ORIGINAL ARTICLE

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Synonymous mutation in adenosine triphosphatase copper-transporting beta causes enhanced exon skipping in Wilson disease

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Abstract

Wilson disease (WD) is caused by biallelic pathogenic variants in adenosine triphosphatase copper-transporting beta (ATP7B); however, genetic testing identifies only one or no pathogenic ATP7B variant in a number of patients with WD. Synonymous single-nucleotide sequence variants have been recognized as pathogenic in individual families. The aim of the present study was to evaluate the prevalence and disease mechanism of the synonymous variant c.2292C>T (p.Phe764=) in WD. A cohort of 280 patients with WD heterozygous for a single ATP7B variant was investigated for the presence of c.2292C>T (p.Phe764=). In this cohort of otherwise genetically unexplained WD, the allele frequency of c.2292C>T (p.Phe764=) was 2.5% (14 of 560) compared to 7.1×10^{-6} in the general population (2 of 280,964 in the Genome Aggregation Database; $p < 10^{-5}$; Fisher exact test). In an independent United Kingdom (UK) cohort, 2 patients with WD homozygous for p.Phe764= were identified. RNA analysis of ATP7B transcripts from patients homozygous or heterozygous for c.2292C>T and control fibroblasts showed that this variant caused high expression of an ATP7B transcript variant lacking exon 8. Conclusion: The synonymous ATP7B variant c.2292C>T (p.Phe764=) causes abnormal messenger RNA processing of ATP7B transcripts and is associated with WD in compound heterozygotes and homozygotes.

INTRODUCTION

Wilson disease (WD) is an autosomal recessive disorder of copper transport, utilization, and storage with variable presentation.[1] Diagnostic criteria based on clinical, biochemical, and genetic findings have been proposed and adopted in society guidelines.[2,3]

Adenosine triphosphatase copper-transporting beta (ATP7B) analysis is important in the diagnostic workup, especially if clinical symptoms or laboratory findings are ambiguous. The detection of two pathogenic or likely pathogenic variants on both chromosomes confirms the diagnosis of WD. However, in large cohorts of Caucasian patients with WD, pathogenic variants of both ATP7B alleles were only detected in about 80% of tested patients.[4,5] Patients with suspected WD and only a single heterozygous ATP7B variant detected represent a diagnostic challenge. In such patients, diagnosis can be ascertained if typical clinical symptoms or laboratory abnormalities are present. A clear distinction between patients with WD and true heterozygotes can be difficult if symptoms or laboratory tests are ambiguous. In

patients with WD-typical clinical presentation and laboratory findings, pathogenic ATP7B variants can also be located in the promoter or in intronic regions of the gene.[6–8]

To date, 1332 exonic and intronic ATP7B sequence variants have been classified (in UniProt, VarSome, ClinVar, and MEDLINE) based on scoring by American College of Medical Genetics and Genomics (ACMG) criteria[9]; 455 of those are considered pathogenic, 163 are possibly pathogenic (including one synonymous variant), 531 are variants of unknown significance (including 68 synonymous variants), 105 are possibly benign, and 99 are benign (VarSome,[10] as of April 18, 2021).

Synonymous variants that do not modify the amino acid sequence of the protein product are frequently considered benign but can be disease causing if they affect highly conserved nucleotides or splice consensus sequences.[9] Two patients with WD from Bulgaria were reported as heterozygous for variant c.2292C>T (p.Phe764=).[11] This synonymous variant is listed with conflicting interpretations on the functional consequences in the clinical genetic database

ClinVar (variation, VCV000157937.7, as of July 12, 2021),[12] but no functional studies on this variant have yet been reported. The private synonymous variant c.4014T>A (p.Ile1338=) in *ATP7B* was recently shown to cause WD by skipping of exon 19.[13] A recent analysis of the effects of synonymous *ATP7B* variants identified 11 synonymous variants that disrupted RNA splicing, where two of these variants (c.1620C>T and c.3888C>T) caused complete exon skipping but the *in vitro* analysis did not include c.2292C>T.[14] Synonymous sequence variants can affect messenger RNA (mRNA) splicing not only by abolishing canonical splice-site motifs but also by affecting *cis*-regulatory sequences or by abnormally creating splicing elements. Alternatively, synonymous sequence variants can also cause ribosomal stalling through codon usage bias, changes in RNA folding, and epigenetic changes, which all result in altered protein function or gene expression.[15] Splicing prediction algorithms are recommended to predict the functional effects of synonymous sequence variants.[9] However, the functional consequences of specific synonymous sequence variants are best demonstrated by transcript or protein analyses *in vivo* or by functional assays.[16]

Here, we report the synonymous *ATP7B* sequence variant c.2292C>T (ENST00000242839.10) to account for ~0.5% (14 of 2816) of *ATP7B* alleles in a large cohort of patients with WD.[4] This variant increases the rate of exon 8 skipping from the canonical *ATP7B* transcript, predicting an ATP7B protein lacking transmembrane domains 3 and 4.

PATIENTS AND METHODS

Patients

The prevalence of c.2292C>T (p.Phe764=) was analyzed by Sanger sequencing in a cohort of 1408 patients with WD. The clinical and biochemical characteristics of 1357 patients in this cohort have been published.[4] Since this publication, 51 additional patients were included for the present study. All patients included were diagnosed based on the diagnostic score that was proposed by the working party at the Eighth International Meeting on Wilson Disease, Leipzig, 2001. Leipzig scores of 4 or above were considered diagnostic of WD. The age range of our patients was 5-58 years (median age, 36 years). All patients and families provided written informed consent for participation in the study. The study protocol was approved by the Ethics Research Committee of the Medical University of Innsbruck (protocol number 1030/2020). Additional patients with WD with the c.2292C>T variant were identified from a cohort at Addenbrooke's Hospital Liver Center, Cambridge University Hospital, by one of the authors (W.G.).

Genetic analysis

The presence and zygosity of the p.His1069Gln mutation had been determined by allele-specific polymerase chain reaction (PCR). Patients with WD who were nonhomozygous for p.His1069Gln underwent Sanger sequencing of PCR amplicons of all exons and exon–intron boundaries of *ATP7B* from DNA isolated from whole-blood samples using published primers (for details see Ferenci et al.[4]). For the present study, exon 8 sequencing results were reviewed for the presence of c.2292C>T. Whole-exome sequencing in patients #1 and #13 (Roche NimbleGen SeqCap EZ Exome v2.0 enrichment kit; HiSeq 2000 sequencing; Illumina, San Diego, CA) was performed by Atlas Biolabs (Berlin, Germany). Sequence alignment against the reference human genome (GRCh37) and variant calling was performed by the authors (A.J. and R.J-H.) as described.[17]

Data were analyzed by the software tools GeneTalk and MutationDistiller.[18,19] For each called variant, the Genome Aggregation Database (gnomAD) v.2.1.1 population frequency and the combined annotation dependent depletion (CADD) score were obtained.[20] To study the effect of the c.2292C>T variant on RNA splicing *in silico*, the Human Splicing Finder tool was used (http://www.umd.be/HSF/).

Cell culture and transcript analysis

Cultured fibroblasts from skin biopsies were available from 1 patient homozygous for the c.2292C>T variant and patients compound heterozygous for the c.2292C>T variant and either c.3207C>A ENST00000242839; (p.His1069Gln) or c.2128G>A ENST00000242839; (p.Gly710Ser) mutation. Fibroblasts were cultivated as described.[21] To assess if the variant under investigation affected RNA stability, puromycin was added at a concentration of 20 mg/12 mL to patient and control fibroblast cultures 6 hours before harvesting for total RNA extraction. RNA was isolated from cultured fibroblasts using the RNeasy Mini kit in accordance with the manufacturer's instructions (QIAGEN, Hilden, Germany). RNA was converted to complementary DNA (cDNA) using the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. PCR with Platinum II Taq polymerase (Invitrogen) was performed using a forward primer in *ATP7B* exon 7 (ENSE00001797943) and a reverse primer in exon 9 (ENSE00003473904). PCR products were separated on 1% agarose gel and

were analyzed by Sanger sequencing (Microsynth, Balgach, Switzerland). Primers used for reverse transcription (RT)-PCR are listed in Table S1. The amounts of transcripts with and without exon 8 were quantified by real-time PCR using SYBR Select Master Mix (Applied Biosystems, Foster City, CA) according to manufacturer's instructions, with primers spanning exon–exon junctions specific for either the canonical transcript (ENST00000242839.10) or the transcript where exon 8 is skipped (ENST00000673772.1; Figure S2). Samples were analyzed on a StepOnePlus instrument (Applied Biosystems). TATA box binding protein (TPB) was used as a housekeeping gene. Primers used for quantitative real-time PCR are listed in Table S2.

RESULTS

In a cohort of 1408 Caucasian patients with WD[4] (from Austria, Germany, Switzerland, Hungary, and the Czech Republic), the allele frequency of the synonymous variant c.2292C>T (p.Phe764=, rs372979339) was 0.5% (14 of 2816). The allele frequency was 2.5% (14 of 560) in the subgroup of patients with a single pathogenic missense variant detected. By comparison, the allele frequency of c.2292C>T in gnomAD is significantly lower with 7.12×10^{-6} (2 of 280,964 alleles; $p < 10^{-5}$; Fisher exact test).[22] The c.2292C>T variant was not detected in 731 patients homozygous or compound heterozygous for other pathogenic ATP7B variants (Figure 1).

Family studies were carried out to test if patients heterozygous for a known pathogenic ATP7B sequence variant and for c.2292C>T both variants are in *trans* on different parental alleles. In 3 patients with WD, compound heterozygosity for p.Phe764= and either p.His-1096Gln or p.Gly710Ser was shown by sequencing of

parental samples (Figure S1). Family studies further showed that the father of patient #1, who is heterozygous for p.Phe764= without an additional variant on the other ATP7B allele, had reduced ceruloplasmin (CP) without clinical evidence of WD (Figure S1).

Next, we investigated if homozygosity for the variant c.2292C>T was also associated with WD. International investigators (W.G., A.B., P.F., S.H.) were contacted to search for patients homozygous for c.2292C>T, and 2 patients with WD from the United Kingdom were identified. Demographic, clinical, and biochemical characteristics of patients who were compound heterozygous and homozygous for c.2292C>T are summarized in Table 1.

The clinical presentation of patients was variable and included neurologic as well as hepatic WD, including a case with fulminant hepatic failure. The majority had a reduced plasma concentration of CP. There was an increase in 24-hour urinary copper excretion in 10 of 16 patients for whom this parameter was available.

To study how this synonymous sequence variant could cause WD, *in silico* analysis of the predicted functional effect of c.2292C>T was carried out. As a first step, the phylogenetic conservation of cytosine in position 2292 was conducted using the CADD–Public Health Review Evaluation Database score, where a score of 14.7 suggested pathogenicity of this variant. As this single-nucleotide variant is not predicted to affect the amino acid sequence of the ATP7B protein, the effect on mRNA splicing was next investigated by using the Human Splicing Finder tool,[23] which predicted that the c.2292C>T variant creates a novel exonic splicing silencer in a position where the normal sequence encodes an exonic splicing enhancer.

To test how RNA splicing was affected by this variant, RNA samples of cultured fibroblasts from patient #1 and a healthy control subject were isolated. RT-PCR analysis of these RNA samples with a forward primer in exon 7 and a reverse primer in exon 9 produced two
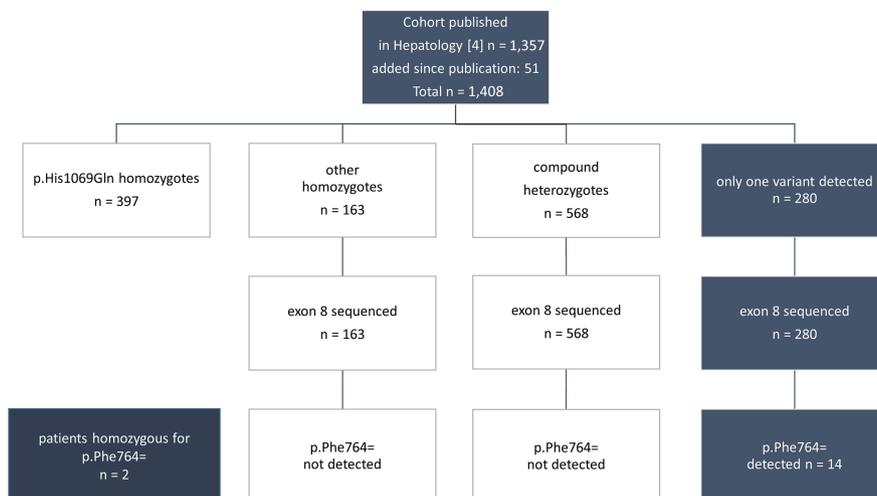

FIGURE 1　Consort diagram

TABLE 1 Demographic, clinical, biochemical, genetic, and histologic parameters of patients with WD with compound heterozygosity for c.2292C>T and disease-associated missense variants in ATP7B or homozygosity for c.2292C>T

| Patient Identification Number	Sex	Age at Diagnosis (Years)	Age at Onset of Symptoms (Years)	CP (mg/dL) (20-60[a])	U-Cu (µg/24 hours) (10-60[a])	K-F Rings	Hepatic Cu (µg/g) (<250 µg/g[a])	Liver Histology	Presentation	Genotype	Country
1	F	27	27	10.8	384	+	NA	NA	N	His1069Gln/Phe764=	AUT
2	M	8	7.5	<10	84.2	–	570	Steatosis	H	Met769Val/Phe764=	GER
3	F	58	54	NA	NA	–	667	F3	H	Arg1319Ter/Phe764=	AUT
4	F	15	15	11	NA	+	113	Cirrhosis	H	His1069Gln/Phe764=[b]	AUT
5	M	40	8	6	NA	–	645	Cirrhosis	H	Arg1319Ter/Phe764=[b]	AUT
6	F	45	41	6.2	NA	+	475	Cirrhosis	N	Lys35Ter/Phe764=	AUT
7	F	49	26	<10	NA	+	NA	NA	N	His1069Gln/Phe764=	HUN
8	F	26	26	9.1	272	–	1128	Cirrhosis	H	His1069Gln/Phe764=[b]	AUT
9	M	39	39	10	1026	–	595	Cirrhosis	H	His1069Gln/Phe764=[b]	AUT
10	F	18	18	29	>1000	+	NA	Cirrhosis	H	Gly710Ser/Phe764=[b]	AUT
11	M	16	16	15.7	238	–	321	Steatosis	H	Gly710Ser/Phe764=	AUT
12	F	33	30	24	179	+	681	Fibrosis	H	Arg616Gln/Phe764=	CH
13	F	45	45	11	620	+	645	Cirrhosis	N	His1069Gln/Phe764=	AUT
14	F	5	5	13	NA	–	791	Steatosis	H	Thr1092Pro/Phe764=	AUT
15	M	48	48	5	82.6	–	641	Steatosis/F3	H	Phe764=/Phe764=	GBR
16	M	43	43	5	174.2	–	NA	Cirrhosis	H	Phe764=/Phe764=[b]	GBR

Abbreviations: AUT, Austria; CH, Switzerland; GBR, Great Britain; H, hepatic; HUN, Hungary; K-F, Kayser-Fleischer; N, neurologic; NA, not available; U-Cu, urine copper.

[a]Reference ranges.

[b]orthotopic liver transplantation.
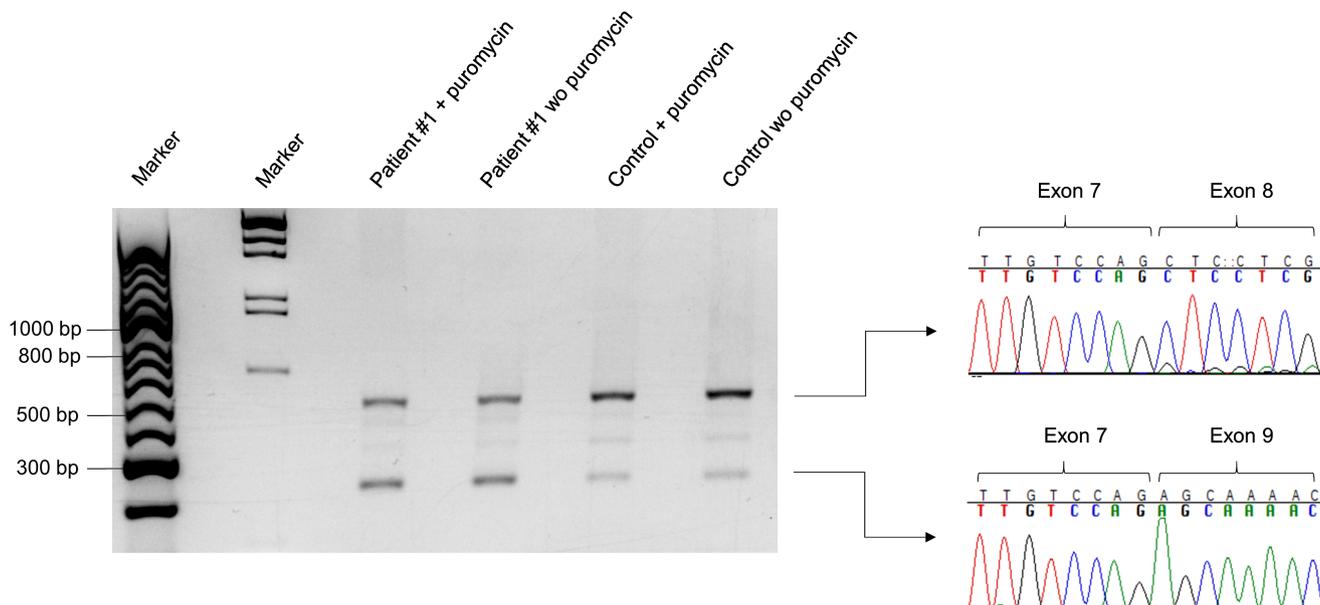
FIGURE 2 RT-PCR analysis from mRNA extracted from fibroblasts from the index patient (#1) and controls. Qualitative analysis shows that amplification with primers located in exon 7 and exon 9 produces two amplicons in the patient heterozygous for the c.2292C>T mutation and in controls. Sanger sequencing of the ~550-bp amplicon aligns with the canonical ATP7B transcript ENST00000242839.10. Sanger sequencing of the ~250-bp amplicon aligns with the shorter ATP7B transcript ENST00000673772.1. The sequence of the ~550-bp amplicon is homozygous for the normal C allele in position c.2292, which indicates that this amplicon is exclusively derived from the transcript of the maternal allele. wo, without
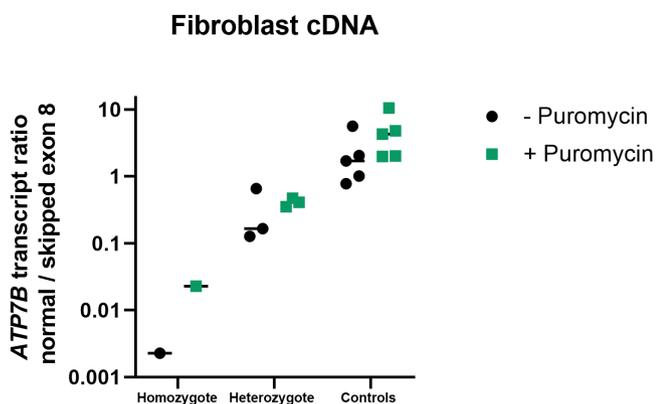
FIGURE 3 Quantitative analysis of ATP7B transcripts by TaqMan PCR in RNA extracted from 1 homozygous patient (#16) and 3 patients compound heterozygous for p.Phe764= (c.2292C>T) and either p.His1069Gln (#1, #13) or p.Gly710Ser (#10) and from controls. Horizontal lines show median

distinct amplicons of ~250 base pairs (bp) and ~550 bp. Sanger sequencing showed that the ~550-bp amplicon aligned with the canonical *ATP7B* transcript ENST00000242839.10, which contains exons 7, 8, and 9. In contrast, the ~250-bp amplicon aligned a shorter *ATP7B* transcript (ENST00000673772.1) where exon 8 was skipped. Both amplicons were detectable by RT-PCR in RNA extracted from fibroblasts of patient #1 and controls (Figure 2).

As both amplicons were detected in patients and controls, the expression of each ATP7B mRNA variant was quantified and expressed as a ratio between the two transcripts. In controls, the ratio between the canonical *ATP7B* transcript and the transcript lacking exon 8 was 1.53 without puromycin (4.27 with puromycin). In fibroblast cDNA from a patient homozygous (patient #16) for the c.2292C>T variant, the ratio was lowest at 0.0023 without puromycin (0.0229 with puromycin). In patients compound heterozygous for c.2292C>T and either p.His1069Gln (patients #1 and #13) or p.Gly710Ser (patient #10), an intermediate ratio of 0.1650 without puromycin and 0.4100 with puromycin was observed (Figure 3).

This finding indicates that the c.2292C>T variant is associated with increased skipping of exon 8. Incubation of fibroblasts with puromycin did not indicate that the RNA stability of the transcript lacking exon 8 was reduced (Figure 3). These findings indicate that fibroblasts with the c.2292C>T variant express more of the *ATP7B* transcript that lacks exon 8 than of the canonical transcript.

As shown by quantitative mass spectrometry where no tryptic ATP7B peptide fragments were found in peripheral blood from patient #1,[24] it can be concluded that the observed differences in mRNA processing also result in reduced ATP7B protein expression.

DISCUSSION

Test interpretation and WD diagnosis are guided by the Leipzig scoring system.[25] If two pathogenic or likely

pathogenic *ATP7B* variants are detected, the diagnosis is established, but previous studies have shown that no or only one *ATP7B* pathogenic variant can be identified in 1%-21% of patients with WD.[4,5,26] Possible explanations for missing variants on Sanger sequencing of all coding *ATP7B* exons and exon–intron boundaries include larger genetic deletions/duplications, which can be detected by multiplex ligation-dependent probe amplification, or mutations in untranslated regions, the promoter region, or deep intronic region.[27–29]

We show that *in silico* filtering for rare and potentially damaging variants and reinterpretation of sequencing results identified the synonymous variant c.2292C>T as likely pathogenic in 16 patients with WD initially categorized with none or only one known disease-causing *ATP7B* variant. Synonymous sequence variants are increasingly recognized as pathogenic or likely pathogenic in a number of monogenic disorders (reviewed in Sauna and Kimchi-Sarfaty[30]). Although such variants do not apparently affect the linear amino acid sequence of the respective protein, diverse molecular disease mechanisms have been described that indicate how such variants indeed affect RNA stability, splicing, secondary structure, and ultimately translation.[15,31]

The c.2292C>T variant causes skipping of exon 8. Skipping of exon 8 is predicted to result in an in-frame deletion of residues 708-785 of the ATP7B protein, which corresponds to part of the protein region beginning in transmembrane domain 2 and reaches to the end of transmembrane domain 4.[32] Of note, the transcript without exon 8 is present in control fibroblasts at lower amounts than the canonical transcript, whereas fibroblasts from patients homozygous or heterozygous for c.2292C>T express mainly the noncanonical transcript. These findings also raise the question why the transcript lacking exon 8 is expressed at all in control fibroblasts. As no protein fragments were detectable by mass spectrometry in leukocytes from patients with compound heterozygosity for c.2292C>T and another pathogenic variant in *ATP7B*, it is unlikely that the transcript lacking exon 8 is actually translated into protein. The biologic function of the transcript variant lacking exon 8 is therefore highly speculative but could include regulatory functions or specific expression in certain tissues.

Quantification of both transcripts in liver and brain of affected patients and controls would be of great interest but would require fresh or snap-frozen tissue samples from affected patients, which were not available in this study. The significantly higher allele frequency of this variant in the Vienna WD cohort than in the gnomAD and the finding of two c.2292C>T homozygotes without other pathogenic *ATP7B* variants further support the classification of rs372979339 as pathogenic. By manual adjustment based on the ACMG guidelines,[9] the c.2292C>T variant can be classified as PS3 (functional defect) and PS4 (prevalence in affected significantly increased vs. controls).

Other synonymous variants of *ATP7B* may also be relevant in WD. The synonymous sequence variant c.4014C>T (p.Ile1338=) was reported in compound heterozygosity with a missense variant in *ATP7B* in a Chinese patient with WD. The molecular disease mechanism of the c.4014C>T variant was found to be skipping exon 19.[13] Recent data from China indicate that the c.1620C>T (p.Leu540=) and c.3888C>T (p.Ala1296=) variants could also lead to abnormal splicing of the corresponding exons, resulting in complete skipping of exon 4 and a 25% increase in skipping of exon 18, respectively.[14]

In this study, only patients with a definitive WD diagnosis (Leipzig score ≥4 without considering the gene-test results) were included. The majority of patients presented with late-onset WD (age at diagnosis >35 years). Two patients presented with fulminant hepatic failure and 1 as decompensated cirrhosis. All 3 underwent liver transplantation. These findings further support the notion that WD presentation is determined by age and sex rather than by genotype.[4]

The findings presented in this study can improve interpretation of diagnostic *ATP7B* gene sequencing results in clinical genetics and highlight that synonymous sequence variants could indeed be pathogenic. During the diagnostic evaluation of patients with suspected WD, it is important to remember that disease-causing variants in other genes may be involved in diseases with copper accumulation and cause WD-like phenotypes. Patients with rare congenital disorders of glycosylation that have been associated with genetic defects in coiled-coil domain containing 115 (*CCDC115*) can result in a disease phenotype very similar to WD with hepatic copper accumulation, neurologic impairment, and liver disease.[33–35] Similarly, patients with aceruloplasminemia present with low or undetectable serum CP and a wide range of movement disorders, which are also typical signs and symptoms of WD.[36] However, whole-exome sequencing did not identify pathogenic variants in *CCDC115* or *CP* (data not shown) in 2 patients in this study.[37–39]

Our study shows that the synonymous sequence variant c.2292C>T in *ATP7B* is pathogenic by affecting mRNA splicing. This variant provides an explanation for the lack of two detectable variants in some patients with WD and accounts for approximately 0.5% of WD disease alleles. Compound heterozygosity for c.2292C>T with other pathogenic variants or homozygosity for this sequence variant are associated with WD. These findings have implications for the diagnosis of WD carrier testing and family screening.

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CONFLICT OF INTEREST

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