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ORIGINAL ARTICLE

Mutation analysis of 73 southern Chinese Wilson's disease patients: identification of 10 novel mutations and its clinical correlation

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This study was designed to investigate the molecular basis and the correlation between genotype and phenotype in the southern Chinese patients with Wilson's disease (WD). Genotypes of the *ATP7B* gene in 73 WD patients were examined by denaturing high-performance liquid chromatography (DHPLC) and DNA sequencing. A total of 38 different disease-causing mutations were identified, including 10 novel mutations: missense mutations (p.Gln707Arg, p.Cys1079Phe, p.Gly1149Glu, p.Ser855Tyr, p.Ala874Pro and p.Ser921Arg), nonsense mutation (p.Arg1228Stop), splice-site mutations (2121+3A>T and 3244-2A>G) and frameshift mutation (1875_1876insAATT). We found that a pair of siblings carried the same genotype but different clinical type, and two patients were found to have three mutations. In addition, we compared the clinical data for p.Arg778Leu homozygotes and compound heterozygotes. Our research has enriched the mutation spectrum of the *ATP7B* gene in the Chinese population and can serve as the basis for genetic counseling and clinical/prenatal diagnosis to prevent WD in China.
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Keywords: *ATP7B*; genotype; novel mutation; phenotype; southern Chinese; Wilson disease

INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder with a copper metabolism defect, including deficient incorporation of copper into ceruloplasmin and impaired biliary copper excretion. The metabolism lesions lead to toxic accumulation of copper in various tissues, principally the liver and brain, which can cause complex clinical manifestation, such as hepatic, neurological or psychiatric impairment and Kayser–Fleischer (K–F) ring. The worldwide prevalence of WD is estimated to be 1 in 30 000, with a corresponding gene frequency of 0.56% and a carrier frequency of ~1 in 90.[1] Early diagnosis and treatment are essential in decreasing morbidity and mortality because WD is lethal if untreated.[2]

WD is caused by mutations of the *ATP7B* gene, which maps at chromosome 13q14.3 and encodes a copper-transporting P-type ATPase. This protein is composed of 1465 amino-acid residues that form 6 copper-binding domains, 8 transmembrane domains, the phosphatase domain TGEA, the phosphorylation domain DKTGT, the ATP-binding domain TGDN and the MxGDGxNDxP domain, which connects the ATP-binding domain to the transmembrane segment.[3] Mutation analysis has been investigated in many WD patients in different ethnic populations and >500 different *ATP7B* mutations have been identified (http://www.wilsondisease.med.ualberta.ca/database.asp).[4] More than 100 of these mutations have been identified in Chinese populations. p.Arg778Leu in exon 8 was the first hot point mutation found in China.[5,6] There are few reports of mutations of the *ATP7B* gene and the genotype–phenotype correlation in southern Chinese patients.

Here, we examined the genotypes of the *ATP7B* mutant alleles in 73 WD patients using denaturing high-performance liquid chromatography (DHPLC)[7,8] and DNA sequencing. A total of 38 different mutations were identified and 10 of these are novel. The characteristics of the mutations were compared with the clinical symptoms to find the genotype–phenotype correlation in southern Chinese WD patients.

MATERIALS AND METHODS

Subjects

A total of 73 WD patients (41 males and 32 females including 2 affected siblings, onset age was from 3 to 48 years) from 70 unrelated families of ethnic

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Han Chinese originating from Guangdong and Guangxi provinces were enrolled in this study. The diagnosis of WD was based primarily on clinical symptoms including the presence of K–F ring in the cornea, acute or chronic liver failure and/or typical neurological symptoms, and on biochemical parameters including low serum ceruloplasmin ($<0.2\,g\,l^{-1}$) and high-level excretion of urinary copper ($>100\,\mu g$ per 24 h).[9] A total of 200 healthy Chinese individuals were enrolled as a control group. Informed, written consent was obtained from all patients and controls for participation in this study.

Genomic DNA preparation and PCR of the *ATP7B* gene

Genomic DNA was extracted from peripheral blood using the standard phenol/chloroform extraction procedure. All the coding exons of *ATP7B* and their associated splice-site junctions were amplified by the PCR using published primers[7,10] or newly designed primers (Table 1). PCR was done in a total volume of 25 µl containing PCR buffer, 200 µM dNTP, 2 pmol of each primer, 1 U of Taq polymerase and 100 ng of genomic DNA. The conditions for all PCR runs were: 94 °C for 5 min; then 35 cycles of 94 °C for 30 s, appropriate annealing temperature for 30 s (varying between 54 and 64 °C), 72 °C for 30 s; and a final extension step at 72 °C for 7 min. The size and quantity of PCR products were verified by electrophoresis in 1.5% (w/v) agarose gel.

Analysis of the *ATP7B* gene

All samples were analyzed by DHPLC. The PCR products and wild-type PCR product mixture were denatured at 95 °C for 5 min and then re-annealed to form a complete duplex by decreasing the temperature to 45 °C at a rate of 1 °C per min. Heteroduplex analysis was done by DHPLC on the WAVE Nucleic Acid Fragment Analysis System (model 4500 HT, Transgenomic, Omaha, NE, USA). PCR products showing abnormal peaks by DHPLC were sequenced directly.

Statistical analysis

Data were analyzed using the SPSS statistical package (version 8; SPSS, Chicago, IL, USA) and the results are presented as mean ± s.d. Statistical analysis was done using Student's *t*-test or the χ^2 test. The level of statistical significance was set at $P \leqslant 0.05$.

RESULTS

Analysis of mutation and polymorphism in the *ATP7B* gene

We investigated 73 patients (146 chromosomes) with WD from 70 families, and identified 38 different *ATP7B* mutations that included 24 missense mutations, 3 splice-site mutations, 5 nonsense mutations and 6 frameshift mutations (Table 2). In all, five mutations were identified on exon 8 or 12, four on exon 16, three on exons 3, 7, 11 or 13, whereas none was found on exons 1, 4, 5, 9, 20 or 21. c.2333G>T (p.Arg778Leu) is a common mutation in the Chinese population and it was the most common mutation in this study. It was detected in 32 patients (2 homozygous, 16 compound heterozygous and 14 single heterozygotes) and accounted for 23.29% of the alleles studied. The second most frequent mutation was c.3443T>C (p.Ile1148Thr), which accounted for 9.59% of the alleles studied.

In addition to the mutations, 13 polymorphisms were identified, and details of them are given in Table 3. These base substitutions were defined as polymorphisms because they either did not modify the amino-acid sequence or led to conservative changes in the amino-acid residues or existed in healthy chromosomes. Among the 13 polymorphisms, 11 were found in the present study and 2 of them are novel. The novel polymorphisms c.1839C>T (p.Ile613Ile) and c.2922G>A (p.Thr974Thr) are synonymous and, although they were not detected in the 200 healthy controls, we still consider them to be polymorphisms.

Evaluation of the genotype–phenotype correlation

Mutations in *ATP7B* were identified in 69 of the 73 patients (3 homozygotes, 30 compound heterozygotes and 36 single heterozygotes) and no mutation was detected in 4 patients. The mutation detection rate was 69.86% (102 disease alleles found in 146 chromosomes). The mean age of patients at disease manifestation was 15.10 ± 9.01 years. Of the 73 patients, 45 had a primary hepatic manifestation, 27 showed a primary neurological and psychiatric manifestation and 1 had combined hepatic and neurological manifestation.

Evaluation of the genotype–phenotype correlation was possible only in the 33 patients who were found to carry at least two mutations (the clinical features and molecular basis are shown in Supplementary Table 1). Two homozygotes of p.Arg778Leu and one homozygote with p.Arg919Gly were identified. The age of onset and status of K–F ring were similar among these three homozygotes. However, one homozygote of p.Arg778Leu displayed symptoms in the liver and the other displayed symptoms in the brain.

Interestingly, we found that a pair of siblings carried the same genotype but were different clinical type (Figure 1a). The proband was a 19-year-old female who displayed WD neurological and psychiatric type with serum ceruloplasmin $0.08\,g\,l^{-1}$; she was 13 years old at onset and was diagnosed at 16 years of age. She was a compound heterozygote of c.314C>A mutation (inherited from her father) and c.3443T>C mutation (inherited from her mother). Her younger brother had no significant clinical symptom of WD except K–F ring. According to the family history of WD and WD-related check-up, he was diagnosed as WD hepatic type at 12 years of age with serum ceruloplasmin of $0.08\,g\,l^{-1}$ and abnormal aminotransferase (alanine aminotransferase was $68\,U\,l^{-1}$, aspartate aminotransferase was $58\,U\,l^{-1}$) and his genotype was the same as that of his sister. Both patients were treated with sodium 2,3-dimercapto-1-sulfonate and their symptoms were improved.

In addition, two patients were found to have three mutations (Figure 1b). They carried both c.3443T>C and c.3426G>C on the same allele and another mutation, c.2924C>A or c.2333G>A, on the

Table 1 Primers and conditions used for PCR and DHPLC analysis of *ATP7B* exons

Exon	*5′ Primer*	*3′ Primer*	*Product length (bp)*	*PCR temperature (°C)*	*Column temperature (°C)*
2–2	CAGTCATGTGTGAAGTCC	AGCCACTTTGCTCTTGATG	438	54	56
2–3	AGCAACCAAGAGGCCGTCAT	CCGGCAATGGCAATCAGAGT	566	59	61
2–4	CCGAAGGGAGTGGGACAGAT	CAGGACATGCCTCAAACACACT	322	62	60
3	CACCAAGAGCCCTGAAACC	CGCAGCATTCCTAAGTTCAAC	381	54	60
10–11	GCCATGTGAGTGATAAGTG	CCCAGAACTCTTCACATAAT	492	56	60
13	CACCTGCCTCTGACTCT	GGGAAAGCCGTGCTACA	250	61	63
18–19	GCATTGCCTTCCTTTTGTC	CAGTGGGTAAGAGCTGCCTA	448	57	61

Abbreviation: DHPLC, denaturing high-performance liquid chromatography.
Primers are given in the 5′→3′ orientation.

Table 2 Distribution and frequency of mutations detected in the *ATP7B* gene

Nucleotide change	*Amino-acid change*	*Type*	*Affected protein domain*	*Exon*	*Mutation frequency (%)*
c.2333G>T	p.Arg778Leu	Missense	TM4	8	23.29 (34/146)
c.3443T>C	p.Ile1148Thr	Missense	ATP loop	16	9.59 (14/146)
c.2755C>G	p.Arg919Gly	Missense	Td/TM5	12	2.74 (4/146)
c.2828G>A	p.Gly943Asp	Missense	TM5	12	2.74 (4/146)
c.525dupA	p.Val176fs	Frameshift	Cu2	2	2.74 (4/146)
c.2804C>T	p.Thr935Met	Missense	TM5	12	2.05 (3/146)
c.2662A>C	p.Thr888Pro	Missense	Td/TM5	11	2.05 (3/146)
c.314C>A	p.Ser105Stop	Nonsense	Cu1	2	1.37 (2/146)
c.2332C>T	p.Arg778Trp	Missense	TM4	8	1.37 (2/146)
c.3426G>C	p.Gln1142His	Missense	ATP loop	16	1.37 (2/146)
c.3446G>A	**p.Gly1149Glu**	**Missense**	**ATP loop**	**16**	**1.37 (2/146)**
c.3809A>G	p.Asn1270Ser	Missense	ATP hinge	19	1.37 (2/146)
c.3244-2A>G	**NA**	**Splice-site**	**ATP loop**	**15**	**1.37 (2/146)**
c.2304dupC	p.Met769fs	Frameshift	TM4	8	1.37 (2/146)
c.2120A>G	**p.Gln707Arg**	**Missense**	**TM2**	**7**	**0.68 (1/146)**
c.2294A>G	p.Asp765Gly	Missense	TM4	8	0.68 (1/146)
c.2333G>A	p.Arg778Gln	Missense	TM4	8	0.68 (1/146)
c.2564C>A	**p.Ser855Tyr**	**Missense**	**Td**	**10**	**0.68 (1/146)**
c.2620G>C	**p.Ala874Pro**	**Missense**	**Td/TM5**	**11**	**0.68 (1/146)**
c.2621C>T	p.Ala874Val	Missense	Td/TM5	11	0.68 (1/146)
c.2761A>C	**p.Ser921Arg**	**Missense**	**Td/TM5**	**12**	**0.68 (1/146)**
c.2924C>A	p.Ser975Tyr	Missense	TM6	13	0.68 (1/146)
c.2939G>A	p.Cys980Tyr	Missense	TM6	13	0.68 (1/146)
c.2975C>T	p.Pro992Leu	Missense	TM6/Ph	13	0.68 (1/146)
c.3236G>T	**p. Cys1079Phe**	**Missense**	**ATP loop**	**14**	**0.68 (1/146)**
c.3316G>A	p.Val1106Ile	Missense	ATP loop	15	0.68 (1/146)
c.3532A>G	p.Thr1178Ala	Missense	ATP loop	16	0.68 (1/146)
c.3646G>A	p.Val1216Met	Missense	ATP binding	17	0.68 (1/146)
c.1470C>A	p.Cys490Stop	Nonsense	Cu5	3	0.68 (1/146)
c.1531C>T	p.Gln511Stop	Nonsense	Cu5	3	0.68 (1/146)
c.3682A>T	**p.Arg1228Stop**	**Nonsense**	**ATP binding**	**17**	**0.68 (1/146)**
c.3955C>T	p.Arg1319Stop	Nonsense	ATP hinge/TM7	19	0.68 (1/146)
c.1543+1G>T	NA	Splice-site	Cu5	3	0.68 (1/146)
c.2121+3A>T	**NA**	**Splice-site**	**TM2**	**7**	**0.68 (1/146)**
c.1875-1876insAATT	**p.Gly626fs**	**Frameshift**	**Cu6**	**6**	**0.68 (1/146)**
c.2097-2099delCTT	p.Phe700del	Frameshift	TM2	7	0.68 (1/146)
c.2810delT	p.Val937fs	Frameshift	TM5	12	0.68 (1/146)
c.3700-1delG	NA	Frameshift	ATP binding	18	0.68 (1/146)

Abbreviation: NA, no amino acid change.
Novel mutations are highlighted in bold.

Table 3 Polymorphisms identified in the *ATP7B* gene

				Frequency (%)	
Polymorphism	*Nucleotide change*	*Exon*	*Evidence of polymorphism*	*Healthy individuals* (n=*200*)	*Patients* (n=*73*)
p.Ile613Ile	**c.1839C>T**	**5**	**No amino acid change**	**0**	**1.4**
p.Leu770Leu	c.2310C>G	8	No amino acid change	0	45.2
p.Gln951Gln	c.2853G>A	12	No amino acid change	0	1.4
p.Thr974Thr	**c.2922G>A**	**13**	**No amino acid change**	**0**	**1.4**
IVS14+38A>G	c.3243+38A>G	14	No amino acid change	0	4.1
IVS1-75 A>C	c.-75A>C	5′ UTR	Found in normal chromosomes	47.5	53.4
p.Ser406Ala	c.1216T>G	2	Found in normal chromosomes	53.0	54.8
p.Leu456Val	c.1366G>C	3	Conservative amino acid change	44.0	49.3
p.Lys832Arg	c.2495A>G	10	Found in normal chromosomes	36.5	27.4
p.Lys952Arg	c.2855A>G	12	Conservative amino acid change	42.5	41.1
IVS12-13G>C	c.2866-13G>C	13	Found in normal chromosomes	8.0	4.1
p.Ala1140Val	c.3419C>T	16	Conservative amino acid change	42.5	39.7
IVS18+6T>C	c.3903+6T>C	18	Found in normal chromosomes	30.5	4.1

The novel polymorphisms are highlighted in bold.

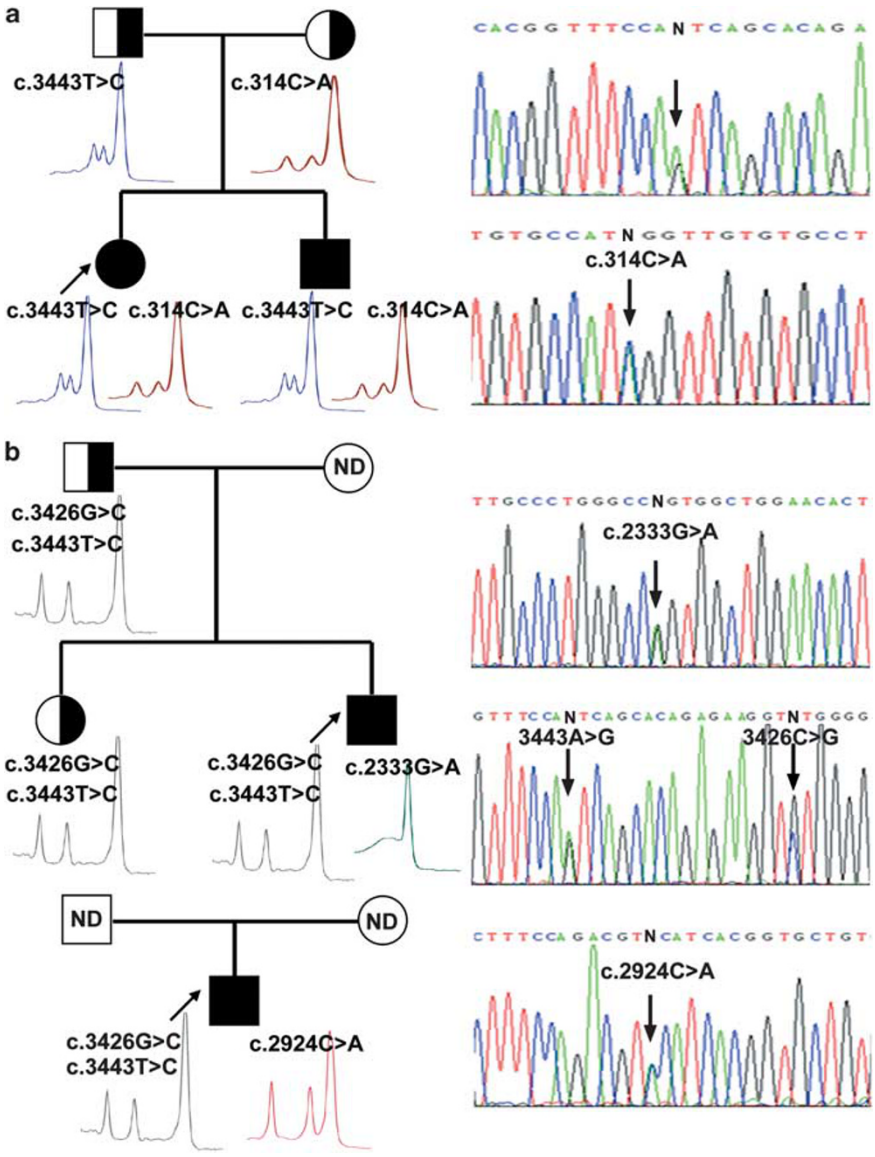

Figure 1 Predigree, DNA sequencing results and denaturing high-performance liquid chromatography (DHPLC) peaks of three patients. The three probands are labeled by the arrows. (**a**) Predigree analysis of a pair of siblings. (**b**) Predigree analysis of two probands carried three disease-causing mutations. ND, not done.

other allele. The parental genotypes of one patient confirmed that c.3426G>C cosegregated *in cis* with c.3443T>C and the compound heterozygosity on the same chromosomes. Both patients were diagnosed as WD neurological and psychiatric type with low serum ceruloplasmin (one was 0.08 g l^{-1} and the other was 0.09 g l^{-1}). One male patient was first diagnosed at 13 years of age and the main clinical manifestations were clumsiness, tremor and voice vague with no apparent cause. The other male patient was first diagnosed at 37 years of age and the main symptom was tremor, which became more serious, and 1 year ago he presented with fatigue and anorexia. Both c.3443T>C and c.3426G>C were described as disease-causing mutations by Loudianos *et al.*[11,12] Neither of these mutations was found in the 200 healthy control subjects.

Identification of 10 novel *ATP7B* mutations

In this study, 10 novel mutations were found, including 6 missense mutations (c.2120A>G, c.2564C>A, c.2620G>C, c.2761A>C, c.3236G>T and c.3446G>A), 2 splice-site mutations (2121+3A>T and 3244-2A>G), 1 nonsense mutation (c.3682A>T) and 1 frameshift mutation (1875_1876insAATT). The results of sequencing and DHPLC analysis of the novel mutations are shown in Figure 2. Among these novel mutations, c.3446G>A and 3244-2A>G were detected on two alleles and the others were found on one allele only. None of the novel mutations was found in the 200 healthy controls. These mutations were identified as novel to the WND database (http://www.wilsondisease.med.ualberta.ca/database.asp).

DISCUSSION

WD is a lethal genetic disorder if untreated, and early diagnosis of asymptomatic individuals is extremely important. In this study, 73 individuals affected with WD were analyzed by DHPLC and direct sequencing of the exon sequences of the *ATP7B* gene. In all, 38 mutations were detected and 10 of these are novel. The novel mutations included 6 missense, 2 splice-site, 1 nonsense and

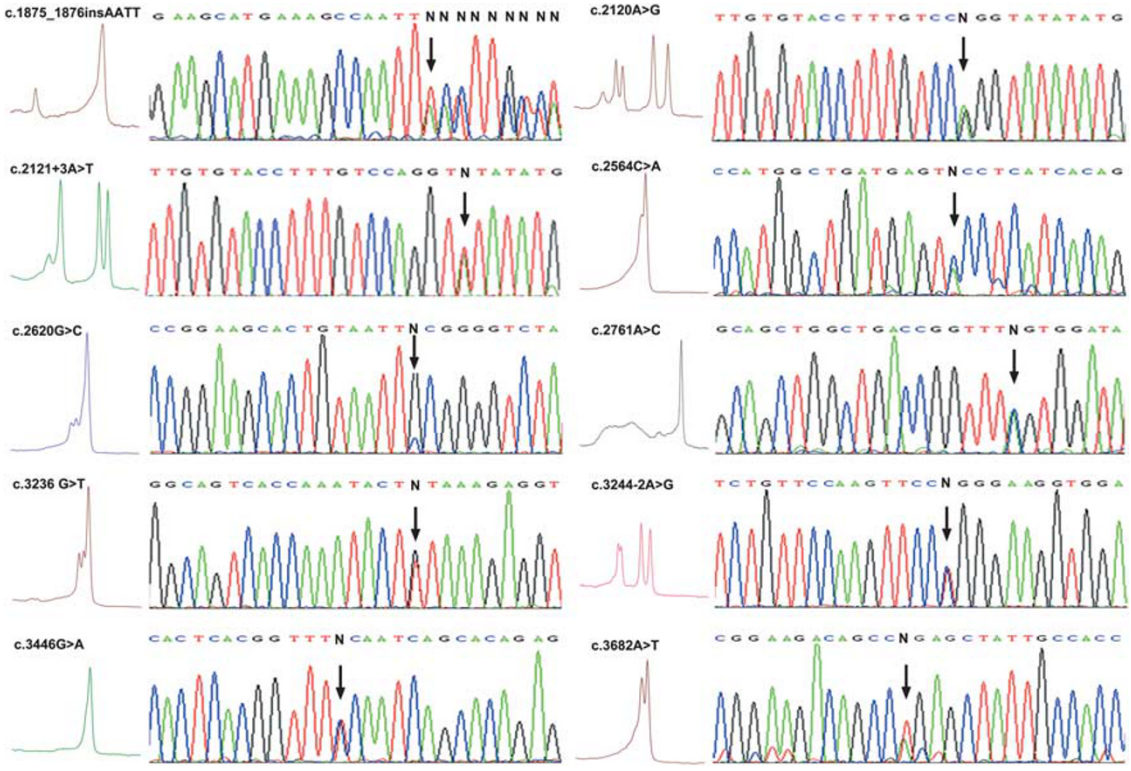

Figure 2 The DNA sequencing results and denaturing high-performance liquid chromatography (DHPLC) peaks of 10 novel *ATP7B* mutations. DNA sequences of the sense strand or the antisense strand are shown with arrows indicating the mutation nucleotides (overlapping peaks are indicated by N).

1 frameshift. They were classified as disease-causing mutations and none was found in the 200 healthy controls. According to our data, a missense mutation is the most frequent type in southern Chinese patients; 102 WD chromosomes were detected among a total of 146 alleles. In 36 patients, only 1 mutation was found, suggesting that the other unknown mutation might be located on the outside exons and the flanking regions, such as the promoter, introns or other DNA control regions.

The p.Arg778Leu mutation was the most frequent in our patients, accounting for 23.29% of alleles studied. It is the most prevalent mutation in other Chinese populations, such as eastern Chinese (50%)[13] and Taiwanese (27%).[14] It is also the most common mutation in Asian populations, including Korean (37.9%),[15] Japanese (13.4%)[16] and Thai (10.52%).[17] The second most frequent mutation was p.Ile1148Thr (9.59%) in exon 16, but the other hot mutation, p.Thr935Met in exon 12, was detected in only one allele, which differs from the published results of earlier research.[18] The p.His1069Gln mutation is the most common mutation in European populations,[19–21] but it was not found in our patients. All other mutations were identified at a low frequency. Only 3 among 73 patients were homozygotes, indicating that the *ATP7B* mutation is characteristic of a compound heterozygous mutation and a rare homozygous mutation.

The mean onset age of 73 patients was 15.10 ± 9.01 years. More patients displayed a hepatic manifestation than those who displayed a neurological manifestation (45 vs 27). K–F ring was observed in 66 patients; the detection rate was 90.41%. Seven patients without K–F ring were diagnosed in early childhood with abnormal aminotransferase. We observed three especially interesting cases. In case 1, a pair of siblings carried the same genotype but different clinical types, which suggested that the genotype–phenotype correlation of WD had great genetic heterogeneity and there might be other factors that affect the phenotype, which needs further study. In cases 2 and 3, both patients carried three mutations and had been diagnosed as neurological and psychiatric type WD. We found that c.3426G>C cosegregated *in cis* with c.3443T>C and the parental genotypes confirmed compound heterozygosity of c.3426G>C and c.3443T>C on the same chromosomes. This phenomenon had been described only in WD patients in Hong Kong Chinese,[22] which suggested that this *cis*-mutation might be specific for southern Chinese WD patients.

Evaluation of the genotype–phenotype correlation was done with 33 homozygotes and compound heterozygotes (Supplementary Table 1). Patients with only one detected mutant allele were excluded. It is difficult to investigate the potential correlation between genotypes and phenotypes because many different mutations are detected and most patients are compound heterozygotes with different genotypes. We therefore restricted our investigation to a comparison between p.Arg778Leu homozygosity and compound heterozygosity with the clinical data (Supplementary Table 2). No significant difference was found in the levels of serum copper or ceruloplasmin in these two groups. We observed that the average onset age in compound heterozygotes was significantly earlier (P=0.002) than that in the homozygotes, which is in contradiction to the findings reported by Ye *et al.*[13] and Wu *et al.*[23] This difference might be because the patient groups in the different studies were from different geographic areas of China and there are only two homozygotes in the present study.

In conclusion, we had identified 10 novel mutations. It expanded the range of mutations in Chinese population and enriched the mutation spectrum of the *ATP7B* gene worldwide. We also found several interesting cases that could provide valuable information for genetic counseling and clinical/prenatal diagnosis to prevent WD in China. According to our study, the genetic background of WD has great

heterogeneity, and we can not speculate the phenotype by genotype. Other factors that affected the phenotype need further study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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