A recurrent WARS mutation is a novel cause of autosomal dominant distal hereditary motor neuropathy

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Words in the main body: 5,793

Abstract words count: 237

Running title: A recurrent WARS mutation causes dHMN

Summary

Distal hereditary motor neuropathy (dHMN) is a heterogeneous group of inherited neuropathies characterized by distal limb muscle weakness and atrophy. Although at least 15 genes have been implicated in dHMN, the genetic causes remain elusive in many families. To identify an additional causal gene for dHMN, we performed exome sequencing for two affected individuals and two unaffected members in a Taiwanese family with an autosomal dominant dHMN in which mutations in common dHMN-implicated genes had been excluded. The exome sequencing revealed a heterozygous mutation, c.770A>G (p.His257Arg), in the cytoplasmic tryptophanyl-tRNA synthetase (TrpRS) gene (WARS) that co-segregates with the neuropathy in the family. Further analyses of WARS in additional 79 Taiwanese pedigrees with inherited neuropathies and 163 index cases from Australian, European, and Korean dHMN families identified the same mutation in another Taiwanese dHMN pedigree with different ancestries and one additional Belgian dHMN family of Caucasian origin.

Cell transfection studies demonstrated a dominant-negative effect of the p.His257Arg mutation on aminoacylation activity of TrpRS, which subsequently compromised protein synthesis and reduced cell viability. His257Arg TrpRS also inhibited neurite outgrowth and led to neurite degeneration in the neuronal cell lines and rat motor neurons. Further in vitro analyses showed that the WARS mutation could potentiate the angiostatic activities of TrpRS by enhancing its interaction with vascular endothelial-cadherin (VE-cadherin). Taken together, these findings establish WARS as a gene whose mutations may cause dHMN and alter canonical and non-canonical functions of TrpRS.

Keywords: distal hereditary motor neuropathy, dHMN, exome sequencing, *WARS*, tryptophanyl-tRNA synthetase

Abbreviations:

AMP = adenosine monophosphate; ARSs = Aminoacyl-tRNA synthetases; β-Gal = beta-galactosidase; CMT = Charcot-Marie-Tooth disease; Co-IP = co-immunoprecipitation; dHMN = distal hereditary motor neuropathy; dSMA = distal spinal muscular atrophy; FL = full-length; HUVECs = human umbilical vein endothelial cells; PPi = pyrophosphate; siRNAs = small interfering RNAs; TrpRS = tryptophanyl-tRNA synthetase; UTR = untranslated region; VE-cadherin = vascular endothelial-cadherin; VEGF = vascular endothelial growth factor; WT = wild-type.

Introduction

Distal hereditary motor neuropathy (dHMN), also known as distal spinal muscular atrophy (dSMA), comprises a clinically and genetically heterogeneous group of inherited neuropathies characterized by slowly progressive distal limb muscle weakness and atrophy with no or minimal sensory involvement (Harding, 2005; Rossor et al., 2012). Phenotypically, dHMNs are very similar to axonal Charcot-Marie-Tooth disease (CMT) and both entities may share common causative genes (Murphy et al., 2012; Rossor et al., 2013). Traditionally, dHMNs are divided into seven subtypes based on age at onset, mode of inheritance, and presence of additional features (i.e. vocal cord palsy, predominate upper limbs involvement, and pyramidal signs) (Harding, 2005; Rossor et al., 2012). However, there are substantial genetic heterogeneities for a clinically homogenous dHMN subtype actually being attributed ~~by~~ to mutations in different genes. To date, mutations in at least 15 genes have been implicated in dHMN (http://neuromuscular.wustl.edu), but the genetic diagnosis for more than 70% of patients with dHMN remains elusive (Dierick et al., 2008; Murphy et al., 2012; Rossor et al., 2012; Rossor et al., 2013).

Aminoacyl-tRNA synthetases (ARSs) are ubiquitously expressed, essential enzymes responsible for the first step of translation process, in which ARSs charge amino acids to cognate tRNA molecules (Delarue, 1995). Mutations in six ARS genes have been implicated in hereditary polyneuropathies. These genes include glycyl- (*GARS*; MIM *600287) (Antonellis et al., 2003), tyrosyl- (*YARS*; MIM *603623) (Jordanova et al., 2006), alanyl- (*AARS*; MIM *601065) (Latour et al., 2010), histidyl- (*HARS*; MIM *142810) (Vester et al., 2013; Brozkova et al., 2015), methionyl-

(*MARS*; MIM *156560) (Gonzalez et al., 2013), and lysyl- (*KARS*; MIM *601421) (McLaughlin et al., 2010) tRNA synthetase genes. All of them are associated with axonal or intermediate CMT, and three of them (GARS, AARS and HARS) are also linked to dHMN. Especially for *GARS, YARS, AARS* and *HARS* the evidence in favor of pathogenicity is strong ~~for *GARS, YARS, AARS* and *HARS*~~ with multiple mutations and families reported, supporting a critical role of ARSs in maintaining peripheral nerve function. Except for KARS mutations leading to a recessively inherited CMT, neuropathies related to other five ARS genes are autosomal-dominantly inherited and each of the implicated ARSs functions as dimers or tetramers (Ibba and Soll, 2000). These common features imply a dominant-negative mechanism underlying these ARS-related neuropathies (Wallen and Antonellis, 2013).

Here, we report a recurrent p.His257Arg mutation in *WARS* (MIM *191050), initially identified by whole exome sequencing and segregating with an autosomal-dominantly inherited dHMN in two unrelated Taiwanese pedigrees and later found in one additional dHMN family of European ancestry in Belgium. *WARS* encodes the human cytoplasmic tryptophanyl-tRNA synthetase (TrpRS), which is an essential enzyme responsible for charging $tRNA^{trp}$ molecules with tryptophan (Shen et al., 2006; Yang et al., 2006) and also possesses a non-canonical effect on angiostasis (Wakasugi et al., 2002; Kise et al., 2004). We characterize the phenotypic features and functional influences of the WARS mutation and postulate that WARS causes dHMN when mutated.

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Materials and methods

Subjects

Eleven individuals of two unrelated Taiwanese families of Han Chinese origin afflicted with a molecularly unassigned autosomal-dominantly inherited dHMN were enrolled into the study, including 7 patients and 4 unaffected individuals (Families A and B in Fig. 1A). Additional 78 unrelated Taiwanese index patients including 73 with molecularly unassigned axonal CMT and 5 with dHMN were also recruited. Genomic DNA was isolated from peripheral blood leukocytes following a standard protocol. Pathogenic variants in 20 genes known to be associated with dHMN or other hereditary motor neuropathies, including AARS, ATP7A, BICD2, BSCL2, DNAJB2, DYNC1H1, FBLN5, GARS, HARS, HINT1, HSPB1, HSPB3, HSPB8, IGHMBP2, MYH14, PLEKHG5, SBF1, SETX, SLC5A7 and TRPV4, had been excluded in the index patients by a targeted multi-gene next-generation sequencing (Hsiao et al., 2016). The lower limbs of patient A-III-3 and one healthy control were studied with MRI by using a Signa EXCITE 1.5-tesla system (GE Medical Systems). T1-weighted images (repetition time 517–613 milliseconds, echo time 7.98–8.34 milliseconds, 10-mm slice thickness) were used to look for the presence of muscle atrophy with fatty substitution.

In addition, we screened for mutations in the candidate gene in the index patients of 82 European, 57 Australian and 24 Korean families with molecularly unassigned dHMN, in whom pathogenic variants in genes implicated in inherited neuropathy had been excluded by exome sequencing. The Ethical Review Boards of the participating institutions approved this study. All patients or their legal representatives signed informed consent prior to enrolment.

Genetic analyses

In family A, exome sequencing was performed in two affected and two unaffected individuals using Agilent SureSelect Human All Exon v2 kit for exome enrichment and the Illumina HiSeq2000 platform with a paired-end 2 x100 bp protocol for sequencing. Since the dHMN in pedigree A is inherited in an autosomal dominant manner, only heterozygous variants shared by the two patients but absent in any of the two unaffected individuals were taken for further analyses. Variants present in the Single Nucleotide Polymorphism Database (dbSNP144; https://www.ncbi.nlm.nih.gov/snp), the 1000 Genomes Project (1000 Genomes Project Consortium et al., 2012), Exome Aggregation Consortium (ExAC version 0.3) (Lek et al., 2016), the 24 in-house exome controls, and those that do not alter coding sequences were excluded. Sanger sequencing was then performed to determine whether any of the remaining variants completely co-segregated with dHMN in pedigree A (Supplementary Table 1). Mutation Taster (Schwarz et al., 2014) and Combined Annotation Dependent Depletion (CADD) (Kircher et al., 2014) were used to predict the pathogenicity of the identified variants. Sanger sequencing of the coding exons and the exon-intron boundaries of *WARS* were performed in additional 79 unrelated Taiwanese index patients with axonal CMT or dHMN, including the one from family B. Haplotype analysis was conducted in families A and B by genotyping seven single nucleotide polymorphism (SNP) markers flanking *WARS* c.770A>G and covering a region of 83.5 kb (Fig. 1A).

Exome sequencing in the additional cohorts of total 163 dHMN index patients was performed using Agilent SureSelect Human All Exon kit v2, 4, 5, or Roche-NimbleGen SeqCap EZ v3.0 for exome enrichment and Illumina HiSeq2000 or HiSeq2500 platform for sequencing. The exome data of 82 European and 10 Australian patients with dHMN were imported and re-annotated into the GENESIS

(gem.app) platform, a web-based tool for next generation sequencing data analysis (http://thegenesisprojectfoundation.org/) (Gonzalez et al., 2015). The *WARS* coding variants revealed by exome sequencing were further confirmed by Sanger sequencing.

Expression plasmids

A 1.4 kb PCR-amplified fragment containing the full-length (FL) coding region of WARS (NM_004184.3) was cloned into pcDNA3.1/myc-His (Invitrogen) or pEGFP-N1 (Clontech). The CDH5 cDNA clone (BC096363) was purchased from transOMIC. The expression vector for Flag-tagged vascular endothelial-cadherin (VE-cadherin) was generated by subcloning the coding sequence of CDH5 into pFlag-CMV5a (Sigma-Aldrich). For the production of recombinant His-tagged TrpRS in Escherichia coli (E. Coli), the coding sequences of WARS encoding FL and T2-TrpRS (residues 94-471 of FL TrpRS) were separately cloned into pQE-30 vector (Qiagen). QuikChange Site-Directed Mutagenesis kit (Stratagene) was used to introduce the c.770A>G (p.His257Arg) mutation into the expression plasmids. All the primers and plasmids used in this study are listed in Supplementary Tables 2 and 3, respectively.

Cell culture, growth and transfection

Human embryonic kidney 293 (HEK293) cells and human SH-SY5Y neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Mouse Neuro-2a (N2a) neuroblastoma cells were maintained in minimal essential medium (MEM; Invitrogen) supplemented with 10% FBS. Human umbilical vein endothelial cells (HUVECs) were maintained in medium M200 (Invitrogen) containing low serum growth supplement and 5% FBS. Rat ventral spinal cord neurons, extracted and

isolated from Wistar rat ventral spinal cords at the 14th embryonic day, were purchased from ScienCell Research Laboratories, grown according to the supplier instruction, and used for primary motor neuron culture. Specificity of the motor neurons was attested by immunocytochemistry with antibodies against the motor neuron marker, non-phosphorylated neurofilament H (SMI-32), choline acetyl transferase (ChAT) and islet I. The motor neuron culture medium consisted of neuronal medium (ScienCell) supplemented with neuronal growth supplement, 10 ng/ml brain derived neurotrophic factor (BDNF), 50 ng/ml nerve growth factor (NGF), and 2% FBS. Cells were transfected using Lipofectamine 2000 (Invitrogen) in the following transfection experiments. For endogenous WARS knockdown, siRNAs specifically targeting the 3' untranslated region (UTR) of WARS (Supplementary Table 2) were transfected into cells by using Lipofectamine 2000 (Invitrogen). N2a cells were differentiated by incubation in MEM supplemented with 1 mM dibutyryl cAMP, 10 μM retinoic acid and 1% FBS. For differentiation, SH-SY5Y cells were treated with 10 μM retinoic acid.

Reporter assays for assessing protein synthesis

HEK293 cells were co-transfected with wild-type (WT) or mutant TrpRS expression construct along with beta-galactosidase (β-Gal) reporter plasmid pCMV-lacZ (Clontech) or firefly luciferase reporter plasmid pCMV-Red Firefly Luc (Thermo Scientific). At 48 hours after transfection, cell lysates containing equal amounts of protein (2.5 μg) were analyzed for β-Gal or luciferase activities by using β-Galactosidase Reporter Gene Activity Detection Kit (Sigma-Aldrich) or Pierce Firefly Luciferase Glow Assay Kit (Thermo Scientific), respectively.

Production and purification of WT and His257Arg TrpRSs

WT and His257Arg TrpRSs with a C-terminal in-frame His tag were expressed in Escherichia coli (E. Coli) M15 and purified by affinity chromatography with nickel resin according to the manufacturer's instructions (Novagen). Protein concentration was determined by the Bradford method (Bio-Rad) using bovine serum albumin as a standard.

Aminoacylation activities of WT and His257Arg TrpRSs

The aminoacylation activities were estimated by quantifying the release of pyrophosphate (PPi) and adenosine monophosphate (AMP) during aminoacylation. The reactions were performed in 50 μl of the reaction mixture containing TrpRS proteins (crude protein extract or purified protein) with 100 mM Tris-HCl (pH7.6), 10 mM $MgCl_2$, 40 mM KCl, 1mM dithiothreotol, 40 U/ml bovine liver tRNA (Sigma-Aldrich), 1 mM ATP, 0.2 mM tryptophan, and 5 U/ml of yeast inorganic pyrophosphatase (Sigma-Aldrich) at 37°C for 1 hour. The PPi produced in the reaction was hydrolyzed by the coupling enzyme pyrophasphatase and the free orthophosphates (Pi) were measured in a colormetric assay using Biomol Green reagent (Enzo Life Sciences). The production of AMP was quantified using the AMP-Glo Assay (Promega) method with a microplate luminometer (TECAN).

Co-immunoprecipitation (Co-IP) analysis

HEK293 cells were co-transfected with constructs to express EGFP-tagged WT or His257Arg mutant TrpRS as well as Myc-tagged WT or His257Arg mutant TrpRS. Cell extracts were prepared 48 hours post-transfection using Pierce IP Lysis Buffer (Thermo Scientific). Except for the 10% of sample volume loaded for input, the

lysates were incubated with anti-Myc antibody-conjugated Dynabeads (Invitrogen) at 4°C overnight with rotation. After washing with RIPA buffer, bound proteins were eluted in 1X SDS loading buffer at 95 °C for 5 min and the eluates were subjected to Western blotting with antibodies against TrpRS. Antibodies used in this study are summarized in Supplementary Table 4.

Cell viability assay

At 24 hours after transfection, the HEK293 cells transfected with different TrpRS constructs were treated with *WARS* siRNAs (25nM) specifically targeting the 3'UTR (Supplementary Table 2). After incubation for another 24 hours, cell viability of the transfected cells was examined using the Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich).

Neurite outgrowth assays and immunocytochemistry

Cells were grown on coverslips precoated with poly-L-lysine (100 μg/mL) and laminin (50 μg/mL) and transfected with WT or His257Arg TrpRS expression construct. Sixteen hours after transfection, the N2a cells or SH-SY5Y cells were induced to differentiate for 48 hours and the neurite outgrowth phenotypes were observed under a phase-contrast light microscope (PrimoVert, Zeiss) prior to proceed with immunostaining. The differentiation step was omitted for rat primary neuron cells but culture medium was changed to remove residual Lipofectamine. For immunocytochemistry, cells were fixed in 4% paraformaldehyde, permeablized with 0.2% Tween-20 and blocked with 5% BSA before incubation in primary antibody overnight at 4°C (Supplementary Tables S4). Bound primary antibodies were detected using Alexa-conjugated secondary antibodies. Images were taken with an Olympus

FluoView FV10i confocal laser scanning fluorescence microscopy system. Transfected cells bearing neurite-like extensions with length of at least one cell body were counted and scored as the percentage of total transfected cells. The determination of the average lengths of primary neurites was performed through the analysis of microscopic images using the Image-Pro PLUS (Meyer Instruments) measurement tool.

Yeast complementation assays

Please see the Supplementary Methods.

In vitro pull-down assay

Protein pull-down experiments were performed using Pierce Pull-Down PolyHis Protein:Protein Interaction Kit (Thermo Scientific) according to the manufacturer's instructions. In brief, His-tagged TrpRS was expressed in E. coli M15 and purified by Ni-NTA affinity chromatography. Bead-bound His-tagged TrpRS (40 nM) was mixed with equal amounts of lysates (2 mg) of the transfected HEK293 cells expressing Flag-tagged VE-cadherin, and then incubated for 2 hours with 5 mM $CaCl_2$. Pull-down mixtures and inputs were analyzed by western blotting.

In vitro angiogenesis assays

HUVEC migration assays were performed using 24-well Millicell inserts (8 μm pore size; Merck Millipore) with fibronectin coating. HUVECs were seeded on the upper chamber with 1 x 10^5 cells per well, while vascular endothelial growth factor$_{165}$ ($VEGF_{165}$) (10 ng/ml) was added in the lower chamber. For inhibition assays, recombinant TrpRS (500 nM) was added to both upper and lower chambers and

HUVECs migration was allowed to proceed at 37°C, 5% CO_2 for 16 hours. Migrant cells were fixed, stained with crystal violet, photographed at 100x magnification, and counted in three random views. For Matrigel angiogenesis assay, HUVECs were seeded in Matrigel (BD Biosciences)-coated 24 well plates at a density of 8.5 x 10^4 cells/well and treated with $VEGF_{165}$ (10 ng/ml). After incubation with recombinant TrpRS (500 nM) for 16 hours, HUVECs were microscopically evaluated for tube formation under 40x magnification. An average of vessel coverage (the total microscopic area covered by HUVEC vasculature) from 4 random fields in each well was calculated by ImageJ software (NIH).

Results

Exome sequencing and in silico analyses

We initially studied a three-generation Taiwanese family of Han Chinese origin afflicted with an autosomal-dominantly inherited dHMN, of which four affected individuals and two unaffected ones were enrolled (pedigree A in Fig. 1A). Whole exome sequencing was performed in two affected (A-II-1 and A-III-2) and two unaffected individuals (A-II-7 and A-III-1). On average, 23 Gb of sequence data was generated from each individual, resulting in an average coverage depth of 206x per targeted base (Supplementary Table 1). After analysis and filtering, four heterozygous coding variants were found to be shared by both affected individuals but not by either of the unaffected individuals and were not present in dbSNP build 144, 1000 genome project, ExAC version 0.3, or the 24 in-house exome controls. Sanger sequencing of the 4 variants in all 6 individuals of pedigree A showed that only one variant, c.770A>G (p.His257Arg) in WARS (RefSeq NM_004184.3) (Fig. 1B), perfectly segregates with the dHMN phenotype.

To ascertain the pathogenicity of WARS p.His257Arg, this mutation was screened and not found in 1112 Taiwanese healthy controls (2224 chromosomes). This mutation is also absent in the genome Aggregation Database (gnomAD; www://gnomad.broadinstitute.org). In addition, it was predicted to be a disease-causing mutation by two bioinformatics programs, Mutation Taster and CADD. The CADD v1.2 Phred score for this mutation was 22.3, ranking WARS p.His257Arg in the top 0.59% most deleterious variants in the genome (Kircher et al., 2014). Mutation Taster showed that this variant seemed to be disease-causing with a strong probability value, 0.99999998 (Schwarz et al., 2014). The His257 residue of TrpRS protein is evolutionarily conserved from human to zebrafish (Fig. 1C).

Human TrpRS consists of 471 amino acids, containing an N-terminal domain (residues 1-153), two catalytic domains (residues 154-362 and 453-471) and a C-terminal anticodon-binding domain (residues 363-452) (Fig. 1C) (Shen et al., 2006; Yang et al., 2006). From a structural viewpoint, human TrpRS functions as a homodimer and the His257 residue is situated within the connective polypeptide 1 (CP1) insertion (residues 247-292) of the catalytic domain (Fig. 1C). CP1 is known to play an essential role in dimer interface interaction and forms the substrate-binding pocket to grasp tRNA acceptor arm (Shen et al., 2006). The His257 is very close to the residues Thr259 and Asn261, which are directly involved in the recognition and binding of the tRNA acceptor arm (Fig. 1D) (Shen et al., 2006; Yang et al., 2006). These bioinformatics approaches support that WARS p.His257Arg may compromise the enzymatic activity of human TrpRS.

WARS p.His257Arg is a recurrent mutation in dHMN pedigrees

To further investigate the pathogenic role of WARS mutations in inherited

neuropathies, we screened additional 79 unrelated Taiwanese individuals, including 73 with molecularly unassigned axonal CMT and 6 with dHMN, and the same c.770A>G mutation accompanied with WARS c.1327G>T (p.Ala443Ser) in cis was found in one family with dHMN (pedigree B; Fig. 1A). Among the 5 members of pedigree B, WARS [c.770A>G; c.1327G>T] was present in all 3 affected individuals. One unaffected individual of pedigree B carried only WARS c.1327G>T without WARS c.770A>G (B-II-4; Fig. 1A). WARS c.1327G>T variant, denoted as rs139914390, may be a benign polymorphism because it had been previously identified in 1 of 104 southern Han Chinese samples and 1 of 103 Japanese samples in 1000 genome project. Sanger sequencing demonstrated that WARS c.1327G>T variant was not present in pedigree A (Fig 1A), suggesting that WARS p.His257Arg mutation in pedigree A and pedigree B originated from different ancestors. Haplotype analysis using seven SNP markers flanking the p.His257Arg mutation revealed two distinct haplotypes (Fig 1A). These findings strongly suggested that this identical mutation has independent origins in the 2 pedigrees and provided further evidence for the pathogenic role of WARS p.His257Arg mutation in dHMN.

We further checked other dHMN cohorts from different populations to affirm that a *WARS* mutation is indeed a cause of dHMN. In 163 unrelated index cases from Australia, Korea and the GENESIS collection in the USA, we identified one additional family of European ancestry with the same *WARS* p.His257Arg mutation in Antwerp, Belgium (pedigree C; Fig.1A).

Clinical features

Among the two Taiwanese dHMN pedigrees, individuals B-I-1 and B-I-2 both passed away at octogenarian age without distal limb muscle atrophy or weakness during their

lifetime. Individual A-II-1 has an overt dHMN phenotype with disease onset at 12 years of age but refused to receive clinical evaluation. The clinical manifestations and electrophysiological features of the other 6 affected individuals in the two pedigrees harboring WARS p.His257Arg mutation are similar (Table 1). All of them are affected by a juvenile-onset, slowly progressive, distal predominant, pure motor neuropathy. The average age of disease onset was 12.2 ± 1.2 years (range: 10-13). The affected individuals had normal developmental milestones at early childhood but developed muscle weakness and atrophy in the feet/legs followed by hand muscle involvement. The symptoms mainly were restricted to distal limbs and there was no subjective sensory complaint. Except for A-II-3, who was wheelchair-bound at fifties, the other 5 affected individuals could walk without assistance. All the affected individuals had high arches, a stork leg appearance and a variable degree of hand muscle atrophy and weakness (Fig. 2A). Paralysis and profound atrophy of intrinsic hand muscles were noted in the 2 older affected individuals (A-II-3 and B-II-2). Nerve conduction studies showed a pure motor axonal neuropathy with preserved conduction velocities and no sensory involvement. Skeletal muscle MRI demonstrated that the affected individual A-III-3 had a widespread muscle atrophy with fatty infiltration in the leg muscles, but the muscles in the thighs were spared (Fig. 2C). This finding is concordant with the clinical observation of predominantly distal limb involvement in all the affected individuals. The clinical video of individual A-III-3 is available in the Supplementary Material (Supplementary Video 1).

The female index patient (C-II-1) of the Belgian dHMN family has a disease onset at 9 years with distal weakness in the lower limbs, markedly progressive from the age of 20 years onwards with also weakness in the hands. At the age of 45 years, there was a severe distal weakness in all limbs and no sensory involvement (Fig. 2B).

Nerve conduction studies revealed a pure motor axonal neuropathy (Table 1). Sural nerve biopsy at the age of 32 years revealed normal pattern and muscle biopsy at the same time was compatible with neurogenic atrophy. The proband's father and younger brother (both deceased at the time of evaluation) were reported to have a similarly clinical presentation. Additional family members were not available for segregation studies. The patient died at the age of 66 years to illness unrelated to the dHMN phenotype.

His257Arg TrpRS compromises protein synthesis in a dominant-negative manner

Since TrpRS catalyzes tRNA aminoacylation, which is an essential step of translation process, we constructed WT and His257Arg mutant TrpRS expression plasmids to assess the effect of this mutation on protein synthesis. Two distinct reporter assays were utilized to examine the influence of this mutation on each reporter protein. We first analyzed the β-Gal activities in HEK293 cells co-transfected with β-Gal reporter plasmids along with WT or mutant TrpRS expression construct. As shown in Fig. 3A, the transfectants expressing His257Arg TrpRS had a decreased β-Gal activity in comparison to those expressing WT TrpRS. Interestingly, the β-Gal activity in cells expressing mutant TrpRS was noticeably lower than that in cells without exogenous TrpRS (i.e. cells transfected with empty vector). Moreover, the β-Gal activity was lower in HEK293 cells transfected with half-dose of WT and half-dose of mutant TrpRS expression plasmid than those transfected with half-dose of WT TrpRS expression plasmid only (Fig. 3A). These findings suggested that the exogenous His257Arg mutant TrpRS had a dominant-negative effect to disturb the functions of both endogenous and exogenous WT TrpRSs. Similar results were obtained when experiments were performed using firefly luciferase as a reporter (Fig. 3B). The

differences of the reporter activities between the studied cell groups appeared to be more evident in β-Gal assay than those in firefly luciferase assay given that β-Gal protein contains a higher proportion of tryptophan than firefly luciferase dose (3.8% vs. 0.36%, respectively). Both assays supported that WARS p.His257Arg mutation possesses a dominant-negative effect on protein synthesis.

His257Arg TrpRS has an impaired aminoacylation activity

The negative effect on protein synthesis might be attributed to the defective aminoacylation activity of mutant TrpRS. To test this hypothesis, the aminoacylation activities were analyzed in cell lysates of HEK293 cells co-transfected with different combinations of WT and mutant TrpRS expression plasmids along with 25 nM of the WARS siRNAs knocking down approximately 70% of endogenous TrpRS in HEK293 cells (data not shown). The aminoacylation reaction was simulated in vitro using bovine tRNA as substrate, and the TrpRS activity was estimated by measuring the production of reaction byproducts - AMP and PPi (the schematic equation is shown in Fig. 3C) (Jordanova et al., 2006; Cestari and Stuart et al., 2013).

We first tested the TrpRS aminoacylation activity via measuring PPi concentration from lysates of the transfected HEK293 cells. Similar to the results obtained from the β-Gal or firefly luciferase experiments, the TrpRS aminoacylation activities were lowest in the cells transfected with His257Arg mutant construct only, followed by those transfected with half-dose of WT and half-dose of mutant constructs, and then those transfected with half-dose of WT constructs only (Fig. 3D). The highest enzyme activity was observed in the cells over-expressing WT TrpRS.

Meanwhile, to specifically assess the enzymatic activity of mutant TrpRS, we purified recombinant His-tagged TrpRS proteins from E. Coli. This cell-free assay

also demonstrated that His257Arg mutant TrpRS has a significantly lower aminoacylation activity than WT TrpRS, no matter which measurement of aminoacylation activity (AMP or PPi) was used (Fig. 3E). These findings indicated that WARS p.His257Arg mutation has a direct and dominant-negative effect to compromise the aminoacylation activity of TrpRS, which subsequently perturbs protein synthesis.

His257Arg TrpRS retains the ability to form dimers with WT TrpRS

Since TrpRS functions as a homodimer (Fig. 1D), it would be intriguing to know whether the His257Arg mutation alters the dimerization properties of TrpRS. Co-IP experiments performed with HEK293 cells expressing EGFP-tagged WT or His257Arg mutant TrpRS as well as Myc-tagged WT or His257Arg mutant TrpRS revealed that the mutant TrpRS retained the ability to form dimers with WT proteins (Fig. 3F). The heterophilic association between the mutant and WT molecules might be a key precondition for the dominant-negative effect of WARS p.His257Arg mutation.

His257Arg TrpRS leads to attenuated cell viability

The mutant TrpRS may have a deleterious effect on cell viability due to insufficient protein synthesis. Therefore, we investigated the viability of HEK293 cells transfected with different TrpRS constructs and WARS siRNAs against endogenous TrpRS expression. Compared to cells transfected with empty vector and non-targeting siRNA control, knockdown of the endogenous TrpRS expression by the siRNAs significantly attenuated the cell viability, which could be rescued by expressing exogenous WT TrpRS but not His257Arg mutant TrpRS (Fig. 3G). Notably, cells expressing

exogenous mutant TrpRS had a poorer viability than those transfected with empty vector, indicating that this mutation has a toxic effect rather than simply haplo-insufficiency. These cellular experiments clearly demonstrated a dominant-negative effect of WARS p.His257Arg mutation on cell viability.

His257Arg TrpRS inhibits neurite outgrowth and leads to neurite degeneration

To investigate the effects of mutant TrpRS on neuronal cell differentiation, N2a cells were transiently transfected to overexpress WT or His257Arg TrpRS under retinoic acid-induced differentiated conditions. At 72 hours after transfection, cells expressing WT TrpRS or those transfected with empty vectors (control cells) both had extensive neurite formation, and WT TrpRS overexpression further enhanced neurite outgrowth (Fig. 4A). Conversely, in cells expressing His257Arg mutant TrpRS, we observed a noticeable reduction in both the number of cells bearing neurite and neurite length, compared with cells expressing WT TrpRS (Fig. 4A). Similar results were obtained when TrpRS constructs were expressed in SH-SY5Y cells (Fig. 4B).

We then examined the effect of His257Arg TrpRS on neurite extension utilizing primary culture of rat embryonic motor neurons. Concordant to the results from the neuronal cell lines, motor neurons transfected with mutant TrpRS construct had shorter neurites than those expressing WT TrpRS or those transfected with empty vectors (control cells) (Fig. 4C). Interestingly, as judged by SMI-32 neurofilament immunostainings, we found that motor neurons expressing mutant TrpRS had prominent degenerative changes in the form of irregular swelling and beading of neurites, while this was not the case in cells expressing WT TrpRS or control cells (Fig. 4C). To further determine whether these abnormal phenotypes could be a sign of axonal degeneration, WT or mutant TrpRS-transfected motor neurons were stained

with antibodies against amyloid precursor protein (APP), a marker widely used to denote neuro-degeneration (Irobi et al., 2010). Motor neurons expressing WT TrpRS showed a faint or punctate APP staining (Fig. 4D), indicative of normal and active axonal transport, comparable to that observed in control cells. For mutant TrpRS-expressing motor neurons, there was abundant accumulation of APP in the neurites, which might occur as a consequence of neurite disruption and impaired axonal transport (Fig. 4D). These data suggested that mutant TrpRS compromises neurite growth in primary motor neurons and leads to neurite degeneration.

Yeast complementation assays and aminoacylation activities with yeast tRNA

Human FL WT TrpRS failed to complement *wrs1* deficiency in yeast cells and had a defective aminoacylation activity using yeast tRNA as substrates, whereas His257Arg TrpRS could partially complement *wrs1* deficiency in yeast cells and had a significantly higher aminoacylation activity with yeast tRNA (Supplementary Fig. 1B, 1C, and 1D). These findings support that *WARS* His257Arg mutation may significantly change the structure of human TrpRS so that the mutant TrpRS acquires exceptional catalytic ability toward yeast $tRNA^{Trp}$ and can partially complement *wrs1* deficiency in yeast cells. Please see Supplementary Results for the details.

His257Arg TrpRS acquires an enhanced angiostatic effect

T2-TrpRS, generated by proteolysis of FL TrpRS in physiological condition, has a potent angiostatic activity through binding to VE-cadherin (Tzima et al., 2005; Zhou et al., 2010) and inhibiting VEGF-induced angiogenesis (Wakasugi et al., 2002; Yang et al., 2004). Previous study showed that the binding affinity between VE-Cadherin and FL TrpRS was only 5% of that between VE-Cadherin and T2-TrpRS (Zhou et al.,

2010). However, removal of the N-terminal WHEP domain exposes the essential residues for cell signaling and gives short forms of TrpRS (T2-, T1-, and mini-TrpRSs) an additional angiostatic function (Kise et al., 2004; Yang et al., 2004). Since His257Arg mutant TrpRS showed a similar, even augmented effect akin to mini- and T1-TrpRSs in yeast complementation experiments (Supplementary Result), we performed pull-down assays to investigate whether WARS p.His257Arg mutation altered the binding affinity between TrpRS and VE-Cadherin. As shown in Fig. 5A, the p.His257Arg mutation significantly enhanced the interaction between VE-cadherin and FL TrpRS as well as the bindings between VE-cadherin and T2-TrpRS. To examine whether the mutant TrpRS had an augmented angiostatic activity than WT TrpRS, transwell migration assay and Matrigel angiogenesis assay were performed using HUVECs under $VEGF_{165}$ stimulation (Wakasugi et al., 2002; Zhou et al., 2010). The VEGF-induced migration of HUVECs was significantly reduced in cells treated with mutant FL TrpRS, mutant T2-TrpRS, and WT T2-TrpRS proteins, but not by WT FL TrpRS (upper panel of Fig. 5B and Fig. 5C). Matrigel angiogenesis assay showed a similar pattern of tube formation between HUVECs treated with $VEGF_{165}$ only and those treated with $VEGF_{165}$ plus WT FL TrpRS protein, supporting that FL TrpRS had no or minimal angiostatic effect (lower panel of Fig. 5B and Fig. 5D). However, mutant FL TrpRS, mutant T2-TrpRS, and WT T2-TrpRS significantly inhibited angiogenesis of HUVECs in comparison to those treated with WT FL TrpRS. These findings indicated that WARS p.His257Arg mutation had an enhanced angiostatic effect.

Discussion

We identified a novel heterozygous mutation, WARS p.His257Arg, in seven

individuals with dHMN from two Taiwanese families of Han-Chinese extraction but with different ancestries demonstrated by haplotype analysis. In addition, the same mutation was also identified in a Belgian kinship of Caucasian origin ~~after~~ through screening 163 unrelated dHMN cases from Korea, Australia and the GENESIS collection in the USA. The *WARS* p.His257Arg mutation is not present in 1122 Taiwanese control subjects as well as in the gnomAD, which contains approximately 126,000 exome sequences and 15,000 whole-genomes from unrelated individuals (Lek et al., 2016). The recurrence of *WARS* p.His257Arg in dHMN patients under a completely different genetic background and its absence from a very large ~~huge~~ number of controls provide a strong evidence supporting the pathogenic role of *WARS* mutations in dHMN. Moreover, this study reveals that the *WARS* p.His257Arg mutation does not affect protein dimerization but possesses a damaging and dominant-negative effect on aminoacylation activity of TrpRS, which subsequently disturbs protein synthesis and reduces cell viability. The expression of mutant His257Arg TrpRS also inhibited neurite outgrowth and led to neurite degeneration in ~~the~~ neuronal cell lines ~~or~~ and rat motor neurons. Furthermore, we showed that the p.His257Arg mutation significantly changes the properties of TrpRS to facilitate normal function of ~~fit for~~ yeast translational machinery and acquires an enhanced angiostatic activity. This study clearly demonstrates that WARS p.His257Arg mutation causes dHMN and alters the canonical and non-canonical functions of TrpRS.

The derangement of canonical and non-canonical TrpRS functions may both contribute to dHMN pathology. His257Arg TrpRS has a reduced aminoacylation activity, which results in disturbed protein synthesis and defective cell viability. The impaired aminoacylation activity may cause secondary neurotoxicity since a large

volume of uncharged tRNAs would interfere with the function of eukaryotic initiation factor 2 (eIF2) and subsequently leads to a general suppression of protein translation (Dong et al., 2000; Wallen and Antonellis, 2013). Analogously, an impaired translation efficacy that fails to meet the high metabolic demand of protein synthesis in long axons has also been implicated in inherited neuropathies caused by mutations in other ARS genes (Jordanova et al., 2006; McLaughlin et al., 2012; Griffin et al., 2014). Multiple pathogenic mechanisms that are not mutually exclusive could jointly contribute to axonal degeneration. A loss-of-function effect has been documented in majority of disease-associated ARS mutations. However, haplo-insufficiency solely does not warrant the development of neuropathy. For example, mice heterozygous for a complete null allele caused by a gene-trap insertion in the second intron of *Gars* ($Gars^{XM256/+}$) did not develop neuropathy (Sebum et al., 2006). Our study shows a dominant-negative toxicity of WARS His257Arg mutation. Similar findings have been reported in neuropathies related to mutations in GARS and HARS (Brozkova et al., 2015; Malissovas et al., 2016). The ability of mutant His257Arg TrpRS to form dimers with WT TrpRS could be an important prerequisite for its dominant-negative effect. Recently, a zebrafish model illustrated that dimerization is required for the dominant GARS mutations to manifest a neuromuscular phenotype. The zebrafishes heterozygous for Gars p.Thr209Lys mutation, which resulted in loss of dimerization, did not have any phenotype (Malissovas et al., 2016).

In addition, the enhanced angiostatic effects of His257Arg mutant FL and T2-TrpRSs may interfere with the original neurotrophic effects of angiogenic factors, including regulating the growth, migration and survival of neurons as well as guidance of the axonal outgrowth (Greenberg and Jin, 2005; Mackenzie and Ruhrberg, 2012). In physiological condition, short forms of TrpRS are the major ones

that bind to VE-cadherin and inhibit the expression of downstream pro-angiogenic genes (Tzima et al., 2005; Zhou et al., 2010). However, WARS p.His257Arg mutation enables and enhances the bindings of both FL and T2- mutant TrpRSs to VE-cadherin leading to inhibition of angiogenesis and migration of HUVECs. Similarly, GARS mutations which antagonize the physiological signaling between VEGF and neuropilin-1 were found to play a pathogenic role in CMT type 2D (CMT2D) and over-expression of VEGF improved the motor deficit of a CMT2D mouse model (He et al., 2015). These findings collectively support a link between derangement of angiogenesis pathway and axonal neuropathy.

The molecular epidemiology of dHMN in Taiwan remains obscure, and there is only one dHMN study in Taiwan before, reporting a dHMN patient with a *BSCL2* p.Arg96His mutation (Hsiao et al., 2016). Nevertheless, in Caucasian populations, dHMN has been well studied and clinically categorized into 7 subgroups (Harding, 2005). The patients harboring WARS p.His257Arg mutation manifest a slowly progressive, lower limb onset, length-dependent motor neuropathy starting at age 9-13 years. Such clinical features conform to dHMN type I, which presents as juvenile onset, autosomal-dominantly inherited, lower limbs predominant and pure motor neuropathy (Harding, 2005). Among the other ARS genes implicated in inherited neuropathy, *AARS*, *HARS* and *GARS* may also result in dHMN type I when mutated (Dubourg et al., 2006; Zhao et al., 2012; Brozkova et al., 2015). In addition to dHMN type I, the clinical phenotypes associated with these ARS genes include axonal CMT, intermediate CMT, and dHMN type V which features an upper-limb predominant motor syndrome. Why different alleles of these functionally similar genes result in variable phenotypes is still unclear. Mutations in *AARS*, *DYNC1H1*, *GARS*, *HARS*, HSPB1 and HSPB8 have been identified to cause dHMN type I (Evgrafov et al.,

2004; Rossor et al., 2012), and WARS mutations should also be considered in dHMN type I patients with unknown genetic diagnosis.

In conclusion, this study demonstrates a recurrent WARS mutation causing autosomal dominant dHMN and altering the canonical aminoacylation activity and non-canonical angiostatic functions of TrpRS. These findings establish WARS as a gene whose mutations can cause dHMN and extend our knowledge of the pathogenic role of tRNA-synthetases in inherited neuropathies.

Funding

This work was supported by the grants from Ministry of Science and Technology, Taiwan, ROC (MOST102-2628-B-075-006-MY3 and MOST105-2628-B-075-002-MY3), and Taipei Veterans General Hospital (V104C-041 and V105C-027). Grants by the Association Belge contre les Maladies Neuromusculaire (ABMM) - Aide à la Recherche ASBL and the EU FP7/2007-2013 under grant agreement number 2012-305121 (NEUROMICS). I.M. is supported by a PhD fellowship of the agency for Innovation by Science and Technology (IWT). JB is supported by a Senior Clinical Researcher mandate of the Research Fund - Flanders (FWO).”

Supplementary material

Supplementary material, including the method, result and figure of the yeast study, one video clip of clinical features of a Taiwanese dHMN patient, and four tables can be found at *Brain* online.

Acknowledgments

We would like to thank the patients who participated in this study. We thank the High-throughput Genome and Big Data Analysis Core Facility of National Core Facility Program for Biotechnology, the Ministry of Science and Technology, Taiwan (MOST104-2319-B-010-001), for the NGS service and bioinformatics support.

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Figure 1. Genetic analysis of families with distal hereditary motor neuropathy (dHMN)

(A) Pedigrees of the two Taiwanese and one Belgian dHMN families harboring WARS c.770A>G (p.His257Arg). Haplotype analysis of seven single nucleotide polymorphism markers flanking the WARS variant revealed two distinct disease-associated haplotypes in the two Taiwanese families. Open symbol: unaffected; filled symbol: affected; symbol with a diagonal line: deceased; arrow: proband. (B) Sanger sequencing to confirm the c.770A>G (p.His257Arg) variant. (C) Alignment of multiple tryptophanyl-tRNA synthetase (TrpRS) orthologs showing conservation of the His257 residue throughout vertebrate species, but not yeast. The domain diagram of human TrpRS shows that His257 residue is localized in the connective polypeptide 1 insertion (CP1). WHEP: a helix-turn-helix motif, responsible for protein-protein interaction. (D) The structural model of human TrpRS homodimers (PDB ID: 2AKE) showing that His257 is situated at dimer interface (left panel) as well as in the catalytic domain (green) close to the recognition sites (Thr259 and Asn261) for tRNA acceptor arm (grey) (middle and right panel).

Figure 2. Clinical and muscle magnetic resonance imaging (MRI) features in patients with WARS p.His257Arg mutation

(A) Atrophy of the intrinsic hand muscles and muscles in the feet and legs seen in patient A-III-3. (B) Atrophy of the intrinsic hand muscles and muscles in the feet and legs seen in patient C-II-1. **(C)** T1-weighted MRI of patient A-III-3 revealing distal lower limb muscle atrophy with fatty infiltration (arrowheads). (D) T1-weighted lower limb MRI of a 30-year-old healthy man for comparison. Arrow: corresponding cross-section level of the axial view images.

Figure 3. In vitro characterization of wild-type (WT) and His257Arg (H257R) mutant TrpRS proteins

(A) β-galactosidase (β-Gal) reporter assay and (B) firefly luciferase reporter assay demonstrating that H257R TrpRS has a dominant-negative effect on protein synthesis. HEK293 cells co-transfected with β-Gal or firefly luciferase reporter plasmids, along with different ratios of WT and H257R TrpRS expression plasmids were lysed and assayed for β-Gal or firefly luciferase activities at 48 hours after transfection. The error bars indicate standard errors of the mean (n = 3) and asterisks indicate statistically significant differences (** $p < 0.01$,* $p < 0.05$). Vector: empty vector control. (C) Aminoacylation of $tRNA^{trp}$ mediated by TrpRS generating adenosine monophosphate (AMP) and pyrophosphate (PPi), which can be used to assess aminoacylation activities. (D) Analyses of aminoacylation activities of TrpRSs in the cells. HEK293 cells transfected with different ratios of WT and mutant TrpRS constructs as well as the siRNAs specifically depleting endogenous WARS were lysed at 48 hours after transfection, and then assayed for aminoacylation activities by assessing the amount of the generated PPi. The data were displayed relative to the values detected in the control vector-transfected cells (n = 4). (E) Analyses of aminoacylation activities of purified TrpRSs. His-tagged WT and H257R TrpRS proteins were produced in E. coli and purified by Ni-NTA affinity chromatography. Both PPi and AMP generated through aminoacylation were quantified to estimate the aminoacylation activities (n = 3). (F) Investigating the dimerization properties of WT and H257R TrpRSs by co-immunoprecipitation (Co-IP) assay. HEK293 cells co-transfected with Myc- or/and GFP-tagged WT or/and H257R TrpRS expression plasmids were lysed at 48 hours after transfection. Dimerization was demonstrated via

pull-down of GFP-tagged WT or H257R TrpRS with the Myc-tagged TrpRS using anti-Myc antibody in Co-IP. These data showed that the H257R TrpRS could form dimers with WT TrpRS in cells. (G) Cell viability assays. Knockdown of endogenous WARS in HEK293 cells resulted in a significantly compromised cell viability, which could be rescued by expressing exogenous WT TrpRS but worsened by H257R TrpRS. Percentage of viable cells was displayed relative to cells transfected with empty vector along with non-targeting siRNA control (Ctrl siRNA) (n = 7).

Figure 4. His257Arg (H257R) TrpRS inhibits neurite outgrowth and leads to neurite degeneration

Neuro-2a (N2a) **(A)** or SH-SY5Y **(B)** cells were transfected with expression vector containing wild-type (WT), or H257R TrpRS, or empty vector (vector control), grown under differentiation conditions, and immunostained against Myc (exogenous TrpRS staining) and neurofilament heavy polypeptide (NFH; neurite staining) at 72 hours after transfection. The representative fluorescence-immunostaining and phase-contrast images were shown, along with statistical results of percent of cells bearing neurites and average primary neurite length. The scale bar is 50 μm. At least 100 cells from three independent experiments were measured for each preparation and data were expressed as the mean ± standard error of the mean (SEM). *p value < 0.05; **p value < 0.01 when compared to WT. **(C)** Rat motor neurons (embryonic day 14) were transfected with expression vector containing WT, or H257R TrpRS, or empty vector, and processed for immunostaining using antibodies against Myc and SMI-32 (staining for motor neurons and their neuritis) at 72 hours after transfection. The representative fluorescence-immunostaining and phase-contrast images were shown. Neurites of motor neurons were traced and measured, and the average primary neurite length and

percent of cells bearing beaded neurites were estimated. Error bars represent the mean ± SEM of three independent experiments with at least 50 cells.

(D) Rat motor neurons expressing above TrpRS construct were processed for immunofluorescence analysis using antibody against amyloid precursor protein (APP; neurodegeneration staining) at 72 hours post-transfection. Motor neurons expressing WT TrpRS showed only a faint or punctate staining of APP in the neurites, while motor neurons expressing TrpRS appear to accumulate APP at neurites (arrow).

Figure 5. Pull-down assay and in vitro angiogenesis assays showing that human His257Arg (H257R) mutant TrpRS has an augmented angiostatic effect through binding to vascular endothelial-cadherin (VE-cadherin)

(A) An in vitro pull-down assay utilized to assess the ability of His-tagged TrpRS to bind to Flag-tagged VE-cadherin. The immunoblots showed that both the full-length (FL) and the T2-H257R TrpRSs had a substantial increase in affinity to VE-cadherin compared to wild-type (WT) TrpRS. (B) Effect of the p.His257Arg mutation on the angiostatic activity of TrpRS evaluated by transwell migration assay and Matrigel angiogenesis assay using human umbilical vein endothelial cells (HUVECs) under 16 hours of $VEGF_{165}$ stimulation with or without TrpRS treatment. Top panel: representative microscopic images of the undersides of the transwell assay membranes; bottom panel: representative microscopic images of tube formation on Matrigel. (C) Quantification of migration of HUVECs after 16 hours treatment with $VEGF_{165}$ and the indicated conditions of three independent experiments. Treatment with FL H257R TrpRS, T2-H257R TrpRS, or T2-WT TrpRS significantly reduced in vitro cell mobility, while FL WT TrpRS application did not further inhibit the cell migration compared to a control treatment of $VEGF_{165}$ alone. The error bars represent

standard errors of the mean (n = 3) and asterisks indicate statistically significant differences (** $p < 0.01$,* $p < 0.05$, NS: non-significant). (D) Quantification of vessel coverage (the total microscopic area covered by HUVEC vasculature) after 16 hours treatment with $VEGF_{165}$ and the indicated conditions of three independent experiments. The data revealed that FL H257R TrpRS, T2-WT or T2-H257R TrpRS could significantly inhibit angiogenesis. Treatment of FL WT TrpRS did not show inhibitory effect on tube formation.

Table 1. Clinical manifestations of the affected individuals carrying *WARS* p.His257R

	Pedigree A			Pedigree B			Pedigree C
Subject	**A-II-3**	**A-III-2**	**A-III-3**	**B-II-2**	**B-III-1**	**B-III-2**	**C-II-1**
Age at onset (years)	12	10	13	12	13	13	9
Age at clinical evaluation (years)	64	25	30	58	22	20	45
Gender	male	female	male	male	male	male	female
Limb weakness UL[a] (MRC)	distal: 0	distal: 4	distal: 3	distal: 0	distal: 3	distal: 3	distal: 0
Limb weakness LL[a] (MRC)	distal: 0	distal: 1	distal: 3	distal: 0	distal: 3	distal: 3	distal: 0
Muscle atrophy UL[a]	distal: severe	distal: mild	distal: moderate	distal: severe	distal: mild	distal: mild	distal: severe
Muscle atrophy LL[a]	distal: severe	distal: moderate	distal: moderate	distal: severe	distal: moderate	distal: moderate	distal: severe
Pinprick sensation	normal	normal	normal	normal	normal	normal	normal
Knee/Ankle DTRs	absent/absent	+/absent	absent/absent	absent/absent	absent/absent	+/absent	weak/absent
Age at NCS (years)	64	25	13	50	14	13	45
Ulnar nerve MNCV (m/s)	46	55.6	57.9	NR	51.8	56.8	63
Ulnar nerve cMAP (mV)	0.1	5.3	5.2	NR	5.6	5.9	0.9
Sural nerve SNAP (uV)	17.0	46.0	34.0	8.9	13.2	11.9	11.0

The following abbreviations are used: MRC, Medical Research Council scale; UL, upper limbs; LL, lower limbs; DTR, deep tendon reflex; MCS, nerve conduction studies; MNCV, motor nerve conduction velocity; cMAP, compound motor action potential; SNAP, sensory nerve action potential; NR, not recordable; and ND, not done.