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NUBPL mitochondrial disease: new patients and review of the genetic and clinical spectrum

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ABSTRACT

Background The *NUBPL* gene was first reported as a cause of mitochondrial complex I deficiency (MIM 613621, 618242) in 2010. To date, only eight patients have been reported with this mitochondrial disorder. Five other patients were recently reported to have *NUBPL* disease but their clinical picture was different from the first eight patients. Here we report clinical and genetic findings in five additional patients (four families).
Methods Whole exome sequencing was used to identify patients with compound heterozygous *NUBPL* variants. Functional studies included RNA-Seq transcript analyses, missense variant biochemical analyses in a yeast model (*Yarrowia lipolytica*), and mitochondrial respiration experiments on patient fibroblasts.
Results The previously reported c.815-27T>C branch-site mutation was found in all four families. In prior patients, c.166G>A [p.G56R] was always found *in cis* with c.815-27T>C, but only two of four families had both variants. The second variant found *in trans* with c.815-27T>C in each family was: c.311T>C [p.L104P] in three patients, c.693+1G>A in one patient, and c.545T>C [p.V182A] in one patient. Complex I function in the yeast model was impacted by p.L104P but not p.V182A. Clinical features include onset of neurological symptoms at 3-18 months, global developmental delay, cerebellar dysfunction (including ataxia, dysarthria, nystagmus, and tremor), and spasticity. Brain magnetic resonance imaging showed cerebellar atrophy. Mitochondrial function studies on patient fibroblasts showed significantly reduced spare respiratory capacity.
Conclusion We report on five new patients with *NUBPL* disease, adding to the number and phenotypic variability of patients diagnosed worldwide, and review prior reported patients with pathogenic *NUBPL* variants.

INTRODUCTION

Complex I deficiency is the third most common mitochondrial disorder and is highly heterogeneous, clinically and genetically. Human complex I has 44 different subunits that are encoded by nuclear and mitochondrial genes[1]. To date, pathogenic variants have been identified in 34 genes[2] and a molecular diagnosis is commonly achieved in ~50% of affected individuals[3]. Additionally, there are at least 16 genes that play a role in the assembly of complex I, of which 12 have been identified to cause autosomal recessive disease[4, 5]. One such assembly factor is nucleotide binding protein like (*NUBPL*), which was first identified as Ind1 in the yeast *Yarrowia lipolytica*[6]. Depletion of human or yeast NUBPL protein leads to decreased complex I activity[6, 7].

Variants in the *NUBPL* gene (MIM 613621) were first associated with complex I deficiency (MIM 618242) in 2010 in a whole exome sequencing (WES) study of over 100 patients with clinical and biochemical evidence of complex I deficiency[3]. One patient was identified initially with a point mutation in the paternal copy of *NUBPL* (c.166G>A [p.G56R]), while the maternal copy had a large chromosomal rearrangement that disrupts *NUBPL*. However, a protein variant carrying only the p.G56R substitution was able to rescue complex I activity in patient fibroblasts, suggesting it was not pathogenic[8]. Further sequence analysis revealed an intronic variant, c.815-27T>C on the paternal copy, which affects a splicing branch site[3, 8]. A subset of the c.815-27T>C transcripts lack exon 10, leading to a frameshift and truncated protein product. Interestingly, the c.815-27T>C variant is found in ~1% of European ancestry subjects (Finnish plus non-Finnish) in the Genome Aggregation Database (gnomAD)[9].

Subsequently, six additional patients were identified from a magnetic resonance imaging (MRI) database of more than 3,000 subjects with unclassified leukoencephalopathy. These cases were recognized by specific MRI features including diffuse leukodystrophy involving the cerebellar cortex, periventricular deep and subcortical white matter and corpus callosum with some cystic changes[10]. All patients from this study had c.815-27T>C *in cis* with c.166G>A plus a second deleterious *NUBPL* variant *in trans*, with the exception of one patient that is presumed to be homozygous for c.815-27T>C and c.166G>A. With recent reports on three more

families, there are now 14 patients (in 11 families) reported to have *NUBPL* disease[3, 10-15]. Here, we now report clinical features of five additional patients in four families, plus functional and biochemical experiments as supporting evidence for complex I deficiency in these patients. We also review the spectrum of genotypes and phenotypes found in all known cases of *NUBPL* disease.

MATERIALS AND METHODS

Patients

Institutional Review Board (IRB) approval was obtained from the University of California, Irvine for this study. Informed consents were obtained from the parents of the children who participated in this study, in addition to consent from one patient over the age of 18 years.

WES was used for identification of the *NUBPL* variants at Baylor Genetics (Houston, TX), at GeneDx (Gaithersburg, MD), or as described previously[12, 16, 17], with the exception of Patient 1B (Sanger sequencing). Thoughout the manuscript, all *NUBPL* variants are described based on NM_025152.3. Brain MRIs for all patients and a cerebellar brain biopsy for Patient 1A were provided by the referring physicians.

RNA splicing analysis

Splicing analysis of *NUBPL* variant c.815-27T>C was performed using CloneSeq, an RNA sequencing (RNA-Seq) assay based on cloning of RT-PCR products followed by massively parallel sequencing of the cloned transcripts[18]. Briefly, total RNA was isolated from whole blood from the proband, both parents, an affected sibling, and an unaffected sibling of Family 1. RT-PCR was perfomed on cDNA derived from mRNA only using the following primers: 5'-ATGGTATTGCTTGTATGTCTATGG-3', and 5'-GTTCCATCACCACATTGCTG-3'. Transcript levels (Sashimi plots) and the percentage of aberrantly spliced transcripts was determined as described previously[18].

Mitochondrial respiration assays

The Seahorse assay system (Agilent, Santa Clara, CA), which directly measures the oxygen consumption rate (OCR), was used for mitochondrial respiration studies[19]. Human fibroblasts from three *NUBPL* patient cell lines (patients 1A, 1B, and 2) and one control line were cultured in DMEM supplemented with 10% fetal bovine serum, 5 mM Glucose, 1 mM pyruvate, and 1X non-essential amino acids, penicillin, streptomycin, and amphotericin B (Gibco). The day prior to the assay, patient and control fibroblasts were seeded onto 24-well Seahorse XF24 Cell Culture Microplates at a density of 30,000 cells/well (~$1x10^5$ cells/cm^2) and incubated overnight in a humidified 5% CO_2 37°C incubator.

After 18 hours incubation, cells were assayed using a Seahorse XF Cell Mito Stress Test Kit and XFe24 analyzer according to the manufacturer's protocol[19]. Cellular bioenergetic profiles were measured by three serial injections of four reagents at 1 μM final concentration each: oligomycin, which inhibits ATP synthase (complex V); carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), a proton ionophore that induces uncoupling of ATP synthesis from electron transfer and is thus a measure of maximal respiration; and a mixture of rotenone plus antimycin A, which completely inhibits electron transport to enable measurement of non-mitochondrial respiration. Data were normalized to total protein per well using the Bio-Rad DC protein assay. The XF Mito Stress Test report generator was used to calculate basal respiration, ATP turnover rate, proton leak, and maximal and spare respiratory capacity for each cell line (patient's fibroblasts were assayed in triplicate).

Yeast model functional studies

The functional effect of the *NUBPL* variants was tested in *Yarrowia lipolytica* as previously described[12, 20].

RESULTS

We report on genetic and clinical findings in four families, three of which contain individuals with mitochondrial dysfunction symptoms consistent with complex I deficiency due to autosomal recessive *NUBPL* mutations. Pedigree charts for the

families are shown in Figure 1A (Family 1 has two patients) and brain MRIs for the patients are shown in Figure 1B. Cerebellar brain biopsy results are shown in Figure 1C for Patient 1A. Detailed clinical descriptions for each patient are reported in the Supplementary Data file. Below, molecular, clinical, and functional findings are reported for the five new patients plus genotype-phenotype comparisons to all previously reported *NUBPL* patients.

Molecular diagnosis of new patients

Genetic analysis using WES revealed compound heterozygous variants in the *NUBPL* gene (Tables 1 and 2). All patients were heterozygous for the common c.815-27 T>C variant (present in ~1% of European ancestry subjects) found in the first reported case[3]. The missense variant c.166G>A [p.G56R], present *in cis* with c.815-27T>C in all previously reported cases, was found in Families 2 and 3 (Figure1A, Table 1). However, we report, for the first time, patients without c.166G>A, see Families 1 and 4 (Figure 1A, Table 1).

Missense variant c.311T>C [p.L104P] was found *in trans* with c.815-27T>C in Families 1 and 3. It is predicted to be damaging/deleterious (Table 2) and was shown to be pathogenic in a yeast model[12]. It was first reported in a 2014 WES study[16] to cause complex I deficiency in Patient 1A; however, no clinical details were provided. Subsequently, it was reported for two conditions[11, 13] with overlapping phenotypes (see below) wherein patients are compound heterozygotes with missense variants p.D96V or p.F242L (Tables 1, 2) instead of c.815-27T>C.

In Family 2, the patient was found to be compound heterozygous for previously reported variants[10], c.815-27T>C and c.166G>A *in cis* plus the splice donor mutation c.693+1G>A *in trans* (Figure 1A, Table 1). In Family 4, the patient was found to be compound heterozygous for c.815-27T>C and newly reported variant c.545T>C [p.V182A], which is predicted to be damaging/deleterious (Table 2) but our yeast model *in vitro* functional assays (see below) do not support its pathogenicity.

Table 1. Clinical summary for 5 new patients and previous 14 patients reported with autosomal recessive *NUBPL* disease.

Family Country	Patient	Age[a] (yr)	Age of onset (mo)	Gender	*NUBPL* variants	Inh.[b]	Dev. delay	Clinical features[c]	Cerebellar atrophy (MRI)[d]	CI def. defect[e]	High lactate[f]	Other clinical details[g]	Ref
New patients – compound heterozygotes with c.815-27T>C													
1 USA	1A	16	3	F	c.815-27T>C (no p.G56R) c.311T>C [p.L104P]	pat na mat	+	A, D, N, T	+, +	na, –	na, na		16, New[h]
	1B	6	3	F			+	A, D, N, T	+, +	na, na	na, na		New
2 USA	2	7	13	F	c.815-27T>C c.166G>A [p.G56R] c.693+1G>A	mat mat pat	+	A, T	+	–, na	na, na	INAD considered	New
3 USA	3	19	18	M	c.815-27T>C c.166G>A [p.G56R] c.311T>C [p.L104P]	mat mat pat	+	A, D, N, S	+	na, –	na, +	Leigh-like	17, New[h]
Suspected patient – compound heterozygote with c.815-27T>C													
4[i] USA	4	3	birth	M	c.815-27T>C (no p.G56R) c.545T>C [p.V182A]	mat na pat	+	N	–	na, na	–, na	16p12.1 deletion	New
Previously reported patients – compound heterozygotes with c.815-27T>C													
5[j] Australia (NZ)	5	12	24	M	c.815-27T>C c.166G>A [p.G56R] del/dup (240Kb/137Kb)	pat pat mat	+	A, D, N, S	–	+, +	+, +		3, 8
6[k] France	6	23	toddler	M	c.815-27T>C (p.G56R genotype unknown) c.205_206delGT [p.V69Yfs*80]	unk unk unk	+	A, D, N	+	na, +	na, na		14
7[l] Argentina	7	9	8	M	c.815-27T>C c.166G>A [p.G56R]	unk unk	+	A, N, S	+	na, na	–, na		10

					(hemi. or hom. suspected)								
8	8	12	8	M	c.815-27T>C	pat	+	A, D, N, S	–, +	+, na	+, +		10
Germany					c.166G>A [p.G56R]	pat							
					c.667_668ins[m] [p.E223Afs*4]	mat							
9	9A	9	13	F	c.815-27T>C	pat	+	A, D, N	+	+, –	–, na		10
Canada					c.166G>A [p.G56R]	pat							
					c.313G>T [p.D105Y]	mat							
	9B	7	13	F			+	A, D, N	–, +	+, +	+, +		
10	10	7	21	F	c.815-27T>C	pat	+	A, D, N, S	–	na, na	+, na		10
USA					c.166G>A [p.G56R]	pat							
					c.693+1G>A	unk							
11	11	4	10	F	c.815-27T>C	unk	+	A, D, S	–	+, +	+, +		10
Netherlands					c.166G>A [p.G56R]	unk							
					c.579A>C [p.L193F]	unk							
12	12	na	na	na	c.815-27T>C	unk	na	na	na	na, na	na, na		12
Germany					(p.G56R genotype unknown)	unk							
					c.859G>T [p.G287C]	unk							
Previously reported patients – compound heterozygotes without c.815-27T>C													
13	13A	25	toddler	F	c.311T>C [p.L104P]	mat	+	D, H	+	na, na	na, na	dystonia,	11
UK					c.287A>T [p.D96V]	pat						BSN	
	13B	17	toddler	F			+	A, T	+	na, na	na, na	dystonia,	
												BSN	
14	14	13	18	F	c.311T>C [p.L104P]	mat	+	H	–, –	na, +	+, na	multi-	13
UK					c.726C>G [p.F242L]	pat						systemic	
15[n]	15A	2	4	M	c.693+1G>A	pat	+	H, N, S	+, +	+, na	+, na	thalamic	15
USA					c.351G>A [p.M117I]	mat						involvement	
	15B	2	4	M			+	H, N, S	+, +	+, na	+, na	thalamic	
												involvement	

[a] Age at last clinical assessment (rounded to whole year)

[b] Inheritance (Inh.) indicated as paternal (pat), maternal (mat), unknown (unk); not applicable (na) listed for patients that did not have the c.166G>A [p.G56R] variant

[c] Common clinical features: A = ataxia, D = dysarthria, H = hypotonia, N = nystagmus, S = spasticity, T = tremor (intention)

[d] Cerebellar atrophy determined from early and/or late MRI (a single entry indicates only one MRI was available/done): + = present, – = absent, na = MRI not available or not done

[e] Complex I (CI) deficiency (def.) defect reported for fibroblast (first entry) or muscle (second entry): + = present, – = absent, na = test data not available or not done

[f] High lactate reported for plasma (first entry) or CSF (second entry): + = present, – = absent, na = test data not available or not done

[g] Other clinical details: Family 2 patient, infantile neuroaxonal dystrophy 1 (INAD) considered but genetic testing was negative; Family 13 patients have dystonia and bilateral striatal necrosis (BSN); Family 14 patient is reported to have multi-systemic involvement, including renal tubular acidosis, osteoporosis, hepatomegaly, growth hormone deficiency (short stature present), and interstitial lung disease

[h] Patient mutations previously reported but without clinical information: Patient 1A, Farwell et al. 2015 Supplemental data[16]; Patient 3, Posey et al. 2016 Supplemental data[17]

[i] Patient 4 was suspected to have *NUBPL* disease, but his brain MRI and p.V182A functional experiments (yeast model) are not supportive of complex I deficiency (see text)

[j] Family 5 was reported to be from Australia by Kevelam et al. 2013[10], but is actually from New Zealand (NZ) per personal communication from D. Thorburn

[k] Family 6 patient's c.815-27T>C variant was reported as c.815-217T>C (likely a typographical error for c.815-27T>C) and p.D273Qfs*32 (likely p.D273Qfs*31), see Tenisch et al. 2012[14]

[l] Family 7 patient is thought to be hemizygous or homozygous for c.166G>A [p.G56R] and c.815-27T>C [p.D273Qfs*31], see Discussion

[m] Full variant name is c.667_668insCCTTGTGCTG

[n] Patients 15A and 15B are monozygotic twins

Table 2. Variants reported in autosomal recessive *NUBPL* disease[a]: functional impact and population frequency.

DNA variant	Protein effect	Functional impact		Family number (Table 1)													gnomAD EUR subjects[h]	
		in silico[b]	Yeast model[c]	5	7[d]	6[e]	13	14	1	3	9	15	4[f]	11	8	2	10	12[g]	Hom/Het/Total	Allele frequency
Del/Dup	Disrupted	LOF	na	+															Novel	0
c.166G>A	p.G56R	+, +	na	+	+	?				+	+			+	+	+	+	?	1/37/64,144	0.0003040
c.205_206delGT	p.V69Yfs*80	LOF	na			+													0/0/56,617	0
c.287A>T	p.D96V	+, –	na				+												Novel	0
c.311T>C	p.L104P	+, +	+, +				+	+	+	+									0/30/64,317	0.0002332
c.313G>T	p.D105Y	+, +	+, +								+								0/5/64,314	0.0000389
c.351G>A	p.M117I	+, +	na									+							Novel	0
c.545T>C	p.V182A	+, +	–, –										+						1/541/64,243	0.0042260
c.579A>C	p.L193F	+, +	+, +											+					Novel	0
c.667_668ins[i]	p.E223Afs*4	LOF	na												+				Novel	0
c.693+1G>A	Splicing defect[j]	LOF	na									+				+	+		0/3/56,513	0.0000265
c.726C>G	p.F242L	+, +	na					+											0/3/56,090	0.0000267
c.815-27T>C	Splicing defect[j]	LOF	+, +	+	+	+			+	+	+		+	+	+	+	+	+	3/568/64,114	0.0044760
c.859G>T	p.G287C	+, +	–, +															+	Novel	0

[a] See Table 1 Reference column (Ref) for patient case report citations.

[b] *In silico* prediction (LOF or Polyphen2, SIFT): loss-of-function (LOF); Polyphen2, + = possibly or probably damaging, – = benign; SIFT, + = deleterious, – = tolerated

[c] Yeast model (*Yarrowia lipolytica*) study, see Maclean et al. 2018[12] and Figure 4: first entry, + = severely or slightly decreased complex I level, – = normal complex I level; second entry, + = impaired growth in cold, – = normal growth in cold; na = not applicable or not done

[d] Family 7 patient is thought to be hemizygous or homozygous for c.166G>A [p.G56R] and c.815-27T>C [p.D273Qfs*31], see Discussion

[e] Family 6 patient's c.166G>A [p.G56R] genotype is unknown (?); his c.815-27T>C variant was reported as c.815-217T>C (likely a typographical error for c.815-27T>C) and p.D273Qfs*32 (likely p.D273Qfs*31), see Tenisch et al. 2012[14]

[f] Patient 4 was suspected to have *NUBPL* disease, but his brain MRI and p.V182A functional experiments (yeast model) are not supportive of complex I deficiency (see text)

[g] Family 12 patient's c.166G>A [p.G56R] genotype is unknown (?)

[h] gnomAD is the Genome Aggregation Database, see Karczewski et al. 2019[9]: EUR subjects correspond to Non-Finnish European (NFE) ancestry; population information reports the number of homozygous (Hom), heterozygous (Het), and total (Total) subjects with the variant and the corresponding allele frequency

[i] Full variant name is c.667_668insCCTTGTGCTG

[j] Splicing defects are: c.693+1G>A impacts a splice donor site; c.815-27T>C is a branch migration variant that causes skipping of exon 10 and a frameshift (p.D273Qfs*31)

Clinical findings of new patients

Primary clinical features of Patients 1-5 (3 females and 2 males) are summarized in Table 1; detailed clinical descriptions for each patient are provided in the Supplementary Data file. All patients are of European ancestry (USA-based) and at last evaluation are aged 3-19 years. Onset of neurological symptoms ranged from birth to 18 months and included global developmental delay, cerebellar dysfunction (ataxia, dysarthria, dysmetria, nystagmus, and intentional tremor), hyperreflexia with clonus, and, in Patient 3, a Leigh-like phenotype. Patient 2 was initially thought to have infantile neuroaxonal dystrophy 1 (*INAD*, MIM 256600) and Patient 4 also has a 16p12.1 deletion[21], which may be contributing to his symptoms.

All five patients had delays in their gross motor skills and are able to walk with assistance. Gaits of Patients 1A and 2 were described as wide-based and ataxic, while Patient 3 walked with a narrow/scissoring (diplegic) gait. All patients have difficulties with their fine motor skills (especially with tremor) except Patient 2, whose tremor has improved with the help of occupational therapy and, potentially (parental report, see Supplementary data), from treatment with EPI-743. Patients 1A and 4 have language delay while the others have slurred speech. Patient 3 has right sensorineural hearing loss. Cognition level varies, but is impaired for all except for Patient 2 who has a normal IQ. Brain MRIs for all five patients are shown in Figure 1B. Cerebellar atrophy was noted in all patients except for Patient 4. Cerebellar biopsy results for Patient 1A are shown in Figure 1C.

Functional studies of new patients

Aberrant splicing is observed for *NUBPL* c.815-27T>C carriers

Transcript variants in Family 1 carriers and non-carriers of *NUBPL* c.815-27T>C were assessed by CloneSeq analysis[18]. The two most abundant abnormal transcripts were r.815-72_815-1ins72 (partial intron 9 inclusion) and r.815_897del83 (exon 10 skipping), as shown in the Sashimi plots (Figure 2A). Transcript variant r.815-72_815-1ins72 was

essentially not observed in non-carriers of c.815-27T>C, but was found in the carriers (observed reads were 239 for Sister+, 198 for Proband+, and 129 for Father+). Whereas transcript variant r.815_897del83 was observed in all members of Family 1, but higher levels were found in carriers of c.815-27T>C (observed reads were 629 for Sister+, 410 for Proband+, and 801 for Father+) compared to non-carriers (observed reads were 245 for Mother- and 59 for Sister-). Relative levels of the two transcript variants, r.815-72_815-1ins72 and r.815_897del83, are reported as PSI (Figure 2B), a measure of how efficiently sequences of interest are spliced into transcripts. This analysis shows that 5-20% of transcripts expressed by c.815-27T>C carriers contain partial intron 9 inclusion (r.815-72_815-1ins72) but it is not observed in non-carriers. Skipping of exon 10 (r.815_897del83) was observed in ~25% of transcripts expressed by the carriers, but only ~5% of non-carriers. These results are consistent with aberrant splicing data previously reported for a complex I deficiency patient with the c.815-27T>C variant[8].

Mitochondrial function is impaired in patient fibroblasts

Fibroblasts from three *NUBPL* patient cell lines (Patients 1A, 1B, and 2) and one control cell line were used to perform *in vitro* mitochondrial respiration studies (Figure 3). Basal respiration was not significantly different in the *NUBPL* patient fibroblast cells compared to control cells, nor was ATP production (post-oligomycin injection) or non-mitochondrial respiration (post-rotenone + antimycin A injection). However, spare respiratory capacity, which is the difference between maximal respiration (post-FCCP injection) and basal respiration, was significantly reduced in all three *NUBPL* patient fibroblast cells as compared to the control cells (Figure 3B). This is in contrast to the negative results found by the clinical electron transport chain analyses of Patient 1A's muscle or Patient 2's fibroblasts (see Table 1).

Functional studies of *NUBPL* variants in the yeast model *Yarrowia lipolytica*

The c.815-27T>C variant, found in all four families, was previously shown to result in synthesis of a truncated protein, p.D273QfsX31, in addition to reduced amounts of normal NUBPL protein[8]. The equivalent truncated protein product in Yarrowia, p.N271QfsX31, is less stable and severely affected the assembly of complex I[20].

The p.L104P substitution, present in Families 1 and 3, changes a highly conserved amino acid in a protein motif involved in ATP hydrolysis. The equivalent substitution caused instability of the Yarrowia Ind1 protein and decreased complex I levels to ~30% of the control line[12].

The p.V182A substitution is only found in Family 4. In the Yarrowia Ind1 protein, valine is semi-conserved and aligns with a methionine (Figure 4A). Therefore, to match the human sequence, first methionine was substituted by valine (p.M180V), both of which have large non-polar side chains. Next, methionine was changed to alanine (p.M180A), which has a much shorter side chain. These changes were neutral with respect to complex I levels (Figure 4B).

When the Yarrowia *IND1* gene is deleted (*ind1*Δ), a 160-kDa assembly intermediate containing the NUCM subunit (NDUFS2 in human) accumulates[12]. This intermediate has been observed in all Ind1 protein variants tested so far, including the p.G136D variant (p.G138D in human), which has otherwise no discernable phenotypes. In contrast, the assembly intermediate was not observed in p.M180V or p.M180A (Figure 4C). Another feature of ind1 mutants is a striking growth defect at low temperature[12]. When the p.M180V and p.M180A variants were grown at 10 C, colonies grew to the same size as in the control strain, whereas the *ind1*Δ mutant did not grow at all (Figure 4D). Taken together, the biochemical and phenotypic tests in Yarrowia yeast show that substitution of M180 with valine or alanine in Ind1 does not have an effect on complex I, but they do not rule out a potential effect of the valine to alanine substitution on the function of human NUBPL. We note that the V182A variant is predicted to be possibly damaging/deleterious (Table 2).

Clinical comparison of all *NUBPL* patients

Table 1 summarizes the main clinical features in our five new patients reported here, plus twelve previously reported patients (19 patients total in 15 families). Patients are sub-grouped based on whether they have the c.815-27T>C branch-site mutation, which is found in 14 of 19 patients. Phenotypes for this subset of patients have greater overlap than with patients without the c.815-27T>C variant (Families 13-15). We note that clinical information was not available for German Patient 12 and USA Patient 4 is listed separately as *NUBPL* disease is suspected but not supported by his brain MRI (Figure 1B) or functional experiments on the p.V182A variant (Figure 4).

Notably, all patients had developmental delay with onset as infants or toddlers. Cerebellar dysfunction symptoms (ataxia, dysarthria, nystagmus, and tremor) were prevalent in patients with the c.815-27T>C branch-site mutation, but absent (Patient 14) or minimal (Patients 13A,13B, 15A, and 15B) in patients without this mutation. Spasticity was reported in 6 of 14 patients with c.815-27T>C and 3 of 5 patients without this mutation. Brain MRIs revealed cerebellar atrophy in 13 of 18 patients who underwent at least one assessment but this symptom may not be present at an early age (e.g., Patients 8 and 9B were only positive in a later MRI and Patients 5, 10 and 11 were negative in an early MRI but no late MRI results were available).

Biochemical assays for complex I defects (electron transfer) and lactate levels (plasma and/or CSF) can be inconclusive. For example, results were reported for 12 of 19 patients but only 9 of 12 patients tested positive for a complex I defect and/or a high lactate level.

Patient 2 is the first unrelated case of *NUBPL* disease that has identical mutations to those reported in a previous case (Patient 10, Table 1)[10]. Ataxia is the main overlapping cerebellar symptom for these two patients and both were noted to have normal intelligence. Cerebellar atrophy was noted in Patient 2's MRI obtained at age 3 years (Figure 1B), but not in Patient 10's MRI obtained at age 1.5 years old (no late MRI was available). Previously reported Patients 15A and 15B[15] also have c.693+1G>A (but lack c.815-27T>C) and have thalamic involvement in addition to cerebellar atrophy.

Patients 1A, 1B, and 3 share nearly identical variants (patients 1A and 1B do not have c.166G>A [p.G56R]) and their overlapping cerebellar symptoms include ataxia,

dysarthria, and nystagmus, while spasticity was only reported in Patient 3. All three patients showed cerebellar atrophy in their MRIs (Figure 1B) and electron transport chain assays for Patients 1A and 3 were both negative for a complex I defect (Table 1). Interestingly, while previously reported Patients 13A, 13B, and 14[11, 13] share the c.311T>C [p.L104P] mutation (but lack c.815-27T>C) with Patients 1A, 1B, and 3, their main clinical features are distinct (Table 1). Patients 13A and 13B have dystonia and bilateral striatal necrosis (BSN) and Patient 14 lacks cerebellar symptoms but has renal tubular acidosis, osteoporosis, hepatomegaly, and growth hormone deficiency.

Genetic comparison of all *NUBPL* patients

Table 2 summarizes the genetic findings for our 5 new patients plus 14 prior reported patients (19 patients total in 15 families). As noted above, only two patients (Patients 2 and 10) are compound heterozygous for identical mutations (c.166G>A *in cis* with c.815-27T>C plus c.693+1G>A *in trans*).

Nearly all families (12 of 15) carry the c.815-27T>C branch-site mutation, which is not surprising since they are of European descent and this is the highest frequency *NUBPL* variant amongst known pathogenic variants (Table 2), based on the gnomAD database (~0.45% in non-Finnish Europeans)[9]. Despite its relatively high frequency in the general population, aberrant splicing data, demonstrated in Patient 5[8] and in newly reported Patients 1A and 1B and their father (Figure 2), supports its pathogenicity.

While the c.815-27T>C branch-site mutation was reported to be hemizygous or homozygous in Patient 7[10], parental DNA was unavailable for testing so it is possible the patient is heterozygous for c.815-27T>C and has another yet to be characterized pathogenic *NUBPL* variant. We note that the gnomAD database reports ten c.815-27T> homozygotes (three non-Finnish European and seven Finnish subjects). It is unlikely that subjects with early-onset *NUBPL* disease would be included in a population database. We hypothesize that homozygous subjects have a mild form of *NUBPL* disease (e.g., many may be undiagnosed) or, as Tucker et al.[8] suggested, may be at higher risk of developing late-onset neurological disorders such as Parkinson's disease.

As noted above, we report, for the first time, three patients without the c.166G>A [p.G56R] variant *in cis* with c.815-27T>C. The allele frequency difference between the c.166G>A (0.03%) and c.815-27T>C (0.45%) variants infers that they are not always found on the same haplotype. Despite *in silico* prediction that c.166G>A [p.G56R] is damaging/deleterious (Table 2), *in vitro* functional assays do not, thus far, support pathogenicity[8].

Besides c.815-27T>C and c.693+1G>A, c.311T>C [p.L104P] (allele frequency 0.02%) is the only other pathogenic variant found in two or more families (see Families 1, 3, 13, and 14). As noted above, for patients that share the c.311T>C [p.L104P] variant, their clinical symptoms are quite variable depending on which of the three pathogenic *NUBPL* variants (c.815-27T>C, c.287A>T [p.D96V], or c.726C>G [p.F242L]) they carry *in trans* (Tables 1, 2). Finally, we note that 6 of 15 families (5, 8, 11, 12, 13, and 15) carry a novel *NUBPL* variant (i.e., not reported in the gnomAD) *in trans* with a variant found in the general population.

DISCUSSION

Primary mitochondrial diseases (PMD) are caused by mutations in a large number of mitochondrial and nuclear genes, resulting in a broad range of phenotypes that are often present in other diseases[22]. As a subclass of PMD, complex I deficiency is one of the most common, with 34 nuclear genes now recognized to cause autosomal recessive disease[2]. Complex I assembly factor gene *NUBPL* (MIM 613621), 1 of 12 assembly factors known to cause disease[2, 5] was first reported to cause complex I deficiency (MIM 618242) in 2010[3]. Prior to this report on clinical and genetic findings in 5 new patients, only 14 cases were known worldwide[3, 10-15].

We have presented evidence that four of our five patients have *NUBPL* complex I deficiency symptoms consistent with other known patients (all patients are summarized in Table 1). In addition to cerebellar atrophy (Table 1), brain MRIs (Figure 1B) showed progressive global cerebellar hypoplasia with both vermis and cortex involved, in addition to hypoplasia of the medulla and pons. Consistent with cerebellar involvement, ataxia, dysarthria, nystagmus, and tremor were usually found, which was also noted in

prior patients, along with spasticity. Biochemical findings (e.g., complex I defects and high lactate), when available, were not a consistent indicator of mitochondrial dysfunction in our patients or prior patients. Patient 4 was suspected of having *NUBPL* disease, but neither his brain MRI (Figure 1B) or yeast model functional experiments for his c.545T>C [p.V182A] variant (Figure 4) are supporting evidence. He also has a 16p12.1 deletion (Figure 1A, Table 1) and this possibly explains his neurological symptoms[21].

None of the four *NUBPL* variants found in our five new patients (1A, 1B, 2, and 3) with *NUBPL* disease are novel. Variants c.815-27T>C and c.166G>A [p.G56R], reported to be *in cis* for most patients[3, 10], were found in Patients 2 and 3. However, Patients 1A and 1B (and suspected Patient 4) are the first reported patients that do not have c.166G>A. This supports the conclusion by Tucker et al.[8] that c.815-27T>C, by itself, is a pathogenic variant as their *in vitro* experiments did not show that p.G56R was deleterious. Our Patient 2 is the first reported case with identical mutations (c.815-27T>C and c.166G>A *in cis* plus c.693+1G>A *in trans*) as a prior patient[10]. Variant c.311T>C [p.L104P], found in Families 1 and 3 (Figure 1A), was originally reported for Patient 1A without a clinical description[16]. Recently it was reported in compound heterozygous patients that had either c.287A>T [p.D96V][11] or c.726C>G [p.F242L][13], but their phenotypes (see Table 1, Patients 13A, 13B, and 14) were distinct from our patients and prior patients that all had in common the c.815-27T>C variant. Similarly, Patients 15A and 15B, who also lack c.815-27T>C, have distinctive clinical features (e.g., thalamic involvement)[15]. They have the same splicing mutation (c.693+1G>A) as Patients 2 and 10 plus a novel mutation (p.M117I). These findings underscore that, even for a single gene, phenotypes can vary significantly.

On a broader level, there are two observations in common between *NUBPL* and three other complex I assembly factors: *NDUFAF5*, *NDUFAF6,* and *NDUFAF8*. First, *NDUFAF6* has also been linked to dystonia and BSN[23], which are clinical findings in *NUBPL* patients that are compound heterozygous for p.D96V and p.L104P[11]. Second, analgous to *NUBPL* c.815-27T>C, aberrant splicing variants that do not involve a splice donor or acceptor have been reported for *NDUFAF5* (c.223-907A>C)[24] and *NDUFAF8* (c.195+271C>T)[25]. Such variants may be difficult to uncover in WES bioinformatics

pipelines that exclude intronic and/or common variants. This is a particularly important issue for c.815-27T>C due to its ~1% frequency in the European ancestry population (i.e., we suspect that complex I deficiency patients with this splicing mutation are underdiagnosed).

With such a limited number of *NUBPL* disease patients, now eighteen total with our four new patients, there are several open genotype-phenotype questions. First, with additional evidence that c.815-27T>C by itself is pathogenic (Patients 1A and 1B), what phenotypes, if any, are found in c.815-27T>C homozygotes (ten are reported in gnomAD)? Second, does c.815-27T>C confer an alternate clinical picture compared to patients without it? Third, despite lack of evidence thus far from *in vitro* studies, does p.G56R contribute to the complex I deficiency phenotype? Fourth, what genetic backgrounds and/or environmental factors contribute to varying degrees of severity in patients with the same mutations (either siblings or in unrelated patients like Patients 2 and 10 in Table 1)? Finally, new treatments are needed, which are currently limited to mitochondrial cocktails (e.g., supplements of coenzyme Q10, carnitine, and α-lipoic acid) although experimental drugs have shown promise (e.g., EPI-743)[26, 27].

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Contributors

VK and PSE established the study and recruited patients. VK, SP, DAS, and MKK provided patient/family samples and clinical information. LW, KH, ST, MS, and EC performed and analyzed the genetic experiments. AEM, AES, PHS, BB, BRC, SW, RK, and JB performed and/or analyzed the functional and/or biochemical experiments. ANH provided analyses of MRIs. VK, RAD, KH, MS, EC, EH, and PSE analyzed and interpreted clinical and/or genetic data. VK , RAD, and PSE wrote the manuscript; CC, JB, and EH provided critical revisions.

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Competing interests

EH and PSE are employees of Population Bio. BRC, SW, RK, ST, and EC are employees of Ambry Genetics. The other authors declare no competing interests.

Patient consent for publication

Parental/guardian consent obtained.

Ethics approval

Ethics approval for this study was obtained from the University of California, Irvine.

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FIGURE LEGENDS

Figure 1 New patients with *NUBPL* disease. (A) Pedigree charts for five new patients (four families). Circles indicate females and squares males, arrow P = Proband. Family 4 has a suspected patient (see text). (B) Cross section of brain MRIs in all subjects (Patients 1A and 1B show MRIs for two different ages). Cerebellar and pons hypoplasia are noted (and cerebellar atrophy) except in Patient 4, who had normal brain anatomy except slightly prominent Sylvian fissures/subarchnoid spaces. (C) Cerebellar brain biopsy for Patient 1A at age one year with hematoxylin and eosin (H&E) stain (left panel) and neurofilament (NF) antibody stain (right panel, same region as left panel). The biopsy shows abnormal foliar architecture with large and irregularly distributed Purkinje neurons (highlighted with Neurofilament protein immunohistochemistry), Bergmann gliosis, and widespread karyorrhexis of the internal granular cell layer.

Figure 2 Alternatively spliced transcripts levels are higher in *NUBPL* c.815-27T>C carriers. (A) CloneSeq results for the splice variants observed in Family 1 are displayed as Sashimi plots, wherein transcript levels are quantified as Reads Per Kilobase of transcript, per Million mapped reads (RPKM). These provide an absolute number of aligned reads (numbers are indicated for each family member) that enables comparison of exon usage across the carriers (Sister+, Proband+, Father+), non-carriers (Mother-, Sister-), and controls (Blood control-, Brain control-). In addition to normal transcript, two alternate transcripts were observed: r.815_897del83 (exon 10 skipping) and r.815-72_815-1ins72 (partial inclusion of intron 9). (B) The relative expression of the two alternatively spliced transcripts are displayed as Percent Splicing Index (PSI). Higher levels of both alternate transcripts were observed only in c.815-27T>C carriers.

Figure 3 Mitochondrial respiration is impaired in *NUBPL* patient fibroblasts. (A) Oxygen consumption rate (OCR) traces were measured in fibroblast cell lines from *NUBPL* patients 1A, 1B, and 2 and a control cell line using the Seahorse XF Cell Mito Stress Test. Three measurements were taken for each stage of the assay: the initial OCR;

OCR after injection of oligomycin; OCR after injection of FCCP; and OCR after injection of rotenone + antimycin A. The OCR values are normalized by total protein for each well. (B) Basal respiration and spare respiratory capacity for *NUBPL* patient and control fibroblast cell lines, calculated using Mito Stress Test report generator software. Basal respiration is calculated by subtracting the non-mitochondrial OCR (lowest measurement after the injection of rotenone + antimycin A) from the initial OCR (last measurement before the injection of oligomycin). Spare respiratory capacity is calculated by subtracting the initial OCR from the maximal OCR (maximal measurement after injection of FCCP). Error bars are the standard deviation; statistical significance was calculated using student's t-test. The results show that the spare respiratory capacity for all three patients was less than control.

Figure 4 p.V182A substitution does not impact function of the NUBPL homolog in a yeast model. (A) Alignment of the amino acid sequence surrounding valine 182 (V182) in NUBPL from human (NP_079428.2) and the homologous Ind1 protein from *Yarrowia lipolytica* (XP_501064.1). Conserved residues are shaded black. Position V182 is semi-conserved (shaded grey), with a methionine present at the corresponding position in Ind1 (M180). (B) Complex I levels in the Ind1 M180V and M180A variants compared to control (*ind1* deletion mutant expressing the wild-type IND1 gene from a plasmid and an *ind1* deletion mutant *ind1*Δ). Complex I was visualised by NADH/NBT staining of Blue-Native Poly-Acrylamide Gel Electrophoresis (BN-PAGE) gels. Ind1 and Aco1 protein levels were detected by SDS-PAGE and immunoblot analysis. (C) Assembly intermediates of complex I containing the NUCM subunit (NDUFS2 in human), visualised by BN-PAGE and immunoblotting. (D) Growth of the indicated *Yarrowia* yeast strains at normal and cold temperatures. Images show a serial dilution of cultures that were spotted onto agar plates.