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Polymorphisms of metal transporter *genes DMT1* and *ATP7A* in Wilson's disease

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ABSTRACT

Wilson's disease (WND) is an inherited disorder of copper metabolism. Divalent metal transporter1 (DMT1) and ATP7A play important roles in metal transport in humans. The frequency of two single nucleotide polymorphisms of the *DMT1* gene: *DMT1* IVS4 C>A, *DMT1* 11245 T>C and two of the *ATP7A* gene: rs1062472 T>C, *ATP7A* rs 2227291 G>C have been evaluated in a population of 108 Wilson's disease patients and 108 sex- and age-matched healthy volunteers. The *DMT1* IVS4 C(+) allele occurred more frequently in WND than in the healthy controls. The allele frequencies of other studied polymorphisms in WND group were in line with frequencies obtained for healthy volunteers. Neither of the polymorphisms had an impact on the age at onset or clinical phenotype of WND.

Introduction

Wilson's disease (Online Mendelian Inheritance in Man [OMIM] 277900, WND) is an autosomal recessive metabolic disorder characterized by impaired copper metabolism [1]. There is a high variability in the clinical picture of the disease, which can manifest itself with a wide spectrum of hepatic, neurological, psychiatric and even hematological or ophthalmic symptoms. The age at onset of the clinical symptoms is also highly variable [2]. More than 500 mutations have been identified in the *ATP7B* gene; however, only a few genotype–phenotype correlations have been identified [3–6]. Besides the *ATP7B* genotype, an association has been documented between the WND clinical picture and gender, as well as with genetic variability in genes including the apolipoprotein E (*APOE*) gene, human prion-related protein (PrP) gene, methylenetetrahydrofolate reductase (*MTHFR*) gene, X-linked inhibitor of apoptosis (*XIAP/BIRC4*) gene and the interleukin 1 receptor antagonist (*IL1RN*) gene [2,7–13]. Nevertheless, these factors do not completely explain the phenotypic variability of WND.

The characteristic feature of WND is dysfunction of the ATP7B protein. ATP7B is abundant in the hepatocytes, where it is responsible for copper incorporation into ceruloplasmin and for copper excretion into the bile. In untreated WND patients, loss of ATP7B function leads to copper accumulation and organ damage as well as synthesis of unstable forms of cuproenzyme-ceruloplasmin [1].

Existing data suggest that, besides impaired copper metabolism, WND may be characterized with iron dyshomeostasis, since ceruloplasmin plays an important role in iron turnover due to its ferroxidase activity [14]. Copper and iron disequilibrium can lead to oxidative-stress, as both metals stimulate generation of free radicals via Fenton reaction [15].

Transporters of copper and iron play an important role in the distribution of these metals within cells; thus, their dysfunction or misregulation may create an environment triggering production of free radicals and cell damage [16]. Divalent metal transporter 1 (DMT1) transports non-transferrin bound iron (NTBI) into most mammalian cells[17]. Increased expression of DMT 1 has been found in the substantia nigra of Parkinson disease patients and in animal models of the disease [18]. In WND patients, Parkinson syndrome is a frequent manifestation of basal ganglia involvement [1]. It is even speculated that heterozygotes for WND are at increased risk for idiopathic PD [19].

ATP7A is a member of the P-type adenosine triphosphatase (ATPase) family and shares structural and functional similarities with ATP7B [14]. The essential role of ATP7A is best illustrated by the phenotype of Menkes disease (OMIM 30940) [15]. This inherited defect of ATP7A results in impaired copper transport in the intestinal epithelium and overall copper deficiency [16]. A characteristic feature of the disease is severe neurological defects, which are believed to result from reduced activity of cuproenzyme-cytochrome c oxidase within neurons [20]. Although ATP7B and ATP7A differ structurally and functionally, ATP7A may contribute

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Table 1
Polymerase chain reaction oligonucleotide sequences, restriction enzymes and product lengths for metal transporter gene analysis.

Gene polymorphism	Location	Oligonucleotide sequences	PCR product length	Restriction endonuclease (RE)	RE digestion product length
DMT1 IVS4 C>A[a] rs224589	Intron 4	5′GACACATGCAATATCTGACATG 5′AGGCTACTATCCAACATGCAG	352bp	MnlI	Allele A: 217 + 100 + 35 Allele C: 183 + 100 + 35 + 34
DMT1 1254 T>C[a] rs1048230	Exon 16: coding	5′CTTTGCCCGAGTGGTTCTGACTCGCTCGAT 5′TTCCTCTCAATATCCCCC	229BP	MboI	Allele C: 198 + 29 Allele T: 229
ATP7A T>C rs1062472	3′-UTR	5′GGGATGGAGTTCTTCCTTT 5′AAGCATGGAGGATGGCATAG	261BP	NlaIII	Allele T: 181 + 84 Allele C: 181 + 56 + 28
ATP7A G>C rs2227291	Exon 10	5′GCAGTTTTTCGGAGGCTGGTA 5′AGGTCATTTATCCACCCAACA	290BP	BfaI (MaeI)	Allele C: 209 + 81 Allele G: 128 + 81 + 81

[a] According to Kelleher et al. Blood Cells Mol Dis 2004, 35–39.

to adaptation to copper overload resulting from ATP7B dysfunction, as has been shown in toxic milk mice. In this animal model of WND, expression of ATP7A in the brain increased transiently in response to elevated concentrations of free copper [21]. In another mice strain, a localized copper deficit in the heart resulting from the selective knock-out of a gene coding copper importer stimulated expression of ATP7A in the intestine and liver [22].

As DMT1 and ATP7A transport metals, their function is likely to modulate metal dyshomeostasis resulting from *ATP7B* mutations. We hypothesize that variability in the *DMT1* or *ATP7A* genes may modify the iron and copper balance and potentially influence the onset of symptoms of WND. Thus, the objective of this study was to explore whether genetic variation in terms of a single nucleotide polymorphism of these two genes is linked with alterations in copper and iron homeostasis and a clinical phenotype of WND.

Methods

Subjects and methods

Subjects

108 unrelated patients with Wilson's disease diagnosed according to Lepzig score criteria and 108 unrelated, sex- and age-matched healthy controls were included in the study. Control subjects were of a comparable age and sex to WND patients, and had neither family history of WND nor diagnosed neurodegenerative or liver disease (chronic inflammatory disease or infectious disease were also excluded). They were recruited from individuals diagnosed in outpatient clinics, as well as from volunteers from among hospital staff.

All participants gave written informed consent to participate in this study. The study protocol was approved a priori by the Institutional Review Board for the protection of human subjects at our institution and conforms to the ethical guidelines of the 1975 Helsinki Declaration.

WND diagnosis was performed according to the scoring system based on clinical signs and symptoms, the presence of Kayser–Fleischer rings, laboratory evidence of impaired copper metabolism (abnormal results of tests for: serum ceruloplasmin concentration), assessed with an H.A. Ravin colorimetric enzymatic assay [23], serum copper concentration, copper excretion in urine (determined by Atomic Adsorption Spectroscopy) and genetic evidence of the presence of pathogenic mutations in both alleles of *ATP7B*. The study protocol was approved by the Institutional Ethical Review Board for the protection of human subjects at our institution; all study participants gave informed written consent.

Clinical phenotyping

The first onset of WND clinical symptoms was established using a standard patient questionnaire, as well as by evaluating patient medical histories. According to the mode of initial disease manifestation, patients were grouped as either hepatic (HWND) or neuropsychiatric (NWND). Patients were categorized as HWND when presenting signs of chronic or acute liver disease (increased liver enzyme activity with increased blood bilirubin prolonged prothrombin time, and/or changes in liver echogenicity; signs of portal hypertension, decompensated liver cirrhosis or acute liver failure) without neuropsychiatric symptoms. Patients were categorized as NWND when having neurological or psychiatric symptoms.

DMT1 and ATP7A polymorphisms

Genomic DNA was extracted from peripheral blood samples using either the TRI Reagent (SIGMA, Poznan, Poland) or the Promega Maxwell 16 Purification Instrument (Promega – Symbios, Gdansk, Poland). Genotyping for the *DMT1* and *ATP7A* polymorphism was conducted by polymerase chain-reaction-restriction fragment length polymorphism (PCR-RFLP). Primer sequences, restriction enzymes and product lengths of analyzed genes are presented in Table 1.

Statistical analysis

Data were analyzed using the statistical package STATISTICA 10.0 (StatSoft, Cracow, Poland). To test for the Hardy–Weinberg equilibrium (HWE) of studied polymorphisms, the expected genotype numbers were calculated from the allele frequencies and deviations from the observed genotype numbers were determined using the χ^2 test (the Hardy–Weinberg Calculator by Michael H. Court was used). The normality of the analyzed continuous variables was determined using Kolmogorov–Smirnov and Lilliefors tests. Variables that were normally distributed were compared between groups with the Student *t* test. Variables that were not normally distributed were presented as medians and interquartile ranges (IQR) and compared between groups with Kruskal–Wallis ANOVA (with post hoc testing using the Mann–Whitney *U*-test) or by the Mann–Whitney *U*-test (two-way variables). Categorical variables were compared between groups using the χ^2 test or the Fisher exact test. The Bonferroni correction for multiple comparisons was used.

Results

Baseline characteristics of the study population

A total of 108 (55 women and 53 men) patients aged 33.1 ± 11.3 were enrolled in the study. The WND group comprised 38 treatment-naive patients and 70 patients on de-coppering therapy (zinc salts or D-penicillamine). Fifty-four patients (50%) were categorized as HWND and 54 as NWND. The control group comprised of 108 healthy volunteers (61 women and 47 men) aged 35.0 ± 10.5. The baseline characteristics of study participants are presented in Table 2.