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MITOCHONDRIAL DNA DEPLETION SYNDROME DUE TO MUTATIONS IN THE *RRM2B* GENE

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Abstract

Mitochondrial DNA depletion syndrome (MDS) is characterized by a reduction in mtDNA copy number and has been associated with mutations in eight nuclear genes, including enzymes involved in mitochondrial nucleotide metabolism (*POLG*, *TK2*, *DGUOK*, *SUCLA2*, *SUCLG1*, *PEO1*) and *MPV17*. Recently, mutations in The *RRM2B* gene, encoding the p53-controlled ribonucleotide reductase subunit, have been described in 7 infants from 4 families, who presented with various combinations of hypotonia, tubulopathy, seizures, respiratory distress, diarrhea, and lactic acidosis. All children died before 4 months of age.

We sequenced the *RRM2B* gene in three unrelated cases with unexplained severe mtDNA depletion. The first patient developed intractable diarrhea, profound weakness, respiratory distress, and died at three months. The other two unrelated patients had a much milder phenotype and are still alive at ages 27 and 36 months.

All three patients had lactic acidosis and severe depletion of mtDNA in muscle. Muscle histochemistry showed RRF and COX deficiency. Sequencing the *RRM2B* gene revealed three missense mutations and two single nucleotide deletions in exon 6, 8 and 9, confirming that *RRM2B* mutations are important causes of MDS and that the clinical phenotype is heterogeneous and not invariably fatal in infancy.

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1. Introduction

Within the past six years, mtDNA depletion syndrome (MDS) has been attributed to mutations in eight nuclear genes. Five of these (*TK2*, encoding mitochondrial thymidine kinase; [1] *DGUOK*, encoding deoxyguanosine kinase; [2] *POLG*, encoding the catalytic subunit of mitochondrial polymerase γ [3]; *SUCLA2*, encoding the β subunit of succinyl-CoA synthase (SCS-A), [4] and *SUCLG1*, encoding the α subunit of SCS-A [5]) are – directly or indirectly [6] – involved in the homeostasis of the mitochondrial nucleotide pool. Mutations in *PEO1*, encoding the T7-phage-like helicase (twinkle), typically cause autosomal dominant multiple deletions of mtDNA but can also cause autosomal recessive mtDNA depletion. [7, 8] The seventh gene, *MPV17*, encodes an inner mitochondrial protein whose function remains elusive. [9]

The latest gene to be associated with mtDNA depletion is *RRM2B*, which encodes the R2 subunit of a p53-controlled ribonucleotide reductase (p53R2). This enzyme catalyzes the biosynthesis of deoxyribonucleotides by storing organic free radicals required for catalysis in the R2 subunit. [10]

Here, we describe three unrelated patients with five novel *RRM2B* mutations. One of the three had the fatal infantile presentation reported in the seven original patients, [10] whereas the other two had milder clinical phenotypes and are alive at 27 and 36 months of age.

2. Case Reports

Patient 1

This 8-week-old congenitally deaf infant girl, born to non-consanguineous parents after a normal pregnancy and delivery, was admitted with a 2-week history of watery diarrhea, persistent acidosis, progressive weakness, poor head control, and worsening respiratory distress requiring intubation. At admission, she was small for age and hypotonic, with bilateral central sensorineural hearing loss. The following laboratory tests were abnormal: repeat plasma lactate values ranged from 2.8 to 17.4 mmol/L (normal, <2.2); blood pyruvate was 0.434 mmol/L (normal, 0.03–0.107); plasma organic acids were normal except for increased lactate and pyruvate; CSF lactate was 5.2 mmol/L (normal, 0.5–2.8) and CSF protein was 164 (normal, 12–60). Notably, serum CK was normal (126; normal <296). MRS of the brain at 7 weeks of age showed the presence of lactate in the left basal ganglia.

Various attempts to wean her from the ventilator failed and control of her metabolic and respiratory acidosis required ventilator adjustments and intravenous bicarbonate drip. She continued having diarrhea and required total parenteral nutrition. Her strength worsened progressively and at 8 months she had minimal spontaneous movements. After the parents decided to withdraw further care, a premortem muscle biopsy was obtained to confirm the diagnosis of mtDNA depletion obtained from a previous very small biopsy and to establish the molecular etiology.

Patient 2

This 4-year-old boy was born to non-consanguineous healthy parents after a normal pregnancy and delivery. He was normal at birth, but progressive failure to thrive rapidly ensued due to uncoordinated suck and swallow. He failed to gain developmental milestones and was hypotonic and microcephalic when first seen at 4 months of age. He developed respiratory failure, urinary infections, and intolerance to oral feeds: an immune deficient state was ruled out. At 7 months, a G-tube was placed, but he continued losing weight and developed electrolyte imbalance, with hyponatemia, hypochloremia, and hypokalemia,

requiring supplementation. At 8 months, he required intubation and assisted ventilation, followed by tracheostomy at 10 months.

Laboratory tests at 8 months of age showed increased serum lactate (4.3) and normal CK (108 IU; normal <296). Urinary organic acids, plasma amino acids and acylcarnitine profile were normal. There was mild generalized aminoaciduria but renal tubular function was not analyzed in detail. A low plasma carnitine level (21.1 nmol/ml; normal, 25–69) was attributed to malnutrition and corrected by carnitine supplementation. An MRI of the brain at 20 months showed bilateral and nearly symmetrical non-enhancing areas of abnormal signal and reduced diffusion in the white matter. Magnetic resonance spectroscopy (MRS) showed a lactate peak in the basal ganglia and an even higher peak in the CSF.

At 4 years of age, he is wheelchair-bound and is ventilated by tracheostomy. He has a stable encephalomyopathy, with microcephaly and global developmental delay. An ophthalmological exam has revealed peripheral pigmentary retinopathy and tunnel vision, but there is no evidence of optic atrophy. With a feeding tube and a Nissen fundoplication, he is growing well and has no overt liver or renal involvement. A recent electrocardiogram was normal.

Patient 3

This 27-month-old girl was born to non-consanguineous Hispanic parents after a normal pregnancy and delivery and developed normally until 6 months of age, when she was evaluated because of progressively worsening hypotonia, failure to thrive, and microcephaly. Abnormal laboratory tests included serum CK (318 IU/L; normal, <296), blood lactate (ranging from 4.1 to 7.2 mmol/L; normal <2.2), and liver function tests (AST, 81 IU/L; normal, 12–27; ALT, 46 IU/L; normal, 7–28). She had multiple respiratory infections needing admissions to the hospital, but no major episodes of decompensation. After placement of a G-tube at 9 months of age and supplementation of vitamins and cofactors, including L-carnitine and coenzyme Q₁₀, she has maintained adequate weight gain and growth and has acquired normal head circumference. At 27 months, she can sit with assistance and walk with a pony walker, grabs, reaches, and has a 12-word vocabulary. Overall, she has continued to make progress.

3. Methods

3.1. Histochemistry and biochemistry

Histochemical studies of muscle using 8-mm-thick frozen sections were carried out as described. [11] Biochemical analysis was performed in 10% muscle extracts as previously described. [12]

3.2. Molecular analysis

Total DNA was extracted from muscle by standard protocol (PUROGENE, Gentra System, Inc, Minneapolis, MN) following the manufacturer's instructions. Sequencing of *TK2*, *DGUOK*, *POLG*, *SUCLA2*, *SUCLA1*, and *MPV17*, and quantification of mtDNA were performed by described techniques. [4, 5, 9, 13–15] The entire coding region of the *RRM2B* gene was amplified by polymerase chain reaction using specific intronic primers described by Bourdon et al., [10] with initial denaturation at 95°C for 7 min, followed by 30 cycles at 95°C for 30 sec, at 55°C for 30 sec, at 72°C for 30 sec, and final extension at 72°C for 10 min.

3.3. Protein modeling

The modeling of human p53-ribonucleotide reductase was performed using Swiss Model software and crystal structure of human ribonucleotide reductase as template (PBD_2uw2). To visualize the structure and predict the effect of missense mutations and deletions on the conformation, we used RasWin Molecular Graphics Windows Version 2.6-ucb. Protein alignments have been done by ClustalW.

4. Results

Clinical features, muscle morphology and biochemistry

We “revisited” 9 patients with severe mtDNA depletion in muscle but without mutations in the two genes associated with muscle mtDNA depletion (*TK2* and *SUCLA2*) or in the four genes more typically associated with liver mtDNA depletion (*DGUOK*, *POLG*, *SUCLG1*, and *MPV17*). When we sequenced the gene encoding the p53-controlled ribonucleotide reductase (*RRM2B*), we identified five apparently pathogenic mutations in 3 unrelated patients (33%).

The degree of mtDNA depletion in muscle was severe in all three patients, but especially in patient 1, who had a fatal infantile presentation (Table 1). Accordingly, the activities of respiratory chain complexes containing mtDNA-encoded subunits (I, I+III, II+III, and IV) were markedly decreased in patient 1, with a disproportionate deficit of complex IV and a massive increase in the activities of the nuclear DNA-encoded enzymes, citrate synthase and succinate dehydrogenase. In contrast, respiratory chain enzymes were much less severely affected in muscle from the other two patients, except for a severe defect of COX and an unexplained decrease of citrate synthase in patient 3 (Table 1).

Histochemical findings were in general agreement with biochemical data. In patient 1, there was a profusion of ragged-red fibers (RRF) with the modified Gomori trichrome stain (Figure 1) and ragged-blue fibers with the SDH stain. All RRF were cytochrome *c* oxidase (COX)-negative with the superimposed SDH/COX stains. The oil-red-O (ORO) stain also revealed an excess of lipid droplets in most RRF. In patient 2, muscle biopsy showed scattered fibers with increased staining for succinate dehydrogenase (SDH): many of these fibers were negative for COX activity in the combined SDH/COX stain (COX-negative ragged-red fibers). The muscle biopsy in patient 3 revealed scattered RRF, which reacted intensely with the SDH stain but were COX-negative or COX-deficient.

Molecular genetic analysis

Molecular genetic analysis showed five novel mutations in *RRM2B*. Patient 1 was homozygous for a c.671 T>G mutation in exon 6, resulting in the substitution of a highly conserved isoleucine to a serine at amino acid position 224. Patient 2 was compound heterozygous for a missense mutation in exon 8 (c.846 G>C) inherited from the mother, and 1-bp deletion in exon 9 (c.920 delA) inherited from the father. The missense mutation changes a highly conserved methionine to an isoleucine at amino acid position 282, whereas the microdeletion alters the reading frame starting at amino acid position 317 by changing a leucine to a stop codon (c.317 L>X) and abrogating the last 34 amino acid residues of the mutated protein. Patient 3 was also compound heterozygous for a missense mutation in exon 9 (c.949 T>G) and a 1-bp deletion in exon 6 (c.584 delG). The missense mutation changes a highly conserved leucine to a valine at amino acid position 317 and the microdeletion alters the reading frame from amino acid 195 and creates a stop codon (L208X), which abrogates the last 144 amino acid residues of the *RRM2B* protein. None of these mutations were found in 192 chromosomes from a mixed North American population.

5. Discussion

Mitochondrial DNA (mtDNA) depletion was identified in 1991[16] in two related infants, one with myopathy, the other with hepatopathy. Although this was a new paradigm of mitochondrial dysfunction due to defective communication between the dominant nuclear DNA (nDNA) and the subservient mtDNA, the molecular cause of the mtDNA depletion in this family was not clarified. It took ten years before mutations in two nuclear genes, one (*TK2*) encoding thymidine kinase, the other (*DGUOK*) encoding deoxyguanosine kinase, were associated with a predominantly myopathic [1] and a predominantly hepatopathic [2] syndrome of mtDNA depletion.

Within the past six years, mtDNA depletion has been attributed to mutations in five more genes. Like *TK2* and *DGUOK*, three of these (*POLG*, encoding the catalytic subunit of mitochondrial polymerase γ [3]; *SUCLA2*, encoding the β subunit of succinyl-CoA synthase (SCS-A) [4], and *SUCLG1*, encoding the α subunit of SCS-A[5]) are – directly or indirectly [6] - involved in the homeostasis of the mitochondrial nucleotide pool. In contrast, *PEO1* encodes a mitochondrial helicase (twinkle), which, when mutated, causes autosomal dominant multiple deletions of mtDNA or autosomal recessive mtDNA depletion. [7, 8] The fifth gene, *MPV17*, encodes an inner mitochondrial protein whose function remains elusive. [9]

The clinical phenotypes associated with the different gene defects vary and partially overlap, but two major presentations have emerged: a hepatocerebral syndrome of infancy or early childhood characteristically accompanies mutations in *POLG*, *DGUOK*, *SUCLG1*, *SUCLA2*, *PEO1*, and *MPV17*, whereas mutations in *TK2* typically cause infantile or childhood myopathy. Mutations in the gene (*ECGF1*) encoding thymidine phosphorylase (TP) can be considered separately because they cause both multiple deletions and depletion of mtDNA and a distinctive clinical syndrome, mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). [17]

Despite this rapid progress, knowledge of the molecular basis of mtDNA depletion syndromes keeps expanding. The latest gene to be associated with mtDNA depletion is *RRM2B*, which encodes a p53-controlled ribonucleotide reductase subunit (p53R2) and has a crucial role in *de novo* nucleotide synthesis. [10] Mutations in *RRM2B* have been described in 7 infants from 4 families, who presented with various combinations of hypotonia, tubulopathy, seizures, respiratory distress, diarrhea and lactic acidosis. [10] All children died before 4 months of age. Muscle biopsy showed severe mtDNA depletion, combined respiratory chain enzyme defects, and, in one patient, abundant ragged-red fibers (RRF) and COX deficiency. As with all new diseases, it is important to establish their frequency and their clinical spectrum. To start this process, we have sequenced *RRM2B* in 9 patients with mtDNA depletion in muscle and without mutations in any of the genes listed above. We identified 5 novel mutations in three children. The first conclusion suggested by our data is that mutations in *RRM2B* are not very rare causes of mtDNA depletion syndrome, at least of the predominantly encephalomyopathic form, as we found them in 30% of molecularly undiagnosed cases.

A second conclusion regards clinical heterogeneity. Whereas the clinical picture of our patient 1, with neonatal onset of diarrhea, hypotonia, severe lactic acidosis, and death at 2 months, closely resembled the early onset and dramatic downhill course of the patients reported by Bourdon et al., [10] and especially their patient 7, our patients 2 and 3 had a distinctly more benign course dominated by encephalomyopathy and are alive and reasonably stable at 2 and 3 years of age. Notably, none of our patients had the proximal tubulopathy described in 5 of the 7 patients of Bourdon et al. and none had overt seizures.

The common clinical feature of all 10 patients with RRM2B deficiency described thus far is the myopathy with lactic acidosis. Only one of our patients had increased serum CK, a common characteristic of the myopathic form of mtDNA depletion, [18] but CK values were not reported by Bourdon et al. [10]

The degree of mtDNA depletion in muscle was very severe (<2% residual amount) in 4 patients studied by Bourdon et al. and in our patient 1, who had a similarly devastating course. Although very low (7.6 % and 11%), residual mtDNA contents in muscle were considerably higher in our patients 2 and 3, which, together with the generally better preserved respiratory chain function, may explain their milder course.

Finding five distinct mutations in addition to the seven already described by Bourdon et al. [10] shows that this form of mtDNA depletion is genetically heterogeneous, which is not surprising, especially considering the different ethnic backgrounds of our patients. Evidence for the pathogenic role of the *RRM2B* gene mutations encountered in our patients includes the following. First, our patients had severe mtDNA depletion in muscle that could not be attributed to any known gene other than *RRM2B*. Second, these mutations affected highly conserved sites and were not present in 192 chromosomes from North American subjects. Third, patients 1 and 2 were each compound heterozygotes for one missense mutation and one microdeletion, resulting in drastically curtailed and probably unstable proteins.

The pathogenic role of the homozygous c.671 T>G mutation in patient 1 is less obvious. This mutation changes a highly conserved isoleucine to a serine at amino acid position 224. Class I reductases, such as human ribonucleotide reductase, typically have a tyrosyl free radical in their structure, which is indispensable for enzyme activity. In the R2 subunit of *E. coli* ribonucleotide reductase, a tyrosyl radical is localized at amino acid 122, where it is stabilized by a nearby iron center. In the human p-53 controlled ribonucleotide reductase R2 subunit, the Tyr residue is buried within a structural pocket at position 138 and its –OH radical keeps it coupled to the magnetic field of the iron atom located at 5.3 Å. Thus, the tyrosine side chain establishes Van der Waals interactions with the side chains of neighboring residues. As shown by the predicted 3D structure of this enzyme, residue 224 is facing this Tyr138 (Figure 2). Given its proximity to Tyr138, the conserved serine at position 224 is probably part of the structural pocket responsible for the stabilization of the free radical. A change in the side chain of the serine at position 224 would distort the conformation and stability of the active site, impairing enzyme activity. Therefore, a change in the side-chain at this key position caused by the I224S mutation could alter the spatial situation of the free radical or the accessibility of Tyr138 to the iron center. The fact that this radical generator site is required for the first step of the enzyme reaction can explain the unusual severity of the mtDNA depletion and of the clinical phenotype.

It is easier to explain the pathogenic role of mutations that alter the reading frame and result in stop codons that abrogate the C-terminal domain of the protein (patients 2 and 3). These are likely to affect the interaction of the R2 and the R1 subunits, thus interfering with the active conformation of the enzyme.

Finally, the three missense mutations alter evolutionarily conserved amino acids (Figure 3) and also appear to be deleterious. The L317V mutation changes a leucine, which is not only itself highly conserved but is in close proximity with two other highly conserved amino acids, Ser316 and Tyr331, which are considered important for the R1-R2 interaction. The M282I mutation substitutes Met282 in the human R2 subunit, and the corresponding Met304Leu in the *E. coli* drastically impairs the activity of the ribonucleotide reductase. [19]

Although the molecular basis for the mtDNA depletion in muscle of six of the nine patients that we “revisited” remains obscure, our data show that mutations in *RRM2B* are relatively common causes of MDS.

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Figure 1. Modified Gomori trichrome stain of a muscle specimen (cross section) from patient 1 shows many ragged-red fibers (arrows). There are also numerous atrophic fibers and increased perimysial and endomysial connective tissue. X120.

Figure 2.

The 3D structure of the wild-type protein (left upper panel) shows in red the part of this subunit missing in the mutant protein (right upper panel) due to the nonsense L208X mutation in patient 3. A detail of the wild-type conformation (left lower panel) shows how the spatial proximity of residue Ile224 (highlighted in yellow) to the Tyr138 residue (highlighted in red) could explain the effect of the I224S mutation (right lower panel) on the free radical position in the active pocket.

Human					
p53R2				MGDPER	PEAAGLDQDE
Mouse				MGDPER	PEAARPEKGE
E.coli					
S.pombe	MGLEHLEEF	YPKEHGEEVE	YDSEQGVRKI	YVKSIIKETFN	FDNVSEEEKQ
Human	...SMTKAAA	PGVEDEPLLR	ENPRRFVIFP	IEYHDIWQMY	KKAEAS.FWT
p53R2	RSSSDTNESE	IKSNEEPLLR	KSSRRFVIFP	IQYPDIWKMY	KQAQAS.FWT
Mouse	QLCSETEENV	VRSNEEPLLR	KSSRRFVIFP	IQYPDIWRMY	KQAQAS.FWT
E.coli	.AYTTFSTQT	NDQLKEPMFF	GQPVNVARYD	QKKYDIFEKL	IEKQLSFFWR
S.pombe	EGGDYYLGKK	EDELDEVVLR	PNPHRFVLFP	IKYHEIWQFY	KKAEAS.FWT
Human	66	AEEVDLSKDI	QHWELSLKPE	ERYFISHVLA	FFAASDGIVN
p53R2		AEEVDLSKDL	PHWN.KLKAD	EKYFISHILA	FFAASDGIVN
Mouse		AEEVDLSKDL	PHWN.KLKSD	EKYFISHILA	FFAASDGIVN
E.coli		PEEVDVSRDR	IDYQ.ALPEH	EKHIFISNLK	YQTLDSIQG
S.pombe		AAEIDLKDL	VDWDNKLNAD	ERYFISTVLA	YFAASDGIVN
Human		VQITEARCFY	GFQIAMENIH	SEMYSLIDT	YIKDPKERE
p53R2		VQVPEARCFY	GFQILIENVH	SEMYSLIDT	YIRDPKKREF
Mouse		VQVPEARCFY	GFQILIENVH	SEMYSLIDT	YIRDPKKREF
E.coli		ISIPLELTWV	ETWAFSETIH	SRSYTHIIRN	IVNDPS...V
S.pombe		VQIPEARCVY	GFQIMIENIH	SETYSLLD	YIREPKKQR
Human		VKKKADWALR	WIGDKEATYG	ERVVAFAAVE	GIFFSGSFAS
p53R2		VKKKADWALR	WIADRKSTFG	ERVVAFAAVE	GVFFSGSFAS
Mouse		VKKKADWALR	WIADRKSTFG	ERVVAFAAVE	GIFFSGSFAS
E.coli		IQKRAEGISS	YYDELIEMTS	YWHLLGEGTH	TVNGKTVTVS
S.pombe		IKAKAKWALR	WINDEDSTYA	IRLVAFAAVE	GIFFSGSFAS
Human		PGLTFSNELI	SRDEGLHCDF	AC.LMPKHLV	HKPSEERVRE
p53R2		PGLTFSNELI	SRDEGLHCDF	AC.LMFQYLV	NKPSEERVRE
Mouse		PGLTFSNELI	SRDEGLHCDF	AC.LMFQYLV	NKPSEERVRE
E.coli		CLMSVNALEA	IR...FYVSF	ACSFAPAERE	LMEGNAKIIR
S.pombe		PGLTFSNELI	CRDEGLHTDF	AC.LMFSLHK	HRPGRKVVEA
Human		EFLTEALPVK	LIGMNCITMK	QYIEFVADRL	MELGFSKVF
p53R2		EFLTEALPVG	LIGMNCITMK	QYIEFVADRL	LVELGFSKVF
Mouse		EFLTEALPVG	LIGMNCITMK	QYIEFVADRL	LVELGFSKVF
E.coli		TGTQHMLNLL	RSGADDPFMA	EIAEACKQEC	YDLFVQAAQQ
S.pombe		EYFTDALPVS	LLGMNKDLMC	QYIEFVADRL	LVALGNDKYY
Human		NISLEGKTNF	FEKRVGEYQR	MGVMS....	SPTENSFTLD
p53R2		NISLEGKTNF	FEKRVSEYQR	FAVMA....	ETTDNVFTLD
Mouse		NISLEGKTNF	FEKRVSEYQR	FAVMA....	ETTDNVFTLD
E.coli		DGSMIGLNKD	ILCQVVEYIT	NIRMQAVGLD	LPFQTRSNPI
S.pombe		NISLAGKTNF	FEKKVSDYQI	AGVMSGTKRA	EKDDHTFTID

Figure 3.

Alignment of the R2 subunit in human and three other species showing the positions of the α -helices (denoted as A-H) and the three missense mutations found in our patients (wild-type amino acids are highlighted and circled in red). Note that all mutations affect evolutionarily highly conserved sites.

Table 1

Activities of respiratory chain enzymes ($\mu\text{mol}/\text{min}/\text{g}$ tissue) and residual mtDNA in muscle from the three patients and 69 control human muscles (control values $\pm$ SD).

I, II, III, IV denote complexes of the respiratory chain.

	Controls	Pt. 1	Pt. 2	Pt. 3
NADH-cyt. c reductase (I+III)	1.02 $\pm$ 0.38	0.31 (30%)	0.68 (67%)	0.74 (73%)
NADH dehydrogenase (I)	35.48 $\pm$ 7.07	14.97 (42%)	29.63 (83%)	21.12 (60%)
Succinate-cyt. C reductase (II+III)	0.70 $\pm$ 0.23	0.25 (36%)	0.63 (90%)	0.31 (44%)
Cytochrome c oxidase (IV)	2.80 $\pm$ 0.52	0.11 (4%)	1.99 (71%)	0.18 (6%)
Succinate dehydrogenase (II)	1.00 $\pm$ 0.53	3.58 (358%)	1.76 (176%)	1.12 (112%)
Citrate synthase		43.03 (435%)	9.01 (91%)	5.27 (53%)
Residual mtDNA, % of normal		1.8	11	7.6