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Alterations in the expression of the *Atp7a* gene in the early postnatal development of the mosaic mutant mice (*Atp7a*$^{mo\text{-}ms}$) – An animal model for Menkes disease

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ABSTRACT

Copper is a trace element that is essential for the normal growth and development of all living organisms. In mammals, the ATP7A Cu-transporting ATPase is a key protein that is required for the maintenance of copper homeostasis. In both humans and mice, the ATP7A protein is coded by the X-linked *ATP7A/Atp7a* gene. Disturbances in copper metabolism caused by mutations in the *ATP7A/Atp7a* gene lead to severe metabolic syndromes Menkes disease in humans and the lethal *mottled* phenotype in mice. *Mosaic* is one of numerous *mottled* mutations and may serve as a model for a severe Menkes disease variant. In Menkes patients, mutations in the *ATP7A* gene often result in a decreased level of the normal ATP7A protein. The aim of this study was to analyse the expression of the *Atp7a* gene in *mosaic* mutants in early postnatal development, a critical period for starting copper supplementation therapy in both Menkes patients and mutant mice. Using real-time quantitative RT-PCR, we analysed the expression of the *Atp7a* gene in the brain, kidney and liver of newborn (P0.5) and suckling (P14) mice. Our results indicate that in *mosaic* P0.5 mutants, the *Atp7a* mRNA level is decreased in all analysed organs in comparison with wild-type animals. In two week-old mutants, a significant decrease was observed only in the kidney. In contrast, their hepatic level of *Atp7a* tended to be higher than in wild-type mice. We speculate that disturbance in the expression of the *Atp7a* gene and, consequently, change in the copper concentration of the organs, may contribute to the early fatal outcome of *mosaic* males.

1. Results and discussion

In all living organisms, copper is one of the most important biometals. Organisms require copper as a cofactor in numerous copper-dependent enzymes such as cytochrome *c* oxidase, Zn, Cu superoxide dismutase and lysyl oxidase. Although copper is a trace element that is essential for normal growth and development, an excess of copper is highly toxic. The production of strongly reactive free radicals in the presence of copper can lead to the oxidative damage of proteins, lipids and nucleic acids. Therefore, copper homeostasis is tightly regulated and living organisms have developed complex control mechanisms for maintaining the balance between essential and toxic copper levels.

The mouse *mottled* mutants exhibit alterations in copper metabolism and have been proposed as an animal model of Menkes disease, a fatal metabolic syndrome in humans. In both Menkes patients and *mottled* mice, the disturbance in copper metabolism is caused by mutations in the *ATP7A/Atp7a* gene (La Fontaine et al., 1999; Llanos et al., 2006). The *Atp7a* gene, cloned in 1994 (Cecchi and Avner, 1996; Levinson et al., 1994), is the mouse homologue of the *ATP7A* gene in humans and has been found to have a high homology (>90%) to the human locus (Kim and Petris, 2007; Reed and Boyd, 1997). Both the *ATP7A* and *Atp7a* genes are located on the X chromosome and encode for a protein called ATP7A, which belongs to the P-type ATPase family. ATP7A is located in the trans-Golgi network and is involved in the ATP-dependent transport of copper across plasma or intracellular membranes. Many *mottled* mutants have arisen spontaneously or have been induced by chemical or radiation mutagenesis. Hemizygous *mottled* males exhibit a severe and often lethal phenotype. However, the severity of the phenotype is dependent on the *mottled* alleles and varies between *mottled* mutations. In general, affected males belong to one of three classes of phenotypic severity: (i) mutant males that are

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dead in utero, e.g., *dappled*, *tortoiseshell* and *11H* (Cecchi et al., 1997; Kim and Petris, 2007), (ii) mutant males that die in the third week of postnatal life, e.g., *brindled*, *mosaic*, *macular*, and *13H* (Grimes et al., 1997; Reed and Boyd, 1997; Lenartowicz et al., 2003), (iii) mutant males that die within a few postnatal months, e.g., *blotchy* and *viable-brindled* (Grimes et al., 1997; La Fontaine et al., 1999).

The *mosaic* mutation (*Atp7a*$^{mo\text{-}ms}$) arose spontaneously in the outbred colony of the Department of Genetics and Evolution of the Jagiellonian University in Kraków (Styrna, 1977). In *mosaic* mutant males, many clinical features that are characteristic of defective copper metabolism have been observed, including defects in pigmentation and hair structure, a decrease in body weight, poor viability and progressive paresis of the hind limbs. *Mosaic* mutant males die at about day 16. Analysis of the copper concentration in the organs of the mutants indicates that copper accumulates in the small intestine and kidneys. In contrast, brain, liver and heart are Cu-deficient (Lenartowicz and Sasuła, 2000; Lenartowicz et al., 2003; Styrna, 1977). Although the phenotype of *mosaic* mutants closely resembles that of *brindled* mice, the molecular basis of the mosaic mutation is different. The *brindled* phenotype is caused by a deletion of 6 bp in the 11th exon of the *Atp7a* gene (Grimes et al., 1997; Reed and Boyd, 1997). Analysis of the coding region of the *mosaic* mice indicate that exon 11 is intact, but there are an additional 3 bp at the end of the 4th exon, as a result of alternative splicing of the *Atp7a* gene (Lenartowicz et al., 2004). About 50% of the mutations in the *ATP7A* gene in patients with Menkes disease are associated with splicing abnormalities, and most of them result in the decrease of ATP7A mRNA levels (Das et al., 1994; Levinson et al., 1994; Hsi and Cox, 2004; Moller et al., 2000. However, it is largely unknown whether the expression of the *ATP7A* gene changes during the neonatal period of these patients. Such knowledge could be useful for starting early copper supplementation therapy.

In this study, we investigate the developmental and postnatal changes in the expression of the *Atp7a* gene in *mosaic* mutant and wild-type mice. The analysis was performed in neonatal P0.5 and young (suckling) P14 animals. We analysed the expression of the *Atp7a* gene in the organs that are usually affected in Menkes disease patients and in *mottled* mutant mice, such as the brain and kidneys. In both patients and mutant mice, the copper concentration in the brain is strongly decreased. In contrast, the kidneys show a copper overload (Lenartowicz et al., 2003; Niciu et al., 2006, 2007). In addition, we investigated *Atp7a* expression in the liver, the main organ responsible for copper metabolism (Wijmenga and Klomp, 2004). To find a relationship between the expression of the *Atp7a* gene and the copper concentration in the investigated organs, we also measured the copper concentration in the brain, kidneys and liver.

1.1. Atp7a expression and copper concentration in the brain

Analysis of the abundance of ATP7A mRNA in the brain indicates that in *mosaic* mutants this expression is decreased in comparison to wild-type (wt) mice (Fig. 1A). In the group of neonatal mice this decrease was especially clear as the transcript level of *Atp7a* gene in wild-type males was three fold higher than in *mosaic* mice. In P14 animals ATP7A mRNA level tended to be lower in mutants although the difference is not significant. Another interesting observation is that in wild-type mice the abundance of ATP7A mRNA in the group of P0.5 animals is significantly higher than in P14 mice (Fig. 1A). Interestingly, in mutants ATP7A mRNA expression remains at the same low level throughout two first weeks of life (Fig. 1A).

Analysis of the copper content in the brain of the *mosaic* mutants and wild-type males indicates that in both age groups, copper

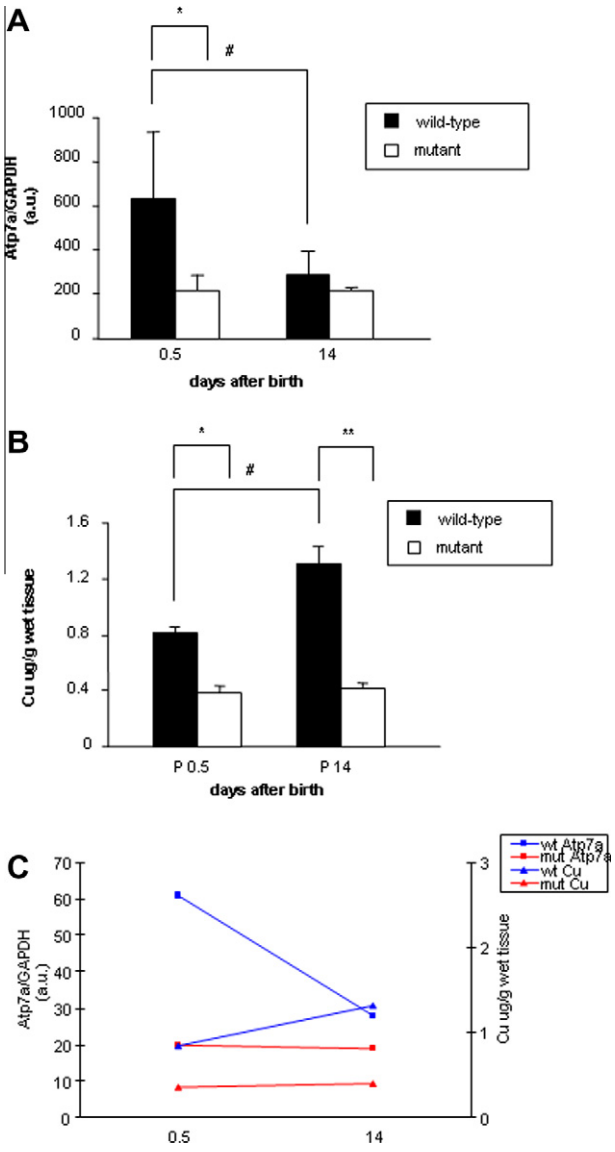

Fig. 1. Analysis of the *Atp7a* gene expression and copper concentration in the brains of the wild-type and *mosaic* mutant mice. (A) *Atp7a* mRNA level was high in the P0.5 wild-type mice and decreased during the first two-week period. In the P0.5 mutants, expression was significantly lower $P < 0.05$ than in wild-type mice and was similar in both investigated groups of mutants. Transcript abundance in brains was measured by real-time RT-PCR using the specific primer pair shown in Table 1. *Atp7a* mRNA was normalised to GAPDH. Each column represents the mean ± SD of two amplification reactions, performed using a single cDNA sample reverse-transcribed from RNA prepared from four mice of each genotype. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis. (B) Copper concentration (μg/g wet tissue) in the brain of neonatal P0.5 and suckling (P14) wild-type and mutant mice. Values are means ± SD, $n = 5$ animals were analysed per group. *Denotes a significant difference between mutant and wild-type males ($P < 0.05$), **($P < 0.01$); $^{\#}$denotes a significant difference ($P < 0.05$) between wild-type groups. (C) Relationship between copper concentration and *Atp7a* gene expression in the brain of wild-type and *mosaic* mutants.

concentration in mutants was lower than in wild-type mice (Fig. 1B). In the neonatal *mosaic* mutants brain copper concentration was more than twofold lower that in wild-type mice (Table 1). It clearly indicates that mutants were born with copper deficiency in the brain. In the suckling 14-day old mutants copper concentration in the brain was about one third of that found in wild genotype males (Table 1). Moreover, in 14-day-old mutants we often observed neurological problems e.g. ataxia, paralysis of the hind limbs and tremor (Authors' observations). We also found that in