure ataxia caused by mutations in *TTBK2* and *PRKCG*) and in other cases the phenotype was more



**Figure 4** (A) Splice predictions for *MRE11* c414 + 4\_314 + 7 show loss of donor site of exon 4 in four splice prediction programs [Alamut version 2.3 (Interactive Biosoftware, Rouen, France)]. (B) Retrospective western blot of Case 37 (Lane 6) showing a slight reduction of *MRE11* but a clearer reduction of the interacting proteins hRAD50 and Nbs1, which is consistent with a diagnosis of ataxia-telangiectasia-like disorder caused by mutations in *MRE11*. Ataxia NGS panel identified two mutations, N117S and a splice mutation 314 + 4\_314 + 7del. The normal size of *MRE11* protein in the patient lane suggests that N117S produces a normally sized but abnormally functioning product, also reported by Stewart *et al.* (1999).

recognizable but associated with mutations in several genes (e.g. ataxia-telangiectasia-like disorder with mutations in *SETX* and *MRE11*; ataxia and spasticity with mutations in *DARS2* and *SACS*). In one case the mutation was *de novo* and would be almost impossible to identify without NGS. Genetic testing using NGS is therefore an efficient and cost-effective alternative to other testing methods even in highly heterogeneous ataxia cases and therefore is likely to have significant clinical utility.

Even after NGS testing many patients still did not have a molecular diagnosis made and there may be several explanations for this. We analysed a phenotypically heterogeneous cohort, and some patients had other neurological features (e.g. spasticity, neuropathy, retinitis pigmentosa), which overlap with other diagnostic categories such as mitochondrial disorders, hereditary spastic paraparesis and neurodegenerative metabolic conditions. These conditions should always be considered in the differential of a patient with ataxia and appropriate investigations performed. However, the majority of patients in this cohort were extensively investigated before referral and most common genetic causes of these conditions had already been ruled out. One patient (Case 39), presenting with a pure cerebellar atrophy, was found to have a neuronal ceroid lipofuscinosis caused by mutations in a known gene using Sanger sequencing. A further patient, Case 43, presented with cerebellar atrophy, cognitive decline and retinitis pigmentosa and was found on exome sequencing to have an exceptionally rare neuronal ceroid lipofuscinosis caused by mutations in *CTSD* (Siintola *et al.*, 2006; Steinfeld *et al.*, 2006). Four other patients have been investigated with exome sequencing on a research basis and no mutations in known genes have been found. New ataxia genes have been identified since the original design and could have been missed; these have been added to the newer designs (Vermeer *et al.*, 2010; Corbett *et al.*, 2011; Doi *et al.*, 2011; Gulsuner *et al.*, 2011; Bourassa *et al.*, 2012; Guergueltcheva *et al.*, 2012; Onat *et al.*, 2012; Winkelmann *et al.*, 2012; Zanni *et al.*, 2012), however, the mitochondrial genome was not included in the capture. The reasons for this include its size, services being offered elsewhere, and difficulties with analysis, although recent new Bioinformatics tools for analysing the mitochondrial genome from exome data are beginning to address this latter issue (Picardi and Pesole, 2012; Guo *et al.*, 2013). Mutations may be missed because GC-rich regions are inefficiently captured by all current techniques and there may be regions of low coverage due to low complexity or inefficient design. Our metrics indicated generally efficient capture and good depth of coverage for target regions. Nevertheless, newer capture techniques have been introduced and will improve these metrics further. Our bioinformatics pipeline used stringent criteria to classify sequence variants as pathogenic, and the functional data support the accuracy of these data (few false positive mutations as shown by confirmation using functional assays). However, the false negative rate is unknown and it is possible that some truly pathogenic mutations were misclassified as benign. Additional work is in progress to analyse the negative cases using exome sequencing and copy number variation analysis. Another possibility is that all sequencing technologies have very limited ability to detect copy number variants, such as larger deletions and insertions. Mutations in *MARS2* (Bayat *et al.*, 2012) and *CAMT1* (Thevenon *et al.*, 2012) have recently been reported in ataxia, and in both

cases high density single nucleotide polymorphism arrays or array comparative hybridization were required. There are many new bioinformatic programs attempting to address these issues, but at present, in both clinical practice or research, single nucleotide polymorphism arrays or array comparative hybridization should be performed if no diagnosis is made using sequencing. Many groups are evaluating exome sequencing for diagnostics as the number of genes investigated is much larger. However, although exome capture appears an attractive option, the large number of variants identified (in excess of 20 000/subject) may require sequencing of additional family members for filtering which will increase the cost and expand the amount of experimental analysis necessary to confirm pathogenicity (Qi *et al.*, 2012; Wooderchak-Donahue *et al.*, 2012). In addition, exome capture suffers from highly variable coverage, cannot be optimized for specific genes of interest, does not include introns, which are likely to be a rich source of disease-associated variation and as noted above present difficulties with analysis of the mitochondrial genome (Picardi and Pesole, 2012; Guo *et al.*, 2013). In the future whole genome sequencing, which avoids any capture phase, has more even coverage and is better suited for copy number variation detection, is likely to supersede any form of sequencing with capture (targeted or exome). It is also possible that 'third generation' sequencing technology which has the potential to detect trinucleotide repeat expansions will offer a more comprehensive solution for molecular diagnosis (Loomis *et al.*, 2013).

Meanwhile, if the proportion of mutations in known genes is applicable to our entire group then up to 50% of the cases might have mutations in known genes that are amenable to targeted screening. This approach represents a very cost-effective and efficient method that can be applied using new, rapid sequencing technology (MiSeq or Ion Torrent, for example) and offer a diagnosis within a few days. However, very importantly, it is highly likely that novel ataxia genes have not yet been discovered and will continue to be identified on a research basis, either by exome/genome sequencing or other technologies. This work should provide the impetus to continue the search for these unknown genes.

In summary, we illustrate the success and challenges of using NGS in one complex group of neurological disorders and suggest that our experience is likely to be applicable to many other conditions such as peripheral neuropathy, spasticity, dementia, parkinsonism and dystonia.

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## Supplementary material

Supplementary material is available at *Brain* online.

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