

Phenotypic spectrum of disorders associated with glycyI-tRNA synthetase mutations

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We describe clinical, electrophysiological, histopathological and molecular features of a unique disease caused by mutations in the glycyI-tRNA synthetase (GARS) gene. Sixty patients from five multigenerational families have been evaluated. The disease is characterized by adolescent onset of weakness, and atrophy of thenar and first dorsal interosseus muscles progressing to involve foot and peroneal muscles in most but not all cases. Mild to moderate sensory deficits develop in a minority of patients. Neurophysiologically confirmed chronic denervation in distal muscles with reduced compound motor action potentials were features consistent with both motor neuronal and axonal pathology. Sural nerve biopsy showed mild to moderate selective loss of small- and medium-sized myelinated and small unmyelinated axons, although sensory nerve action potentials were not significantly decreased. Based on the presence or absence of sensory changes, the disease phenotype was initially defined as distal spinal muscular atrophy type V (dSMA-V) in three families, Charcot-Marie-Tooth disease type 2D (CMT2D) in a single family, and as either dSMA-V or CMT2D in patients of another large family. Linkage to chromosome 7p15 and the presence of disease-associated heterozygous GARS mutations have been identified in patients from each of the five studied families. We conclude that patients with GARS mutations present a clinical continuum of predominantly motor distal neuronopathy/axonopathy with mild to moderate sensory involvement that varies between the families and between members of the same family. Awareness of these overlapping clinical phenotypes associated with mutations in GARS will facilitate identification of this disorder in additional families and direct future research toward better understanding of its pathogenesis.

Keywords: Charcot-Marie-Tooth disease; distal spinal muscular atrophy; genotype–phenotype relationships; glycyI-tRNA synthetase; hand-predominant muscle atrophy

Abbreviations: CAP = cytoskeleton-associated protein; CMAP = compound motor action potential; CMT = Charcot-Marie-Tooth disease; dHMN = distal hereditary motor neuronopathy; dSMA = distal spinal muscular atrophy; EM = electron microscopy; EMG = electromyography; MNF = myelinated nerve fibre; SNAP = sensory nerve action potential; UMNf = unmyelinated nerve fibre

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Introduction

Distal spinal muscular atrophy (dSMA) or distal hereditary motor neuronopathy (dHMN) represents a genetically heterogeneous group characterized by slowly progressive muscle weakness and atrophy in the distal parts of the

limbs caused by progressive anterior horn cell degeneration (Pearn *et al.*, 1978; Harding and Thomas, 1980; Harding, 1993). Twelve chromosomal loci and seven disease-causing genes have been associated with some of the dSMA variants (Irobi *et al.*, 2004*a, b*). Charcot-Marie-Tooth disease type 1 (CMT1) is a peripheral motor and sensory demyelinating neuropathy caused by degeneration of Schwann cells, while CMT type 2 (CMT2) is characterized by primary axonal degeneration (Dyck and Lambert, 1968*a, b*). CMT2 is manifested by distal muscular atrophy, reduced compound motor action potentials (CMAPs), and reduced sensory nerve action potentials (SNAPs), but normal or mildly slowed motor nerve conduction velocity. Ten genetic loci and four genes have been associated with various forms of CMT2 (Berciano and Combarros, 2003; Pareyson, 2004; Zuchner *et al.*, 2004).

A unique variant of dSMA, in which weakness and wasting primarily affects the hands and forearms with no sensory involvement (Lander *et al.*, 1976; O'Sullivan and McLeod, 1978) has been classified as dSMA type V (Harding, 1993). Studies of a large dSMA-V Bulgarian family genetically mapped this disorder to chromosome 7p15 (Christodoulou *et al.*, 1995). Independently, a hereditary motor and sensory neuropathy causing weakness, atrophy and sensory impairment in the hands more than in the feet was discovered in a North American family from Nebraska, termed CMT type 2D (CMT2D), and mapped to the same locus (Ionasescu *et al.*, 1996). The relationship between dSMA-V and CMT2D was closely examined in a large family from Mongolia that included patients with either dSMA-V or CMT2D clinical phenotypes segregating with an identical haplotype. The disease was mapped to a 3 cM interval between the North American CMT2D and the Bulgarian dSMA-V candidate regions, suggesting a possibility that dSMA-V and CMT2D are allelic disorders (Sambuughin *et al.*, 1998; Ellsworth *et al.*, 1999). The glycyI-tRNA synthetase gene (*GARS*) located in this chromosomal region was eventually identified as the gene causing dSMA-V and CMT2D syndromes; *GARS* mutations were detected in each of the original dSMA-V, CMT2D and dSMA-V/CMT2D families (Antonellis *et al.*, 2003). Linkage to chromosome 7p was identified in smaller families with features of CMT2D (Pericak-Vance *et al.*, 1997).

In this report, we summarize clinical, electrophysiological, pathological and genetic data collected in studies of the three prototype pedigrees (Bulgarian with dSMA-V, North American with CMT2D and Mongolian with both dSMA-V and CMT2D phenotypes), and two additional newly identified families with mutations in the *GARS* gene, with the goal of delineating the phenotypic spectrum of this disorder.

Patients and methods

We conducted a comparative analysis of 60 patients from the originally reported Bulgarian, North American and Mongolian families, a family from France (Antonellis *et al.*, 2003) and a newly identified family from England currently residing in Australia (Antonellis *et al.*, manuscript in preparation), all genetically linked to markers on chromosome 7p15 and harbouring mutations in the *GARS*

gene. Institutional Review Boards of each participating institution approved the genetic studies. After obtaining informed consent, affected and unaffected members of these families were evaluated by at least one of the authors. Standard clinical, neurophysiologic and histopathological methods were used. Neurological examination was performed on 60 individuals, and electrophysiological study on 26 individuals. Neurological examination included assessment of mental status, cranial nerves, muscle strength (MRC scale), reflexes, coordination and gait. Evaluation of sensory impairment included clinical testing for pain and temperature sensation, vibration and position sense. Motor and sensory nerve conduction velocities, CMAPs, sensory amplitudes, distal motor latencies, and *F*-wave latencies were recorded under standard conditions from median, ulnar, peroneal, tibial and sural nerves. The distances for distal motor stimulation and interpretation of the findings were based on the normative values used at the geographic region of the patient's origin. Needle electromyography (EMG) was performed in index cases of each phenotype to confirm the neurogenic nature of distal muscle disease. EMG was also used to phenotype at-risk individuals when muscle weakness and wasting were not readily detectable. Concentric needle EMG was performed in proximal and distal muscles of the upper and lower limbs. Denervation was inferred when there was spontaneous muscle fibre activity (fibrillations, positive sharp waves or complex repetitive discharges) in at least one insertion with long duration high amplitude motor units or reduced recruitment pattern.

Nerve biopsy

Sural nerve biopsy was performed on two patients from the Mongolian family, one with a dSMA-V-like phenotype and another with a CMT2D-like phenotype, and one patient from the Bulgarian family with dSMA-V, with informed consent. A segment of nerve was fixed in 3% glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate buffer. Cross-sections of 1 mm thickness were post-fixed in 0.1 M osmic tetroxide for 2 h, dehydrated in a series of graded ethanols and propylene oxide, and embedded in epoxy resin (LX-112). Semithin sections were stained with toluidine blue or paragon. Thin sections were stained with lead citrate and uranyl acetate, and examined under a Philips electron microscope (CM-100).

Myelinated nerve fibre density and size distribution

Four images of each nerve from the Mongolian family at a magnification of $\times 800$ were captured using a Gatan 673 camera. Digital micrograph software was used to determine the diameter (mean of long and short axis) and the endoneurial area to measure and count myelinated nerve fibres (MNFs). MNF diameter determined from the measurements of long and short axis shows results similar to those obtained by methods based on circle equivalent area (Dyck *et al.*, 1984). Histograms of patients' fibre densities and fibre diameters (at 1.0 μm intervals) were compared to control sural nerve biopsies processed in each participating laboratory. The *G*-ratio (axon diameter/fibre diameter) was determined on semi-thin sections. Myelinated fibre morphometry in the Bulgarian patient was carried out by using an IBAS 2 image analyser (Kontron, Oberkochen, Germany).

Unmyelinated nerve fibre density

Sixteen images of endoneurial areas were captured at a magnification of $\times 2600$. The area of 0.001 mm^2 for a total area of 0.159 mm^2 in

Mongolian dSMA-V patient's nerve and 0.0173 mm² in Mongolian CMT2D patient's nerve were analysed on each image. We used previously established criteria to distinguish unmyelinated nerve fibres (UMNFs) from Schwann cell processes (Johnson *et al.*, 1994).

Sural nerves of five individuals, mean age 58 years, showing no apparent abnormalities served as controls. Sural nerve analyses in five further individuals with no apparent abnormalities, mean age 44 years, previously used as the basis in our published report on UMNF density (Johnson *et al.*, 1994), were added as controls in this study. Unmyelinated axon density in the Bulgarian patient was estimated on electron micrographs at magnification of $\times 10\,000$.

Genetic testing

A total of 40 affected individuals from the five studied pedigrees, 43 of their unaffected relatives, 62–184 control individuals from each geographic area where the affected families originated, and 88–134 DNA samples from North American 'mixed-ethnic' controls with removed personal identification were tested for each *GARS* mutation that we have identified in the affected families. Genomic DNA was extracted by standard procedures and served as template for PCR amplification with intronic primers constructed to amplify each exon. Amplification was carried out by using an optimal procedure designed for each segment. The resulting DNA fragments were purified by QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and directly sequenced using BigDyeTerminator™ sequencing protocol on an automated 3100 ABI Prism® Genetic Analyzer (Applied Biosystems, Foster City, CA). Data was extracted and analysed using Sequencing Analysis software (Applied Biosystems) and aligned using Sequencher software (GeneCodes, Ann Arbor, MI). Sequences of primers used in this study are available on request.

Results

Clinical findings

The pattern of inheritance was autosomal dominant with male-to-male transmission observed in each of the five

studied families. Clinical information obtained from evaluation of 60 patients is summarized in Table 1. Although the disease was initially diagnosed as dSMA-V in some families and CMT2D in others, a significant overlap of clinical findings is evident. The presenting symptom was muscle weakness in the hands that occurred between 8 and 36 years of age, with most patients (75%) developing symptoms during the second decade of life. The earliest manifestation of illness in many patients was transient cramping and pain in the hands on exposure to cold, and cramping in calf muscles on exertion.

Progressive weakness and atrophy of the thenar and first dorsal interossei muscles were the major complaints in each patient. In some patients, hand weakness started unilaterally, but the other side soon became involved. The lower extremity involvement when present varied in severity from weakness and atrophy of the extensor digitorum brevis (EDB) and weakness of toe dorsiflexors to classic peroneal muscular atrophy with foot drop. Peroneal muscles were affected earlier and more severely than the calf muscles. Proximal limb muscle weakness was not observed in the upper or lower extremities. Patients with lower leg involvement had a high steppage gait. Sensory examination was either normal or showed mild to moderate impairment limited in many cases to vibration deficits in the hands and feet, but in some patients we observed reduction of pinprick, temperature, touch and vibration perception in a stocking and less often glove pattern. Reflexes at the ankles were lost mostly in patients who developed leg muscle weakness and sensory deficits. The presence or absence of sensory deficits has traditionally been used for distinguishing between dSMA defined as exclusively motor distal involvement and CMT, a distal motor and sensory neuropathy (Harding, 1993). Among patients with *GARS*-mutation-associated disease, we observed patterns that corresponded to dSMA-V-like and CMT2D-like clinical variants (Table 1).

Table 1 Phenotypic features of *GARS* mutation-associated disease in five studied families

	Family origin					
	Bulgaria	North America	Mongolia		UK/Australia	France
Clinical variant	dSMA-V	CMT2D	dSMA-V	CMT2D	dSMA-V	dSMA-V
<i>GARS</i> mutation	L129P	G240R	E71G	E71G	H418R	G526R
Number of examined patients	21	14	11	6	6	2
Average age at onset	16.9	23.0	18.3	17.0	26	19.5
Weakness in the hands on presentation	21/21	14/14	11/11	6/6	6/6	2/2
Progressive weakness and wasting of thenar and FDI* muscles	21/21	14/14	11/11	6/6	6/6	2/2
Peroneal weakness	9/21	14/14	9/11	6/6	5/6	0/2
Peroneal atrophy	5/21	2/14	2/11	5/6	5/6	0/2
Pes cavus/hammertoes	0/21	14/14	0/11	2/6	4/6	
Reduced or absent ankle reflexes	16/21	14/14	9/11	3/6	0/6	0/2
Generalized hyporeflexia	0/21	2/14	0/11	3/6	0/6	0/2
Pyramidal dysfunction	5/21	0/14	0/11	0/6	0/6	0/2
Reduced sensation for touch	0/21	14/14	0/11	6/6	0/6	0/2
Reduced sensation for pain and temperature	0/21	14/14	0/11	6/6	0/6	0/2
Reduced vibration sense	13/21	14/14	0/11	6/6	0/6	2/2

*FDI, first dorsal interosseus.

Patients with the dSMA-V variant were observed in the Bulgarian, Australian and French families, while all patients of the North American family showed the CMT2D phenotype. The Mongolian family included patients with either dSMA-V or CMT2D phenotypes. Of the 40 patients with the dSMA-V variant, 21 (53%) eventually developed demonstrable weakness in the evertors and dorsiflexors of the feet, while the remaining 19 (47%) patients with the same dSMA-V phenotype have not developed lower extremity weakness within an average of 18.5 years after the disease onset. In contrast, all 20 patients showing the CMT2D phenotype developed bilateral foot and peroneal weakness and atrophy at an average of 3.3 years after the hand atrophy became evident. Thus, patients with the CMT2D phenotype developed hand onset motor symptoms with relatively quick progression to peroneal and foot involvement, while only about half of the patients with dSMA-V phenotype developed foot atrophy at later stages of illness. Severe hand and leg involvement with gross peroneal and calf muscle atrophy was observed in six dSMA-V and six CMT2D patients, all but one older males with mean duration of illness 32 years. Each patient with the CMT2D-like phenotype developed moderate reduction of the perception of pain, touch, temperature, position and vibration in the feet and mild sensory deficits in the hands. In contrast, only vibration sense was mildly decreased in 13/40 (32%) of patients diagnosed with dSMA-V, most frequently in those with prolonged duration of illness (mean, 30 years).

Hyperactive reflexes were recorded in only five dSMA-V patients from a single branch of the Bulgarian family; two of the five exhibiting upgoing toes. Muscle tone was normal in all patients including those with pyramidal signs. Pes cavus deformity and/or hammertoes correlated with the severity of weakness at the ankles. Scoliosis was seen in four patients. The peripheral nerves were not enlarged. No fasciculations, tremors, foot ulcers or vocal cord paralysis were seen. Cranial nerve function was intact. Hearing was unaffected. No signs indicative of ocular, retinal, cerebellar or extrapyramidal involvement were present. None of the patients developed bulbar or respiratory problems or obvious cognitive decline in later life. No systemic abnormalities were detected. Although the disease was slowly progressive, the majority of patients functioned independently up to 40 years after the disease onset. Only five patients needed support for ambulation. There was no obvious cardiovascular, hepatic or renal pathology in these patients.

Electrophysiology

After the clinical pattern was established, electrophysiological testing was performed in selected patients (Table 2). The affected individuals showed markedly reduced CMAPs recorded from the abductor pollicis brevis (APB) by median nerve stimulation, i.e. they were absent or measured below 1 mV. In contrast, CMAPs recorded from the abductor digiti minimi (ADM) by ulnar nerve stimulation were preserved. Motor

Table 2 Results of electrophysiological studies in patients with GARS mutation-associated disease

Results of electrophysiological studies	Phenotype	
	dSMA-V	CMT2D
Motor nerve conduction studies		
Upper extremity		
Compound muscle action potential		
Median-APB, <4.5 mV*	9/9	2/2
Ulnar-ADM, <3.5 Mv	0/13	0/2
Distal motor latency		
Median, >5.6 ms	0/9	0/9
Ulnar, >4.5 ms	1/9	0/2
Nerve conduction velocity		
Median and ulnar, <39 m/s	0/17	0/5
Lower extremity		
Compound muscle action potential		
Peroneal-EDB, <2 mV	5/8	3/3
Tibial-AH, <2.5 mV	1/2	0/1
Distal motor latency		
Peroneal and tibial, >7.5 ms	0/14	0/3
Nerve conduction velocity		
Peroneal and tibial, <29 m/s	0/12	0/3
Sensory nerve conduction studies		
SNAP		
Median, <10 μ V and ulnar, <8 μ V	2/17	0/4
Sural, <6 μ V	4/14**	1/6***

APB, abductor pollicis brevis; ADM, abductor digiti minimi; EDB, extensor digitorum brevis; AH, adductor hallucis. The number of patients showing abnormalities appears as numerator and the number of patients studied as denominator. *Normal values derived from published data. **Abnormal sural SNAP responses were within the range of 3.5–5.4 μ V. ***Abnormal sural SNAP response was 5 μ V.

nerve conduction velocities and distal latencies were normal in all nerves of the arms and legs wherever CMAP amplitudes were >1 mV, thus excluding a demyelinating neuropathy. Infrequently, motor conduction velocities were mildly reduced (at CMAP >1 mV), but never <25% of the predicted value. Median nerve entrapment at the wrists was excluded by orthodromic transcarpal studies in selected patients. CMAP amplitude recorded by stimulation of the peroneal and tibial nerves was <1 mV in patients having clinically evident leg atrophy.

Median SNAP amplitudes and conduction velocities were normal in most patients, even those with mildly prolonged distal motor latency (Table 2). Of 17 dSMA-V patients, 2 had evidence of mild ulnar neuropathy with amplitudes of 5 and 4.5 μ V and normal conduction velocities. Sural SNAP amplitudes were mildly reduced in a minority of patients with both clinical phenotypes, but never completely lost: 4 of the 14 studied dSMA-V patients showed sural SNAP amplitudes varying from 3.5 to 5.4 μ V, and 1 of the 6 patients with the CMT2D phenotype had 5 μ V. All other patients in both groups had normal sural nerve amplitudes.

In patients with overt disease, needle EMG showed no voluntary motor activity in the abductor pollicis and first dorsal interossei due to marked atrophy. Spontaneous activity

was often seen in these muscles. EMG was also carried out in order to identify subclinical disease in at-risk individuals. Large long-duration motor unit potentials with reduced recruitment pattern were observed in the thenar muscles of 1 of the 3 tested mutation-carrying presymptomatic individuals. Similarly, chronic partial denervation was seen in the ADM and extensor muscles of the legs in the absence of apparent muscle atrophy.

Nerve biopsy studies

Sural nerve biopsy findings are summarized in Table 3.

Case 1

A 40-year-old Mongolian female diagnosed as dSMA-V, developed symptoms of progressive hand weakness and wasting at the age of 15 years. On examination, there was marked atrophy of thenar and interosseus muscles, but spared hypothenar muscles (Fig. 1A). The toe extensors and evertors of the ankles were mildly weak. Calf muscles were hypertrophic. Pes cavus was present, although no significant peroneal muscle weakness was detected. CMAPs were absent in the thenar eminences, but normal in the hypothenars. Sensory nerve conduction amplitudes were normal in the hands and feet.

Table 3 Clinical, electrophysiological and pathologic features of three individual patients in which nerve biopsy was carried out

Age/sex	dSMA-V (Mongolian) 40/F	CMT2D (Mongolian) 47/M	dSMA-V (Bulgarian) 34/M	Healthy controls (Mean = 44)
Clinical features				
Thenar and FDI weakness/atrophy	+++	+++	+++	
Hypothenar weakness/atrophy	–	++	–	
Peroneal atrophy	+	+++	+	
Pes cavus	++	+++	++	
Ankle reflexes	+	–	–	
Reduced vibration sense in the hands	–	+	–	
Reduced vibration sense in the feet	–	++	+	
Pin prick and temperature deficits				
In the hands	–	+	–	
In the feet	–	++	–	
Motor nerve electrophysiology				
Median				
Distal latency (ms)	0	0	0	<4.5
Conduction velocity (m/s)	0	0	0	>49
CMAP in APB (mV)	<0.5	<0.5	<0.5	>4.5
Ulnar				
Distal latency (ms)	2.9	0	3.2	<3.5
Conduction velocity (m/s)	52	0	50.4	>49
CMAP in ADM (mV)	7.2	<0.5	5	>4.5
Peroneal				
Distal latency (ms)	5.6	0	0	<6
Conduction velocity (m/s)	37	0	0	>39
CMAP in EDB (mV)	1.2	<0.5	<0.5	>2.5
Tibial				
Distal latency (ms)	4.9	0	0	<6
Conduction velocity (m/s)	4.0	0	0	>39
CMAP in AH (mV)	6.9	<0.5	<0.5	>2.5
SNAPs (μV)				
Radial	50	28	ND	>12
Median	58	ND	52	>10
Ulnar	45	ND	5.0	>8
Sural	20	22	70	>6
Electromyogram				
PL and TA	CN +	SA (no units)	CN ++	
MG	Normal	CN ++	CN +	
FDI and APB	SA, CN +++	SA (no units)	SA (no units)	
ADM	Normal	CN ++	Normal	
MNF density per mm ²	7945	5291	8562	7440–9680
Proportion of fibres <7 μ m in diameter (%)	51.7	46	56	65
Unmyelinated fibre density per mm ²	20 176	24 331	Presumed normal	20 041–40 912*

–, absent; +, present; ++, increased; +++, severely increased; FDI, first dorsal interossei; APB, abductor pollicis brevis; ADM, abductor digiti minimi; EDB, extensor digitorum brevis; AH, adductor hallucis; PL, peroneus longus; TA, tibialis anterior; MG, M. gastrocnemius; CN, chronic neurogenic changes; SA, spontaneous activity; ND, not done. *Johnson et al., 1994.

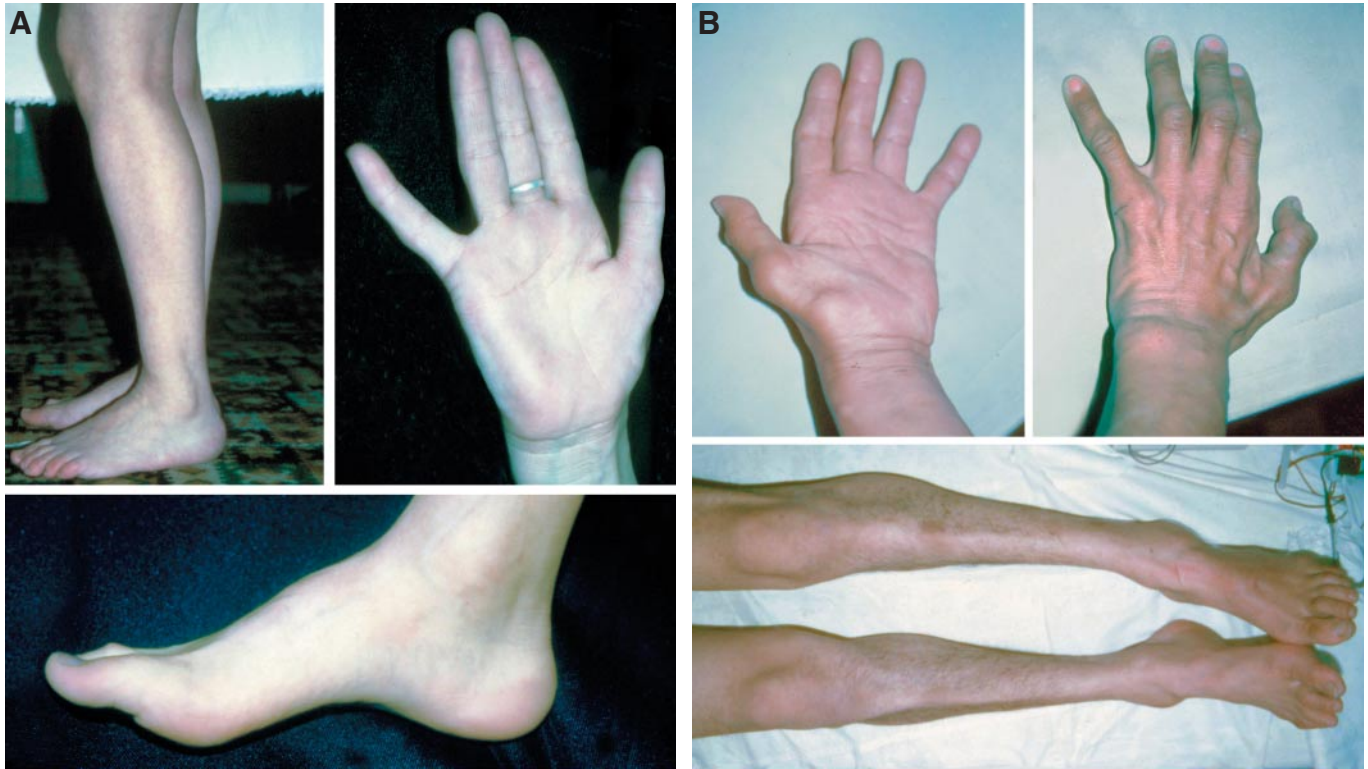


Fig. 1 (A) Patient with dSMA-V-like phenotype. Thenar and first dorsal interosseus muscle wasting, preserved hypothenar. Pes cavus and early development of toe deformity in the absence of significant peroneal atrophy. (B) Patient with CMT2D-like phenotype. Wasting of thenar, first dorsal interosseus and hypothenar muscles. Peroneal atrophy, pes cavus and hammerhead toes.

EMG showed distal chronic partial denervation in weak and atrophic muscles.

Nerve biopsy showed clear signs of axonal pathology with two or more regenerative clusters per fascicle (Fig. 2A). No evidence of active degeneration and no obvious signs of demyelination or typical onion bulb formation were present. Myelin structures appeared normal. Overall myelinated fibre density was at 7945/mm² (controls: 7440–9680, mean 8389/mm²). Fibres below 7 µm in diameter represented 52% of the overall number of fibres in the patient compared to 65% in control specimens. The fibre diameter frequency histogram, although bimodal, reflects this trend and suggests a reduction of small- and medium-size fibres (Fig. 3). Electron microscopy (EM) showed denervated Schwann cell subunits as indicated by an increased number of profiles, suggesting damage to small unmyelinated fibres. The UMNF density was at the low normal level of 20 176/mm² (controls: 20 041–40 912/mm²).

Case 2

A 47-year-old Mongolian male with the diagnosis of CMT2D, developed symmetrical weakness and atrophy of the hands at the age of 20 years; gait disturbance and leg weakness became evident 5 years after the disease onset. On examination, hand muscles, including the hypothenar eminence, were weak and atrophic; bilateral peroneal weakness and atrophy were

prominent (Fig. 1B). Reflexes were absent throughout. There was moderate reduction of perception to touch, pain and temperature in stocking distribution and moderate reduction of vibration sense in the feet. Sensory deficits were definite but less prominent in the hands. Motor nerve stimulation could not elicit CMAPs in any distal muscle. Signs of denervation in distal muscles on EMG suggested severe motor axonopathy. The SNAPs were normal in the hands and feet (Table 3).

Nerve biopsy showed clear evidence of axonal pathology that was qualitatively more prominent than in Case 1. Axonal swelling with filamentous accumulations (Fig. 2D) and at least four, often up to eight, regenerative clusters per fascicle were observed (Fig. 2E). Pseudo onion bulb formations and a few thinly myelinated fibres were seen. Myelin structures appeared intact. Overall myelinated fibre density was reduced to 5291/mm². The proportion of fibres <7 µm in diameter was limited to 46%. The fibre size histogram (Fig. 3) shows relative reduction of small-size (<7 µm) and middle-size (7–11 µm in diameter) fibres, sparing the large myelinated fibres. Denervation of Schwann cell subunits as indicated by an increased number of profiles is seen on EM (Fig. 2C). The UMNF density was 24 176/mm².

Case 3

A 34-year-old Bulgarian dSMA-V patient, presented at age 14 years with weakness and atrophy of hand muscles. Within a

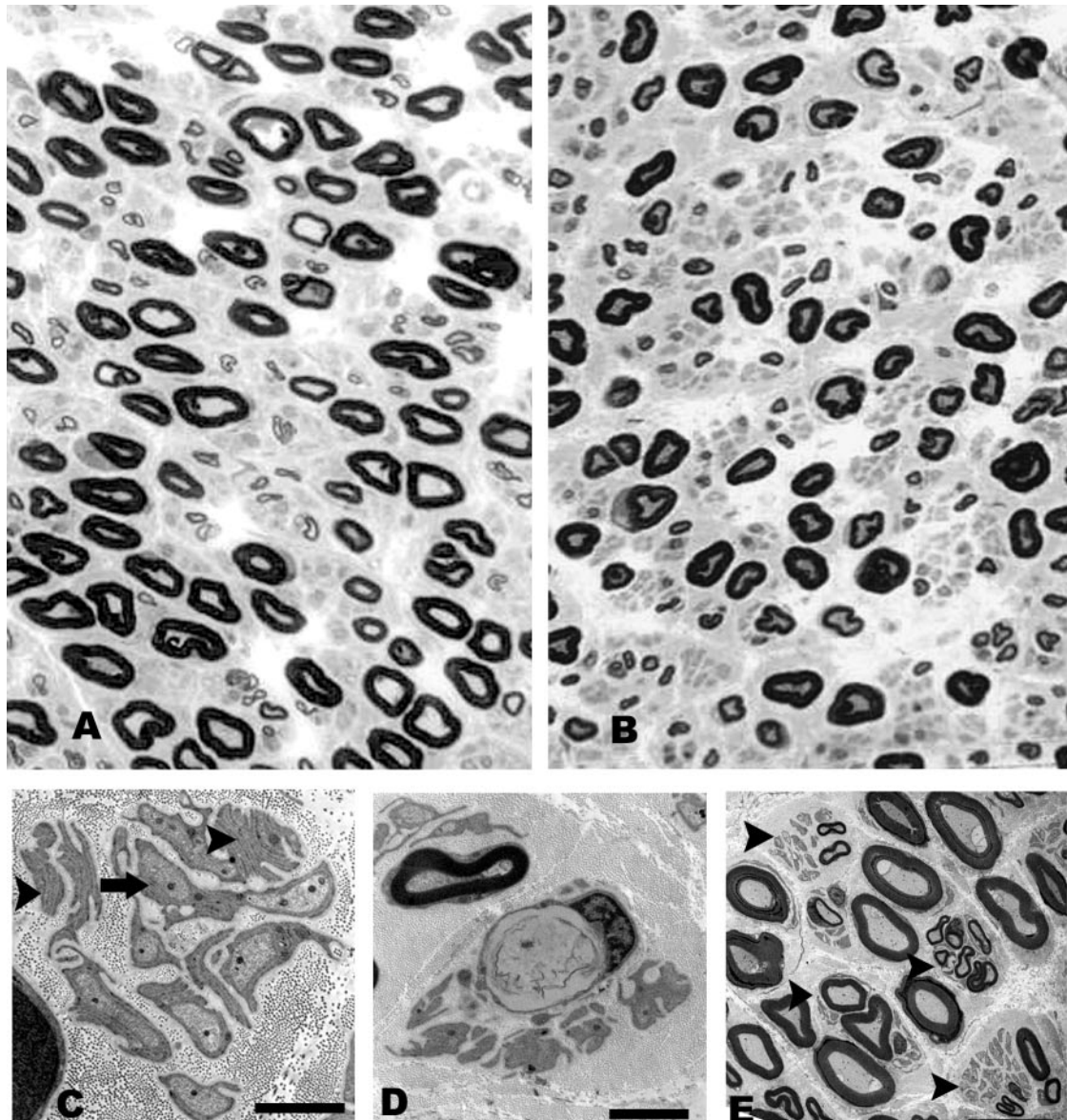


Fig. 2 Sural nerve morphology in GARS-related dSMA-V and CMT2D phenotypes. **(A)** dSMA-V, Mongolian family. Epon embedded, paragon stain, original magnification $\times 400$. Pathologic changes are minimal with a near-normal MNF density. **(B)** CMT2D, Mongolian family. Epon embedded, paragon stain, original magnification $\times 400$. MNF density is moderately reduced. A regenerating sprout cluster and several thinly myelinated fibres are seen. **(C)** CMT2D, Mongolian family. EM (bar = $2\ \mu\text{m}$). Unmyelinated fibre cluster. Two lamellar stacks of denervated Schwann cell processes (arrowheads), indicative of previous unmyelinated axon degeneration. Arrow points to a normal Schwann cell unit with axons. **(D)** CMT2D, Mongolian family. EM (bar = $3\ \mu\text{m}$). Active axonal degeneration of MNF. **(E)** CMT2D, Mongolian family. EM (bar = $3\ \mu\text{m}$). Multiple regenerative clusters (arrowheads).

year, he developed weakness of foot muscles. On examination, the patient had weakness and wasting of the thenar eminence and first dorsal interosseus and, to a lesser extent, the hypothenar eminence. There was mild wasting in the distal leg muscles. Ankle jerks were absent, with proximal reflexes preserved. There was mild impairment of vibration sense in the toes and no sensory deficit in the hands. On motor nerve conduction studies and EMG, CMAPs were absent in the APB and EDB, but present in ADM muscles. Sensory nerve conduction amplitudes were preserved in the hands and feet (Table 3).

Nerve biopsy showed clear evidence of regenerative activity, with up to four regenerative clusters per fascicle. No evidence of active degeneration or onion bulb formation was observed. Myelin structures were normal; thinly myelinated axons were rare. No abnormal axonal or Schwann cell inclusions were seen. Overall myelinated fibre density was normal at $8562/\text{mm}^2$. Evidently, regenerative clusters maintained the myelinated fibre density in the normal range. The proportion of fibres $< 7\ \mu\text{m}$ in diameter was reduced to 56%. The frequency histogram of fibre diameters was interpreted as normal. The UMNF density was not quantified

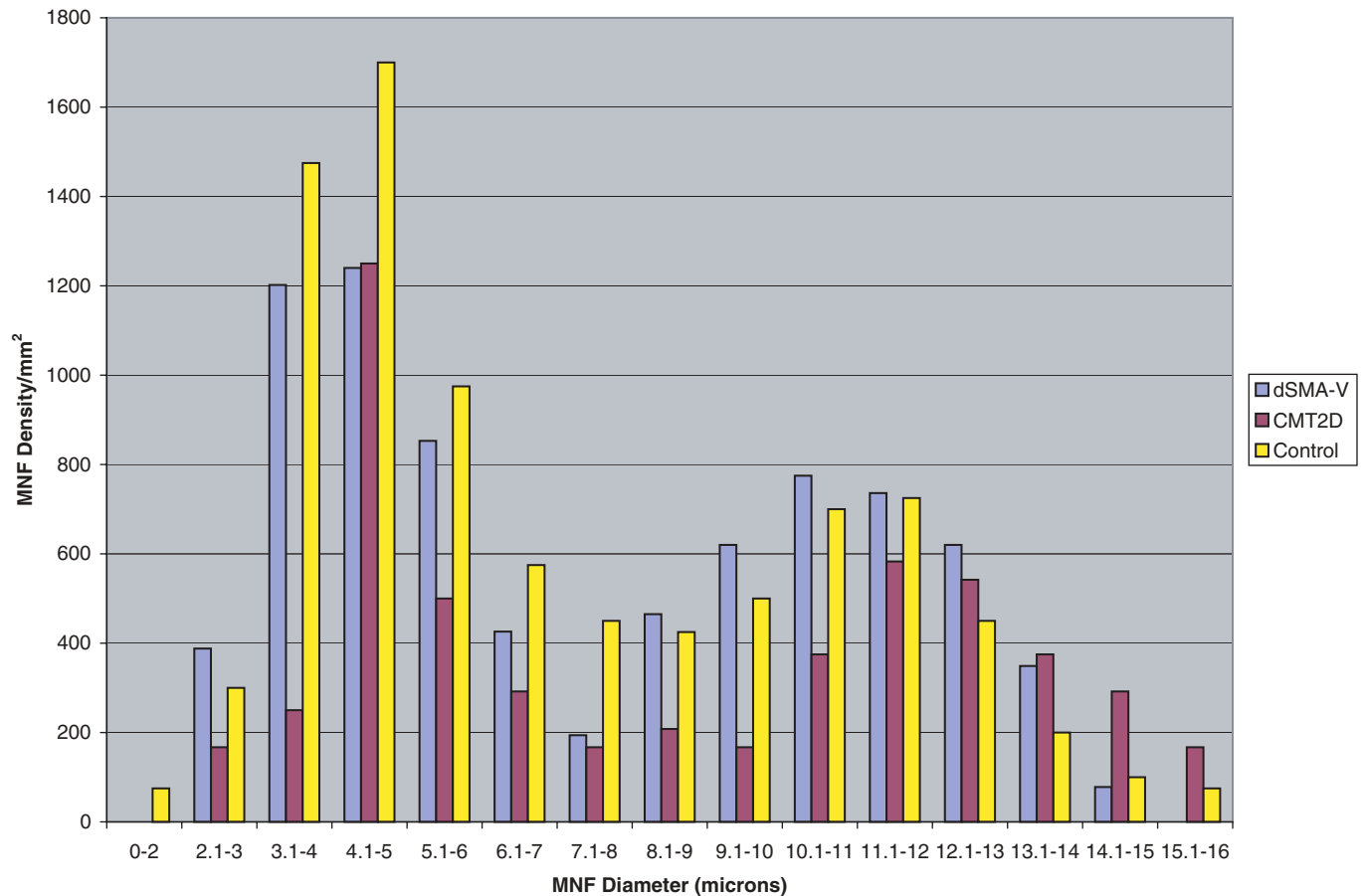


Fig. 3 Size distribution of myelinated fibres in patients with *GARS* mutations associated disease, dSMA-V and CMT2D phenotypes.

in this patient; however, the light microscopy did not suggest a loss.

Comparative analysis suggests that while there is clear evidence of sensory axonal pathology in each studied patient, the abnormalities are quantitatively greater in the patient with the CMT2D-like phenotype, as indicated by the presence of regenerative clusters, pathologic changes within axons and the decrease in myelinated fibre density, involving fibres of small and medium size. There was also evidence of degeneration in unmyelinated fibres.

GARS mutations

Forty patients representing five families were tested for mutations in the *GARS* gene. Exon-by-exon mutation screening detected heterozygous point mutations in each of the 40 individuals affected with either the dSMA-V or CMT2D disease variant (Table 4). L129P mutation was identified in patients from the Bulgarian family, G240R in North American patients, E71G in patients from Mongolia, and G526R in the French, as previously reported (Antonellis *et al.*, 2003). A novel *GARS* H418R mutation was identified in the UK/Australian family. The identified missense mutations consistently segregated with the disease. Thirteen at-risk family members, some of them at the age beyond the average

of disease onset, also show the presence of *GARS* mutations. Most probably, this indicates that the disease is incompletely penetrant. A three-generation branch of the Bulgarian family (grandfather, daughter and granddaughter) carrying the L129P mutation remained asymptomatic. To investigate whether *GARS* mutations are not common DNA variations, we screened control individuals in geographic regions close to each family's place of origin, and additionally in a 'mixed-ethnic' North American population (Table 4). This 'mixed-ethnic' North American population served as a sole control for the North American family. All tested controls were negative for each mutation, including the newly identified H418R, making it unlikely that any of these mutations represent a normal human polymorphism. Furthermore, the affected amino acids are conserved between species ranging from human to yeast. Three of the five mutations are located within or in close proximity to the functional domains.

Discussion

The disorder caused by *GARS* mutations is characterized by autosomal dominant inheritance and adolescent onset of disease with unique patterns of motor and sensory

Table 4 GARS mutations in patients, family members and control individuals

	Family origin				
	Bulgaria	North America	Mongolia	UK/Australia	France
Mutation*	L129P	G240R	E71G	H418R	G526R
Affected individuals	5/5	8/8	17/17	8/8	2/2
Unaffected family members	6/12	0/4	7/22	0/5	ND**
Population controls (from the geographic area of family origin)	0/100	0/184	0/65	0/62	0/80
'Mixed-ethnic' North-American population controls	0/88	0/184	0/134	0/100	0/100

*Codon numbering according to Ge et al., 1994. **ND, not done.

manifestations. Patients with the dSMA-V-like form of disease show predominant thenar and first dorsal interosseus muscle atrophy and sparing of the hypothenar eminence until later in the illness. In about one-half of the patients, there is lower limb involvement, and in one-third a mild vibration sense loss observed in advanced disease. In contrast, patients with the CMT2D-like variant show consistent presence of weakness and atrophy in the feet and distal leg muscles, pes cavus and moderate sensory abnormalities. These patients typically develop peroneal weakness and sensory deficits relatively early in the disease. The finding that sensory involvement in CMT2D is not related to the duration of illness, but rather the disease severity suggests that a modifying factor may operate in some individuals predisposing them to the development of one or the other phenotype.

The results of electrophysiological studies are consistent with motor axonopathy as evidenced by denervation on EMG predominantly in the distal muscle groups at normal distal latencies and conduction velocities. It is notable that the motor axonal loss in these patients does not follow a classic length-dependent pattern, since in both phenotypes the small muscles of the hands were involved earlier than the small muscles of the feet. Furthermore, the fact that motor neuron pools for the thenar and hypothenar muscles are nearby in the spinal cord with little or no difference in the number of motor neurons that innervate each muscle group (Kuwabara et al., 1999) or in their axonal lengths, also argues against a primary length-dependent distal axonopathy and is more in favour of a motor neuronopathy.

We also obtained clear evidence that GARS mutations cause sensory axonal loss, it was established at histopathological examination of a sensory nerve from a CMT2D patient. Similar albeit milder changes were seen in dSMA-V cases. Although sensory deficits are uniform findings in CMT2D patients, only one of six showed reduction in SNAPs on nerve conduction studies and none had completely absent sensory responses. The preservation of larger-size myelinated fibres correlates with the preserved SNAPs, as previously observed in hereditary sensory neuropathies (Low et al., 1978; Dyck et al., 1983; Donaghy et al., 1987). The possibility that GARS mutations cause sensory ganglionopathy in addition to axonal degeneration similar to the deficits produced by vitamin B12 deficiency (Fine and Hallett, 1980) may be

assumed but cannot be validated without postmortem studies.

Our data suggest that dSMA-V and CMT2D are variants of a clinical continuum associated with GARS mutations and challenge the nosological relationship between dSMA and CMT2 as independent disorders. In fact, sensory abnormalities have been known in other motor neuronopathies, such as the infantile SMA type 1 (Rudnik-Schoneborn et al., 2003) and X-linked recessive bulbospinal neuronopathy (Sobue et al., 1989). The existing clinical classification was challenged in another confusing situation, in which Silver syndrome exhibiting a markedly different phenotype was also classified as dSMA-V (distal HMN type V). Silver (1966) described this syndrome as familial spastic paraplegia with non-selective wasting of small muscles of the hands commencing between 15 and 30 years. Mutations in the *BSCL2* (*Seipin*) gene have now been identified in several families with Silver syndrome (Windpassinger et al., 2004; Irobi et al., 2004c), stressing a generic distinction between GARS mutation-associated dSMA-V and dSMA-V caused by *Seipin* mutations.

The mechanism by which mutations in a ubiquitously expressed housekeeping protein result in selective neuronal damage and axonal loss with early thenar muscle involvement is not clear. Glycyl-tRNA synthetase, a class II aminoacyl-tRNA synthetase, performs an essential function in protein synthesis by catalysing aminoacylation of glycyl-tRNA, which is required in the process of insertion of glycine into proteins (Ge et al., 1994). The enzyme must properly recognize the tRNA species and the amino acid in order to maintain fidelity of translation. In accordance with its function, glycyl-tRNA synthetase encoded by the GARS gene has three domains: a catalytic core, a C-terminal anticodon recognition domain and a domain that interacts with the acceptor stem of glycyl-tRNA (Freist et al., 1996). The fact that three of the known five GARS mutations occurred within or next to the catalytic core of the synthetase indicates that they may interfere with the ability of glycyl-tRNA to interact with the receptor of the cognate tRNA.

Some cytoskeleton-associated proteins (CAPs) and motor proteins involved in axonal transport contain a highly conserved glycine-rich CAP-Gly motif that is responsible for binding to microtubules during long-range transport (Li et al., 2002). CAP-Gly motif mutations in dynactin and

tubulin-specific chaperone B are responsible for the development of motor neuron disease in humans (Puls *et al.*, 2003) and mice (Martin *et al.*, 2002). The high glycine content may at least partly explain the vulnerability of proteins of this class and the resulting axonal damage from glycyl-tRNA synthetase defects. Another pathogenic mechanism, the susceptibility of motor neurons to defects in RNA processing is currently attracting increased attention (Anderson and Talbot, 2003; Irobi *et al.*, 2004a).

In conclusion, glycyl-tRNA synthetase mutations cause a distinct syndrome generally characterized by autosomal dominant inheritance and adolescent onset of progressive weakness and wasting of the thenar and first dorsal interosseus muscles, variable involvement of the lower limb muscles and sensory impairment. Electrophysiological data disclosed motor axonopathy affecting distal muscles. Sensory nerves show mild-to-moderate axonal degeneration corresponding to the clinically apparent sensory deficits. Studies of additional affected families would be important to establish a population frequency of this disorder and further define the type and degree of motor and sensory involvement in patients carrying various GARS mutations.

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