Novel mutations of the *ATP7B* gene in Han Chinese families with pre-symptomatic Wilson's disease

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Background: **Wilson's disease (WD) is an autosomal recessive genetic disorder of copper metabolism, caused by mutations in the *ATP7B* gene, resulting in copper accumulation in the liver, brain, kidney, and cornea and leading to significant disability or death if untreated. Early diagnosis and proper therapy usually predict a good prognosis, especially in pre-symptomatic WD. Genetic testing is the most accurate and effective diagnostic method for early diagnosis.**

Methods: **The clinical and biochemical features of three unrelated Han Chinese families with pre-symptomatic WD were reported. The molecular defects in these families were investigated by polymerase chain reaction and DNA sequencing. Hundred healthy children with the same ethnic background served as controls. Bioinformatic tools (polymorphism phenotyping-2, sorting intolerant from tolerant, protein analysis through evolutionary relationships, and predictor of human deleterious single nucleotide polymorphisms) were combined and used to predict the functional effects of mutations.**

Results: **We identified 2 novel *ATP7B* mutations (p.Leu692Pro and p.Asn728Ser) and 3 known mutations (p.Met769fs, p.Arg778Leu and p.Val1216Met) in these Chinese WD families. These mutations were not observed in the 100 normal controls. The bioinformatic method showed that p.Leu692Pro and p.Asn728Ser mutations are pathogenic.**

Conclusions: **Our research enriches the mutation spectrum of the *ATP7B* gene worldwide and provides valuable information for studying the mutation types and mode of inheritance of *ATP7B* in the Chinese population. Liver function analysis and genetic testing in young children with WD are necessary to shorten the time to the initiation of therapy, reduce damage to the liver and brain, and improve prognosis.**

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Original article

Introduction

Wilson's disease (WD) is an autosomal recessive genetic disorder of copper metabolism, characterized by defective incorporation of copper into ceruloplasmin and reduced biliary copper excretion. WD results in copper accumulation preferably in the liver, brain, kidney, and cornea and leads to a series of complex clinical manifestations like acute fulminant hepatic failure, motor dysfunction, neuropsychiatric symptoms, Kayser-Fleischer (K-F) ring and associated sunflower cataract. The prevalence of WD is about 1/30 000 worldwide, but higher in China and Japan (about 1/10 000).[1] Early diagnosis and proper therapies are important to reduce the damage of the liver and brain and improve the prognosis. Delayed diagnosis and therapy may lead to poor prognosis or even death.[2] Clinically, WD is diagnosed based on clinical manifestations and biochemical detection of copper metabolic disorder. However, not all patients exhibit typical clinical manifestations and biochemical findings at the early stage of the disease. Clinical manifestations in children with WD are usually atypical and render the diagnosis difficult. Thus, in patients (especially children) with mild or pre-symptomatic WD, genetic detection is the most accurate and effective diagnostic method for early diagnosis.[3]

WD is caused by mutation in the *ATP7B* gene, which maps to chromosome 13q14.3, extends 78 kb in length, and contains 21 exons and 20 introns. It encodes copper-transporting P-type ATPase, which is a group of

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transmembrane copper transport proteins that mediate the uptake and excretion of copper. This protein is composed of 1465 amino acids that contain 6 copper-binding domains, 8 transmembrane segments, 1 adenosine triphosphate (ATP)-binding domain (with a P-domain and N-domain), and 1 A-domain.[4] To date, more than 600 *ATP7B* gene mutations in WD have been identified along the entire length of the *ATP7B* gene.[3] These mutations are mainly missense mutations, and most patients have compound heterozygous mutations. Meanwhile, in a recent study, Squitti et al[5] found the potential role of *ATP7B* genetic variation in Alzheimer's disease, expanding the pathogenic spectrum of *ATP7B* in neurodegenerative diseases.

Here, we identified 2 new mutations and detected 3 known mutations of *ATP7B* in 3 unrelated Han Chinese families (all the probands being children), thus expanding the mutation spectrum of *ATP7B* and further proving the importance of genetic testing for early diagnosis of WD in children, especially in pre-symptomatic WD.

Methods

Patients

Three Han Chinese families with WD from Children's Hospital, Zhejiang University School of Medicine (Hangzhou, China) were studied. The diagnostic criteria of WD were mainly based on clinical and biochemical findings: low serum ceruloplasmin (<0.2 g/L), increased urinary copper excretion (>40 μg per 24 hours in children), presence of K-F ring in the cornea, abnormal liver function and neuropsychiatric symptoms.[6]

To assess the possible occurrence of polymorphisms in any detected nucleotide substitutions, we analyzed 100 healthy children with the same ethnic background as controls. For each patient we collected an informed consent form signed by at least one of the patients. The study was approved by the Ethics Committee of Children's Hospital of Zhejiang University School of Medicine.

Genetic analysis

We used a standard DNA extraction method to extract genomic DNA from peripheral blood samples of members of the families and 100 normal controls. All the exons of *ATP7B* and their associated boundary regions were amplified by polymerase chain reaction (PCR) using the previously reported primers.[7,8] The PCR reaction mixture (total volume: 50 μL) contained 100 ng genomic DNA, 1.5 mmol/L magnesium chloride, 200 μmol/L deoxyribonucleotides, 20 pmol each of the forward and reverse primers, and 2.5 UEx Taq enzyme (TaKaRa) in the reaction buffer. The amplification reactions consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles at 94°C for 30 seconds, appropriate annealing temperature for 30 seconds (between 54°C-64°C) and 72°C for 45 seconds, with a final extension step at 72°C for 7 minutes. After amplification, the PCR products were separated on 1.5% agarose gel for 1 hour at 100 V and sequenced by using an ABI3730 system (Bio Basic Inc, Shanghai, China). Exons that showed a mutation in the first round of sequencing were amplified and sequenced again to exclude the mismatching caused by the low fidelity of Taq DNA polymerase. Repeated sequencing was performed to confirm the mutations.

Prediction of the influence of gene mutation on protein function

We applied an algorithm (proposed by Squitti and colleagues) which merged the four most used bioinformatic tools to predict the pathogenicity and functional impact on protein of missense mutation.[9] These bioinformatic tools are polymorphism phenotyping (PolyPhen)-2 (http://genetics.bwh.harvard.edu/pph2/), sorting intolerant from tolerant (SIFT) (http://sift.jcvi.org/), protein analysis through evolutionary relationships (Panther) (http://www.pantherdb.org/tools/csnpScoreForm.jsp) and predictor of human deleterious single nucleotide polymorphisms (PhD-SNPs) (http://snps.uib.es/phd-snp/phd-snp.html). Using a simple statistical procedure, information from the four tools was combined to gain an outcome that was not biased by statistical manipulation and maintain the reliability of the prediction.[9] Accordingly, the combined probability to be deleterious was calculated with the following equation: Combined $P_{deleterious}$ (cP_{del})=mean [$Score_{PolyPhen-2}$; 1-$Score_{SIFT}$; $P_{deleterious\_Panther}$; $Prediction_{PhD-SNP}$ (Neutral=0; Disease=1)].[9] This cP_{del} ranges from 0 (no functional impact) to 1 (complete loss of function).

Results

Clinical phenotype analysis

Family 1

The proband was a girl aged 11 years and referred to our hospital with possible liver dysfunction diagnosed at a routine physical examination. No symptoms or signs of digestive and neurological disease were identified at the physical examination. Biochemical and serological testing showed an increase in alanine aminotransferase (ALT) (192, normal value: 5-50 U/L), a slight reduction in serum copper (10, normal value: 12-39 μmol/L), a significant decrease in ceruloplasmin (0.02, normal value: 0.2-0.6 g/L) and a marked increase in 24-hour urine copper (268, normal value: 0-40 μg/24 hours). Specific immunoglobulin M antibodies

(enzyme-linked immuno sorbent assay) against hepatitis virus A, B, C, and D infections were negative. Slit-lamp examination showed corneal K-F rings in both eyes. Cranial magnetic resonance imaging (MRI) was normal; abdominal ultrasonography showed a hyperechoic liver but no hepatomegaly or splenomegaly. Her brother was 3.5 years old and also had an increase in ALT (93 U/L), a slight reduction in serum copper (11 μmol/L), a significant decrease in ceruloplasmin (<0.02 g/L), and a marked increase in 24-hour urine copper (215 μg/24 hours). The K-F rings were also observed. Cranial MRI and abdominal ultrasonography were normal. Their parents had no clinical manifestations and no biochemical evidence of disease except for a slight reduction in serum ceruloplasmin (father: 0.18; mother: 0.17).

Family 2

The proband was a boy aged 5 years and 3 months. He was referred to our hospital with a tic disorder but no other symptoms and signs related to the primary disease. Analysis of trace elements showed a reduction in serum copper (10.5 μmol/L). Subsequent examinations revealed normal ALT (14 U/L), significantly low serum ceruloplasmin (0.04 g/L), and elevated 24-hour urine copper (102 μg/24 hours).The corneal K-F ring test was negative. Cranial MRI and abdominal ultrasonography were normal. Their parents had no clinical manifestations and biochemical evidence of the disease except for a slight reduction in serum copper (father: 10.1; mother: 10.9) and ceruloplasmin (father: 0.18; mother: 0.19).

Family 3

The proband was a girl aged 1 year and 3 months. She was referred to our hospital with a reduction in her serum copper level (9.5 μmol/L) but no symptoms and signs of disease. Subsequent examinations suggested that normal ALT (38 U/L), significantly low serum ceruloplasmin (0.02 g/L), and slightly elevated 24-hour urine copper (68 μg/24 hour). The K-F rings were not observed. Cranial MRI and abdominal ultrasonography were normal. Her older brother had normal clinical and biochemical findings. Their parents had no clinical manifestations nor biochemical evidence of disease except for a slight reduction in serum ceruloplasmin (father: 0.13; mother: 0.18).

Mutation analysis

In family 1, direct sequencing of the 21 exons of the *ATP7B* gene revealed compound heterozygous mutations in proband and her brother; these mutations included a heterozygous T-to-C transition at nucleotide 2075 (c.2075T>C) in exon 7 that resulted in the conversion of leucine to proline at amino acid position 692 (p.Leu692Pro) for one allele, and a heterozygous G-to-A transition at nucleotide 3646 (c.3646G>A) in exon 17 that resulted in the conversion of valine to methionine at amino acid position 1216 (p.Val1216Met) for another allele. The proband's father and mother had the heterozygous mutations p.Val1216Met and p.Leu692Pro, respectively. The p.Leu692Pro is a novel mutation and p.Val1216Met is a known mutation. The mutation of p.Leu692Pro was not found in the 100 controls (Fig. 1).

In family 2, DNA sequencing revealed compound heterozygous mutations in the proband, these mutations included a heterozygous A-to-G transition at nucleotide 2183 (c.2183A>G) in exon 8 that resulted in the conversion of asparagine to serine at amino acid position 728 (p.Asn728Ser) for one allele, and a heterozygous G-to-T transition at nucleotide 2333 (c.2333G>T) in exon 8 that resulted in the conversion of arginine to leucine at amino acid position 778 (p.Arg778Leu) for another allele. The proband's parents had the heterozygous mutations p.Arg778Leu and p.Asn728Ser, respectively. The p.Asn728Ser is a novel mutation and p.Arg778Leu is a known mutation. No such mutation of p.Asn728Ser was found in the 100 controls (Fig. 2).

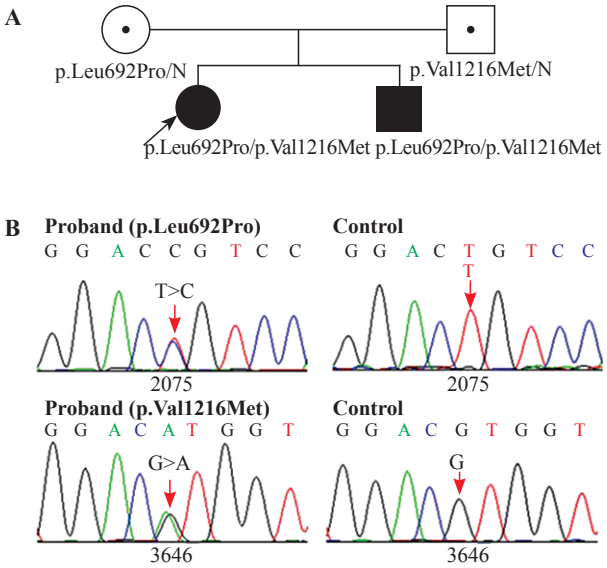

Fig. 1. The pedigree and DNA sequencing results of family 1. **A:** The pedigree of family 1, the filled symbols indicate affected individuals, the open symbol with a central black spot indicates the carrier. The arrow points to the proband, showing that the proband and her brother had compound heterozygous mutations (p.Leu692Pro and p.Val1216Met) and that the parents were carriers; **B:** Sequencing results showed a heterozygous T-to-C transition at nucleotide 2075 (c.2075T>C) in exon 7 (p.Leu692Pro) and a heterozygous G-to-A transition at nucleotide 3646 (c.3646G>A) in exon 17 (p.Val1216Met) in the proband. No such mutations were observed in the controls. N: normal.

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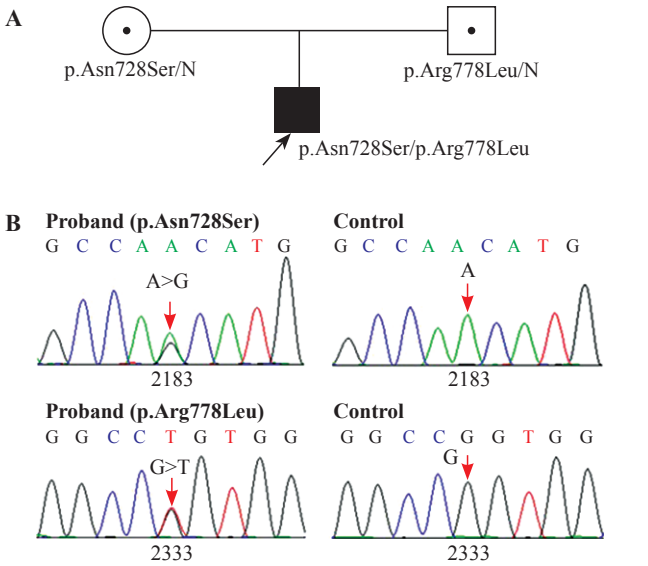

Fig. 2. The pedigree and DNA sequencing results of family 2. **A:** The pedigree of family 2 showed that the proband had compound heterozygous mutations (p.Asn728Ser and p.Arg778Leu) and that the parents were carriers; **B:** Sequencing results showed a heterozygous A-to-G transition at nucleotide 2183 (c.2183A>G) in exon 8 (p.Asn728Ser) and a heterozygous G-to-T transition at nucleotide 2333 (c.2333G>T) in exon 8 (p.Arg778Leu) in the proband. The controls, however, showed no similar mutations at either of these sites. N: normal.

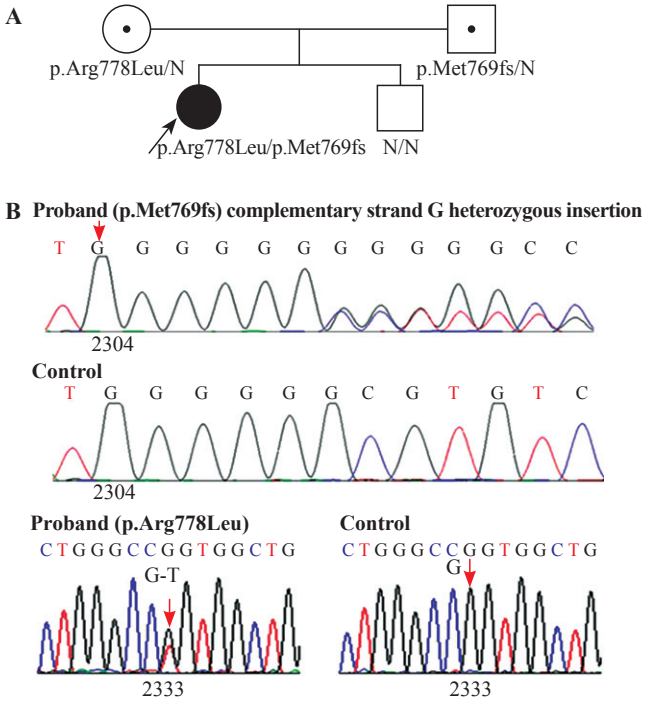

Fig. 3. The pedigree and DNA sequencing results of family 3. **A:** The pedigree of family 3 showed that the proband had compound heterozygous mutations (p.Met769fs and p.Arg778Leu) and that the parents were carriers and her brother was normal; **B:** Sequencing results showed a heterozygous C insertion (complementary strand G insertion) at nucleotide 2304 (c.2304dupC) in exon 8, resulting in a frameshift mutation (p.Met769fs) and a heterozygous G-to-T transition at nucleotide 2333 (c.2333G>T) in exon 8 (p.Arg778Leu) in the proband. No such mutations were observed in the controls. N: normal.

In family 3, DNA sequencing revealed a frameshift mutation and a heterozygous mutation in the proband, these mutations included a heterozygous C insertion (complementary strand G insertion) at nucleotide 2304 (c.2304dupC) in exon 8 that resulted in the frame shift at amino acid position 769 (p.Met769fs) for one allele, and a heterozygous G-to-T transition at nucleotide 2333 (c.2333G>T) in exon 8 that resulted in the conversion of arginine to leucine at amino acid position 778 (p.Arg778Leu) for another allele. The proband's father and mother had the mutations p.Met769fs and p.Arg778Leu, respectively. No mutation was found in her brother. The p.Met769fs and p.Arg778Leu are known mutations (Fig. 3).

Prediction of the functional effects of mutations

1) c.2075T>C, p.Leu692Pro (cP_{del}=0.977); 2) c.2183A>G, p.Asn728Ser (cP_{del}=0.673). The cP_{del} score of p.Leu692Pro was high, and it suggested that p.Leu692Pro may alter the structure and function of *ATP7B* and contribute to the disease. Although the cP_{del} of p.Asn728Ser was not a particularly high score, it was located in the region of 3rd transmembrane domain (TM) of exon 8. According to Squitti and colleagues' observation,[9] the non-synonymous SNPs located in the TM3 had the highest probability of being deleterious. Hence, we concluded that this *ATP7B* variant is also a disease-causing mutation.

Discussion

Early diagnosis and proper therapy usually predict a good prognosis in WD.[2] However, the clinical symptoms of WD are complex, the initial symptoms are usually atypical, and the biochemical profiles of healthy subjects, carriers, and patients are frequently overlapping. Thus, WD is often misdiagnosed, especially when the symptoms are not present or atypical. In addition, clinical manifestations of WD are usually not typical[1,4,10] and early diagnosis is more difficult in children. Thus, clinicians should use all possible clues and genetic detection to reduce the misdiagnosis rate.

Geng et al[1] investigated 11 Chinese children with WD and found K-F rings in 3 patients, manifestations of the neurological disorder in 2, and hepatosplenomegaly in 1, and an increase in ALT (but no clinical manifestations) in 8. Genetic analysis confirmed the presence of pathogenic mutations in these children. Li et al[4] investigated 27 WD children younger than 8 years, and found no neurological symptoms of disease in 26 children and no K-F rings in 25. The diagnosis in 26 (96%) of the children was based on ALT increase, copper biochemistry assays, and mutation in the *ATP7B* gene. In a study in Hong Kong

by Hui et al[10] of 10 children with pre-symptomatic WD, ALT increase was identified as an important clue for early detection of WD and serum copper was reduced. The levels of serum copper were also reduced in 68.9% of 133 WD patients according to Lin et al,[11] in 15 of 16 WD patients according to El Balkhi et al.[12] In England, Coffey et al[2] found a pathogenic mutation of the *ATP7B* gene in 98% of 181 WD patients, suggesting that WD could be accurately diagnosed by genetic testing. In addition, Seo et al[13] proposed the importance of genetic testing for WD in young children. In our study, 3 WD families were investigated. WD was diagnosed in the probands before the onset of symptoms. In family 1, the proband and her brother were referred to our hospital after abnormal ALT was detected in the physical examination and reduced serum copper was detected in subsequent examinations. However, in the probands of families 2 and 3, serum copper was reduced and WD was suspected based on subsequent biochemical findings. WD was diagnosed in these patients based on rapid genetic analysis, resulting in more prompt initiation of therapy. On the basis of previous findings and our results, we conclude that ALT and genetic testing are important for early diagnosis of WD in young children, especially in pre-symptomatic WD. Meanwhile, reduction in serum copper is also an important clue for diagnosis of WD. Thus, in clinical practice, WD should be considered when ALT is increased and serum copper is reduced, and subsequent copper biochemical examination and genetic testing should be recommended for differential diagnosis.

More than 600 mutations of the *ATP7B* gene have been reported to be distributed in 21 exons,[3] especially in exons encoding the transmembrane domain and ATP functional domain.[14] The mutation sites also vary among regions and races. The most common mutation of the *ATP7B* gene in Europe is p.His1069Gln at exon 14 (found in about 50%-80% of patients with WD),[15] in Brazil is p.His1069Gln and 3402delC at exon 15 with an allele frequency of 37.1% and 30.8%, respectively.[16,17] In Chinese patients, the p.Arg778Leu missense mutation at exon 8 is the most prevalent one with an allele frequency of 37.7%-55%.[18,19] It is also one of the most common mutations in Asian populations, including Korean (with an allele frequency of 37.9%),[20] Japanese (13.4%)[21] and Thai (10.52%).[22] The most common mutation in Japanese is 2871delC in exon 13 with an allele frequency of 15.9%.[21] The p.Arg778Leu mutation (especially the homozygous mutation) may cause clinical manifestations of liver injury at the early stage.[4] The reasons for the discrepancy in hot mutation sites among different regions and races are still unclear. In addition, the clinical manifestations of WD are also heterogeneous, and variation in clinical phenotype between patients with the same genotype may be due to environmental factors (copper intake, infection, drugs, and toxins) and regulatory genes of the *ATP7B* gene.

In family 1, both the proband and her brother had two heterozygous mutations (p.Leu692Pro and p.Val1216Met). p.Leu692Pro is a never-reported novel missense mutation and not found in WD mutation database (http://www.wilsondisease.med.ualberta.ca/database.asp), the human genome mutation database, 1000 genomes project (http://browser.1000genomes.org/index.html), and SNP databases. It occurs in the TM2 of exon 7 and causes a change in the structure of the transmembrane domain, affecting copper transport. Bioinformatic analysis showed that Leu692 is highly conserved evolutionarily and p.Leu692Pro mutation is pathogenic. p.Val1216Met is located in the ATP-binding domain of exon 17. Studies[4,23] have reported that this mutation is pathogenic because it can interfere with binding to ATP and reduce the activity of copper-ATPase. The mutation was simple heterozygous in both parents of this proband, had little effect on the protein's function, and only mildly reduced the serum ceruloplasmin level. The compound heterozygous mutations detected in the proband and his brother had a significant influence on the protein's function, causing liver dysfunction.

In family 2, the proband had two heterozygous mutations (p.Asn728Ser and p.Arg778Leu). The mutation p.Asn728Ser was located at a novel site in the TM3 of exon 8 where it might cause conformational change in the transmembrane part of the protein, reducing its copper transport capability and causing WD. Bioinformatic analysis also showed that Asn728 is highly conserved evolutionarily and p.Asn728Ser mutation is pathogenic. p.Arg778Leu is the most common mutation in Chinese patients and may cause conformational change in the TM4, as previously reported.[4,18,19] This child had no clinical symptoms, the pathogenicity of compound heterozygous mutations was not obvious and it might be ascribed to his young age.

In family 3, the proband had two heterozygous mutations (p.Met769fs and p.Arg778Leu). p.Met769fs (c.2304dupC) is a frameshift mutation that occurs in the TM4 of exon 8.[4] This frameshift causes numerous amino acids in the protein sequence to change, leading to protein dysfunction and disease.[4] p.Arg778Leu has been described above. The proband had no clinical symptoms, the pathogenic effect of mutations was not obvious and it might be due to her young age.

In conclusion, we discovered 2 novel *ATP7B* mutations (p.Leu692Pro and p.Asn728Ser) and 3 known mutations (p.Met769fs, p.Arg778Leu and p.Val1216Met) in these Chinese WD families. Our research enriches the mutation spectrum of the *ATP7B*

gene worldwide and provides valuable reference data for studying the mutation types and mode of inheritance of *ATP7B* in the Chinese population. Liver function analysis and genetic testing in young children with WD are important, especially in those with pre-symptomatic WD, thereby shortening the time to therapy initiation, reducing damage to organs (such as the liver and brain), and improving the prognosis.

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Ethical approval: The study was approved by the Ethics Committee of Children's Hospital, Zhejiang University School of Medicine.

Competing interest: The authors declare no conflict of interest.

Contributors: Yuan ZF, Yu YL and Gao F contributed to the study design, and Yuan ZF drafted the manuscript. All the other authors contributed to the acquisition of data and approved the final version of the manuscript.

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