ORIGINAL ARTICLE

Kearns–Sayre syndrome caused by defective R1/p53R2 assembly

Robert D S Pitceathly,[1] Elisa Fassone,[2,3] Jan-Willem Taanman,[4] Michael Sadowski,[5] Carl Fratter,[6] Ese E Mudanohwo,[7] Cathy E Woodward,[7] Mary G Sweeney,[7] Janice L Holton,[1,8] Michael G Hanna,[1,8] Shamima Rahman[1,2,9]

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[1]MRC Centre for Neuromuscular Diseases, UCL Institute of Neurology and National Hospital for Neurology and Neurosurgery, London, UK
[2]Mitochondrial Research Group, Clinical and Molecular Genetics Unit, UCL Institute of Child Health, London, UK
[3]Dino Ferrari Centre, Department of Neurological Sciences, University of Milan, I.R.C.C.S. Foundation Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy
[4]Department of Clinical Neurosciences, UCL Institute of Neurology, London, UK
[5]Division of Mathematical Biology, National Institute for Medical Research, London, UK
[6]Oxford Regional Molecular Genetics Laboratory, Oxford Radcliffe Hospital NHS Trust, Oxford, UK
[7]Neurogenetics Unit, National Hospital for Neurology and Neurosurgery, Queen Square, London, UK
[8]Department of Molecular Neuroscience, UCL Institute of Neurology, London, UK
[9]Metabolic Unit, Great Ormond Street Hospital for Children, London, UK

Correspondence to
Dr S Rahman, MRC Centre for Neuromuscular Diseases, National Hospital for Neurology and Neurosurgery, 8-11 Queen Square, London WC1N 3BG, UK; s.rahman@ich.ucl.ac.uk

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ABSTRACT

Background Mutations in *RRM2B* encoding ribonucleotide reductase (RNR) p53R2 subunit usually cause paediatric-onset mitochondrial disease associated with mitochondrial DNA (mtDNA) depletion. The importance of RNR dysfunction in adult mitochondrial disease is unclear.

Objective To report the *RRM2B* mutation frequency in adults with multiple mtDNA deletions and examine RNR assembly in a patient with Kearns–Sayre syndrome (KSS) caused by two novel *RRM2B* mutations.

Methods 50 adult patients with multiple mtDNA deletions in skeletal muscle were studied. DNA sequencing of *RRM2B* was performed in patients without mutations in mtDNA maintenance genes *POLG* and *C10orf2*. RNR protein was studied using western blot and Blue-native polyacrylamide gel electrophoresis (BN-PAGE).

Results Four per cent (two unrelated cases) of this adult cohort harboured *RRM2B* mutations. Patient 1 had KSS and two novel missense mutations: c.122G→A; p.Arg41Gln and c.391G→A; p.Glu131Lys. BN-PAGE demonstrated reduced heterotetrameric R1/p53R2 RNR levels compared with controls, despite normal steady-state p53R2 levels on western blot, suggesting failed assembly of functional RNR as a potential disease mechanism. Patient 2 had late-onset progressive external ophthalmoplegia and fatigue. A heterozygous deletion c.253_255delGAG; p.Glu85del was identified. Muscle histology in both cases showed significant numbers of necrotic muscle fibres, possibly indicating enhanced apoptotic cell death.

Conclusion These data indicate that 4% of adult mitochondrial disease with multiple deletions is caused by RNR dysfunction. KSS has not previously been linked to a nuclear gene defect. Evidence that disease pathogenesis may be caused by defective RNR assembly is given. *RRM2B* screening should be considered early in the differential diagnosis of adults with multiple mtDNA deletions.

INTRODUCTION

There is increasing recognition that genetic disruption of the molecular processes that control the maintenance of mitochondrial DNA (mtDNA), including those that regulate nucleotide pools, are important in causing human mitochondrial diseases. Mutations in the ribonucleotide reductase (RNR) M2 B gene (MIM 604712, *RRM2B*) encoding p53R2 have been reported to cause severe mtDNA depletion in children, with encephalomyopathy and lactic acidosis, frequently associated with renal tubulopathy.[1–4] Patients with mtDNA depletion syndromes typically die in infancy or childhood.[5] *RRM2B* mutations have also been shown to cause mitochondrial neurogastrointestinal encephalopathy (MNGIE) with mtDNA depletion,[6] and a heterozygous truncating mutation in *RRM2B* has been found to be associated with autosomal dominant progressive external ophthalmoplegia (PEO) with multiple mtDNA deletions.[7] Depletion and deletions of mtDNA may result from unbalanced deoxyribonucleotide triphosphate (dNTP) pools with subsequent misincorporation of nucleotides into mtDNA.

p53R2 is a subunit of RNR, a heterotetrameric enzyme that catalyses the rate-limiting step in the de novo synthesis of dNTPs by direct reduction of ribonucleotide diphosphates to their corresponding deoxyribonucleotides. It is called p53R2 because its transcription is tightly regulated by the tumour suppressor protein p53.[8 9] It was initially suggested that p53R2 translocates to the nucleus during DNA damage-induced cellular arrest and was important for local DNA repair.[8 10] However, the observation of constant low levels of both R1 and p53R2 in post-mitotic cells and the clear correlations between depletion of mtDNA and functionally important mutations in *RRM2B* have expanded the role of p53R2 beyond that of nuclear DNA replication and repair to include an essential role in the supply of dNTPs for the synthesis of mtDNA. This process supplements the dNTPs produced by the mitochondrion's own deoxynucleotide salvage pathway, several defects of which have been implicated in mtDNA depletion syndromes in childhood.[5]

In this study we analysed nuclear mtDNA maintenance genes, including *RRM2B*, in a large cohort of 50 adults with mitochondrial neurological phenotypes. All cases were shown to have significant levels of multiple mtDNA deletions in skeletal muscle. We identified two cases, including one with Kearns–Sayre syndrome (KSS (MIM 530000)), with *RRM2B* mutations and studied their molecular pathogenesis. The frequency of and phenotypes associated with *RRM2B* mutations have not previously been evaluated in an adult population with multiple mtDNA deletions.

PATIENTS AND METHODS

Clinical data

Patient 1 was born after a normal pregnancy by assisted breech delivery to non-consanguineous parents of European descent. There were no

neonatal problems and development was normal up to the age of 4 years when she developed bilateral sensorineural deafness. Over the next 2 years she developed PEO and proximal muscle weakness and had poor weight gain, associated with malaise and tiredness. During adolescence the fatigue prevented her from keeping up with her peers during exercise. She had short stature and delayed puberty. Parents are now in their late 60s and do not have PEO or clinical symptoms suggestive of mitochondrial disease and there is no other relevant family history.

On examination aged 14 she was found to have pigmentary retinopathy, sensorineural deafness, PEO and proximal fatigable myopathy MRC grade 4/5. Serum creatine kinase (CK) was 281 IU/l (reference range 26–140 IU/l). Blood lactate was normal at 1.1 mmol/l but cerebrospinal fluid (CSF) lactate was mildly elevated at 2.6 mmol/l (reference range <2.0 mmol/l) and CSF protein was raised at 1.60 g/l (reference range 0.13–0.40 g/l). The combination of PEO and pigmentary retinopathy with onset before 20 years of age, in association with raised CSF protein and sensorineural deafness confirmed the clinical diagnosis of KSS. Electrocardiogram (ECG) did not show any conduction abnormalities and although echocardiogram (ECHO) showed increased thickness of the interventricular septum and left posterior ventricular wall, left ventricular function was normal. She was found to have a right hydronephrosis but there was no suggestion of renal tubulopathy (tubular reabsorption of phosphate was normal at 98% at 15 years). Cranial MRI demonstrated abnormal high signal in the deep cerebral white matter, posterior limbs of the internal capsules and the region of the anterior commissure, and diffuse changes in the lower half of the midbrain dorsally.

Continued enlargement of the kidney necessitated nephrectomy 2 years later. During the procedure a biopsy sample of the rectus abdominus was taken which showed fibre size variation and frequent necrotic fibres (figure 1A). Numerous fibres showed enhanced succinate dehydrogenase (SDH) activity (figure 1B) some of which showed the classic morphology of ragged red fibres (RRF) (7.8% of fibres) (figure 1C–E). Most RRF and fibres with enhanced SDH activity stained negatively for cytochrome *c* oxidase (COX) (10.3% of fibres) (figure 1F). Electron microscopy demonstrated mitochondrial aggregates, many with abnormal morphology including type I paracrystalline inclusions (figure 1G). She had continuing problems with appetite and nutrition and despite nutritional supplements body mass index aged 16 was 12.9 (weight 33.7 kg, height 161.5 cm). Patient 1 died aged 22 years following an episode of severe pneumonia.

Patient 2 presented aged 58 with a 3-year history of diplopia on a background of longstanding fatigue, having been born to non-consanguineous parents of European descent. Birth history and early development were normal and there was no history of neurological or neuromuscular disease in her parents, two siblings or other relatives. Cranial nerve examination revealed mild bilateral ptosis, restricted extraocular movements and slowing of saccades with no evidence of retinopathy. Other than slight wasting of biceps there were no abnormal findings on motor and sensory assessment of limbs.

Investigation showed normal CK. MRI brain revealed areas of T2 high signal bilaterally in the pons and some further small foci in both frontal lobes in the anterior limb of the left internal capsule, thought to be vascular in nature. Nerve conduction studies and an electromyogram were normal. Muscle histology aged 58 years showed five RRF (0.6% of fibres) and frequent COX-deficient fibres (4.1% of fibres), compatible with a mitochondrial myopathy (figure 1H). Similar to patient 1, fibre necrosis was also observed with two necrotic fibres in the small biopsy specimen available, a feature not usually reported in mitochondrial disease (figure 1H inset).[11] She died aged 66 of urinary sepsis.

Patient cohort

We identified 50 unrelated adults with multiple mtDNA deletions. In 14 of these patients we found mutations in *POLG* (MIM 174763, encoding the catalytic subunit of DNA polymerase γ) and three harboured *C10orf2* (MIM 606075, encoding the DNA helicase Twinkle) mutations. We sequenced *RRM2B* in the remaining 33 patients in this cohort, in whom the underlying nuclear gene defect had not been identified, in order to determine the prevalence of *RRM2B* mutations in this adult population. Each patient was characterised in detail clinically and was found to have multiple mtDNA deletions in skeletal muscle detectable either by long-range PCR or Southern blot analysis.

DNA sequencing

The nine exons and flanking intronic regions of *RRM2B* were amplified from total genomic DNA using specific primers (online supplementary table 1). PCR cycling conditions were: initial denaturation at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s and a final extension at 72°C for 7 min.

Multiplex ligation-dependent probe analysis (MLPA)

The SALSA MLPA kit P089-A1 TK2 (MRC-Holland, Amsterdam, The Netherlands) was used in patient 2 to exclude a trans-acting large-scale rearrangement of *RRM2B*. DNA was mixed with TE (10 mM Tris/1 mM EDTA, pH 8) and denatured by heating to 98°C for 5 min, 25°C for 1 min. The P089-A1 probe mix was added to the DNA and heated to 95°C for 1 min, followed by incubation at 60°C for 16 h for hybridisation. Annealed probes were then ligated by adding Ligase-65 at 54°C for 15 min. Ligation products were amplified by PCR (35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min) with SALSA PCR primers (labelled with Cy5.0). The resulting PCR fragments were separated by capillary electrophoresis on an ABI 3730XL using 'GeneMapper Generic' protocol. Data were analysed using GeneMarker analysis software. Relative copy number was obtained after normalisation of peaks against controls, with values between 0.75 and 1.25 considered to be within the normal range.

Protein analysis

Western blot analysis was performed as described elsewhere[12] on 10 μg of muscle protein extracted from total cell lysate from patients 1 and 2. The p53R2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins were detected using specific rabbit polyclonal and mouse monoclonal antibodies, respectively (Abcam, Cambridge, UK), and visualised using secondary mouse anti-rabbit and goat anti-mouse antibodies conjugated to horseradish peroxidise (Dako, Glostrup, Denmark).

Assembled heterotetrameric R1/p53R2 RNR protein complexes were detected using Blue native polyacrylamide gel electrophoresis (BN-PAGE), performed as previously described for respiratory chain complexes, modified to examine total cell extracts.[12] Samples were obtained from the muscle biopsies of patient 1 and two controls. No further muscle tissue was available from patient 2 for BN-PAGE studies. Skeletal muscle tissue was pulverised in liquid nitrogen. After thawing, the powder was immediately mixed with 0.75 M 6-aminocaproic acid, 37.5 mM bistris (pH 7.0), 2.5% (w/v) n-dodecyl-β-D-maltoside and protease inhibitors, followed by centrifugation at 16 000 *g*, 4°C for 20 min. The supernatant was transferred to a new tube and mixed with 0.1 volume of 1 M 6-aminocaproic acid and 5% (w/v) Serva blue G. Supernatant (15–20 μl) was then loaded, resolved on 4–12%

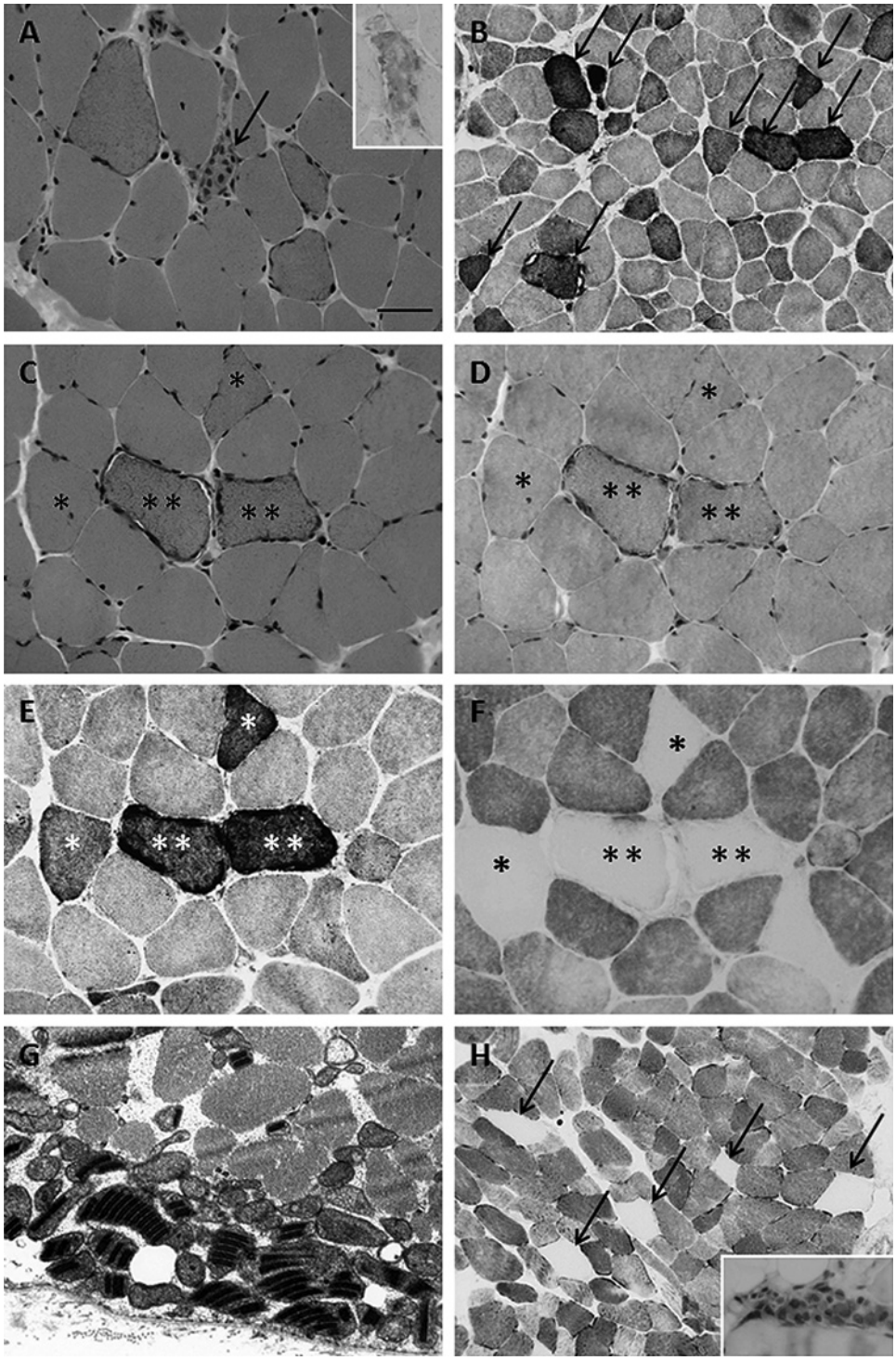

Figure 1 The muscle biopsy specimen from patient 1 showed increased variation in fibre diameter and fibre necrosis (A, arrow) confirmed by acid phosphatase histochemistry (A, inset). Frequent fibres showed increased succinic dehydrogenase (SDH) activity (B, arrows). Ragged red fibres (RRF) were readily apparent (C, D, **) and these displayed an increase in SDH activity (E, **) with cytochrome *c* oxidase (COX)-deficiency (F, **). Many of the fibres with a normal appearance in the haematoxylin and eosin (H&E) (C, *) and Gomori trichrome (D, *) preparations showed enhanced SDH activity (E, *) and COX-deficiency (F, *). Ultrastructural examination showed abnormal mitochondria containing type I paracrystalline inclusions (G). Examination of the muscle biopsy specimen from patient 2 also showed increased numbers of COX-deficient fibres (H, arrows) and a small number of necrotic fibres (H, inset). The bar in A represents 50 μm in A, C–F and insets in A & H; 100 μm in B and H; 800 nm in G. A and C, H&E; B & E, SDH; D, Gomori trichrome; F and H, COX; insets in A & H, acid phosphatase.

polyacrylamide gels, electrotransferred to Hybond-P membrane (GE Healthcare, Chalfont St Giles, UK) and probed with specific polyclonal antibodies to p53R2 (Abcam). The secondary antibody was mouse anti-rabbit conjugated to horseradish peroxidise (Dako). A loading control was performed in parallel using SDHA, a major subunit of the nuclear-encoded Krebs cycle enzyme succinate dehydrogenase (also known as complex II), which is present in both the cytosol and the mitochondrion.

Protein function prediction

The three mutations observed in these two patients were modelled using the published p53R2 crystal structure (pdb code 3hf1),[13] monomers A and B for the mono- and di-iron bound forms, respectively. Residues 1–29 and 318–351 were not observed in the crystal structure and these regions were therefore omitted from the model. Models were built using the automodel routine of the MODELLER software package (version 9v3),[14] with no additional refinement. Figure 2A shows an overview of the model with the locations of the three mutations shown on the monomer B structure.

RESULTS

Long-range PCR and Southern blot analysis of DNA from the muscle of patient 1 both revealed multiple deletions of mtDNA

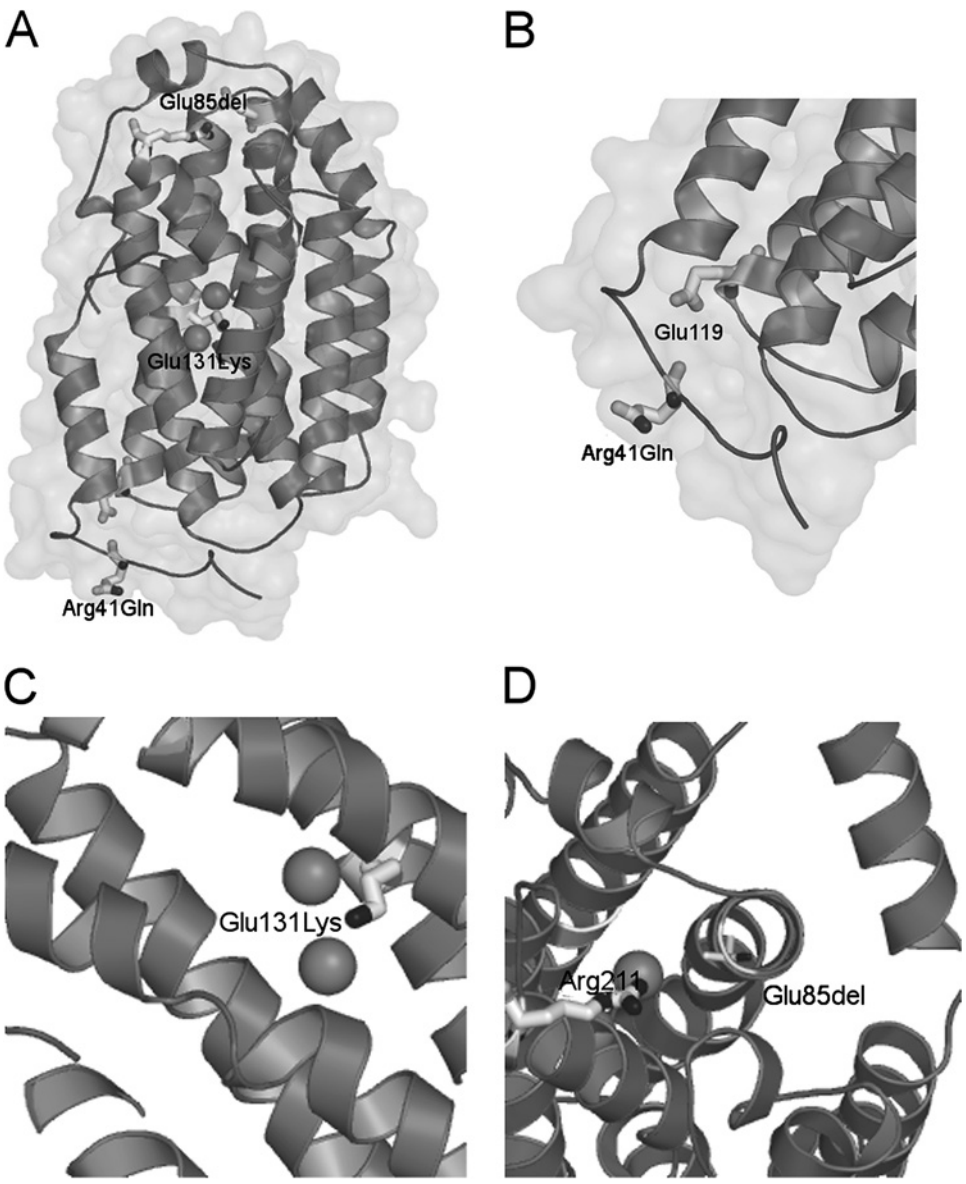

Figure 2 Mutations visualised on the p53R2 structure (PDB code 3hf1) showing details for all three mutations (A); Arg41Gln (B); Glu131Lys (C) and Glu85del (D), respectively.

(figure 3A,B), while multiple deletions of mtDNA were detectable only by long-range PCR in patient 2 (figure 3A,B). The common mtDNA point mutations m.3243A→G, m.8344A→G and m.8993T→G/C were excluded in both patients in DNA extracted from muscle, using PCR-RFLP. Both patients had a normal mtDNA copy number compared with age-matched controls, measured by quantitative real-time PCR as previously described (figure 3C).[15]

Mutation screening by direct sequencing of the coding region and exon–intron boundaries of *POLG* and targeted regions of *C10orf2* to include nucleotides c.690-1592 (3′ end of exon 1, exons 2 and 3) was performed to exclude mutations in these genes, which are known to cause multiple mtDNA deletion formation in adults. No pathogenic mutations were identified in both genes in either patient 1 or patient 2.

Sanger sequence analysis of the amplified fragments revealed deleterious mutations in *RRM2B* in two of the 33 patients, while the remaining 31 patients were not shown to have pathogenic *RRM2B* variations. Patient 1 had two novel heterozygous missense mutations in *RRM2B*: c.122G→A; p.Arg41Gln in exon 2 (figure 4A) and c.391G→A; p.Glu131Lys in exon 4 (figure 4B). The c.122G→A; p.Arg41Gln change in exon 2 segregated to the paternal allele (figure 4C) and the c.391G→A; p.Glu131Lys in exon 4 to the maternal allele (figure 4D). The c.122G→A; p.Arg41Gln change was not present in 332 ethnically matched control chromosomes and c.391G→A; p.Glu131Lys was not present in 308 ethnically matched control chromosomes. Patient 2 had a heterozygous three base-pair deletion in exon 3: c.253_255delGAG; p.Glu85del (figure 4E). No DNA samples from other family members were available for study. The c.253_255delGAG; p.Glu85del mutation has previously been reported in compound heterozygous state with the missense mutation c.707G→T; p.Cys236Phe[1] and also as a homozygous mutation (R Van Coster *et al*, July 2010, *Acta Myologica*, abstract) in neonates with a very severe mtDNA depletion. This mutation has previously been shown to be absent from over 220 ethnically matched control chromosomes.[1] These amino acid residues occur in highly conserved regions across species (figure 5A–C). Patient 2 had normal copy numbers for all eight *RRM2B* MLPA probes at the default threshold level (0.75–1.25), excluding a large-scale rearrangement of this gene on the other allele in this patient.

Immunoblot analysis of both patients revealed normal steady-state levels of p53R2 levels, indicating that none of the three *RRM2B* mutations seen in this study had any effect on p53R2

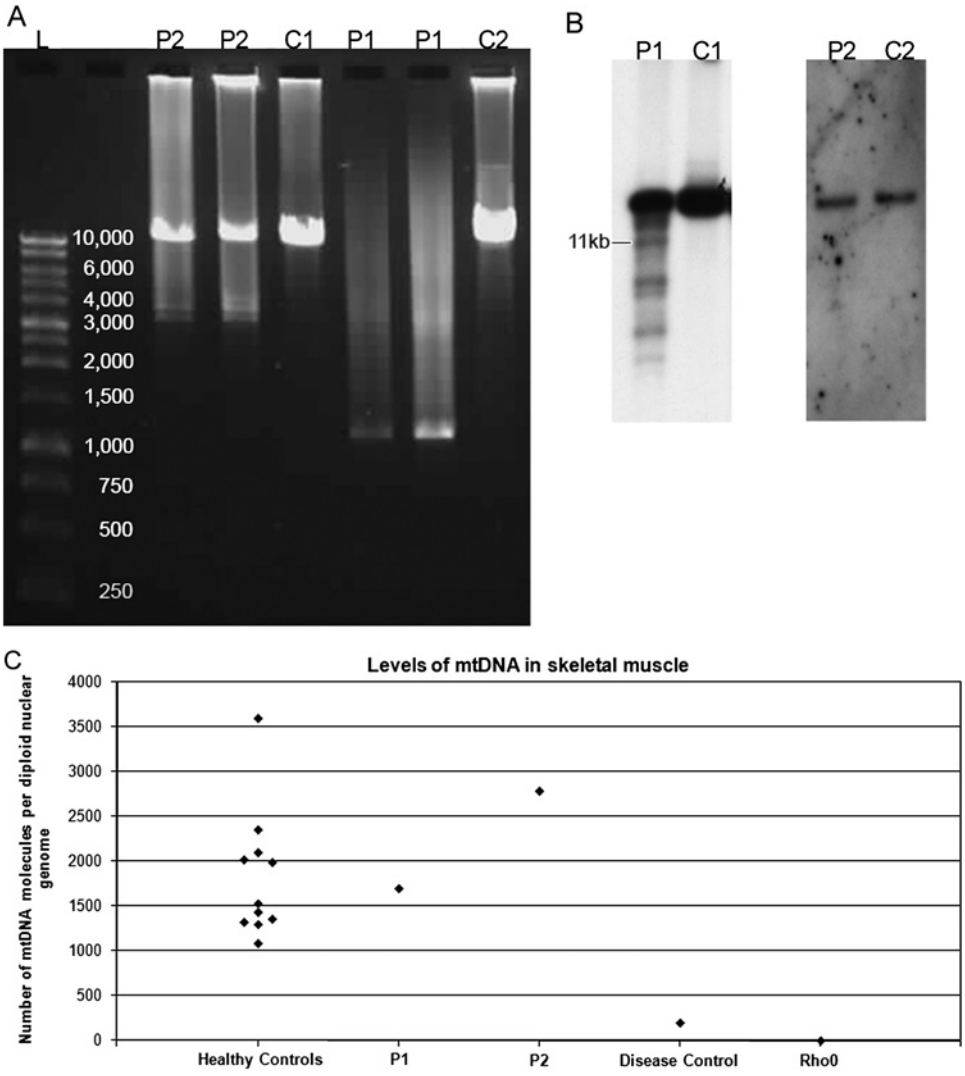

Figure 3 (A) Long-range PCR analysis shows multiple mtDNA deletions in muscle samples of patients 1 and 2 (P1 and P2) compared with ladder (L) and normal control subjects (C1 and C2). (B) Southern blot analysis using *Pvu*II restriction endonuclease shows multiple mitochondrial DNA (mtDNA) deletions (with common 5 kb mtDNA deletion annotated) in muscle sample of patient 1 (P1) compared with control (C1) and only wild-type mtDNA band in muscle sample of patient 2 (P2) compared with control (C2). (C) MtDNA copy number is normal in muscle samples of patient 1 and patient 2 compared with age-matched healthy controls, disease controls and cells depleted of mtDNA (Rho0) measured by quantitative real-time PCR.

stability (figure 6A,B). BN-PAGE analysis demonstrated reduced levels of the heterotetramer R1/p53R2 in the muscle sample from patient 1 in comparison with control muscle tissue (figure 7A), suggesting impaired heterotetramer assembly despite the normal p53R2 levels seen on western blot and BN-PAGE. BN-PAGE of complex II in the muscle sample from patient 1 when compared with control muscle tissue showed equal protein loading (figure 7B).

We modelled all three mutations on the previously published p53R2 crystal[13] (figure 2A) in order to illustrate their structural implications. This enabled a significant insight into the likely functional effects on RNR function, particularly as a result of an impaired interaction between the two p53R2 monomers within the heterotetrameric protein complex. Residue Arg41 is located within the N-terminal swivel region as defined by Smith *et al*[13] at the most N-terminal visible portion of the protein (figure 2B). Conformational change of this region is associated with the formation of a salt bridge between residues Arg41 and Glu119 and the stabilisation of residues in helix B in the required conformation for formation of the di-iron bound monomer as a prerequisite for generation of the active tyrosyl-radical-bearing form of the subunit. Since glutamine would be unable to form a salt bridge, the mutant would not be stable in the di-iron form and this would result in significant reduction of the active form of the enzyme. Glu131 is found in the centre of the iron-binding pocket (figure 2C). In the wild-type (WT) p53R2 this residue bridges both ferric irons and is therefore required for binding one or possibly both ions. Mutation to the larger, positively charged lysine would abolish di-iron binding completely and might prevent iron binding altogether. The effect of both mutations on RNR activity explains the clinical phenotype and is most probably the reason for the multiple mtDNA deletions seen in patient 1.

Glu85 is located at the top of helix B at the opposite end to Glu119, the partner of Arg41 in the swivel mechanism (figure 2D). It forms a salt bridge to Arg211 in helix D. Deletion of the Glu85 residue would preclude the formation of this salt bridge and cause a significant loss of stability, of the order of a few kcal/mol, potentially reducing levels of correctly folded protein. Correctly folded proteins might also be functionally impaired: removal of the link might favour translocation of helix B over conformational change in helix D in response to movement of the swivel region, strongly destabilising the di-iron form and leading to a loss of native function. Alternatively it may interfere with the adoption of the conformation of helix D required to form the channel leading to the iron-binding site.[13] Since the mutant protein appears to exert a dominant effect, it is likely to compete with WT p53R2 to form the heterotetrameric R1/p53R2 RNR complex. The result of this competitive binding would be a dominant-negative effect on R1/p53R2 function.

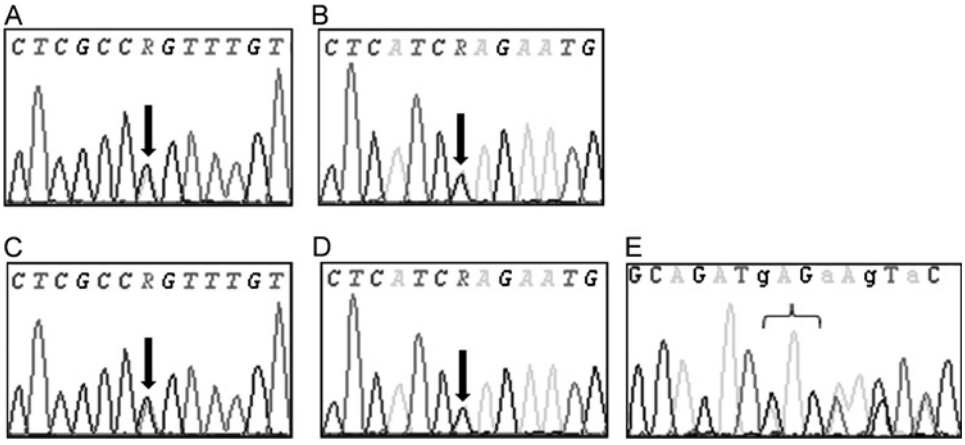

Figure 4 *RRM2B* sequencing data for patient 1 showing: c.122G→A; p.Arg41Gln in exon 2 (A) segregating to the paternal allele (C) and c.391G→A; p.Glu131Lys in exon 4 (B) segregating to the maternal allele (D). *RRM2B* sequencing data for patient 2 showing: c.253_255delGAG; p.Glu85del in exon 3 (E).

The heterozygous nature of the defect is likely to account for the milder phenotype seen in our patient in comparison with the two previously reported cases of the same mutation observed in compound heterozygous[1] and homozygous (R Van Coster *et al*, July 2010, *Acta Myologica*, abstract) states. The presence of the normal allele would be expected to partially compensate for impaired RNR activity.

DISCUSSION

We report the first case of KSS to result from a nuclear genetic defect: two novel missense mutations in *RRM2B*. Single large-scale deletions of mtDNA are normally found to be the cause of KSS and were first reported in this condition in 1988.[16] It was subsequently shown that the causative mtDNA deletions may differ considerably in size and location, although a 5 kb 'common' deletion is present in approximately one-third of patients.[17] The mechanism for the formation of deletions is poorly understood. An initial hypothesis proposed that slipped replication occurs between homologous base-pair repeat sequences within mtDNA,[18] since most deletions appear to occur within the major arc between the two origins of mtDNA replication. The validity of this model has since been questioned.[19] Irrespective of the mechanism, single large-scale deletions are generally considered sporadic events with a low inheritance risk. The present discovery suggests that KSS can also follow Mendelian inheritance patterns as a result of a nuclear gene mutation associated with multiple mtDNA deletions. This potentially has significant clinical implications, particularly during genetic counselling, and analysis of *RRM2B* should be a priority in KSS when multiple mtDNA deletions are present.

As far as we know, this is the first prevalence study investigating *RRM2B* mutations in such a large adult cohort with multiple mtDNA deletions of unknown aetiology. We suggest

Figure 5 Amino acid alignment of the human p53R2 enzyme reference sequence versus p53R2 enzyme sequences of various species for: c.122G→A; p.Arg41Gln in exon 2 (A); c.391G→A; p.Glu131Lys in exon 4 (B) and c.253_255delGAG; p.Glu85del in exon 3 (C).

A

p53R2	33	34	35	36	37	38	39	40	**41**	42	43	44	45	46	47	48	49
Patient 1									**Q**								
Human	P	L	L	R	K	S	S	R	**R**	F	V	I	F	P	I	Q	Y
Chimp	P	L	L	R	K	S	S	R	**R**	F	V	I	F	P	I	Q	Y
Macaque	P	L	L	R	K	S	S	R	**R**	F	V	I	F	P	I	Q	Y
Mouse	P	L	L	R	K	S	S	R	**R**	F	V	I	F	P	I	Q	Y
Dog	P	L	L	R	K	S	S	R	**R**	F	V	I	F	P	I	Q	Y
Chicken	P	L	L	R	K	N	P	R	**R**	F	V	I	F	P	I	Q	H
Frog	P	F	L	R	K	N	P	Q	**R**	F	V	I	F	P	I	H	Y
Tetraodon	P	L	L	Q	E	N	P	R	**R**	F	V	I	F	P	I	Q	Y
C. Elegans	P	M	L	Q	D	L	D	N	**R**	F	V	I	F	P	I	K	H

B

p53R2	123	124	125	126	127	128	129	130	**131**	132	133	134	135	136	137	138	139
Patient 1									**K**								
Human	F	Y	G	F	Q	I	L	I	**E**	N	V	H	S	E	M	Y	S
Chimp	F	Y	G	F	Q	I	L	I	**E**	N	V	H	S	E	M	Y	S
Macaque	F	Y	G	F	Q	I	L	I	**E**	N	V	H	S	E	M	Y	S
Mouse	F	Y	G	F	Q	I	L	I	**E**	N	V	H	S	E	M	Y	S
Dog	F	Y	G	F	Q	I	L	I	**E**	N	V	H	S	E	M	Y	S
Chicken	F	Y	G	F	Q	I	L	I	**E**	N	V	H	S	E	M	Y	S
Frog	F	Y	G	F	Q	I	L	I	**E**	N	V	H	S	E	M	Y	S
Tetraodon	F	Y	S	Y	Q	V	L	I	**E**	S	V	H	S	E	M	Y	S
C. Elegans	F	Y	G	F	Q	I	A	I	**E**	N	I	H	S	E	M	Y	S

C

p53R2	37	78	79	80	81	82	83	84	**85**	86	87	88	89	90	91	92	93
Patient 2									**Del**								
Human	H	W	N	K	L	K	A	D	**E**	K	Y	F	I	S	H	I	L
Chimp	H	W	N	K	L	K	A	D	**E**	K	Y	F	I	S	H	I	L
Macaque	H	W	N	K	L	K	A	D	**E**	K	Y	F	I	S	H	I	L
Mouse	H	W	N	K	L	K	S	D	**E**	K	Y	F	I	S	H	I	L
Dog	H	W	N	K	L	K	S	D	**E**	K	Y	F	I	S	H	I	L
Chicken	H	W	N	K	L	K	A	D	**E**	K	Y	F	I	S	H	V	L
Frog	H	W	E	K	L	K	P	E	**E**	R	N	F	I	S	H	I	L
Tetraodon	H	W	D	S	L	K	P	E	**E**	K	H	F	I	S	H	V	L
C. Elegans	D	W	E	K	M	N	G	D	**E**	Q	Y	Y	I	S	R	I	L

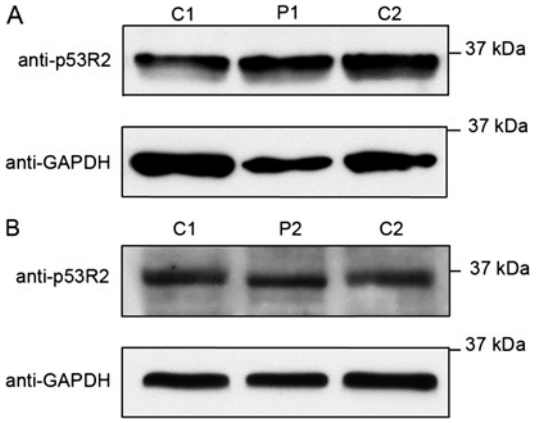

Figure 6 Western blot analysis of p53R2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins in muscle samples of patient 1 (A) and patient 2 (B) shows normal p53R2 levels versus controls (C1 and C2).

that RNR dysfunction is considered early in the genetic diagnostic evaluation of adults with multiple mtDNA deletions, since these data indicate that *RRM2B* mutations are an important cause of multiple mtDNA deletions in adults. We also show that a significant number of our cohort do not harbour mutations in *POLG*, *C10orf2* or *RRM2B*. Analysis is continuing to identify the genetic basis of multiple mtDNA deletions in these patients.

There are 20 published pathogenic mutations in *RRM2B*.[1–4 6 7 20] Before this study all but two mutations were associated with severe mtDNA depletion syndromes presenting in childhood, often with encephalopathy and significant renal impairment. One mutation was associated with multiple deletions of mtDNA in adult patients in two families with autosomal dominant PEO.[7] Despite the cachexia seen in patient 1, diagnostic criteria for MNGIE were not met; however, it is interesting to note that *RRM2B* mutations have also previously been associated with MNGIE.[6] That 4% of our total cohort of 50 adult patients with mtDNA deletions had deleterious mutations in *RRM2B* suggests that there is further genetic

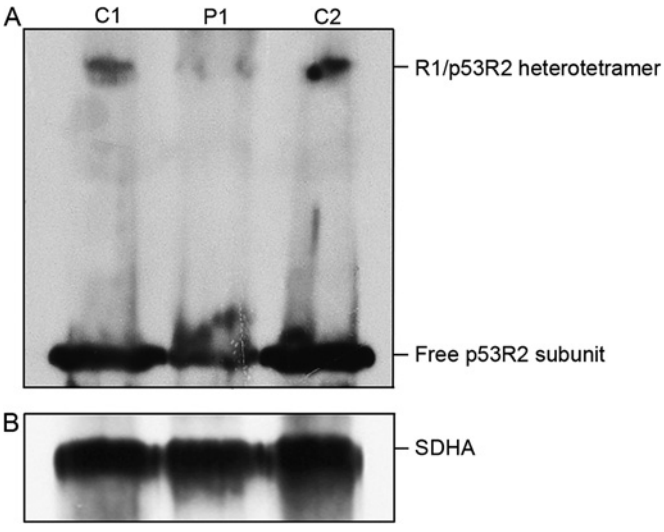

Figure 7 Blue-native polyacrylamide gel electrophoresis analysis of p53R2 subunit, R1/p53R2 heterotetramer (A) and complex II (B) in muscle sample of patient 1 (P1) shows reduced native R1/p53R2 levels despite normal p53R2 levels versus controls (C1 and C2). SDHA, succinate dehydrogenase complex, subunit A.

heterogeneity underlying multiple mtDNA deletion formation in this population.

In mammalian cells RNR exists as a heterotetrameric enzyme consisting of one large homodimeric R1 subunit which contains the catalytic site and one small homodimeric R2 subunit which supplies the tyrosyl free radical required for catalysis. The R2 subunit is produced exclusively during S-phase in cycling cells when RNR provides the nucleus with dNTPs required for DNA replication.[21] The R2 homologue p53R2, however, is detectable at low levels throughout the cell cycle in both proliferating and post-mitotic cells. It is thought that it is the R1/p53R2 heterotetramer which is important in providing dNTPs for nuclear DNA repair and/or mtDNA synthesis. A recent study localised p53R2 exclusively to the cytosol during both cell proliferation and DNA damage, suggesting that the RNR enzyme itself does not translocate into the nucleus or mitochondrion to effect DNA repair locally as was initially thought, but rather that dNTPs diffuse into the nucleus and mitochondria to provide building blocks for DNA repair/synthesis.[22] The role of RNR in repair was also challenged since DNA damage does not lead to significant increases in dNTP pools[23] and induction of p53R2 by p53 peaks around 24 h, yet repair of damaged DNA is complete in a much shorter time period.[8 24] It is possible that p53R2 increases flux through the dNTP pool, rather than the absolute size of the dNTP pool itself.[23] Furthermore, post-translational modification of p53R2 by phosphorylation at Ser72 by the ataxia telangiectasia mutated protein kinase confers p53R2 protein stability and resistance to DNA damage within 30 min of genotoxic stress.[25] This would allow rapid repair of DNA by activating existing pools of p53R2 protein without reliance on slow transcriptional induction of p53R2.

BN-PAGE was used to demonstrate the deleterious effects of *RRM2B* mutations on heterotetramer assembly. This is a technique previously used to assess intact mitochondrial respiratory chain complexes. We present data that expand the application of this method to include analysis of cytosolic multiprotein complexes, and also provide greater understanding of mutational effects on protein complex assembly. Impaired R1/p53R2 heterotetramer assembly is a potential pathogenic mechanism by which mutations in *RRM2B* cause disease. It is important that further attempts are made to replicate this experiment in adults with *RRM2B* mutations to fully understand the frequency of impaired R1/p53R2 assembly in the pathogenesis of these disorders. We suggest that BN-PAGE should be considered to demonstrate native multiprotein complexes when impaired assembly is suspected.

The presence of necrotic fibres alongside the more typical histopathological features of mitochondrial disease in our two patients with *RRM2B* mutations is intriguing, particularly since both patients exhibit this finding. They may be a consequence of impaired nuclear DNA repair caused by the defective p53R2; it has been shown that p53R2-null mice demonstrate enhanced frequency of spontaneous mutations and activation of p53-dependent apoptotic pathways.[26] Identification of necrotic fibres in muscle could be a useful guide when prioritising which nuclear genes to sequence in patients with multiple mtDNA deletions.

In conclusion, we have found that mutations in *RRM2B* are an important cause of multiple mtDNA deletions in adults and we report the first case of KSS related to nuclear genomic dysfunction, as a result of two novel *RRM2B* mutations. The heterotetrameric structure of R1/p53R2 RNR means that disease may arise from heterozygous, homozygous or compound heterozygous mutations which reduce RNR activity and from

Web resources

The URL for data presented herein is as follows: Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim

impaired assembly of the heterotetramer. In addition, there is a dosage effect, dependent on the presence or absence of WT p53R2. Finally, we show that BN-PAGE is a valuable method in the structural analysis of native R1/p53R2 RNR and present a mechanistic insight into the effect of dysfunctional *RRM2B* on R1/p53R2 RNR assembly.

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Kearns–Sayre syndrome caused by defective R1/p53R2 assembly

Robert D S Pitceathly, Elisa Fassone, Jan-Willem Taanman, et al.

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