A novel *AARS* mutation in a family with dominant myeloneuropathy

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ABSTRACT

Objective: To determine the genetic cause of neurodegeneration in a family with myeloneuropathy.

Methods: We studied 5 siblings in a family with a mild, dominantly inherited neuropathy by clinical examination and electrophysiology. One patient had a sural nerve biopsy. After ruling out common genetic causes of axonal Charcot-Marie-Tooth disease, we sequenced 3 tRNA synthetase genes associated with neuropathy.

Results: All affected family members had a mild axonal neuropathy, and 3 of 4 had lower extremity hyperreflexia, evidence of a superimposed myelopathy. A nerve biopsy showed evidence of chronic axonal loss. All affected family members had a heterozygous missense mutation c.304G>C (p.Gly102Arg) in the alanyl-tRNA synthetase (*AARS*) gene; this allele was not identified in unaffected individuals or control samples. The equivalent change in the yeast ortholog failed to complement a strain of yeast lacking AARS function, suggesting that the mutation is damaging.

Conclusion: A novel mutation in AARS causes a mild myeloneuropathy, a novel phenotype for patients with mutations in one of the tRNA synthetase genes. ***Neurology*® 2015;84:2040–2047**

GLOSSARY

5-FOA = 5-fluoroorotic acid; **ARS** = aminoacyl-tRNA synthetases; **CMT** = Charcot-Marie-Tooth disease; **CMTNS** = Charcot-Marie-Tooth neuropathy score; **HMSN** = hereditary motor and sensory neuropathy; **PNS** = peripheral nervous system.

Charcot-Marie-Tooth disease (CMT) and hereditary motor and sensory neuropathy (HMSN) are alternative names for inherited neuropathies that are not part of more complex syndromes. With an estimated prevalence of 1 in 2,500 persons, CMT/HMSN is one of the commonest neurogenetic diseases and is subdivided according to clinical, electrophysiologic, histologic, and genetic features.[1] CMT2/HMSN-II is a dominantly inherited axonal neuropathy, with nerve conduction velocities greater than 38 m/s.

Mutations in more than 20 different genes cause autosomal dominant CMT2. With the exception of some *MPZ* mutations, these mutations are thought to produce a neuropathy through their direct effects in neurons. Mutations in genes that encode aminoacyl-tRNA synthetases (ARS) cause some forms of CMT2. These enzymes charge tRNAs with their cognate amino acids, establishing the genetic code. Mutations in the glycyl-tRNA synthetase (*GARS*) gene were the first to be described, found in patients with dominant purely motor (hereditary motor neuropathy 5A) or dominant motor predominant (CMT2D) neuropathy.[2] Mutations in 5 additional ARS loci have been found in various forms of CMT disease—tyrosyl-tRNA synthetase (*YARS*) in dominant intermediate CMT type C (DI-CMTC)[3]; alanyl-tRNA synthetase (*AARS*) in dominant CMT2N[4–7]; lysyl-tRNA synthetase (*KARS*) in recessive, intermediate CMT (CMTRIB)[8]; histidyl-tRNA synthetase (*HARS*) in a patient with sporadic, presumed dominant neuropathy[9]; and methionyl-tRNA synthetase (*MARS*) in late-onset, dominant

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CMT2 with incomplete penetrance.[10] A growing number of rare recessive developmental disorders have also been linked to tRNA synthetase genes, including the mitochondrial alanyl tRNA synthetase gene, *AARS2*.[11–25] Several of these recessive disorders involve the CNS and non-nervous organ systems, but the dominant tRNA synthetase mutations predominantly affect the peripheral nervous system (PNS).[21] An example of this pronounced distinction is seen with the glycyl-tRNA synthetase gene, *GARS*, where dominant mutations cause distal axonopathies[2] and compound heterozygous mutations cause systemic mitochondrial disease.[24]

METHODS Standard protocol approvals, registrations, and patient consents. Institutional review board approval was obtained from the University of Pennsylvania for these studies. Written informed consent was obtained from each patient who participated.

Clinical data and sample collection. The family was seen by one of the authors (S.S.S.) in an outpatient clinic, where clinical neurophysiology was also performed to generate a Charcot-Marie-Tooth neuropathy score (CMTNS).[26] A sural nerve biopsy was performed as part of the proband's prior diagnostic workup from another institution. We obtained the epoxy blocks and recut and imaged semi-thin and thin sections with transmitted light and electron microscopes.

Mutation analysis. A collection of 94 families with dominant forms of peripheral neuropathies (17 CMT1, 37 CMT2, 9 CMTDI, 31 unspecified type) were screened for mutations in *GARS*, *AARS*, and *YARS* by Sanger sequencing. Genomic DNA was extracted from peripheral blood using standard procedures. All exons and exon-intron boundaries were amplified using primers designed with Primer3 v0.4.0 (primer sequences and PCR conditions available upon request). PCR products were purified with ExoSAP-IT (USB, Cleveland, OH) and bidirectionally sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Fragments were electrophoretically separated on an ABI3730xl DNA Analyzer (Applied Biosystems) and analyzed with SeqMan II (DNASTAR Inc., Madison, WI). The numerical amino acid position for the human mutation is based on GenBank accession number BAA06808.1, and the equivalent yeast amino acid positions were determined by a ClustalW comparison to the sequence for GenBank accession number EDV10897.1. Our description of the mutation follows HGVS nomenclature guidelines (http://www.hgvs.org/mutnomen).

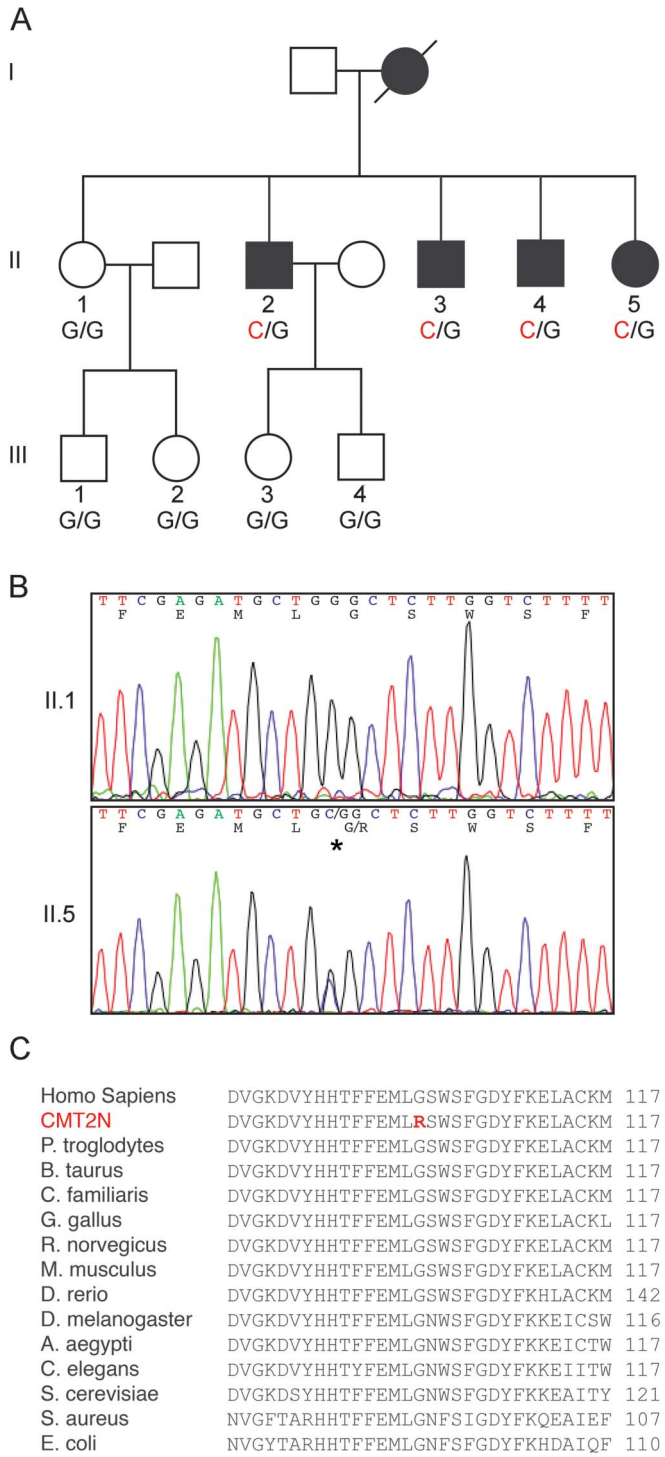
Yeast complementation assay. Yeast complementation assays were performed as previously described.[4] Briefly, mutation-containing oligonucleotides were designed and used with the QuickChange II XL Site-Directed Mutagenesis Kit (per the manufacturer's instructions; Stratagene, Santa Clara, CA) using forward (5′-TTTTTTGAAATGCTGCGTAACTGGTCGTTTG-3′) and reverse (5′-CAAACGACCAGTTACGCAGCATTTCAAAAAA-3′) primers to model the p.Gly102Arg *AARS* mutation in the yeast ortholog *ALA1*, with a c.316G>C nucleotide change, in a pDONR221 Gateway entry clone (Invitrogen, Carlsbad, CA). Plasmids were isolated from individual clones and sequenced to confirm mutagenesis and exclude polymerase errors. The p.Gly102Arg *ALA1*/pDONR221 entry clone was recombined into a Gateway-compatible *LEU2*-bearing pRS315 destination vector. Resulting clones were purified and digested with *Bsr*GI (New England Biolabs, Ipswich, MA) to confirm recombination. The Δ*ala1* haploid yeast strain (harboring a pRS316 maintenance vector to express wild-type *ALA1* and *URA3*) was transformed with wild-type or mutant *ALA1* in a *LEU2*-bearing pRS315 vector and selected on medium lacking uracil and leucine (Teknova, Hollister, CA). For each transformation, 2 colonies were selected for further analysis. Each colony was grown to saturation in selection media for 48 hours. Next, 10 μL of undiluted and diluted (1:10 and 1:100) samples from each culture were spotted on plates containing 0.1% 5-FOA complete medium or SD -leu -ura growth medium (Teknova) and incubated at 30°C for 72 hours.

RESULTS Clinical assessment reveals evidence of peripheral neuropathy and myelopathy. The family pedigree is shown in figure 1A. There are 5 affected members of the family: the proband (II-5), her 3 brothers (II-2, II-3, and II-4), and their mother (who is deceased but had severe neuropathic pain). When first seen in our clinic at 22 years old, the proband reported frequent ankle sprains during her adolescence, including a fractured left ankle at age 21 that ultimately led to a triple arthrodesis at age 25. At age 21 years, she developed burning pain (worse in arms than legs) and painful electric shocks (worse in legs than arms) in her extremities. Over the next several years she tried amitriptyline, nortriptyline, gabapentin, carbamazepine, and tramadol before subsequently getting some relief with long-acting opioids.

Examination at age 22 years revealed slight difficulty standing on the heels, slight weakness in extensor hallucis longus (4+/5), a stocking-type pattern of decreased cold and pinprick, and reduced vibratory sensation at the toes. Reflexes were 3+ at the knees with crossed adduction and absent at the ankles. Plantar responses were muted. The examination raised the possibility of a superimposed myelopathy. MRIs of the brain and cervical spine were unremarkable, and a B_{12} level was normal. While the patient's motor findings have remained largely unchanged from 1999 to 2013, her sensory deficits progressed: pinprick sensation is now decreased to above her knees and vibration sensation is absent in the toes. Plantar responses have remained flexor. Her CMTNS at age 34 years is 14.

At age 24 years, the tibial motor responses had reduced amplitudes, and the peroneal response was absent. All of the measured sensory amplitudes were reduced. Repeat conduction studies at age 34 years showed little progression—the sensory amplitudes were stable, and 2 of 3 motor amplitudes were smaller (table). EMG of the tibialis anterior showed moderate, chronic denervation at both 24 and 34 years. The

Figure 1 Pedigree and genotype of family



(A) Four of 5 siblings in the second generation (II) have a c.304G>C substitution and have clinical and electrophysiologic evidence of neuropathy. The genotypes of individuals whose DNA was collected are indicated below their respective symbol. (B) Normal sequence of the *AARS* gene is shown from the proband's sister (II-1), who does not have myeloneuropathy. Sequence of the proband's *AARS* gene demonstrates a heterozygous c.304G>C substitution, which is predicted to result in p.Gly102Arg. (C) The sequences of the activation domains of the AARS proteins from a range of divergent species, compared with ClustalW multiple sequence alignment tool. The mutated region of the protein, and in particular the residue that is mutated in this family, is highly conserved (the substitution is shown in red).

above findings support the diagnosis of a mild to moderate axonal neuropathy and a possible superimposed myelopathy.

Evaluation at age 21 years included a sural nerve and quadriceps muscle biopsy. The muscle biopsy was reported as showing neurogenic changes. We recut semi-thin and thin sections of the sural nerve biopsy (figure 2). These revealed a mildly reduced density of normally myelinated axons; no myelin debris was observed, indicating that the axonal loss was indolent. There are a few clusters of regenerated axons. Electron microscopy revealed Schwann cell processes that were not related to unmyelinated axons, indicating prior loss of myelinated or unmyelinated axons.

The oldest brother (II-2) reported difficulty with his gait and balance since age 25 years and was aware of numbness in the distal calves. He previously had ankle surgery. At age 45 years, he was strong except for difficulty standing on his heels; bilateral tibialis anterior and abductor pollicus brevis were mildly weak (4+/5). Vibration sensation (scored throughout this work with a Rydell-Seiffer tuning fork) was not felt from the toes, and was reduced at the ankles (3), but not at the knees (5). Pinprick was decreased to the distal calves. Reflexes were 2+ at the ankles, 3+ at the knees (with crossed adduction), and 3+ at the biceps. Plantar responses were extensor. The radial, median, and ulnar sensory responses had reduced amplitudes; the tibial and peroneal motor responses and the sural sensory response were absent (table). His CMTNS was 11. Thus, in addition to a mild axonal neuropathy, his clinical examination indicated that he had a myelopathy. A B_{12} level and a cervical MRI were normal.

The second brother (II-3) did not report any neurologic symptoms, but examination at age 46 years revealed mild weakness (4+/5) in bilateral tibialis anterior and absent vibration at the toes (0), reduced at the ankles (<5), and normal at the knees (>5). Reflexes were 2+ at the ankles, 3+ at the knees (with crossed adduction), and 3+ at the biceps. Plantar responses were extensor on the right and muted on the left. His radial, median, and ulnar sensory amplitudes were reduced, and sural sensory response was absent. His CMTNS was 5. Thus, in addition to a mild axonal neuropathy, his clinical examination indicated that he had a myelopathy.

The third brother (II-4) did not report any motor or sensory deficits. At age 44 years, his examination had normal results, except for diminished vibration in his toes (less than 5). Reflexes were trace at the ankles and 1+ at the knees and biceps. Plantar responses were muted. His sural, median, and ulnar sensory responses, however, had reduced amplitudes. His CMTNS was 1. Thus, in spite of a lack of symptoms, his electrophysiology demonstrated that he has a mild axonal neuropathy.

Table Nerve conduction studies performed in patients after they were examined

	I-1 48 y		II-2 46 y		II-3 45 y		II-4 44 y		II-5 24 y		II-5 34 y		Normal values	
	μV	m/s	μV	m/s	μV	m/s	μV	m/s	μV	m/s	μV	m/s	μV	m/s
CMTNS	7		11		5		1		ND		14		0	
Sensory														
Sural	7.	44	NR	—	NR	—	3.4	35	5.2	35	3.3	45	≥6	≥40
Radial	32	57	7.6	55	7.8	53	19.	48	9.5	63	14.5	66	≥15	≥50
Median[a]	13	59	3.0	47	3.5	49	4.6	45	6.1	52	ND	ND	≥10	≥50
Ulnar[a]	11	55	2.2	45	1.9	51	3.1	39	3.5	48	ND	ND	≥7	≥50
	I-1 48 y		II-2 46 y		II-3 45 y		II-4 44 y		II-5 24 y		II-5 34 y		Normal values	
	mV	m/s	mV	m/s	mV	m/s	mV	m/s	mV	m/s	mV	m/s	mV	m/s
Motor[b]														
Peroneal	1.9	43	NR	—	ND	ND	ND	ND	NR	—	NR	—	≥2.0	≥41
Tibial	20.	52	NR	—	ND	ND	ND	ND	0.9	43	0.2	45	≥4.0	≥41
Median	10.	59	5.6	37	7.9	46	8.5	39	9.6	49	9.7	45	≥4.0	≥49
Ulnar	12	68	7.9	42	11.7	42	9.9	45	16.2	50	9.5	53	≥6.0	≥49
EMG tibialis anterior	ND		Moderate chronic denervation		ND		ND		Moderate chronic denervation		Moderate chronic denervation			

Abbreviations: CMTNS = Charcot-Marie-Tooth neuropathy score; ND = not done; NR = no response.
[a] Orthodromic.
[b] The amplitudes of the distal motor responses are shown.

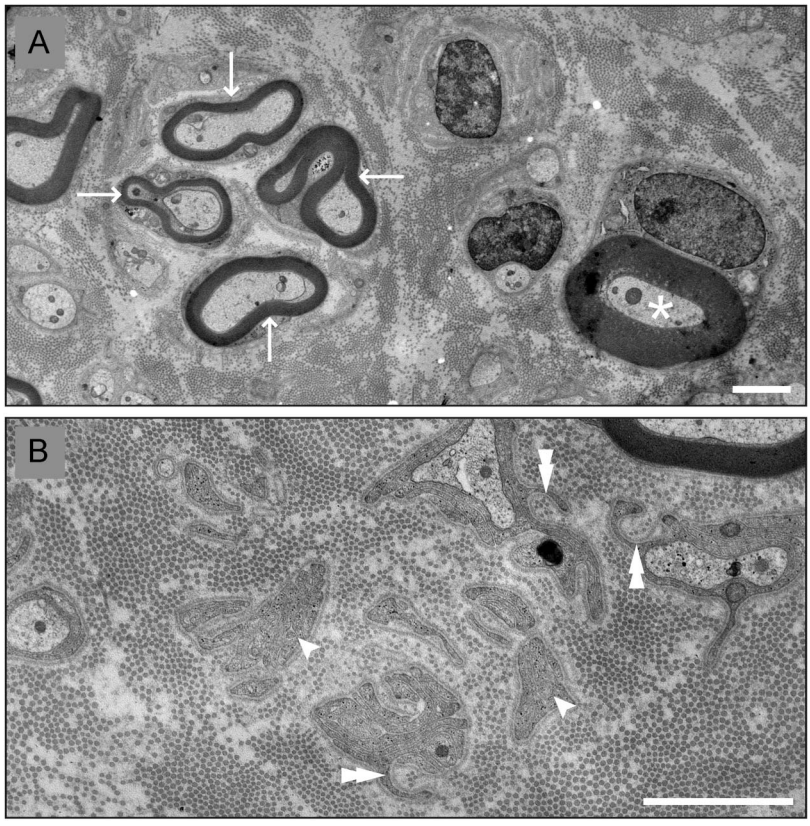
The proband's sister (II-1) was evaluated at age 48 years. She reported numbness in her toes for several years. Her strength was normal, and reflexes were present and symmetric. Pinprick sensation was reduced to the knees, and vibration was reduced at the knees (4), ankles (3), and toes (2). Plantar responses were flexor. Her nerve conductions were normal except for borderline amplitudes of the peroneal motor response (table). Her CMTNS was 7. Her B_{12} and glucose tolerance tests were normal. Although she had sensory symptoms that resulted in her CMTNS of 7, her sensory amplitudes were normal, and we did not consider her to be affected like her other siblings; we made this assessment before the genetic testing was done.

Sequencing of tRNA synthetase genes reveals a novel mutation in *AARS*. Genetic testing for known CMT loci was conducted on the proband. *GJB1*, *MPZ*, *NEFL*, *GDAP1*, and *MFN2* mutations were not found upon sequencing in 2007. At this point, the family was added to a group of samples for sequencing of *GARS*, *YARS*, and *AARS*. No mutations were identified in *GARS* or *YARS*, but a novel sequence variant c.304G>C was found in *AARS* (figure 1B). This variant is present in all living, affected members of the family and absent in unaffected family members. The variant is not present in 186 Caucasian control chromosomes, dbSNP, or any sequences included in the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS/), 1000 genomes (http://www.1000genomes.org/), or Genome Variant Database for Human Disease. Thus, c.304G>C *AARS* is a rare variant that segregates with electrophysiologic evidence of peripheral neuropathy and has not been observed in the general population.

This nucleotide variant results in a missense substitution of the glycine residue at position 102 with an arginine (p.Gly102Arg). This mutation is in the highly conserved activation domain of the AARS protein, where ATP is attached to alanine to make an alanyl adenylate (Ala-AMP) intermediate, completing the first step in tRNA charging. This residue is highly conserved and is present in species as evolutionarily distant as *Saccharomyces cerevisiae* and *Escherichia coli* (figure 1C). In silico predictions indicated that the mutation was probably damaging (PolyPhen2 score = 1.000[27]; SIFT score = 0)[28] and disease-causing (MutationTaster score = 0.999) (http://www.mutationtaster.org/).

Mutant alanyl-tRNA synthetase is unable to complement yeast lacking ALA1. To determine whether the p.Gly102Arg amino acid substitution alters the function of the AARS protein, we used a yeast complementation assay (figure 3), which has been used to identify loss-of-function properties of CMT-associated *AARS* mutations.[4] To assess the functional consequences of p.Gly102Arg *AARS*, we modeled this mutation in the yeast ortholog *ALA1* (residue Gly102 in AARS is equivalent to residue Gly106 in ALA1, and hereafter

Figure 2 Sural nerve biopsy



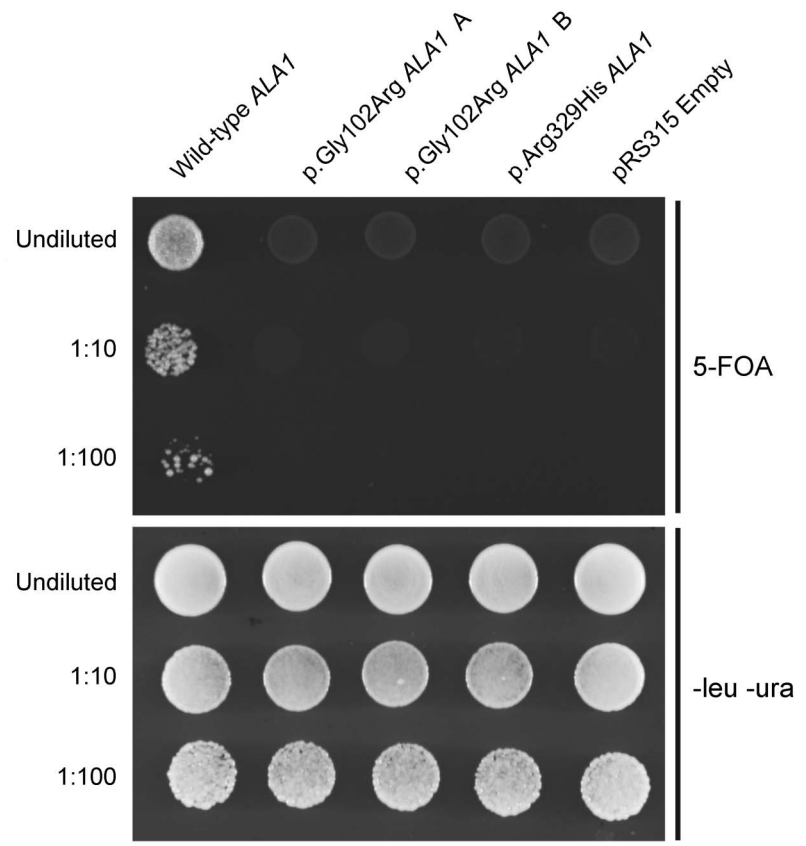
Digital electron micrographs from the sural nerve biopsy of the proband (patient II-5) at age 21 years. (A) This micrograph shows a cluster of regenerated axons (arrows) and a myelinated axon with myelin that is proportionally thicker than expected (asterisk). (B) This micrograph shows Schwann cell processes that are not associated with axons (arrowheads), and some that partially surround collagen fibers (double arrowheads). Scale bars: 2 μm.

referred to by the human substitution) and tested for the ability to support yeast cell growth compared to wild-type and p.Arg329His *ALA1*.[4] A previously validated haploid yeast strain (with the endogenous *ALA1* gene deleted and a vector that expresses wild-type *ALA1* and *URA3* to maintain viability) was transformed with a separate vector harboring a *LEU2* selection marker and either no insert, wild-type *ALA1*, p.Gly102Arg *ALA1*, or the previously reported CMT-associated p.Arg329His *ALA1* allele. Yeast were then selected on media containing 5-fluoroorotic acid (5-FOA), which is toxic to yeast expressing *URA3* and thus selects for cells that have spontaneously lost the maintenance vector. Only yeast cells expressing a functional *ALA1* allele from the *LEU2*-bearing vector will survive on 5-FOA. The wild-type *ALA1* expression vector sustained yeast viability, while the empty vector was unable to complement the knockout allele, consistent with *ALA1* being an essential gene (figure 3). Yeast expressing p.Gly102Arg or p.Arg329His *ALA1* were unable to survive on 5-FOA media (figure 3). These results suggest that p.Gly102Arg *AARS* represents a loss-of-function allele.

DISCUSSION We report a novel mutation in *AARS* that is associated with an indolently progressive, mild myeloneuropathy—a phenotype not previously associated with *AARS* mutations. The p.Gly102Arg mutation affects a conserved residue in an important domain, and segregates with disease in a pedigree with 9 living individuals. This phenotype is different from the p.Arg329His *AARS* mutation that was first identified in 2 French families[5] and subsequently in an Australian family.[4] All 3 families had a dominantly inherited axonal neuropathy affecting motor and sensory axons. A different mutation, p.Asn71Tyr, was found in a large Chinese family that was said to have late-onset, mild CMT2, but the relevant clinical and electrophysiologic findings demonstrating sensory axon involvement, and thus justifying the diagnosis of CMT2, were not reported.[7] Thus, the p.Asn71Tyr mutation could be a distal hereditary motor neuropathy, which was the reported phenotype of the p.Asp893Asn mutation in another Chinese family.[29] Like the p.Gly102Arg mutation, the p.Asn71Tyr and the p.Arg329His mutations affect highly conserved amino acids.

The electrophysiologic data demonstrated that 4 of 5 siblings had a mild axonal neuropathy, and we made the clinical diagnosis of a myelopathy in 3 of them. Interestingly, upgoing toes or hyperactive reflexes have been noted in 2 kindreds of patients with *GARS* mutations.[30,31] In these cases, like the family we report, one supposes that both descending axons from the cortex and brainstem, as well as the PNS axons themselves, are affected in a length-dependent manner. Involvement of both PNS and CNS axons is not surprising: an axonal neuropathy is a common feature of many forms of hereditary spastic paraplegias, spinocerebellar ataxias, and more complex syndromes. Rather, it is surprising that a myelopathy is not more commonly noted in CMT2 patients, as most of the genes that are implicated in this disorder are expressed in the neurons of the CNS and PNS, and for most disorders, there is no known reason why CNS neurons would be unaffected. AARS is expressed in all cells and performs a central role in protein translation. Others have hypothesized that as the genetic underpinnings of CMT2 and hereditary spastic paraplegias and are linked to genes in similar pathways, the pathophysiologic and phenotypic distinctions may blur to become a spectrum of axonal diseases.[32,33] Whereas the clinical and electrophysiologic findings enable us to deduce that the longest sensory and motor axons are most affected in typical axonal neuropathies (including in this family), exactly which axons are responsible for the clinical phenotype of spasticity is more difficult to infer. In Friedreich ataxia, amyotrophic lateral sclerosis, and hereditary spastic paraplegias,[34] the loss of myelinated axons in

Figure 3 Yeast assay: p.Gly102Arg *ALA1* fails to support yeast cell growth



Haploid Δ*ala1* yeast strains were transformed with a vector containing no insert (pRS315 Empty) or an insert to express wild-type, p.Gly102Arg or pArg329His *ALA1*. Cultures resulting from each transformation condition are spotted undiluted and diluted (1:10 and 1:100) on plates containing 0.1% 5-fluoroorotic acid (5-FOA) complete medium or SD -leu -ura growth medium. Experiments were performed using 2 independently generated *ALA1* expression constructs (labeled p.Gly102Arg A and B) for each allele.

the lateral corticospinal tract (which contains myelinated axons of various diameters[35]) is thought to account for the spasticity, so we postulate that this holds for the family we report. It should be noted, however, that this clinical teaching is at odds with the observation that surgical disruption of the corticospinal track at the pyramidal decussation does not cause spasticity in other primates.[36]

Results from our yeast complementation assay demonstrated loss-of-function characteristics of p.Gly102Arg *AARS*, which also suggests that the mutation causes disease. Importantly, while tRNA synthetases harboring disease-causing mutations have successfully complemented yeast lacking the equivalent tRNA synthetase, no benign missense variants have been shown to cause a loss of function in this assay. Therefore, loss-of-function properties in yeast are a reliable predictor of those tRNA synthetase mutations that are associated with peripheral neuropathy. The tRNA synthetase mutations that fail to complement their yeast orthologs (yeast genes in parentheses) *GARS* (*GRS1*), *YARS* (*TYSI*), *AARS* (*ALA1*), *HARS* (*HTS1*), *KARS* (*KRS1*), and *MARS* (*MES1*) also demonstrate loss of function in cell-free tRNA charging assays (for those mutations that have been examined using both techniques), which suggests that this assay is a corollary for tRNA charging function. Now 3 pathogenic *AARS* mutations that have been examined using this assay demonstrated loss-of-function properties (p.Arg329His, p.Asn71Tyr, and now p.Gly102Arg), while the fourth (p.Asp893Asn) has not been examined.

There is little insight into how mutations in alanyl-tRNA synthetase cause neuropathy or a host of other conditions.[37] We favor a shared pathogenic mechanism between *AARS* mutation and other tRNA synthetase mutations that cause peripheral neuropathy. The most insight into the mechanism of tRNA synthetase–linked neuropathy comes from the study of *GARS*-linked dominantly inherited CMT2D.[2] Two *Gars* mutations cause peripheral neuropathy in mice,[38,39] and overexpression of wild-type human *GARS* failed to mitigate severity of the disease in these models, suggesting a toxic gain-of-function mechanism.[40] Despite this, the mechanism of toxicity has not been identified and loss-of-function assays in yeast continue to be among the most specific tests for disease pathogenesis.

Identification of new disease-associated mutations with strong genetic evidence for disease causation and careful evaluation of clinical phenotypes is important to further understand the mechanism of tRNA synthetase-associated CMT disease. Our findings indicate that additional functional characterization of this variant is warranted and may help uncover the relative vulnerabilities of peripheral and CNS axons to tRNA synthetase mutations.

AUTHOR CONTRIBUTIONS

Dr. Motley: study concept and design, analysis and interpretation of data, preparation and revision of the manuscript. L.B. Griffin: acquisition of data, analysis and interpretation of data, preparation and critical revision of the manuscript. I. Mademan: acquisition of data, analysis and interpretation of the data. Dr. Baets: study concept and design, acquisition of data, analysis and interpretation of the data. E. De Vriendt: acquisition of data, analysis and interpretation of the data. Dr. De Jonghe: study concept and design, data acquisition, analysis and interpretation of the data. Dr. Antonellis: study supervision, analysis and interpretation of data, critical revision of the manuscript. Dr. Jordanova: study supervision, study concept and design, analysis and interpretation of the data, critical revision of the manuscript. Dr. Scherer: study concept and design, analysis and interpretation of the data, preparation and revision of the manuscript, study supervision.

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DISCLOSURE

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