Laboratory and clinical evaluation of a microarray for the detection of \textit{ATP7B} mutations in Wilson disease in China

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Abstract

\textbf{Background and objective:} Wilson disease (WD) is an autosomal recessive copper metabolic disorder caused by mutations in \textit{ATP7B}. Sanger sequencing is currently used for \textit{ATP7B} variant identification. However, the \textit{ATP7B} gene contains 21 exons, which makes sequencing of the entire gene both complex and time-consuming. Therefore, a simpler assay is urgently needed.

\textbf{Methods:} We performed a laboratory and clinical evaluation of an oligonucleotide microarray for the detection of 24 \textit{ATP7B} recurrent mutations (except p.P992L) in Chinese patients with WD.

\textbf{Results:} The accuracy of the microarray was evaluated by screening for \textit{ATP7B} mutations in 126 patients including 106 suspected WD samples and 20 patients with other liver diseases as negative control. Results were confirmed by Sanger sequencing. We established a reliable microarray system for the rapid detection of the 24 \textit{ATP7B} mutations, with a sensitivity of 30ng/test genomic DNA and specificity of 100\% for all loci; the coefficient of variation in repeatability tests was <10\%. Clinical evaluation showed an overall concordance between the microarray detection and sequencing of 100\%, and 81.13\% (86/106) of suspected WD cases showed \textit{ATP7B} mutations by microarray detection. Microarray and Sanger sequencing identified p.R778L (50.94\%), p.A874V (17.92\%), p.P992L (11.32\%), p.V1106I (11.32\%), and p.I1148T (6.60\%) as the most common mutations in WD patients.

\textbf{Conclusions:} Our microarray system is customizable and easily used for high-throughput detection of certain recurrent \textit{ATP7B} mutations, providing a simpler method suitable for WD genetic diagnosis in China.

\textbf{KEYWORDS}
\textit{ATP7B}, DNA microarray, mutation detection, Wilson disease

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1 | INTRODUCTION

Wilson disease (WD), also called hepatolenticular degeneration syndrome, is an autosomal recessive disorder of copper metabolism, with a worldwide prevalence of 0.5–3/100,000. Disease-causing variants in ATP7B (which encodes the copper-transporting P-type ATPase) lead to protein dysfunction and copper ion accumulation in organs, especially in the liver. The European Association for the Study of the Liver clinical practice guidelines for WD, released in 2012, show that if the mutations were detected on both chromosomes, the Leipzig score would ≥4, which can meet the WD diagnostic criteria. However, in China, clinical guidelines for the diagnosis of WD with hepatic phenotypes have not been defined. The Leipzig score was applied currently for the diagnosis of WD in China. Therefore, the detection of biallelic ATP7B pathogenic variants is required for the diagnosis of WD, especially in patients with atypical clinical symptoms. To date, more than 1000 ATP7B gene variants have been reported (http://www.hgmd.cf.ac.uk/ac/index.php). Furthermore, the spectrum of ATP7B mutations varies between China and Western countries. Our previous research identified p.R778L (c.2333G>T) and p.A874V (c.2621C>T) as the two most prevalent ATP7B variants in Chinese patients with WD. Other studies in Chinese patients with WD reported recurrent ATP7B mutations with varying prevalence rates, such as p.I1148T (3.88%-13.24%), p.V1106I (0.21%-9.09%), p.T935M (1.30%-6.09%), and p.N1270S (1.94%-5.88%). However, in Western countries, p.H1069Q (c.3207C>G) is the most common ATP7B variant. Most variants in patients with WD are hotspot variants, and therefore, the detection of common ATP7B variants will be helpful for rapid clinical diagnosis. Various approaches, including Sanger sequencing and next-generation sequencing technology, have been used to detect ATP7B gene mutation. However, the ATP7B gene contains 21 exons, and these approaches have the disadvantages of being time-consuming and/or cost prohibitive, making them unsuitable for rapid clinical genetic screens. Therefore, novel and faster diagnostic methods are urgently needed.

In this study, we designed an efficient and accurate microarray assay to detect hotspot mutations in ATP7B. The WD microarray included the 24 most common ATP7B mutant loci, covering up to 70% of Chinese patients with WD. Clinical evaluation of the microarray in Chinese patients with WD demonstrated that this microarray assay could increase the speed of screening potential WD patients. Our results indicate the potential utility of the WD microarray in clinical diagnosis laboratories.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

We recruited 139 patients from the China Registry of Genetic/Metabolic Liver Diseases Group, including 33 WD patients (Table S1) with the 24 most common ATP7B mutations for the determination of the cut-off value and 106 patients (Table S2) with suspected WD for clinical evaluation. Suspected WD was defined by the presence of key features of WD including liver disease and cirrhosis, neuropsychiatric disturbances, Kayser-Fleischer rings in Descemet’s membrane of the cornea, and acute hemolytic attacks that are usually associated with acute liver failure. In addition, 20 patients (Table S3) with other liver diseases but without evidence of WD were included as a negative control group. Peripheral blood was collected from patients and genomic DNA was extracted for ATP7B genotyping. All patients provided written informed consent. This study was approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University (Number 2019-P2-217-02).

2.2 | Primers and probes

The DNA sequence of ATP7B was downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genomes/). Primers and probes were designed by software DNAMAN (https://www.lynnon.com/dnaman.html) and Primer Premier (http://www.premierbiosoft.com/primerdesign/), according to the location of the detected sites. Forward and reverse primers were chemically synthesized by Tsingke Biological Technology and the 5’ end of the reverse primer was labeled with the Cy3 fluorophore. The PCR product lengths varied between 84 and 286 bp (Table S4).

Probes were the reverse complement of target sequences. For each mutation site, four probes were designed (A, T, C, and G at the mutation site). Twelve thymine residues were added to the 3’ end of the probe to improve its flexibility, and the amino group through modification of the probes condensed with the aldehyde group and fixed the probe to the surface of the chip.

2.3 | Plasmid construction

For optimization and quality control of the microarray system, three ATP7B DNA fragments containing the 24 mutated sites were chemically synthesized (Table S5) and ligated into the PGM-T vector (Tsingke). The primer-targeted sequences for multiplex asymmetric PCR were included in each fragment.

2.4 | Microarray preparation

Probes were dissolved to 50μmol/L in RNase-free water and mixed with an equal volume of spotting buffer (6× sodium citrate buffer (SCC), 0.1% sodium dodecyl sulfate (SDS)). Probes were then spotted on the surface of the aldehyde group–based microarray slide (CapitalBio) using a PersonalArrayer™ 16 spotting instrument (CapitalBio). The chip contains 10 reaction zones, and 96 probes were designed as an 8×12 matrix in each reaction zone to detect 24 loci. The four probes corresponding to each variant were arranged
horizontally in the matrix, in the order of A, T, C and G, in duplicate. The positive control (mixture of all probes), negative control 1 (spotting buffer), negative control 2 (RNase-free water) and indicator probes (probes of T12 oligonucleotides with Cy3 fluorophore (5’ end) and amino group modification (3’ end)) were spotted at the bottom of the matrix. Genotyping and the arrangement of the microarray are shown in Figure 1. The slides were dried for 12 h and stored at 4°C until hybridization.

2.5 | Multiplex asymmetric polymerase chain reaction (PCR)

Genomic DNA was amplified using three independent multiplex asymmetric PCR reactions (Platinum Multiplex PCR Master Mix, Thermo Fisher). The PCR components are listed in Table S6. Multiplex asymmetric PCR was performed using the following parameters: pre-denaturing at 95°C for 2 min, followed by 40 cycles of denaturing at 98°C for 20 s and annealing extension at 65°C for 90 s, and a final extension at 72°C for 5 min. The products were denatured at 98°C for 5 min and then incubated at 4°C for 10 min in the dark.

2.6 | Microarray detection

Polymerase chain reaction products were mixed with hybridization reaction buffer (5× SSC, 0.5% SDS) at a ratio of 1:4. The hybridization mixture (10 μl) was added to the microarray reaction zone and uniformly distributed. The microarray slide was protected against exposure to light, placed in a hybridization container, and hybridized in a 60°C water bath for 1 h. The slide was then transferred into pre-warmed (42°C) washing buffer A (1x SSC, 0.2% SDS) and then washing buffer B (0.2% SSC). The slide was centrifuged at 1000 rpm for 1 min and dried at room temperature. Hybridization images were obtained using the Molecular Devices GenePix 4000B chip scanner (CapitalBio) at 532 nm.

2.7 | Statistical analysis

The signal value of each probe was analyzed using ArrayVision 7.0 software. Ratios of the signal value of wild-type probes divided by the signal value of mutant probes as well as the coefficient of variation were calculated to determine the cut-off value for the judgement of each mutation. All statistical analyses were performed using SPSS 16.0 software (SPSS Inc.).

3 | RESULTS

3.1 | Design and preparation of the DNA microarray

We designed the DNA microarray containing 96 probes to detect the 24 common recurrent ATP7B mutations in Chinese patients with WD. The 24 mutations cover more than 70% of WD patients in Chinese clinical practice (Table 1). The mutations and prevalence rates include p.R778L (18.93%–45.59%), p.A874V (0.49%–18.83%), p.I1148T (3.88%–13.24%), p.V1106I (0.21%–9.09%), p.T935M (1.30%–6.09%), and p.N1270S (1.94%–5.88%).

For the optimization of probes, we tested the DNA microarray for all 24 mutations for WD genotyping using synthesized plasmids as positive samples. The plasmids were amplified by multiplex asymmetric PCR, and the amplified oligonucleotides were identical to those using patient genomic DNA as template. The specificity of the 96 probes was ensured by adjusting their length, the side-to-side movement around the mutations, and optimization of hybridization buffer components to reduce the false-positive and false-negative results. The probe sequences are listed in Table S7.

3.2 | Cut-off value determination

For determination of the cut-off value of each probe, a total of 33 clinical samples with known ATP7B variants covering all selected

![FIGURE 1 Schematic representation of the 24 ATP7B loci and the arrangement of probes in the WD microarray.](image)
mutations were used. The samples were hybridized to the WD microarray. Eight spots per site can be used for mutation analysis in each matrix, which significantly reduces non-specific signals. The fluorescence intensity of each site was collected. For genotypes detected in fewer than 10 patients, the fluorescence intensity was recorded by repeating the detection 10 times for statistics and analysis. For cases in which the homozygotes were unavailable, the corresponding plasmids were used for the cut-off determination. Ratios of the fluorescence intensity of wild-type probes divided by the mutant probes when the DNA concentration was 20–50 ng/μl, and the signal was low when the concentration was lower than 10 ng/μl. A 10 ng/μl (30 ng/test) wild-type sample result is shown in Table 2.

The reproducibility of the microarray was also investigated by calculating the coefficient of variation (CV) value of the hybridization signal of each probe, and the fluorescence ratio was calculated with the 33 cases with the 24 different known ATP7B variants. Different matrices of the same microarray were applied to test the same samples. All samples were tested 10 times to calculate the CV value (Table 2). The CV values of the fluorescence ratio of all mutations were less than 10%, confirming good reproducibility of the WD microarray.

### 3.3 Microarray sensitivity, specificity and repeatability

Genomic DNA from 10 patients was subjected to gradient dilution to obtain DNA solutions of 5, 10, 20 and 50 ng/μl, with a total amount of 15, 30, 60 and 150 ng DNA (The amount added to each tube in multiplex asymmetric PCR). The probe hybridization signal represented by each DNA concentration was the mean of all positive probes in the 10 samples involved in the hybridization. The results indicated that the signal value of the microarray hybridization probes was high when the DNA concentration was 20–50 ng/μl, and the signal was low when the concentration was lower than 10 ng/μl. A 10 ng/μl (30 ng/test) wild-type sample result is shown in Figure 2A.B. The test was considered as failed when the genomic DNA was 5 ng/μl (15 ng/test). Thus, the sensitivity (minimum detection limit) is 30 ng/test. The results detected by the microarray showed the coincidence rate of 100% with that detected by Sanger sequencing (Table 2).

### 3.4 Clinical evaluation of WD microarray accuracy

We assessed our WD microarray using DNA from 126 samples, including 106 suspected WD samples and 20 patients with other liver diseases as a negative control for clinical evaluation. The microarray results revealed that 86 of the 106 (81.13%) suspected WD cases...
| No. | Genotype  | Homozygotes | | | | Heterozygotes | | | | Wild-type | | |
|-----|-----------|-------------|---|---|---|---|---|---|---|---|---|---|---|
|     |           | n | Mean (SD) | CV (%) | Mean ± 3SD | n | Mean (SD) | CV (%) | Mean ± 3SD | n | Mean (SD) | CV (%) | Mean ± 3SD |
| 1   | p.D196E   | 10* | 3.11 (0.11) | 3.5 | 2.78–3.44 | 10 | 0.86 (0.02) | 2.3 | 0.80–0.92 | 52 | 0.32 (0.01) | 3.1 | 0.29–0.35 |
| 2   | p.G250R   | 10* | 14.32 (0.50) | 3.5 | 12.82–15.82 | 10 | 0.84 (0.02) | 2.4 | 0.78–0.90 | 52 | 0.17 (0.01) | 5.9 | 0.14–0.20 |
| 3   | p.C490X   | 10* | 3.03 (0.24) | 7.9 | 2.31–3.75 | 10 | 0.95 (0.04) | 4.2 | 0.83–1.07 | 51 | 0.33 (0.02) | 6.1 | 0.27–0.39 |
| 4   | c.1708-5G>T| 10* | 3.12 (0.26) | 8.3 | 2.34–3.90 | 10 | 1.06 (0.04) | 3.8 | 0.94–1.18 | 51 | 0.32 (0.01) | 3.1 | 0.29–0.35 |
| 5   | p.I592F   | 10* | 10.13 (0.15) | 1.5 | 9.68–10.58 | 10 | 0.92 (0.08) | 8.7 | 0.68–1.16 | 51 | 0.20 (0.01) | 5 | 0.17–0.23 |
| 6   | p.L692P   | 10* | 8.71 (0.51) | 5.9 | 7.18–10.24 | 10 | 0.85 (0.04) | 4.7 | 0.73–0.97 | 51 | 0.12 (0.01) | 8.3 | 0.09–0.15 |
| 7   | p.Y741C   | 10* | 3.17 (0.20) | 6.3 | 3.11–3.77 | 10 | 0.89 (0.02) | 2.2 | 0.83–0.95 | 52 | 0.32 (0.02) | 6.3 | 0.22–0.38 |
| 8   | p.D765G   | 10 | 9.13 (0.28) | 3.1 | 8.29–9.97 | 10 | 1.03 (0.05) | 4.9 | 0.88–1.18 | 51 | 0.21 (0.01) | 4.8 | 0.18–0.24 |
| 9   | p.R778L   | 10* | 3.23 (0.08) | 2.5 | 2.99–3.47 | 14 | 1.17 (0.22) | 8.5 | 0.51–1.83 | 36 | 0.31 (0.02) | 6.5 | 0.25–0.37 |
|     | p.R778Q   | 10* | 3.11 (0.06) | 1.9 | 2.93–3.29 | 11 | 1.14 (0.07) | 6.1 | 0.93–1.35 | 52 | 0.20 (0.01) | 5 | 0.17–0.23 |
| 10  | p.G869R   | 10* | 9.34 (0.11) | 1.2 | 9.01–9.67 | 10 | 0.86 (0.02) | 2.3 | 0.80–0.92 | 51 | 0.31 (0.01) | 3.2 | 0.28–0.34 |
| 11  | p.A874V   | 10 | 4.49 (0.12) | 2.7 | 4.13–4.85 | 10 | 1.27 (0.05) | 3.9 | 1.12–1.42 | 50 | 0.42 (0.03) | 7.1 | 0.33–0.41 |
| 12  | p.T888P   | 10 | 2.28 (0.20) | 8.8 | 1.68–2.88 | 10 | 0.84 (0.01) | 1.2 | 0.81–0.87 | 51 | 0.43 (0.02) | 4.7 | 0.37–0.49 |
| 13  | p.R919G   | 10* | 2.87 (0.15) | 5.2 | 2.42–3.32 | 10 | 1.01 (0.01) | 1 | 0.98–1.04 | 52 | 0.34 (0.01) | 2.9 | 0.31–0.37 |
| 14  | p.T935M   | 10* | 2.24 (0.17) | 7.6 | 1.70–2.78 | 10 | 1.65 (0.02) | 3.3 | 1.59–1.71 | 51 | 0.63 (0.05) | 7.9 | 0.48–0.78 |
| 15  | p.G943D   | 10* | 10.07 (0.04) | 0.4 | 9.95–10.19 | 10 | 1.79 (0.04) | 2.2 | 1.67–1.91 | 50 | 0.70 (0.01) | 1.4 | 0.67–0.73 |
| 16  | p.G948C   | 10* | 4.25 (0.35) | 8.2 | 3.20–5.30 | 10 | 1.06 (0.02) | 1.9 | 1.00–1.12 | 52 | 0.74 (0.03) | 4.1 | 0.65–0.83 |
| 17  | p.S975Y   | 10* | 6.28 (0.22) | 3.5 | 5.62–6.94 | 10 | 0.92 (0.07) | 7.6 | 0.71–1.13 | 49 | 0.36 (0.01) | 2.8 | 0.33–0.39 |
| 18  | p.G1000R  | 10 | 12.94 (0.08) | 0.6 | 12.7–13.18 | 10 | 1.50 (0.03) | 2 | 1.41–1.59 | 51 | 0.38 (0.01) | 2.6 | 0.35–0.41 |
| 19  | p.V1106I  | 10 | 3.26 (0.27) | 8.3 | 2.45–4.07 | 10 | 0.85 (0.02) | 2.3 | 0.79–0.91 | 49 | 0.31 (0.01) | 3.2 | 0.28–0.34 |
| 20  | p.I1148T  | 10 | 3.20 (0.16) | 5 | 2.72–3.68 | 10 | 0.81 (0.03) | 3.7 | 0.72–0.90 | 51 | 0.31 (0.01) | 3.2 | 0.28–0.34 |
| 21  | p.E1173K  | 10* | 4.99 (0.10) | 2 | 4.69–5.29 | 10 | 0.88 (0.02) | 2.2 | 0.82–0.94 | 52 | 0.20 (0.01) | 5 | 0.17–0.23 |
| 22  | p.I1230T  | 10 | 16.97 (0.14) | 0.8 | 16.55–17.39 | 10 | 1.29 (0.03) | 2.3 | 1.20–1.38 | 51 | 0.46 (0.01) | 2.2 | 0.43–0.49 |
| 23  | p.N1270S  | 10* | 3.72 (0.27) | 7.3 | 2.91–4.53 | 10 | 1.03 (0.04) | 3.9 | 0.91–1.15 | 51 | 0.27 (0.01) | 3.7 | 0.24–0.30 |
| 24  | p.T1286I  | 10* | 7.13 (0.48) | 6.7 | 5.69–8.57 | 10 | 1.02 (0.04) | 3.9 | 0.90–1.14 | 51 | 0.54 (0.02) | 3.7 | 0.48–0.60 |

*Plasmids were used for the cases without homozygous mutation. Mean = average of ratio, ratio = signal value of wild-type probes divided by signal value of mutant probes.
JIA et al. harbored ATP7B mutations. Sanger sequencing of the other 20 cases identified other mutations in ATP7B not included in the microarray. No ATP7B mutation was identified in patients with other liver diseases. For the 24 mutations included in the microarray, the coincidence rate was 100% between the results detected by the microarray and the sequencing results (Table 3). We also calculated the mutation frequency of the 106 WD cases. The results showed that the mutation frequency of p.R778L (50.94%), p.A874V (17.92%), p.P992L (11.32%), p.V1106I (11.32%), and p.I1148T (6.60%) were over 5% among all 24 mutations. The frequency of the identified mutations is shown in Figure 3. In addition, 20 mutations not included in the microarray were identified by sequencing (Table 3), including c.3659-3660insTGA, which was reported for the first time.

Among the 106 WD cases, 7, 2 and 1 cases had a homozygous mutation of p.R778L, p.R992L, and p.V1106I, respectively, and the other cases harbored heterozygous mutations (Table 3).

We also analyzed the correlation between the representative clinical presentations such as gender, age, symptoms at onset, ceruloplasmin level of the 106 suspected WD cases (Table S2) and the prevalence of the top five hotspot mutations (p.R778L, p.A874V, p.P992L, p.V1106I, and p.I1148T). The results showed that there was no significant correlation between the clinical parameters with the individual mutations (Figure S1).

4 | DISCUSSION

In the present study, we developed and analyzed a microarray method, with advantages of simplicity, efficiency, cost-effectiveness, and user-friendliness, for screening hotspot ATP7B variants in Chinese WD patients. Twenty-four of the most ATP7B mutations, covering up to 70% of Chinese patients with WD reported to date, were simultaneously detected by the microarray. This microarray is a reliable detection system with an approximate 6 h testing time. Clinical evaluation of the microarray showed that accuracy for the detection of ATP7B mutations reached 100%.

Different from the mutations detected in WD cases in Asia, p.H1069Q (c.3207C > A) is the most common mutation in WD patients from European countries. In Asia, p.R778L (c.2333G > T, exon 8) is the most common ATP7B mutation, with a frequency of 10.52% in Thailand, similar to that in other Asian countries. In a recent study of WD cases in South China, ATP7B mutation mainly occurred in exon 8 (23.30%) and exon 16 (12.14%), and the top three mutations were p.R778L (18.93%), p.I1148T (8.74%), and p.P992L (4.37%). This result is consistent with a previous study in eastern China, in which the top three mutations in WD cases were p.R778L (31.9%), p.P992L (11.2%), and p.A874V (5.17%). In Hong Kong and Taiwan, p.R778L accounted for 17.3% and 29.63% of the reported ATP7B mutations, respectively. A systematic literature review of 345 Chinese cases showed that the most common ATP7B mutations were located in exons 8, 13, 12 and 16, accounting for 74.0% of the reported ATP7B mutations and the most common ATP7B mutations were p.R778L and p.P992L, accounting for 50.43% of all reported ATP7B alleles. Similarly, in Korea, the allele frequency of p.R778L was 39.2%. This is in sharp contrast to a study in Japan that revealed that the most common of the 13 mutations in 23 Japanese WD cases was c.2874delC (exon 13, 30%) and p.R778L (exon 8, 25%).

FIGURE 2 Representative result of multiplex asymmetric PCR and microarray detection. (A) Twelve bands of PCR products covering all detection sites are distributed in three tubes. The length of the bands and the target fragments in the three tubes are as follows: Tube 1, 287bp (p.Y741C, p.D765G, p.R778L/Q), 250bp (p.N1270S, p.T1286I), 172bp (p.L692P), 137bp (p.I1448T, p.E1173K); tube 2, 248bp (p.R919G, p.T935M, p.G943D, p.G948C), 212bp (p.G869R, p.A874V, p.T888P), 166bp (p.S975Y, p.G1000R), 130bp (p.V1106I); and tube 3, 260bp (p.D196E, p.G250R), 226bp (p.C490X), 196bp (c.1708-5 T > G, p.I592F), and 84bp (p.I1230T). (B) A representative genotyping result of a clinical sample by microarray detection.
TABLE 3  Results of microarray detection of ATP7B mutation in 106 suspected WD cases and comparison with that by sequencing.

| No. | Cases No. | WD microarray | Ratio of signal values | Positive/Negative | Sanger sequencing | Coincidence of tested mutations |
|-----|-----------|---------------|------------------------|-------------------|------------------|----------------------------------|
| 1   | SWD1      | p.G250R       | 0.83                   | Positive          | p.G250R          | Yes                              |
|     |           | p.R778L       | 1.22                   | Positive          | p.R778L          |                                  |
|     |           | NA            | NA                     | NA                | p.R616Q          |                                  |
| 2   | SWD2      | NA            | NA                     | NA                | p.G881D          | Yes                              |
| 3   | SWD3      | p.R778L       | 0.62                   | Positive          | p.R778L          | Yes                              |
| 4   | SWD4      | p.R778L       | 1.59                   | Positive          | p.R778L          | Yes                              |
| 5   | SWD5      | p.Y741C       | 0.93                   | Positive          | p.Y741C          | Yes                              |
|     |           | p.N1270S      | 1.07                   | Positive          | p.N1270S         |                                  |
| 6   | SWD6      | p.R778L       | 1.68                   | Positive          | p.R778L          | Yes                              |
| 7   | SWD7      | p.R778L       | 1.04                   | Positive          | p.R778L          | Yes                              |
| 8   | SWD8      | p.R778L       | 1.44                   | Positive          | p.R778L          | Yes                              |
|     |           | p.V1106I      | 0.91                   | Positive          | p.V1106I         |                                  |
| 9   | SWD9      | p.A874V       | 1.26                   | Positive          | p.A874V          | Yes                              |
| 10  | SWD10     | p.A874V       | 1.28                   | Positive          | p.A874V          | Yes                              |
|     |           | p.V1106I      | 0.80                   | Positive          | p.V1106I         |                                  |
| 11  | SWD11     | p.R778L       | 1.50                   | Positive          | p.R778L          | Yes                              |
| 12  | SWD12     | p.T935M       | 1.63                   | Positive          | p.T935M          | Yes                              |
|     |           | p.I1148T      | 0.87                   | Positive          | p.I1148T         |                                  |
| 13  | SWD13     | p.V1106I      | 0.85                   | Positive          | p.V1106I         | Yes                              |
| 14  | SWD14     | p.I1148T      | 0.79                   | Positive          | p.I1148T         | Yes                              |
| 15  | SWD15     | p.R778L       | 1.37                   | Positive          | p.R778L          | Yes                              |
|     |           | p.E1173K      | 0.86                   | Positive          | p.E1173K         |                                  |
| 16  | SWD16     | NA            | NA                     | NA                | p.V1297I         | Yes                              |
|     |           | NA            | NA                     | NA                | p.G205V          |                                  |
| 17  | SWD17     | p.N1270S      | 1.07                   | Positive          | p.N1270S         | Yes                              |
| 18  | SWD18     | p.R778L (Homo)| 3.22                   | Positive          | p.R778L (Homo)   | Yes                              |
|     |           | p.V1106I      | 0.84                   | Positive          | p.V1106I         |                                  |
| 19  | SWD19     | p.R778L (Homo)| 3.06                   | Positive          | p.R778L (Homo)   | Yes                              |
| 20  | SWD20     | p.G943D       | 1.73                   | Positive          | p.G943D          | Yes                              |
| 21  | SWD21     | p.I1148T      | 0.75                   | Positive          | p.I1148T         | Yes                              |
| 22  | SWD22     | p.R778L       | 1.16                   | Positive          | p.R778L          | Yes                              |
|     |           | p.I1148T      | 0.73                   | Positive          | p.I1148T         |                                  |
| 23  | SWD23     | p.R778L (Homo)| 3.47                   | Positive          | p.R778L (Homo)   | Yes                              |
|     |           | p.V1106I      | 0.85                   | Positive          | p.V1106I         |                                  |
| 24  | SWD24     | p.R778L (Homo)| 3.24                   | Positive          | p.R778L (Homo)   | Yes                              |
| 25  | SWD25     | p.I1148T      | 0.89                   | Positive          | p.I1148T         | Yes                              |
| 26  | SWD26     | p.R778L       | 1.12                   | Positive          | p.R778L          | Yes                              |
|     |           | p.G943D       | 1.81                   | Positive          | p.G943D          |                                  |
| 27  | SWD27     | p.A874V       | 1.13                   | Positive          | p.A874V          | Yes                              |
| 28  | SWD28     | NA            | NA                     | NA                | p.P663R          | Yes                              |
|     |           | NA            | NA                     | NA                | p.L1188F         |                                  |
| 29  | SWD29     | p.R778L       | 1.19                   | Positive          | p.R778L          | Yes                              |
| 30  | SWD30     | p.R778L       | 1.37                   | Positive          | p.R778L          | Yes                              |
| 31  | SWD31     | p.R778L       | 1.25                   | Positive          | p.R778L          | Yes                              |
| 32  | SWD32     | p.R778L (Homo)| 3.11                   | Positive          | p.R778L (Homo)   | Yes                              |
| 33  | SWD33     | p.R778L       | 1.37                   | Positive          | p.R778L          | Yes                              |
|     |           | NA            | NA                     | NA                | p.R992L          |                                  |
| 34  | SWD34     | NA            | NA                     | NA                | K1010T           | Yes                              |
| No. | Cases No. | WD microarray | Sanger sequencing | Coincidence of tested mutations |
|-----|-----------|---------------|-------------------|-------------------------------|
| 35  | SWD35     | p.A874V       | 1.33 Positive     | p.A874V                       | Yes                           |
| 36  | SWD36     | p.R778L       | 1.61 Positive     | p.R778L                       | Yes                           |
| 37  | SWD37     | NA            | NA                |                               |                               |
| 38  | SWD38     | p.R778L       | 1.27 Positive     | p.R778L                       | Yes                           |
| 39  | SWD39     | p.R778L       | 1.31 Positive     | p.R778L                       | Yes                           |
| 40  | SWD40     | p.R778L       | 1.29 Positive     | p.R778L                       | Yes                           |
| 41  | SWD41     | p.R778L       | 1.26 Positive     | p.R778L                       | Yes                           |
|     |           | p.V1106I      | 0.85 Positive     | p.V1106I                      |                               |
| 42  | SWD42     | p.A1148T      | 0.76 Positive     | p.A1148T                      | Yes                           |
| 43  | SWD43     | NA            | NA                |                               |                               |
| 44  | SWD44     | p.A874V       | 1.31 Positive     | p.R778L                       | Yes                           |
| 45  | SWD45     | p.R778L       | 1.34 Positive     | p.R778L                       | Yes                           |
| 46  | SWD46     | p.R778L       | 1.25 Positive     | p.A874V                       | Yes                           |
| 47  | SWD47     | p.A874V       | 1.35 Positive     | p.R778L                       | Yes                           |
|     |           | NA            | NA                |                               |                               |
| 48  | SWD48     | p.R778L       | 1.35 Positive     | p.R778L                       | Yes                           |
| 49  | SWD49     | p.R778L       | 1.34 Positive     | p.R778L                       | Yes                           |
| 50  | SWD50     | p.A874V       | 1.32 Positive     | p.A874V                       | Yes                           |
| 51  | SWD51     | p.A874V       | 1.39 Positive     | p.A874V                       | Yes                           |
| 52  | SWD52     | p.R778L       | 1.33 Positive     | p.R778L                       | Yes                           |
| 53  | SWD53     | NA            | NA                |                               |                               |
| 54  | SWD54     | p.A874V       | 1.29 Positive     | p.A874V                       | Yes                           |
| 55  | SWD55     | NA            | NA                |                               |                               |
| 56  | SWD56     | p.V1106I      | 0.83 Positive     | p.V1106I                      | Yes                           |
| 57  | SWD57     | NA            | NA                |                               |                               |
| 58  | SWD58     | p.R778L       | 1.32 Positive     | p.R778L                       | Yes                           |
|     |           | p.V1106I      | 0.80 Positive     | p.V1106I                      |                               |
| 59  | SWD59     | p.R778L       | 1.22 Positive     | p.R778L                       | Yes                           |
| 60  | SWD60     | p.R778L       | 1.26 Positive     | p.R778L                       | Yes                           |
| 61  | SWD61     | p.R778L       | 1.81 Positive     | p.R778L                       | Yes                           |
|     |           | p.D196E       | 2.87 Positive     | p.D196E                       |                               |
|     |           | p.V1106I      | 0.83 Positive     | p.V1106I                      |                               |
|     |           | (Homo)        | NA                |                               |                               |
| 62  | SWD62     | p.R778L       | 1.65 Positive     | p.R778L                       | Yes                           |
|     |           | p.V1106I      | 1.65 Positive     | p.R778L                       |                               |
|     |           | (Homo)        | NA                |                               |                               |
|     |           | c.3903+6C>T   | Positive          |                               |                               |
| 63  | SWD63     | p.R778L       | 1.65 Positive     | p.R778L                       | Yes                           |
|     |           | p.V1106I      | 0.86 Positive     | p.V1106I                      |                               |
|     |           | (Homo)        | NA                |                               |                               |
|     |           | c.3903+6C>T   | Positive          |                               |                               |
|     |           | p.A874P       | NA                |                               |                               |
|     |           | p.C154S       | NA                |                               |                               |
| 64  | SWD64     | NA            | NA                |                               |                               |
| 65  | SWD65     | NA            | NA                |                               |                               |
| 66  | SWD66     | p.V1106I      | 0.86 Positive     | p.V1106I                      | Yes                           |
|     |           | NA            | NA                |                               |                               |
|     |           | NA            | NA                |                               |                               |
|     |           | NA            | NA                |                               |                               |
|     |           | NA            | NA                |                               |                               |
| 67  | SWD67     | NA            | NA                |                               |                               |
| 68  | SWD68     | p.R778L       | 1.18 Positive     | p.R778L                       | Yes                           |
| 69  | SWD69     | p.R778L       | 3.46 Positive     | p.R778L                       | Yes                           |
### TABLE 3 (Continued)

| No. | Cases No. | WD microarray | Mutation | Ratio of signal values | Positive/Negative | Sanger sequencing | Coincidence of tested mutations |
|-----|------------|---------------|----------|------------------------|------------------|------------------|---------------------------------|
| 70  | SWD70      |               | p.G943D  | 1.80                   | Positive         | p.G943D         | Yes                             |
| 71  | SWD71      |               | p.R778L  | 1.66                   | Positive         | p.R778L         | Yes                             |
| 72  | SWD72      |               | NA       | NA                     | NA               | NA               |                                 |
| 73  | SWD73      |               | p.A874V  | 1.18                   | Positive         | p.A874V         | Yes                             |
| 74  | SWD74      |               | p.A874V  | 1.34                   | Positive         | p.A874V         | Yes                             |
| 75  | SWD75      |               | NA       | NA                     | Positive         | NA               |                                 |
| 76  | SWD76      |               | p.R778L  | 0.70                   | Positive         | p.R778L         | Yes                             |
| 77  | SWD77      |               | p.R778L  | 1.74                   | Positive         | p.R778L         | Yes                             |
| 78  | SWD78      |               | p.A874V  | 1.30                   | Positive         | p.A874V         | Yes                             |
| 79  | SWD79      |               | p.A874V  | 1.40                   | Positive         | p.A874V         | Yes                             |
| 80  | SWD80      |               | p.V1106I | 0.87                   | Positive         | p.V1106I        | Yes                             |
| 81  | SWD81      |               | NA       | NA                     | NA               | c.3903+6C>T     | Yes                             |
| 82  | SWD82      |               | p.R778L  | 1.39                   | Positive         | p.R778L         | Yes                             |
| 83  | SWD83      |               | p.R778L  | 0.55                   | Positive         | p.R778L         | Yes                             |
| 84  | SWD84      |               | p.A874V  | 1.33                   | Positive         | p.A874V         | Yes                             |
| 85  | SWD85      |               | NA       | NA                     | NA               | p.P992L         | Yes                             |
| 86  | SWD86      |               | p.R778L  | 1.19                   | Positive         | p.R778L (Homo)  | Yes                             |
| 87  | SWD87      |               | p.R778L  | 0.96                   | Positive         | p.R778L         | Yes                             |
| 88  | SWD88      |               | NA       | NA                     | NA               | p.W779X         | Yes                             |
| 89  | SWD89      |               | p.R778L  | 1.07                   | Positive         | p.R778L         | Yes                             |
| 90  | SWD90      |               | p.R778L  | 1.61                   | Positive         | p.R778L         | Yes                             |
| 91  | SWD91      |               | p.V1106I | 0.89                   | Positive         | p.V1106I        | Yes                             |
| 92  | SWD92      |               | NA       | NA                     | NA               | p.P992L         | Yes                             |
| 93  | SWD93      |               | p.A874V  | 1.32                   | Positive         | p.A874V         | Yes                             |
| 94  | SWD94      |               | p.R778L  | 0.74                   | Positive         | p.R778L         | Yes                             |
| 95  | SWD95      |               | p.R778L  | 1.58                   | Positive         | p.R778L         | Yes                             |
| 96  | SWD96      |               | p.R778L  | 0.58                   | Positive         | p.R778L         | Yes                             |
| 97  | SWD97      |               | p.I1148T | 0.77                   | Positive         | p.I1148T        | Yes                             |
| 98  | SWD98      |               | NA       | NA                     | NA               | p.P992L         | Yes                             |
| 99  | SWD99      |               | p.R778L  | 1.06                   | Positive         | p.R778L         | Yes                             |
| 100 | SWD100     |               | p.R778L  | 1.35                   | Positive         | p.R778L         | Yes                             |
| 101 | SWD101     |               | p.A874V  | 1.27                   | Positive         | p.A874V         | Yes                             |
| 102 | SWD102     |               | p.S975Y  | 0.98                   | Positive         | p.S975Y         | Yes                             |
| 103 | SWD103     |               | p.R778L  | 0.88                   | Positive         | p.R778L         | Yes                             |
| 104 | SWD104     |               | NA       | NA                     | NA               | p.P992L         | Yes                             |
| 105 | SWD105     |               | p.R778L  | 1.74                   | Positive         | p.R778L         | Yes                             |
| 106 | SWD106     |               | p.R778L  | 0.66                   | Positive         | p.R778L         | Yes                             |

Abbreviations: Homo, Homozygote; NA, not available as the loci were not included in the microarray.
Our microarray results showed that the mutation frequency of p.R778L (50.94%), p.A874V (17.92%), p.V1106I (11.32%), and p.I1148T (6.60%) were over 5% in Chinese patients with WD. In addition, p.G943D (2.83%), p.T888P (1.89%), and p.N1270S (1.89%) showed relatively high mutation frequency. p.D196E, p.G250R, p.Y741C, p.G250R, p.D196E, p.T1286I, p.I1230T, p.G1000R, p.G948C, p.R919G, p.G869R, p.D765G, p.L692P, and p.I592F were each identified in one sample. No samples contained any of the remaining 11 mutations. These results were consistent with the previous literature and related mutation database reported in Chinese patients with WD. In addition, Sanger sequencing showed that the most frequent mutation, outside of the 24 mutations examined, was p.R992L (11.32%), followed by the splicing mutation c.3903+6C>T (5.56%), p.V1297I (2.83%), and p.A874P(1.89%). Our results identified 21 mutations that were each present in one sample, which may further improve the mutation database (Figure 3). p.V1106I was one of the most common ATP7B mutation reported by our previous study. However, our results need to be confirmed in a larger cohort of WD cases. We also analyzed the correlation between the clinical parameters such as gender, age, symptom at onset and ceruloplasmin level of the WD cases and the prevalence of the top five hotspot mutations (p.R778L, p.A874V, p.P992L, p.V1106I, and p.I1148T). Our results showed that there was no significant correlation between the clinical parameters and the individual mutation, possibly due to the small sample size.

Early diagnosis of WD has a major impact on the prognosis of the disease. Misdiagnosis can cause severe liver failure or even death.
WD diagnosis is currently based on a range of clinical features, including Kayser-Fleischer rings, abnormal liver function, hepatic failure, and neurological symptoms. To date, Sanger sequencing remains the most widely used method for detecting potential ATP7B genetic variants. However, with 21 exons in the ATP7B gene, Sanger sequencing is not convenient for independent sample analysis. NGS that enables high-throughput and full-sequence processing has been used in research and clinical practice. However, this approach has a long running time, high cost, a complicated procedure and data pruning and analysis. The time periods for analysis are generally three to 5 days, or longer, from sampling to sequencing. WD has relatively concentrated pathogenic mutations, and thus it is wasteful to use NGS to detect the entire genome or exome. Furthermore, traditional genetic testing methods are time-consuming and costly, and only several loci can be detected in one test; therefore, these methods are not suitable for rapid clinical genetic screening. A simple, low-cost, efficient, and highly targeted detection method is required for WD genetic testing to better assist clinical diagnosis and treatment.

As there are common ethnic-specific mutations in ATP7B among Chinese WD patients, the microarray is an efficient, cost-effective and convenient method for simultaneous detection of a series of ATP7B variants in multiple independent DNA samples. A previous study reported a microarray for the genotyping of ATP7B variants in Czech and Slovak populations using an arrayed primer extension (APEX) reaction method. However, this approach requires 24h, leading to complicated operation and difficulties in primer set design and microarray preparation, all of which increase the direct cost. Moreover, this technology has high DNA quality requirements, which increases the associated time and cost because the DNA needs to be extensively purified. Manjula and co-workers reported a microarray that was based on the amplified PCR products that labelled with digoxigenin. The sample preparing was complicated and time-consuming because of the nested PCR and the following digoxigenin labelling. Moreover, the designed probes only focused on the wild-type and most common mutated bases, the precise genotype was impossible. Finally, and most importantly, the use of colorimetric detection and a simple home-made flat-bed scanner was not as sensitive as the fluorescence based methods. In our WD microarray, the Cy3 fluorophore is directly added to the 5’ end of the reverse primer; Cy3-labeled single-stranded DNA fragments can be easily harvested using multiplex asymmetric PCR, and the fluorescence intensity is captured by a microarray scanner. Our WD microarray takes approximately 6 hrs from DNA extraction to identification of the 24 most common ATP7B mutations. This approach is also highly automated, standardized, and easy to operate, making it suitable for clinical diagnosis. Indeed, for future commercial applications in clinics, the microarray needs to be validated by other laboratories. We will conduct the validation of the microarray in other laboratories in the near future.

Notably, this microarray method is highly dependent on existing understanding of WD. Furthermore, we failed to detect any mutations in several patients included in our study group. Both false-positive and false-negative results cannot be avoided if patients harbor some specific rare mutations. For example, because of the short distance from the hotspot sites, the p.W779X variant may reduce the accuracy of the probes for the p.R778L variant, resulting in aberrant fluorescence intensity. Moreover, the p.P992L variant is also a hotspot in Chinese WD patients, but the GC content in the sequences near the mutation site is extremely high. This makes it difficult to balance probe hybridization temperature, and the hybridization temperature required for a probe at this site is much higher than other probes. Therefore, the p.P992L variant was not included in our WD microarray. However, based on the high-resolution melting curve (HRM), we have developed a detection method for p.P992L and applied for a patent in order to make up for the shortcomings of this site. With the identification of novel recurrent ATP7B mutation and specifically, hotspot mutations in other countries such as p.H1069Q in the western countries, more probes can be included in the microarray, and this method can be used for the detection of more ATP7B mutations in patients worldwide.

In summary, our WD microarray represents a potential convenient tool for the clinical screening of ATP7B gene mutations, especially in Chinese WD patients. This method will be helpful in the diagnosis, genetic counseling and treatment of WD patient.

AUTHOR CONTRIBUTIONS

All authors accept responsibility for the entire content of this manuscript and approved its submission. JH and DHZ designed the study. SYJ and DHZ analyzed and interpreted the data and wrote the manuscript. SYJ, DHZ, XJL, and BZ performed the experiments. WZ, WD and ZW provided patient samples and clinical data. XJO, JH, and DHZ advised on the conception of the study and supervised the study. JH and DHZ revised the paper. All authors vouch for the data and analysis, approved the final version, and agree to publish the manuscript.

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CONFLICT OF INTEREST

The authors have no conflict of interest related to this study.

DATA AVAILABILITY STATEMENT

The data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.
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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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