Adults with *RRM2B*-related mitochondrial disease have distinct clinical and molecular characteristics

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ABSTRACT

Mutations in the nuclear-encoded mitochondrial maintenance gene RRM2B are an important cause of familial mitochondrial disease in both adults and children and represent the third most common cause of multiple mitochondrial DNA (mtDNA) deletions in adults, following POLG (pol γ) and PEO1 (also called C10orf2, encoding the Twinkle helicase) mutations. However, the clinico-pathological and molecular features of adults with RRM2B-related disease have not been clearly defined. In this multicentre study of 26 adult patients from 22 independent families, including 5 additional cases published in the literature, we show that extra-ocular neurological complications are common in adults with genetically confirmed RRM2B mutations. We also demonstrate a clear correlation between the clinical phenotype and the underlying genetic defect. Myopathy was a prominent manifestation, followed by bulbar dysfunction and fatigue. Sensorineural hearing loss and gastrointestinal disturbance were also important findings. Severe multisystem neurological disease was associated with recessively-inherited, compound heterozygous mutations with a mean age of disease onset at 7 years. Dominantly-inherited heterozygous mutations were associated with a milder, predominantly myopathic phenotype with a later mean age of disease onset at 46 years. Skeletal muscle biopsies revealed subsarcolemmal accumulation of mitochondria and/or cytochrome c oxidase deficient fibres. Multiple mtDNA deletions were universally present in patients who underwent a muscle biopsy. We identified a total of 18 different heterozygous RRM2B mutations within our cohort of patients, including five novel mutations that have not previously been reported. Despite marked clinical overlap between the mitochondrial maintenance genes, key clinical features such as bulbar dysfunction, hearing loss and gastrointestinal disturbance should help prioritise genetic testing towards RRM2B analysis and sequencing of the gene may preclude performance of a muscle biopsy.
Key Words: Mitochondrial DNA; mtDNA maintenance; mtDNA depletion; multiple mtDNA deletions; RRM2B

Abbreviations: C10orf2 = Chromosome 10 open reading frame 2; PEO1 = Progressive external ophthalmoplegia 1 protein; POLG = Polymerase (DNA directed), gamma; POLG2 = Polymerase (DNA directed), gamma 2, accessory subunit; RRM2B = Ribonucleotide reductase subunit M2 B (TP53 inducible)
INTRODUCTION

Approximately half of all adult mitochondrial disease results from genetic dysfunction of the nuclear-encoded mitochondrial genes. A significant number of these cases are due to disruption of genes involved in maintenance (replication and repair) of mitochondrial DNA (mtDNA), leading to qualitative (accumulation of multiple mtDNA deletions) and/or quantitative (depletion of mtDNA copy number) downstream mitochondrial genomic effects. To date, almost all known nuclear maintenance genes fall into one of two categories: 1) genes whose protein products function directly at the mtDNA replication fork (Fratter et al., 2009, Spelbrink et al., 2001, Longley et al., 2006), or 2) genes which encode proteins involved in supplying mitochondria with deoxyribonucleoside triphosphate (dNTP) pools required for DNA replication (Fratter et al., 2011, Bourdon et al., 2007, Nishino et al., 1999, Kaukonen et al., 2000, Mandel et al., 2001, Saada et al., 2001, Ostergaard et al., 2007). The most common presenting neurological feature seen in adults with mitochondrial DNA maintenance disorders is progressive external ophthalmoplegia (PEO) and ptosis. Of the twelve known maintenance genes, eight have been associated with PEO, although the clinical phenotype is not necessarily restricted to the extra-ocular muscles.

Ribonucleotide reductase M2B (TP53 inducible), RRM2B (MIM 604712), encodes the p53-inducible small subunit (p53R2) of ribonucleotide reductase (RNR), a heterotetrameric enzyme that catalyses de novo syntheses of dNTPs by direct reduction of ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates. This process supplements the dNTPs produced by the mitochondrion’s own dNTP salvage pathway, defects of which cause many of the mtDNA depletion syndromes (Rahman and Poulton, 2009), and is essential for mtDNA synthesis. Transcription of RRM2B is tightly regulated by the tumour suppressor protein p53. Mutations in the gene have been associated with both recessively- and dominantly-inherited mitochondrial disease. Spliced variant transcripts have also been reported (Spinazzola et al., 2009).

The first reported human diseases linked to RRM2B dysfunction were associated with mtDNA depletion (Bourdon et al., 2007, Spinazzola et al., 2009, Bornstein et al., 2008, Kolberg et al., 2009, Acham-Roschitz et al., 2009) and caused severe, early-onset, multisystem disease with infant mortality. RRM2B mutations have since been recognised to cause adult-onset syndromes. This was first demonstrated in a patient with mitochondrial...
neurogastrointestinal encephalopathy (MNGIE) and mtDNA depletion (Shaibani et al., 2009). Tyynismaa et al. later published a large autosomal dominant (ad) progressive external ophthalmoplegia (PEO) family with multiple mtDNA deletions whose disease was not explained by mutations in any of the known adPEO genes (Tyynismaa et al., 2009). The disease locus in the family was mapped using genome-wide linkage and found to contain RRM2B, and a heterozygous nonsense mutation in exon 9 was detected and demonstrated in another unrelated family. The mutant mRNA was shown to escape nonsense mediated decay and resulted in a truncated protein that was postulated to cause a dominant-negative or gain-of-function effect on the heterotetrameric structure of the RNR enzyme. The mechanistic importance of truncating exon 9 mutations was further expanded by Fratter et al. where three further novel pathogenic variants in exon 9 were shown to cause familial adPEO in seven unrelated probands. In addition, clinically more severe, recessively-inherited, compound heterozygous mutations were demonstrated (Fratter et al., 2011). Kearns-Sayre syndrome (KSS) resulting from compound heterozygous RRM2B mutations with secondary multiple mtDNA deletions has also been reported, thus confirming the more severe nature of recessively-inherited adult RRM2B mutations, and expanding the clinical phenotype associated with RRM2B-related mitochondrial disease beyond that of PEO and PEO plus syndromes (Pitceathly et al., 2011).

Despite emerging evidence that RRM2B mutations are the third most common cause of multiple mtDNA deletions in adults, following POLG and PEO1 (Fratter et al., 2011, Pitceathly et al., 2011), and RRM2B dysfunction being an important cause of neurological disease, the adult phenotypic and molecular spectrum is not fully understood. We therefore conducted a systematic clinical and molecular study of adult patients with RRM2B mutation(s) to define the phenotypic spectrum of adult RRM2B-related mitochondrial disease and establish any genotype-phenotype correlations.
METHODS

Patient cohorts

Newcastle-Oxford-Halle cohort
We selected a cohort of adult patients who had been referred to the NHS Specialised Services-funded Mitochondrial Diagnostic Centres in Newcastle and Oxford, or the Mitochondrial Centre in Halle, Germany, for investigation of suspected mitochondrial disease, due to their clinical presentation, evidence of a mitochondrial biochemical defect and/or presence of mtDNA deletions. RRM2B sequencing was performed when initial screens for POLG, POLG2, PEO1 and SLC25A4 were found to be negative.

The subject’s consent was obtained according to the Declaration of Helsinki and institutional ethical committee approval was obtained in each centre in which the work was performed.

Additional published adult cases
We conducted a systematic review of the literature to identify all previous published adult cases with confirmed RRM2B mutations. These were included in our analysis to determine the complete clinical spectrum and genotype-phenotype correlates seen in adult RRM2B-related mitochondrial disease (Shaibani, et al., 2009; Tyynismaa et al., 2009, Fratter et al., 2011, Pitceathly et al., 2011, Takata et al., 2011).

Phenotypic evaluation
To define the extent and severity of their mitochondrial disease, a neurologist at each centre comprehensively assessed each patient harbouring pathogenic RRM2B mutations and/or examined their medical records.

Mitochondrial histochemistry
Skeletal muscle biopsies were collected from 21 patients with pathogenic RRM2B mutations. Cryostat sections (10 µm) were cut from transversely orientated muscle blocks and subjected to cytochrome c oxidase (COX), succinate dehydrogenase (SDH), and sequential COX-SDH histochemical staining to assess the numbers of COX-deficient fibres as a measure of respiratory chain deficiency, as previously described (Taylor et al., 2004). The SDH reaction was used to ascertain the number of fibres exhibiting increased levels of enzyme activity in
the subsarcolemmal region, namely “ragged-blue” fibres, in addition to data obtained from the histological modified Gomori trichrome stain.

**Mitochondrial DNA analysis**

Where available, total muscle DNA was extracted by standard techniques and screened for mtDNA rearrangements by Southern blotting and/or long-range PCR protocols, as described previously (Murphy et al., 2008). The presence of clonally-expanded mtDNA deletions in individual COX-positive and COX-deficient fibres were further screened in muscle biopsies from nine patients with either dominantly-inherited or recessively-inherited RRM2B mutations (Patients 1.1, 3, 5, 9, 10, 14, 19 and 20), by previously-published protocols, using primers and Taqman probes to assess the simultaneous amplification of the MTND1 (rarely deleted) and MTND4 (often deleted) genes (He et al., 2002, Krishnan et al., 2007). Absence of mtDNA depletion in muscle was confirmed in all cases by real-time quantitative PCR (qPCR) as described previously (Blakeley et al., 2008).

**Identification of pathogenic RRM2B mutations**

The coding exons and intron-exon boundaries of RRM2B (GenBank Accession number (NM_015713) were sequenced as previously described (Tyynismaa et al., 2009). RRM2B exon copy number (exons 1-8) was assessed by MLPA (MRC-Holland kit P089-A1) in all patients with dominantly-inherited, heterozygous, missense, RRM2B mutations. Total RNA from patients with the c.48G>A RRM2B variant (patients 1, 1.1 and 2) was extracted from EDTA-blood with the QIAamp RNA Blood Mini kit (Qiagen, Crawley, UK) and reverse transcribed using the Thermoscript RT-PCR system (Invitrogen, Paisley, UK). The resulting cDNA was PCR amplified across the RRM2B exon 1-2 boundary using exonic primers, leading to a normally spliced 303bp product. The PCR products were separated by agarose gel electrophoresis under standard conditions. Gel stabs of discreet bands were taken for secondary PCR using the same primers, which were M13-tagged for subsequent dideoxy Sanger sequencing utilising universal M13 primers, Big Dye terminator kit 3.1 (Applied Biosystems, Foster City, CA), and capillary electrophoresis on an ABI Prism 3730 Genetic Analyzer.
Modelling missense *RRM2B* mutations on the p53R2 crystal structure

The 2.6 Å X-ray crystal structure of human p53R2 (PDB: reference 3HF1) (Smith et al., 2009) was viewed in ViewerLite v.4.2 (Accelrys Inc.) and the amino acids known to have been substituted were isolated and saved as a separate pdb file. The p53R2 crystal structure and the isolated amino acids were then rendered using PyMOL v.0.97 (Warren L. DeLano "The PyMOL Molecular Graphics System" DeLano Scientific LLC, San Carlos, CA, USA. http://www.pymol.org).

RESULTS

Patient cohorts

Clinical data is presented on 31 patients (Table 1). Twenty six patients were identified via the NHS Specialised Services-funded Mitochondrial Diagnostic Centres in Newcastle and Oxford, or the Mitochondrial Centre in Halle, Germany (Newcastle-Oxford-Halle cohort), and are described here in greater clinical and molecular detail. Clinical information from five further patients was obtained from the literature review. One previously published case (Patient 12, Fratter et al., 2011) has been omitted from the current analysis as the pathogenic nature of the reported heterozygous mutation is uncertain following segregation studies.

Newcastle-Oxford-Halle cohort

The clinical and molecular features of all 26 patients from our Newcastle-Oxford-Halle cohort are summarised in Table 2. Molecular data from 11 of these subjects have been previously reported in brief (Fratter et al., 2011). The mean whole group age at onset was 40 years (age range: birth-70 years); patients with autosomal recessive PEO presented considerably earlier at 7 years (age range: birth-14 years), whilst those with adPEO presented later in the fifth decade of life (mean age of onset: 46 years; age range: 15-70 years).

Additional published adult cases

A systematic review of the literature to date (1st June, 2012) revealed 18 published *RRM2B* positive adult families, shown in supplemental table 1. These 33 individuals have been extensively genotyped and harbour *RRM2B* gene mutations characterized by either autosomal recessive mtDNA depletion syndrome (Shabani et al., 2009) or recessive and dominant mutations that cause the accumulation of multiple mtDNA deletions (Tyynismaa et al., 2009, Fratter et al., 2011, Pitceathly et al., 2011, Takata et al., 2011).
Major clinical features

Evaluation of the clinical features of all 31 adult patients harbouring pathogenic RRM2B mutations showed that PEO was universal and frequently associated with ptosis (28 patients). Neuromuscular features were common and included: proximal muscle weakness (16 patients); bulbar dysfunction (13 patients); and fatigue (11 patients). Additional neurological manifestations included ataxia (12 patients) and sensorineural hearing loss (SNHL) (11 patients). Non-neurological sequelae such as gastrointestinal disturbance, including irritable bowel syndrome (IBS)-like symptoms and low body mass index (BMI) (six patients), and endocrinopathy, specifically hypothyroidism, hypoparathyroidism, diabetes and hypogonadism (seven patients) were also important clinical findings. Malignancy was detected in two patients (patients 4 and 18).

Other less common problems included: cognitive impairment (four patients); cardiac dysfunction, including non-fatal arrhythmia and diastolic dysfunction (two patients); encephalopathy and stroke-like events (three patients); cataracts (two patients); migraine (two patients); renal disturbance (three patients); distal muscle weakness (one patient); neuropathy (three patients); pigmentary retinopathy (two patients); short stature (one patient); glaucoma (one patient); and depression (three patients).

Mitochondrial histochemical studies

Twenty-one patients with confirmed RRM2B mutations underwent a diagnostic muscle biopsy for the investigation of suspected mitochondrial disease. Patient 6.1 was diagnosed on the basis of RRM2B gene screening in a blood-derived DNA sample without performing a muscle biopsy. All showed histochemical evidence of a COX mosaic defect, with many exhibiting evidence of subsarcolemmal mitochondrial accumulation (ragged-red or ragged-blue fibres) following Gomori trichrome staining or SDH enzyme histochemistry. The severity of the associated COX mosaic defect varied widely, with a more pronounced histochemical defect associated with autosomal recessive RRM2B mutations than autosomal dominant mutations (Figure 1).

Mitochondrial DNA deletion analysis

All 21 patients who underwent muscle biopsy had multiple mtDNA deletions detectable either by long range PCR assays across the major mtDNA arc (Figure 2) or by Southern blot
analysis (undertaken in 11 of the 21 patients, with positive findings reported in all). A small number of patients (five patients with autosomal dominant missense or truncating RRM2B mutations, one patient with an autosomal dominant splicing defect and two patients with autosomal recessive RRM2B mutations) were also investigated by real-time PCR to further characterise the presence of clonally-expanded mtDNA deletions within single muscle fibres (Figure 2). In all cases, the majority of COX-deficient fibres revealed very high levels (>80% mutated mtDNA) of clonally-expanded mtDNA deletion involving the MTND4 gene region (He et al., 2002) whereas all COX-positive reacting fibres had levels of mtDNA deletion below this level. No difference in the distribution of mtDNA deletion levels was observed between different RRM2B mutation types.

Identification of pathogenic RRM2B mutations

We identified a total of 18 (12 dominantly- and six recessively-inherited) different RRM2B mutations within our cohort of patients, including five novel mutations that have not previously been reported (Figure 3). Thirteen mutations were missense variants predicted to alter conserved amino acids (Supplemental Figure 1), four were truncating mutations in exon 9, and one was a novel c.48G>A variant predicted to cause aberrant splicing.

Eleven patients (from 9 families) were found to have truncating mutations in exon 9 of RRM2B, three with the p.Leu317X mutation, one with a p.Glu318X mutation, five with a p.Asn322LysfsX4 frameshift mutation and two patients from one family (Patient 17 and her clinically-affected daughter, patient 17.1) with the p.Arg327X mutation which was the first mutation in this gene to be described as a cause of adPEO (Tyynismaa et al., 2010). All nine probands had affected family members and a family history consistent with a dominantly-inherited disorder (Table 2). Eight patients (from 7 families) harboured single, heterozygous RRM2B missense changes implying autosomal dominant inheritance. In all 7 families, MLPA was used to exclude exonic copy number variation in trans with further supporting evidence of pathogenicity provided by the fact that these changes affected conserved residues (E-Supplemental Figure 1). Four of these RRM2B mutations were unreported. Patient 4 harboured a novel p.Arg41Trp mutation and whilst there was no relevant family history to support dominant transmission, mutation of this particular amino acid (p.Arg41Gln) was reported in another patient (patient 5). Patient 12 had a novel p.Asp70Asn mutation, and a clinically-affected sister. Patient 18 had a novel mutation (p.Ala349Gly) in exon 9 which is a
recognized mutation hotspot (Fratter et al., 2011). The single heterozygous p.Ile224Ser mutation reported in patient 8 is of particular interest given this has previously been reported as a recessive \textit{RRM2B} mutation in a case of mtDNA depletion myopathy (Bornstein et al., 2008) and as such seems to be able to behave as either a recessive or dominant allele. Similarly, p.Arg41Gln and p.Arg211Lys function as dominant alleles in patients 3 and 7 respectively, but have also been identified in compound heterozygotes with more severe recessively-inherited disease (Pitceathly et al., 2011, and patient 21). Four patients harboured two recessively-inherited compound heterozygous \textit{RRM2B} mutations associated with an earlier-onset of disease and more pronounced COX histochemical defects (Figure 1 and Table 2). The p.Thr144Ile and p.Gly273Ser mutations were reported \textit{in trans} in two of the four patients, whilst the p.Arg186Gly and p.Thr218Ile mutation (patient 20) were both novel. Within this family, the p.Thr218Ile mutation was detected in the heterozygous state in the clinically-unaffected mother and a sister, whilst the second heterozygous mutation, p.Arg186Gly, was identified in another clinically-unaffected sister. Segregation studies were also performed in the family of patient 19; the p.Gly273Ser mutation was seen in the heterozygous state in the clinically-unaffected mother, whilst a clinically-unaffected sister was heterozygous for the p.Thr144Ile mutation alone. Paternal testing was not performed.

Finally, an unreported c.48G>A \textit{RRM2B} variant was identified in three patients (Patient 1, patient 1.1 and patient 2). This is not predicted to alter the amino acid sequence, resulting in a predicted p.Glu16Glu synonymous change. However, it was noted that the c.48G>A variant occurs at the last nucleotide of exon 1, and was therefore considered likely to result in aberrant splicing (Figure 4). Analysis of blood RNA extracted from patients 1, 1.1 and 2 across the exon 1-2 junction demonstrated that normally spliced RNA arises exclusively from the normal c.48G allele (Figure 4). Furthermore, the three patients had additional RNA species associated with the c.48G>A allele in which various portions of the 5’ region of intron 1 were retained resulting in the introduction of a termination codon 26 codons beyond exon 1. All three patients had RNA incorporating the first 268 nucleotides of intron 1. Patient 1.1 also had RNA with the first 518 nucleotides of intron 1, and patient 1 also had RNA with the first 467 and 518 nucleotides of intron 1 (Figure 4). Thus, these results demonstrate that c.48G>A abolishes normal splicing of exons 1 to 2, leading to partial intron retention and premature termination of translation, thereby confirming pathogenicity of c.48G>A.
DISCUSSION

RRM2B mutations are emerging as one of the leading causes of both paediatric and adult-onset mitochondrial disease associated with disruption of mtDNA maintenance. RRM2B mutations represent the third most common cause of Mendelian PEO and multiple mtDNA deletions in adults (13%), following mutations in POLG (27%) and PEO1 (14%), based on data from both Oxford and Newcastle centres. It is clear there is significant clinical overlap between the multiple mitochondrial maintenance genes. In our cohort, ophthalmoparesis was universal, usually severe, and often associated with ptosis (90% of patients). Other prominent myopathic features included proximal muscle weakness (52% of patients) and bulbar dysfunction (42% of patients). The latter manifested as dysarthria, dysphagia, dysphonia, facial weakness and neck weakness. SNHL (36% of patients) and gastrointestinal disturbance (19% of patients), including IBS-like symptoms and low BMI, were also relatively common findings. This is perhaps unsurprising given that they are prominent features in children with mtDNA depletion secondary to RRM2B mutations. The presence of significant bulbar weakness, hearing loss and gastrointestinal symptoms should guide clinicians towards RRM2B genetic analysis prior to POLG and PEO1, given that these key features are more common components of the clinical spectrum (bulbar dysfunction: 42% versus 37% and 12%; SNHL: 36% versus 11% and 9%; and gastrointestinal symptoms: 19% versus unreported and 9% in RRM2B versus POLG and PEO1-related PEO syndromes respectively) (Horvath et al., 2006, Fratter et al., 2011).

Although cerebellar ataxia was present in 39% of cases, other central features of mitochondrial disease were seen less frequently, such as cognitive impairment (13% of patients) and encephalopathy/stroke-like events (10% of patients). Cardiac complications were rare (10%) and non-fatal. Disturbance in renal function was present in three patients resulting from obstructive uropathy (two patients) and glomerulonephritis (one patient) with no reports of proximal renal tubulopathy, a common finding in children with RRM2B mutations and mtDNA depletion.

Two patients developed solid tumours (patient 4 oral carcinoma and patient 18 breast carcinoma). RRM2B is a gene not only involved in mtDNA replication but also plays a critical role in DNA damage repair. P53R2, a p53-inducible homologue of the R2 subunit of ribonucleotide reductase, has been evaluated in different cancer types, and is known to play a critical role in DNA damage repair and cancer cell proliferation (Zhang et al., 2011). These
data may suggest abnormalities in DNA repair; however, it is not possible to conclude if RRM2B mutations are oncogenic, due to small sample size and lack of mutational analysis of tumour tissue.

To further understand the functional consequence of the RRM2B mutations identified, we mapped the positions of the mutated amino acids on the tertiary p53R2 structure (Smith et al., 2009) (Figure 5). Many of the missense mutations identified appear likely to affect the iron-binding properties of p53R2, and hence impair the catalytic capability of the functional heterotetramer (two p53R2 subunits and two R1 subunits). Gly195, Phe202 and Ile224 are located around the iron-binding pocket. Whilst the effect of Phe202Leu may be orchestrated through subtle hydrophobic contacts, the effect of amino acids Gly195 and Ile224 is more blatant. Positioned adjacent to amino acids that contribute to the iron coordination environment, substitutions at these locations (p.Gly195Arg and p.Ile224Ser) will influence their amino acid neighbours and alter the coordination of the iron atom(s). Previous molecular modelling has indicated that p.Arg41Gln prevents formation of a salt bridge that is important in conformational changes that control iron binding (Pitceathly et al., 2011, Smith et al., 2009). p.Arg41Trp is also predicted to prevent formation of this salt bridge. Arg211 forms a salt bridge to Glu85 which is thought to be important in stabilisation of the di-iron form (Pitceathly et al., 2011, Smith et al., 2009), and so p.Arg211Lys may also destabilise the di-iron subunit.

Thr144, Arg186, Thr218 and Gly273 are all located at the end of, or between in the case of Gly273, α-helices and appear to stabilise the orientation of the helices. Mutation of these 4 amino acids may reduce protein folding efficiency, and is associated with autosomal recessive disease in our cohort. The effect of the p.Asp70Asn mutation cannot readily be predicted, as Asp70 lies in a poorly understood region of the protein between two helices. p.Ala349Gly could not be modelled since the crystal structure does not include the C-terminal portion of the protein. However, Ala349 is located within a conserved heptapeptide (amino acids 345-351) required for interaction with the R1 subunit (Tyynismaa et al., 2009), and loss of this heptapeptide has been proposed as the pathological basis of the exon 9 truncating mutations (Tyynismaa et al., 2009).
There was a clear relationship between phenotypic severity and genotype in the patients studied. Individuals harbouring recessively-inherited compound heterozygous RRM2B mutations (patients 19, 20, 21 and 22) presented at an earlier age (mean age of onset 7 years) with a more severe and multisystem disorder, whereas patients with single heterozygous mutations, inferring autosomal dominant transmission, had a later average age of disease onset (46 years), as is seen with PEO1 mutations (Horvath et al., 2006), and developed a predominantly myopathic phenotype consisting of PEO, ptosis, proximal muscle weakness and bulbar dysfunction with exceptions noted (patients 3 and 14). The distinction between recessively- and dominantly-inherited mutations was also evident on histochemical analysis of skeletal muscle tissue, in which COX deficient fibres were much more widespread in patients with recessively-inherited compound heterozygous mutations (Figure 1).

Clinical syndromes caused by dysfunction of the nuclear maintenance genes can be broadly classified into two groups: 1) mutations that cause mtDNA depletion, which are at the most severe end of the phenotypic spectrum; and 2) mutations that predispose to accumulation of multiple mtDNA deletions. The latter is further subdivided into: a) recessively-inherited disease, which presents during childhood with multisystem involvement; and b) dominantly-inherited disease, which is milder, typically develops in adulthood, and is often tissue-specific. A review of the literature suggests RRM2B generally conforms to these basic principles. The most severe form of RRM2B-related mitochondrial disease is associated with mtDNA depletion (Bourdon et al., 2007, Spinazzola et al., 2009, Bornstein et al., 2008, Kolberg et al., 2009, Acham-Roschitz et al., 2009). Clinical presentation occurs in the first 6 months of life with a multisystem disorder characterised by: muscle hypotonia and weakness; seizures; gastrointestinal dysmotility; respiratory insufficiency; hearing loss; lactic acidosis; renal tubulopathy; and early childhood mortality. The first reported adult-onset case was in a 30 year old woman with MNGIE who harboured compound heterozygous missense mutations in RRM2B and mtDNA depletion (Shaibani et al., 2009). RRM2B mutations were subsequently associated with multiple mtDNA deletions in two large unrelated families with autosomal dominant PEO and an identical heterozygous nonsense mutation that caused truncation of the translated p53R2 protein (Tyynismaa et al., 2009). Further reports have demonstrated RRM2B mutations with KSS and sporadic/familial PEO (Fratter et al., 2011, Pitceathly et al., 2011).

There are, however, notable exceptions to these rules: firstly, RRM2B-related mtDNA depletion can potentially cause a relatively mild clinical phenotype (Shaibani et al., 2009);
and secondly, identical RRM2B mutations are associated with a varied phenotypic severity depending on whether they exist in homozygous, compound heterozygous or heterozygous states. We attempt to explain the latter finding using molecular modelling of the RRM2B missense mutations identified in this study which suggests the variants can be broadly divided into two groups: mutations which severely impair RNR activity and cause autosomal dominant disease via a dominant negative effect; and mutations associated with autosomal recessive disease, which are predicted to result in moderately decreased catalytic activity or decreased levels of functional protein via reduced protein folding efficiency. This is likely a consequence of the heterotetrameric structure of RNR, which predisposes the enzyme to both a dominant negative effect (competitive binding and inactivation of the enzyme) or gain of function (competitive binding with altered function of the enzyme), alongside the loss of enzymatic activity that occurs with recessively-inherited disease. There is also evidence that impaired assembly of the multiprotein structures occurs with some mutations, and there may be a dosage effect, whereby wild-type p53R2 appears to partially compensate for the mutant allele, thus ameliorating the clinical phenotype when present in a heterozygous state (Pitceathly et al., 2011). This phenomenon is demonstrated in the present study by the p.Ile224Ser, c.48G>A, p.Arg41Gln and p.Arg211Lys variants. These 4 variants are associated with relatively late onset PEO (4th-8th decades) and multiple mtDNA deletions when present as the only heterozygous change, as in patients 1, 1.1, 2, 3, 7, and 8. However, when homozygous or compound heterozygous, these variants are associated with much more severe multisystem disease, either with mtDNA depletion in the case of p.Ile224Ser (homozygote reported by Bornstein et al., 2008) and c.48G>A (compound heterozygous with another pathogenic RRM2B mutation, Oxford Molecular Genetics Laboratory, unpublished data), or with multiple mtDNA deletions in the case of p.Arg41Gln and p.Arg211Lys (compound heterozygous patient reported by Pitceathly et al., 2009, and patient 19 in this study, respectively).

Our data support previous reports that RRM2B mutations can present with a PEO plus/KSS phenotype akin to single mtDNA deletion disorders (Pitceathly et al., 2011). Patients 19, 20, 21 and 22 all developed symptoms before the age of 20 years with PEO and other features suggestive of KSS such as hearing loss, cerebellar ataxia, and endocrine disturbance, although pigmentary retinopathy and heart block were absent. We would, therefore, recommend RRM2B over POLG and PEO1 genetic analysis in patients with PEO plus or KSS before a muscle biopsy is performed to exclude a single mtDNA deletion, if there is a
Mendelian pattern of inheritance. *RRM2B* analysis should also be considered in patients with MNGIE if blood/urinary deoxyuridine and thymidine levels are undetectable and thymidine phosphorylase activity is normal in white cells and platelets, due to the previous report of MNGIE secondary to compound heterozygous missense mutations in *RRM2B* (Shaibani *et al.*, 2009), case 20 reported here and because gastrointestinal symptoms were a prominent finding in our cohort of adult patients.

Finally, we report the novel c.48G>A *RRM2B* variant, and provide evidence to support its pathogenicity through aberrant splicing, partial intron retention, premature termination of translation, and hence predicted absence of any functional protein from this allele. We report 2 families (patients 1, 1.1 and 2) where heterozygosity for c.48G>A is associated with adult onset mitochondrial disease. Since all other *RRM2B* mutations reported to date in this disease group are either missense or exon 9 truncating, our data further expands the molecular heterogeneity of *RRM2B*-related adult mitochondrial disease.

**CONCLUSION**

Prioritising which nuclear-encoded mitochondrial maintenance genes to screen in adults with multiple mtDNA deletions is challenging. These data provide the physician with important clinical data regarding the phenotypic spectrum of *RRM2B*-related adult mitochondrial disease, and should help guide both genetic diagnosis and enable tailored counselling regarding potential disease progression. PEO, ptosis, and proximal muscle weakness, are well recognized as the predominant clinical features seen in adult patients with *POLG* and *PEO1* (Twinkle) mutations. Bulbar dysfunction, hearing loss and gastrointestinal problems, including IBS-like symptoms and low BMI, are also additional discriminatory features seen in *RRM2B*-related mitochondrial disease, and appear to occur more often than with the other individual nuclear-encoded mitochondrial maintenance genes. CNS involvement, characteristic of other syndromic presentations of mitochondrial disease, is present less frequently. Thus, the prominence of bulbar dysfunction, gastrointestinal problems and hearing loss, in the absence of conspicuous CNS features, would support early prioritisation of screening of *RRM2B* over *POLG* and *PEO1* in adults with PEO and muscle restricted multiple mtDNA deletions. Furthermore, *RRM2B* gene analysis should be considered early in KSS where there is evidence of multiple mtDNA deletions in skeletal muscle or when there appears to be a Mendelian pattern of inheritance. Finally, *RRM2B* screening should be
considered in patients with MNGIE when deoxyuridine and thymidine levels in both blood and urine are negative, and thymidine phosphorylase activity is normal in white cells and platelets and analysis of the *TYMP* gene does not identify causative mutations.
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REFERENCES


TABLES

**Table 1:** Summary of clinical data from 31 patients with pathogenic *RRM2B* mutations

**Table 2:** Clinical, muscle histopathological and molecular genetics findings of 26 adult patients with *RRM2B* mutations

**Supplemental Table 1:** A summary of the published clinical data for adults with *RRM2B* mutations

FIGURES

**Figure 1:** Mitochondrial histochemical changes associated with *RRM2B* mutations. Representative, sequential COX-SDH histochemistry demonstrates a mosaic distribution of COX deficient muscle fibres (blue) amongst fibres exhibiting normal COX activity (brown). Illustrated are the images for a, patient 5; b, patient 10; c, patient 19; d, patient 20. Patients 5 and 10 have autosomal dominant *RRM2B* mutations and a milder histochemical COX defect compared to patients 19 and 20 (autosomal recessive *RRM2B* mutations) in which a more severe biochemical defect is clearly apparent.

**Figure 2:** Characterisation of multiple mtDNA deletions in muscle from patients with *RRM2B* mutations. a, Representative long-range PCR amplification (15.4 kb fragment) across the major mtDNA arc shows evidence of multiple mtDNA deletions in patient muscle. Lane 1, size marker; lane 2, patient 5; lane 3, patient 10; lane 4, patient 19; lane 5, patient 20; lane 6, control. Patients with autosomal dominant mutations (lanes 2 and 3) show amplification of wild-type, full length mtDNA amplimers in addition to mtDNA deletions whereas those with recessive *RRM2B* mutations (patients 19 and 20) display a more severe, secondary mtDNA defect. b, Quantitative, single fibre real-time-PCR reveals the majority, but not all, of COX-deficient fibres contain high levels of a clonally-expanded mtDNA deletion involving the *MTND4* gene. Autosomal dominant missense or truncating mutations are represented by patients 3, 5, 9, 10, 14. Patient 1.1 has a dominant splicing mutation whilst patients 19 and 20 harbour recessive *RRM2B* mutations.

**Figure 3:** Schematic representation of the *RRM2B* gene structure illustrating the 18 different mutations identified in this study. Coding exons are numbered 1-9. Missense mutations are shown in pink boxes, exon 9 truncating mutations are shown in green boxes,
and the c.48G>A splice mutation is shown in a yellow box. \textit{RRM2B} mutations associated with adPEO in this study which have also been associated with more severe autosomal recessive disease (either early onset arPEO or mtDNA depletion syndrome - refer to Discussion for further details) are highlighted in red. Novel, unreported \textit{RRM2B} mutations are highlighted in blue.

\textbf{Figure 4: Molecular analysis of the novel c.48G>A (p.Glu16Glu) \textit{RRM2B} mutation.} 
\textbf{a,} Schematic showing the location of primers and the c.48G>A mutation within \textit{RRM2B} exons 1 and 2. \textbf{b,} Agarose gel electrophoresis of amplified cDNA from Patient 1 (P) and a normal, age-matched control (N) alongside a 123bp molecular weight marker (M), with the position of normally spliced (303bp) and aberrantly spliced (571, 770 & 821bp) products indicated. \textbf{c,} Sequencing of the normally-spliced cDNA product from Patient 2 reveals that this product is exclusively derived from the normal c.48G allele (equivalent results for patients 1 and 1.1 not shown). \textbf{d,} Example of a sequencing trace of the aberrantly-spliced products (571bp fragment from patient 2 is shown) demonstrating that these are almost exclusively derived from the mutant c.48G>A allele

\textbf{Figure 5:} Location of missense \textit{RRM2B} mutations on crystal structure. Shown is an image of the p53R2 dimer structure (PDB code 3HF1). The locations of 11 of the 12 amino acids altered by missense mutations identified in this study are shown on the di-iron bound (active) subunit (it has not been possible to illustrate Ala349, since the C-terminus is absent from the crystal structure). The physical space occupied by these 11 amino acids is illustrated by a multi-coloured mesh, with blue indicating positive charge, red negative charge and yellow neutral. Iron atoms are represented as red spheres.

\textbf{Supplemental Figure 1:} Evolutionary conservation of mutated amino acids detected in our \textit{RRM2B} patient cohort.