Loss of function mutations in HARS cause a spectrum of inherited peripheral neuropathies

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Inherited peripheral neuropathies are a genetically heterogeneous group of disorders characterized by distal muscle weakness and sensory loss. Mutations in genes encoding aminoacyl-tRNA synthetases have been implicated in peripheral neuropathies, suggesting that these tRNA charging enzymes are uniquely important for the peripheral nerve. Recently, a mutation in histidyl-tRNA synthetase (HARS) was identified in a single patient with a late-onset, sensory-predominant peripheral neuropathy; however, the genetic evidence was lacking, making the significance of the finding unclear. Here, we present clinical, genetic, and functional data that implicate HARS mutations in inherited peripheral neuropathies. The associated phenotypic spectrum is broad and encompasses axonal and demyelinating motor and sensory neuropathies, including four young patients presenting with pure motor axonal neuropathy. Genome-wide linkage studies in combination with whole-exome and conventional sequencing revealed four distinct and previously unreported heterozygous HARS mutations segregating with autosomal dominant peripheral neuropathy in four unrelated families (p.Thr132Ile, p.Pro134His, p.Asp175Glu and p.Asp364Tyr). All mutations cause a loss of function in yeast complementation assays, and p.Asp364Tyr is dominantly neurotoxic in a Caenorhabditis elegans model. This study demonstrates the role of HARS mutations in peripheral neuropathy and expands the genetic and clinical spectrum of aminoacyl-tRNA synthetase-related human disease.

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Introduction

Inherited peripheral neuropathies (IPNs) represent a common, heterogeneous group of disorders that affect about 1 in 2500 individuals worldwide (Skre, 1974). A common feature of these diseases is progressive, length-dependent axonal degeneration of the peripheral nervous system resulting in impaired motor and sensory function in the distal extremities. IPNs are clinically subdivided based on the involvement of different types of peripheral nerve fibres. The most common type is hereditary motor and sensory neuropathy (HMSN), also known as Charcot–Marie– Tooth (CMT) disease, which affects both motor and sensory fibres. Less frequent subtypes display more selective involvement of nerve fibres and include hereditary motor neuropathy (HMN) and hereditary sensory and autonomic neuropathy (HSAN). The common HMSN/CMT group is further classified based on electrophysiological studies with motor nerve conduction velocities in the median nerve <38 m/s (normal >49 m/s) indicating demyelinating neuropathy (CMT1 or HMSN-I) and nerve conduction velocities >38 m/s indicating axonal neuropathy (CMT2 or HMSN-II) (Harding and Thomas, 1980). In addition, an intermediate group is defined as having nerve conduction velocities between 25 and 45 m/s among patients in the same family (Baets et al., 2014). Interestingly, IPNs display a high level of clinical heterogeneity, even among patients that carry an identical genetic lesion.

The genetic diversity of IPN is extensive with >75 genes identified to date (Baets et al., 2014). The transmission of the disease can be autosomal dominant, autosomal recessive, or X-linked. Dominantly inherited CMT1 is the most common type and also the easiest to diagnose genetically with mutations in three loci accounting for at least 80% of cases (Saporta et al., 2011; Rossor et al., 2013). In contrast, for axonal forms (CMT2) the genetic cause is only found in ~25% of patients because there are no major gene(s) accounting for a substantial proportion of patients (with the possible exception of mitofusin 2, MFN2), and the locus and allelic heterogeneity of CMT2 is extensive with many genes still undiscovered (Murphy et al., 2012).

Aminoacyl-tRNA synthetases (ARSs) are ubiquitously expressed, essential enzymes that charge tRNA molecules with cognate amino acids—the first step of protein translation (Antonellis and Green, 2008). To date, mutations in six genes encoding ARSs have been identified in patients with IPN phenotypes (Antonellis et al., 2003; Jordanova et al., 2006; Latour et al., 2010; McLaughlin et al., 2010; Gonzalez et al., 2013a; Vester et al., 2013). Three of these genes have been convincingly implicated in disease via linkage analysis, with multiple families and patients described in independent studies: (i) glycyl-tRNA synthetase mutations (GARS) cause CMT2D and HMN5A (Antonellis et al., 2003); (ii) tyrosyl-tRNA synthetase mutations (YARS) cause an intermediate form of CMT (DI-CMTC) (Jordanova et al., 2006); and (iii) alanyl-tRNA synthetase mutations (AARS) cause CMT2N and also a form of HMN (Latour et al., 2010; Zhao et al., 2012). Interestingly, extensive functional studies have shown that disease-associated ARS mutations cause a loss-of-function effect in tRNA charging and yeast viability assays, suggesting that peripheral nerves are uniquely sensitive to tRNA charging deficits (Wallen and Antonellis, 2013).

Recently, a p.Arg137Gln variant in the histidyl-tRNA synthetase gene (HARS) was found by whole exome sequencing in an isolated patient with a sporadic, late-onset predominantly sensory axonal neuropathy (Vester et al., 2013). While functional studies in yeast revealed that the variant behaved similarly to other disease-implicated ARS variants, the lack of convincing genetic findings and the detection of the variant in the general population made it impossible to conclude that this was a disease-causing mutation (Vester et al., 2013). Here, we present 23 patients from four unrelated families with HARS mutations that segregate with axonal or intermediate neuropathy phenotypes. Our functional studies show that all identified

Keywords: hereditary motor and sensory neuropathies; molecular genetics; neurodegeneration; RNA processing; whole-exome sequencing

Abbreviations: ARS = aminoacyl-tRNA synthetase; CMT = Charcot–Marie–Tooth; HMN = hereditary motor neuropathy; HMSN = hereditary motor and sensory neuropathy; IPN = inherited peripheral neuropathy
mutations are unable to support viability in yeast complementation assays and that one mutation is dominantly toxic in a worm model system. Combined, our data clearly establish HARS as a neuropathy-associated locus and further expand the genetic and phenotypic spectrum of ARS-related human disease.

Patients and methods

Patients

In total, 23 patients from four unrelated families with a dominantly inherited peripheral neuropathy are described (Fig. 1). The Ethical Review Boards of the participating institutions approved this study. All patients or their legal representatives signed informed consent prior to enrolment.

Linkage analysis

To define the molecular genetic basis of the disease in Families A and D, a whole genome scan using single nucleotide polymorphism (SNP) arrays was carried out. Genomic DNA samples from patients and unaffected relatives were hybridized to GeneChip® Human Mapping Nsp1 250 K arrays (Family A, seven individuals) and GeneChip® Human Mapping 50 K arrays (Family D, 12 individuals) (Affymetrix) according to the manufacturer protocols. Genotypes were called using GeneChip® Genotyping Analysis Software (Version 4.1) and default thresholds. To identify the linkage regions, the parametric multipoint logarithm of the odds (LOD) scores and haplotypes were obtained using a subset of SNPs (distance between markers >50 kb and heterozygosity >0.15) with the MERLIN program (v 1.1.2) with the assumption of an autosomal dominant mode of inheritance and fully penetrant model (Abecasis et al., 2002).

For Family B, an in-house developed multiplex genome-scan panel was used consisting of 422 polymorphic short tandem repeat (STR) markers, subsequently PCR amplified with fluorescently labelled primers and size-separated on an ABI3730xl DNA Analyzer. Results were scored with an in-house developed software program, Local Genotype Viewer (LGV). Two-point parametric linkage analysis was calculated with EasyLINKAGE software package under a fully penetrant autosomal dominant model, equal female/male recombination rates, and a disease frequency of 0.0001.

Sanger sequencing

Prior to linkage analysis, candidate gene sequencing, or whole-exome sequencing, the chromosome 17 duplication (CMT1A) was excluded in all four families. Subsequently, various sets of IPN associated genes were tested negative in these families: GJB1, MPZ, BSCL2, NEM2, MFN2, HSP22, HSP27, RAB7, GARS, YARS, DNM2, and TRPV4 in Family A; MPZ, PMP22, GJB1, GARS, AARS, and GDAP1 in Family B; PMP22 in Family C; GJB1, MPZ, HSP22, HPS27, SETX, and BSCL2 in Family D.

For the index patient of Family A, all 13 coding exons and adjacent exon-intron boundaries of HARS were amplified as well as a cohort of 61 index patients with genetically unresolved HMN (primers available upon request). To validate whole-exome sequencing results (Families B, C and D) and to demonstrate segregation, the mutated exons of HARS were Sanger sequenced in all available individuals. Primer pairs were designed with the Primer3 program (sequences available upon request) (Rozen and Skaletsky, 2000). Total

Figure 1  Pedigrees of the families with HARS mutations. Female family members are indicated with a circle and male family members are indicated by squares. Filled symbols indicate affected individuals, while empty symbols indicate unaffected individuals. The number of the individual is shown in Arabic numerals if the DNA was available for genotyping.
Whole-exome sequencing

Index patients from Families B and C and two distant relatives from Family D (Subjects IV.1 and IV.6) were selected for whole-exome sequencing. Exome capture was performed using the Agilent SureSelect Human All Exon V5 kit (50 Mb), followed by sequencing on a HiSeq 2000 platform (Illumina). Sequence alignment was performed using the BWA-v0.5.9rc1 tool. GATK-v1.4-37 was used for variant calling. Further data analysis was performed in the Genomes Management Application database (GEM-app) (Gonzalez et al., 2013b). Variants were filtered for the regions with suggestive linkage for Families B and D, no occurrence in the normal population [absent in the Exome Variant Server (EVs)], predicted impact on the encoded protein (missense, nonsense, frame shift, inframe indels and essential splice variants), conservation (Genomic Evolutionary Rate Profiling (GERP) score > 4, or PhastCons score > 0.9, or PhyloP Score > 1.5), and predicted damaging amino acid substitution [at least in one: SIFT, PolyPhen-2, MutationTaster, Mutation Assessor, Likelihood Ratio Test (LRT), Functional Analysis through Hidden Markov Models (FATHMM)], and quality (GATK GQ score > 75). An overview of the general outcome after performing whole-exome sequencing (number of reads, coverage etc.) can be found in Supplementary Table 1. Confirmation of the possible pathogenic variants and segregation analysis in all available family members was performed using Sanger sequencing.

Yeast complementation assays

Yeast complementation assays were performed as previously described (Vester et al., 2013). Briefly, mutation-containing oligonucleotides were designed to model the p.Thr132Ile, p.Thr132Ser, p.Pro134His, p.Asp175Glu, or p.Asp364Tyr HARS missense variants in the yeast orthologue HTSI. The QuickChange® II XL Site-Directed Mutagenesis Kit (Stratagene) was used (per manufacturer’s instructions) to mutate the HTSI locus in a pDONR221 Gateway® entry clone (Invitrogen). Resulting clones were purified and sequenced to confirm successful mutagenesis and exclude polymerase-induced mutations. The mutated HTSI/pDONR221 entry clone was subsequently recombined into a Gateway®-compatible LEU2-bearing pRS315 destination vector. Resulting clones were purified and digested with BsrGI (New England Biolabs) to confirm successful recombination.

Two independently generated haploid Δhts1 strains (harbouring a pRS316 maintenance vector to express wild-type HTSI and URA3) were transformed with a LEU2-bearing pRS315 vector containing no insert (‘Empty pRS315’ in Fig. 2) or containing a wild-type or mutant HTSI allele (Vester et al., 2013). Subsequently, yeast strains were selected on medium lacking uracil and leucine (Teknova) to select for the presence of both vectors. For each transformation, four colonies were grown to saturation in selective medium for 48 h. Next, 10 μl of undiluted and diluted (1:10 and 1:100) samples from each culture were spotted on plates containing 0.1% 5-fluoroorotic acid (5-FOA) or SD -leu -ura growth medium (Teknova) and incubated at 30°C for 48 h. Survival was determined by visual inspection of growth. Experiments were performed using two independently generated HTSI expression constructs for each allele (designated as ‘A’ and ‘B’ in Fig. 2).

Caenorhabditis elegans plasmids and strains

Nematode strains were provided by the Caenorhabditis Genetic Centre. Strains were raised at room temperature on nematode growth media plates with OP50 Escherichia coli as the food source per standard protocols (Brenner, 1974). Plasmids and transgenic worms were constructed as previously described (Mello et al., 1991; Vester et al., 2013). The human p.Asp364Tyr mutation was created by PCR-based site directed mutagenesis into the equivalent C. elegans hars-1 residue D383Y using the oligonucleotide primers: D383Y_FWD: TAGCTGCGGCTGACGATACTAT; and D383Y_REV: ATAGATCGTCCACCAGGCAGCTA.

Morphological and behavioural analysis in C. elegans

Quantification of motor neuron and behavioural defects were performed as previously described (Vester et al., 2013). Quantification was performed on the following strains: EG1285: oxIs12 (Punc-47::GFP; lin15b) X; BNG616: oxIs12 (Punc-47::GFP; lin15b) X; aabEx12 (Punc-25::hars-1 [D383Y], Pmyo-2::mCherry). L4 stage worms were synchronized by bleaching and grown at 20°C. Morphological defects were quantitated in > 100 worms/genotype at each developmental time point. Animals exhibiting at least one aberrant neuronal process were scored as positive. Behavioural thrash assays were performed as previously described (Miller et al., 1996; Vester et al., 2013). At least 40 animals/genotype were tested. Briefly, single animals were picked to a 35 mm agarose-coated dish filled with 2 ml of M9 media. Animals were allowed to acclimate for 2 min and then a 1-min video was recorded using a Leica IC80HD camera. The movies were slowed to one-quarter speed and the total number of body bends per minute was manually scored offline using ImageJ software.

Microscopy

All morphological quantitation was performed on a Leica DM16000B compound microscope with a CCD camera (DFX360, Leica Microsystems Inc.) using a x40 objective. High-resolution confocal images were obtained on a Nikon A1R microscope with a x20 and x60 objective (Nikon Corporation).
Results

Mutations in HARS are identified in patients with IPN

Linkage analysis, Sanger sequencing, and whole-exome sequencing revealed four distinct heterozygous mutations in HARS in four unrelated families with dominantly inherited peripheral neuropathy.

Genome-wide linkage analysis in Family A, including four affected and three unaffected family members, revealed one chromosomal interval on chromosome 5q tentatively linked to the disease, with a maximal LOD score of 2.107. All affected individuals, but none of the unaffected subjects, shared a haplotype consisting of 93 SNP alleles over a region of 7.9 Mb. This interval contained 161 positional RefSeq genes including HARS, which was considered as the most plausible candidate gene (Vester et al., 2013). Sanger-sequencing of HARS revealed the p.Thr132Ile variant. This variant also segregated in all additional family members previously not included in the linkage study. At the same position another amino acid change p.Thr132Ser was listed in dbSNP database (rs143473232), this variant is present in 1 of 13 006 chromosomes in the EVS.

In Family B, linkage analysis revealed five plausible regions with suggestive but inconclusive linkage (LOD score >1) on chromosomes 4, 5, 6, 11, and 13.

Searching these five regions of interest for variants identified by whole-exome sequencing in the index patients, promising variants in six candidate genes were found: CDC42BPG, KLHDC1, MYH7, PYGL, PCDHB1, and the p.Pro134His variation in HARS as the most likely segregating candidate.

In Family C, whole-exome sequencing data filtering yielded promising variants in 66 genes including a p.Asp175Glu variant in HARS, which was found to segregate with the disease in the pedigree.

In Family D, linkage analysis including seven affected and five healthy individuals delineated six genomic regions of interest: four on chromosomes 4, 5, 8, 9 (LOD score 2.4) and two located on the X chromosome (LOD score 1.8). Whole-exome sequencing data from two affected patients combined with linkage analysis revealed only two possible variants, one in LHX6 and one in HARS. After confirmation with Sanger sequencing, the variant in LHX6 was excluded because of presence also in a healthy family member not included in the original linkage analysis, thus leaving the segregating p.Asp364Tyr variant in HARS as the only probable disease cause.

All variants detected in HARS (p.Thr132Ile, p.Pro134His, p.Asp175Glu, p.Asp364Tyr) are in the heterozygous state and segregate with disease in all available family members from Families A–D (Fig. 1). In silico prediction programs classified all four missense variants as pathogenic: ‘probably damaging’ (PolyPhen-2) (Adzhubei et al., 2010), ‘damaging’ (SIFT) (Ng and Henikoff, 2001), ‘disease causing’ (Mutation Taster) (Schwarz et al., 2014) and ‘high’ (Mutation Assessor) (Reva et al., 2011). The same apply also for the variant p.Thr132Ser; the prediction programs assessed it as pathogenic. None of the four HARS variants are present in dbSNP, the Exome Variant Server, or 1000 genomes database (Supplementary Table 2). No additional HARS mutations were found in a cohort of 62 index patients with genetically unresolved HMN to which Family D belonged. Families B, C, and D were part of large whole-exome sequencing effort tackling a heterogeneous cohort of 128 autosomal dominant families with genetically undefined neuropathies (axonal and intermediate CMT and...
Clinical findings

Clinical and electrophysiological findings in all studied individuals are summarized in Table 1 and Supplementary Table 3. Photos of selected patients from Families A and D are in Supplementary Fig. 1. The phenotype is variable, with disease onset ranging from early childhood to late adulthood. Some individuals had clinical signs and electrophysiological abnormalities without subjective symptoms (Subjects A-IV.3, A-IV.6, D-IV.2, and D-IV.6). Especially in Family D, most patients are mildly affected, often only displaying steppage gait and sensory symptoms/signs at later stages of the disease (Supplementary Fig. 1). Two asymptomatic individuals (Subjects D-IV.2 and D-IV.6) have brisk patellar reflexes with absent ankle jerks. Based on electrophysiological studies, the phenotypes of the families were classified as axonal neuropathy in Families A, C, and D and intermediate neuropathy in Family B. Four young individuals did not have sensory symptoms/signs and normal sensory nerve conduction studies consistent with a diagnosis of HMN (Subjects A-V.1, D-IV.2, D-IV.3 and D-IV.6). Older individuals of the same families were diagnosed with CMT2.

HTSI mutations are associated with decreased cell viability in yeast

Yeast complementation assays have been employed to test mutations in ARS genes for a loss-of-function effect, including p.Arg137Gln in HARS (Antonellis et al., 2006; Jordanova et al., 2006; McLaughlin et al., 2010; Stum et al., 2011; Gonzalez et al., 2013a; Vester et al., 2013; Griffin et al., 2014). To test the functional consequences of the four HARS missense variants that segregate with disease (p.Thr132Ile, p.Pro134His, p.Asp175Glu and p.Asp364Tyr) and one rare variant listed in dbSNP without diseases association (p.Thr132Ser), we modelled these missense variants in the yeast orthologue HTS1 (Supplementary Table 4) and independently tested each mutation for the ability to support yeast cell growth compared to wild-type HTS1 or an empty vector. Mutations in the text and Fig. 2 are referred to by the position in the human protein. Briefly, a haploid yeast strain (with the endogenous HTS1 locus deleted and a maintenance vector to express wild-type HTS1 and URA3) was transformed with either a pRS315 vector with no insert (‘Empty pRS315’ in Fig. 2) or a pRS315 vector harbouring wild-type, p.Thr132Ile, p.Thr132Ser, p.Pro134His, p.Asp175Glu, or p.Asp364Tyr HTS1. Yeast cells were then selected on media containing 5-FOA, which is toxic to yeast expressing URA3 and therefore selects for cells that have spontaneously lost the maintenance vector (Boeke et al., 1987). Only yeast cells expressing a functional HTS1 allele from pRS315 will grow in this assay.

Yeast transformed with a wild-type HTS1 expression vector demonstrated significant growth, while those transformed with the empty vector did not (Fig. 2). These data are consistent with HTS1 being an essential gene (Vester et al., 2013). Regarding the novel, CMT-associated HARS mutations described here, yeast expressing p.Thr132Ile, p.Pro134His and p.Asp364Tyr HTS1 were unable to grow on 5-FOA media (Fig. 2) indicating that these are complete loss-of-function alleles. Additionally, yeast expressing p.Asp175Glu HTS1 showed a significant reduction, but not complete abrogation, of yeast viability compared to wild-type HTS1 (Fig. 2; note pronounced differences in growth at 1:10 and 1:100 dilutions) indicating that this is a partial loss-of-function allele. Unlike CMT-associated HARS mutations, the p.Thr132Ser HARS variant supported yeast growth to the same extent as wild-type HARS, indicating that this variant has no significant effect on HARS activity.

p.Asp364Tyr causes late-onset motor neuron defects and behavioural impairments in C. elegans

C. elegans was previously established as a model system to differentiate the pathogenicity of potential mutant hars-1 (the C. elegans orthologue of HARS) variants (Vester et al., 2013). In C. elegans, 19 GABA motor neurons innervate body wall muscles and are required for locomotion via reciprocal inhibition (Schuske et al., 2004). These GABA motor neuron cell bodies reside in the ventral nerve cord and extend circumferential axons that form the dorsal nerve cord—these axons are easily visualized with fluorescent proteins facilitating visual assessment of axon morphology and integrity (Fig. 3A). To determine if p.Asp364Tyr expression resulted in morphological and functional toxicity to motor neurons, we specifically expressed the C. elegans hars-1 (p.Asp364Tyr) transgene in GABA motor neurons, which were labelled with green fluorescent protein (GFP). Expression of p.Asp364Tyr caused morphological neurotoxicity denoted by dorsal and ventral nerve gaps, axonal blebbing, and severely aberrant axonal processes that were not present in control animals (Fig. 3B). Axonal morphological defects increased over time (L4 to 7-day adult) in those animals expressing the p.Asp364Tyr variant (Fig. 3C). To determine if the axonal pathology produced behavioural defects, we tested animals in liquid thrash assays, which measure the fidelity of neuromuscular motor performance (Miller et al., 1996). Transgenic animals expressing the p.Asp364Tyr variant exhibited significantly decreased thrashing rates compared to control in 4- and 7-day adults, which mirrored the increased axonal pathology (Fig. 3D). Although p.Asp364Tyr expressing L4 and 1-day adult animals exhibited a significant increase in axonal pathology, there were no overt behavioural defects,
### Clinical findings in patients with HARS mutations

<table>
<thead>
<tr>
<th>Individual (mutation, origin)</th>
<th>Onset (age, years)</th>
<th>Disease duration, years</th>
<th>Walking</th>
<th>Weakness (proximal/distal)</th>
<th>Sensory loss</th>
<th>Other features</th>
<th>NCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-III.3 p.Thr132Ile German</td>
<td>Weakness of foot dorsiflexion (25)</td>
<td>15</td>
<td>Steppage gait, no aids</td>
<td>UL 5/5</td>
<td>Reducted vibration sense distally (LL)</td>
<td>Pes cavus</td>
<td>CMT2</td>
</tr>
<tr>
<td>A-III.5</td>
<td>Pes cavus, hammer toes (childhood)</td>
<td>60</td>
<td>Steppage, uses wheeled walker</td>
<td>UL 5/3</td>
<td>Distal loss of vibration sense (LL)</td>
<td>Pes cavus</td>
<td>CMT2</td>
</tr>
<tr>
<td>A-IV.3</td>
<td>No complaints at age 62</td>
<td>No</td>
<td>Mild steppage gait, no aids</td>
<td>UL 5/5-LL 5/3</td>
<td>Reduced vibration sense distally (LL)</td>
<td>Pes cavus</td>
<td>CMT2</td>
</tr>
<tr>
<td>A-IV.4</td>
<td>Slender hands and feet (childhood)</td>
<td>40</td>
<td>Steppage, no aids</td>
<td>UL 5/4</td>
<td>Hypoesthesia of the feet, vibration sense reduced distally (LL)</td>
<td>Pes cavus</td>
<td>CMT2</td>
</tr>
<tr>
<td>A-IV.6</td>
<td>No complaints at age 58</td>
<td>No</td>
<td>Normal gait, no heel walking</td>
<td>UL 5/5</td>
<td>Distal loss of vibration sense (LL)</td>
<td>Pes cavus</td>
<td>CMT2</td>
</tr>
<tr>
<td>A-IV.7</td>
<td>Weakness in his hands (26)</td>
<td>10</td>
<td>Normal gait, is still able to walk on heels a few steps</td>
<td>UL 5/3</td>
<td>Hypoesthesia of the 1st toe, reduced vibration sense distally (LL)</td>
<td>Pes cavus</td>
<td>CMT2</td>
</tr>
<tr>
<td>A-V.1</td>
<td>Weakness in his hands, hammer toes (20)</td>
<td>4</td>
<td>Normal gait, no heel walking</td>
<td>UL 5/4</td>
<td>No</td>
<td>No</td>
<td>HMN</td>
</tr>
<tr>
<td>B-II.2 p.Pro134His Moroccan</td>
<td>Gait difficulties (childhood) &gt; 40</td>
<td>4</td>
<td>Severe steppage, crutch</td>
<td>UL 5/3</td>
<td>Panmodal distal</td>
<td>No</td>
<td>CMT1</td>
</tr>
<tr>
<td>B-II.5</td>
<td>Gait difficulties (childhood) &gt; 35</td>
<td>8</td>
<td>Steppage, no aids</td>
<td>UL 5/4</td>
<td>Distal loss of vibration and pinprick sense</td>
<td>Hip dysplasia R</td>
<td>CMT1</td>
</tr>
<tr>
<td>B-III.3</td>
<td>Gait difficulties (10)</td>
<td>7</td>
<td>Steppage, no aids</td>
<td>UL 5/4+LL 5/1</td>
<td>Distal loss of vibration sense (LL)</td>
<td>No</td>
<td>CMT2/CMT-INT</td>
</tr>
<tr>
<td>B-III.5</td>
<td>Gait difficulties, weakness hands (&gt; 20)</td>
<td>&gt; 8</td>
<td>Slight steppage, no aids</td>
<td>UL 5/4+LL 5/-</td>
<td>Distal paresthesia and loss of vibration sense (LL)</td>
<td>No</td>
<td>CMT-INT</td>
</tr>
<tr>
<td>C-I.1 p.Asp175Glu Czech/Belgian</td>
<td>Subjectively no weakness at age 80</td>
<td>No</td>
<td>No heel walking</td>
<td>UL 4/3+LL 4/3+</td>
<td>Reduced distal vibration sense (LL), cold feet</td>
<td>Brisk patellar reflexes</td>
<td>CMT2</td>
</tr>
<tr>
<td>C-II.1 Pain, positive sensor symptoms (39)</td>
<td>8</td>
<td>Is able walk on heels and tiptoes</td>
<td>UL 5/5</td>
<td>Reduced distal vibration sense (LL), cold feet</td>
<td>No</td>
<td>CMT2</td>
<td></td>
</tr>
<tr>
<td>C-II.2 Pain, positive sensor symptoms (&gt; 37)</td>
<td>11</td>
<td>Slight steppage, no aids</td>
<td>UL 5/5-LL 5/4</td>
<td>Distal dysesthesia and reduced vibration sense (LL)</td>
<td>Intact/brisk reflexes</td>
<td>CMT2</td>
<td></td>
</tr>
<tr>
<td>C-III.1 Foot deformities (12)</td>
<td>15</td>
<td>Steppage, in-soles, no other aids</td>
<td>UL 5/5-LL 5/2</td>
<td>Distal, panmodal</td>
<td>Pes cavus/hammer toes</td>
<td>CMT2</td>
<td></td>
</tr>
<tr>
<td>C-II.2 p.Asp364Tyr Czech</td>
<td>Gait difficulties (20)</td>
<td>56</td>
<td>Steppage, crutches</td>
<td>UL 5/3+LL 5/0</td>
<td>Distal panmodal</td>
<td>Areflexia, atrophic intrinsic hand muscles</td>
<td>(CMT)</td>
</tr>
<tr>
<td>C-III.2 Gait difficulties (26)</td>
<td>21</td>
<td>Steppage</td>
<td>UL 5/5</td>
<td>Distal reduced vibration sense and hypeaesthesia</td>
<td>Brisk patellar reflexes</td>
<td>CMT2</td>
<td></td>
</tr>
<tr>
<td>C-III.4 Gait difficulties (19)</td>
<td>34</td>
<td>Steppage, crutches</td>
<td>UL 5/4</td>
<td>Hypoesthesia distal, Reduced vibration sense</td>
<td>Areflexia (LL)</td>
<td>(CMT)</td>
<td></td>
</tr>
</tbody>
</table>

(continued)
suggesting p.Asp364Tyr expression imparts a progressive loss of motor neuron function and neuromuscular coordination (Fig. 3C and D).

Discussion

The advent of next-generation, high-throughput sequencing technologies has allowed rapid identification of disease-associated variants. However, these same advances have caused human geneticists to be increasingly faced with variants of unknown significance in single patients and small families (Schabhuttl et al., 2014). Several ARS family members have been implicated in IPNs; however, the simple identification of a missense variant in a gene encoding one of these enzymes is not sufficient evidence of pathogenicity. To date, mutations in three ARSs (GARS, YARS and AARS) have strong genetic evidence supporting a role in dominantly inherited peripheral neuropathy (Antonellis et al., 2003; Jordanova et al., 2006; Latour et al., 2010; Zhao et al., 2012). Our study now establishes the same level of genetic evidence for the role of HARS in IPN.

Previously, a missense variant in histidyl-tRNA synthetase (p.Arg137Gln HARS) was identified in a single patient with late-onset, sensory-predominant axonal neuropathy; however, segregation studies could not be performed and this variant was also identified at a very low rate in the general population (Vester et al., 2013). Despite demonstrating a loss of function and dominant toxicity in established functional assays, the lack of segregation studies and failure to identify additional unrelated families with IPN and HARS mutations made it difficult to establish a causal link between HARS and IPN (Vester et al., 2013). In the current study, next-generation sequencing was used to identify four HARS mutations in four large unrelated families with IPN. All four mutations are missense alterations that segregate with disease status and that are predicted to be pathogenic using several in silico tools (Supplementary Table 2). In the current study no large systematic cohort screenings have been performed so an accurate estimation of HARS mutation frequencies is not possible. Based on the series that were studied we estimate that the frequency is in the order of 1.6–2.3% (1/62 in HMN cases and 3/128 in GEMapp). Given the extensive phenotypic diversity associated with HARS mutations we expect multiple additional cases are likely to be identified in future whole-exome sequencing studies.

A growing body of evidence suggests that impaired enzyme function is an important component of ARS-mediated CMT disease. Fifteen of 19 CMT-associated mutations in GARS, YARS, and AARS demonstrate loss-of-function characteristics in aminoacylation assays and/or in yeast complementation assays. Importantly, all mutations that cause a loss of function in yeast growth assays also demonstrate loss of tRNA charging in kinetic assays (Jordanova et al., 2006; McLaughlin et al., 2010, 2012; Griffin et al., 2014). By using a yeast model to test the
function of ARS variants in vivo, we determined that all four disease-associated HARS variants result in a severe reduction in yeast viability. In contrast, although affecting the same residue as p.Thr132Ile, the p.Thr132Ser HARS variant, which is not associated with disease, complements loss of endogenous HTS1, indicating that p.Thr132Ser is not a loss-of-function allele (Fig. 2). This supports the notion that impaired function is an important component of ARS-mediated disease pathogenesis. In addition, we used a C. elegans model to show that over-expression of p.Asp364Tyr HARS causes morphological and functional motor deficits, consistent with the dominant IPN phenotype.
observed in patients carrying this mutation. This also confirms that yeast and C. elegans data are consistent with the previously identified variant (Vester et al., 2013) and in favour of pathogenicity allowing the use of genetic and yeast data alone to implicate the remaining alleles.

Phenotypic diversity in IPN is well documented and next generation sequencing techniques have helped reveal the allelic heterogeneity of IPN-associated mutations that may, at least partially, explain the phenotypic variations observed. Our current study demonstrates the diverse types of IPN—in terms of presentation, severity and electrophysiology—that are associated with HARS mutations. Family A is diagnosed with an adult onset CMT2 phenotype of moderate severity. Family B on the other hand displays a more severe disease course with marked slowing of nerve conduction velocities that are in the range of CMT1 for the older individuals. This finding suggests a progressive slowing of nerve conduction velocities. Subject B-III.5 had positive sensory symptoms under the form of paraesthesia. In Family C the disease presentation and severity is highly variable with the male index (Subject C-III.1) presenting a typical CMT phenotype in adolescence while his mother (Subject C-II.2) has unusual late onset features with positive sensory symptoms. Electrophysiology is again in keeping with CMT2 although the effect of progressive slowing of nerve conduction velocities seems to be present as well. In Family D, an initial diagnosis of pure motor axonal neuropathy was made in some patients (HMN) but on progression of the disease in older individuals, clear sensory symptoms and signs and abnormalities of the sensory nerve conductions were noted. Follow-up over time will show if sensory involvement becomes apparent in the mildly affected individuals in the first and second generation of the corresponding family this raises the question of a genotype–phenotype correlation. However Subject C-III.1 has a more typical CMT phenotype in adolescence so this correlation is certainly not straightforward. At the same time several individuals in Family A (Subjects A-IV.3 and A-IV.6) and Family D (Subjects D-IV.2 and D-IV.6) are very mildly affected as well although their respective mutations (Thr132Ile and Asp364Tyr) are complete loss of function alleles. Based on this observation and also our extensive previous experience with other ARS genes (GARS and AARS), we are confident that the yeast assay is a robust predictor of disease but does not allow for strong correlations with disease severity.

ARSs are ubiquitously expressed enzymes that perform the essential first step of protein translation. It is therefore interesting that mutations in genes encoding these enzymes have been implicated in tissue-specific diseases such as peripheral neuropathy. There is currently a preponderance of data suggesting that impaired ARS function is a component of dominant ARS-related IPN; however, to date only missense and in-frame deletions have been associated with these diseases suggesting that the mutant protein must be expressed. This apparent discrepancy may be explained by two non-mutually exclusive possibilities. First, as GARS, AARS, YARS, and HARS holoenzymes function as homodimers, the loss-of-function protein may deplete the function of the remaining wild-type protein via a dominant-negative effect (Freist et al., 1999). In this scenario, dramatically reduced ARS function may breach a threshold of tRNA charging required for protein translation in axons, leading to the axonal phenotype (Wallen and Antonellis, 2013). Second, impaired ARS function (i.e. reduced catalytic activity or decreased tRNA binding) may be a prerequisite for an as-yet undiscovered toxic gain-of-function effect; for example, mutant ARS may now be free to inappropriately bind to axonal RNAs or proteins (Motley et al., 2010, 2011; Wallen and Antonellis, 2013). The first possibility is supported by the over 20 loss-of-function mutations identified in the dimeric GARS, YARS, AARS and HARS enzymes in patients with IPN (Wallen and Antonellis, 2013; Griffin et al., 2014) and the fact that each HARS mutation described here maps to the catalytic domain of the enzyme. The second possibility is supported by the apparent lack of a neuropathy phenotype in patients with Usher Syndrome that are homozygous for a presumably hypomorphic HARS mutation (p.Tyr454Ser) (Puffenberger et al., 2012). However, it is important to note that patients homozygous or compound heterozygous for null and/or hypomorphic mutations in other ARS enzymes do present with a peripheral neuropathy (Isohanni et al., 2010; McLaughlin et al., 2010; Schwartzentruber et al., 2014). While the mechanistic link between ARS mutations and IPN remains unclear, there is abundant evidence that reduced ARS function is an important component of the molecular pathology.

Here we present clinical, genetic and functional data that implicate HARS mutations in inherited peripheral neuropathy. These findings expand the locus, allelic, and phenotypic spectrum of ARS-related human disease and further support a pathogenic role for these enzymes in diseases of the peripheral nerve. Future efforts aimed at teasing out the precise molecular pathology of ARS mutations will be critical for assessing if improving enzyme function or decreasing the activity of mutant ARS alleles will be relevant therapeutic strategies for patients with dominant ARS-related disease.
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Supplementary material

Supplementary material is available at Brain online.

References


