Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

# Genetic variability in the methylenetetrahydrofolate reductase gene (*MTHFR*) affects clinical expression of Wilson's disease

Grażyna Gromadzka[1,2,*], Magdalena Rudnicka[2], Grzegorz Chabik[1], Adam Przybyłkowski[2], Anna Członkowska[1,2]

[1]*Institute of Psychiatry and Neurology, Second Department of Neurology, Warsaw, Poland;*
[2]*Medical University, Department of Experimental and Clinical Pharmacology, Warsaw, Poland*

See Editorial, pages 753–755

**Background & Aims**: Wilson's disease (WND) is an autosomal recessive disorder of copper (Cu) transport, resulting from pathogenic mutations in the *ATP7B* gene. The reason for the high variability in phenotypic expressions of WND is unknown. Hepatotoxic and neurotoxic effects of homocysteine (Hcy), as well as interrelationships between Hcy and Cu toxicity, were documented.

**Methods**: We genotyped the two 5,10-methylenetetrahydrofolate reductase (one of the key folate/Hcy pathway enzymes) gene (*MTHFR*) polymorphisms: C677T and A1298C in 245 WND patients. Next, we tested the modulation of WND phenotypes by genotypes of *MTHFR*.

**Results**: *MTHFR* C677T genotype distribution deviated from that expected from a population in Hardy–Weinberg equilibrium (C677T, $\chi^2 = 12.14$, $p = 0.0005$). Patients with the *MTHFR* 1298C allele were younger at symptoms' onset than those without this allele (median (IQR) age, 24.9 (14.0) years *vs.* 28.5 (12.0) years, $p = 0.006$). Carriers of *MTHFR* "high activity" diplotype (double wild-type homozygotes 677CC/1298AA) manifested WND at older age, than non-carriers (median (IQR) age, 33.5 (9.0) years *vs.* 25.0 (13.0) years, $p = 0.0009$). Patients with the *MTHFR* 677T allele less frequently exhibited the neurological WND phenotype (31 (29.5%) *vs.* 36 (48.0%)), and more frequently presented with hepatic WND (44 (41.9%) *vs.* 22 (29.3%)), compared with subjects *MTHFR* 677T(−).

**Conclusions**: We postulate that *MTHFR* polymorphism contributes to the phenotypic variability of WND.



## Introduction

Wilson's disease (WND) (OMIM #277900) is an autosomal recessive copper (Cu) storage disease [1–3]. The WND gene *ATP7B* encodes a Cu-transporting P-type ATPase (ATP7B) (OMIM ∗606882) [4]. In persons with pathogenic mutation in both *ATP7B* alleles, a severe dysfunction of ATP7B results in excessive Cu accumulation mainly in the hepatocytes and in the brain, resulting in hepatic and/or neuropsychiatric signs and symptoms of WND [1–3].

Considerable phenotypic variability was observed among WND patients possessing the same type of mutations in *ATP7B* [5–8], as well as among WND-affected family members [9,10], including monozygotic twins [11,12]. So, it is suggested that the phenotypic variability in WND is due to other modifying factors. Three previous studies revealed association between genetic variation at the *APOE* (apolipoprotein E) structural gene locus [13,14], as well as at the Prp (human prion protein) gene (*PRNP*) codon 129 polymorphism (Val129Met) [15] and age at onset of WND symptoms. None of the analyzed genetic variabilities had an impact on the mode of clinical manifestation of the disease.

Irrelevant of etiology, liver disease accounts for disturbances in homocysteine (Hcy) metabolism and hyperhomocysteinemia (HHcy) [16], which maintain liver damage, as increased cellular concentrations of Hcy create a strong oxidative stress. On the other hand, the ability of Hcy to pass the blood–brain barrier (BBB), and its dose-dependent neurotoxic effects were documented [17,18]. Some observations suggest a strong interrelationships between Hcy and Cu metabolism and toxicity [18–21]. Hcy metabolism is under genetic control. Two common polymorphisms have been identified in the gene encoding the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) (OMIM

Keywords: ATPase7B (ATP7B); Copper; Genotype; Homocysteine; 5,10-Methylenetetrahydrofolate reductase (MTHFR); Phenotype; Polymorphism; Wilson's disease.
*Received 23 February 2010; received in revised form 21 December 2010; accepted 4 January 2011; available online 18 February 2011*
☆ DOI of original article: 10.1016/j.jhep.2011.02.025.
∗ Corresponding author. Address: Institute of Psychiatry and Neurology, Second Department of Neurology, Sobieskiego 9, 02-957 Warsaw, Poland. Tel.: +48 22 4582593; fax: +48 22 4582593.
*E-mail addresses:* gromadz@ipin.edu.pl, gragrom@gmail.com (G. Gromadzka).
*Abbreviations:* WND, Wilson's disease; OMIM, Online Mendelian Inheritance in Man; Cu, copper; ATP7B, ATP-ase 7B; ApoE, apolipoprotein E; Prp, human prion protein; Hcy, homocysteine; HHcy, hyperhomocysteinemia; BBB, blood–brain barrier; MTHFR, 5,10-methylenetetrahydrofolate reductase; NCBI, National Center for Biotechnology Information; SNP, single nucleotide polymorphism; HWND, hepatic form of WND; NWND, neuropsychiatric form of WND not associated with liver disease; HWND, neuropsychiatric form of WND associated with liver disease; DNA, deoxyribonucleic acid; PCR/RFLP, polymerase chain reaction/restriction fragments length polymorphism; HWE, Hardy–Weinberg equilibrium; ANOVA, analysis of variance; IQR, interquartile range.

*607093), one of the key folate/Hcy pathway enzymes: C677T in exon 4 (NCBI SNP ID: rs 1801133) [22] and A1298C in exon 7 (NCBI SNP ID: rs 1801131) [23]. Both polymorphisms are thought to be associated with decreased MTHFR activity resulting in HHcy [24,25]. The *MTHFR* C677T polymorphism results in an alanine to valine amino acid substitution (A222V) within the N-terminal catalytic domain of the enzyme. The MTHFR form, encoded by the 677T allele, has decreased enzymatic activity (by approximately 30% in heterozygotes and 70% in homozygotes compared to controls) [22,25]. The *MTHFR* A1298C polymorphism results in a substitution of glutamate for alanine (E429A) within the C-terminal regulatory domain [26]. This polymorphism is thought to affect the regulation of MTHFR, possibly by *S*-adenosylmethionine, an allosteric inhibitor of MTHFR that binds to the C-terminal region [23,26].

The objective of this study was to evaluate the *MTHFR* genotype as a modifier of Cu metabolism as well as of phenotypic effects of Cu toxicity, represented by age of patients at WND onset and mode of disease manifestation.

## Patients and methods

### *Patients*

Two hundred and forty-five consecutive WND patients were included in this study. The scoring system for the diagnosis of WND based on clinical signs and symptoms was used. WND diagnosis was based on the presence of Kayser–Fleischer rings, laboratory evidence of impaired Cu metabolism (abnormal results of tests for: serum ceruloplasmin concentration (assessed with colorimetric enzymatic assay by Ravin [27]), serum Cu concentration, and Cu excretion in urine (determined by Atomic Adsorption Spectroscopy)) and genetic evidence of the presence of pathogenic mutations in both *ATP7B* alleles [28]. All patients 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 Declaration of Helsinki.

### *Clinical phenotyping*

The first onset of WND clinical symptoms was established by WND experts using a standard patient questionnaire as well as evaluating whole available patients' medical history (including laboratory tests results as well as data on the hepatic and neuropsychiatric signs and symptoms at diagnosis or in the history); data was collected according to the protocol established for the EuroWilson project database (http://www.eurowilson.org). According to the mode of initial disease manifestation, patients were grouped as hepatic (HWND), or neuropsychiatric presenting (NHWND) or not presenting (NWND) with liver disease. Patients were categorized as HWND when presenting any signs of chronic or acute liver disease (increased liver enzyme activity with increased blood bilirubin and abnormal INR, and/or changes in liver echogenicity; signs of portal hypertension, decompensated liver cirrhosis or acute liver failure) without neurological symptoms. Patients were categorized as NWND when having typical neurological symptoms, such as tremor, dystonia, ataxia, dysarthria, rigidity, or psychiatric symptoms, including behavioral abnormalities, depression, manic psychosis or cognitive impairment, and not manifesting with clinical signs of liver cirrhosis or impaired liver function. Patients were classified as NHWND when having neuropsychiatric presentation associated with symptomatic liver disease. Some patients were diagnosed presymptomatically (they were identified by family screening).

### *Genetic studies*

#### *Mutational analysis of ATP7B*

DNA analysis for the three frequent *ATP7B* mutations in the Polish population [8] was performed using polymerase chain reaction – restriction fragments length polymorphism method (PCR-RFLP), as previously described (mutation p.H1069Q [c.3207C >A]) [5] or using primers: 5′-CCTTTCACTTCACCCCTCTT-3′ and 5′-GCTTTTGTCTCTGCAGCT-3′, and restriction endonuclease *Fau*I (New England Biolabs) (p.A1135fs [c.3402delC]), or 5′-GTCACGTTGTGTCCAGTGC-3′ and 5′-GAGTGGCTCTCAGGCTTTTC-3′, and restriction endonuclease *Pst*I (Eurx, Gdansk, Poland) (p.Q1351X [c.4051C >T]). Such strategy did not allow the identification of mutations in one allele in 15 patients (6.1%), and in both alleles in 1 patient (0.4%). In previous studies, 100% mutation detection rate by *ATP7B* gene DNA sequencing was never reached in WND patients. For example, in the study by Ferenci *et al.*, no mutation was found in 6 out of the 46 (13.0%) tested patients and only 1 mutation was detected in additional 13 patients (28.8%) [29]. In another study, DNA sequencing did not allow the identification of 19.7% of *ATP7B* mutations among studied WND patients [5]. It is hypothesized that unknown molecular defects may be present in the intronic regions of the *ATP7B* gene, and may have an effect on splicing.

#### *Genotyping of MTHFR C677T and A1298C polymorphisms*

Two functional *MTHFR* gene variants were genotyped in genomic DNA samples: C677T and A1298C. *MTHFR* C677T genotypes were determined using the TaqMan genotyping assay as described by Ulvik *et al.* [30]; genotyping of *MTHFR* A1298C polymorphism was performed using the PCR/RFLP technology by Weisberg *et al.* [31].

### *Statistical analysis*

Data were analyzed using the statistical package STATISTICA 8.0 (licensed by StatSoft PL, Cracow, Poland). To test for 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 $\chi^2$ test (Hardy–Weinberg Calculator by Michael H. Court was used). The normality of analyzed continuous variables was determined using Kolmogorov–Smirnov and Lilliefors tests. Variables that were normally distributed were compared between groups by Student *t* test. Variables that were not normally distributed were presented as a median and interquartile range (IQR) and compared between groups with Kruskal–Wallis ANOVA (with post hoc testing using Mann–Whitney *U*-test) or by Mann–Whitney *U*-test (two-way variables). Categorical variables were compared between groups using $\chi^2$ test or Fisher exact test. We stratified the data by individual, as well as combined *MTHFR 677* and *1298* genotypes, and estimated their relation with the individual phenotypic features of WND in our patients' cohort. The combined effects of *MTHFR 677* and *1298* were calculated using individuals who were wild-type homozygous for the enzyme activity at both *loci* ("high activity diplotype" 677CC/1298AA), as the referent group. To examine the independent effect of C677T, we repeated our analysis after patients' stratification into homozygotes AA at codon 1298 and carriers of the 1298C allele (AC + CC genotypes). To examine the independent effect of A1298C, we performed additional analysis with patients' stratification into homozygotes 677CC and carriers of the 677T allele (CT + TT genotypes). To test for the difference in genotype frequency between WND patients and two control groups of Polish origin (data from published papers [32,33]), an application "other tests for significance", available in the Statistica package, was used. The criterion for significant difference was $p < 0.05$.

## Results

### *Baseline genotypic and phenotypic patients' characteristics*

A total of 245 patients were enrolled in this study (Table 1). In 14 patients, the mode of initial disease manifestation was not noticed, and in 11 symptomatic patients, the age at WND onset was not established (lack of enough information). Fifty-one patients (22.1%) were diagnosed presymptomatically. Of the 180 symptomatic patients, 67 (37.2%) were classified as HWND, 66 (36.7%) as NWND, and 47 (26.1%) as NHWND.

The most frequent mutations we found in the *ATP7B* were: c.3207C >A [p.H1069Q] (73.0 % of all alleles; 135 individuals (55.3%) were homozygous for this mutation), c.4051C >T [p.Q1351X] (5.3%), c.3402delC [p.A1135fs] (4.0 %), c.2962G >C [p.G988R] (1.5%), and c.3818C >T [p.1273L] (1.1%). The other mutations were detected in less than 1% of alleles.

Patients with WND showed a distribution of the *MTHFR* C677T genotype that deviated significantly from a distribution expected in a population with genotype distribution in HWE ($p = 0.0005$;