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Small amounts of functional ATP7A protein permit mild phenotype

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SUMMARY

Mutations in *ATP7A* lead to at least three allelic disorders: Menkes disease (MD), Occipital horn syndrome and X-linked distal motor neuropathy. These disorders are mainly seen in male individuals, but a few affected females have been described. More than 400 different mutations have been identified in the *ATP7A* gene. We have conducted several studies in the hope of uncovering the relationship between genotype and phenotype. We have examined the X-inactivation pattern in affected females, the effect of exon-deletions and—duplications, and splice-site mutations on the composition and amount of *ATP7A* transcript, and we have examined the structural location of missense mutations. The X-inactivation pattern did not fully explain the manifestation of MD in a small fraction of carriers. Most of the affected females had preferential inactivation of the X-chromosome with the normal *ATP7A* gene, but a few individuals exhibited preferential inactivation of the X-chromosome with the mutated *ATP7A* gene. The observed mild phenotype in some patients with mutations that effect the composition of the *ATP7A* transcript, seems to be explained by the presence of a small amount of normal *ATP7A* transcript. The location of missense mutations on structural models of the ATP7A protein suggests that affected conserved residues generally lead to a severe phenotype. The ATP7A protein traffics within the cells. At low copper levels, ATP7A locates to the Trans-Golgi Network (TGN) to load cuproenzymes with copper, whereas at higher concentrations, ATP7A shifts to the post-Golgi compartments or to the plasma membrane to export copper out of the cell. Impaired copper-regulation trafficking has been observed for ATP7A mutants, but its impact on the clinical outcome is not clear. The major problem in patients with MD seems to be insufficient amounts of copper in the brain. In fact, prenatal treatment of mottled mice as a model for human MD with a combination of chelator and copper, produces a slight increase in copper levels in the brain which perhaps leads to longer survival and more active behavior. In conclusion, small amounts of copper at the right location seem to relieve the symptoms.

Introduction

Menkes disease (MD [OMIM 309400]) is a rare X-linked recessive disorder with an incidence of about 1:300,000 [1]. The disease is caused by mutations in the *ATP7A* gene [OMIM 300011]. The ATP7A protein belongs to the superfamily 1B of membrane proteins termed P-type ATPases. This family includes the Na^+/K^+-, H^+/K^+- and Ca^{2+}-pumps [2]. A multiple alignment covering the full length of the different P-type ATPases demonstrates that the cores that define the essential catalytic motifs of the protein-sequences align very well, suggesting that the proteins are homologous. ATP7A contains eight trans-membrane domains (TM1-TM8) and three cytoplasmic domains; the actuator domain (A-domain), the phosphorylation domain (P-domain) and the nucleotide binding domain (N-domain). Six metal binding sites (MBS) are located in the amino-terminal of the protein (Fig. 1). The ATP7A protein transports copper across membranes and has two functions: it is responsible for (1) the copper-loading of several cuproenzymes, and (2) the efflux of copper from the cell [1,3]. Humans mainly acquire their essential amounts of copper from food. Patients with MD have an insufficient amount of copper in their blood due to an impaired efflux from the enterocytes in the gastrointestinal tract. The copper influx to the brain is also reduced, so that these patients suffer from severe developmental and neurological impairment [4,5]. The brain is especially sensitive to a defective ATP7A protein and copper deficiency because several cuproenzymes, *e.g.* peptidylglicine alfa-amidating monooxygenase, Cu, Zn-superoxide dismutase, and dopamine-beta-monooxygenase [5–7] are essential for normal brain function. Furthermore, as the activity of several other cuproenzymes expressed throughout the body, *e.g.* lysyloxidase, sylfhydryloxidase, tyrosinase, and cytochrome *c* oxidase is also reduced, MD patients also exhibit skeletal and connective

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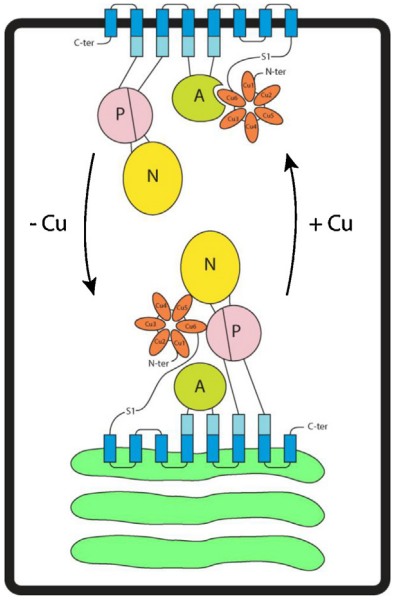

Fig. 1. Illustration of hypothetic conformation shifts in the ATP7A protein during copper (Cu)-dependent trafficking. The location of the N-domain (N), P-domain (P) and A-domain (A) are indicated. The 6 copper binding sites (MBS) are numbered Cu1-Cu6. The stalk region between the MBS sites and the first transmembrane domain (TMA) is marked S1. The transmembrane segments (TM1-6) are indicated with blue bars. The stalk regions between TM4, TM5 and the A-domain, and between TM6 and TM7 and the P-domain are indicated with light blue bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tissue abnormalities, hypo-pigmented skin, and abnormal hair (kinky hair or pili torti). Paradoxically, although less copper is delivered to the blood and to major tissues including the brain, copper is accumulated in other tissues such as the duodenum, kidney, spleen, pancreas and skeletal muscles. Thus the clinical features of MD are the combined consequences of impaired copper distribution and the malfunction of a large number of copper-dependent enzymes [3].

Impaired copper efflux can also be demonstrated biochemically in fibroblasts. Cultured fibroblasts isolated from skin biopsies from MD patients, accumulate 2–5 times more copper when compared to control fibroblasts [8,9].

Symptoms

The phenotypic features of patients with mutations in *ATP7A* can be divided into at least three allelic forms: early onset of MD and death in early childhood, occipital horn syndrome (OHS [OMIM 304050]) with survival into adulthood, and the adult onset of X-linked distal motor neuropathy [OMIM 300489]. The neurological symptoms of OHS patients are milder than those seen in patients with classical MD. OHS patients have less severe neurological symptoms and predominantly exhibit connective tissue abnormalities. Patients with X-linked distal motor neuropathy have – in contrast to patients with MD or OHS – no signs of abnormal copper metabolism [10,11].

The majority of patients with mutations in the *ATP7A* have the severe form of classical MD and die at a very young age. A fraction of the patients (5–8%) have a milder form of MD and survive longer. About 2% of the patients have OHS, and so far only two families with X-linked distal motor neuropathy have been identified [9,10].

Affected females

As the *ATP7A* gene is X-linked, most of the affected patients are males and only few females are affected. Female carriers are heterozygous with a mutation in one of their two *ATP7A* copies. Because only one X-chromosome is active in each cell, female carriers are mosaics. In one fraction of a female's cells, the X-chromosome with the wild-type *ATP7A* is active, whereas in the other fraction, the X-chromosome with the mutated *ATP7A* gene is active [12]. Whereas hypo-pigmentation of the skin, and pili torti hair have been observed in a large number of female carriers, only few have mental defects [13]. In a cohort of 517 MD families, we identified nine carriers with symptoms of MD. A mutation in the *ATP7A* gene was identified in eight of the nine carriers. Three of the patients had a deletion of one or more exons, whereas five had a single base-pair substitution that led to a missense mutation or a premature termination codon. Although the female patients had clear symptoms of MD, the symptoms were milder than those in the affected males from the same families with the same mutations [13]. The phenotypes of the female patients ranged from mild to severe mental retardation.

In an effort to find an explanation for the symptoms of MD, we analysed the X-inactivation on samples from the affected females. We looked at the X-inactivation pattern in DNA extracted from fibroblast cultures from skin biopsies taken from the females. We found that the normal X-chromosome was inactivated or preferentially inactivated in four of the females, which appears to be consistent with the symptoms. However, unexpectedly we found one affected female with equal inactivation of the two X-chromosomes. Furthermore, we found two females with inactivation or preferential inactivation of the affected X-chromosome. Thus, the inactivation pattern, at least in fibroblast cultures, cannot explain the symptoms. For comparison, we studied the X-inactivation pattern in neurologically normal female carriers. We found that the X-chromosme with the normal *ATP7A* was active in 94% of 49 investigated females [13]. Thus, while almost all non-affected females had an active X-chromosome that encoded for the normal *ATP7A*, only about half of the affected females had an active X-chromosome with the mutated *ATP7A*. Thus, predicting whether a female fetus or newborn girl, will develop symptoms of MD is not possible by analyzing the X-inactivation pattern.

Identification of mutations

Over the last 15–20 years, The Kennedy Center has received DNA samples from patients worldwide who are suspected of suffering from MD. So far we have identified more than 400 different mutations in the *ATP7A* gene (partly unpublished).

The mutations were identified by combining Multiplex Ligation-dependent amplification (MLPA) with Sanger Sequencing of all 23 encoding exons, including 20 base-pair flanking sequences. MLPA was used for screening for exon-deletions or -duplications and Sanger Sequencing for identifying base-pair substitutions, small base-pair deletions, -duplications and -insertions. These methods enabled us to identify mutations in about 95% of the patients.

The mutations can be divided into several subtypes; indels, insertions and/or deletions of few base-pairs: 22%, nonsense mutations: 18%, missense mutations and exon deletions: 17% each, splice site mutations: 16%, and about 4% of the mutations were exon duplications [10].

Splice-site mutations

Splice-site mutations are changes in the DNA sequence that alter or abolish correct mRNA splicing during the maturation of