RESEARCH NOTE

Middle-aged heterozygous carriers of Wilson's disease do not present with significant phenotypic deviations related to copper metabolism

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Introduction

Wilson disease (WD) is an autosomal recessive disorder of copper (Cu) metabolism (OMIM 277900 (Online Mendelian Inheritance In Man, URL: http://www.ncbi.nlm.nih.gov/omim/)). The WD gene, *ATP7B*, is located on chromosome 13 (13q14.3) (Petrukhin *et al*. 1994) and encodes the copper-transporting P-type ATPase (ATP7B) (OMIM 606882). Over 300 mutations have been described in the *ATP7B* (http://www.medgen.med.ualberta.ca/database.html). Pathogenic mutation in *ATP7B* leads to the synthesis of functionally abnormal ATP7B that has a shorter lifetime and/or improper intracellular localization (Forbes and Cox 2000; Huster *et al*. 2003; Cater *et al*. 2007). Severe Cu metabolism abnormalities are observed in individuals with pathogenic mutations in both alleles of the ATP7B (Ala *et al*. 2007; de Bie *et al*. 2007). This results in accumulation of toxic Cu mainly in the liver and in brain leading to a high spectrum of hepatic and/or neuropsychiatric signs and symptoms (Ala *et al*. 2007; de Bie *et al*. 2007; Barbosa *et al*. 2009).

In some instances, slight phenotypic deviations were observed in heterozygous carriers of recessive alleles. In the general population, the frequency of heterozygous carriers of WD (WDHzc) is about 1%–2% (Das and Ray 2006). It seems to be of importance to evaluate if WDHzc are predisposed to any abnormalities in Cu metabolism and, if so, whether these abnormalities are related to any symptomatic manifestations. It has been hypothesized, for example, that WDHzc may be at high risk of developing Parkinson disease (PD) (Johnson 2001).

It is known for a long time that studies on the phenotypic effects in WDHzc are difficult because of a high genetic heterogeneity. Cox *et al*. (1972) distinguished three genetic types of WD. According to them, the WDHzcs had normal serum ceruloplasmin concentrations in two of these types: the 'juvenile' (mainly affecting Western Europeans), and 'Slavic' (occurring mainly among Eastern European Slavic language speakers). The third type 'atypical' could be characterized by decreased serum ceruloplasmin in WDHzc. At that time, the WD gene had not yet been discovered. Currently we can suppose that probably the three WD types were based on genetic background of *ATP7B* with a Slavic type probably related to the p.H1069Q mutation which is most common among Eastern Europeans, and is generally thought as a phenotypically 'mild' mutation (Gromadzka *et al*. 2006). In the present study, we aimed to evaluate whether WDHzc with different p.H1069Q *ATP7B* mutational backgrounds differ with regards to phenotypic expression.

Initial disease signs develop early in WD patients before they reach their twenties (Ala *et al*. 2007). As WDHzc we studied were on about 2–3 times older than most WD patients manifesting first clinical symptoms, we might expect to observe any phenotypic signs due to cumulative over-time effects in case of abnormalities in Cu metabolism. Thus, we analysed non-invasive indices of liver function as well as evaluated the prevalence of neurological signs among studied WDHzc.

Materials and methods

Clinical assessment

Sixty-eight WDHzc were recruited from a group of our WD patients, parents. Additionally, 31 individuals of comparable age and sex who had no family history of WD diagnosed neurodegenerative or liver disease (chronic inflammatory disease or infectious disease were excluded), were chosen as control subjects (CS). All WDHzc were subjected to neurological examination. They were questioned about history of any liver

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disease and subjected to abdominal palpation. The research was approved by the local Ethics Committee.

Biochemical and genetic studies

Routine biochemical studies (blood alanine transaminase (ALT) and aspartate transaminase (AST)), as well as hematologic (international normalized ratio (INR) and blood platelets) evaluation were performed by standardized methods in the clinical laboratory of our institution. AST/ALT ratio (AAR) was also calculated. Serum ceruloplasmin concentration was measured using ceruloplasmin assay (with p-phenylenediamine as a substrate) following Ravin (1961). Serum Cu concentration and urinary Cu excretion were determined by atomic adsorption spectroscopy. All biochemical investigations were performed at the same laboratory according to the standardized procedures.

DNA analysis for the presence of the suspected mutation in *ATP7B* (based on the type of *ATP7B* mutations found in an index case within a given family) was performed in all cases included in this study. DNA analysis for the presence of the most frequent *ATP7B* mutations (c.3207C>A (p.H1069Q) and c.3402delC (p.A1135fs)) (Gromadzka *et al*. 2005) in the Polish population was performed using polymerase chain reaction / restriction fragments length polymorphism (PCR/RFLP), as described previously (Vrabelova *et al*. 2005) (c.3207C>A (p.H1069Q)), or according to our own method c.3402delC (p.A1135fs) (using primers: 5′-CCTTTCACTTCACCCCTCTT and 5′-GCTTTTGTCTCTGCAGCT, and restriction endonuclease *Fau*I (New England Biolabs). DNA analysis for other mutations in *ATP7B* was performed by direct sequencing of the respective exons, as previously described (Gromadzka *et al*. 2005).

Statistical analysis

Data were analysed using statistical package STATISTICA 8.0 (StatSoft, Cracow, Poland). The normality of analysed variables was determined using Kolmogorov–Smirnov and Lilliefors tests. As individual continuous variables were all not normally distributed among study subgroups, we present data as mean and standard deviation, as well as median (interquartile range; IQR), respectively. Data were subsequently compared among groups with parametric (Student's *t*-test) or nonparametric (Mann–Whitney U test) analysis. Categorical variables were compared among groups by the χ^2 test (with Yates' correction, if appropriate). The criterion for statistical significance was $P < 0.05$.

Results

Baseline characteristics of study participants

The mean age of study subjects was 55.5 years (range: 43–74) for *ATP7B* heterozygous individuals (42 females; 61.8% and 26 males; 38.2%), and 57.5 years (range: 34–74 years) for CS (17 females; 54.8% and 14 males; 45.2%). Age and gender differences among study groups were not statistically significant. Among WDHzc, 49 (72.1%) possessed the p.H1069Q mutation, and 19 (27.9%) had other non-p.H1069Q mutation (c.1958C>A (p.S653X), c.2975C>T (p.P992L), c.4022G>A (p.G1341D), c.2332C>G (p.R778G), c.3402delC (p.A1135fs), c.2304insC (p.M769fs), c.4051C>T (p.Q1351X), c.2337G>A (p.W779X)) in one *ATP7B* allele.

Cu metabolism parameters among WDHzc and CS

Using currently accepted cut-off points for normality (<20 mg/dL for serum ceruloplamin, < 70 μg/dL for serum Cu, and < 50 μg/24 h for Cu excretion in urine), Cu metabolism parameters were within normal ranges among CS. Among WDHzc, three had a little decreased serum ceruloplasmin (19.3 mg/dL, 19.0 mg/dL, and 19.5 mg/dL); of them, one had slightly decreased total serum Cu (66.0 μg/dL). Serum Cu and ceruloplasmin concentrations were significantly lower among WDHzc, and Cu excretion in urine were higher in them, than in CS (figure 1, a–c).

Among WDHzc, individuals having the p.H1069Q mutation in one allele of *ATP7B* had significantly higher serum ceruloplasmin than those having any other mutation (figure 1, d–f).

Non-invasive liver function parameters among WDHzc and CS

Among 31 CS, four had slightly increased blood ALT level (62.0 IU/L, 47 IU/L, 45 IU/L, 43 IU/L), and two had increased AST level (62 IU/L and 50 IU/L). INR was increased in two CS (1.23 and 3.8). None of the CS had abnormal blood platelets. Among WDHzc, two had slightly elevated ALT (42.5 IU/L, and 55.6 IU/L; normal: ≤ 38 IU/L)), one of them also had a little elevated AST (60.4 IU/L; normal: ≤ 40.0 IU/L), and INR (1.24; normal: < 0.9–1.2). Two other WDHzc had only a little elevated INR (1.25 and 1.9). Slightly decreased blood platelets were detected in two WDHzc (138×10^9/L, and 135×10^9/L; normal: $140–440\times10^9$/L). WDHzc had significantly lower blood ALT and tend to have lower AST, compared with CS (table 1). WDHzc possessing the p.H1069Q mutation had significantly lower median ALT (19.0 (IQR 9.4) versus 22.0 (IQR 18.0), respectively), as well as higher mean AAR (1.2 ± 0.4 versus 0.9 ± 0.2, respectively), than individual possessing other, non-p.H1069Q mutation.

Neurological findings in WDHzc

Neurological examination of heterozygotes revealed a slight postural hand tremor in five individuals, and a slight head tremor in one. Among these five individuals, in whom hand tremor was detected, one was alcoholic, two were previously diagnosed to have hyperthyreosis, and one had neurosis. All of them had the p.H1069Q mutation in one allele of *ATP7B*. None of these subjects had abnormal Cu metabolism

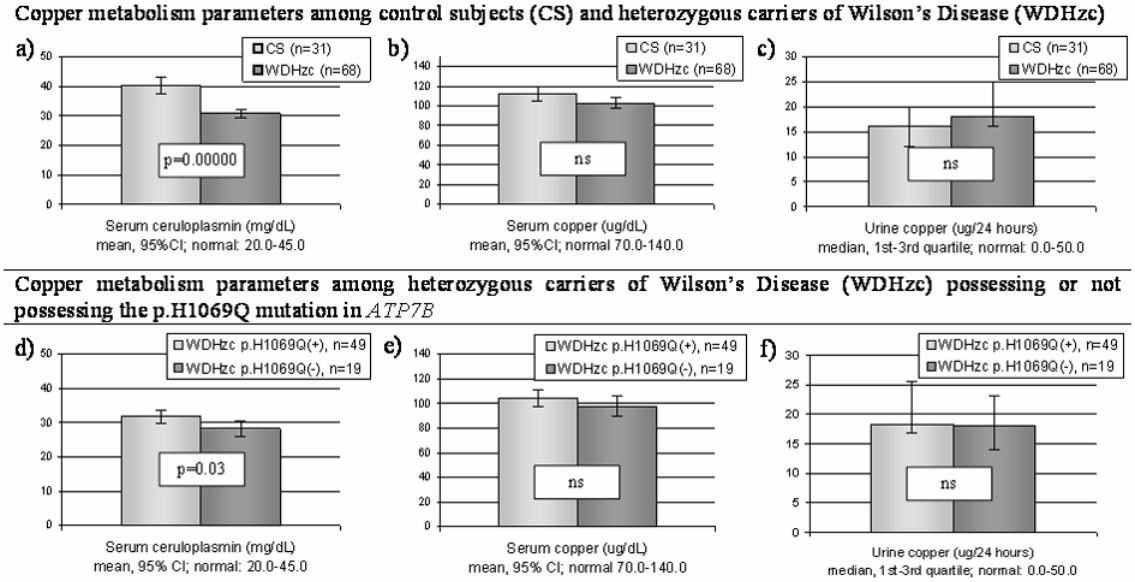

Figure 1. Copper metabolism parameters among control subjects and heterozygous carriers of Wilson's disease (WDHzc) with or without the p.H1069Q mutation in *ATP7B*.

Table 1. Non-invasive liver function parameters among heterozygous carriers of Wilson's disease (WDHzc) and control subjects.

Parameter	Control subjects (n = 31)	WDHzc (n = 68)	P
Alanine aminotransferases (ALT), IU/L (normal: 0.0–38.0)	26.2 (14.8)	20.0 (11.0)	0.014
Asparagine aminotransferase (AST), IU/L (normal: 0.0–40.0)	23.5 (8.6)	21.0 (4.0)	0.07
AST/ALT ratio	1.0 ± 0.3	1.1 ± 0.3	ns
International normalized ratio (INR) (normal: 0.9–1.2)	1.0 (0.1)	1.0 (0.1)	ns
Platelets $\times 10^9$/L (normal: 140.0–440.0)	234.1 ± 60.8	245.7 ± 66.7	ns

Mean ± SD (standard deviation) is presented for normally distributed variables, median (IQR) (interquartile range) is presented for not normally distributed variables; Student's t-test was used for group comparisons of normally distributed variables; Mann–Whitney U test was used for group comparisons of not normally distributed variables; ns, not significant.

parameters, and abnormal laboratory liver function parameters (data not shown).

Discussion

To date, some studies have been published on phenotypic manifestations in heterozygous carriers of WD. Cartwright *et al*. (1960) reported an increased serum concentration of non-ceruloplasmin bound Cu in one of four parents of their WD patients. Two other reports described abnormally low serum Cu levels in two of eight parents of WD cases (Heuyer *et al*. 1953; Neale and Fischer-Williams 1958); none of them exhibited hypercupriuria. Sternlieb *et al*. (1961) reported that out of 19 WD parents, two presented with decreased (< 20 mg/dL) serum ceruloplasmin (Sternlieb *et al*. 1961). In the study of 20 Icelandic heterozygotes (parents, siblings or children of WD patients) Tórsdóttir *et al*. (2009) did not observe difference in serum concentrations of ceruloplasmin and of total Cu compared with CS. In contrast, in the study

by Haghighat *et al*. (2008), of 31 parents of WD patients, six (19.4%) had decreased serum ceruloplasmin (< 25.0 mg/dL) and 14 (45.2%) had increased Cu excretion in urine (> 75.0 μg/24 h). However, the studies mentioned above seem to be weak in some aspects. First, number of studied heterozygotes was low in most of them. Second, individuals of different age were investigated, as parents, siblings and children of the WD patients were included. Third, study participants were not genetically characterized for mutations in *ATP7B* in any of these reports. It is of importance, because discrepancies between the results of individual studies might be a result of differences in *ATP7B* mutational background among individual populations.

Studying relatively high number of genetically confirmed WDHzc, all being parents of our WD patients, we documented that most of WDHzc had Cu metabolism parameters within normal range. However, they had significantly lower serum Cu and ceruloplasmin and significantly higher Cu excretion in urine, compared with CS.

Moreover, we firstly documented, that WDHzc possessing the p.H1069Q mutation in *ATP7B* have significantly higher serum ceruloplasmin than carriers of any other mutation. This result suggests that other mutations may be more deleterious and may predispose WDHzc to any phenotypic manifestations more strongly than the p.H1069Q. Perhaps this study should be repeated among populations with a higher frequency of mutations considered 'severe', since in WDHzc possessing such mutations may have more distinct phenotypic expressions.

When analysing laboratory indices of liver function, we observed that WDHzc had lower median blood ALT, and tend to have lower median AST than CS. This observation needs confirmation in a larger population, as it may be simply due to chance. However, it seems be in line with the theory formulated by Nevsímalová *et al*. (1986) who hypothesized that any control mechanisms may play a role in the normalization of biochemical and clinical findings in WDHzc. However, it has to be remembered that liver function tests are strongly influenced by genetic factors, body mass index, gender, age and alcohol, and all these factors might confound our study results.

In a previous study, we detected metabolic alterations within basal ganglia in 12 WDHzc, using proton magnetic resonance spectroscopy (^{1}H MRS) (Tarnacka *et al*. 2009). The profile of these changes had suggested that they might be related to toxic Cu accumulation in the brain. In the current study, we looked for neurological signs among WDHzc. We have detected slight hand or head tremor in six of them. However, in four of them the tremor was probably related to other health problems, and not to mutation in *ATP7B* allele. In only two WDHzc the reason of tremor was difficult to explain. However, it has to be remembered that the prevalence of essential tremor in normal middle-aged population is about 3%–6% (Benito-León 2009). A previous report on this topic, described non-progressive predominantly postural tremor of both arms in a mother of a WD patient (Nicholl *et al*. 2001). The other study documented akinetic-rigid syndrome and cognitive deficits in a 36-year-old sister of a WD patient, and a third described dystonia in patients with metabolic abnormalities suggestive of being heterozygotic for WD (Quinn and Marsden 1986). Unfortunately, genetic studies were not performed in any of these individuals to definitively exclude WD.

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