

## RESEARCH NOTE

# Compound mitochondrial DNA mutations in a neurological patient with ataxia, myoclonus and deafness

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## Introduction

Mitochondrial diseases caused by mitochondrial dysfunction are, clinically and genetically, a very heterogeneous group of disorders. Most mitochondrial diseases involve multiple organs, particularly tissues with high-energy demand, e.g., nerve and muscle. Ataxia, myoclonus and deafness (AMDF), also called May–White syndrome, is one of these clinically and genetically heterogeneous mitochondrial neuropathic disorders. It also frequently exhibits multi-organ involvement and overlapping of symptoms with other mitochondrial diseases, e.g. sensorineural deafness, mental retardedness, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), and myoclonic epilepsy with ragged-red fibers (MERRF). The present study used whole mitochondrial DNA (mtDNA) sequencing to determine underlying causes of a severe AMDF polyneuropathy patient with additional symptoms of dysarthria.

AMDF was first described as a syndrome of familial myoclonus, cerebellar ataxia and deafness with autosomal dominant inheritance by May and White (1968). Thereafter, maternally inherited AMDF has also been reported (Vaamonde *et al.* 1992; Tiranti *et al.* 1995; Jaksch *et al.* 1998). Mutations in several mtDNA genes have been reported to be associated with the mitochondrially inherited AMDF or sensorineural deafness: *tRNA-Ser(UCN)* (Tiranti *et al.* 1995), *12S rRNA* (Prezant *et al.* 1993), *CO1* (Pandya *et al.* 1999), *tRNA-Val* (Tiranti *et al.* 1998), *tRNA-His* (Crimi *et al.* 2003), *ND1* (Leveque *et al.* 2007) and *tRNA-Ile*.

In the present study, we examined a Korean AMDF patient whose parents and siblings were unaffected. We determined the nucleotide sequences of complete mtDNA and several nuclear genes of the patient, from which two causative mutations in the *12S rRNA* and *tRNA-Ser(UCN)* genes, and a putative secondary genetic cause in the *CO2* gene were identified as the underlying cause or association factor of the clinical phenotype.

## Materials and methods

### Subjects

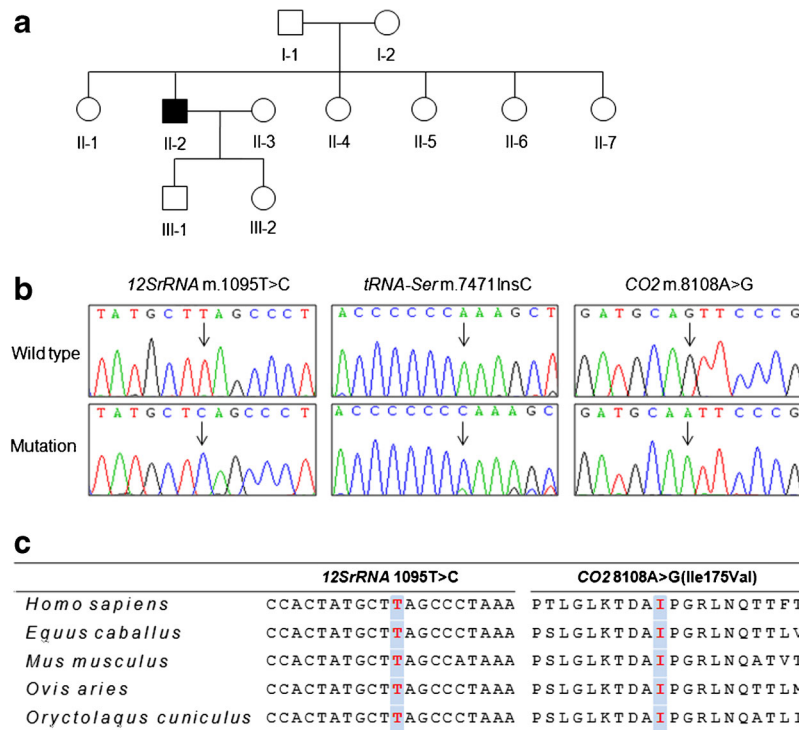
This study examined a Korean AMDF patient with additional symptoms of dysarthria and sensorimotor neuropathy (figure 1a). Paternity was confirmed by genotyping of 15 microsatellites using the PowerPlex 16 System (Promega, Madison, USA). This study also included 300 healthy control individuals. All participants provided written informed consent according to the protocol approved by the ethics committee of Ewha Womans University School of Medicine.

### Clinical and electrophysiological assessments

Motor nerve conduction velocities (MNCVs) of the median and ulnar nerves were determined by stimulating at the elbow and wrist, while recording compound muscle action potentials (CMAPs) over the abductor pollicis brevis and adductor digiti quinti, respectively. In the same way, the MNCVs of peroneal and tibial nerves were determined by stimulating the knee and ankle, while recording CMAPs over the

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**Figure 1.** Pedigree and sequencing analysis of an AMD patient. (a) Pedigree of the patient's family (family id: MT106). The open symbols represent unaffected individuals and filled symbol represents affected individuals. (b) Sequencing chromatograms of three mtDNA mutations: m.1095T>C in 12S rRNA, m.7471insC in tRNA-Ser(UCN), and m.8108A>G (Ile175Val) in CO2 shown in the patient (II-2). Vertical arrows indicate the mutation sites. (c) Conservation of nucleotides or amino acids in the mutation regions. Multiple alignments revealed that two mutation sites are highly conserved among different species (*H. sapiens*: NC\_012920.1, YP\_003024029.1; *E. caballus*: NC\_001640.1, NP\_007163.1; *M. musculus*: NC\_005089.1, NP\_04331.1; *O. aries*: NC\_001941.1, NP\_008409.1; and *O. cuniculus*: NC\_001913.1, NP\_007552.1).

extensor digitorum brevis and adductor hallucis, respectively. Sensory nerve conduction velocities (SNCVs) and sensory nerve action potentials (SNAPs) were obtained over a finger-wrist segment from the median and ulnar nerves by orthodromic scoring. Magnetic resonance imaging (MRI) of the brain was obtained using a 3.0-T system (Achieva, Philips, Best, The Netherlands).

#### Molecular genetic studies

Total DNA was extracted from whole blood samples using a QIAamp Blood DNA mini kit (Qiagen, Hilden, Germany). The mtDNA was completely sequenced by PCR by using 46 primer sets of the MitoSeqr resequencing system (Life Technology, Foster City, USA). The PCR condition consisted of initial denaturation at 96°C for 5 min, followed by 32 cycles at 94°C for 30 s, 60°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 10 min. PCR products were sequenced by an automatic genetic analyzer (ABI3130XL, Life Technology) using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Life Technology). The mtDNA sequences were compared with the revised Cambridge reference sequence (NC\_012920.1) using a SeqScape software ver. 2.1 (Life Technology). The coding exons of the *PEO1*, *TYMP*, *ANT1*, *POLG1*, *POLG2*,

*DGUOK* and *TK2* nuclear genes, which have been reported to be involved with nuclear-mtDNA intergenomic disorders, were also amplified by the PCR method. PCR products were sequenced by the same method of mtDNA analysis. The primer sequences and PCR conditions used are available on request from the corresponding author.

#### Determination of mtDNA heteroplasmy

The mtDNA fragments obtained by PCR amplification were cloned into the pGEM-T Easy vector (Promega), which was then used to transform *Escherichia coli* (DH5α). Plasmid DNA was isolated from about 100 colonies per mutation using a Plasmid DNA Isolation Kit (SolGent, Daejeon, Korea), and the mutation was determined by sequencing the insert DNA. The mutation load was measured by counting the clones those had a mutant allele.

#### Haplogrouping, in silico and conservation analysis

The mtDNA haplogroup was determined by the HaploGrep (<http://haplogrep.uibk.ac.at/>) and the mtDNAMANAGER (<http://mtmanager.yonsei.ac.kr/>) programs. Multiple alignments of amino acid sequences among different species were

**Table 1.** Nerve conduction studies of the AMDF patient.

	Patient (II-2)	Normal value
Age at examination (years)	57	
Median motor nerve		
TL (ms)	3.8	< 3.9
CMAP (mV)	11.6	> 6.0
MNCV (m/s)	52.2	> 50.5
Ulnar motor nerve		
TL (ms)	2.4	< 3.0
CMAP (mV)	11.3	> 8.0
MNCV (m/s)	52.7	> 51.1
Peroneal nerve		
TL (ms)	3.4	< 5.3
CMAP (mV)	4.0	> 1.6
MNCV (m/s)	<b>39.5</b>	> 41.2
Tibial nerve		
TL (ms)	5.1	< 5.4
CMAP (mV)	12.6	> 6.0
MNCV (m/s)	<b>38.6</b>	> 41.1
Median sensory nerve		
SNAP ( $\mu$ V)	<b>5.0</b>	> 8.8
SNCV (m/s)	<b>34.7</b>	> 39.3
Ulnar sensory nerve		
SNAP ( $\mu$ V)	<b>6.7</b>	> 7.9
SNCV (m/s)	<b>22.4</b>	> 37.5
Sural nerve		
SNAP ( $\mu$ V)	<b>2.5</b>	> 6.0
SNCV (m/s)	<b>28.0</b>	> 32.1

Bold values indicate abnormal values. Nerve conduction studies were done on right side of the patient. TL, terminal latency; CMAP, compound muscle action potential; MNCV, motor nerve conduction velocity; SNAP, sensory nerve action potential; SNCV, sensory nerve conduction velocity.

performed using the MEGA5 program ver. 5.05 by exerting the ClustalW algorithm (Thompson *et al.* 1994).

## Results

### Clinical and electrophysiological features

The patient (57-year-old man) was initially admitted to Ewha Womans University Mokdong Hospital with a progressive ataxia. Ten years ago, he noticed an ataxia, myoclonus in

both upper and lower extremities, and increasing gait imbalance. At the age of 52 years, he had bilateral deafness, and revealed dysarthria. During the past four years, he experienced jerking of his arms when engaged in purposeful movements. At 56 years, he was not able to walk without cane. However, he did not reveal muscle atrophy.

On neurological examination, he showed marked gait and limb ataxia, negative myoclonus on both arms, touch-sensitive myoclonus on both legs, bilateral sensorineural hearing loss and dysarthria. Also, he had muscle weakness on ankle dorsiflexion (G4/G4), and impaired heel gait. He had mild distal loss of vibration sense in the distal lower limbs. Stretch reflexes were decreased in the upper extremities, and abolished in the lower extremities. Audiometry confirmed bilateral sensory hearing loss. There was no sign of retinal or pyramidal degeneration. His other family members, including his parents, siblings, and progenies revealed no symptoms of the disease.

The neuroelectrophysiological findings are shown in table 1. MNCVs were diminished in the peroneal and posterior tibial nerves. SNCVs and SNAPs were diminished in all tested nerves including the median, ulnar and sural nerves. Evoked somatosensory potentials showed a prolonged latency of cortical potentials. Electroencephalography showed frequent spike discharges in the occipital areas. His brain MRI revealed no atrophy of the cerebellar hemispheres.

### Identification of several pathogenic mutations in mtDNA

Complete mtDNA sequencing (GenBank acc. no.: KC709481) revealed three specific mutations: m.1095T>C in *12S rRNA*, m.7471insC in *tRNA-Ser(UCN)*, and m.8108A>G (Ile175Val) in *CO2* genes (table 2; figure 1b). These three mutations were not found in 300 controls, and the mutation sites are highly conserved among different species (figure 1c). The three mutations have been reported in human mitochondrial genome database (<http://mitomap.org/MITOMAP>). The m.7471insC in *tRNA-Ser(UCN)* exhibited heteroplasmy with a mutation load of 85.7%; however,

**Table 2.** MtDNA mutations specific to the MT106 patient.

Gene	Mutation			Reference
	Nt change <sup>a</sup>	AA change	Heteroplasmy (%)	
<i>12S rRNA</i>	m.1095T>C	—	100	Tessa <i>et al.</i> (2001) Zhao <i>et al.</i> (2004) Wang <i>et al.</i> (2005)
<i>tRNA-Ser(UCN)</i>	m.7471insC	—	85.7	Jaksch <i>et al.</i> (1998) Verhoeven <i>et al.</i> (1999) Hutchin <i>et al.</i> (2001)
<i>CO2</i>	m.8108A>G	Ile175Val	100	Wang <i>et al.</i> (2005)

<sup>a</sup>Nt numbers are from the revised Cambridge reference sequence (NC\_012920.1).

the other two mutations were homoplasmic. DNA testing was done only for the patient, since other familial members refused sampling for DNA analysis.

The m.7471insC (also reported as m.7472insC) has been reported to be associated with the AMDF phenotype (Tiranti et al. 1995; Jaksch et al. 1998; Toompuu et al. 2002; Cardaioli et al. 2006; L  v  que et al. 2007). The m.1095T>C in *12S rRNA* has been reported to be associated with maternally inherited sensorineural deafness (SNHL) (Thyagarajan et al. 2000; Tessa et al. 2001). In contrast, it was also suggested that this might be a haplogroup M11 specific variation, rather than being pathogenic (Yao et al. 2006). The m.8108A>G (Ile175Val) in *CO2* was identified in an auditory neuropathy patient concurrent with the 1095T>C mutation (Wang et al. 2005). Neither large deletion of mtDNA nor causative mutations in nuclear genes was observed.

The mtDNA sequencing analysis identified a total of 45 variations in the mtDNA of the MT106 patient (see table 1 in electronic supplementary material at <http://www.ias.ac.in/jgenet/>). Except for the above mentioned three mutations, all other variants were not considered as the genetic cause of the AMDF, because they have been reported as polymorphisms in human mitochondrial genome databases (<http://mitomap.org/MITOMAP> or <http://www.mtddb.igp.uu.se>). The phylogenetic analysis assigned the patient's mtDNA sequence to the haplogroup M11c (figure 1 in electronic supplementary material).

## Discussion

The multi-organ involvement and overlapping symptoms for mitochondrial diseases make exact diagnosis difficult. Therefore, mutation screening is an important method for the diagnosis of rare mitochondrial disorders. In this study, we identified three mtDNA mutations which seemed to be associated with the AMDF phenotype. These mutations were not found in healthy controls, and these mutation sites are highly conserved among different species. The m.1095T>C in *12S rRNA* and m.7472insC in *tRNA-Ser(UCN)* have frequently been reported to be associated with AMDF or mitochondrially inherited sensorineural deafness (Jaksch et al. 1998; Verhoeven et al. 1999; Hutchin et al. 2001; Tessa et al. 2001; Zhao et al. 2004; Wang et al. 2005). Therefore, we believe that the AMDF phenotype was caused by these two mutations. Although direct evidence of *CO2* involvement was not provided, the m.8108A>G in *CO2* has also been proposed to be related to deafness as a secondary factor (Wang et al. 2005). The *12S rRNA* and *tRNA-Ser(UCN)* genes are known to be mutational hotspots for sensorineural hearing loss, since several deafness-associated mtDNA mutations have been identified in these genes (Jaksch et al. 1998; Verhoeven et al. 1999).

The mtDNA haplotype analysis of six Western European families with the m.7471insC mutation in *tRNA-Ser(UCN)* were assigned to haplogroups H (four families), K and V (Hutchin et al. 2001). Since haplogroup H is the most

common European haplogroup (approximately 40%), the m.7471insC mutation seems not to be linked to haplogroup H in the European population. In our case, the haplogroup was determined to be M11c. Haplogroup M is frequently seen in the Korean population.

We suggest that compound mutations of *12S rRNA* and *tRNA-Ser(UCN)* played a major role in the development of this AMDF disease, and that the m.8108A>G mutations in *CO2* may function as secondary genetic factors. This study may be the first report linking the phenotype of severe AMDF patients with complex pathogenic mtDNA mutations.

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