Short Communication

Copper Toxicity Associated With an *ATP7A*-Related Complex Phenotype

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ABSTRACT

Background: The *ATP7A* gene encodes a copper transporter whose mutations cause Menkes disease, occipital horn syndrome (OHS), and, less frequently, *ATP7A*-related distal hereditary motor neuropathy (dHMN). Here we describe a family with OHS caused by a novel mutation in the *ATP7A* gene, including a patient with a comorbid dHMN that worsened markedly after being treated with copper histidinate.
Methods: We studied in detail the clinical features of the patients and performed a genomic analysis by using TruSight One Expanded Sequencing Panel. Subsequently, we determined the ATP7A and ATP7B expression levels, mitochondrial membrane potential, and redox balance in cultured fibroblasts of Patient 1.
Results: We found a novel *ATP7A* late truncated mutation p.Lys1412AsnfsX15 in the two affected members of this family. The co-occurrence of OHS and dHMN in Patient 1 reveals the variable phenotypic expressivity of the variant. A severe clinical and neurophysiologic worsening was observed in the dHMN of Patient 1 when he was treated with copper replacement therapy, with a subsequent fast recovery after the copper histidinate was withdrawn. Functional studies revealed that the patient had low levels of both ATP7A and ATP7B, the other copper transporter, and high levels of superoxide ion in the mitochondria.

Conclusions: Our findings broaden the clinical spectrum of *ATP7A*-related disorders and demonstrate that two clinical phenotypes can occur in the same patient. The copper-induced toxicity and low levels of both ATP7A and ATP7B in our patient suggest that copper accumulation in motor neurons is the pathogenic mechanism in *ATP7A*-related dHMN.

Introduction

ATP7A is a transmembrane protein that (1) exports copper across the plasma membrane to maintain intracellular copper homeostasis and (2) provides copper to copper-dependent enzymes in the trans-Golgi network.[1] Mutations in the *ATP7A* gene may lead to three distinct X-linked recessive clinical phenotypes: Menkes disease (MD, MIM #309400), occipital horn syndrome (OHS, MIM #304150), and *ATP7A*-related distal hereditary motor neuropathy (dHMN, MIM #300489). The dHMN phenotype is a pure motor neuropathy in which, unlike MD and OHS, the copper-related biochemical parameters are normal.[2,3] Although copper is relevant to maintain the integrity of motor neurons (MNs), the precise mechanisms by which dysfunctional ATP7A leads to the specific length-dependent axonal degeneration observed in MNs of patients with *ATP7A*-related dHMN remain unknown.

Here we present a family with OHS due to a novel *ATP7A* late truncating variant. In addition to OHS, the older patient has a dHMN that severely worsened while treated with copper replacement therapy, which may be key to understanding the pathogenic mechanism in *ATP7A*-related dHMN.

Patient Descriptions

Patient 1 is a 24-year-old male, the maternal uncle of Patient 2. He had experienced gait difficulties since age three years along with learning difficulties and symptoms of dysautonomia (syncope related to orthostatic hypotension and temperature instability). Physical examination at age 10 years revealed a high forehead, large ears, pectus excavatum, and normal hair (Fig 1A). Trichoscopy was not performed. His skin was lax and hyperextensible, and his joints were hypermobile. Distal muscle weakness in his lower limbs was detected (tibialis anterior: Medical Research Coucil scale grade 4-/5). Serum copper levels were 313 µg/L (normal range: 620 to 1544 µg/L) and ceruloplasmin level was 103 mg/L (normal range: 170 to 460 mg/L). Occipital exostoses at the tendinous insertions of the sternocleidomastoid and trapezius muscles were observed in skull radiographs (Fig 1F). The brain angio-magnetic resonance imaging showed that anterior and middle cerebral arteries were elongated and tortuous (Fig 1B). The first electroneurography test (ENG), performed when the patient was aged 10 years, showed reduced amplitude of the compound motor action potentials (CMAPs) with normal velocity, whereas sensory nerve conductions were normal. Positive waves and fibrillations were observed via electromyography (EMG). All these findings confirmed an axonal neuropathy predominantly affecting motor nerves (Table). A frameshift variant *ATP7A* c.4236delA; p.Lys1412AsnfsX15 that causes a truncated peptide that preserves all ATP7A domains except the carboxyl terminal (Fig 1G) was detected after genomic DNA was analyzed using the TruSight One Expanded Sequencing Panel in a NextSeq 500 platform (Illumina, San Diego, California, USA). The unaffected mother was heterozygous for the variant. No pathogenic variants were identified in any of the other known causative genes for inherited neuropathies.

Patient 2 is a 10-year-old boy, son of Patient 1's sister. Examination at age 10 months showed global hypotonia, down-slanting eyes, internal epicanthus, prominent large ears, hypermobile joints, lax skin, and hair that was hypopigmented, dull, and friable (Fig 1D). Pili torti was observed through optic microscopy (Fig 1C). Mild motor delay was detected. Serum copper and ceruloplasmin were in the normal range (1123 µg/L [normal range: 620 to 1544 µg/L] and 225 mg/L [normal range: 220 to 470 mg/L], respectively). Vascular tortuosity and dilatation of the Willis polygon and basilar and vertebral arteries were observed in a cerebral magnetic resonance imaging (Fig 1E). His ENG/EMG evaluation was normal, with peroneal CMAP and nerve conduction velocity within the normal range (2.6 mV and 55.4 m/s, respectively). It is not known if Patient 2 had occipital exostoses because a skull x-ray was not performed.

Because some patients with MD had proven to be responsive to early copper replacement therapy[4,5] and since copper supplementation may be potentially beneficial in OHS,[6] we treated Patient 1 with copper histidinate at age 15 years, after having obtained an informed consent from the family. Doses of 20 µg/kg were administered three times per week and increased to four times per week after serum levels of copper and ceruloplasmin were tested. The treatment was withdrawn after four months due to a marked worsening of the motor symptoms, with increased distal muscular weakness in all four limbs. His gait worsened considerably, and significant difficulties to perform daily activities appeared. Actions such as pulling out a book from his bag, writing, or picking up a glass of water became very difficult for him, if not impossible. The ENG/EMG evaluation was repeated after four months of treatment, and it demonstrated a lower CMAP and clear signs of denervation (Table). Once the copper histidinate treatment was withdrawn, the adverse side effects disappeared progressively and rapidly until the functional status of the patient was completely recovered. Four months after the copper was withdrawn, the neurophysiologic evaluation showed similar results to those obtained in the ENG/EMG performed before the start of treatment (Table).

Functional studies

Because ATP7A and ATP7B are two copper transporters that act in a coordinated manner in neurons,[7] we quantified their levels in fibroblasts of Patient 1 and compared them with the levels in fibroblasts of two patients with the main inborn errors of copper metabolism: MD and Wilson disease (WD), as well as the levels in fibroblasts of control individuals (Fig 2A and B). ATP7A showed small size and was poorly expressed in Patient 1 fibroblasts, absent in MD fibroblasts, and normal in controls and WD fibroblasts. Intriguingly, ATP7B was also significantly lower in Patient 1 than in the other individuals. Exome re-analysis of the *ATP7B* gene did not detect any pathogenic variants in Patient 1, which suggests that this lower level of ATP7B could be related to the low expression of ATP7A[Lys1412AsnfsX15].

As ATP7A is essential for redox balance in mitochondria, we quantified the levels of mitochondria superoxide (MitoSOX Red
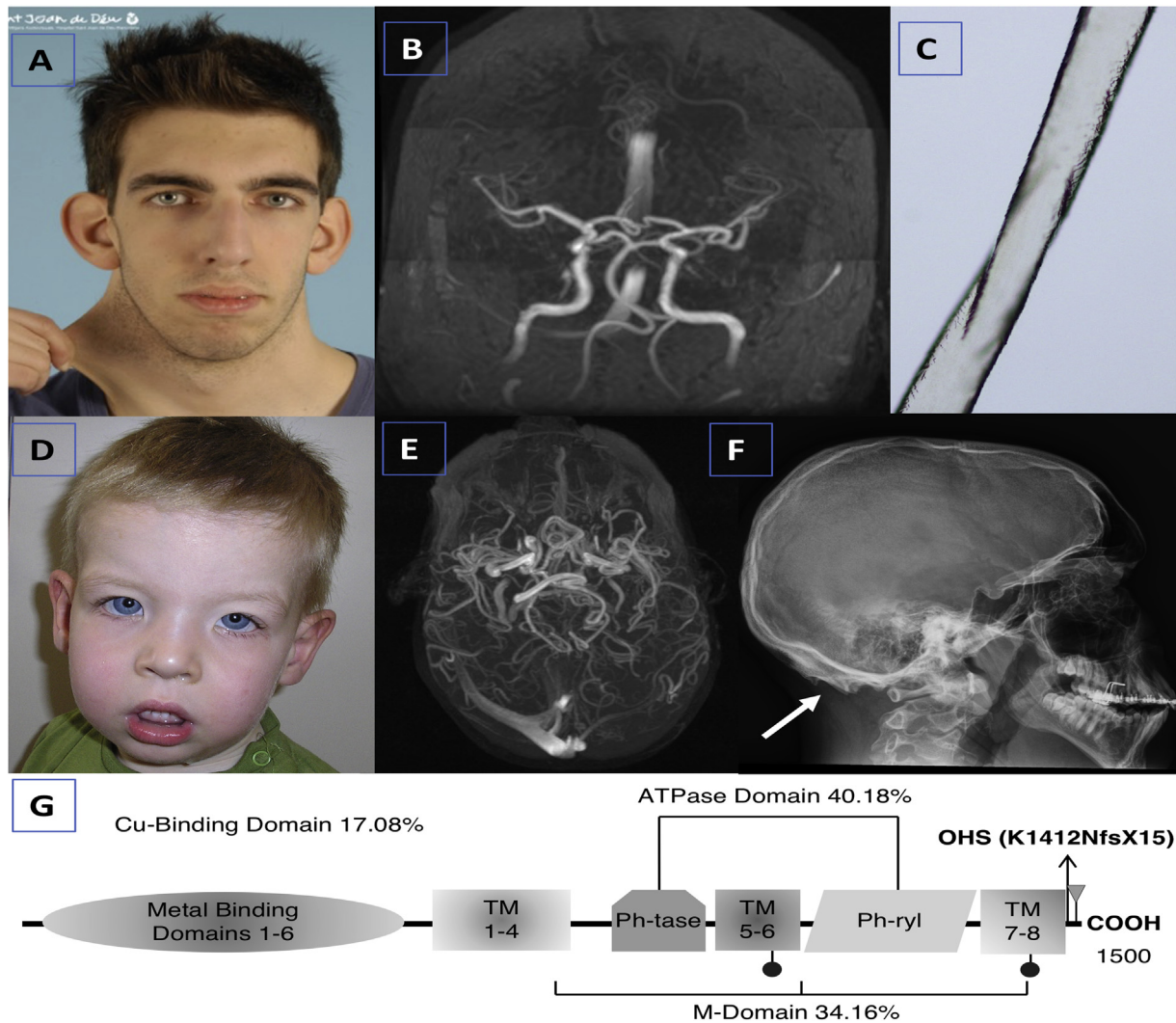
FIGURE 1. (A) Frontal view of Patient 1 at age 16 years. Note the large ears and lax and hyperextensible skin. (B, E) Vascular tortuosity and dilatation of the Willis polygon and basilar and vertebral arteries were observed in the cerebral angio-magnetic resonance imaging of both Patient 1 (B) and Patient 2 (E). (C) *Pili torti* of Patient 2 was revealed through optical microcopy. (D) Frontal view of Patient 2 at age three years. Note down-slanting eyes, internal epicanthus, prominent large ears, and hypopigmented, dull, and friable hair. (F) Typical occipital exostoses at the tendinous insertions of the sternocleidomastoid and trapezius muscles were observed in lateral skull radiograph of Patient 1. (G) ATP7A structure (NP_000043.4). Note the location of the novel p.Lys1412AsnfsX15 mutation, as well as those previously reported missense mutations in patients with CMT phenotype (black dots). TM, transmembrane domains; Ph-tase, phosphatase domain; Ph-ryl, phosphorylation domain. The color version of this figure is available in the online edition.

reagent, Molecular Probes) and membrane potential (tetramethylrhodamine methyl ester perchlorate, Sigma-Aldrich) in patients' fibroblasts using flow cytometry. We observed high levels of superoxide ion in the mitochondria of all patients' fibroblasts (Fig 2C). In contrast, membrane potential was only affected in MD fibroblasts (Fig 2D). Further characterization of fibroblasts' redox status using glutathione quantification revealed normal levels of both reduced glutathione (GSH) and oxidized glutathione (GSSG) (GSH/GSSG-Glo Assay, Promega) in fibroblasts of Patient 1 and the patient with WD, whereas the patient with MD showed significantly higher levels of GSSG (Fig 2E and F). Collectively, these data showed important differences in both the correlation of ATP7A/ATP7B expression and in oxidative status across patients with OHS, MD, and WD.

Discussion

Here we describe two related patients with OHS, one of them with comorbid dHMN. Both patients carry the frameshift mutation p.Lys1412AsnfsX15 in *ATP7A*, which affects the last intracytoplasmic ATP7A domain. Unlike other truncating *ATP7A* variants, which cause MD, late truncating variants located at the C-t lead to an OHS phenotype by removing the distal di-leucine motif $L^{1477}L^{1478}$.[8] The lack of this motif, which is an endocytic signal for ATP7A recycling between the trans-Golgi network and the plasma membrane, leads to a truncated ATP7A predominantly located at the plasma membrane.[9] The resulting ATP7A$^{p.Lys1412AsnfsX15}$ shortage at the trans-Golgi network would limit the copper supply to the copper-dependent enzymes lysyl oxidase and dopamine-ß-hydroxylase, eventually leading to the typical signs of OHS. The finding of pili torti in Patient 2, with normal serum copper and ceruloplasmin, would also be explained by the intracellular mislocation of ATP7A. Pili torti structure is caused by malformation of keratin due to lack of sulfhydryl oxidase.[10] Sulfhydryl oxidase typically functions in the endoplasmic reticulum, catalyzed by copper, to promote disulfide bond-forming reactions, which are necessary for keratin cross-linking. The prevalence of pili torti in patients with OHS is still unknown. On the other hand, normal

TABLE.
Patient 1 Neurophysiologic Examinations

Biochemical and Neurophysiological Parameters	Before Cu Therapy (Basal Values)	During Cu Therapy (4 Months on Treatment)	After Cu Therapy (4 Months Without Treatment)
Copper serum levels (μg/L)			
Normal range (620-1544 μg/L)	313	604	359
Nerve conduction study			
R peroneal nerve CMAP (mV)	0.6	0.3	0.6
R peroneal NCV (m/s)	47.3	35.8	34.5
L suralis SNAP (μV)	9.1	6.2	2.2
L suralis sensory NCV (m/s)	56.7	48.2	62.5
EMG study			
Fibrillations	2/10	2/10	NP
Positive waves	2/10	2/10	NP
Amplitude	++	++	NP
Duration	++	+++	NP
Firing	-	–	NP

Abbreviations:
CMAP = Compound motor action potential
Cu = Copper
L = Left
NCV = Nerve conduction velocity
NP = Not performed
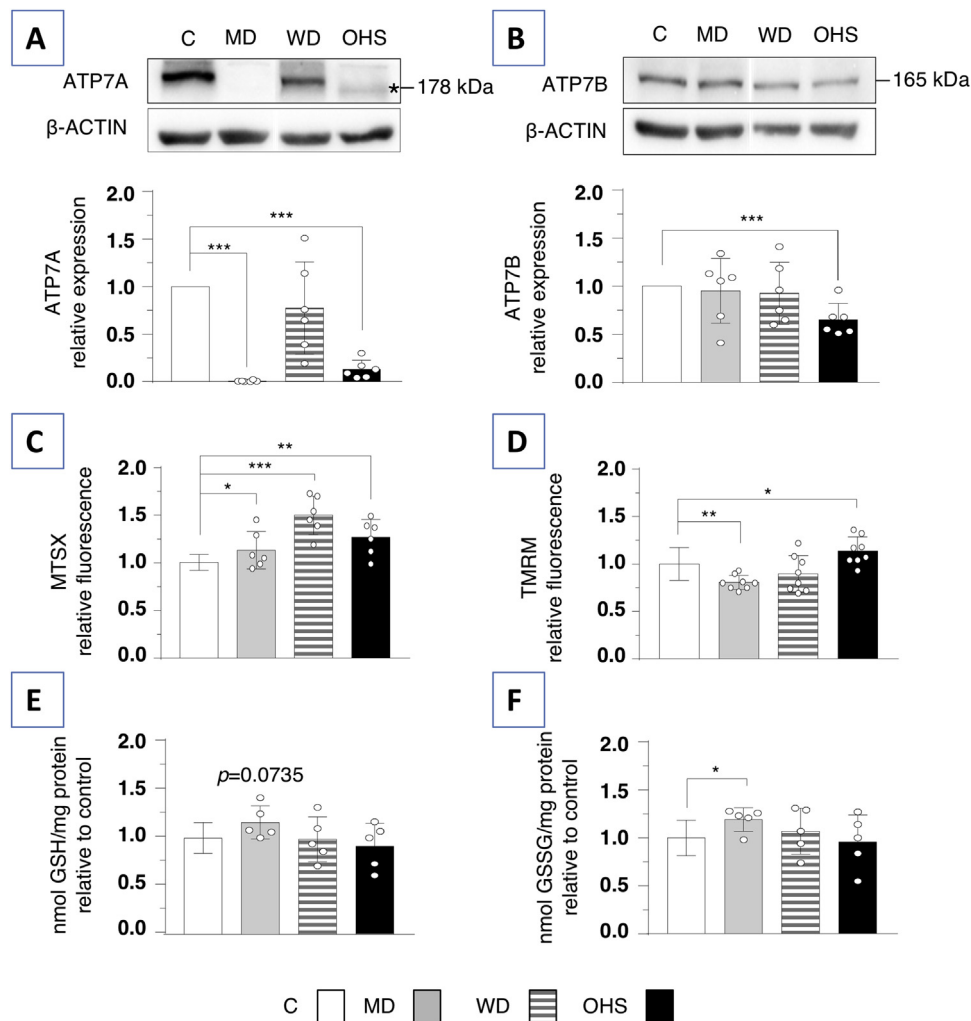R = Right
SNAP = Sensory nerve action potential

FIGURE 2. Functional studies of mitochondria and oxidative stress parameters in fibroblast. (A, B) Western blot and quantification of ATP7A (A) and ATP7B (B) in fibroblast. (C, D) Quantification of MitoSOX (MTSX) and TMRM staining to detect mitochondrial superoxide and mitochondrial membrane potential, respectively. (E) Reduced (GSH) and (F) oxidized (GSSG) glutathione (nmol/mg protein). Data are means + SEM from 6 independent experiments related to three control samples. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. MD, Menkes disease (*ATP7A* genotype: p.Arg980fs); WD, Wilson disease (*ATP7B* genotype: p.Met645Arg/p.His1069Glu); OHS, occipital horn syndrome (*ATP7A* genotype: p.Lys1412AsnfsX15).

serum values of copper and ceruloplasmin have been reported in 29% and 26% of patients with OHS, respectively.[11] This variability could be due to differences in the reduced amount of ATP7A variants and in their location, which could prevent the reduction of these biomarkers in some patients, in contrast to what is observed in the total absence of ATP7A in Menkes disease. In our family, the differences in copper management between Patients 1 and 2, although they are uncles and nephews, may be related to individual genomic background.

The three missense variants in transmembrane domains that have been previously documented in patients with *ATP7A*-related dHMN also results in a preferential location of ATP7A at the plasma membrane.[12] Therefore the accumulation of ATP7A at the plasma membrane seems a common pathophysiologic mechanism to explain the unique association of OHS and dHMN observed in Patient 1. Moreover, one of the previously reported dHMN-causing variants (ATP7A^{Pro1386Ser}) has been associated with reduced calcium (Ca^{2+}) removal and synapses recovery in NSC-34 cells.[13] Since mitochondria contribute to Ca^{2+} buffering and energy production at synapses,[14] it could be hypothesized that *ATP7A*-related dHMN variants impair mitochondrial transport, similar to what occurs in *MFN2*-related Charcot-Marie-Tooth disease.[15] Putative mislocation of ATP7A at the plasma membrane would cause a decrease in the number of mitochondria at synapses, leading to synaptic dysfunction and neuropathy. Our comparison of the mitochondrial and cellular oxidative state in patients with MD and OHS supports the idea that impaired mitochondrial mobility, and not oxidative stress, would be the cause of dHMN in Patient 1.

Last, the finding of marked worsening of neuropathy in Patient 1 after copper supplementation could be also explained by the impaired trafficking of ATP7A, which would be unable to properly remove the excess of copper[16,17] and would eventually lead to a copper accumulation in MNs and axons, which is a known pathogenic mechanism in neurodegenerative disorders. Patient 1, who carries the p.Lys1412AsnfsX15 variant in *ATP7A* and has low levels of the other copper transporter ATP7B, would have a copper homeostasis defect in MNs that would have eventually resulted in both dHMN and toxicity after copper supplementation. In neurons, ATP7B is found in vesicles distributed throughout the cytosol where it sequesters copper and regulates the amount of cytosolic copper available for ATP7A.[7] Further studies of the ATP7A/ATP7B ratio in additional patients' samples are necessary to better understand the biology of the functional relationship of these copper transporters. In any case, we propose that Patient 1 has a global defect in the regulation of intracellular copper levels that causes this particular OHS phenotype and eventually leads to axonal neuropathy that is highly susceptible to copper-induced toxicity. In contrast, we found in MD a total absence of ATP7A and normal levels of ATP7B. These differences in ATP7A/ATP7B ratio in MD and OHS could explain why copper replacement therapy may be beneficial in patients with MD and be toxic in patients with OHS. Therefore copper replacement therapy should be considered and monitored with caution in patients with dHMN and/or OHS according to the distinct pathophysiologic mechanisms discussed here.

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