Functional mRNA analysis reveals aberrant splicing caused by novel intronic mutation in *WDR45* in NBIA patient

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ABSTRACT

WDR45 gene-associated Neurodegeneration with Brain Iron Accumulation (NBIA), referred to as beta-propeller protein-associated neurodegeneration (BPAN), is a rare disorder that presents with a very non-specific clinical phenotype in children constituting global developmental delay. This case report illustrates the power of a combination of trio exome sequencing, *in silico* splicing analysis and mRNA analysis to provide sufficient evidence for pathogenicity of a relatively intronic variant in *WDR45*, and in so doing, find a genetic diagnosis for a 6-year old patient with developmental delay and seizures, a diagnosis which may otherwise have only been found once the characteristic MRI patterns of the disease became more obvious in young adulthood.

INTRODUCTION

Mutations in the *WDR45* gene are associated with an X-linked dominant form of Neurodegeneration with Brain Iron Accumulation (NBIA) [Haack et al, 2012], referred to as beta-propeller protein-associated neurodegeneration (BPAN). The term NBIA, encompasses several single-gene disorders characterized by abnormal iron deposition in the basal ganglia [Gregory et al 2017].

To date, there are 10 genes established to be associated with NBIA, the three most common forms being caused by mutations in the *PANK2* (OMIM 606157), *C19orf12* (OMIM 614297), and *PLA2G6* (OMIM 603604) genes, which encode mitochondria-associated proteins with no clear link to iron homeostasis. The X-linked *WDR45* gene encodes a beta-propeller scaffold protein with a putative role in autophagy [Carvill et al 2017]. Eight of the ten genetically defined types of NBIA are inherited in an autosomal recessive manner. Based on observed

cases in the population, the prevalence of NBIA is estimated at 1-3/1,000,000 [Gregory et al 2005] of which BPAN constitutes only one to two percent [Horvath 2013].

Typical clinical features of BPAN include early-onset global developmental delay remaining essentially static until young adulthood when there is further neurological deterioration (Parkinsonism, dystonia, and dementia). Seizures and sleep disorders are also a relatively common feature. During the early years, the clinical picture is very non-specific making diagnosis difficult [Gregory et al 2017].

A characteristic pattern observed on MRI in BPAN patients, thought not to become apparent until the further neurological deterioration of young adulthood has occurred, is T1 hyperintensity surrounding a central linear region of signal hypointensity within the substantia nigra and cerebral peduncles. Cerebral and cerebellar atrophy are also observed.

The majority of mutations reported in *WDR45* to-date are truncating mutations predicted to result in a loss of function. Mutations at the canonical splice site positions of several *WDR45* exons have also previously been reported. Based on the positions of the mutations reported to date, there is no apparent mutational hotspot.

Here we describe how a genetic diagnosis of BPAN was reached in a 6-year old child with a typical non-specific phenotype, via the use of trio exome sequencing, *in silico* splicing analysis and functional mRNA characterisation demonstrating aberrant splicing as the result of a novel, relatively intronic variant in *WDR45*.

CLINICAL REPORT

The proband is the second child of healthy non-consanguineous, White European parents with no significant family history of note. The pregnancy was uncomplicated with normal

antenatal scans. She was born at term with a birth weight of 4130 g and was in a good condition immediately after birth. There were no concerns immediately after birth but she was noted to have developmental delay in the first year of life.

At one year of age, she was only sitting unsupported, was not able to stand without support and just about being able to transfer objects. She also developed seizures from 4-months of age, initially managed conservatively but requiring medical management soon thereafter.

This patient was initially referred to the Paediatric Neurology clinic at 10-months of age with developmental delay and seizures. She was evaluated and found to have no focal neurological abnormality and no evidence of regression. An MRI-brain scan at 1-year of age demonstrated abnormal high signal in white matter around dentate nuclei bilaterally with overall decreased white matter bulk with ex-vacuo ventricular dilatation. Investigations at the time included extensive Neurometabolic screen including testing for leukodystrophy and neurodegenerative conditions (VLCFA profile, urine organic and plasma amino acids, purine and pyrimidine assays and isoelectric focussing of transferrins) which were negative. She went on to develop epileptic spasms around 18-months of age and EEG at the time showed documented evidence of hypsarrhythmia. She was diagnosed with epilepsy at 2-years of age and initially trialled with Vigabatrin and steroids; and a trial of pyridoxine all with only a partial response. She was subsequently treated with Topiramate with good response. However, she went on to have intermittent clusters of epileptic spasms and commenced on ketogenic diet therapy with better response. She also developed astigmatism and myopia needing glasses. In terms of her general health, she developed recurrent urinary tract infections needing prophylactic antibiotics and pyelonephritis needing stenting at 3-years of age.

This patient was initially seen in the Genetics clinic at a year of age, when she was noted to have metopic sutural craniosynostosis, deep-set eyes, a small mouth with thin upper lip and

broad forehead with bilateral low-set ears (Figure 1a-b). Her growth parameters included head circumference~47.5 cms (75^{th}-91^{st} centile), weight and height~98^{th}-99^{th} centile. Genetic investigations at the time included arrayCGH, mitochondrial testing, *MECP2* and targeted epilepsy and craniosynostosis panel, which were all negative. She was subsequently reviewed again at 3-years of age and noted to have similar findings as before (Figure 1c-d) with some improvement in her development. She was recruited to the Deciphering Developmental Disorders (DDD) Project for trio exome analyses which identified a *WDR45* intronic variant.

Further to this result the patient had MRI-brain scan performed at 4-years of age which showed mildly reduced T2 signal in bilateral globus pallidus on SWI and persistent white matter signal changes (Figure 2a-b). Repeat MRI at 5-years of age (Figure 2c-d) demonstrated mineralisation within the dentate nuclei, globus pallidus and substantia nigra, possibly in keeping with NBIA.

Her most recent clinical examination at 6-years of age demonstrated a very sociable young girl with good eye contact, developmental delay with moderate intellectual disability (needing special educational support). She was able to stand with support but had difficulty with her balance; she was crawling but unable to walk with support. She was using a wheelchair, remained non-verbal but had a very happy, friendly personality. She was wearing glasses for astigmatism and myopia but her hearing was reported to be normal. She continued to have multi-focal frequent myoclonic seizures needing treatment with multiple anti-epileptic medications including Lamotrigine, Topiramate and more recently started on trial of sodium valproate in addition to the ketogenic diet to attain seizure-control. Her growth parameters included head circumference~52 cms (50^{th}-75^{th} centile), weight~24.2 kg (98^{th} centile) and height~111.5cms (50^{th}-75^{th} centile).

MATERIALS AND METHODS

This patient was recruited to the Deciphering Developmental Disorders (DDD) study, trio-based exome sequencing was performed on the affected individual and their parents, as previously described [Wright et al., 2014]. Each affected individual has also had a high-resolution analysis for copy number abnormalities using array-based comparative genomic hybridization (aCGH). Putative *de novo* mutations were identified from exome data using DeNovoGear software [Ramu et al., 2013] and were validated using targeted Sanger sequencing.

In silico splicing analysis: the following *in silico* tools were utilised, accessed via Alamut Visual v2.9. SpliceSiteFinder-like (http://www.umd.be/HSF/) , MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), GeneSplicer (http://www.cs.jhu.edu/~genomics/GeneSplicer/) , NNSPLICE (https://omictools.com/nnsplice-tool), Human Splicing Finder (http://www.umd.be/HSF3/).

mRNA analysis: Epidermal fibroblasts were received as a confluent monolayer growth in a T25 flask. Cells were washed once using PBS then detached using a Trypsin-EDTA solution and centrifugation at 1500 RPM for 5 minutes. Cells were washed again once in PBS, and RNA extracted from half of the total volume using the RNeasy Mini Kit (Qiagen, Hilden, Germany) on the Qiacube® (Qiagen) as according to the manufacturer's instructions. cDNA was synthesised from 25µl of total RNA using the High Capacity cDNA RT kit (Thermo Fisher Scientific Waltham, MA) and random oligo priming in a total volume of 50 µL (10× Random Primers 5.0 µL, 10× Buffer 5.0 µL, dNTPs (100 mm) 2.0 µL, Multiscribe Reverse Transcriptase 2.5 µL, RNase Inhibitor 2.5 µL, RNA 25 µL, RNase Free Water 8.0µl). All PCR was conducted using Phusion® DNA polymerase (New England Biolabs, Ipswich, MA) as according to the manufacturer's instructions, with an annealing temperature of 61°C and

15s extension time. PCR primers were designed using Primer-BLAST. Primers were designed to amplify a region spanning exon 3 to exon 8: Primer sequences for PCR and for sequencing were as follows:

WRD45 fwd: 5′ AACAGCCACTTCGAGGAGTG 3′

WDR45 rev: 5′ GGAACACTAGCAGTTGCTTC 3′

Products were separated by 3% agarose gel electrophoresis (50% Nusieve® (Lonza, Basel, Switzerland): 50% Agarose MP (Roche, Basel, Switzerland)) using 1× TBE buffer and sized using a 50 bp GeneRuler ladder (Thermo Fisher Scientific).

Amplicons were extracted from agarose gels using a Monarch® DNA Gel Extraction Kit (New England Biolabs) as according to the manufacturer's instructions. Products were sequenced using a BigDye® Terminator kit v3.1 (Applied Biosystems) and analysed using an ABI 3730 DNA sequencer (Applied Biosystems). ABI sequence scanner v1.0 was used for electropherogram analysis.

RESULTS

DNA analysis: Trio exome sequencing through the DDD Project identified the *de novo* heterozygous ChrX(GRCh37):g.48934430T>C, c.236-18A>G variant in intron 5 of the *WDR45* gene (variant nomenclature according to Human Genome Variation Society guidelines with reference transcript NM_007075.3). This particular variant has not been reported previously in the literature.

In silico splicing analysis: *in silico* tools (see methods section) indicated this variant may interfere with normal splicing at the exon 6 splice acceptor site. Although the NNSPLICE tool failed to offer a prediction, the other 4 tools all indicated activation of a cryptic exon 6

acceptor splice site at c.236-17 with abolition of the natural acceptor splice site at c.236. This variant was confirmed by Sanger sequencing on a genomic DNA sample from the proband, and Sanger sequencing on parental samples confirmed the *de novo* status of this variant. This variant was completely absent from population databases (gnomAD, 1000 genomes project). Given the variant's *de novo* status, the *in silico* prediction, the absence from population databases and the phenotype and MRI-imaging being consistent with the genotype, we undertook mRNA functional analysis on a blood sample and cultured fibroblasts in search of further evidence of an effect on splicing.

mRNA Analysis: After PCR across exons 3 to 8, gel electrophoresis revealed two major bands from both patient and wild-type control samples (See Figure 3.). All samples showed a band corresponding to an amplicon of approx. 500bp in size, consistent with that expected from the wild-type (WT) transcript. Sanger sequencing of this band from the K562 control (Figure 3; lane 6) was consistent with the *WDR45* reference transcript NM_001029896.1, which lacks 3 nucleotides at the beginning of exon 6 relative to the NM_007075.3 transcript. The corresponding band from the affected patient (Figure 3; lane 1) appeared fainter and less discreet than that of the WT controls and sequencing did not produce a reliably interpretable result, other than to determine that it was *WDR45*-derived.

A secondary band corresponding to an amplicon of approx. 400bp in size could be seen in the PCR product generated from the affected patient (Figure 3; lane 1). Sanger sequencing of this band revealed an aberrant transcript exhibiting exon 5 (75bp) skipping with the incorporation of 17bp of intron 5 (Figure 4). A secondary band corresponding to an amplicon of approx. 375 bp could also be seen in the PCR product generated from the WT controls (Figure 3; lanes 2-6). Sanger sequencing of the secondary band from the K562 control showed it to comprise a transcript exhibiting exon 5 skipping and lacking the 3 nucleotides at the beginning of exon 6 as does the NM_001029896.1 reference transcript, but not containing the

17bp of intron 5 seen in the aberrant transcript from the patient sample. This indicates that the skipping of exon 5 likely represents a normal variant transcript not related to the effect of the c.236-18A>G mutation. Sanger sequencing of total PCR product from the patient sample was also carried out and results showed the sequence corresponding to the aberrant transcript to be by far the predominant PCR product present.

The presence of this aberrant transcript may indicate that it does not undergo nonsense-mediated decay, resulting in the production of a truncated protein product, however further analysis to demonstrate the presence of a truncated protein would be required to establish this. Other investigators have demonstrated, from cells of patients with *WDR45* truncating mutations, no evidence of truncated protein product, suggesting the mutant proteins are structurally unstable and undergoes degradation [Saitsu et al 2013].

DISCUSSION

For changes at canonical splice site positions (±2 bp from the ends of exons), it is generally safe to assume a deleterious effect unless there is evidence to the contrary, but variants that lie deeper within an intron are much less likely to have a significant impact on splicing and so require further evidence to support a conclusion of pathogenicity, and for a clinical diagnosis *in silico* predictions cannot be relied upon alone.

The c.236-18A>G variant, although relatively intronic, was close enough to the exon-intron boundary to be picked up via the particular exome sequencing protocol used by the DDD Project. However, the extent of intronic sequence coverage achieved for any given gene by exome sequencing is variable and dependent on the protocol used, amongst other factors.

Cases such as this serve as a reminder of the potential importance of this coverage, particularly the context of recent research [Vaz-Drago et al 2017] and illustrate the utility of *in silico* splicing analysis to help identify variants with a potential to disrupt splicing.

Though end-point PCR was used for the cDNA analysis, which does not provide an accurate indication of relative transcript abundance, our results do suggest the possibility of skewing of X-inactivation in favour of the mutant allele. Other investigators have shown similar results suggestive of skewed X-inactivation [Saitsu et al 2013] suggesting this may form an inherent aspect of the disease mechanism. The number of female patients reported to date significantly outweighs that of males but the phenotype is similar between the sexes. Haack et al. (2012) have previously concluded that males with *WDR45* mutations must be somatic mosaic, which was demonstrated in 1 affected male. It is thought therefore that males with germline *WDR45* mutations may be nonviable. However, there are now several reports of surviving males with germline WDR45 variants [Takano et al 2017; Redon et al 2017].

This case also serves as another example of the power of trio exome sequencing to find a relatively early diagnosis for a patient with very non-specific clinical features, however it also demonstrates that for some variants further work is required in order to provide sufficient evidence for pathogenicity beyond *de novo* status.

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COMPETING INTERESTS

No competing interest to declare.

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FIGURE LEGENDS

Figure 1a-b: Photographs of patient (frontal and profile) at 1 year of age demonstrating metopic synostosis, flat nasal bridge with a pointed chin, broad forehead and bilateral low-set ears.

Figure 1c-d: Photographs of patient at age 3 demonstrating evolution of facial dysmorphism of age.

Figure 2a-d:

2a and b: Selected 3T MRI-brain images done at 4-years of age demonstrating low signal intensity on susceptibility weighted imaging. Image involving substantia nigra and lentiform nucleus.

2c and d: 3T MRI done at 5-years of age showing progressive increase in the low signal intensity on susceptibility weighted imaging involving substantia nigra and lentiform nucleus secondary to iron deposition.

Figure 3: 3% agarose gel displaying *WDR45* cDNA exon 3-8 PCR products. Lane 1; affected c.[236-18A>G] patient epidermal fibroblast derived cDNA, Lane 2; Wildtype (WT) patient control 1 cDNA derived from epidermal fibroblasts, Lane 3; WT patient control 2 cDNA derived from EDTA peripheral blood, Lane 4; WT patient control 1 cDNA derived from PAX gene peripheral blood, Lane 5; WT patient control 2 cDNA derived from PAX

gene peripheral blood, Lane 6; K562 cell line derived cDNA control, Lane 7; negative control H_20.

Figure 4: Schematic to illustrate the effect of the *WDR45* c.[236-18A>G] variant on mRNA transcription. A; genomic sequence (exons represented by coloured boxes , successive introns represented by lines), B; aberrantly spliced mRNA transcript displaying exon 5 skipping and incorporation of 17bp of intron 5, C; Electropherogram trace obtained from the Sanger sequencing of mRNA transcript B.