Mutations in Twinkle primase-helicase cause Perrault syndrome with neurologic features

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ABSTRACT

Objective: To identify the genetic cause in 2 families of progressive ataxia, axonal neuropathy, hyporeflexia, and abnormal eye movements, accompanied by progressive hearing loss and ovarian dysgenesis, with a clinical diagnosis of Perrault syndrome.

Methods: Whole-exome sequencing was performed to identify causative mutations in the 2 affected sisters in each family. Family 1 is of Japanese ancestry, and family 2 is of European ancestry.

Results: In family 1, affected individuals were compound heterozygous for chromosome 10 open reading frame 2 (*C10orf2*) p.Arg391His and p.Asn585Ser. In family 2, affected individuals were compound heterozygous for *C10orf2* p.Trp441Gly and p.Val507Ile. *C10orf2* encodes Twinkle, a primase-helicase essential for replication of mitochondrial DNA. Conservation and structural modeling support the causality of the mutations. Twinkle is known also to harbor multiple mutations, nearly all missenses, leading to dominant progressive external ophthalmoplegia type 3 and to recessive mitochondrial DNA depletion syndrome 7, also known as infantile-onset spinocerebellar ataxia.

Conclusions: Our study identifies Twinkle mutations as a cause of Perrault syndrome accompanied by neurologic features and expands the phenotypic spectrum of recessive disease caused by mutations in Twinkle. The phenotypic heterogeneity of conditions caused by Twinkle mutations and the genetic heterogeneity of Perrault syndrome call for genomic definition of these disorders.

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GLOSSARY

CLPP = caseinolytic mitochondrial matrix peptidase proteolytic subunit; **C10orf2** = chromosome 10 open reading frame 2; **HARS2** = histidyl-tRNA synthetase 2; **HSD17B4** = 17- β -hydroxysteroid dehydrogenase IV; **¹²³I-IMP** = *N*-isopropyl-*p*-[¹²³I] iodoamphetamine; **IOSCA** = infantile-onset spinocerebellar ataxia; **LARS2** = leucyl-tRNA synthetase 2; **mtDNA** = mitochondrial DNA; **MTDPS7** = recessive mitochondrial DNA depletion syndrome 7; **NHLBI** = National Heart, Lung, and Blood Institute; **PEOA3** = dominant progressive external ophthalmoplegia type 3.

Perrault syndrome (MIM 233400) is a recessive disorder characterized by sensorineural hearing loss in both females and males and gonadal dysfunction in females.¹ Neurologic symptoms, including ataxia, sensory neuropathy, muscle weakness, ophthalmoplegia, nystagmus, and intellectual disability, may also be present but vary among families and patients.^{2–4} Although hearing loss and neurologic symptoms may develop in childhood, Perrault syndrome is usually diagnosed when a failure of puberty, with primary amenorrhea, or secondary amenorrhea is noted in a young woman. The phenotypic heterogeneity of Perrault syndrome is accompanied by, and is likely a result of, genetic heterogeneity. *HSD17B4* (MIM 601860), *HARS2* (MIM 600783), *LARS2* (MIM 604544), and *CLPP* (MIM 601119) have been identified as genes causing Perrault syndrome.^{5–8} The *HSD17B4* protein is involved in fatty acid β -oxidation and steroid metabolism; *HARS2*, *LARS2*, and *CLPP* proteins have important functions in mitochondrial gene translation and protein homeostasis.

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In the present study, we sought to determine the cause of Perrault syndrome in 2 families, each with 2 affected sisters. Using exome sequencing, we identified compound heterozygous mutations in *C10orf2*, encoding the Twinkle protein, a DNA helicase acting in the mitochondria.

METHODS **Standard protocol approvals and patient consents.** This study included 11 members of 2 families, 4 of whom had Perrault syndrome. The study was approved by the human subjects committees of Hiroshima University and of the University of Washington; all subjects provided written informed consent.

Patients. Family 1 is of Japanese ancestry. The parents are from different regions of Japan and are unrelated. Two sisters, presently ages 40 and 34 years, were born healthy at full term after normal pregnancies, and their psychomotor development proceeded normally. They first presented as teenagers with lack of secondary sexual characteristics, primary amenorrhea, and gonadal dysgenesis. Both have 46,XX karyotypes. They were diagnosed with sensorineural hearing loss at ages 13 and 8 in Hiroshima University Hospital, and slowly progressing ataxia beginning at ages 20 and 16. Their clinical courses were similar, but the older sister was more severe.

Family 2 is a nonconsanguineous American family of paternal Greek ancestry and maternal mixed European ancestry. Two sisters presented as teenagers with primary amenorrhea, with the presence of streak ovaries indicating gonadal dysgenesis. Both have 46,XX karyotypes. The older sister had been diagnosed at age 7 with sensorineural hearing loss, which was severe by age 36. The younger sister was diagnosed at approximately the same age with hearing loss that was moderate by age 31, when she received a diagnosis of auditory neuropathy. The older sister experienced tonic-clonic epileptic seizures at age 7, which were resolved by treatment with phenobarbital for 3 years. She developed ataxia as a teenager and peripheral neuropathy after age 20. The younger sister also developed ataxia and peripheral neuropathy with hyporeflexia and difficulty balancing with eyes closed.

Exome sequencing and filtering of causative mutations. Exome sequencing for individuals 1-II-1 and 1-II-3 (figure 1A) was performed in the Kawakami laboratory at the University of Hiroshima, using previously described methods.⁹ Median coverage for the sequenced individuals was >85-fold. Exome sequencing for individuals 2-I-1, 2-I-2, 2-II-2, and 2-II-4 (figure 1B) was performed in the King laboratory at the University of Washington, also using previously described methods.¹⁰ Median coverage for the sequenced individuals was >100-fold. In each laboratory, artifacts and common variants were excluded by comparison with exomes sequenced in the same laboratory for other conditions, and by filtering with dbSNP v137, 1000 Genomes Project, and the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project. Variants were classified by predicted function as nonsense mutations, frameshift mutations, variants within 1 base pair of a splice site, and missense variants. Missense variants were evaluated with PolyPhen-2, an online tool that uses amino acid conservation, protein structure, and amino acid properties to predict the possible impact of amino acid substitutions on protein structure and function.¹¹ PolyPhen-2 scores range from zero, indicating a variant most likely to be benign, to 1.0, indicating a variant most likely to be damaging to protein function. All variants predicted to lead to protein truncation

and all missense variants with PolyPhen-2 scores of at least 0.6 were retained as candidate alleles. Candidate variants were validated by Sanger sequencing, then tested for cosegregation with the phenotype in all family members.

Protein structure modeling. The structure of T7 bacteriophage primase-helicase gp4 (PDB 1Q57) was used to predict the structural roles of Twinkle residues. Orthologous residues were determined by amino acid sequence alignment with ClustalW-2. Structures were analyzed and cartoons rendered using the PyMOL Molecular Graphics System, version 1.5.0.4, Schrödinger, LLC.

RESULTS **Clinical manifestations of family 1.** The 2 affected sisters in family 1 (1-II-1 and 1-II-3; figure 1A) exhibited high-arched palate and pes cavus. Neurologic examination revealed mild external ophthalmoplegia, gaze-evoked nystagmus, bilateral sensorineural hearing loss, hyporeflexia of lower extremities, mild movement abnormalities of upper and lower extremities, ataxic gait, positive Romberg sign, and hyporeflexia of temperature and vibration. Sensory axonal neuropathy was confirmed by EEG. Laboratory evaluations revealed mild elevation of serum creatine kinase (1-II-1, 243 mg/dL; 1-II-3, 146 mg/dL; and normal range, 5–140 mg/dL), elevation of luteinizing hormone (1-II-1, 31 mIU/mL; 1-II-3, 34 mIU/mL; and normal range, 2.4–12.6 mIU/mL) and follicle-stimulating hormone (1-II-1, 95 mIU/mL; 1-II-3, 200 mIU/mL; and normal range, 3.5–12.5 mIU/mL), and low levels of estradiol (1-II-1, 10.5 pg/mL; 1-II-3, 10.3 pg/mL; and normal range, 25–195 pg/mL). The older sister showed high levels of lactate and pyruvate at rest, and aerobic exercise for 15 minutes increased the levels of lactate and pyruvate (figure 2A). The younger sister did not show elevation of lactate and pyruvate at rest, but with aerobic exercise showed marked increases in lactate and pyruvate levels and in the lactate/pyruvate ratio (figure 2B). MRI of the brain did not indicate cerebellar atrophy in either sister (figure 2, C and D), but *N*-isopropyl-*p*-[¹²³I]iodoamphetamine (¹²³I-IMP) SPECT revealed hypoperfusion of the cerebellum of both sisters (figure 2, E and F). A brother has none of the above signs and is fertile.

Clinical manifestations of family 2. Neurologic examination of the older affected sister in family 2 (2-II-2; figure 1B) at age 41 indicated vertical and horizontal direction-changing nystagmus, ataxic gait, distal atrophy of the hands and feet, hyporeflexia of the lower extremities, reduced sensitivity to vibration, and positive Romberg sign. Axonal neuropathy was confirmed by EEG. Laboratory examination indicated mildly elevated lactate after 15 minutes of rest. A muscle biopsy showed type II myofiber atrophy and levels of the mitochondrial enzymes cytochrome oxidase (3.9 U/min/g), succinate dehydrogenase (2.0 U/min/g), ubiquinone cytochrome *c* reductase

Figure 1 Identification of mutations in *C10orf2*, encoding Twinkle, in families with Perrault syndrome

(A, B) Pedigrees and Sanger sequences of mutations in *C10orf2* in families 1 and 2. Affected individuals are indicated by filled circles. (C) Conservation of protein sequence at the 4 residues with mutations in families 1 and 2. Mutated residues are in red. Residues 507 (red) and 514 (green) form a pair with a complementary structural relationship, as described in the text. (D) Diagram of the Twinkle primase-helicase. The protein forms a heptameric or hexameric ring structure of primase domains (blue) and helicase domains (orange). (E) Domain architecture of Twinkle with mutations responsible for 3 phenotypes: dominant progressive external ophthalmoplegia type 3 (PEOA3, MIM 609286); recessive mitochondrial DNA depletion syndrome 7 (MTDPS7, MIM 271245), also known as infantile-onset spinocerebellar ataxia (IOSCA)³⁴⁻³⁸; and recessive Perrault syndrome.

(9.5 U/min/g), and NADH ubiquinone reductase (4.4 U/min/g) were within normal ranges. Brain MRI revealed nonspecific white matter changes in both affected sisters. Two other sisters, one a dizygous twin of the older affected sister, have none of the above signs. Both unaffected sisters are fertile.

Compound heterozygous mutations in *C10orf2*. In both families, the appearance of Perrault syndrome was consistent with recessive inheritance, so variants carried by affected sisters were filtered to identify shared homozygous or compound heterozygous potentially damaging mutations. No homozygous damaging

variants were detected in either family. In each family, potentially damaging mutations in only one gene were compound heterozygous in the affected individuals and either heterozygous or absent from unaffected relatives. In family 1, the 2 affected sisters were compound heterozygous for chr10:102,749,139 G>A, corresponding to *C10orf2* c.1172G>A (p.Arg391His) (NM_021830; MIM 606075), inherited from their father, and for chr10:102,752,966 A>G, corresponding to *C10orf2* c.1754A>G (p.Asn585Ser), inherited from their mother (figure 1A). Their unaffected brother was heterozygous for *C10orf2* c.1172G>A. PolyPhen-2 scores were 0.995 for p.Arg391His and

Figure 2 Clinical and pathologic features of affected individuals in family 1

(A, B) Elevated lactate and pyruvate levels during and after aerobic exercise. The x-axes indicate time course (minutes); exercise occurred during the first 15 minutes. The left y-axes indicate the level of lactate (mg/dL, blue diamond) and the lactate/pyruvate ratio (green circle); the right y-axes indicate the level of pyruvate (mg/dL, red square). Normal ranges of lactate, pyruvate, and lactate/pyruvate ratio are 0.30–0.90 mg/dL, 3.7–16.3 mg/dL, and <20, respectively. (C, D) MRI of the brain in both patients of family 1. T2-weighted images show no cerebellar atrophy. (E, F) ^{123}I -IMP SPECT in both patients of family 1 shows decreased cerebellar perfusion.

1.000 for p.Asn585Ser. In family 2, the 2 affected sisters were compound heterozygous for chr10:102,749,478 T>G, corresponding to *C10orf2* c.1321T>G (p.Trp441Gly), inherited from their mother, and for chr10:102,750,227 G>A, corresponding to *C10orf2* c.1519G>A (p.Val507Ile), inherited from their father (figure 1B). The 2 unaffected sisters were homozygous for wild-type alleles at both sites. PolyPhen-2 scores were 0.965 for p.Trp441Gly and 0.637 for p.Val507Ile. The p.Val507Ile variant was present once and the other variants were absent from >6,500 exome sequences in the NHLBI Exome Sequencing Project. All variants were absent from in-house control exomes.

The amino acid residues of one mutation in each family, Asn585 in family 1 and Trp441 in family 2, are conserved throughout vertebrates (figure 1C).

Sites of the other mutation in each family, Arg391 in family 1 and Val507 in family 2, are less conserved: identical in most, but not all, mammals. Of particular note, isoleucine at amino acid 507, a mutant residue in family 2, is the wild-type residue at this site in Rhesus monkey. Given that both parents in each family are unaffected, it is very likely that both mutations contribute to the Perrault phenotype. We explored this possibility by evaluating protein structure.

Structural roles of mutated Twinkle residues. *C10orf2* encodes the Twinkle protein, a DNA helicase of 684 amino acids that is essential for the replication of mammalian mitochondrial DNA (mtDNA).^{12–14} Twinkle contains 3 functional domains: an N-terminal primase; a linker region involved in oligomerization and required for helicase activity; and a C-terminal helicase

Figure 3 Structure of the Twinkle ortholog gp4 from bacteriophage T7 (PDB ID 1Q57)

Three subunits of the heptameric primase-helicase gp4 are highlighted in pink, green, and cyan; remaining subunits are in gray. The helicase domain of the green subunit is rendered as a cartoon, while other helicase and primase domains are shown in surface representation. The linker regions of the pink and green subunits are shown as cartoons in dark pink and dark green, respectively. Residues corresponding to the Twinkle mutations in families 1 and 2 are shown as purple spheres and indicated with the Twinkle residue numbers. Relevant predicted structural interactions of Arg391 and Trp441 (dashed white line), Val507 with Ile514 (yellow spheres) and Ile367 (magenta spheres) (solid white line), and Asn585 with Asn608 (yellow spheres) (yellow line) are circled and described in the text. Arg609 is shown in red sticks.

(figure 1E).¹⁵ Twinkle proteins likely assemble into heptameric or hexameric ring structures^{16,17} (figure 1D). The mutations in families 1 and 2 affect residues in the Twinkle helicase domain and could disrupt function by affecting enzyme activity or by interfering with formation of the helicase ring structure.

To investigate the possible consequences of the Twinkle mutations in families 1 and 2, we examined the crystal structure of the bacteriophage T7 primase-helicase gp4, an ortholog of mammalian Twinkle. The linker domain at residues 261–284 of gp4 (Twinkle residues 346–384) makes key interactions to close the helicase ring structure and provides flexibility that allows it to adapt to different intersubunit interactions.¹⁶ Three of the 4 mutations in families 1 and 2 likely affect interactions of the linker. Two of the gp4 residues corresponding to mutated Twinkle residues, Leu288 (Twinkle Arg391) and Trp329 (Twinkle Trp441), interact with each other and are proximal to the linker domain (figure 3). The p.Arg391His substitution may alter the flexibility or an interaction of the linker. Trp329 is buried and loss of the large tryptophan side chain as a result of the p.Trp441Gly substitution may destabilize the helicase domain and alter the position of the helicase domain relative to the linker. The observation that 2 mutated residues, Arg391 and Trp441, directly interact supports the hypothesis that mutations at these sites are damaging.

Met412 of gp4 (Twinkle Val507) interacts with Ile421 (Twinkle Ile514) in the same subunit and with

Val265 (Twinkle Ile367) in the linker of the neighboring subunit. p.Val507Ile would juxtapose a large branched isoleucine across from another isoleucine, causing a shift that would be transmitted back to the linker of the neighboring subunit. Although isoleucine is present at position 507 of Rhesus Twinkle, Rhesus Twinkle has a compensatory substitution of valine at position 514 (figure 1C). While some mammals have valine residues at both positions 507 and 514, the large isoleucine residue is never seen at both of these positions, supporting the hypothesis that p.Val507Ile is a damaging mutation in human Twinkle.

The fourth mutation, p.Asn585Ser in family 1, likely affects enzyme activity. Residue Thr498 of gp4 (Twinkle Asn585) interacts with residue Cys521 (Twinkle Asn608), which is adjacent to Arg522 (Twinkle Arg609). This completely conserved arginine is critical for enzymatic activity, acting as an “arginine finger” by donating its side chain to activate nucleotide hydrolysis by a neighboring subunit.^{16,18} Substitution of serine for the larger asparagine residue is likely to cause a shift that would be translated to the arginine finger, thus lowering the effectiveness of hydrolysis.

DISCUSSION Mutations of *C10orf2* are responsible for multiple severe phenotypes (figure 1E): dominant progressive external ophthalmoplegia type 3 (PEOA3, MIM 609286),¹⁷ in some families accompanied by late-onset dementia¹⁹; and recessive mtDNA depletion syndrome 7 (MTDPS7, MIM 271245),

also known as infantile-onset spinocerebellar ataxia (IOSCA).^{20,21} Mutations of *C10orf2* have been described that cause multiple mtDNA deletions or mtDNA depletion without detectable deletions that are detectable by muscle biopsy.^{17,21–24} These mutations lead to dominantly inherited PEO3 or to the more severe recessive phenotypes, such as the hepatocerebellar form of MTDP57. In contrast, in cases of the less severe IOSCA phenotype, mtDNA depletion is detectable in brain and liver, but not in muscle, and muscle mitochondrial enzyme activities are normal.^{20,25,26} Deletion and/or depletion of mtDNA, leading to mitochondrial dysfunction, explain the involvement of multiple organ systems in these disorders.

The role of mitochondrial dysfunction in these patients is consistent with the high values of lactate and pyruvate after aerobic exercise observed in our patients. Before the identification of genes causing Perrault syndrome, some cases with nervous system involvement were speculated to involve mitochondrial dysfunction, based on their clinical and pathologic features.⁴ Three of the genes now known to

cause Perrault syndrome, *HARS2*, *LARS2*, and *CLPP*, are associated with mitochondrial function. *C10orf2* is now added as a fourth mitochondria-associated gene causing Perrault syndrome.

The clinical features of the diseases caused by mutations in *C10orf2* overlap considerably (table). Hearing loss, a defining feature of Perrault syndrome, is also observed in some patients with PEOA3²⁷ and in most patients with MTDP57/IOSCA.²⁸ Ataxia, myopathy, neuropathy, and ophthalmoplegia are observed in some patients with each of these conditions. High levels of lactate and pyruvate after aerobic exercise are features of our patients and of some patients with PEOA3 and with IOSCA. Female hypergonadotropic hypogonadism is observed in most patients with MTDP57/IOSCA who survive to puberty.²⁵

Understanding the relationship between PEOA3, MTDP57/IOSCA, and Perrault syndrome phenotypes and mutant Twinkle genotypes presents a daunting challenge. Virtually all mutant genotypes are missenses. Perrault syndrome is a less severe phenotype than PEOA3 or MTDP57/IOSCA, and all 4 Twinkle mutations in Perrault syndrome patients lie in the helicase domain. Many of the mutations causing dominant PEOA3 are clustered within or near the linker region, and mutations causing MTDP57/IOSCA are widely distributed. But mutation locale is not fully explanatory. Twinkle helicase activity is affected by efficiency of ATP hydrolysis, by DNA binding affinity, and by protein stability. Mutations causing PEOA3 or MTDP57/IOSCA have been evaluated regarding these parameters.^{29–32} In particular, a thorough evaluation of 20 mutations of Twinkle, causing both dominant and recessive disease, included analyses of helicase activity, kinetics of ATP hydrolysis, DNA binding affinity, and thermal stability, using optimized protein purification and assay conditions.³³ Compared with wild-type Twinkle, the individual mutant proteins varied from normal levels of all these properties to approximately 3-fold differences from normal for one or more properties. For example, p.Tyr508Cys, the Finnish founder mutation causing MTDP57/IOSCA, differed from wild-type Twinkle only in a subtle reduction in DNA binding affinity. Among these 20 mutations, no simple relationship between severity of clinical phenotypes and biochemistry of Twinkle mutants emerged. Both these authors and we speculate that subtle changes in enzyme activity, DNA binding affinity, interaction of subunits in the ring structure, overall stability, or a combination of these, are enough to cause disease, with clinical outcome affected by the specific allelic combination. There is no reported overlap between alleles causing dominant and recessive disease. While several dominant alleles have been shown to cause mtDNA defects when overexpressed in cell culture or transgenic

Table Features of conditions caused by mutations in <i>C10orf2</i>			
	PEOA3	MTDP57	Perrault
Clinical symptoms			
Age at onset, y	17–73	<2	7–13
Hearing loss	+	++	++
Female hypogonadism	–	++	++
Ataxia	+	++	++
Myopathy	++	++	+
Neuropathy	+	++	++
Hyporeflexia	+	++	++
Ophthalmoplegia	++	++	++
Optic atrophy	–	++	–
Intellectual disability	–	++	–
Dementia	+	–	–
Epilepsy	+	++	+
Liver involvement	–	+	–
Laboratory findings			
Lactate elevation	+	+	++
CK elevation	+	+	++
Abnormal neuroimaging	+	++	++
mtDNA			
Deletion	++	–	NT
Depletion	+	++	NT

Abbreviations: CK = creatine kinase; mtDNA = mitochondrial DNA; MTDP57 = recessive mitochondrial DNA depletion syndrome 7; NT = not tested; PEOA3 = dominant progressive external ophthalmoplegia type 3.

Symbols: – absent; + present in some cases; ++ present in most cases.

mice,^{30,33} accurate modeling of recessive mutations in vivo will require replacing wild-type gene function with the appropriate mutant genotype, expressed at endogenous levels. Understanding the consequences at the cellular level of specific mutations of the Twinkle protein, including the mutations reported here, would be helpful to the understanding of pathology of multiple organ systems.

Our results indicate that Perrault syndrome can be caused by recessive mutations of *C10orf2*, leading to dysfunction of Twinkle and hence to predicted aberrations of mtDNA replication. These results expand the phenotypic spectrum resulting from Twinkle dysfunction and suggest that *C10orf2* mutations be considered in women with ovarian dysgenesis, hearing loss, and neurologic features.

Web resources. The URLs for data presented herein are as follows:

PolyPhen-2: <http://genetics.bwh.harvard.edu/pph2/>
dbSNP: <http://www.ncbi.nlm.nih.gov/SNP/>
1000 Genomes Project: <http://www.1000genomes.org/>
NHLBI Exome Sequencing Project (ESP): <http://evs.gs.washington.edu/EVS/>

AUTHOR CONTRIBUTIONS

Hiroyuki Morino and Sarah B. Pierce: study concept and design, data acquisition, analysis, and interpretation, manuscript preparation and revision. Yukiko Matsuda and Tom Walsh: data acquisition and analysis. Ryosuke Ohsawa, Keiko Hiraki, Masahito Kuramochi, and Hirofumi Maruyama: data acquisition and manuscript revision. Marta Newby: data acquisition and sample collection. Ming K. Lee: data analysis. Rachel E. Klevit: protein structure analysis. Alan Martin: data acquisition and patient evaluation. Mary-Claire King: study concept and design, data analysis and interpretation, and manuscript revision. Hideshi Kawakami: study concept and design and manuscript revision.

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DISCLOSURE

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