Loss-of-function mutations in Lysyl-tRNA synthetase cause various leukoencephalopathy phenotypes

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Abstract

Objective

To expand the clinical spectrum of lysyl-tRNA synthetase (*KARS*) gene-related diseases, which so far includes Charcot-Marie-Tooth disease, congenital visual impairment and microcephaly, and nonsyndromic hearing impairment.

Methods

Whole-exome sequencing was performed on index patients from 4 unrelated families with leukoencephalopathy. Candidate pathogenic variants and their cosegregation were confirmed by Sanger sequencing. Effects of mutations on *KARS* protein function were examined by aminoacylation assays and yeast complementation assays.

Results

Common clinical features of the patients in this study included impaired cognitive ability, seizure, hypotonia, ataxia, and abnormal brain imaging, suggesting that the CNS involvement is the main clinical presentation. Six previously unreported and 1 known *KARS* mutations were identified and cosegregated in these families. Two patients are compound heterozygous for missense mutations, 1 patient is homozygous for a missense mutation, and 1 patient harbored an insertion mutation and a missense mutation. Functional and structural analyses revealed that these mutations impair aminoacylation activity of lysyl-tRNA synthetase, indicating that defective *KARS* function is responsible for the phenotypes in these individuals.

Conclusions

Our results demonstrate that patients with loss-of-function *KARS* mutations can manifest CNS disorders, thus broadening the phenotypic spectrum associated with *KARS*-related disease.

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Glossary

ARS = aminoacyl-tRNA synthetase; 5-FOA = 5-fluoroorotic acid; OXPHOS = oxidative phosphorylation system.

Aminoacyl-tRNA synthetases (ARSs) play key roles in charging specific tRNAs with cognate amino acids and are critical for enabling protein translational fidelity and cellular integrity. Pathogenic mutations in different ARSs have been reported in patients with a variety of clinical presentations including cardiomyopathy, cancer, autoimmune disorders, and diabetes.^{1–3} Of interest, a number of mutations in genes encoding ARSs have been linked to neurologic diseases, including inherited peripheral neuropathy, sensorineural deafness, leukodystrophies, or leukoencephalopathies, summarized in table e-1, links.lww.com/NXG/A143.^{4–27}

KARS is one of the 3 bifunctional ARSs and catalyzes the specific attachment of L-lysine to cognate tRNA molecules. Compound heterozygous disease-associated KARS mutations were first identified in a single patient with Charcot-Marie-Tooth disease.²⁸ Later, other KARS mutations were also reported in 3 unrelated families with autosomal recessive nonsyndromic hearing impairment,¹⁹ in 1 patient with a suspected mitochondrial disorder,²⁹ in 2 siblings with severe infantile visual loss and progressive microcephaly,¹⁵ and in a boy with combined respiratory chain complex deficiencies (I and IV).³⁰ However, dysfunctions of CNS involved in leukoencephalopathy have not been reported or observed as a major clinical presentation in these affected individuals.

In this study, 4 unrelated nonconsanguineous families with leukoencephalopathy were identified by whole-exome sequencing analysis. Functional and structural analyses revealed that each variant was a loss-of-function KARS mutation. Our findings highlight the association of KARS mutations in patients with CNS involvement and broaden the phenotypic spectrum associated with KARS-related disease.

Methods

Standard protocol approval, registrations, and patient consents

Patient blood specimens were submitted to Baylor Genetics, previously Medical Genetics Laboratories at Baylor College of Medicine, Houston, TX, for WES-based analyses. Additional patient specimens were from Fudan University Huashan Hospital, Shanghai, China. The ethical review boards of the participating institutions approved this study.

Exome sequencing in probands and family studies

Library preparation, exome capture, HiSeq next-generation sequencing, and data analyses were conducted as described.^{31–33} Variants identified in KARS were further validated by Sanger sequencing in these patients. Family

members were also tested to evaluate the mode of inheritance and disease segregation.

Functional studies

Aminoacylation assays in vitro were performed as previously described.^{28,34,35} The initial rate of aminoacylation as a function of tRNA concentration was fit to a hyperbola equation, from which the Michaelis constant (K_m) for tRNA and the catalytic turnover (k_{cat}) were derived. Analysis of the catalytic efficiency (k_{cat}/K_m) of aminoacylation was presented for each mutant enzyme.

Yeast complementation assays were performed using a haploid *Saccharomyces cerevisiae* strain with the endogenous *KRS1* gene deleted ($\Delta KRS1$)²⁸ in both solid and liquid -Leu-Ura media (Teknova, Hollister, CA) containing 0.1% 5-fluoroorotic acid (5-FOA) (Boeke, Trueheart et al. 1987). Each human KARS variant (GenBank accession number AAG30114.1, NM_005548) was modeled in yeast *KRS1* (GenBank accession number AAA66916.1) using Gateway technology (Invitrogen). The human KARS residues p.L233, p.E427, p.R505, p.P533, p.T587, and p.L596 correspond to the following yeast residues, respectively: p.L208, p.E403, p.R480, p.P508, p.T562, and p.L571.

Results

Patient history and clinical presentations

Patient 1 is a 26-year-old woman who developed progressive neurocognitive decline at age 25 years. Her key clinical features included hypotonia, mild intellectual disability, slurred speech, ataxia, and abnormal movement, as well as congenital hearing loss. Two KARS variants c.1514G>A (p.R505H) and c.1597C>T (p.P533S) were identified in the compound heterozygous state. The EMG and histochemical analysis of muscle biopsy did not reveal any myogenic or neurogenic damage. Brain MRI showed symmetric hyperintensity in bilateral frontal white matter, extending along the anterior limb of inner capsule on FLAIR and DWI (figure 1, A and B). Magnetic resonance spectroscopy was performed with Stimulated Echo Acquisition Mode sequence, and data were analyzed with the LC Model (version 6.3). The spectroscopy showed distinctly reduced N-acetylaspartate and slightly elevated lactate peak in the right frontal lesion (figure 1E) compared with that in the ipsilateral normal white matter (figure 1F). The brother of patient 1, who also harbored the same 2 variants, had hearing loss, and his brain MRI performed at age 16 years showed bilateral abnormality in the periventricular white matter (figure 1C). The parents were clinically unaffected and had normal MRI scans, and each was heterozygous for one of these 2 variants. Therefore, variants

Figure 1 Representative cases for novel *KARS* mutation with leukoencephalopathy

Characteristics of patients for novel *KARS* mutations with leukoencephalopathy. (A,B) Brain imaging of Patient 1 showed bilateral FLAIR and DWI signal hyperintensity in the white matter of the frontal lobe. (C) The affected brother of Patient 1 has also abnormality in the white matter. (D) Brain MRI of Patient 4 showed bilateral T2 signal hyperintensity in the white matter of periventricular area. (E) The MRS showed reduced NAA and elevated lactate peak in the right frontal lesion of Patient 1. (F) The MRS showed the presence of NAA and lactate in the normal white matter of Patient 1.

c.1514G>A (p.R505H) (from the mother) and c.1597C>T (p.P533S) (from the father) segregate with leukoencephalopathy in an autosomal recessive manner in the family of patient 1. She was deceased after 2 years of neurologic features appeared.

Patient 2 is a 35-year-old man who developed progressive neurocognitive decline, hypertonia, seizures, ataxia, and abnormal movement over 3 years. Other clinical features included congenital hearing loss, likely secondary hypothyroidism and possible left eye blindness. Brain MRI revealed abnormality in the white matter, and EEG was normal. Family history indicated a sister with congenital deafness and hydrocephaly and 2 second cousins with congenital deafness. He was compound heterozygous for a novel missense variant c.881T>C (p.I294T) and a reported variant c.1760C>T (p.T587M).

Patient 3 is a 3-year-old boy present with developmental delay and regression, failure to thrive, microcephaly, and progressive hypotonia. Other clinical presentations include hearing and vision loss, nystagmus, hyperreflexia, progressive joint contractures, febrile seizures, dysphagia, renal tubular acidosis type I, mild hydronephrosis of the left kidney, and abnormal liver ultrasound. Head CT showed increasing periventricular and cerebellar nuclei calcifications and cerebral atrophy. He was

compound heterozygous for a novel insertion variant c.1281_1282insAGA (p.E427_L428insR) and a novel missense variant c.1786C>T (p.L596F).

Patient 4 is an 11-year-old girl whose clinical presentations include global developmental delay, hypotonia, mild intellectual disability, congenital bilateral profound sensorineural hearing loss, history of seizure disorder, mildly elevated lactate, elevated CSF total protein, and developmental regression. Family history was remarkable for a sister with bilateral sensorineural hearing loss. Her brain MRI and CT showed normal at age 1 year. However, the second MRI at age 11 years showed extensive abnormality of the deep white matter of both cerebral hemispheres with increased T2 hyperintensity and involvement of the corticospinal tracts with sparing of the subcortical U fibers (figure 1D). Mild cerebellar volume loss with prominence of the fourth ventricle was also observed. The patient died at age 12 years after a rapid deterioration in her neurologic status over a period of 2 years. Her autopsy showed severe bilateral spongiform leukodystrophy involving the internal capsule, frontal, parietal, and occipital white matter. There was symmetric white matter loss in the descending corticospinal tracts, brainstem, and spinal cord. Dystrophic calcifications were noted in basal

ganglia, frontal, and parietal lobes. She harbored a homozygous missense variant c.697C>G (p.L233V). Her mother was found to be heterozygous for this variant. But the father's sample was not enough for further testing.

Computational evaluation of KARS variants

Six novel and 1 known *KARS* variants were identified by WES in the index patients described here (table). All variants are rare, and none is found in the homozygote state in the ExAC database (table e-2, links.lww.com/NXG/A144). Sequence alignments of *KARS* proteins from bacteria to human showed that all the affected amino acids are highly conserved (figure 2F). A variety of in silico prediction programs were used to predict the possible effect of each amino acid substitution (table e-2). All 8 novel variants were considered as likely pathogenic, based on predictions by PolyPhen-2, Sorting Intolerant from Tolerant, MutationTaster, MutationAssessor, etc. (table e-2).

Functional studies

As shown in figure 3, all *KARS* mutations studied (p.L233V, p.I294T, p.R505H, p.P533S, p.T587M, and p.L596F) reduced enzyme kinetics by at least 13-fold compared with that of wild-type *KARS*, indicating that these mutations impaired *KARS* aminoacylation activity.

To further evaluate the deleterious effects of *KARS* mutations, yeast complementation assays were performed by modeling each *KARS* variant in the *S. cerevisiae* ortholog *KRS1*. As shown in figure 4A, yeast expressing p.E427_Lins428R and p.R505H *KRS1* demonstrated dramatically reduced, but not ablated, growth. In addition, yeast expressing p.L233V, p.P533S, p.T587M, and p.L596F *KRS1* showed a slight but significant reduction of yeast viability compared with wild-type *KRS1*. These results suggested that p.L233V, p.P533S, p.T587M, p.L596F, p.R505H, and p.E427_Lins428R are hypomorphic alleles. Similar results were obtained from growth curve analyses in liquid media containing 5-FOA (figure 4B).

Mechanisms of pathogenicity for impaired function of mutated residues

Patient 1 harbors 2 novel *KARS* missense variants (c.1514G>A [p.R505H] and c.1597C>T [p.P533S]) in the compound heterozygous state. Close examination of the crystal structure revealed that Arg505 forms a hydrogen-bond network with Asp374 and Glu512 of *KARS* and an adjacent water molecule.³⁶ Replacement with the side chain of His would eliminate the positively charged side chain of Arg and thus impair their hydrogen-bond network (figure 2, B and C). Pro533 is located at the transition point of a helix to a β -strand of *KARS* protein consisting of amino acids 520–545 to form dimeric interface. The replacement with Ser would not permit the required peptide bond turn of Pro and thus would be expected to have a detrimental effect on the protein secondary structure. Consistent with the predictions from structural analysis, both mutations decreased in tRNA charging activity

by 20- and 200-fold relative to the wild-type enzyme, respectively (figure 3). Of interest, although p.P533S mutation caused a 10-fold reduction relative to p.R505H, p.R505H mutation showed a more severe reduction in cellular growth in yeast complementation assays. Thus, these 2 mutations identified in this patient affect critical amino acids and reduce tRNA charging capacity, suggesting a pathogenic role in causing the patient phenotype.

Patient 2 has a novel variant c.881T>C (p.I294T) and a known mutation c.1760C>T (p.T587M). The Ile294 residue forms a hydrophobic interaction with Leu597 located within the same monomer to form the dimeric interface. Replacement of Ile294 with Thr introduces an extra hydroxyl group, which may alter the nature of hydrophobic core. The p.I294T mutant showed a reduction in tRNA charging by 13-fold relative to the wild-type enzyme (figure 3). The p.T587M variant, a previously reported mutation, almost completely eliminated the enzyme activity.

Patient 3 has a novel c.1281_1282insAGA (p.E427_L428insR) insertion mutation and a novel c.1786C>T (p.L596F) missense mutation. Structural analysis indicated that Glu427 and Leu428 are located in a helix tightly packed against another helix consisting of residues 468–484. The insertion of a bulky amino acid Arg into the middle of the first helix will disrupt its secondary structure, affecting the enzyme stability. In addition, Leu596 is embedded in the protein interior and packed tightly with adjacent hydrophobic residues. The replacement with Phe introduced a large bulky side chain creating stereochemical clashes and causing protein instability. Yeast expressing p.E427L_ins428R *KRS1* showed a severe reduction of yeast viability compared with wild-type strain, indicating that it is a loss-of-function allele, whereas the p.L596F mutation caused a slight but significant reduction in yeast viability. On the other hand, the tRNA charging activity of p.L596F mutant is reduced by 571-fold, with a 2.2-fold increase in K_m , but nearly a 240-fold reduction in k_{cat} (figure 3).

Patient 4 has a single nucleotide variation, c.697C>G (p.L233V), and a copy number loss of *KARS* at this locus, which were inherited from each parent respectively. The Leu233 is located in the anticodon binding domain. This variant reduced enzyme activity by 13-fold relative to the wild-type protein and slightly decreased yeast viability in yeast complementation studies (figures 3 and 4). Thus, the abnormal kinetic aspect of this mutation affected the catalytic behavior of the enzyme and may likely contribute to disease pathogenesis.

Discussion

KARS mutations have been linked to neurologic disorders with different clinical manifestations. Compound heterozygous mutations p.L133H and p.Y173Sfs*7 were first identified in a patient with Charcot-Marie-Tooth disease.²⁸ Two

Table Summary of key clinical manifestations of affected individuals

Index number	Age at onset	Sex	Summary of clinical presentation of affected individuals						Molecular study		
			CNS	Brain imaging	Hearing loss	EEG	NCV + EMG	Other systems	Allele 1	Allele 2	Status
1	26 y	F	Hypotonia/spasticity, mild intellectual disability, slurred speech, ataxia, and abnormal movement	Bilateral frontal white matter	Y	Normal	Normal	N	c.1514G>A	c.1597C>T	Compound heterozygous
									p.R505H	p.P533S	
2	35 y	M	Neurocognitive decline, spasticity, seizures, ataxia, and abnormal movements	Bilateral periventricular white matter	Y	Diffuse slow activity	UN	Primary hypothyroidism	c.881T>C	c.1760C>T	Compound heterozygous
									p.I294T	p.T587M	
3	3 y	M	Failure to thrive, developmental delay, developmental regression, microcephaly, nystagmus, hypotonia, hypertonia/spasticity, hyperreflexia, febrile seizures, and dysphagia	Cerebellar nuclei calcifications	Y	UN	UN	Vision loss, abnormal renal function, abnormal liver ultrasound, and progressive joint contractures	c.1281_1282insAGA	c.1786C>T	Compound heterozygous
									p.E427_L428insR	p.L596F	
4	11 y	F	Developmental delay, hypotonia/spasticity, mild intellectual disability, seizures, bilateral hand tremor and ataxia, mildly elevated lactate, elevated CSF total protein, and slurred speech	Bilateral deep white matter	Y	Diffuse slow activity with spike activity	Normal	N	c.697C>G	c.697C>G	Homozygous
									p.L233V	p.L233V	
BAB564 ²⁸	UN	UN	Developmental delay and dysmorphic features	UN	UN	UN	Neurogenic damages	Charcot-Marie-Tooth, self-abusive behavior, and vestibular schwannoma	c.398T>A	c.524_525insTT	Compound heterozygous
									p.L133H	p.Y173Sfs*7	
1098 ²⁹	6 m	M	Hypotonia, global developmental delay, strabismus, ophthalmoplegia, dystonia, elevated plasma alanine, and CSF lactate	N	Y	N	UN	Increased mtDNA levels in muscle and abnormal brainstem auditory-evoked potential ^a	c.683C>T	c.1760C>T	Compound heterozygous
									p.P228L	p.T587M	
4338 V1 ¹⁹	UN	M	N	UN	Y	UN	UN	Autosomal recessive nonsyndromic hearing impairment (ARNSHI)	c.1129G>A	c.1129G>A	Homozygous
									p.D377N	p.D377N	

Continued

Table Summary of key clinical manifestations of affected individuals (*continued*)

Index number	Age at onset	Sex	Summary of clinical presentation of affected individuals					Molecular study			
			CNS	Brain imaging	Hearing loss	EEG	NCV + EMG	Other systems	Allele 1	Allele 2	Status
Brother¹⁵	9 m	M	Visual impairment/pendular nystagmus, microcephaly, seizures, and global developmental delay	Thinning of the central cerebral white matter and corpus callosum	N	Generalized epileptiform discharges	UN	N	c.1312C>T	c.1573G>A	Compound heterozygous
Sister¹⁵	4 m	F	Visual impairment/pendular nystagmus, microcephaly, seizures, and global developmental delay	Thinning of the central cerebral white matter and corpus callosum	N	Generalized epileptiform discharges	UN	N	c.1312C>T	c.1573G>A	Compound heterozygous

Abbreviations: M = male; F = female; Y = yes; N = no; UN = unknown.
In the original text is "brainstem auditory-evoked potential study at 10 months was markedly abnormal, suggesting severe peripheral conduction defects in the auditory pathways bilaterally."

mutations p.D377N and p.Y173H were found in the homozygous state in patients with autosomal recessive nonsyndromic hearing impairment.¹⁹ Compound heterozygous mutations p.P228L and p.T587M were identified in a patient presenting with development delay, hypotonia, and ophthalmoplegia.²⁹ Meanwhile, patients with congenital visual impairment and progressive microcephaly were reported to be compound heterozygous for mutations p.R438W and p.E525K.¹⁵ Recently, compound heterozygous mutations (p.V448D and p.I318T) were identified in a boy with combined mitochondrial complex deficiencies.³⁰ In this study, we identified 6 novel KARS mutations in patients with leukoencephalopathy. We also found that the previously reported mutation p.T587M is associated with leukoencephalopathy. Each mutation exerts a loss-of-function effect in at least 1 of the following assays: aminoacylation assay and yeast complementation assay, suggesting that defective KARS charging function is an important component of leukoencephalopathy pathogenesis in our patients. Our results are consistent with the notion that impaired enzyme function is a common characteristic of disease-associated ARSs mutations.

In the patients examined here, CNS involvement is the main clinical presentation. Common clinical features include impaired cognitive ability, seizure, hypotonia, and ataxia. Brain MRI or CT revealed abnormalities in the white matter in all patients. Our results suggest that leukoencephalopathy in these patients is caused by KARS defects and that there are novel clinical features from the previously described KARS-associated neurologic diseases.

Like other bifunctional ARSs, KARS has cytoplasmic and mitochondrial isoforms, which result from alternative splicing of the first 3 exons.^{36,37} Previously, KARS mutations have been implicated in peripheral neuropathies and sensorineural diseases. In this study, we showed that KARS is also involved in CNS diseases. The mechanisms underlying the tissue specificity of KARS-associated neurologic disorders are unclear. To date, 4 cytoplasmic ARSs (*YARS*, *AARS*, *HARS*, and *MARS*), but not their mitochondrial counterparts (*YARS2*, *AARS2*, *HARS2*, and *MARS2*), are associated with peripheral neuropathy, indicating that this disease might be caused by dysfunctions of cytoplasmic protein translation. Mutations in KARS and another bifunctional enzyme (*GARS*) had also been implicated in peripheral neuropathy. We propose that the dysfunction of cytoplasmic KARS or *GARS*, but not of mitochondrial KARS or *GARS*, is involved in peripheral neuropathy. Conversely, most of the mitochondrial ARSs defects have been found to affect the CNS. A functional CNS has an extremely high demand for energy. The oxidative phosphorylation system (OXPHOS) is a key functional unit in mitochondria, and it is the major source of cellular adenosine triphosphate. Defects in mitochondrial ARSs affect mitochondrial protein synthesis and lead to mitochondrial OXPHOS dysfunction³⁸ (tables e-1 and e-2, links.lww.com/NXG/A143 and links.lww.com/NXG/A144). As expected, CNS involvement as the main or sole clinical presentation had

Figure 2 Summary of KARS mutations

been reported in most patients with pathogenic mutations in mitochondrial ARSs, including *DARS2*, *EARS2*, *MARS2*, *FARS2*, *RARS2*, *VARS2*, *TARS2*, *NARS2*, and *CARS2*. Meanwhile, the elevated level of lactate observed in some of our patients is also consistent with mitochondrial dysfunction. Therefore,

dysfunction of mitochondrial KARS, but not cytoplasmic KARS, more likely to be contributing to the pathogenesis of leukoencephalopathy reported in this study. This work provides a framework to link the dysfunction of mitochondrial KARS with leukoencephalopathy associated with disorders of the CNS.

Figure 3 Summary of the mutations identified in KARS and their effects on tRNA charging

	$K_m(\mu\text{M})$	$k_{cat}(s^{-1})$	$k_{cat}/K_m(\mu\text{M}^{-1}s^{-1})$	Ratio to WT	
Wild-type	3.2 ± 0.2	1.2 ± 0.1	0.4	1	
L233V	9.7 ± 2.2	0.3 ± 0.1	0.03	1/13	
I294T	6.8 ± 2.0	0.20 ± 0.02	0.03	1/13	
R505H	11.0 ± 1.0	0.2 ± 0.1	0.02	1/20	
P533S	10 ± 0.7	0.02 ± 0.01	0.002	1/200	
T587M	—	—	5.6×10^{-8}	$1/(7 \times 10^6)$	
L596F	7.1 ± 1.4	0.005 ± 0.001	0.0007	1/571	

The column "ratio to WT" indicates the decrease in tRNA charging relative to the WT enzyme for each mutant. All these mutations have a deleterious effect, ranging from 13- to nearly 10^7 -fold.

Figure 4 Yeast complementation analysis of mutant *KRS1* alleles

(A) A haploid yeast strain deleted for endogenous *KRS1* was transformed with a *LEU2*-bearing pRS315 vector containing wild-type *KRS1*, the indicated mutant form of *KRS1*, or no insert ("Empty"). Cultures for each strain (labeled along the top) were grown for 2 days in liquid medium and spotted on solid medium containing 5-FOA to determine whether the *KRS1* alleles complement loss of *KRS1* at 30°C. (B) Cultures for each indicated strain (labeled at right) were grown for 2 days in liquid medium and then diluted in liquid medium containing 5-FOA to determine whether the *KRS1* alleles complement loss of *KRS1* at 30°C. The optical density (OD₆₀₀, y-axis) was evaluated for each culture at the indicated time points (x-axis), and error bars indicate SD. 5-FOA = 5-fluoroorotic acid.

Author contributions

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Disclosure

Baylor College of Medicine (BCM) and Miraca Holdings Inc. have formed a joint venture with shared ownership and governance of the Baylor Genetics Laboratories (BMGL), which performs clinical exome sequencing. The Department

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