Familial screening of children with Wilson disease
Necessity of screening in previous generation and screening methods

Huamei Li, MD, Lifang Liu, MD, Yun Li, MD, Shendi He, MD, Yujie Liu, MD, Jinhong Li, MD, Ran Tao, MD, Wei Li, MD, Shiqiang Shang, MD.*

Abstract
Wilson disease (WD) is an autosomal recessive genetic disorder associated with copper metabolism. Early diagnosis and therapy can result in good prognosis of WD. Thus, it is highly recommended to perform familial screening. In this study, we aimed to investigate the range of familial screening of children with WD and determine the appropriate screening methods.

We enrolled 20 children with WD and 50 family members of each of these patients (40 parents and 10 siblings). All the subjects underwent a physical examination, Kayser–Fleischer (K-F) rings in the cornea, abdominal ultrasonography (Abdl Ur), cranial magnetic resonance imaging (MRI), serum ceruloplasmin, serum copper, 24-hour urine copper, blood alanine transaminase (ALT) and aspartate transaminase (AST), and ATP7B gene.

Two new patients with presymptomatic WD (1 mother and 1 brother) in 2 families were found by screening. They had no clinical symptoms and K-F rings in corneal. Biochemical examination indicated decreased serum ceruloplasmin and serum copper in the mother and decreased serum ceruloplasmin in the brother. Gene sequencing revealed compound heterozygous mutations in them. In addition, 48 heterozygous carriers of Wilson disease (WHDzc) were found in this study. The levels of ceruloplasmin and serum copper in patients of WD were significantly less than WHDzc and 24-hour urinary copper were significantly higher than WHDzc (P<.000). The biochemical profiles of WD and WHDzc overlapped in range of 0.8 to 1.6 g/L in ceruloplasmin, above 9 μmol/L in serum copper and below 100 μg/24 h in urinary copper. Gene sequencing showed 2 pathological mutations in all patients with WD and 1 pathological mutation in all WHDzc.

Not only siblings but also the previous generation of children probands with WD should be screened. Genetic testing should be conducted for the diagnosis of presymptomatic patients with WD.

Abbreviations: AASLD = American Association for Study of Liver Diseases, Abdl Ur = abdominal ultrasound, ALT = alanine transaminase, AST = aspartate transaminase, ATP7B = P-type adenosine triphosphatase gene, DNA = deoxyribonucleic acid, EASL = European Association for Study of Liver, h = hour, K-F = Kayser–Fleischer, MRI = magnetic resonance imaging, PCR = polymerase chain reaction, WD = Wilson disease, WHDzc = heterozygous carriers of Wilson disease.

Keywords: ATP7B, children, family screening, hepatolenticular degeneration, Wilson disease

1. Introduction
Wilson disease (WD; also known as hepatolenticular degeneration) is an autosomal recessive disease associated with copper metabolism and characterized by the reduced capability of copper into ceruloplasmin and copper excretion. Copper accumulates in various organs, including liver, central nervous system, kidney, and cornea and results in various clinical manifestations, such as hepatic failure, motor dysfunction, neuropsychiatric symptoms, and Kayser–Fleischer (K-F) rings in corneal. WD occurs at a frequency of approximately 1 in 30,000 to 50,000 worldwide, but 1 in 10,000 in China and Japanese.[1,2] The frequency of heterozygous carriers of Wilson disease (WHDzc) is about 1% to 2%.[3] The diagnosis of WD is mainly based on clinical manifestations and detection of biochemical levels of copper. In particular, low ceruloplasmin and serum copper levels and elevated urinary copper excretion and liver copper content are associated with WD. However, several patients did not exhibit typical clinical symptoms and biochemical findings at the early stage of the disease. Thus, genetic detection is an important diagnostic method for early diagnosis stage of WD.[4–6]

WD is caused by mutation in the P-type adenosine triphosphatase (ATP7B) gene, which is located in chromosome 13q14.3 and composed of 21 exons and 20 introns. Copper-transporting P-type ATPase encoded by ATP7B gene is a group of transmembrane copper transport proteins that mediate copper metabolism. Currently, more than 600 gene mutations are found in ATP7B, and new mutations are constantly reported worldwide.[7] Some
hot mutations show regional and ethnic difference. The most common mutation of ATP7B gene is p.His1069Gln at exon 14 in Europe, while in Asia is p.Arg778Leu missense mutation at exon 8,[8,9] and in Brazil is p.His1069Gln and 3402delC at exon 15.[8,9] WD has high mortality and disability rate. However, it is one of the treatable hereditary diseases. Irreversible tissue injury can be prevented if WD is diagnosed and treated at an early stage.[10] Family members of WD subjects have different risk degree of developing clinical disease. Thus, it is essential to screening family members of WD subjects. In this study, we investigated the crowd range and determine the appropriate methods of familial screening of children with WD.

2. Materials and methods

2.1. Subjects

Twenty children with WD from 20 unrelated families (male, n = 12; female, n = 8; age range, 2–15 years old) are recruited from Children’s Hospital, Zhejiang University School of Medicine (Hangzhou, China) from February 2015 to October 2017. The diagnosis of WD was based on clinical examination, biological tests (low serum ceruloplasmin level and increased 24-hour urinary copper excretion), the presence of K-F rings, and genetic tests.[11] We also recruited 50 family members from these families, including 40 parents (male, n = 20; female, n = 20; age range, 28–46 years) and 10 siblings (male, n = 4; female, n = 6; age range, 3–15 years). Patients with WD were measured at the time of diagnosis and before therapy. Informed consent was obtained from participants included in the study. The study was approved by the Ethics Committee of Children’s Hospital of Zhejiang University School of Medicine.

2.2. Physical examination

All enrolled subjects’ medical history was collected and then simultaneously they were underwent related physical examination, including examination of neural symptoms, abdominal palpation, evaluation of K-F rings of the cornea, abdominal ultrasonography (Abdl Ur), and brain magnetic resonance imaging (MRI).

2.3. Biochemical studies

Blood alanine transaminase (ALT) and aspartate transaminase (AST) were performed on the biochemical analyzer (BECKMAN COULTER, AUS800, Brea), serum ceruloplasmin concentration was measured using immune turbidimetry assay (SIEMENS, BNII, Munich, Germany). Serum copper concentration and 24-hour urinary copper excretion were determined by atomic absorption spectrometry (PERSEE, MAS-60, Peking, China). Serum copper concentration and 24-hour urinary copper were measured using immune turbidimetry assay (SIEMENS, BNII, Brea), serum ceruloplasmin concentration was measured using immune turbidimetry assay (SIEMENS, BNII, Munich, Germany). Serum copper concentration and 24-hour urinary copper excretion were determined by atomic absorption spectrometry (PERSEE, MAS-60, Peking, China). Serum copper concentration and 24-hour urinary copper excretion were determined by atomic absorption spectrometry (PERSEE, MAS-60, Peking, China). Serum copper concentration and 24-hour urinary copper excretion were determined by atomic absorption spectrometry (PERSEE, MAS-60, Peking, China).

2.4. Analysis of ATP7B

We extracted genomic DNA from peripheral whole blood samples of members of the families using a DNA extractor kit (AxyPrep Blood Genomic DNA QIA Miniprep kit; AXYGEN, San Jose). The 21 exons of ATP7B and their associated boundary regions were performed via polymerase chain reaction (PCR) using the previously reported primers.[11,12] After amplification, the PCR products were subjected to DNA sequencing using an ABI3730 system (Bio Basic Inc, Shanghai, China). Repeated sequencing was performed to confirm the mutations.

2.5. Statistical analysis

Data analysis was performed using SPSS software (SPSS 19.0, SPSS Inc, Chicago, IL). Independent-samples t test or nonparametric test was used to compare means between patients with WD and WDHzc. The criterion for statistical significance was P < .05.

3. Results

3.1. Examination results of new patients with WD diagnosed by family screening

Two new patients with WD (1 parent and 1 sibling) in 2 families were found by screening. In family 1, the new patient was a mother. She had no symptoms of digestive and neurological diseases. Biochemical examination suggested normal ALT and AST, and significantly low serum ceruloplasmin and serum copper. The K-F rings were not observed. Gene sequencing revealed compound heterozygous mutations in her. In family 2, the new patient was the brother of a proband. He had no clinical manifestations and no biochemical evidence of disease except for a reduction in serum ceruloplasmin. The K-F rings were not observed. Gene sequencing revealed compound heterozygous mutations in him (Table 1, Fig. 1).

3.2. Examination results of WD and WDHzc

This study found 48 WDHzc. No symptoms or signs of digestive and neurological disease were identified in them. Gene sequencing showed 1 pathological mutation in all them. The levels of ceruloplasmin and serum copper in patients of WD were significantly less than WHDzc and 24-hour urinary copper was significantly higher than WHDzc (P = .000) (Fig. 2). The biochemical profiles of WD and WDHzc overlapped in range of 0.8 to 1.5 g/L in ceruloplasmin, above 9 µmol/L in serum copper, and below 100 µg/24h in urinary copper. The study indicated 31 (64.6%) of 48 of the WDHzc had less than 0.2 g/L of ceruloplasmin.

| Table 1 |
|---|---|---|
| Examination results of new patients with WD diagnosed by family screening. | | |
| Patient 1 | Patient 2 |
| Age, y | 41 | 6 |
| Gender | Female | Male |
| Relationship with proband | Mother | Brother |
| Clinical symptoms | No | No |
| Serum ceruloplasmin (0.02–0.2 g/L) | 0.02 | 0.07 |
| Urinary copper (<40 or 100 µg/24h) | 34.7 | 38.4 |
| Serum copper (12–39 µmol/L) | 1.8 | 12.8 |
| Serum ALT (<50 U/L) | 12 | 46 |
| Serum AST (15–60 U/L) | 20 | 30 |
| Serum ALP (42–362 or 30–120 U/L) | 78 | 308 |
| K-F ring | No | No |
| Cranial MRI | Normal | Normal |
| Abdul | Normal | Normal |
| Gene mutations | p.Arg778Leu/ | p.Arg778Leu/ |
| | p.Arg150His | p.Val909Met |

*Abdul = abdominal ultrasound, ALP = alkaline phosphatase, ALT = alanine transaminase, AST = aspartate transaminase, h = hour, MRI = magnetic resonance imaging.

*The reference interval for urinary copper is <45 µg/L in children and <100 µg/L in adults, for ALP is 42–362 U/L in children and 30–120 U/L in adults.
serum ceruloplasmin levels but none below 0.8 μg/L, 19 (39.6%) of 48 had greater than 40 μg/24h of urinary copper but none greater than 100 μg/L, and 21 (43.8%) of 48 had less than 12 μmol/L of serum copper but none less than 9 μmol/L. Another study showed that 1 (4.5%) of 22 patients of WD had greater than 12 μmol/L in serum copper and 2 (9.1%) of 22 had less than 40 μg/24h in urinary copper and 3 (13.6%) of 22 less than 100 μg/24h in urinary copper (Table 2). Gene sequencing showed 2 pathological mutations in all them, which are known mutations.

4. Discussion

WD is highly recommended to perform familial screening; American Association for Study of Liver Diseases (AASLD) and European Association for Study of Liver (EASL) recommend screening first-degree relatives of the proband, suggesting siblings or offspring only. It is usually considered that WD not only occurs in siblings (2.5%) and the offspring (0.5%), but it also occurs in the previous generation (0.5%), although rarely reported. Brunet et al reported a 43-year-old asymptomatic father diagnosed with WD after his daughter was diagnosed with a typical WD. Similarly, in Korea, a 14-year-old girl was diagnosed of having WD. Further study for her family members revealed that her father, a paternal uncle, and a sister were compound heterozygous WD patients. In our study, a 41-year-old mother of a proband was also diagnosed as a presymptomatic patient with WD through whole ATP7B gene sequencing. Although < 40 years old has been considered the upper age limit for WD, several studies reported old patients who were diagnosed with WD in their early 70s. Moreover, the ages of most parents of children with WD are less than the upper age limit. In our study, more than half of the parents are less than 40 years old. Meanwhile, WD show different clinical symptoms, including hepatic, neurological, psychiatric disorder, or asymptomatic, and the phenotype often differs among patients with the same genotype, even within a single family. Considering incidence equaling to the next generation, the possibility of late-onset and asymptomatic, and differing phenotype of the same genotype, it seems necessary to screen parents of children with WD.

On the basis of clinical symptoms and copper biochemical examination, diagnosing typical patients with WD through familial screening is uncomplicated. However, clinical symptoms of WD are complex and usually atypical, and the biochemical examination results of patients, carriers, and healthy individuals frequently overlap. Thus, WD is frequently misdiagnosed, especially in presymptomatic and atypical patients. Thus, clinicians should use an accurate and effective diagnostic method for the reduction of the misdiagnose rate during family screening. Serum ceruloplasmin, serum copper, 24-hour urinary copper, and hepatic copper content are important indicators for WD diagnosis but limited. For instance, ceruloplasmin is unstable in the body, and reduced levels of ceruloplasmin are detected in cases of nephritic syndrome, Menkes’ disease, protein-losing enteropathy, and chronic liver disease and observed in 20% of WHDzc.

![Figure 1](image1.png)

**Figure 1.** The pedigrees of 2 families with new patients with WD. The filled symbols indicate affected individuals. The half-filled symbols indicate the carrier. The arrow points to the proband. Family1: The proband and his mother had compound heterozygous mutations (p.Arg778Leu/p.Arg1156His) and his father were carriers. Family2: The proband and her brother had compound heterozygous mutations (p.Leu692Pro/p.Arg778Leu) and that the parents were carriers.

![Figure 2](image2.png)

**Figure 2.** The expression levels of serum ceruloplasmin, serum copper, and urinary copper in the WD patients and WHDzc. (A) The median levels of serum ceruloplasmin in the 2 groups were 0.05, 0.18 g/L, respectively; (B) Serum copper, 6.15, 12.47 μmol/L; (C) Urinary copper, 284.65, 38.3 μg/24 h. P value indicated comparisons between 2 groups.
Urinary copper excretion greater than 100 μg/24h was taken as diagnostic of WD.[22] However, urinary copper excretion may be less than 40μg/24h at presentation in 16% to 23% of patients, especially in children and asymptomatic siblings.[23–25] In our study, 3 (13.6%) of 22 of WD patients contained less than 100 μg/24h of urinary copper (including 2 presymptomatic patients) and 19 (39.6%) of 48 of WDHzc contained more than 40 μg/24h of urinary copper. Moreover, hepatic copper content (more than 250μg/g dry weight), a significant biochemical indicator for WD, significantly increases in established cholestatic disorders and idiopathic copper toxicosis syndromes.[20] Most patients refuse to undergo hepatic copper content detection because it is invasive. No patient underwent hepatic copper content detection in our study. Although the above-mentioned copper biochemical indicators are defective, they can indicate us to do further examination when they were abnormal, especially when it increases or decreases significantly. The copper biochemical profiles of WD and WDHzc are overlapping partly, the proportion of WDHzc with abnormal levels of copper biochemical indicators is high, but ceruloplasmin below 0.08g/L, serum copper below 9μmol/L, and urinary copper above 100 μg/24h were more prone to diagnose WD in our study. This study also suggested that a slightly reduced ceruloplasmin and serum copper and slightly increased 24-hour urinary copper may be a clue to the diagnosis of WHDz. The percentage of WDHzc with reduced ceruloplasmin in our study was significantly higher than that reported,[15] which may be related to different mutation types. In the future, we will expand sample size to analyze the relationship between the level of ceruloplasmin and genotype of WDHzc.

Given that patients diagnosed by family screening are usually presymptomatic, gene analysis is recommended for their diagnosis.[5] Meanwhile, gene analysis differentiates WDHzc from presymptomatic patients, and misdiagnosis of WD and lifelong treatment are prevented. It also avoids the need for continued testing when the results had not been adequate to diagnosis WD or excluded WD. In our study, 2 WD patients diagnosed through screening were both presymptomatic. They were diagnosed with WD by rapid genetic tests, which resulted in an appropriate initiation of therapy.

WD gene analysis methods mainly include direct sequencing and haplotyping.[5] Currently, molecular genetic analysis has become widely available and obtained a high score in WD diagnosis.[5] Given the characteristics of autosomal recessive inheritance diseases, individuals carrying 2 pathogenic mutations are considered to have the disease. Direct sequencing is a standard method used in WD diagnosis and capable of identifying mutation types. Although the cost of detecting the whole ATP7B gene is highly expensive, detecting mutational hotspots first is an economical method that can ensure accurate results. The costs will decline if the

### Table 2

**Proportion of patients with WD and WDHzc in different ranges of biochemical indicators.**

|                | Patients with WD |       | WDHzc |       |
|----------------|------------------|-------|-------|-------|
|                | N                | %     | N     | %     |
| Serum ceruloplasmin |                  |       |       |       |
| < 0.08g/L       | 21               | 95.5  | 5     | 10.4  |
| 0.08–0.15g/L    | 1                | 4.5   | 26    | 54.2  |
| 0.15–0.20g/L    | 2                | 9.1   | 21    | 43.8  |
| ≥0.20g/L        | 1                | 4.5   | 27    | 56.2  |
| Serum copper    |                  |       |       |       |
| < 6μmol/L       | 12               | 54.6  | 29    | 60.4  |
| 6–9μmol/L       | 7                | 31.8  | 19    | 39.6  |
| 9–12μmol/L      | 2                | 9.1   | 19    | 39.6  |
| ≥12μmol/L       | 1                | 4.5   | 27    | 56.2  |
| 24-h urinary copper |                |       |       |       |
| < 40μg/24h      | 2                | 9.1   | 29    | 60.4  |
| 40–100μg/24h    | 1                | 4.5   | 19    | 39.6  |
| ≥100μg/24h      | 19               | 86.4  | 19    | 39.6  |
| AST             |                  |       |       |       |
| 15–60U/L        | 3                | 13.6% | 22    | 100%  |
| > 60U/L         | 19               | 86.4% |       |       |
| ALT             |                  |       |       |       |
| <50U/L          | 3                | 13.6% | 22    | 100%  |
| ≥50U/L          | 19               | 86.4% |       |       |
| ALP             |                  |       |       |       |
| 42–362U/L       | 15               | 68.2% | 19    | 100%  |
| >362U/L         | 7                | 31.8% | 22    | 100%  |

The reference interval for serum ceruloplasmin is 0.2–0.4g/L, for urine copper is <40μg/24h in children and <100μg/24h in adults, for serum copper is 12–39μmol/L, for ALT is <50U/L, for AST is 15–60 U/L, for ALP is 42–362 U/L. in children and 30–120 U/L in adults.

ALP = alkaline phosphatase, ALT = alanine transaminase, AST = aspartate transaminase, WD = Wilson disease, WDHzc = heterozygous carriers of Wilson disease.
proband’s mutations have been identified, allowing mutations analysis for the same mutations occurring in siblings and subsequent generations.[13] However, the screening modalities of parents are different from those for next generations and siblings. The method that only analyzing the 2 mutations of the proband is not sufficient to diagnose WD in parents.[13] Thus, the entire ATP7B genes of suspected presymptomatic and atypical parents should be sequenced. Haplotyping is also a suitable method used for screening the relatives of patients with WD when mutations in index patients are unidentified. This analysis requires the identification of proband with the unquestionable diagnosis of WD within the family.[5] False-positive results can occur if haplotyping is used for low probability gene recombination.[26] With the development of new sequencing technology, gene diagnosis of WD in the future will be more efficient and comprehensive.

Because the sample size is small in our study, the results may be controversial; to get more accurate result, large sample size clinical experiments should be done.

4.1. Conclusion

Siblings and previous generations of children with WD should be screened by suitable modalities. Genetic testing should be conducted for the diagnosis of presymptomatic patients of WD.

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Author contributions

Conceptualization: Shiqiang Shang.

Data curation: Huamei Li, Lifang Liu, Yun Li, Yujie Liu, Shiqiang Shang.

Funding acquisition: Huamei Li, Shiqiang Shang.

Investigation: Huamei Li, Lifang Liu, Shiqiang Shang.

Methodology: Huamei Li, Lifang Liu, Yun Li, Shendi He, Yujie Liu, Jinhong Li, Ran Tao, Wei Li, Shiqiang Shang.

Project administration: Shiqiang Shang.

Resources: Yun Li, Shiqiang Shang.

Software: Huamei Li, Lifang Liu.

Supervision: Shiqiang Shang.

Validation: Shiqiang Shang.

Writing – original draft: Huamei Li.

Writing – review & editing: Lifang Liu, Shendi He, Shiqiang Shang.

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