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Novel heteroplasmic mutation in the anticodon stem of mitochondrial tRNA^{Lys} associated with dystonia and stroke-like episodes

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Objectives – We report a novel heteroplasmic mitochondrial tRNA^{Lys} mutation associated with dystonia, stroke-like episodes, sensorineural hearing loss and epilepsy in a Hungarian family. *Material and methods* – A 16-year-old boy, his brother and mother were investigated. Thorough clinical investigation as well as electrophysiological, neuroradiological and myopathological examinations were performed. Molecular studies included the analysis of the DYT1, DDP1/TIMM8A (deafness-dystonia peptid-1) genes and mitochondrial DNA (mtDNA). *Results* – The mtDNA analysis of the proband revealed a heteroplasmic A8332G substitution in the anticodon stem of the tRNA^{Lys} gene. The mutation segregated in all affected family members. Besides this mutation 16 further mtDNA polymorphisms were detected. Complex I activity of the patient's fibroblast cultures showed decreased activity confirming mitochondrial dysfunction. *Conclusion* – The novel A8332G heteroplasmic mutation is most likely a new cause of dystonia and stroke-like episodes due to mitochondrial encephalopathy. The synergistic effect of the G8697A, A11812G and T10463C single nucleotide polymorphisms may modify the phenotype.

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Introduction

Mitochondrial DNA mutations have been associated with a variety of clinical manifestations, including dystonia. To date 15 different mtDNA mutations in five genes (tRNA^{Leu}, tRNA^{Lys}, ND1, ND3 and ND5) have been described (1) which could result in dystonic features. Interestingly the typical A8344G MERRF (mitochondrial encephalopathy with ragged red fibres) mutation in tRNA^{Lys} (2), and the typical A3243G MELAS (mitochondrial encephalomyopathy lactate acidosis stroke like symptoms) mutation in tRNA$^{Leu(UUR)}$ can also be a cause of dystonia (3). Besides mtDNA defects, the dysfunctions of 17 nuclear genes have been identified as potential causes of dystonia (4). One of these genes (DDP1/TIMM8A – deafness-dystonia peptid-1) encodes a mitochondrial protein, which is responsible for the deafness-dystonia syndrome (DDS) or Mohr-Tranebjaerg syndrome (MTS). This is a rare X-linked recessive neurological disorder characterized by impaired transport and sorting of proteins to the mitochondrial inner membrane (5). The most common causes of dystonia are due to various alterations in the nuclear genome: the DYT-1 gene mutation on chromosome 9 (4) and dopa-responsive dystonia gene defect on chromosome 14 (6). Recently

mutations in the THAP1 gene have been described in a substantial proportion of early-onset dystonia (7). Here we describe a Hungarian dystonic family with a new heteroplasmic pathogenic mtDNA mutation (A8332G), as the cause of dystonia, stroke-like-episodes.

Subjects and methods

A Hungarian patient, his brother and mother are the subjects of this report. Informed consent was obtained from all patients involved in our study. Clinical data were gathered from these patients. Electrophysiological investigations were performed by standard techniques (Dantech Keypoint, Skovlunde, Denmark). Muscle biopsy was taken from the proband for light and electron microscopic examination using standard routine staining. DNA was extracted from blood and skeletal muscle tissue (in case of the proband) by ABI PRISM 6100 Nucleic Acid Isolation Systems (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. As the mother had severe sensorineural hearing loss and mild dystonia, the DDP1/TIMM8A gene was bidirectionally sequenced by Centogene (Rostock, Germany) in Patient 1. The GAG deletion analysis of DYT-1 gene was performed using fluorescent quantitative fragment length polymorphism on an ABI PRISM 3100 genetic analyzer. The 22 mitochondrial tRNA genes, the entire coding region and the hypervariable segment 1 (nt 15,975–16,420) of the mtDNA were sequenced by standard methods (8, 9). Nucleotides 8105–8536 and nt 14,400–14,580 of the mtDNA were sequenced in 150 healthy controls also. The control DNA samples were selected from the biobank of the Clinical and Research Centre for Molecular Neurology, Semmelweis University. Functional tests were performed by measuring Complex I activity from primary fibroblasts collected from skin biopsy samples of the patients and three healthy controls. The fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Invitrogene GmbH, Lofer, Austria) with 20% foetal bovine serum (GIBCO), 0.5% Penicillin-Streptomycin (GIBCO), and 0.3% Fungisone (GIBCO) at 37°C with 5% CO_2 and 95% air. Mitochondrial Complex I was measured by Enzyme Activity Microplate Assay Kits (MitoScience, Eugene, OR, USA) according to the manufacturer's instructions. Complex I activity was determined by detecting the oxidation of NADH to NAD+ and the simultaneous reduction of a dye which leads to increased absorbance at 450 nm.

Clinical profile

The 16-year-old male proband (Patient 1) was investigated for severe dystonic symptoms, which appeared after a long period of fever at age 9. Neurological investigation revealed severe generalized dystonia localized mainly to the muscles of the face, tongue, arms and hands, bilateral horizontal nystagmus, anarthric speech, diffuse atrophy of the skeletal muscles, bilateral pes cavus, moderate, dominantly proximal weakness of the limbs, and weakness of the respiratory muscles, missing deep-tendon reflexes. The dystonia did not respond to levadopa administration. Laboratory results showed normal creatinine kinase activity, elevated serum lactate and ammonia levels, and elevated protein concentration in the cerebrospinal fluid. EMG revealed neurogenic damage in the right deltoid and tibial anterior muscles. ENG, EEG and brain MRI were normal. Ophthalmologic examination found no abnormality above the nystagmus. Muscle biopsy of the left deltoid muscle revealed mild neurogenic atrophy, 6% of the muscle fibres appeared as ragged blue with modified SDH staining. These fibres were COX negative. Electron microscopy found aggregated mitochondria in many muscle fibres. His brother (Patient 2) had transient limb dyskinesia and left-sided hemiparesis at age 4. Serum ammonia and lactate levels were increased as well as protein content in the cerebrospinal fluid at that time. At age 11 he was diagnosed with epilepsy. Clinical investigations at age 19 revealed dysarthric speech, mild dystonic movement in the distal parts of the upper limbs, mild palsy dominantly in the distal muscles of the left lower limb and reduced tendon reflexes. Brain MRI found no abnormality. EEG showed epileptic signs in the left fronto-central region. ENG found axonal neuropathy. Ammonia and lactate levels were in the normal range at age 19. Their mother (Patient 3) had stroke with aphasia and right-sided hemiparesis in early childhood. At the time of examination (age 50 years) she had mild dysarthria, moderate truncal ataxia, focal dyskinesia in the right hand, severe sensorineural hearing loss and mental retardation. Brain MRI showed mild brain atrophy. ENG found axonal neuropathy in the lower limbs, EMG was normal. Audiological examination revealed bilateral sensorineural hearing loss. The investigation of grandparents could not be carried out, as the mother (Patient 3) was adopted in her early childhood.

Molecular genetic results

Screening of the DYT1 gene and the nuclear-encoded mitochondrial DDP1/TIMM8A gene did

not show any alteration in Patient 1. His mtDNA analysis revealed two heteroplasmic mutations, an A to G change at nt 8332 in the anticodon arm of $tRNA^{Lys}$ (Figs 1 and 2) and a C14520G substitution in the ND6 gene. In the case of the A8332G mutation, ratio of heteroplasmy was 55% in the blood and 65% in muscle tissue. Besides these mutations 15 different single nucleotide polymorphisms (SNPs) and an East Asian-specific 9 bp large deletion between nt 8271 and nt 8280 were also found (Tables 1 and 2). The novel A8332G substitution segregated in all affected family members. Ratio of heteroplasmy of this mutation was 17% in Patient 2 and 35% in Patient 3 measured in the blood. The A8332G substitution was not present in 150 healthy controls. In the case of the C14520G substitution ratio of heteroplasmy was 35% in the proband's blood. This substitution was present in all affected family members and in 13 healthy controls. Haplogroup analysis detected the mitochondrial B haplogroup in the investigated family members.

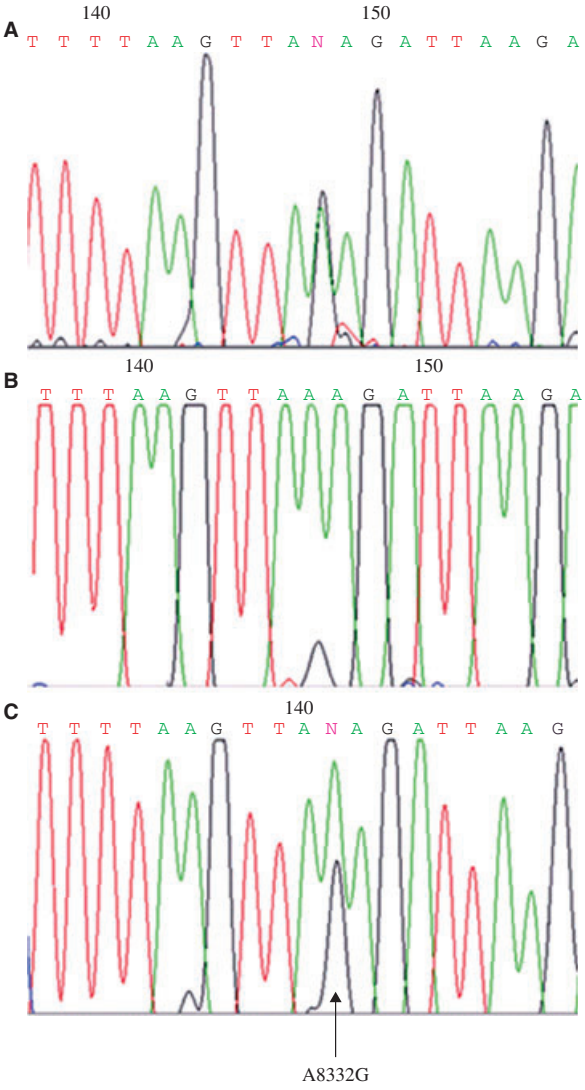

Figure 1. The heteroplasmic A8332G substitution in the sequenogram of Patient 1 (A), Patient 2 (B) and Patient 3 (C).

Functional tests

To confirm the effect of the novel A8332G mutation on mitochondrial function Complex I activity, the largest multi-protein enzyme complex of the mitochondrial respiratory chain was measured from the patient's fibroblasts. Complex I activity was significantly decreased in all patients compared to the values of healthy controls (Patient 1: 42%; Patient 2: 47%; Patient 3: 65%; normal healthy control: 100%).

Discussion

A novel heteroplasmic mitochondrial $tRNA^{Lys}$ anticodon stem (A8332G) mutation with the coexistence of four non-synonymous, 12 synonymous polymorphisms and a 9-bp deletion were detected in a Hungarian family with dystonia and stroke-like episodes (Tables 1 and 2). The $tRNA^{Lys}$ is one of the most mutable genes in the mitochondrial genome. To date 14 different pathogenic mutations and eight polymorphisms have been described in the $tRNA^{Lys}$ as a cause of various clinical symptoms (1). We consider the A8332G substitution as potentially pathogenic, because this mutation was segregated through the maternal lineage in our family, it was heteroplasmic and was absent in 150 Hungarian healthy controls.

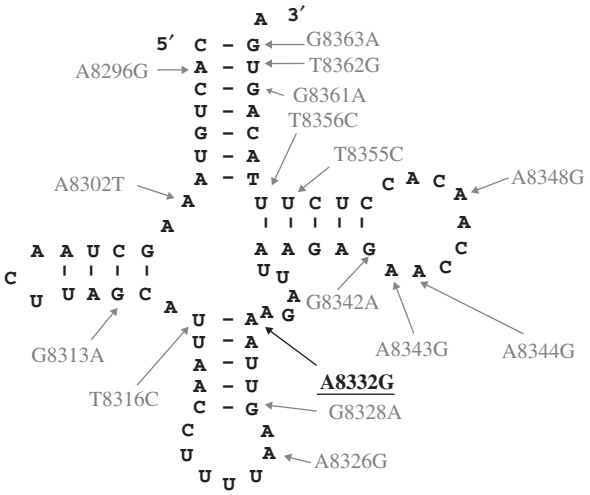

Figure 2. Schematic presentation of the $tRNA^{Lys}$ clover-leaf structure showing the position of the new (8332) (black) and the known pathogenic mutations (grey).

Table 1 The mtDNA alteration in tRNA and protein-coding genes of the investigated family

Locus	Nucleotide position	Nucleotide change	Amino acid change	Form	Patient 1	Patient 2	Patient 3	Qualification	Reference
ND1	4216	T>C	Tyr/His	Homoplasmic	+	+	+	Non-synonymous substitution	Torroni et al. (20)
NC7	8270	C>T	–	Homoplasmic	+	+	+	Synonymous substitution	Ruppert et al. (15)
NC7	Del. nt 8271–8280	–	–	Homoplasmic	+	+	+	Synonymous substitution	Lorenz and Smith (21)
tRNA^{Lys}	8332	A>G	–	Heteroplasmic	55%	17%	35%	Pathogenic substitution	
tRNA^{Lys}	8347	A>C	–	Homoplasmic	+	+	+	Non-synonymous substitution	Coon et al. (18)
ATP6	8697	G>A	–	Homoplasmic	+	+	+	Synonymous substitution	Rieder et al. (22)
tRNA^{Arg}	10,463	T>C	–	Homoplasmic	+	+	+	Non-synonymous substitution	Houshmand et al. (23)
ND4	11,812	A>G	–	Homoplasmic	+	+	+	Synonymous substitution	Howell et al. (24)
ND6	14,520	C>G	Gly/Arg	Heteroplasmic	40%	25%	15%	Non-synonymous substitution	

Table 2 The detected mtDNA alterations in the HVS1 region

Locus	Nucleotide position	Nucleotide change	Form	Patient 1	Patient 2	Patient 3	Qualification	Reference
HVS1	16,126	T>C	Homoplasmic	+	+	+	Polymorphism	Dirienzo and Wilson (25)
HVS1	16,140	T>C	Homoplasmic	+	+	+	Polymorphism	Horai and Hayasaka (26)
HVS1	16,183	A>C	Homoplasmic	+	+	+	Polymorphism	Lahermo et al. (27)
HVS1	16,189	T>C	Homoplasmic	+	+	+	Polymorphism	Horai and Hayasaka (26)
HVS1	16,209	T>C	Homoplasmic	+	+	+	Polymorphism	Horai and Hayasaka (26)
HVS1	16,294	C>T	Homoplasmic	+	+	+	Polymorphism	Horai and Hayasaka (26)
HVS1	16,296	C>T	Homoplasmic	+	+	+	Polymorphism	Horai and Hayasaka (26)
HVS1	16,311	T>C	Homoplasmic	+	+	+	Polymorphism	Horai and Hayasaka (26)

Although the 'A' at position nt 8332 is not highly conserved during evolution, the nucleotide at this particular position is invariably complementary to the nucleotide at position 8316 in various species, resulting in a base pair that is highly conserved throughout evolution (10). Mutation of the complementary 8316 nucleotide may result in mitochondrial myopathy, lactic acidosis and stroke-like episodes (10). The A to G nucleotide change at this position results in the dissolution of the H-bond between nt 8316 and nt 8332, in the first base pair of the anticodon stem of tRNA^{Lys} (Fig. 2). This disruption may alter the double helix of the anticodon stem and secondary structure of the tRNA. The decreased Complex I activity of the patients' fibroblasts confirms the functional effect of this mutation. The higher decreased activity was found in Patient 1, who had the most serious clinical symptoms, and in his muscle sample the A8332G mutation was found in 65% of heteroplasmy. Besides the novel pathogenic mutation 16 polymorphisms were present. In the non-coding region between cytochrome oxidase II (COII) and tRNA^{Lys} genes a 9-bp deletion and a C8270T substitution have also been found. This deletion was originally thought to be an East Asian-specific anthropological marker (11); however, it occurs frequently in Amerindians (12) and rarely in Caucasians as well (13). Redd et al. proposed that this region might have high instability caused by slipped-strand mispairing (SSM) during DNA replication (14). The C8270T substitution in COII-tRNA^{Lys} intergenic region (NC7) has been reported previously as a polymorphism linked to ischaemic heart disease (15), later González et al. (16) and Thangaraj et al. (17) considered that the coexistence of the 9-bp deletion and C8270T substitution determines M subhaplogroups. The haplogroup of the presented family belong to an East Asian B mitochondrial haplogroup, which can be explained by westward migration of the Hungarians from the forest steppes of Western Siberia.

The base substitution at nt 8347 in the pseudouridin loop (T-loop) of tRNA^{Lys} was observed previously by MitoChip resequencing array. It was present more frequently in Alzheimer patients than in healthy controls (18). Lehtonen et al. investigated the sequence variation in mtDNA in maternal sensorineural hearing loss (19). They found increased sequence variation in the patients compared to controls. Three synonymous substitutions (9 bp deletion – nt 8271–8280, G8697A and A11812G) and one non-synonymous polymorphism (T10463C in tRNA^{Arg}) both in their patient cohort and our family (19). The T haplogroup-specific G8697A substitution coexisted in one of their patients with the T10463C non-synonymous SNP (19). Similar coexistence could be observed in our patients. We suppose that the synergistic effect of these sequence variants may have pathogenic potential in sensorineural hearing loss.

In summary, we conclude that the clinical symptoms of this Hungarian family are the result of the novel A8332G heteroplasmic mutation and the synergistic effect of some non-synonymous substitutions (A8347C, T10463C and C14520G).

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