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Original Article

ATP7A mutations in 66 Japanese patients with Menkes disease and carrier detection: A gene analysis

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Abstract ***Background*:** Menkes disease (MNK; MIN 309400) is an X-linked recessive lethal disorder of copper metabolism caused by mutations in *ATP7A* (MIM 300011), which encodes a transmembrane copper-transporting P-type ATPase. This study assessed mutations in *ATP7A* in Japanese patients with MNK and their families using gene analysis.

***Methods*:** A total of 66 patients with MNK born between 1975 and 2013 in Japan were investigated in this study. Definite diagnosis of MNK was carried out on polymerase chain reaction (PCR) amplification and direct sequencing of each exon. Genetic analysis was also performed on 39 women for carrier diagnosis, and on nine fetuses and 10 neonates for the diagnosis of MNK.

***Results*:** We detected 55 different mutations, of which 20 were de novo mutations. The mutations were located around the six copper binding sites, first to third and six transmembrane domains, and the ATP binding site. Of 30 mothers, 23 (76.7%) were carriers. Approximately half of the male siblings of patients with MNK were also diagnosed with MNK.

***Conclusion*:** Mutations in *ATP7A* varied widely across patients, although approximately half of the mutations were located in exons 4, 9, 10, and 15. Approximately 23% of patients did not inherit the mutations from their mothers, but had de novo mutations. An early definite diagnosis is necessary for the early treatment of MNK, and gene analysis serves as an effective method for detecting mutations in *ATP7A*.

Key words *ATP7A*, copper, diagnosis, Menkes disease, Mutation.

Menkes disease (MNK; MIN 309400) is an X-linked recessive lethal disorder of copper metabolism characterized by progressive neurodegenerative symptoms, abnormal hair, and connective tissue abnormalities.[1–3] The disease is caused by mutations in *ATP7A* (MIM 300011), which is located on chromosome Xq13.3 and encodes a transmembrane copper-transporting P-type ATPase.[2,4–7] Most patients with the severe classic form of MNK die in early childhood. *ATP7A* has 23 exons and encodes a protein with 1,500 amino acids. The protein has six amino-terminal copper binding sites and a catalytic transduction core with several functional domains. It is expressed in all tissues, except the liver.[8,9]

ATP7A which is a copper-transporting P-type ATPase, transports copper from the cytosol to the Golgi apparatus, where copper is incorporated into secretory copper enzymes and excreted from cells. In MNK-affected cells, copper accumulates in the cytosol and cannot be excreted. Copper accumulation in the intestine results in a failure of copper absorption that leads to systemic copper deficiency, subsequently resulting in reduced copper-dependent enzyme activity. The dysfunction of copper-dependent enzymes, such as cytochrome c oxidase, lysyl oxidase, and dopamine beta-hydroxylase, explains most of the clinical features of MNK.[10,11] Treatment must be initiated before 2 months of age in order to improve neurological outcomes, given that copper can be transported to neurons through the immature blood–brain barrier during this period.[12–15] Thus, early diagnosis of the disease is important. For this purpose, genetic analysis is particularly useful for diagnosing MNK.

In this study, we performed a mutational analysis of *ATP7A* in 66 Japanese patients with MNK. Genetic analysis was also performed in 39 women for carrier detection, and in fetuses and neonates for early diagnosis of MNK.

Methods

A total of 66 patients with MNK born between 1975 and 2012 in Japan were targeted in this study. Most patients were referred to the Department of Pediatrics of Teikyo University School of Medicine from hospitals throughout Japan. These

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patients had suspected MNK on clinical examination, and/or were diagnosed by measurement of copper concentration in cultured fibroblasts. Four pairs in the 66 patients were siblings or cousins, and the remaining patients were unrelated.

Carrier detection was performed in 30 mothers and in nine female relatives of patients with MNK. Prenatal/neonatal diagnosis was performed in nine fetuses and in 10 neonates/infants by gene analysis when they were identified as male and their mothers were carriers. Clinical data for all patients were obtained from medical records or medical record summaries prepared by their pediatricians.

Prenatal diagnosis was carried out using cultured amniotic fluid cells obtained by amniocentesis at 11–15 weeks of gestation. Amniotic fluid cells were cultured in AmnioMAX™ C-100 medium (Thermo Fisher Scientific, Waltham, MA, USA) or Chang's medium (Irvine Scientific, Santa Ana, CA, USA) as previously described.[16] Fibroblasts obtained by biopsy from patients with MNK or from control subjects were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and penicillin–streptomycin (Thermo Fisher Scientific). Cells were cultured under humidified air at 37°C in 5% CO_2.[11]

Genomic DNA and/or total RNA were isolated from blood, cord blood, Epstein–Barr virus-transformed lymphoblasts, cultured skin fibroblasts, or amniotic fluid cells using standard protocols. Polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) analysis was performed in the 5′-upstream region, in each of the 23 exons, and in adjacent intronic sequences of *ATP7A*. PCR products were then directly sequenced using a genetic analyzer (ABI PRISM 3130xl Genetic Analyzer, Applied Biosystems, Foster City, CA, USA), as described previously.[5,6]

Reverse transcription–PCR (RT-PCR) analysis was performed as described previously.[17] PCR products were analyzed on electrophoresis using agarose gels, and directly sequenced.

The study protocol was approved by the Institutional Review Board of Teikyo University School of Medicine, and a parent or guardian provided written informed consent (TEIRIN No.12-014).

Results

Detection of mutations in ATP7A

We identified 55 different mutations in *ATP7A* in 62 patients during the period from 1975 to 2012. Only four patients (6%) had no detectable mutations in *ATP7A*. Of the 55 mutations, 35 were the same as those previously reported (Leiden Open Variation Database 3.0 [LOVD], https://databases.lovd.nl/shared/genes). In three pairs of patients, each pair had one of three mutations, and the patients in each pair were not related. The three pairs of patients had one each of the following mutations: c.2324G>A (p.Gly727Arg), c.3101 C>T (p.Arg986*), and c.2317+5 g>c. A total of 20 novel mutations were identified (Table 1). The mutations were disease-causing mutations, but not polymorphisms. Based on the mutations in this study and mutations previously reported by us,[5] we classified the mutations into six subtypes: insertions, deletions, nonsense mutations, missense mutations, splice site mutations, and duplications (Fig. 1). We confirmed that three different splice site mutations skipped exons on RT-PCR. The mutations with skipped exons were c.2091+6 (skip exon 8), c.2317+5 (skip exons 9,10), and c.4353-4371+1 deletion (skip exon 22). The most frequent mutations were nonsense mutations and missense mutations, each of which accounted for 24% of the mutations. Deletion mutations accounted for 22% of the mutations. One of the 58 patients had an exon3–exon5 duplication, a previously described mutation.[19] The exon with the highest incidence of mutation was exon 4 (9/57; 16%), and approximately half of the mutations were located in exons 4, 9, 10, and 15. No mutations were found in exons 1, 2, 11, 12, 14, or 17.

Location of mutations in the ATP7A protein

Figure 2 shows the position of each mutation mapped onto the structure of the ATP7A protein. The mutations are distributed across four of the six copper binding sites: the first–third and sixth transmembrane domains, and the ATP binding site. One patient had a mutation in the Cys-Pro-Cys (CPC) motif, which is involved in copper transport in the channel formed by the transmembrane domains (c.3143T>C). One of the mutations, c.3282C>T, is located on a phosphorylated domain (Asp-Lys-Thr-Gly-Thr: DKTGT). Another mutation, c.C4060G, is located on a phosphatase domain (Gly-Asp-Gly-Ile-Asn-AspGDGIND).

Carrier and fetal/neonatal diagnoses

A total of 39 women, including mothers, aunts, and sisters of patients with MNK, were examined for carrier status. Of these, 28 were found to be carriers of heterozygous mutations, which were the same as the mutations in the proband. Seven of the 30 mothers (23.3%) were not carriers, because they did not have a mutation in *ATP7A* (Table 2). Of the mothers of unrelated patients with the same mutation (c.3101C>T), one was a carrier and the other was not. Similar to patients with MNK, carriers had various types of mutations. Women whose proband had splice site mutations were all carriers. The ratio of carriers was high among women whose proband had deletion mutations (88.9%).

The diagnosis of nine fetuses and 10 neonates was performed using gene analysis. Approximately half of the fetuses/neonates (9/19) were diagnosed with MNK (Table 2). Two of the five fetuses diagnosed with MNK were born and promptly received treatment with copper-histidine injections (Table 2).

Discussion

Menkes disease is caused by mutations in *ATP7A*, such as deletions, insertions, missense mutations, nonsense mutations, splice site mutations, and duplications.[4,20] Other types of mutations, such as gross deletions including one or several exons,[21] and exon duplications, have also been reported.[22,23]

Table 1 Menkes disease: Novel mutations in *ATP7A*

Patient ID no.	Exon	Type	Nucleotide	Codon
1	3	Deletion	c.611_612AA del[†]	p.Lys156 Lysfs*5
2	4	Deletion	c.818_819ATdel	p.Met225Glufs*5
3	4	Deletion	c.979Adel[†]	p.Thr 278 Thrfs*27
4	4	Deletion	c.1168Gdel[†]	p.Gly341Gly fs*27
9	9	Insertion	c.2157_2158ATins	p.Met 671Ilefs*1
14	10	Insertion	c.2499_2500Cins[†]	p.Pro785fs*41
17	18	Deletion	c.3778Gdel	p.Arg1211 Argfs*4
18	18	Deletion	c.3802Tdel	p.Asp1219Glufs*6
21	4	Missense	c.1152A>T	p.Glu336Val
22	4	Missense	c.1175A>G	p.Arg344Gly
26	13	Missense	c.2828A>T	p.Asn895Tyr
27	15	Missense	c.3102G>A	p.Arg986Q
29	16	Missense	c.3282C>T	p.Thr1046Ile
31	20	Missense	c.4060C>G	p.Asp1305Glu
34	23	Missense	c.A4394G	p.Ser1417Gly
38	4	Nonsense	c.1403G>T	p.Gly 420*
40	5	Nonsense	c.1618C>A	p.Tyr491*
43	6	Nonsense	c.1793G>T[†]	p.Gly 550*
50	IVS8+6	Splice site	c.2091+6t>c	Skip exon 8
54	IVS19-2	Splice site	c.3803a>g	Not detected

[†]Reported in reviews by us.[18]

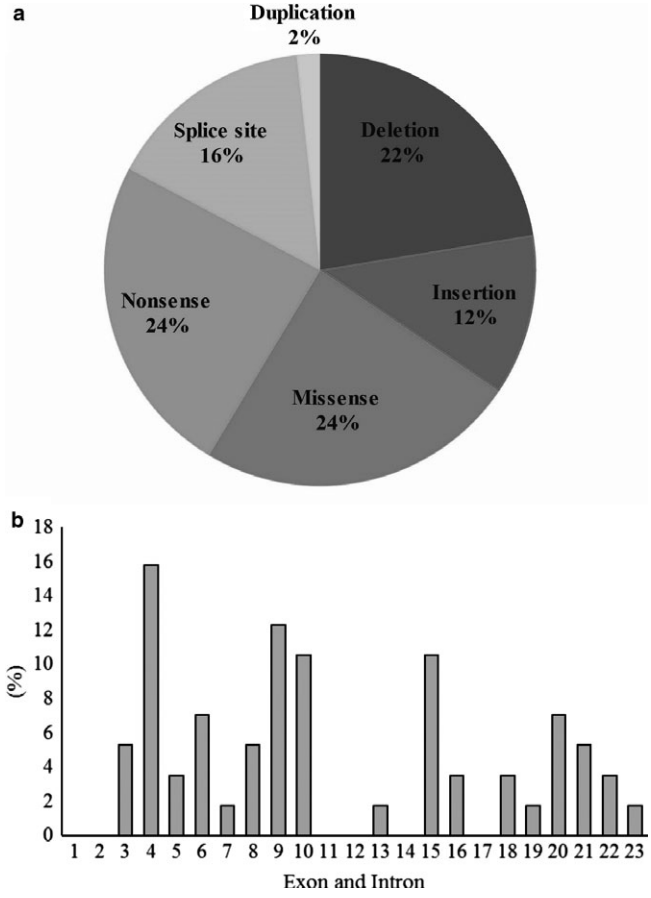

Fig. 1 (a) Incidence of six subtypes of mutations in *ATP7A* in 66 Japanese patients with Menkes disease. (b) Incidence of the mutations in each exon/intron of *ATP7A*.

These mutations have been identified in many countries, and the incidence of MNK has also been discussed.[24] In the present study, we identified 55 different mutations, including mutations previously reported by our group.[5,19] Twenty mutations were novel and are not listed in LOVD (http://www.lovd.nl/3.0/home). We divided the identified mutations into six subtypes, as follows: deletions, 22%; insertions, 12%; missense mutations, 24%; nonsense mutations, 24%; splice site mutations, 16%; and duplications, 2% (Fig. 1). More than 400 different mutations were previously identified by the Kennedy Center, with the following breakdown: insertions/deletions, 22%; nonsense mutations, 18%; missense mutations, 17%; exon deletions, 17%; splice site mutations, 16%; and duplications, 4%.[25] Other studies have reported different ratios of mutation types.[9,23] The differences in the ratio of mutation types between the present study and others may be due to differences in countries. One consistent feature, however, is that deletions and insertions have been found to be the most common types of mutations, and duplications to be the least common.

ATP7A is a copper transporting P-type ATPase family with six copper binding sites at the N-terminus of the cytoplasmic domain, eight transmembrane domains, and three cytoplasmic domains, including an activation (A) domain, a phosphorylation (P) domain, and a nucleotide-binding (N) domain.[24,25] The A domain includes a Thr-Gly-Glu (TGE) motif, which is important for dephosphorylation. The P domain includes a DKTGT motif, which is phosphorylated during the catalytic cycle by ATP. The N domain includes a GDGIND motif and is important for ATP binding. The sixth transmembrane segment includes a conserved CPC motif important for copper transport.[20]

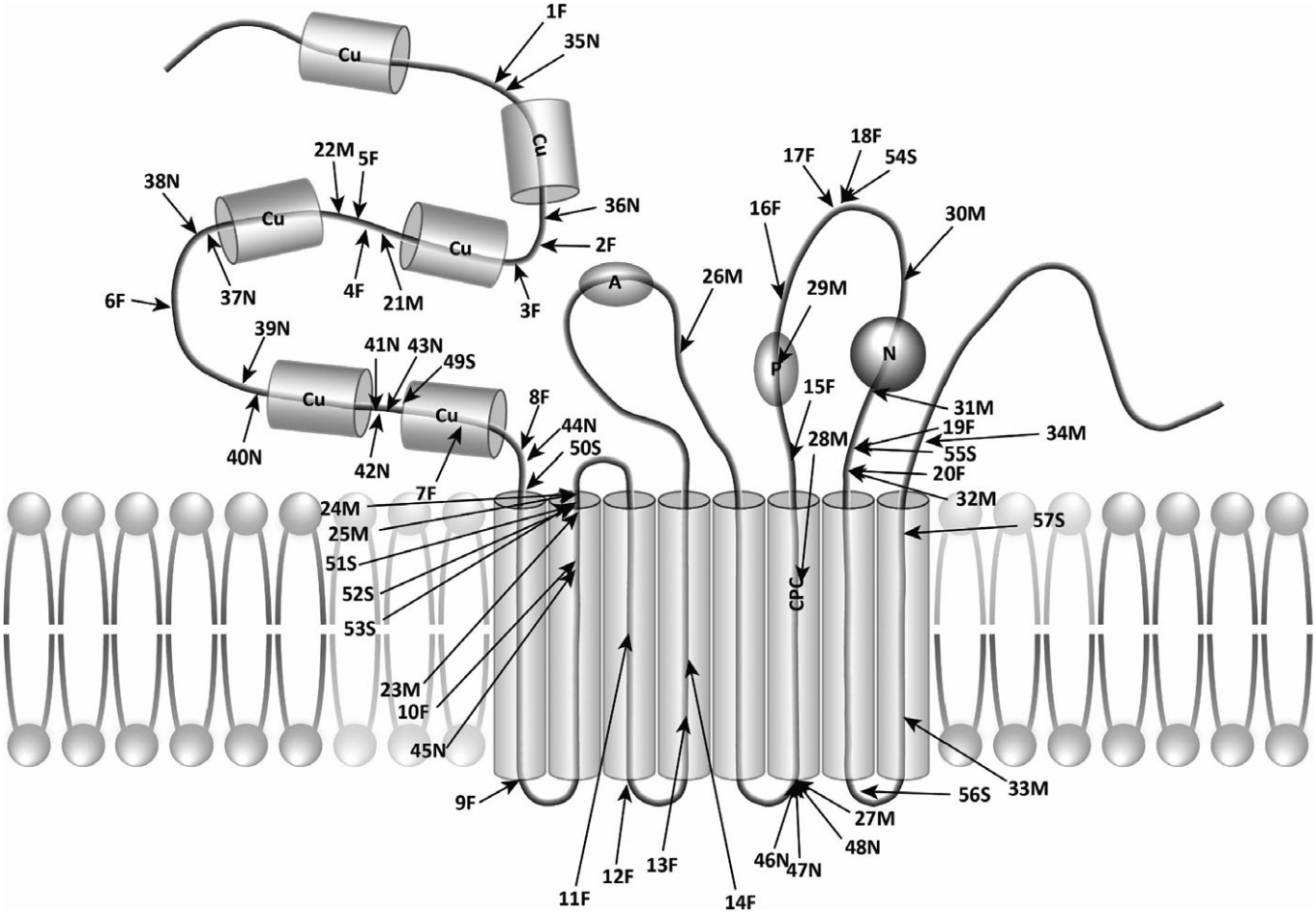

Fig. 2 Position of mutations in the ATP7A protein. ATP7A has six copper binding sites (Cu), eight transmembrane domains, an activation domain (A), a phosphorylation domain (P), and a nucleotide domain (N). Type of mutation: deletion/insertion mutations resulting in a frame shift (F), nonsense mutations (N), missense mutations (M), and splice site mutations (S). The numbers corresponds to the patient ID numbers in Table 1.

Table 2 Analysis of carrier mothers and MNK prenatal/neonatal diagnosis

Proband mutation type	Carrier *n* (%)	Prenatal/Neonatal *n* (%)
Insertion	2/3 (66.7)	1/2 (50)
Deletion	6/6 (100)	4/8 (50)
Missense	6/8 (75)	1/3 (33.3)
Nonsense	4/8 (50)	2/4 (50)
Splice site	5/5 (100)	1/2 (50)
Total	23/30 (76.7)	9/19 (50)

MNK, Menkes disease.

Although *ATP7A* mutations have been reported to be distributed over the entire sequence,[22] it is clear that most mutations in Japanese patients with MNK occur around four of the six copper binding sites: the first–third and the sixth transmembrane domains, and the ATP binding site. Approximately half of the mutations were located in exons 4, 9, 10, and 15.

In the present study, one patient had a mutation in the CPC motif, which could have impaired ATP7A function. Other patients had mutations in the P domain or N domain, both of which are needed for ATP7A function. These mutations were missense mutations involving a c.3282C>T (p.Thr1046Ile) mutation in the P domain and a c.4060C>G (p.Asp1305Glu) mutation in the N domain. While the patient with the P domain mutation had severe symptoms, the patient with the N domain mutation had only mild neurological degeneration. The phenotypes of patients with missense mutations have been reported to depend on the site affected by the mutation.[24] Thus, the differing phenotypes might be attributed to the particular substituted bases, although mutations in similarly important domains have been previously observed.

The patients with same mutation in *ATP7A* had differing symptoms. A previous study reported a patient with ac.3914A>C (p.Asp1305Ala) mutation, and that patient presented with the classic MNK phenotype.[26] The present patient who had the same mutation, however, did not have this phenotype, but achieved normal neurodevelopment and had mild

neurodegeneration. The one patient in the present study who had a c.2998T>C (p.Cys1000Arg) mutation and the classic MNK phenotype died at the age of 1 year 5 months, but this same mutation was previously reported in a patient who presented with the classic MNK phenotype but lived for >6 years.[21]

Nine patients in this study who had splice site mutations had the classic MNK phenotype, but splice site mutations have been previously reported to lead to milder phenotypes, such as atypical MNK.[27] With respect to duplications, having more than three exon duplications reportedly results in a more severe phenotype than having only two exon duplications.[19] The present patient with the exon3–exon5 duplication also had the classic MNK phenotype and had severe neurodegeneration, but lived for >20 years. This indicates that phenotype and clinical course differ even between patients who share the same mutation. These differences may depend on the extent of residual ATP7A activity and differences in treatment.

The carrier diagnosis of mothers and female relatives with a family history of MNK is necessary for an accurate prenatal diagnosis.[23] In the present study, 77% of the 30 mothers of patients with MNK were carriers, and the remaining mothers were not carriers. This suggests that 23% of the mutations in patients with MNK were de novo mutations, consistent with a previous report.[24] Sixty-three percent (5/8) of female relatives of carrier mothers were also carriers. A carrier diagnosis should be performed on direct sequencing rather than simple multiplex PCR, because the normal allele of *ATP7A* will be PCR amplified and mask the deletion.[25] Male siblings of patients are at risk of developing MNK due to the X-linked nature of the disorder. When a mother of a patient with MNK is diagnosed as a carrier, prenatal diagnosis can be performed for the next pregnancy. In the present study, 50% of male siblings of patients with MNK were also diagnosed with MNK.

In conclusion, *ATP7A* gene analysis is useful not only for a definite diagnosis of MNK, but also for the diagnosis of carriers and male siblings of patients with MNK, although mutation was not identified on this gene analysis method in some of the present patients who were diagnosed based on clinical symptoms and biochemistry.

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Disclosure

The authors declare no conflict of interest.

Author contributions

C.F. and H.K. designed the study and collected clinical data. C.F. and T.H. performed genetic analysis and measured copper concentration. Y.A. and M.H. provided conceptual advice. All authors read and approved the final manuscript.

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