Genetic studies discover novel coding and non-coding mutations in patients with Wilson's disease in China

Chenjun Huang1 | Meng Fang2 | Xiao Xiao1 | Zhiyuan Gao1 | Ying Wang2 | Chunfang Gao1

1Department of Clinical Laboratory Medicine Center, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China
2Department of Laboratory Medicine, Shanghai Eastern Hepatobiliary Surgery Hospital, Shanghai, China

Correspondence
Ying Wang, Department of Laboratory Medicine, Shanghai Eastern Hepatobiliary Surgery Hospital, Shanghai 200438, China. Email: nadger_wang@139.com
Chunfang Gao, Department of Clinical Laboratory Medicine Center, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200437, China. Email: gaocf1115@163.com

Funding information
This work was supported by the Innovation Group Project of Shanghai Municipal Health Commission [2019CXJQ03], the National Natural Science Foundation of China [81572072], the Science and Technology Commission of Shanghai Municipality [17JC1404500 and 16441907200], the Shanghai "Rising Stars of Medical Talent" Youth Development Program [2019-72], the Science Foundation of Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine [2021yyq08] and Shanghai Outstanding Youth Clinical Medical (Medical Laboratory Scientists) Training Funding (presided by Xiao Xiao).

Abstract
Objectives: Wilson disease (WD) is a rare autosomal recessive genetic disorder associated with various mutations in the ATP7B gene and leads to significant disability or death if untreated. Early diagnosis and proper therapy usually predict a good prognosis, especially in pre-symptomatic WD. Genetic testing provides an accurate and effective diagnostic method for the early diagnosis of WD.

Methods: We recruited 18 clinically diagnosed WD patients from 16 unrelated families and two independent individuals. The next-generation sequencing of the ATP7B gene was performed. The 293T cell lines were divided into wild-type (WT) ATP7B and mutated ATP7B groups. Cell proliferation was determined by Cell Counting Kit-8 (CCK-8) assay and apoptosis was detected by Annexin V/propidium iodide (PI) assays.

Results: Pedigree analysis showed that compound heterozygous variants (17/18, 94.44%) were present in the majority of WD patients. A total of 33 ATP7B gene variants were identified, including three variants with uncertain significance (VUS) [two splice mutations (c.51+2T>G, c.1543+40G>A) and one frameshift mutation (c.3532_3535del)]. The CCK-8 and apoptosis assays demonstrated that the VUS of ATP7B could significantly affect the transportation of copper.

Conclusions: The study revealed genetic defects of 16 Chinese families and two independent individuals with WD, which enriched the mutation spectrum of the ATP7B gene worldwide and provided valuable information for studying the mutation types of ATP7B in the Chinese populations. Genetic testing in WD patients is necessary to shorten the time to initiate therapy, reduce damage to the liver and improve the prognosis.

KEYWORDS
ATP7B, Chinese population, mutation, next-generation sequence, Wilson disease

Chenjun Huang and Meng Fang have contributed equally to this work.

Correction added on 30th April 2022, after first online publication: the contributor details 'Chenjun Huang and Meng Fang have contributed equally to this work' has been updated.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. Journal of Clinical Laboratory Analysis published by Wiley Periodicals LLC.
1 | INTRODUCTION

Wilson disease (WD), first reported by Kinnear Wilson in 1912, is a rare autosomal recessive inherited disease involving copper metabolism disturbance due to the mutations of ATP7B gene that encodes the P-type ATPase.\textsuperscript{1,2} Defects of ATP7B will reduce the ceruloplasmin (CP) plasma levels and affect the transport of copper to plasma CP with pathological copper accumulation in different organs (liver, kidney, and other tissues).\textsuperscript{3,4} Excess copper exposure in these tissues can lead to secondary organ damage, including liver cirrhosis, limbal Kayser–Fleischer (K-F) ring, neurologic degeneration, and other clinical symptoms.\textsuperscript{2,5}

Early diagnosis and intervention of WD are critical to limit disease progression, and the disease is lethal if the patients is not treated early. The diagnosis of WD is usually conducted by combining the signs, symptoms, laboratory, and imaging information.\textsuperscript{5,7} However, each of the diagnostic strategies has its limitations.\textsuperscript{2} It is reported that due to the clinical heterogeneity of WD, only about 30% of WD patients were accurately diagnosed of the beginning medical consultation.\textsuperscript{8}

The detection of ATP7B gene mutation may be powerful tools for WD accurately diagnosis, especially for patients who have mild-to-moderate disease.\textsuperscript{8,9} The frequency of p.778I (c.2333g\textgt;t, exon 8) reported by China is 17.3%–31.9%, which is the most common of ATP7B mutations.\textsuperscript{10–12} However, because some studies have reported that the frequency of heterozygotes is much higher than that of homozygotes, WD disease shows genetic heterogeneity.\textsuperscript{10,13,14}

Previous studies have usually focused on unrelated individuals,\textsuperscript{10,15,16} case reports,\textsuperscript{17,18} or a limited number of pedigrees.\textsuperscript{19–21} In addition, the functional consequences of these mutations still lack direct experimental evidence. Therefore, we recruited 18 WD Chinese patients and their 43 first-degree relatives from 16 families and two independent individuals for DNA sequencing to systematically analyze the genotypes of Chinese WD patients. We also conducted a series of experiments to elucidation of possible functional consequences of these ATP7B mutations.

2 | METHODS

2.1 | Patients and diagnostic criteria

Patients with WD or suspected WD and their first-degree relatives were recruited from the Shanghai Eastern Hepatobiliary Surgery Hospital (EHBH) and Yueyang Hospital of Integrated Traditional Chinese and Western Medicine of Shanghai University of Traditional Chinese Medicine between January 2019 and June 2021. The diagnosis of WD was established according to the scoring system provided by the 8th International Meeting on Wilson disease and Menkes disease\textsuperscript{6} and the EASL Clinical Practice Guidelines for Wilson disease.\textsuperscript{22} The biochemical parameters, clinical presentation, and medical history were recorded in the Department of Laboratory Medicine of EHBH. All the patients and their relatives provided written informed consent, and this study was approved by the Ethics Committee of the EHBH (EHBHKY2020-02–013).

2.2 | Identification of ATP7B gene variants

2.2.1 | DNA extraction and sequencing

Blood DNA Extraction Kit (Cwbio, CWY049S) was used for isolating genomic DNA from peripheral blood. In the presence of high salt, DNA binds to the surface of silicon-coated magnetic beads. The isolated DNA concentration was determined by the Qubit dsDNA HS Assay Kit (Life Technologies). For each serum ATP7B gene of the samples, ATP7B was sequenced through the MiSeq sequencer using the Illumina paired-end sequencing protocol using the MiSeq Reagent Kit, V3 (Illumina, San Diego, CA, USA), as we previously established and optimized.\textsuperscript{23} The reads were aligned with the hg19 (UCSC) reference genome. All codes for WGBS data analysis are available on GitHub (https://GitHub.com/cemordarun/WilsonDiseaseEpigenome).

2.2.2 | Variant identification

The sequencing results were aligned to referred ATP7B sequence (NM_000053.3) to figure out the mutations. When a genetic variation meets all the following criteria, the variation with uncertain significance (VUS) is identified: (1) no reports in PubMed literatures; (2) no records in Human Gene Mutation Database (HGMD, \textsuperscript{http://www.hgmd.org/}) and WD Mutation Database (\textsuperscript{http://www.wilsonsdisease.med.ualberta.ca/database.asp}).

2.3 | The experiment of functional consequences

2.3.1 | Cell culture

The 293T cell lines were obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China) and cultured in basic DMEM (GIBCO, California, USA) supplemented with 10% fetal bovine serum and 100 U / ml penicillin/streptomycin (Invitrogen). All cultures were stored in a humidified incubator at 37 °C and 5% CO\textsubscript{2}.

2.3.2 | Lentiviral transduction

The construction of lentivirus vector expressing ATP7B and ATP7B mutation is the same as previous studies.\textsuperscript{24,25} Then, the 293T cell lines were transfected with GV341-ATP7B and ATP7B mutant expression vectors. The relative expression of the ATP7B gene was detected by quantitative real-time PCR. The expression of ATP7B protein in 293T cells was detected by Western blot. The 293T cells
transfected with naked GV341 vector without ATP7B sequence and non-transfected 293T cell lines were used as controls.

2.3.3 | ATP7B protein secretion assays

The total protein of 293T cell line was extracted using sample loading buffer (Cat No. P0015; beyotime Biotechnology Institute), and the proteins were determined using BCA Protein Assay Kit (Cat No. P0010S; beyotime Biotechnology Institute). The proteins were separated by SDS-PAGE (10% separation gel and 4% concentrated gel) and transferred to a polyvinylidene fluoride membranes. After blocking with non-fat milk dissolved in Tris-buffered saline with 31490, Thermo Fisher Scientific, Inc.) at room temperature for 1 h. The membrane was incubated with anti-ATP7B (1:1000) antibody at 4°C overnight. The membrane was then incubated with 15 mL of horse-radish peroxidase-labeled secondary antibody (1:2000, Cat No: LI-COR® Odyssey®) at room temperature for 1 h. Signals were visualized with the Odyssey Infrared Imaging System (LI-COR® Odyssey® 700 or 800 channels).

2.3.4 | Cytotoxicity assay

Cytotoxicity of CuCl2 in 293T cell line was assessed using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies Inc., Rockville, MD, USA). Briefly, 4×103 cells were seeded into 96 well plates and allowed to rest for 24 h. CuCl2 was diluted to concentrations ranging from 300 to 500 μM. Cells were incubated with increasing doses of CuCl2 and after 48, 72, and 96 h, cell viability was measured using the CCK-8 kit according to the manufacturer’s protocol.

2.3.5 | Visible assay of apoptosis

According to the manufacturer’s instructions, the apoptosis of 293T cell lines was analyzed by Annexin V-APC propidium iodide (PI) staining (Invitrogen). Briefly, the cells were washed with PBS and re-suspended in 1× binding buffer at a 1×106 cells/mL concentration. Two hundred microliters of the cell suspension, 10 μl of Annexin V-APC, and 5 μl of PI were added. After 15 min of incubation, the cells were analyzed by flow cytometry. Living cells (Annexin V-APC-/PI-), early apoptotic cells (Annexin V-APC+/PI-), late apoptotic cells (Annexin V-APC+/PI+), and necrotic cells (Annexin V-APC-/PI+) were distinguished and quantified. The apoptotic ratio refers to the percentage of cells with Annexin V-APC+/PI- fluorescence and Annexin V-APC+/PI+ fluorescence.

2.4 | Statistical analysis

Continuous variables were reported as mean ± standard deviation (SD). The unpaired two-tailed t-test was used for comparison between the two groups. P values <0.05 were considered statistically significant. The data were analyzed using GraphPad, USA and R language for Windows (http://www.r-project.org/).

3 | RESULTS

3.1 | Variants identified in the ATP7B Gene

By detecting the ATP7B mutation of patients and their immediate family members, 18 WD patients and 43 first-degree relatives (including 33 males and 28 females) were recruited. They were recruited from 16 unrelated families and two independent individuals aged from 2 to 78 years. Table 1 and Figure 1 summarized the clinical characteristics of these patients. Among these, 11 patients had some clinical manifestations of hepatic origin, such as hepatomegaly, splenomegaly, thrombocytopenia, and elevation of serum bilirubin. Three patients had different neurological abnormalities, such as tremor, dysarthria, dystonia, epilepsy, or muscle weakness. Nine patients had K-F rings. All patients had decreased serum ceruloplasmin levels.

3.2 | Mutation analysis

By performing the genetic screening on the 18 WD probands and their relatives, a total of 33 ATP7B gene variants were identified, including 24 missense mutation (c.695C>T, c.1168A>G, c.2111C>T, c.2267C>T, c.2333G>T, c.2605G>A, c.2621C>T, c.2662A>C, c.2804C>T, c.2924C>A, c.2930C>T, c.2975C>T, c.3074T>C, c.3104G>T, c.3166G>A, c.3443T>C, c.3459G>T, c.3517G>A, c.3532A>G, c.3646G>A, c.3836A>G, c.3889G>A, c.3956G>A, c.3960G>C), four splice mutation (c.51+2T>G, c.1543+1G>T, c.1543+40G>A, c.1708+1G>C), four frameshift mutation (c.2304dupC, c.3532_3535del, c.3767_3768insCA, c.525dupA), and one large fragment deletion (c. 52544898_52558635del defined as delEXON2), showed in Table 1. Seventeen patients harbored compound heterozygous variants and one patient had one homozygous variant (c.3074T>C).

As shown in the Table 1, the mutations located in different protein domains, including the ATP bind domains (p.V1216 M, p.D1279G, p.Q1256Hfs*74, p.R1320S, p.V11061, p.I1148T, p.W1153C, p.E1173K, p.T1178fs*12, p.T1178A, p.V1297I), Cu²⁺ binding domains (p.V1765fs*27, delEXON2, p.P223L, p.I390V), phosphorylation domain (p.M1025R, p.G1035V), transduction domain (p.G869R, p.A874V, p.T888P), and the transmembrane domains (p.T704I, p.A756V, p.M769Hfs*25, p.R778L, p.T935 M, p.S975Y, p.T977 M, p.P992L). We reviewed HGMD, WD Mutation Database, and PubMed literatures in our WD cohort, the two splice mutations (c.51+2T>G, c.1543+40G>A) and one frameshift mutation (c.3532_3535del), were being reported for the first time, were defined as variants with uncertain significance (VUS) according to the American College of Medical Genetics and Genomics (Table 1).26
| Patients | Mutation | Change of Nuclear acid | Chromosome | Mutation position | types of mutation | Change of amino acid | Domain of protein |
|----------|----------|------------------------|------------|------------------|------------------|---------------------|-------------------|
| 1        | c.3316G>A | GGTGCAAAAGT            | Ch13       | Exon 15          | Missense         | p.V1106I            | ATP loop          |
|          | c.51+28788_c.1272 | -                      | Ch13       | Exon 2           | Deletion         | -                   | Cu2/Cu3/Cu4       |
| 2        | c.2333G>T | GGCCCGGTGG             | Ch13       | Exon 8           | Missense         | p.R778L             | TM4               |
|          | c.2267C>T | CTGAGAGG               | Ch13       | Exon 8           | Missense         | p.A756V             | TM3/TM4           |
| 3        | c.2662A>C | AGCTAACCAC             | Ch13       | Exon 11          | Missense         | p.T888P             | Td/TM5            |
|          | c.695C>T  | ACTAACCAAA             | Ch13       | Exon 2           | Missense         | p.P232L             | Cu3               |
|          | c.3960G>C | AGGATACGCA             | Ch13       | Exon 19          | Missense         | p.R1320S            | ATP hinge/TM7     |
| 4        | c.2621C>T | TTGGGCGGTTC            | Ch13       | Exon 11          | Missense         | p.A874V             | Td/TM5            |
|          | c.2333G>T | GGCCCGGTGG             | Ch13       | Exon 8           | Missense         | p.R778L             | TM4               |
| 5        | c.3889G>A | CGTGTCTCTT             | Ch13       | Exon 18          | Missense         | p.V1297I            | ATP hinge/TM7     |
|          | c.3532_3535del | ACAGACAACC            | Ch13       | Exon 16          | Frame shift      | p.T1178Pfs*12       | ATP loop          |
|          | c.1543+1G>T | GAATGCTGC             | Ch13 Intron 3 | Splice          | -                | -                   | Cu5               |
|          | c.3646G>A | GTGCGGACTG             | Ch13       | Exon 17          | Missense         | p.V1216M            | ATP bind          |
| 6        | c.3316G>A | GGTGCAAAAGT            | Ch13       | Exon 15          | Missense         | p.V1106I            | ATP loop          |
|          | c.51+2T>G | G TGAGTTTTG            | Ch13       | Intron 1         | Splice           | -                   | before Cu1        |
| 7        | c.3316G>A | GGTGCAAAAGT            | Ch13       | Exon 15          | Missense         | p.V1106I            | ATP loop          |
|          | c.2333G>T | GGCCCGGTGG             | Ch13       | Exon 8           | Missense         | p.R778L             | TM4               |
| 8        | c.3767_3768insCA | TCCACAGAAT          | Ch13       | Exon 18          | Frame shift      | p.Q1256Hfs*74       | ATP bind          |
|          | c.3517G>A | ACAGACACCG             | Ch13       | Exon 16          | Missense         | p.E1173K            | ATP loop          |
| 9        | c.2605G>A | GAAACCGGAG             | Ch13       | Exon 11          | Missense         | p.G869R             | Td                 |
|          | c.1543+1G>T | GAATGCTGC             | Ch13 Intron 3 | Splice          | -                | -                   | Cu5               |
|          | c.3646G>A | GTGCGGACTG             | Ch13       | Exon 17          | Missense         | p.V1216M            | ATP bind          |
| 10       | c.1543+40G>A | GAATGCTGGG           | Ch13       | Intron 3         | Splice           | -                   | Cu5               |
|          | c.3836A>G | CCAGCCAGAC             | Ch13       | Exon 18          | Missense         | p.D1279G            | ATP hinge         |
| 11       | c.1708-1G>C | TATGACAG              | Ch13       | Intron 5         | Splice           | -                   | Cu6               |
|          | c.1168A>G | ATATCGGTGT             | Ch13       | Exon 2           | Missense         | p.I390V             | ATP hinge/TM7     |
|          | c.3956G>A | GACTGTCCGA             | Ch13       | Exon 19          | Missense         | p.R1319Q            | TM6/Ph            |
|          | c.2975C>T | GCCACGGCCA             | Ch13       | Exon 13          | Missense         | p.P992L             | TM6/Ph            |
| 12       | c.3074T>G | CTGAGTAGGT             | Ch13       | Exon 14          | Missense         | p.M1025R            | Ph                |
| 13       | c.2975C>T | GCCACGGCCA             | Ch13       | Exon 13          | Missense         | p.R1319Q            | TM6/Ph            |
|          | c.2111C>T | GTAACCTTTG             | Ch13       | Exon 7           | Missense         | p.T704I             | TM2               |
| 14       | c.3532A>G | ACAGACAGCC             | Ch13       | Exon 16          | Missense         | p.T1178A            | ATP loop          |
|          | c.2975C>T | GCCACGGCCA             | Ch13       | Exon 13          | Missense         | p.P992L             | TM6/Ph            |
3.3 Effect of mutations on copper transport

However, the functional and clinical relevance of many VUS identified through clinical genetic testing is unclear. In order to determine the effect of mutation on ATP7B transport function, we analyzed the biological consequences of the ATP7B variant by constructing lentivirus vector expression plasmid containing wild-type (WT) ATP7B or its mutant variant. It is difficult to construct the lentiviral vector expression plasmids with the splice mutations. Therefore, the fragment deletion mutation of p.T1178fs was used to evaluate the functional consequences of ATP7B variants. The known pathogenic variant delEXON2 was selected as positive control.27

The expression of ATP7B from 293T cell line transformed mutant strains was confirmed by Western blot. ATP7BdelEXON2 and ATP7B1178fs showed lower expression compared with the WT ATP7B (Figure 2A). The CCK-8 assay was used to determine whether CuCl2 caused cytotoxicity in 293T cells after 48, 72, and 96 hours of CuCl2 exposure, respectively. CuCl2 did not cause cytotoxicity in the CCK-8 assay after 96 hours at a concentration of 300 μM. When exposed to 500 μM CuCl2 for the same amount of time, ATP7BdelEXON2 and ATP7B1178fs significantly reduced the viability of cultured cells when compared to WT groups for 72 and 96 hours (Figure 2B).

To clarify the mechanism of CuCl2-induced 293T cell death, annexin V/PI staining was performed to determine quantitatively the apoptotic cell percent by flow cytometry (Figure 2C). The early apoptotic cell rate in the WT group was 2.90 ± 0.17% after 72 hours at 300 μM concentrations. In contrast to the WT group, the ATP7BdelEXON2 (4.37 ± 0.23% and ATP7B1178fs (5.45 ± 0.21%) groups had higher rates of early apoptotic cells (p < 0.05). Similarly, in the 300 μM concentrations, the rate of late apoptotic cells was significantly higher in the ATP7BdelEXON2 (5.60 ± 0.26%) and ATP7B1178fs (6.30 ± 0.00%) groups compared to the WT group (3.37 ± 0.15%; p < 0.05). The CuCl2 at concentrations of 500 μM induced early apoptotic and late apoptosis of the 293T cells at a higher proportion compared with the CuCl2 at concentrations of 300 μM (early apoptotic cell of ATP7BdelEXON2: 11.20 ± 0.44%, ATP7B1178fs: 12.30 ± 0.28%; late apoptotic cell of ATP7BdelEXON2: 21.67 ± 0.86%, ATP7B1178fs: 15.20±0.28%). These results show that CuCl2 caused a remarkable increase in cellular apoptosis in a concentration-dependent manner.

4 DISCUSSION

WD is a treatable monogenic disease.15 Its main symptoms include liver and nervous system diseases, ranging from mild abnormalities to severe progression.28,29 Traditionally, the diagnosis of WD mainly depends on clinical manifestations and routine biochemical indexes, including hepatic copper content, 24-hour elevated urinary copper, and decreased serum CP.30 However, patients with mild symptoms may be misdiagnosed, resulting in delayed treatment.20 Gene detection of ATP7B gene mutation may lead to reliable early diagnosis and treatment to prevent copper accumulation and tissue
Nowadays, ATP7B gene detection is suitable for prenatal diagnosis and neonatal screening. At present, more than 500 mutations of ATP7B have been described. In our study, when we finished sequencing, a total of 33 different mutations were identified in 18 Chinese WD patients, including VUS [two splice mutations (c.51+2T>G, c.1543+40G>A) and one frameshift mutation (c.3532_3535del)]. The mutations were characterized by pervasive compound heterozygous and rare homozygous, and common missense and point mutations. In general, the mutation frequency of exon 8 was 12.00% (9 / 75), ranking the first, followed by exon 16, 10.67% (8 / 75). This result is consistent with the previous study. The p.R778L mutation on exon 8 was the most common in our study populations, accounting for 9.33% (7/75) of the alleles studied. Most studies have shown that p.R778L is the first hot spot mutation in the Chinese population. Exon 8 is located in the transmembrane domain (TM4) of the ATP7B gene. Therefore, the p.R778L may destroy the proper anchoring of transporters in the membrane and eventually damage copper transport.
The second hotspot mutations remained controversial in Chinese populations.\textsuperscript{35,36} Our study demonstrated that p.V1106I on exon 15 was the second frequent mutation, accounting for 6.67% (5/75). Nevertheless, Wu et al. suggested that the p.T935 M was the second hotspot.\textsuperscript{35,36} A study of 62 Chinese WD patients showed that p.P992L was the second most frequent mutation instead of p.T935 M.\textsuperscript{34} Another study also demonstrated that p.P992L was the second hotspot through a study of 65 southern Chinese WD patients.\textsuperscript{37} Our study also found a high mutation frequency of p.P992L on exon 13, accounting for 5.33% (4/75). Li et al. sequenced the DNA from 114 WD patients from northern Chinese populations and showed that p.R778L (21.5%), p.A874V (7.5%), and p.P992L (6.1%) were the most frequent mutations.\textsuperscript{38}

The c.51+2T>G, c.1543+40G>C, and c.3532_3535del (p.T1178Pfs) mutations were newly found. The VUS of c.3532_3535del (p.T1178Pfs) on exon 16 and the known pathogenic variant of delEXON2\textsuperscript{27} were used to evaluate the functional consequences of ATP7B variants. The mutation of p.T1178Pfs occurred in the ATP loop region and presumably disrupted ATP binding to affect the transport of copper.\textsuperscript{16} Similarly, the delEXON2 located in the copper-binding domain from ATP7B is implicated in WD.

The CCK-8 assays showed that the mutations of ATP7B groups, including ATP7B\textsuperscript{delEXON2} and ATP7B\textsuperscript{T1178P}, could significantly inhibit the proliferation than WT control cells when treated with 500μM CuCl\textsubscript{2} for more than 72 h. Apoptosis is a complicated process that involves multiple genes.\textsuperscript{40} Our results indicated that the mutations of ATP7B could induce 293T cell apoptosis with the treatment of CuCl\textsubscript{2} (300 μM and 500 μM) for 72 h. The results matched with the outcome with CCK-8 assays.

In conclusion, this study revealed the genetic pattern of WD in 16 Chinese families and summarized the pathogenic genotypes. Compound heterozygous mutations of different alleles are the most common genotype in WD patients. Three VUS [two splice mutations (c.51+2T>G, c.1543+40G>C) and one frameshift mutation (c.3532_3535del)] were found, and the functional changes associated with the new ATP7B mutation were evaluated by CCK-8 and apoptosis analysis. Our study enriches the mutation spectrum of the ATP7B gene worldwide and provides valuable reference data for studying the mutation type and genetic mode of ATP7B in the Chinese population. However, to understand the pathogenesis of WD more comprehensively, it is necessary to analyze the patients and families with clinically confirmed WD on a larger scale, and it is urgent to study the molecular mechanism of WD pathogenic mutation.

ACKNOWLEDGMENT

We thank all the patients who participated in this research sincerely. We are also grateful to Shanghai Amplicon-gene Bioscience Co., Ltd for their technical assistance in this study.

CONFLICT OF INTEREST

All authors claim that there is no conflict of interest including a desire for financial gain, prominence, professional advancement, or a successful outcome.

DATA AVAILABILITY STATEMENT

Data sources and handling of the publicly available datasets used in this study are described in the Materials and Methods. Further details and other data that support the findings of this study are available from the corresponding authors upon request.

ORCID

Chenjun Huang  https://orcid.org/0000-0003-3836-6262
Xiao Xiao  https://orcid.org/0000-0002-9260-4519
Chunfang Gao  https://orcid.org/0000-0002-4891-2944

REFERENCES

1. Gauthier L, Charbonnier P, Chevallet M, et al. Development, formulation, and cellular mechanism of a lipophilic copper chelator for the treatment of Wilson's disease. Int J Pharm. 2021;609:121193.
2. Merle U, Schaefer M, Ferenci P, Stremmel W. Clinical presentation, diagnosis and long-term outcome of Wilson's disease: a cohort study. Gut. 2007;56(1):115-120.
3. Huster D. Wilson disease. Best Pract Res Clin Gastroenterol. 2010;24(5):531-539.
4. Gupta A, Das S, Ray K. A glimpse into the regulation of the Wilson disease protein, ATP7B, sheds light on the complexity of mammalian apical trafficking pathways. Metallomics. 2018;10(3):378-387.
5. Ferenci P, Caca K, Loudianos G, et al. Diagnosis and phenotypic classification of Wilson disease. Liver Int. 2003;23(3):139-142.
6. Schilsky ML. Wilson disease: genetic basis of copper toxicity and natural history. Semin Liver Dis. 1996;16(1):83-95.
7. Brewer GJ. Recognition, diagnosis, and management of Wilson's disease. Proc Soc Exp Biol Med. 2000;223(1):39-46.
8. Lin LJ, Wang DX, Ding NN, Lin Y, Jin Y, Zheng CQ. Comprehensive analysis on clinical features of Wilson's disease: an experience over 28 years with 133 cases. Neurol Res. 2014;36(2):157-163.
9. Li X, Zhang W, Zhou D, et al. Complex ATP7B mutation patterns in Wilson disease and evaluation of a yeast model for functional analysis of variants. Hum Mutat. 2019;40(5):552-565.
10. Cheng N, Wang H, Wu W, et al. Spectrum of ATP7B mutations and genotype-phenotype correlation in large-scale Chinese patients with Wilson Disease. Clin Genet. 2017;92(1):69-79.
11. Li XH, Lu Y, Ling Y, et al. Clinical and molecular characterization of Wilson's disease in China: identification of 14 novel mutations. BMC Med Genet. 2011;12:6.
12. Wan L, Tsai CH, Hsu CM, et al. Mutation analysis and characterization of alternative splice variants of the Wilson disease gene ATP7B. Hepatology. 2010;52(5):1662-1670.
13. Hua R, Hua F, Jiao Y, et al. Mutational analysis of ATP7B in Chinese Wilson disease patients. Am J Transl Res. 2016;8(6):2851-2861.
14. Xie JJ, Wu ZY. Wilson's disease in China. Neurosci Bull. 2017;33(3):323-330.
15. Coffey AJ, Durkie M, Hague S, et al. A genetic study of Wilson's disease in the United Kingdom. Brain. 2013;136(Pt 5):1476-1487.
16. Dong Y, Ni W, Chen WJ, et al. Spectrum and classification of ATP7B variants in a large cohort of Chinese patients with Wilson's disease guides genetic diagnosis. Theranostics. 2016;6(5):638-649.
17. Diaz J, Fonseca AG, Arboleda R, et al. Case report: the association of Wilson disease in a patient with ataxia and GLUT-1 deficiency. Front Pediatr. 2021;9:750593.
18. Zhang YZ, Jian G, He P, et al. Immunoglobulin a nephropathy as the first clinical presentation of Wilson disease: a case report and literature review. BMC Gastroenterol. 2021;21(1):384.
19. Dufernez F, Lachaux A, Chappuis P, et al. Wilson disease in offspring of affected patients: report of four French families. Clin Res Hepatol Gastroenterol. 2013;37(3):240-245.
20. Yuan ZF, Wu W, Yu YL, et al. Novel mutations of the ATP7B gene in Han Chinese families with pre-symptomatic Wilson's disease. World J Pediatr. 2015;11(3):255-260.
21. Zhang QJ, Xu LQ, Wang C, Hu W, Wang N, Chen WJ. Four-year follow-up of a Wilson disease pedigree complicated with epilepsy and hypopituitarism: case report with a literature review. Medicine (Baltimore). 2016;95(49):e5331.
22. European Association for Study of L. European association for study of L. EASL clinical practice guidelines: Wilson's disease. J Hepatol. 2012;56(3):671-685.
23. Chen S, Zhang Z, Wang Y, et al. Using quasispecies patterns of hepatitis B virus to predict hepatocellular carcinoma with deep sequencing and machine learning. J Infect Dis. 2021;223(11):1887-1896.
24. Giry-Laterriere M, Verhoeyen E, Salmon P. Lentiviral vectors. Methods Mol Biol. 2011;737:183-209.
25. Goncalves MA, Janssen JM, Holkers M, de Vries AA. Rapid and sensitive lentivirus vector-based conditional gene expression assay to monitor and quantify cell fusion activity. PLoS One. 2010;5(6):e10954.
26. Riggs ER, Andersen EF, Cherry AM, et al. Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen). Genet Med. 2020;22(2):245-257.
27. Mameli E, Lepori MB, Chiappe F, et al. Wilson's disease caused by alternative splicing and Alu exonization due to a homozygous 3039-bp deletion spanning from intron 1 to exon 2 of the ATP7B gene. Gene. 2015;560(2):276-279.
28. Ala A, Walker AP, Ashkan K, Dooley JS, Schilsky ML. Wilson's disease. Lancet. 2007;369(9559):397-408.
29. Lepori MB, Zappu A, Incollu S, et al. Mutation analysis of the ATP7B gene in a new group of Wilson's disease patients: contribution to diagnosis. Mol Cell Probes. 2012;26(4):147-150.
30. Li M, Ma J, Wang W, Yang X, Luo K. Mutation analysis of the ATP7B gene and genotype-phenotype correlation in Chinese patients with Wilson disease. BMC Gastroenterol. 2021;21(1):339.
31. Aggarwal A, Bhatt M. Update on Wilson disease. Int Rev Neurobiol. 2013;110:313-348.
32. Kenney SM, Cox DW. Sequence variation database for the Wilson disease copper transporter, ATP7B. Hum Mutat. 2007;28(12):1171-1177.
33. Roberts EA, Schilsky ML. American association for study of liver D. Diagnosis and treatment of Wilson disease: an update. Hepatology. 2008;47(6):2089-2111.
34. Wei Z, Huang Y, Liu A, et al. Mutational characterization of ATP7B gene in 103 Wilson's disease patients from Southern China: identification of three novel mutations. NeuroReport. 2014;25(14):1075-1080.
35. Wu Z, Wang N, Murong S, Lin M. Identification and analysis of mutations of the Wilson disease gene in Chinese population. Chin Med J (Engl). 2000;113(1):40-43.
36. Wu ZY, Wang N, Lin MT, Fang L, Murong SX, Yu L. Mutation analysis and the correlation between genotype and phenotype of Arg778Leu mutation in Chinese patients with Wilson disease. Arch Neurol. 2001;58(6):971-976.
37. Mak CM, Lam CW, Tam S, et al. Mutational analysis of 65 Wilson disease patients in Hong Kong Chinese: identification of 17 novel mutations and its genetic heterogeneity. J Hum Genet. 2008;53(1):55-63.
38. Li K, Zhang WM, Lin S, et al. Mutational analysis of ATP7B in north Chinese patients with Wilson disease. J Hum Genet. 2013;58(2):67-72.
39. Xu P, Liang X, Jankovic J, Le W. Identification of a high frequency of mutation at exon 8 of the ATP7B gene in a Chinese population with Wilson disease by fluorescent PCR. Arch Neurol. 2001;58(11):1879-1882.
40. Ye K, Wei Q, Gong Z, et al. Effect of norcantharidin on the proliferation, apoptosis, and cell cycle of human mesangial cells. Ren Fail. 2017;39(1):458-464.

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

How to cite this article: Huang C, Fang M, Xiao X, Gao Z, Wang Y, Gao C. Genetic studies discover novel coding and non-coding mutations in patients with Wilson's disease in China. J Clin Lab Anal. 2022;36:e24459. doi:10.1002/jcla.24459