Periventricular Calcification, Abnormal Pterins and Dry Thickened Skin: Expanding the Clinical Spectrum of *RMND1*?

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Abstract *Background:* We report a consanguineous Sudanese family whose two affected sons presented with a lethal disorder characterised by severe neonatal lactic acidosis, hypertonia, microcephaly and intractable seizures. One child had additional unique features of periventricular calcification, abnormal pterins and dry thickened skin.

Methods: Exome enrichment was performed on pooled genomic libraries from the two affected children and sequenced on an Illumina HiSeq2000. After quality control and variant identification, rare homozygous variants were prioritised. Respiratory chain complex activities were measured and normalised to citrate synthase activity in cultured patient fibroblasts. *RMND1* protein levels were analysed by standard Western blotting.

Results: Exome sequencing identified a previously reported homozygous missense variant in *RMND1* (c.1250G>A; p.Arg417Gln), the gene associated with combined oxidation phosphorylation deficiency 11 (COXPD11), as the most likely cause of this disorder. This finding suggests the presence of a mutation hotspot at cDNA position 1250. Patient fibroblasts showed a severe decrease in mitochondrial respiratory chain complex I, III and IV activities and protein expression, albeit with normal *RMND1* levels, supporting a generalised disorder of mitochondrial translation caused by loss of function.

Conclusions: The current study implicates *RMND1* in the development of calcification and dermatological abnormalities, likely due to defective ATP-dependent processes in vascular smooth muscle cells and skin. Review of reported patients with *RMND1* mutations shows intra-familial variability and evidence of an evolving phenotype, which may account for the clinical variability. We suggest that COXPD11

should be considered in the differential for patients with calcification and evidence of a mitochondrial disorder.

Introduction

Mitochondrial disorders are clinically heterogeneous and typically affect multiple organ systems. These disorders tend to be severely debilitating, progressive and often fatal. Mitochondrial disorders can be caused by mutations in the maternally inherited mitochondrial genome (mtDNA), which encodes 13 essential proteins of the oxidative phosphorylation (OXPHOS) system, or by mutations in one of >1,300 nuclear genes that encode mitochondrially targeted proteins (Koopman et al. 2012). Mitochondrial diseases have poor genotype-phenotype correlation, and most patients' clinical features do not fall discretely into any one particular syndrome/category, making molecular diagnosis challenging. The introduction of targeted multi-gene panel testing and exome sequencing has greatly increased molecular diagnostic yield (Taylor et al. 2014).

We report a consanguineous family of Sudanese origin whose two affected sons presented with a lethal syndrome characterised by severe neonatal lactic acidosis, hypertonia and intractable seizures (Fig. 1a). One child also had periventricular calcification, abnormal pterins and dry thickened and pigmented skin. After negative single-gene testing, whole-exome sequencing was undertaken to identify the cause of the suspected nuclear-driven mitochondrial disorder in this family.

Methods

Sample Collection

Written informed consent was obtained from the parents for publication of this report, including patient photographs. Genomic DNA was extracted from peripheral blood lymphocytes of the two affected children and unaffected parents. The study protocol was approved by the ethics committee of Temple Street Children's University Hospital (Dublin, Ireland).

Whole-Exome Sequencing

Whole-exome sequencing was undertaken for both affected children. Libraries were prepared from DNA of the two children and pooled in equal amounts (IntegraGen, France). The exonic DNA was enriched with the SureSelect v5 Human All Exon Kit (Agilent Technologies, Santa Clara) and sequenced on an Illumina HiSeq2000 (Illumina, San Diego, California, USA) at IntegraGen (France). The 100 bp paired-end reads were aligned to the hg19 human

reference genome. Quality control and variant identification were performed as previously described (Casey et al. 2015). Assuming an autosomal recessive model, we prioritised variants that were (i) autosomal; (2) absent or present with a frequency <1% in dbSNP130, NHLBI Exome Variant Server database and 1,000 Genomes; (3) homozygous; and (4) absent in our 60 control exomes (Supplementary Table S1). As the two patient samples were pooled pre-capture, only variants for which both affected children are homozygous are called as homozygous in the pooled sample.

Respiratory Chain Complex Analysis

Respiratory chain complex activities were measured and normalised to citrate synthase activity in cultured fibroblasts as previously described (Kirby et al. 2007). Cell lysates were prepared from patient fibroblasts (II:1) and analysed by Western blotting as previously described (Brito et al. 2015). Antibodies against RMND1 (Sigma HPA031399), NDUFB8 (Abcam ab110242), SDHA (Abcam ab14715), UQCRC2 (Abcam ab14745), COXI (Abcam ab14705), COXII (Abcam ab110258), ATP5A (ab14748) and VDAC1 (Abcam ab14734) were used, followed by HRP-conjugated secondary antibodies (DakoCytomation).

Case Reports

Patient II:1

Patient II:1 was born at term, birth weight 2.34 kg (0.4th centile) and occipitofrontal circumference 32 cm (0.4th centile). There was respiratory difficulty requiring ventilation. A pigmented skin rash was noted on the trunk. At 2 h of life, bilateral clonic seizures emerged. There was marked peripheral hypertonia with fisting of the hands. At 6 weeks there was microcephaly (34.5 cm <<0.4th centile), stiffness, excessive startle, rigid lower limbs, stiff flexed upper limbs, dry thickened skin and little spontaneous movement (Fig. 1b, c). There was no visual fixation or following. Thereafter, there was no neurodevelopment. The seizures were refractory to antiepileptic drug treatment with frequent bouts of status epilepticus, and the infant died at 11 months of age.

Investigations

Screen for congenital infections (toxoplasmosis, rubella, cytomegalovirus, herpes simplex and HIV) was negative. Metabolic acidosis was noted on initial blood gases. Blood count and film, blood glucose, serum calcium, serum magnesium, coagulation studies, immunoglobulins, abdominal and pelvic ultrasounds and skeletal survey were normal.

Fig. 1 Clinical, genetic and proteomic analyses. **(a)** Pedigree of a consanguineous Sudanese family who have had two affected sons and one healthy daughter. DNA was not available for II:3. **(b)** Photograph of patient II:1 showing stiffness in both arms, fisting of the hands and flexion of the toes. **(c)** Photograph of patient II:1 showing dry thickened skin with a pigmented skin rash. **(d)** Axial T1 MR image at day 7 of life shows posterior predominant ventricular dilation with bilateral symmetric white matter volume reduction. The periventricular calcifications detected by ultrasound are evident here as bilateral punctate hyperintensities. A focal area of hyperintensity in the left parietal parenchyma is in keeping with an acute haemorrhagic infarction. It was associated with restricted diffusion. **(e)** A CT scan at 4 weeks of age confirms the periventricular calcification bilaterally. Moderate ventricular dilation was unchanged. **(f)** The *RMND1*

NM_017909.3 c.1250G>A variant was validated by Sanger sequence analysis. Traces are shown for parent I:1 and affected child II:1. The inverted triangle indicates the position of the mutated G base which changes Arg (R) to Gln (Q) at residue 417. **(g)** Assessment of individual respiratory chain enzyme activities in fibroblasts identified severe OXPHOS deficiencies involving complexes I, III and IV with sparing of complex II activity in patient II:1 (blue bars) compared to controls (red bars). Mean enzyme activities shown for fibroblast controls ($n = 10$) are set at 100%. **(h)** Western blot analysis of cell lysates from control (C1 and C2) and patient II:1 (P) fibroblasts. Antibodies against RMND1, NDUFB8 (complex I), SDHA (complex II), UQCRC2 (complex III), COXI (complex IV), COXII (complex IV), ATP5A (complex V) and VDAC1 (mitochondrial loading control) were used

Cranial ultrasound (Day 1) showed moderate ventricular dilation with periventricular calcification. Magnetic resonance imaging (MRI) brain (Day 7) showed reduced white matter volume posteriorly and a small acute haemorrhagic infarct (Fig. 1d). Computed tomography (CT) of the brain at 4 weeks showed moderately dilated

lateral ventricles and confirmed the bilateral periventricular calcification (Fig. 1e). Electroencephalography (Day 10) showed slowing of the background with excessive sharp activity. This evolved over the following months to a severely encephalopathic pattern with periodic lateralised epileptiform discharges.

Analysis of urine organic acids identified increased excretion of lactate with marked ketonuria (3-hydroxybutyrate), mild dicarboxylic aciduria and hydroxydicarboxylic aciduria and increased excretion of tricarboxylic acid cycle intermediates fumarate and malate, suggestive of a mitochondrial disorder. Blood lactate was 2–6 mmol/L (normal range 0.6–2.4 mmol/L), CSF lactate was 5.7 mmol/L (normal range 1.0–2.2 mmol/L), and blood lactate-pyruvate ratio was increased at 28, pointing towards an oxidative phosphorylation defect. Assessment of fatty acid β -oxidation flux in cultured fibroblasts showed abnormalities consistent with a primary defect of the mitochondrial respiratory chain (Olpin et al. 1997).

Analysis of cerebrospinal fluid (CSF) neurotransmitters identified abnormal pterins, neopterin 375 (7–65 nmol/L), dihydroneopterin 16.5 (0.4–13.9 nmol/L) and low vitamin B6 at 22 nmol/L (44–89 nmol/L). Serum and CSF alpha interferon levels were normal.

Chromosome analysis showed a normal 46XY karyotype. A number of diagnoses were considered including small-vessel brain disease (*COL4A1*-related disorder) and Aicardi-Goutieres syndromes because of the calcification and abnormal CSF pterins. However, clinical sequencing of *COL4A1*, *TREX*, *RNASEH2B*, *RNASEH2C*, *RNASEH2A* and *SAMHD1* did not identify any pathogenic variants.

Patient II:2

During the second pregnancy, the mother had pedal oedema, high blood pressure and fever at week 33. Regular fetal ultrasound scans did not detect intracranial calcifications. Patient II:2 was born at term by emergency caesarean section due to fetal bradycardia and required resuscitation. From birth, he showed almost identical clinical manifestations to his older brother (II:1): seizures on day 1, axial hypotonia and lactic acidosis. Dysmorphic features included a relatively large anterior fontanelle, small toes and small suboptimally curved pinna. Seizures, initially focal clonic and later myoclonic and tonic, were noted within 1 h of life. Cranial ultrasound on day 1 of life showed a speckled appearance raising the possibility of precalcification, though quality was suboptimal. CT brain scan did not identify any clear evidence of calcification. Despite supportive care, he died on day 4 of life.

Results

Exome Sequencing

Variant prioritisation identified 16 novel or rare homozygous variants shared by both affected children (Supplementary Table S3). Only one of the 16 genes is known to be

mitochondrial, *RMND1* NM_017909.3 c.1250G>A, p.(Arg417Gln). Validation and segregation analysis of the *RMND1* c.1250G>A variant was undertaken by polymerase chain reaction and bidirectional Sanger sequencing (Supplementary Table S2). Sanger sequencing confirmed that both affected brothers are homozygous for the c.1250G>A *RMND1* variant and the parents as obligate carriers (Fig. 1f).

RMND1 encodes a member of the evolutionary conserved sif2 protein family which localises to the mitochondria and is involved in mitochondrial translation (Janer et al. 2012; Garcia-Diaz et al. 2012). Mutations in *RMND1* have been associated with a severe neonatal encephalomyopathy termed combined oxidative phosphorylation deficiency 11 (COXPD11; *614917). COXPD11 is an autosomal recessive disorder characterised by deficiencies of multiple respiratory chain complexes leading to neonatal hypotonia and lactic acidosis.

Respiratory Chain Complex and RMND1 Protein Analyses

Analysis of patient fibroblasts showed a severe biochemical defect involving respiratory chain complexes I, III and IV with sparing of complex II (Fig. 1g), in accordance with a generalised disorder of mitochondrial translation. Steady-state levels of RMND1 protein were similar in patient fibroblasts compared to controls, despite a decrease in protein levels of complex I (NDUFB8) and complex IV (COXI and COXII) subunits (Fig. 1h). These data are consistent with previous work showing that the steady-state levels of RMND1 were unchanged, but formation of an RMND1 complex was impaired in a patient with the same p.(Arg417Gln) mutation (Janer et al. 2012), supporting a loss-of-function mechanism.

Discussion

We report a homozygous missense p.(Arg417Gln) variant in *RMND1* in two Sudanese brothers with a lethal mitochondrial disorder presenting as neonatal lactic acidosis, hypertonia and intractable seizures. The identified *RMND1* c.1250G>A, p.(Arg417Gln) variant has previously been reported by Janer and colleagues in a consanguineous sib-pair of Pakistani origin (Janer et al. 2012). Finding the same disease variant in a second population suggests the presence of a mutation hotspot at cDNA position 1250, given the absence of any known shared ancestry between the Pakistani and Sudanese populations.

To date, 12 patients (8 families) with 4 different *RMND1* mutations have been reported in the literature (Table 1) (Garcia-Diaz et al. 2012; Janer et al. 2012; Taylor et al. 2014). The core features of lactic acidosis, hypertonia and

Table 1 Clinical and biochemical features of patients with *RMND1* mutations

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
Age of onset	2 months	6 days	18 days	n.r.	n.r.	n.r.	6 months	3 months	18 months	6 months	<1 month	18 months	1 day	1 day
Encephalopathy	+	+	+	+	+	+	—	+	+	—	—	—	+	+
Severe lactic acidosis	+	+	+	+	+	+	+	+	+	—	+	—	+	+
Intractable seizures	+	+	+	—	—	—	n.r.	n.r.	n.r.	n.r.	n.r.	+	+	+
Microcephaly	+	+	—	—	—	—	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	+
Hypotonia	n.r.	+	+	+	+	+	+	+	+	+	+	+	+	+
Respiratory failure at birth	—	—	+	+	+	+	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	+
Death	—	—	—	—	—	—	+	+	+	+	+	+	—	—
Tongue fasciculations	n.r.	n.r.	+	—	+	—	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	—
Renal tubular acidosis	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	—	—	—	+	—	+	—	—
Renal dysplasia	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	+	+	—	—	—	—	—
Cardiac anomaly	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	—	—	+	+	—	—	—	—
Bilateral equinus foot deformity	n.r.	n.r.	+	+	n.r.	+	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	—	—
Other	—	—	abs ten ref	—	abs Moro ref, musc skel def	—	—	↓ Hb	—	—	—	—	pv calc, abn pterins, dry thick skin	Large ant font, small toes, small pinna
Blood lactate (0.63–2.44 mM)	n.r.	↑ 3.56	↑ 3.2	↑ 3.5	↑ 2.5–8.4	↑ 5.7	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	↑ 2–6	↑ 3.9
OXPHOS	n.r.	↓ C I, III–V	n.r.	↓ C I–IV	n.r.	n.r.	↓ C I, III, IV	↓ C I, IV	↓ C I, III, IV	↓ C I, III, IV	↓ C I, III, IV	↓ C I, CIV	↓ C I, III, IV	n.p.
Age at time of report/death (†)	13 months†	5 months†	18 months†	12 days†	8 months	4 months	4 years	1 year†	5 years	10 years†	18 months	5 years†	11 months†	4 days†

Comparison of the clinical and biochemical profiles of patients with *RMND1* mutations. Information on previously reported patients was obtained from the studies of Janer et al., Garcia-Diaz et al. and Taylor et al. Note that blood ammonia, urine amino acids and lysosomal enzymes were normal where reported

Abbreviations: ↑, increased compared to normal; ↓, decreased compared to normal; n.p., test not performed; n.r., information not reported; abs ten ref, absent tendon reflexes; abs Moro ref, absent Moro reflex; ant font, anterior fontanelle; ↓ Hb, iron deficient anaemia; musc skel def, muscular skeletal deformities; pv calc, periventricular calcification. Patient identifiers: A, Janer P3; B, Janer P4; C, Garcia-Diez VI-1; D, Garcia-Diez VI-3; E, Garcia-Diez VI-7; F, Garcia-Diez VI-8; G, Taylor P1; H, Taylor P2; I, Taylor P3; J, Taylor P4; K, Taylor P5; L, Taylor P6; M, Current study II-1; N, Current study II-2. Note that the stillborn baby (VI-9) with *RMND1* mutation reported in the study by Garcia-Diaz is not included in this table; † indicates the age of the child when they died (deceased)

seizures are common to all patients. In addition, some patients may have other features including deafness, renal dysplasia and cardiac anomalies. As most of the patients with reported deafness are the oldest of the *RMND1* cohort, it is likely that *RMND1* mutation causes an evolving phenotype where clinical signs manifest with age.

While the patients in this study have the core clinical features of COXPD11, one child (II:1) has a number of additional features not previously associated with COXPD11, namely, periventricular calcification, abnormal pterins and dry thickened skin. Are these features a direct result of the *RMND1* mutation, or could they be due to a second genetic disorder in the eldest patient? To investigate the possibility of a second disorder, we analysed the exome data to look for dominant or recessive variants in genes associated with one or more of the following clinical features; brain calcification, abnormal pterins and dry thickened skin (Supplementary Table S4). We did not identify any rare potentially pathogenic variants in these genes, including Aicardi-Goutieres syndrome genes, which could account for the extra clinical features in the eldest patient. However, we cannot fully exclude the possibility of a mutation in an as yet uncharacterised calcification/dermatological gene or a mutation outside of the exome.

There is evidence in the literature to support a causal role for the *RMND1* mutation in both calcification and dermatological abnormalities. Firstly, there are three previous reports of patients with combined OXPHOS defects and brain calcifications (thalamic, cerebral and basal ganglia) (Kaiming et al. 2014; Robinson et al. 1992; Van Straaten et al. 2005). The relationship between impaired COX activity and excessive vascular calcification has been demonstrated in mutant *Lmna* mice (Villa-Bellosta et al. 2013). The current study suggests that *RMND1* could also be involved in the development of calcification due to defects in oxidative phosphorylation in vascular smooth muscle cells, though it is likely to be a rare feature. Given the speckling observed on cranial ultrasound in the second baby (patient II:2), we postulate that calcification might have developed had he lived longer. It is not unusual for there to be differences in the timing of emergence of clinical features in autosomal recessive disorders.

Secondly, patient II:1 was noted to have pigmentation and dry thickened stiff skin. Histological examination of a skin biopsy was normal. However, the underlying fascia was not biopsied. There are known interactions between mitochondria and cytokeratins, and 10% of patients with mitochondrial disorders present with dermatological features including hair abnormalities, rashes, pigmentation abnormalities and acrocyanosis (Feichtinger et al. 2014). Given the numerous ATP-dependent processes occurring in the skin, it is not surprising that mutations affecting mitochondrial function cause dermatological features. However, it is not clear why

only a subset of patients with OXPHOS deficiencies develop skin changes. Indeed, in this study there was intra-familial variability (not uncommon in mitochondrial disease) with intracranial calcification and dermatological abnormalities present in only one of the two siblings.

In conclusion, our study implicates *RMND1* in the development of calcification and dermatological features and highlights the variable and progressive nature of COXPD11.

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Synopsis

RMND1 mutation can cause calcification, abnormal pterins and dermatological features and should be considered in the differential for patients with mitochondrial disorders that include calcification.

Compliance with Ethics Guidelines

Conflict of Interest

Jillian P. Casey, Ellen Crushell, Kyle Thompson, Eilish Twomey, Langping He, Sean Ennis, Roy K. Philip, Robert W. Taylor, Mary D. King and Sally Ann Lynch declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from the patient's parents for inclusion in the

study. Additional informed consent was obtained from the parents to include identifying information in this article.

Author Contributions

JPC, SAL and EC were responsible for the study concept and design and obtained funding. EC, RKP, MDK and SAL performed clinical assessment and diagnostic investigations and were responsible for obtaining clinical samples and patient management. EC, ET and MDK reviewed and interpreted the biochemical, radiological and neurological findings, respectively. JPC analysed and interpreted the exome data and did a literature review on previous patients. EC, JPC, MDK, SE and SAL assessed the genetic findings in relation to the patient phenotype. KT, LH and RT analysed patient fibroblasts for respiratory chain defects and altered mitochondrial protein levels and interpreted their findings. JPC drafted the manuscript, and all authors were involved in critical revisions.

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