Original Article

Molecular Diagnostics of Copper-Transporting Protein Mutations Allows Early Onset Individual Therapy of Menkes Disease

(Menkes disease / *ATP7A* / copper / cytochrome c oxidase / *ATOX1)*

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Abstract. Menkes disease is a severe X-linked recessive disorder caused by a defect in the *ATP7A* gene, which encodes a membrane copper-transporting ATPase. Deficient activity of the ATP7A protein results in decreased intestinal absorption of copper, low copper level in serum and defective distribution of copper in tissues. The clinical symptoms are caused by decreased activities of copper-dependent enzymes and include neurodegeneration, connective tissue disorders, arterial changes and hair abnormalities. Without therapy, the disease is fatal in early infancy. Rapid diagnosis of Menkes disease and early start of copper therapy is critical for the effectiveness of treatment. We report a molecular biology-based strategy that allows early diagnosis of copper transport defects and implementation of individual therapies before the full development of pathological symptoms. Low serum copper and decreased activity of copper-dependent mitochondrial cytochrome c oxidase in isolated platelets found in three patients indicated a possibility of functional defects in copper-transporting proteins, especially in the ATPA7 protein, a copper-transporting P-type ATPase. Rapid mutational screening of the *ATP7A* gene using high-resolution melting analysis of DNA indicated presence of mutations in the patients. Molecular investigation for mutations in the *ATP7A* gene revealed three nonsense mutations: c.2170C>T (p.Gln724Ter); c.3745G>T (p.Glu1249Ter); and c.3862C>T (p.Gln1288Ter). The mutation c.3745G>T (p.Glu1249Ter) has not been identified previously. Molecular analysis of the *ATOX1* gene as a possible modulating factor of Menkes disease did not reveal presence of pathogenic mutations. Molecular diagnostics allowed early onset of individual therapies, adequate genetic counselling and prenatal diagnosis in the affected families.

Introduction

Menkes disease (MD, OMIM 309400) is a severe, recessive, X-linked disorder caused by mutations in the *ATP7A* gene (Menkes et al., 1962). The *ATP7A* gene spans a genomic region of about 140 kb and is located on chromosome Xq13. Its 8.5 kb transcript encodes a protein of 1500 amino acids (ATPase ATP7A or Menkes protein) and is expressed in all tissues except the liver (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). This 165 kDa membrane protein is essential for transport of copper in cells because it controls the export of copper from non-hepatic tissues. ATP7A also transports copper into the secretory pathway, where it can be incorporated into enzymes. Several proteins including ATOX1 and adaptor protein complexes have been identified to be also involved in copper transport in cells (Yi and Kaler, 2015). At normal copper concentrations, ATP7A is localized to the *trans*-Golgi network; at increased copper levels, the protein is transferred to the plasma membrane (Fig. 1) (Petris et al., 1996).

In its organization and structure, Menkes protein is very similar to Wilson protein (ATP7B), which plays an

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Abbreviations: CP – ceruloplasmin, COX – cytochrome c oxidase, CS – citrate synthase, MD – Menkes disease, MLPA – multiplex ligation-dependent probe amplification, NCCR – cytochrome c reductase (rotenone sensitive), NQR – coenzyme Q reductase (rotenone sensitive), QCCR – cytochrome c reductase, SQR – succinate: coenzyme Q reductase.

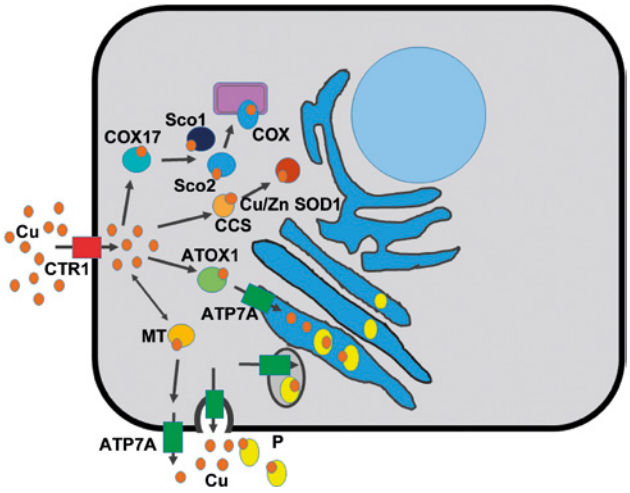


Fig. 1. Copper transport in the cell
Cu^{+} – copper ions; COX – cytochrome c oxidase; P – protein produced in ER pathway (e.g. ceruloplasmin); MT – metallothionein; Cu/Zn SOD1 – Cu/Zn superoxide dismutase 1; CTR1 – copper transporter 1; ATP7A – Menkes protein ATP7A; COX17, Sco1, Sco2, CCS, ATOX1 are metallochaperones (Lutsenko and Petris, 2003).

analogous role in hepatic tissue (Yamaguchi et al., 1993; Tumer et al., 1995; Camakaris et al., 1999). The results of genetic defects in ATP7A are impaired intestinal absorption of copper, low copper levels in the plasma, impaired distribution of copper in tissues and decreased function of important copper-dependent enzymes such as ceruloplasmin or cytochrome c oxidase, which serves as the terminal complex of the mitochondrial respiratory chain. Clinically, these changes manifest as neurodegeneration, connective tissue disorders, arterial abnormalities and grey hair. If there is some residual activity of Menkes protein, the phenotype is less severe and a milder form of the disease, occipital horn syndrome, is observed (Kaler et al., 1994). Another phenotype associated with ATP7A mutations is characterized by distal motor neuropathy without known copper deficiency (Kennerson et al., 2010). MD can be diagnosed based on low copper and ceruloplasmin levels in the serum and high copper levels in cultured fibroblasts (Kodama and Murata, 1999). Without treatment, most patients with MD die before the age of three years (Grover and Scrutton, 1975; Daish et al., 1978; Sarkar et al., 1993). The incidence of MD in Europe is about 1 in 250,000 live births (Tonnesen et al., 1991). Here we report a molecular biology-based strategy that allows early diagnosis of copper transport defects and implementation of individual therapies.

Material and Methods

Patients

All probands with suspected MD were born at term after an uncomplicated pregnancy and delivery. Their early postnatal adaptation was uneventful. Their parents were healthy and their families were not related. The suspected diagnosis of MD was established based on clinical signs and symptoms and biochemical parameters before *ATP7A* gene mutation analyses.

Patient 1

The boy developed central hypotonic syndrome and sporadic seizures since the second month of life. At the age of three months, he had failure to thrive, EEG was pathological with epileptic grapho-elements, and CT scan and brain angiography revealed periventricular, subcortical and stem atrophy and tortuous cerebral arteries. Microscopy examination of hair revealed kinky hair. The blood levels of ceruloplasmin (0.03 g/l, reference range 0.2–0.6) and serum copper (2.1 µmol/l, reference range 14–24) were low. The activities of respiratory chain complex IV (cytochrome c oxidase, COX) and the ratio between COX and control enzyme citrate synthase (CS) were decreased in isolated platelets. During intravenous therapy with copper sulphate (50–150 µg/kg/day), the levels of the copper (16 µmol/l, reference range 14–24) and ceruloplasmin (0.24 g/l, reference range 0.2–0.6) increased and the activity of COX and the COX/CS ratio had normalized. However, despite normalization of biochemical parameters, the boy did not improve clinically and died at the age of 13 months.

Patient 2

The boy developed early onset of central hypotonia. Brain sonography at the age of six months revealed subdural hygroma and the boy was treated at the department of child neurosurgery. At the age of one year, he was hypotrophic with remarkably hypopigmented and loose skin, particularly on the neck and abdomen, his hair was very subtle and grey. He had hypotonic syndrome with acral spasticity, brain atrophy and abnormal EEG. The blood levels of ceruloplasmin (0.14 g/l, reference range 0.2–0.6) and serum copper (4.3 µmol/l, reference range 14–24) were low. The activities of respiratory chain complex IV (cytochrome c oxidase, COX) and the ratio between COX and control enzyme CS were decreased in isolated platelets. Following parents' decision, the therapy with copper sulphate was not applied and the boy died at the age of 16 months.

Patient 3

A 6-week old boy developed tonic-clonic seizures. He had hypotonia, pale skin, sparse hair and eyebrows. Hair microscopy examination revealed kinky hair, supporting the diagnosis of MD. EEG revealed epileptic grapho-elements, and CT scan and brain angiography revealed cortical atrophy and high tortuosity of cerebral and vertebral arteries. The blood levels of ceruloplasmin (0.06 g/l, reference range 0.2–0.6) and serum copper (4 µmol/l, reference range 14–24) were low. The activity of respiratory chain complex IV (cytochrome c oxidase, COX) and the ratio between COX and control enzyme CS were decreased in isolated platelets. Although

the biochemical parameters had normalized on treatment with copper sulphate (Table 2c), his clinical condition did not improve and the boy died at the age of three years.

Bioinformatics

The *ATP7A* gene sequence was retrieved from the NCBI database (Accession number NM_000052) and a set of primers for 23 exons was designed according to ensembl.org (Transcript: ATP7A-201 ENST00000341514.10) and NCBI Chromosome X – NC_000023.11. Sequences of the template structures for model building (Protein databank IDs 2kij, 2kmv, 3j09, 4bbj) were aligned with the sequence of ATP7A (UniProt ID Q04656) using Expresso algorithm (Armougom et al., 2006) and the model built with program Modeller (Sali and Blundell, 1993). The structure was displayed and analysed with BrowserPro (MolSoft L.L.C., Sorrento Valley, CA).

Molecular and genetic analyses

Genomic DNA was extracted from peripheral blood cells according to the standard protocol. Primers specific for 23 exons of the *ATP7A* gene were used to amplify the coding regions and exon/intron boundaries (Yi and Kaler, 2015). PCR amplification was performed in a total volume of 25 µl containing 50 mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 200 nM dNTP, 2 U Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO) and 0.4 mM each primer. PCR conditions were as follows: initial denaturation at 95 °C (2 min) followed by 30 cycles of denaturation at 95 °C (30 s), annealing at 59 °C (30 s) and extension at 72 °C (30 s) and terminated by final extension at 72 °C (3 min). All PCR products were purified from agarose gel using a QIAquick gel extraction kit (Quiagen, Hilden, Germany) and sequenced with the ABI PRISM 3100/3100 Avant Genetic Analyser (Applied Biosystems, Waltham, MA).

The restriction length fragment polymorphism assay was designed and used for each found mutation to analyse mutations in the patients' families. The *ATOX1* gene mutation screening was carried out using primers and reaction conditions as described previously (Moore et al., 2002). Mutation screening of the *ATP7A* gene was performed using the Light Scanner System (Idaho Technology Inc., Salt Lake City, UT) with high-resolution melting analysis based on differences in the melting temperatures of DNA fragments with sequence variations. Standard PCR conditions as described above were used, except that LCGreen Plus+ melting dye (Idaho Technology Inc.) was added to the reaction mixture and the number of reaction cycles was increased to 40. All samples were measured in duplicate and compared with controls using LightScanner software (Idaho Technology Inc.).

Biochemical methods

Serum ceruloplasmin levels were determined by turbidimetric analysis. The copper serum level was determined by atomic absorption spectroscopy.

Isolation of thrombocytes from peripheral blood

Plasma enriched with thrombocytes was isolated from 9 ml of non-coagulable blood (treated with sodium citrate) by centrifugation for 20 minutes at 130 *g* at 25 °C. Thrombocytes were purified by centrifugation without addition of prostacyclin (Fox et al., 1992). Fresh, non-frozen thrombocytes were used to measure the activities of respiratory chain complexes I, II and I + III; part of isolated thrombocytes was stored frozen at –80 °C and used for measurement of complex III, IV and citrate synthase activities (Srere, 1969; Rustin et al., 1994).

Enzymatic activities

Spectrophotometric determination of enzymatic activities was carried out in cuvettes with optical path length of 1 cm at 37 °C in a Shimadzu UV-2101PC double-ray spectrophotometer (Shimadzu Corp., Kyoto, Japan). The final reaction volume in the cuvette was 1 ml and all samples were measured twice. Assessments were performed using a modified procedure based on the work by Rustin (Rustin et al., 1994).

The activity of rotenone-sensitive NADH: coenzyme Q oxidoreductase (complex I) was determined by NADH oxidation at wavelength 340 nm using decylubiquinone as an electron acceptor. In order to break the mitochondrial membrane, 100 µg of thrombocytes was incubated for 3 min in hypotonic conditions of H_2O. Activity was measured in 50 mM TRIS (pH 8.1), 1 mg/ml BSA, 2 mM KCN and 1 mM NADH. The reaction was initialized by addition of 50 µM decylubiquinone. The acquired values were adjusted by subtracting the activity measured with 3 µM rotenone.

The reaction mixture for determining activity of succinate: coenzyme Q reductase (complex II) consisted of 10 mM KPi (pH 7.8), 2 mM EDTA, 1 mg/ml BSA, 200 µg thrombocytes, 3 µM rotenone, 10 mM sodium succinate, 0.2 mM ATP, 0.3 mM KCN, 80 µM DCPIP (2,6-dichlorophenolindophenol) and 1 µM antimycin. The reaction was initiated by adding 50 µM decylubiquinone. The decrease in absorbance at 600 nm as a result of DCPIP reduction was observed for 3 min. The reaction was terminated by 10 mM malonate, a complex II-specific inhibitor.

To measure the activity of NADH: cytochrome c reductase (complexes I+III), the mitochondrial membranes of 100 µg thrombocytes were disrupted by hypotonic treatment with H_2O for 3 min. The reaction mixture consisted of 50 mM TRIS (pH 8.1), 2.5 mg/ml BSA, 40 µM oxidized cytochrome c, 2 mM KCN, and the reaction was initialized by 0.1 mM NADH. The progress of the reaction was observed for 1 min as increased absorbance at 550 nm caused by reduction of oxidized cytochrome c. The acquired values were adjusted by subtracting the activity measured with 3 µM rotenone.

The activity of ubiquinol: cytochrome c reductase (complex III) was analysed in a reaction mixture of 50 mM KPi (pH 7.8), 2 mM EDTA, 1 mg/ml BSA,

0.3 mM KCN, 50 μM cytochrome c and 100 μg thrombocytes. The increase in absorbance at 550 nm caused by reduction of cytochrome c was measured for 3 min.

For measurement of the activity of cytochrome c oxidase (COX, complex IV) and citrate synthase, the thrombocytes were solubilized by incubation with 1.5% LM (n-dodecyl B-d-maltoside) for 15 min at 4 °C, then centrifuged for 10 min 3000 *g*, and the supernatant was used for the analysis. The activity of COX was determined in a reaction mixture consisting of 40 mM KPi (pH 7.0), 1 mg/ml BSA, and 10 μM reduced cytochrome c. The reaction was initiated by addition of 80 μg solubilized thrombocytes and the decrease in absorbance was measured for 1 min.

The activity of CS was determined according to Srere (Srere, 1969). The reaction mixture contained 100 mM Tris/HCl (pH 8.1), 0.1 mM DTNB (5.5'-dithio-bis(2-nitrobenzoic acid)), 80 μg thrombocytes and 0.5 mM acetyl coenzyme A. After measuring the background at 412 nm for 1 min, the reaction was initialized by addition of 0.5 mM oxaloacetate. The activity was counted after subtracting the background values. The concentration of proteins was determined by the Lowry method (Lowry et al., 1951).

Ethics

All analyses were performed according to the guidelines approved by the General Faculty Hospital Ethics Committee in Prague. Informed consent was obtained from each patient's parents and the study was carried out in accordance with the principles of the Declaration of Helsinki.

Results

Identification of patients with Menkes disease

Three patients with symptoms suspected for MD were admitted to the Metabolic Unit of the University Hospital (boys at the age of 3 months, 6 months and 6 weeks) (for details see Material and Methods). Biochemical analysis indicated changes in activities of respiratory chain complexes (Table 1) and decreased levels of serum copper and serum ceruloplasmin in all three examined probands (Table 2).

All exons of the *ATP7A* gene (Accession number NM_000052) were amplified using PCR and sequenced with their adjacent intronic regions.

The sequencing identified three different nonsense mutations: c.2170C>T (p.Gln724Ter); c.3745G>T (p.Glu1249Ter); and c.3862C>T (p.Gln1288Ter) in exon 9, 19 and 20 in patient 1, 2 and 3, respectively (Fig. 2, Fig. 3). Each of these three mutations changes an amino acid codon to a termination codon, thus leading to premature termination of the protein. The molecular model (Fig. 2) shows the position of the mutations in the 3D structure. Two of them occur in the cytosolic phosphorylation domain and one in the membrane domain. It is obvious that the protein chain termination deep inside the structure must lead to complete loss of function of ATP7A.

Analysis of inheritance

To determine the family inheritance, we introduced fast mutation screening of the *ATP7A* gene based on high-resolution melting analysis (Fig. 4). This method allowed us to quickly analyse mutations in the families with MD.

Restriction analysis in the family of patient 1 with c.2170C>T (p.Gln724Ter) revealed this mutation in the patient's mother and sister (Fig. 5).

Implementation of individual therapies

Intravenous therapy with copper sulphate (50–150 μg/kg/day) was applied in patients 1 and 3. In both patients treated with copper sulphate therapy the biochemical values improved (Table 2A and C, respectively). Patient 1 that was diagnosed at 3 months of age and the copper sulphate therapy was applied from the 2nd week of monitoring died at age 13 months, comparable to patient 2 that was diagnosed at 6 months of age and died at age 16 months with no copper therapy. Patient 3 that was diagnosed at the age of 6 weeks and treated with coper sulphate therapy from the 2nd to 16th week of monitoring survived to the age of 3 years. In contrast to biochemical values, the improvement of clinical symptoms of the disease was less obvious.

Discussion

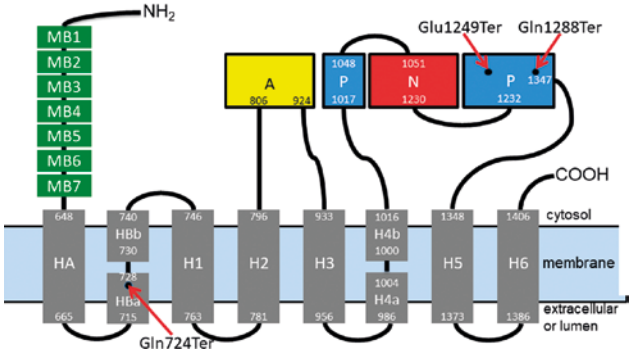
We examined three unrelated Czech males with typical symptoms of MD. The serum copper and ceruloplas-

Table 1. Activities of respiratory chain complexes I, II, I+III, III and IV in isolated thrombocytes from Czech patients with Menkes disease

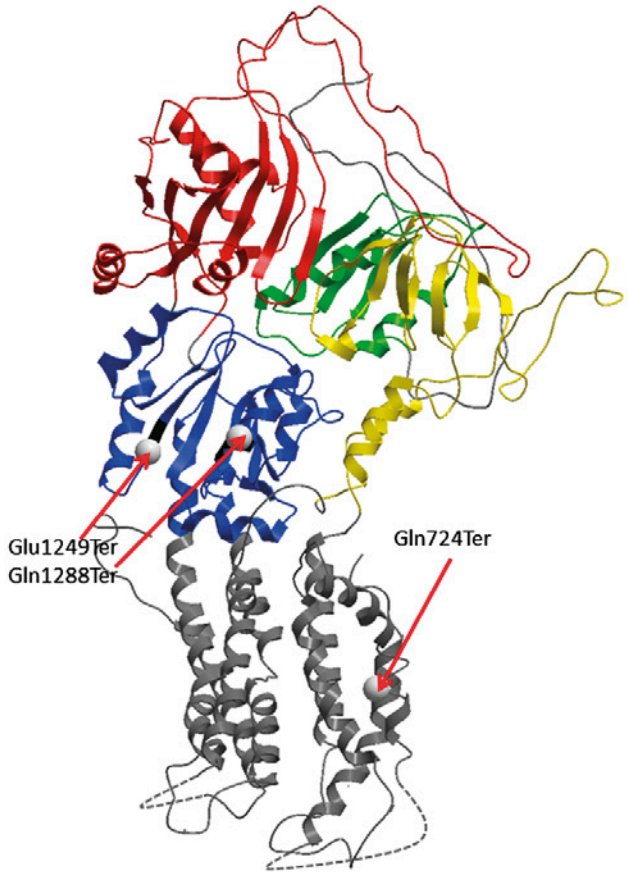
Patient	COX	CS	COX/CS ratio	NCCR	NQR	SQR	QCCR
	nmol/min/mg			nmol/min/mg			
1	**13.80***	60.40	0.22	**38.7***	42.8	**14.9***	10.3
2	**4.45***	90.81	**0.05***	23.0	36.4	**15.6***	**30.5***
3	**4.25***	85.35	**0.04***	**36.6***	35.5	13.1	**8.6***
Age-related controls	*21.9 ± 6.4*	*78.00 ± 18.80*	*0.28 ± 0.07*	*17.59 ± 9.69*	*37.33 ± 18.00*	*9.8 ± 3.8*	*15.34 ± 5.76*

*values different from the reference range

Fig. 2. Position of the patients' mutations in the structure of ATP7A



2a. Domain topology



2b. 3D homology model based on the structures of two domains of ATP7A and two homologous microbial transporters (Allen et al., 2011; Andersson et al., 2014)
Mutated amino acids are represented by their α-carbons and indicated by red arrows. Colour coding of the domains: metal binding MB – green, actuator A – yellow, phosphorylation P – blue, nucleotide binding N – red, transmembrane helices H – grey. Numbers indicate the beginning and end of the domains. Non-cytosolic loops (broken lines) were not modelled due to the lack of homology. The large unstructured mobile segments in the cytosolic domains are not displayed.

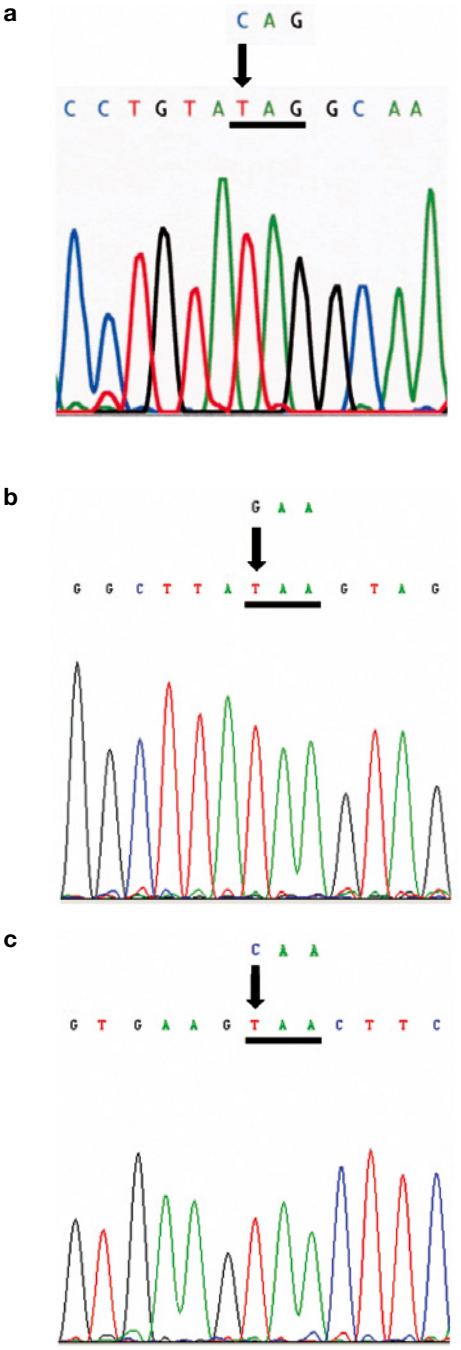


Fig. 3. Mutations in the *ATP7A* gene:
a) Mutation c.2173 C>T, p.Gln724Ter in patient 1
b) Mutation c.3748 G>T, p.Glu1249Ter in patient 2
c) Mutation c.3865 C>T, p.Gln1288Ter in patient 3

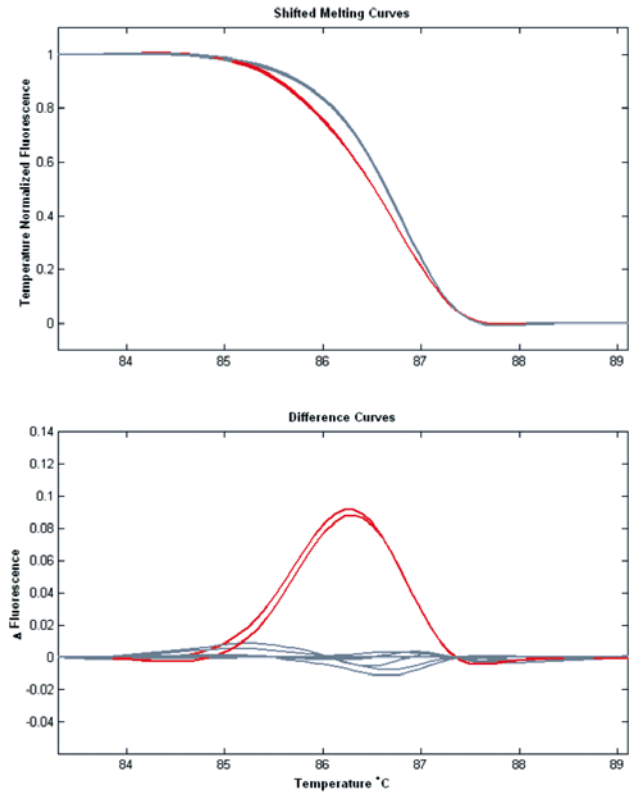


Fig. 4. Melting curves of DNA fragments containing exon 19 of the *ATP7A* gene in patient 2: novel c.3745G>T mutation (red) and controls (grey)

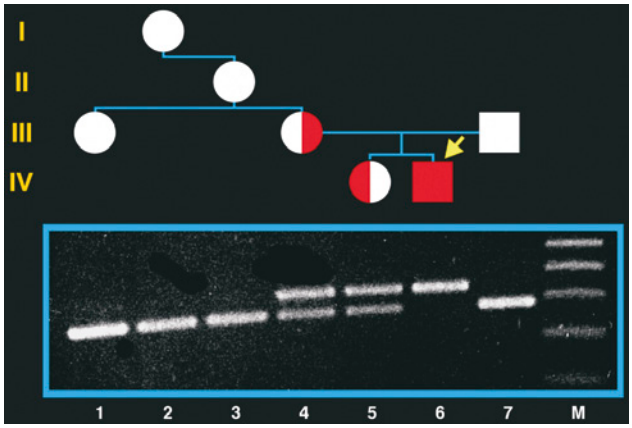


Fig. 5. Restriction analysis using *Bsr*GI in patient's 1 family with p.Gln724Ter mutation in the *ATP7A* gene

min levels and the activity of cytochrome c oxidase were decreased in all three patients (Table 2). Deficient cytochrome c oxidase activity caused by decreased copper levels is likely a major factor in the neuropathology of MD. The effects on the brain are similar to those in individuals with Leigh's disease (subacute necrotizing encephalomyelopathy), in which cytochrome c oxidase deficiency is caused by complex IV respiratory chain defects (Robinson et al., 1987; DiMauro et al. 1990). Cytochrome c oxidase deficiency may also contribute to the hypotonia and muscle weakness evident in MD patients (Kaler, 1998).

Despite normalization of biochemical parameters on treatment with copper, the patients did not improve clinically and died in early childhood.

More than 300 mutations have been identified in the *ATP7A* gene, including chromosome abnormalities involving Xq13.3, deletions, insertions, and missense, nonsense, and splice-site mutations (Das et al., 1995; Tumer et al., 1997). Mutation analysis definitively confirmed the diagnosis of MD in all three patients in this study. We identified three different nonsense mutations: c.2170C>T (p.Gln724Ter); c.3745G>T (p.Glu1249Ter); and c.3862C>T (p.Gln1288Ter) in exon 9, 19 and 20, respectively (Table 3). Each of these three mutations

*Table 2: Activities of cytochrome c oxidase and citrate synthase in isolated thrombocytes, blood copper content and ceruloplasmin levels in patient 1 (**A**), patient 2 (**B**) and patient 3 (**C**) with MD during therapy*

(A)

Week of monitoring	COX	CS	COX/CS ratio	Serum Cu	Serum CP
	nmol/min/mg			µmol/l	g/l
1	**16.80***	**114.9***	**0.15***	**2.10***	**0.03***
2	*Therapy started*				
5	29.01	93.7	0.31	**5.00***	NA
14	31.80	76.8	0.41	16.00	0.24
26	**13.80***	**60.4***	**0.22***	17.46	NA
Age-related controls	*20–40*	*75–110*	*0.23–0.43*	*14.1–29.8*	*0.2–0.6*

(B)

Week of monitoring	COX	CS	COX/CS	Serum Cu	Serum CP
	nmol/min/mg			µmol/l	g/l
1	**4.45***	90.81	**0.05***	**4.3***	**0.14***
Age-related controls	*20–40*	*75–110*	*0.23–0.43*	*14.1–29.8*	*0.2–0.6*

(C)

Week of monitoring	COX	CS	COX/CS	Serum Cu	Serum CP
	nmol/min/mg			µmol/l	g/l
1	**4.00***	85.35	**0.04***	**4.0***	**0.06***
2	**12.00***	95.00	**0.12***	**7.0***	**0.18***
2	*Therapy started*				
3	NA	NA	NA	**9.5***	NA
4	NA	NA	NA	**12.0***	0.33
5	**15.80***	**63.83***	0.24	14.5	0.33
6	24.50	**70.14***	0.35	**12.2***	0.29
7	21.68	**69.92***	0.30	**12.2***	0.25
8	NA	NA	NA	15.5	0.35
13	NA	NA	NA	**12.6***	0.40
15	25.60	85.25	0.30	NA	NA
16	*Therapy discontinued*				
19	**12.30***	**65.12***	**0.19***	NA	NA
32	**9.40***	**71.30***	**0.13***	NA	NA
Age-related controls	*20–40*	*75–110*	*0.23–0.43*	*14.1–29.8*	*0.2–0.6*

*values different from the reference range

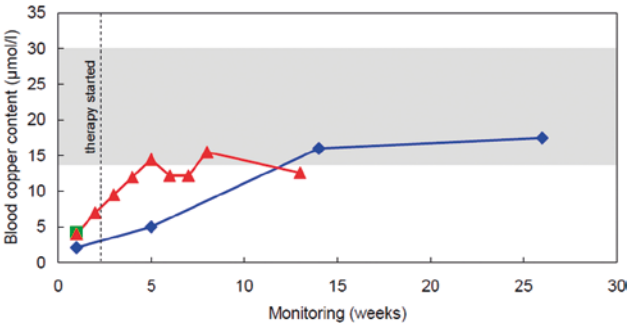


Fig. 6. Serum copper content in patients with MD during copper therapy: patient 1 (red), patient 2 (green) and patient 3 (blue). Age-related control range is indicated in grey.

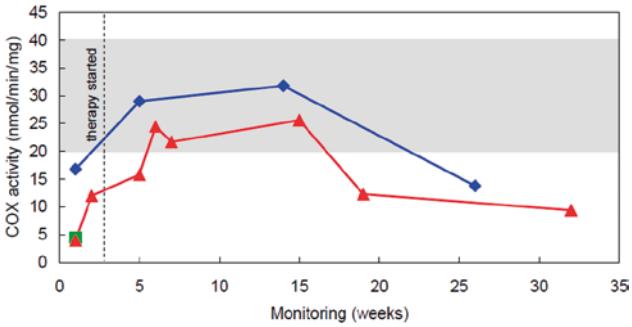


Fig. 7. Activities of COX in isolated thrombocytes in patients with Menkes disease during copper therapy: patient 1 (red), patient 2 (green) and patient 3 (blue). Age-related control range is indicated in grey.

Table 3: Mutations in the ATP7A gene in Czech patients with MD

Patient	Exon	Nucleotide change	Codon change	Amino acid change
1	9	c.2170C>T	CAG → TAG	p.Gln724Ter
2	19	c.3745G>T	GAA → TAA	p.Glu1249Ter
3	20	c.3862C>T	CAA → TAA	p.Gln1288Ter

changes an amino acid codon to a termination codon, thus leading to premature termination of the protein (Fig. 2) and loss of its function.

The mutation c.3745G>T (p.Glu1249Ter) has not been published previously. Restriction analysis in the family of the patient with c.2170C>T (p.Gln724Ter) revealed this mutation in the patient's mother and sister (Fig. 5).

Direct sequencing after PCR is a commonly employed method in mutation analysis of MD patients (Hahn et al., 2001; Liu et al., 2002). Multiplex PCR or multiplex ligation-dependent probe amplification (MLPA) is used to detect long deletions in the gene, which are quite common in this disorder. Pathological mutations causing MD are usually unique and the distribution of these mutations is specific to families with MD (Tumer et al., 2003).

In addition, we also analysed the *ATOX1* gene as a possible modulating factor (Hamza et al., 2001), but we did not find any sequence variants in the coding regions of this gene. No mutation has been found in the *ATOX1* gene screening so far (Moore et al., 2002; Simon et al., 2008). The phenotype of ATOX1 deficiency is not known but may be similar to that of MD, as ATOX1 deficiency may also impair copper homeostasis (Hamza et al., 2001; Simon et al., 2008). Despite the fact that we did not find any mutation in the *ATOX1* gene, this screening may be useful for MD-like phenotypes.

Our results show that early onset treatment with copper sulphate significantly improves the course of MD, but it is not sufficient for long-time survival of the patients.

It is necessary to start treatment in affected children as soon as possible after birth or even *in utero* to prevent irreversible neurological deterioration (Kaler et al., 2008; Haddad et al., 2012; Kaler, 2014). Identification and understanding of the molecular pathology of MD facilitates genetic counselling and prenatal diagnosis in the afflicted families.

The present developments in the CRISPR/Cas9 technology and other related techniques of gene editing are likely to revert this situation.

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