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ASSOCIATION OF VARIANTS IN THE *CP*, *ATOX1* AND *COMMD1* GENES WITH WILSON DISEASE SYMPTOMS IN LATVIA

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ABSTRACT

Wilson's disease (WD) is a copper metabolism disorder, caused by allelic variants in the *ATP7B* gene. Wilson's disease can be diagnosed by clinical symptoms, increased copper and decreased cerulopasmin levels, which could all also be by other genetic variants beyond the *ATP7B* gene, *e.g.,* disturbed ceruloplasmin biosynthesis can be caused by pathogenic allelic variants of the *CP* gene. Copper metabolism in the organism is affected by several molecules, but pathogenic variants and related phenotypes are described with *COMMD1* and *ATOX1* genes. The aim of the study was to test other genes, *CP*, *ATOX1* and *COMMD1*, for possible influence to the manifestation of WD. Patients were enrolled on the basis of Leipzig's diagnostic criteria, 64 unrelated patients with confirmed WD. Direct sequencing of promoter region of the *CP* gene and *ATOX1* and *COMMD1* gene exons was conducted. Statistically significant differences were found between the two variants in the *CP* gene and the *ATP7B* genotype (rs66508328 variant AA genotype and the rs11708215 variant GG genotype) were more common in WD patients with an unconfirmed *ATP7B* genotype. One allelic (intronic) variant was found in the *ATOX1* gene without causing the functional changes of the gene. Three allelic variants were identified in the *COMMD1* gene. No statistically significant differences were found between allele and genotype frequencies and the first clinical manifestations of WD. Different variants of the *CP* gene contributed to a WD-like phenotype in clinically confirmed WD patients with neurological symptoms and without identified pathogenic variants in the *ATP7B* gene. Allelic variants in the *ATOX1* and *COMMD1* genes do not modify the clinical manifestation of WD in Latvian patients. (266 words)

Keywords: Copper metabolism; Decreased blood ceruloplasmin; Wilson's disease (WD).

INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder of copper metabolism, caused by allelic variants of the *ATP7B* gene. Wilson's disease has been established as a monogenic disorder, although heterogeneity in phenotype is observed even in patients possessing the same type of allelic variants in the *ATP7B* gene, leading to the assumption about other genetic modifiers affecting the WD phenotype [1]. There are many studies looking for factors modifying the clinical presentation of WD, such as allelic variants in the genes *COMMD1*, *ATOX1*, *XIAP*, *APOE DMT1* (*SLC11A2*), *ATP7A*, *MTHFR*, *ESD*, *INO80* and *PRNP* genes, as well as changes in epigenetic mechanisms of gene expression regulation [2-4]. Moreover, as the main pathogenetic mechanism is copper metabolism disorder, in this study we analyzed pathogenic variants in the genes affecting copper metabolism; there are previously described pathogenic variants and related phenotypes with the *COMMD1* and *ATOX1* genes [5,6].

One of the criteria of WD diagnostics is decreased ceruloplasmin level. Disturbed ceruloplasmin biosynthesis caused by pathogenic allelic variants of the *CP* gene leads to decreased ceruloplasmin level in the blood, which disrupts iron metabolism, resulting in iron accumulation in various organs, especially basal ganglia, causing serious neuronal damage. Iron accumulation in the brain leads to neurodegeneration and neurological symptoms such as motor disorientation and other motor deficits in the age range of 45 to 55 years [7]. The association of the *CP* gene allelic

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variants and different diseases is described in various studies of Parkinson's disease [8] and atrial fibrillation (AFib) [9,10], concluding that changes in the *CP* gene promoter may be associated with altered levels of ceruloplasmin.

MATERIALS AND METHODS

This study protocol was approved by the Latvian Central Medical Ethics Comittee and was performed according to the Declaration of Helsinki. Informed consent was obtained from all patients. In the study, 64 unrelated patients with confirmed WD, were enrolled on the basis of Leipzig's diagnostic criteria [11]. All patients included in the study carried at least four points according to the WD scoring system. According to the initial symptoms, WD patients were categorized in the following groups, as described elsewhere [12]: asymptomatic, hepatic, neurological/psychiatric and neurological/hepatic.

Genomic DNA was extracted from peripheral blood by a standard phenol/chloroform method. Allelic variant c.3207 C>A; p.H1069Q (rs76151636) was tested by polymerase chain reaction (PCR)-BiPASA (bidirectional PCR of specific alleles) [13].

Direct DNA sequencing of the *ATP7B* gene (promoter, exons and exon-intron boundaries) was performed for the patients with WD symptoms, being either heterozygous for H1069Q or without it on any allele. Methods for WD molecular confirmation and the group of WD patients have been described before in detail [14,15].

Direct Sequences of the *ATOX1*, *COMMD1* and *CP* Genes. For sequencing of the *CP* gene promoter region, *ATOX1* and *COMMD1* gene exons and adjacent introns, primers selected using the open access Primer 3 program (available at http://bioninfo.ut.ee/primer3-0.4.0/) and previously reported primers were used [6,8]. For the genetic variants nomenclature, traditional names were used (if existing), or HGVS nomenclature according to reference sequences: *CP* gene (NM_000096.3, NP_000087.1); *ATOX1* gene (NM_004045.3, NP_004036.1); *COMMD1* gene (NM_152516.3, NP_689729.1). Allele frequencies in the European population were obtained from GnomAD database (https:/gnomad.broadinstitute.org/) [16]. For Linkage data, the 1000 Genome browser (https://www. international genome.org) was used [17]. If a variant was not reported to have a functional effect, it was characterized according to the American College of Medical Genetics and Genomics (ACMG guidelines [18], including *in silico* tools. The *CP* gene promoter region (not all of the gene) sequencing was selected based on information in other studies concluding that changes in the *CP* gene promoter may be associated with altered levels of ceruloplasmin [8].

Statistical Analyses. Data processing was performed using the Statistical Package for the Social Sciences (IBM SPSS®) version 22 (https://www.ibm.com/SPSS-Statistics/Software), and PLINK 1.07 (http://zzz.bwh.harvard.edu/plink/). The comparison between patient groups was done by Fisher exact *t*-test. The frequencies of genotypes in the WD patient population before further data analysis were tested for Hardy-Weinberg equilibrium. The association of the *COMMD1* and *CP* gene variants with WD phenotype was analyzed in the allelic, dominant, recessive and genotypic inheritance model.

RESULTS

The majority of the WD patients expressed hepatological symptoms as the first manifestation (detailed segregation of the WD patients according to their first symptoms and age of onset can be found in Table 1). A detailed description of WD patient genotypes in the *ATP7B* gene, clinical and biochemical findings can be found in the Supplemental Table 1. All identified variants corresponded to the Hardy-Weinberg equilibrium in the analyzed WD patient group ($p > 0.05$).

The *CP* Gene. In the *CP* gene promoter, seven allelic variants were observed: rs66508328, rs67870152, rs16861642, rs73020328, rs73166855, rs66953613 and rs11708215. As the first six of the variants mentioned above are in strong linkage ($r^2 = 1$) (see Table 2), one of them was analyzed, rs66508328 along with rs11708215 ($r^2 = 0.562$ from the 1000 genome project) [17], the frequency of which differed from the other variants.

None of the variants in the WD patients was statistically significantly different from those described in the GnomAD

Table 1. Segregation of Wilson's disease patients in the Latvian population according to their clinical findings.

Parameters	Hepatologiacal ($n = 42$)	Neurological and/or Psychiatric ($n = 17$)	Mixed ($n = 5$)	*p* Value
Level of ceruloplasmin in blood (g/dL) [median (IQR)]	0.11 (0.08-0.13)	0.13 (0.08-0.15)	0.08 (0.07-0.15)	0.562
24-hour urine copper [median (IQR)]	197.0 (136.0-373.0)	163.0 (96.0-268.0)	374.0.0 (365.0-384.0)	0.370
Age of onset [average (±SD)]	20.21(±9.10)	29.82(±13.25)	36.00(±8.75)	0.066

IQR: interquartile range; SD: standard deviation.

database. Next, the frequency of alleles and genotypes (according to different inheritance models) was compared with the first clinical manifestation and genotype of the *ATP7B* gene (see Table 3). No statistically significant differences were found between allele frequencies and the first clinical manifestations of WD. Statistically significant differences were found between the two analyzed variants and the *ATP7B* genotype, rs66508328 variant AA genotype and the rs11708215 variant GG genotype (both according to recessive inheritance model) were more common in WD patients with the unconfirmed genotype by *ATP7B* gene molecular testing.

The *ATOX1* Gene. Four patients (out of 64) were found to have one allelic (intronic) variant, rs571657964 in a heterozygous state [minor alle frequency (MAF) for patients = 0.023; MAF in European (non Finnish) population = 0.0058] [16]. Analyzing the variant in the Human Splicing Finder software (http://www.umd.be/HSF/) [19], it was predicted that the allelic variant had no effect on splicing, thus the allelic variant was not included in further statistical analyses, as it was very unlikely to cause functional changes of the *ATOX1* gene.

The *COMMD1* Gene. Three allelic variants were identified: rs569267407, rs55677935 and rs9096. The frequency of allelic variants of the *COMMD1* gene found in this study was compared to the GnomAD database. See results in Table 4.

Table 2. Frequency of two selected allelic variants of the *CP* gene promoter identified in Wilson's disease patients.

Parameters	**rs66508328**	**rs11708215**
Alleles	G>A	A>G
MAF (this study)	0.0902	0.1750
MAF (GnomAD), European (non Finnish population)	0.1068	0.2078
p Value (odds ratio)	0.550 (1.2070)	0.3771 (1.2369)
Genotypes	2: AA; 7: AG; 52: GG	2: GG; 17: GA; 41: AA
p Value	0.0608	1.0000

MAF: minor allele frequency.

Table 3. Relation of the *CP* gene promoter allelic variants to Wilson's disease phenotype and the *ATP7B* genotype.

	Inheritance Model	***ATP7B* Gene Genotype**		***p* Value**
		Non WD[a] (*n* = 12)	WD[b] (*n* = 49)	
rs66508328	Genotype (AA/AG/GG)	2: AA; 0: AG; 10: GG	0: AA; 7: AG; 42: GG	**0.0289**
	Allele (A/G)	4: A; 20: G	7: A; 91: G	0.2240
	Dominant (AA+AG/GG)	2: AA+AG; 10: AG/GG	7: AA+AG; 42: AG/GG	1.0000
	Recessive (AA/AG+GG)	2: AA/AG; 10: AG+GG	0: AA/AG; 49:AG+GG	**0.0361**
rs11708215	Genotype (GG/GA/AA)	2: GG; 0: GA; 10: AA	0: GG; 17: GA; 31: AA	**0.0016**
	Allele (G/A)	4: G; 20: A	17: G; 79: A	1.0000
	Dominant (GG+GA/AA)	2: GG+GA; 10: GA/AA	17: GG+GA; 31: GA/AA	0.3059
	Recessive (GG/GA+AA)	2: GG/GA; 10: GA+AA	0: GG/GA; 48: GA+AA	**0.0373**

[a] Non WD: patients who did not have two pathogenic variants in the *ATP7B* gene.

[b] WD: patients with two pathogenic variants in the *ATP7B* gene.

Table 4. Variants found in the *COMMD1* gene in Wilson's disease patients.

Variant	**rs569267407**	**rs55677935**	**rs9096**
Reference: NM_152516.3	c.-68_67delTT	c.358C>T	c.492C>T
Reference: NP_689729.1	–	p.Arg120Trp	p.Asp164Trp
Location in the *COMMD1* gene	intron 1	exon 2	exon 3
MAF (this study)	0.0088	0.0440	0.0968
MAF (GnomAD), European (non Finnish population)	0.0020	0.0215	0.1263
p Value	0.1469	0.1553	0.3247

MAF: minor allele frequency.

The frequencies of *COMMD1* variants did not significantly differ from the frequencies listed in the GnomAD database. As the variant rs569267407 is located in the non coding part of the gene, its potential effect on gene splicing was analyzed (using the Human Splicing Finder software) [19]. As the impact was not predicted, the variant was not further analyzed. The frequency of alleles or genotypes (by different inheritance models) was compared to the first clinical symptoms of WD patients (taking into account only those patients with molecularly confirmed WD, $n = 49$). No statistically significant differences were found between allele and genotype frequencies and the first clinical manifestations of WD. Comparing the incidence of both variants (in the dominant inheritance model) and the age of first symptoms, no differences were observed (rs9096 $p = 0.112$; rs55677935 $p = 0.146$); results are shown in Table 5.

Table 5. Relation of the allelic variants in the *COMMD1* gene to the Wilson's disease phenotype.

Variant	Allele 1	Allele 2	Inheritance Model	Neurological Symptoms (*n*=7)	Hepatological Symptoms (*n*=42)	*p* Value
rs55677935	C	T	Genotype (TT/TC/CC)	0: TT; 0: TC; 7: CC	1: TT; 2: TC; 39: CC	1.0000
			Allele (T/C)	0: T; 14: C	4: T; 80: C	1.0000
			Dominant (TT+TC/CC)	0: TT+TC; 7: TC/CC	3: TT+TC; 39: TC/CC	1.0000
			Recessive (TT/TC+CC)	0: TT/TC; 7: TC+CC	1: TT/TC; 41: TC+CC	1.0000
rs9096	T	C	Genotype (CC/CT/TT)	0: CC; 1: CT; 6: TT	1: CC; 6: CT; 35: TT	1.0000
			Allele (C/T)	1: C; 13: T	8: C; 76: T	1.0000
			Dominant (CC+CT/TT)	1: CC+CT; 6: CT/TT	7: CC+CT; 35: CT/TT	1.0000
			Recessive (CC/CT+TT)	0: CC/CT; 7: CT+TT	1: CC/CT; 41: CT+TT	1.0000

DISCUSSION

In our previous study, not all of the WD cases were confirmed molecularly [15]. One explanation of undiagnosed WD in a molecular level could be the fact that possibly WD patients with clinically confirmed WD diagnosis actually might have Wilson's disease-like disease. Wilson's disease clinical and laboratory diagnostic criteria were developed in 2001 and have not changed since. Several studies have been published on the revision of the guidelines and criteria [20] but remain unchanged at the European level [11]. Two of the criteria for WD diagnostics are reduced levels of ceruloplasmin in the blood and neurological symptoms. In case of WD, the levels of ceru-loplasmin are reduced due to copper accumulation, but other causes, such as aceruloplasminemia and malabsorption, may also cause reduced levels of ceruloplasmin [11]. Detection of ceruloplasmin level in the blood is recommended as a first step in WD diagnostics [21]. Very low levels of ceruloplasmin in the blood (<5 mg/dL) are highly associated with WD, but such low levels may also be found in other diseases, *e.g.,* aceruloplasminemia caused by pathogenic allelic variants in the *CP* gene. Neurological symptoms of aceruloplasminemia can mimic WD, but this is actually due to the accumulation of iron [20]. In such cases, to confirm aceruloplasminemia, serum ceruloplasmin levels of first-degree relatives should be checked (serum ceruloplasmin is also reduced in heterozygous patients with pathogenic allelic variants), as well as patients with aceruloplasminemia generally found to have reduced serum iron levels, increased ferritin levels, diabetes and evidence of iron accumulation in magnetic resonance imaging in the brain [22]. In view of lack of the aforementioned changes in symptoms and laboratory parameters in WD patients without a molecularly confirmed diagnosis, the diagnosis of aceruloplasminemia is questionable. The association of allelic variants in the *CP* gene and different diseases are described in various studies: with Parkinson's disease [8] and AFib [9,10], concluding that changes in the *CP* gene promoter may be associated with altered levels of ceruloplasmin. Analyzing the *CP* gene promoter in the current study, it was concluded, that the AA genotype of the rs66508328 variant and the GG genotype of rs11708215 (both according to the recessive inheritance model) were more common in patients who were not confirmed molecularly to have WD according to the *ATP7B* genotype. This may indicate that the above-mentioned variants in the *CP* gene could affect gene expression, resulting in reduced levels of ceruloplasmin in the blood, that in turn, leads to increased accumulation of iron in the brain, causing Parkinson's disease-like symptoms, which are also characteristic of WD. The results observed here points to a further development of the study by increasing the size of the patient group, and although the differences were statistically significant, due to the small size of the groups, the data could have been accidental.

Considering the important interaction of ATOX1 protein with ATP7B protein, there have been several studies looking for possible changes in the *ATOX1* gene in WD

patients, but so far, the results have been negative [5,1]. In this study, there were no identified changes in the *ATOX1* gene causing functional changes of the protein, so we were unable to prove that changes in the *ATOX1* gene could modify the WD phenotype.

In studies of changes in other genes that could affect the clinical manifestations of WD, the researchers focused on the *COMMD1* gene, in which pathogenic variants have been shown to cause copper toxicosis in Bedlington terriers that is clinically similar to WD in humans [23]. There have been several studies analyzing the possible effects of this gene variants on humans. Stuehler *et al.* [24] revealed that the substitution of the nucleotide in the position c.492T>C (p.Asn164 or rs9096) is associated with earlier (about 10 years) expression of clinical symptoms in WD patients. In this study, the above-described variant was also found in Latvian patients. Analyzing its possible association with clinical manifestations of the disease, the relationship was not found, so the above observation was not confirmed. In another study in 2010, Gupta *et al.* [6] reported the effect of another variant, c.521C>T (p.Thr 174Met; rs139775239), on the WD phenotype; they associate this variant with more elevated levels of copper in the urine, as well as with enhanced cell apoptosis in WD patients. In the current study, no such variant was found in the *COMMD1* gene in any of the Latvian patients. There have been several studies with the effect of *COMMD1* gene variants on WD, but unfortunately, no association with these studies was confirmed [25,26], the same as in present study.

CONCLUSIONS

1) The difference variants of the *CP* gene contribute to a WD-like phenotype in clinically confirmed WD patients with neurological symptoms and without pathogenic variants in the *ATP7B* gene, but for confirmation, a larger study group is required; ***2)*** no changes were found in the *ATOX1* gene that would cause functional changes of the protein; ***3)*** no statistically significant differences were found between allele and genotype frequencies in the *COMMD1* gene and the first clinical manifestations of WD; ***4)*** allelic variants in the *ATOX1* and *COMMD1* genes do not modify the clinical manifestation of WD in Latvian patients.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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