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SHORT COMMUNICATION

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Novel mutation of L718X in the *ATP7A* gene in a Japanese patient with classical Menkes disease, and four novel polymorphisms in the Japanese population

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Abstract Menkes disease is an X-linked recessive disorder of the copper membrane transport system caused by mutations in the *ATP7A* gene. While various mutations in the *ATP7A* gene have been reported, a genotype-phenotype correlation has not been clearly defined. A novel mutation in the *ATP7A* gene in a Japanese patient with classical Menkes disease was identified via analysis of reverse-transcriptase polymerase chain reaction products and genomic DNA of the *ATP7A* gene. The nonsense mutation, L718X, was found to result in premature termination and immature ATP7A protein, unlikely to have normal functioning. Therefore, this nonsense mutation of the *ATP7A* gene is proposed to play a causative role in presenting the classical Menkes phenotype. Furthermore, four novel polymorphisms, C1535T (L464L), C2151T (T669I), G2253A (R703H), and C3677T (H1178Y) were also identified.

Key words Menkes disease · *ATP7A* gene · *MNK* gene · Mutation · Polymorphism

Introduction

Menkes disease (OMIM 309400) is an X-linked recessive disorder of copper transport, characterized by neurodegeneration in infancy, failure to thrive, and abnormalities of the connective tissues (Menkes et al. 1962). The Menkes gene (*ATP7A*, previously designated as *MNK*) codes for a protein predicted to be a P-type cation-transporting adenosine triphosphatase (ATPase) (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993), due to homology with a bacterial form and the presence of a putative metal-binding motif at the N-terminus (Vulpe et al. 1993). The clinical spectrum of Menkes disease includes a range of neurological severity from the classical type to the occipital horn syndrome. Although various authors (Das et al. 1994; Ogawa et al. 1999a; Tümer et al. 1997) have found mutations in the *ATP7A* gene obtained from patients with Menkes disease, a genotype-phenotype correlation has not been clearly defined. Thus, it is important to characterize and report any mutations found in this devastating illness.

The purpose of this study was to explore the presence of mutation in the *ATP7A* gene in a Japanese patient with classical Menkes disease.

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Subjects and methods

Subjects

The patient, aged 8 years, was a Japanese boy who was the second child of a healthy, unrelated couple, a 34-year-old father and a 33-year-old mother. The elder sister of the patient was healthy. The patient was vaginally delivered at 37 weeks' gestation after an unremarkable pregnancy, with a birth weight of 2804 g. At birth, he had kinky hair and showed hypotonia, convulsions, and hypothermia. His serum level of copper was 45 μg/dl, and the ceruloplasmin level was 13 mg/dl. The diagnosis of Menkes disease was made on the basis of these clinical and laboratory findings. Once informed consent was obtained, genomic DNA samples were extracted from the peripheral blood of the patient and from 104 healthy unrelated Japanese volunteers, to assess the allelic frequencies and polymorphisms of the *ATP7A* gene.

DNA sequencing and electrophoresis

Peripheral leukocytes were used for poly A+ RNA isolation. The 4.5-kb coding region (Vulpe et al. 1993; GenBank accession number NM 000052) was divided into four over-

lapping segments and each segment was amplified by reverse-transcriptase polymerase chain reaction (RT-PCR). The number of nucleotides present in each of the segments was: segment 1, 106–1308; segment 2, 1088–2450; segment 3, 2330–3608; and segment 4, 3438–4758. Amplified segments were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced using an automated DNA sequencer (DSQ 2000L; Shimadzu, Kyoto, Japan). The identified mutation was confirmed by sequencing of the genomic DNA.

The PCR products amplifying the mutation-bearing exon were digested with *Mse* I and then subjected to electrophoresis on 3% NuSieve 3:1 agarose gels (FMC BioProducts, Rockland, ME, USA).

Polymorphisms

For assessment of the allelic frequencies of the *ATP7A* gene in the Japanese population, restriction enzyme analysis was performed. The G2253A polymorphism is known to remove the *Hha* I site, while the G2444C polymorphism removes the *Bfa* I site. None of the other three of five nucleotide changes, C1535T, C2151T, and C3677T, result in modification of the restriction sites of the *ATP7A* gene. Additional base substitutions were therefore artificially introduced into the region of each nucleotide change site. For the C1535T, an artificial *Mse* I site (**T**TAA; the substitution in the primer sequence is underlined and C1535T is shown in bold type) was introduced into the T1535 sequence, but not into the C1535 sequence. For the C2151T, an artificial *Eco* RV site (GA**T**ATC; formatting conventions as above; C2151T is shown in bold type) was introduced into the T2151 sequence, but not into the C2151 sequence. For the C3677T, an artificial *Acc* I site (G**T**ATAC; formatting conventions as above; C3677T is shown in bold type) was introduced into the T3677 sequence, but not into the C3677 sequence. The PCR products were digested with the appropriate restriction enzymes and then electrophoresed.

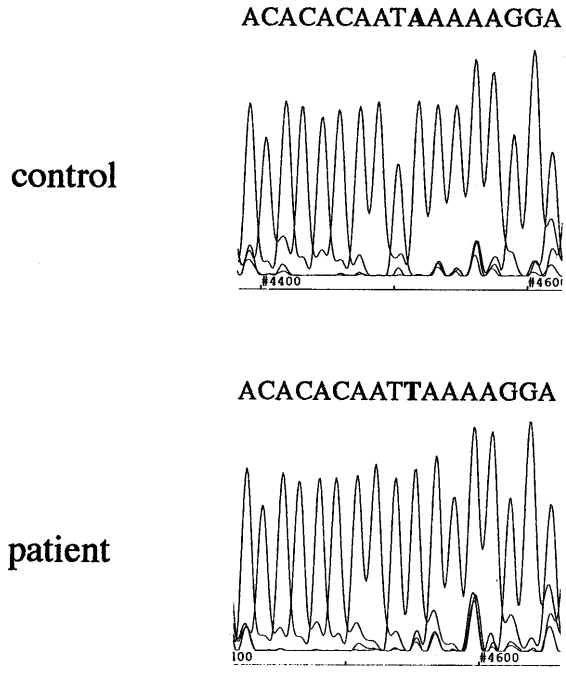

Fig. 1. Partial nucleotide sequences of the normal and mutated *ATP7A* gene. The nucleotide sequence of the mutated region is shown. The single base substitution of T-to-A at position 2298 is indicated in *bold type*

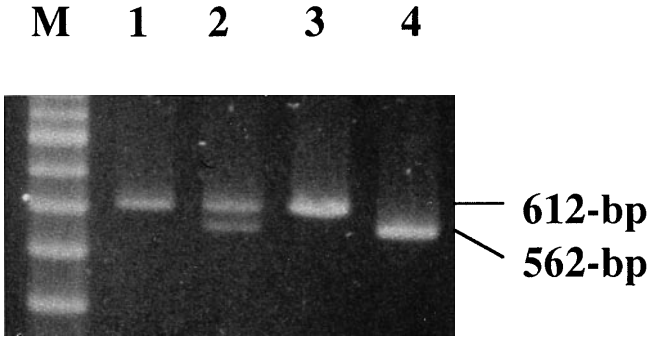

Fig. 2. T2298A carrier analysis. The *Mse* I digestion of the polymerase chain reaction (PCR) product from the genomic DNA of a normal control generated one DNA fragment of 612-bp (*lane 1*). The mutation, T2298A, generated a second *Mse* I site. The *Mse* I digestion of the PCR product from the patient generated two DNA fragments, of 562- and 50-bp (*lane 4*). The *Mse* I digestion of the PCR product from the mother generated a DNA fragment of 612-bp derived from a normal allele that lacks the *Mse* I site, in addition to the 562- and 50-bp fragments (*lane 2*). This result indicates that the mother is heterozygous for the T2298A mutation. The result of the *Mse* I digestion of the PCR product from the sister was the same as that in the control, indicating that the sister is not carrying the T2298A mutation. The 50-bp fragment is not shown in this Fig. *M*, 100-bp Ladder DNA marker

Results and discussion

DNA sequencing

The four RT-PCR products amplified from *ATP7A* cDNA were normal in size. Each fragment was cloned and sequenced. A nonsense mutation, T2298A (L718X), was found in segment 2. Genomic analysis of exon 9 of the *ATP7A* gene, which contains the nucleotide at position 2298, confirmed the presence of this mutation (Fig. 1). The mutation leads to premature termination at codon 718, resulting in an immature ATP7A protein, unlikely to have normal functioning. Therefore, we propose that this nonsense mutation could confer the observed disease state.

Carrier diagnosis

Carrier diagnosis was performed in the patient's mother and sister via extraction of genomic DNA from leukocytes and amplification of the mutation-bearing exon. Analysis of *Mse* I-digested PCR products indicated that the mutation was maternally inherited (Fig. 2). The sister of the patient was found not to carry the mutation. A prenatal molecular-genetic diagnosis may therefore be possible in this family.

Table 1. Polymorphisms in the *ATP7A* gene

Base pair	Polymorphism	Exon	Second structure change	Evidence of each polymorphism	Frequency	Number of chromosomes examined
C1535T	L464L	Exon 5	None	a	1.0	26
C2151T	T669I	Exon 9	None	b, c	1.0	20
G2253A	R703H	Exon 9	None	b, c	0.0095	105
G2444C	V767L (Das et al. 1994)	Exon 10	None	b, c	0.30	76
C3677T	H1178Y	Exon 18	None	b, c	1.0	22
A4535G	I1464V (Ogawa et al. 1999b)	Exon 23	None	b, c	0.57	104

[a] Does not affect the amino acid sequence of the protein product
[b] Results in conservative changes in apparently non-vital residues within the protein
[c] Is present on normal chromosomes in the population

Polymorphisms

During screening for possible disease-causing mutations in the patient and three normal controls, we also identified six polymorphisms in the *ATP7A* gene: four of these (C1535T [L464L], C2151T [T669I], G2253A [R703H], and C3677T [H1178Y]) were novel, while the other two (G2444C [V767L] and A4535G [I1464V]) have been described elsewhere (Das et al. 1994; Ogawa et al. 1999b) (Table 1). The nucleotide changes C1535T, C2151T, and C3677T were observed in the patient and the three controls. The nucleotide change G2253A was observed in one of the three normal controls. Each of these nucleotide changes is considered to be a polymorphism rather than a mutation, in accordance with one of the following criteria: (a) does not affect the amino acid sequence of the protein product; (b) results in conservative changes in apparently non-vital residues within the protein; or (c) is present on normal chromosomes in the population (Thomas et al. 1995). While five of the identified polymorphisms (C2151T, G2253A, C3677T, G2444C, and A4535G) cause an amino acid substitution, prediction analysis, using the Chou-Fasman method, suggests that these nucleotide changes would be unlikely to modify the secondary structure of the product.

To investigate the allelic frequencies in the normal Japanese population, restriction enzyme digestion analysis was performed. Table 1 shows a summary of the frequencies of the polymorphisms observed. The nucleotide changes C1535T, C2151T, and C3677T were found in all Japanese individuals tested, and thus are believed to be due to ethnic variation.

In summary, we have identified a novel mutation in the *ATP7A* gene in a patient with classical Menkes disease. Further analysis of more patients may be necessary to elucidate the relationship between the genotype and the phenotype.

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References

Chelly J, Tümer Z, Tønnesen T, Petterson A, Ishikawa-Brush Y, Tommerup N, Horn N, Monaco AP (1993) Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein [see comments]. Nat Genet 3:14–19

Das S, Levinson B, Whitney S, Vulpe C, Packman S, Gitschier J (1994) Diverse mutations in patients with Menkes disease often lead to exon skipping. Am J Hum Genet 55:883–889

Menkes JH, Alter M, Steigleder GK, Weakley DR, Sung JH (1962) A sex-linked disorder with retardation of growth, peculiar hair and focal cerebral and cerebellar degeneration. Pediatrics 29:764–779

Mercer JF, Livingston J, Hall B, Paynter JA, Begy C, Chandrasekharappa S, Lockhart P, Grimes A, Bhave M, Siemieniak D, Glover TW (1993) Isolation of a partial candidate gene for Menkes disease by positional cloning [see comments]. Nat Genet 3:20–25

Ogawa A, Yamamoto S, Takayanagi M, Kogo T, Kanazawa M, Kohno Y (1999a) Identification of three novel mutations in the *MNK* gene in three unrelated Japanese patients with classical Menkes disease. J Hum Genet 44:206–209

Ogawa A, Yamamoto S, Takayanagi M, Kogo T, Kanazawa M, Kohno Y (1999b) An Ile/Val polymorphism at codon 1464 of the *ATP7A* gene. J Hum Genet 44:423–424

Thomas GR, Forbes JR, Roberts EA, Walshe JM, Cox DW (1995) The Wilson disease gene: spectrum of mutations and their consequences [published erratum appears in Nat Genet 1995 9:451]. Nat Genet 9:210–217

Tümer Z, Lund C, Tolshave J, Vural B, Tønnesen T, Horn N (1997) Identification of point mutations in 41 unrelated patients affected with Menkes disease. Am J Hum Genet 60:63–71

Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J (1993) Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase [published erratum appears in Nat Genet 1993 3:273] [see comments]. Nat Genet 3: 7–13