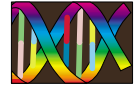


# Identification of a High Frequency of Mutation at Exon 8 of the *ATP7B* Gene in a Chinese Population With Wilson Disease by Fluorescent PCR



Pingyi Xu, MD, PhD; Xiuling Liang, MD; Joseph Jankovic, MD; Wei-dong Le, MD, PhD

**Background:** Wilson disease (WD) is an autosomal recessive disorder of copper transport. Mutation analysis has led to the discovery of more than 100 mutations at *ATP7B*, and most of them are population specific.

**Objectives:** To verify the high frequency of mutation at exon 8 of *ATP7B* in Chinese patients with WD and to establish a DNA diagnostic method for WD.

**Setting:** University medical centers.

**Patients and Methods:** Screening for mutations at exon 8 of *ATP7B* by fluorescent polymerase chain reaction analysis and restriction analysis was conducted in 106 unrelated Chinese patients with WD and in 55 individuals from 10 Chinese families with WD.

**Results:** Five homozygotes and 32 heterozygotes were identified. Sequence analysis showed a missense mutation (2273G→T) and a nonsense mutation (2250C→G) together at exon 8. The rate of gene mutation in 106 patients was 35% (5% homozygous and 30% heterozygous). Samples of DNA from 55 individuals from 10 Chinese families with WD were examined by fluorescent polymerase chain reaction. We found that 13 siblings were carriers (24%).

**Conclusions:** A high frequency of mutation at exon 8 of the *ATP7B* gene exists in the Chinese population, and fluorescent polymerase chain reaction analysis may be an effective and accurate assay in detection of the WD gene.

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**W**ILSON DISEASE (WD) is an autosomal recessive disorder of copper transport. Because of decreased biliary copper excretion and reduced copper incorporation into ceruloplasmin, copper accumulates in tissues, particularly the liver, basal ganglia, cornea, and kidney. Clinical presentation usually occurs in the first or second decade of life and is characterized by liver disease, extrapyramidal and psychiatric symptoms, renal disturbance, or Kayser-Fleischer ring in the cornea.<sup>1</sup> The worldwide frequency of WD is 1 in 35 000 to 1 in 100 000 live births.<sup>2</sup> The epidemiologic features vary considerably in different countries and ethnic backgrounds. The prevalence of WD in China seems to be much higher than that in Western countries.<sup>3</sup>

The WD gene has been cloned and found to encode a copper-transporting p-type adenosine triphosphatase (*ATP7B*). To date, more than 100 mutations, including small insertions and deletions and missense, nonsense, and splice site mutations, have been described in *ATP7B*.<sup>4-6</sup> Most of

these mutations are population specific.<sup>7-11</sup> There have been several studies<sup>12-17</sup> of mutations of the *ATP7B* gene in mainland China, and all indicated that the mutation of Arg778Leu at exon 8 is a hotspot in the Chinese population with WD. Because of the limited number of patients with WD enrolled in these studies, the frequencies reported varied considerably (range, 17%-30%).<sup>13-17</sup> In this study, we present the results of mutation screening by fluorescent polymerase chain reaction (PCR) analysis and restriction enzyme digestion analysis of exon 8 of the *ATP7B* gene in 106 Chinese patients with WD and 55 nonsymptomatic siblings from 10 Chinese families with WD. Our objectives were (1) to verify the high frequency of mutation at exon 8 of *ATP7B* in Chinese patients with WD and (2) to establish a DNA diagnostic method for WD using fluorescent PCR analysis.

## RESULTS

In 55 healthy individuals, analysis of fluorescent PCR amplification showed only an increment of green fluorescent line in the

From the Department of Neurology, Baylor College of Medicine, Houston, Tex.

## PARTICIPANTS AND METHODS

### STUDY POPULATION

The study included 106 unrelated Chinese patients with WD and classical symptoms and signs of WD (69 males and 37 females; age range, 6-25 years; mean  $\pm$  SD age,  $12.4 \pm 3.8$  years), 55 nonsymptomatic siblings from 10 Chinese families with WD (38 males and 17 females; age range, 9-28 years; mean  $\pm$  SD age,  $16.4 \pm 4.1$  years), and 55 unrelated healthy individuals (30 males and 25 females; age range, 10-25 years; mean  $\pm$  SD age,  $14.7 \pm 3.7$  years) (**Table 1**). In haplotype data published on these families,<sup>18</sup> no evidence was found for locus heterogeneity in the linkage analysis. Of 106 patients with WD, 35 had a family history of WD. At the early stage of disease, 40 patients (23 males and 17 females; age range, 5-21 years; mean  $\pm$  SD age,  $11.3 \pm 3.1$  years) had the primary symptom of hepatic dysfunction, 31 (18 males and 13 females; age range, 7-24 years; mean  $\pm$  SD age,  $14.6 \pm 4.7$  years) had neurological manifestations, and 37 (23 males and 14 females; age range, 12-25 years; mean  $\pm$  SD age,  $14.4 \pm 2.8$  years) had both as their first complaint. All patients were treated customarily with D-penicillamine, 0.75 to 1.50 g/d. Once improvement occurred and decreased total body copper content was demonstrated, patients were placed on maintenance therapy at half the initial dose. At 2-year follow-up, 68 patients experienced significant improvement, 28 had moderate improvement, and 10 showed no improvement after D-penicillamine treatment.

### EXAMPLE CASE REPORT

A 12-year-old schoolboy had "infectious hepatitis" at age 5 years but no symptoms or signs of liver disease or any other serious illness since then. He started with intentional tremor in the right arm at age 10 years, and then developed a progressive "wing beating" in 2 arms. Physical examination showed that his liver and spleen were palpable, his facial expression was fixed, and his smile was

turned to a grin. Kayser-Fleischer rings were detected in his cornea by the naked eye. He had severe rigidity in all limbs, but his mind remained clear. His sensation and reflexes were normal, and pyramidal signs were absent. Laboratory findings showed that his serum copper level was elevated to 30  $\mu\text{g/dL}$  ( $4.86 \mu\text{mol/L}$ ) and his serum ceruloplasmin concentration was less than 1 mg/dL, but 24-hour urine copper values were greater than 1105  $\mu\text{g}$ . Computed tomographic examination demonstrated hypodense lesions in bilateral basal ganglia. The patient was previously treated with a short course of zinc acetate, followed by D-penicillamine, 1.0 g/d for 6 months and 0.5 g/d for 1 year. His clinical symptoms were remarkably improved after D-penicillamine treatment.

### DNA ANALYSIS

Genomic DNA samples were extracted from blood cells using standard techniques. Two fluorescent probes were synthesized based on the wild-type and mutant-type sequences at exon 8 of the *ATP7B* gene. The wild fluorescent probe sequence was 5'-CAGCCACCGGCCAGGG-3' and the mutant fluorescent probe sequence was 5'-AGCCACAGGCCAGGG-3'. Fluorescent PCR analysis was carried out in the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Instruments, Wellesley, Mass). After 20-minute denaturation at 95°C, the PCR consisted of 40 cycles at 93°C for 45 seconds and 65.5°C for 2 minutes. Using the 5' nuclease assay, a specific fluorescent signal was generated and measured at every cycle during a run. Fluorescent readings were quantitatively analyzed in the system: the normal sequence of exon 8 of the *ATP7B* gene was marked as green and the mutant as red. Two curve reaction lines were automatically drawn according to the numbers of amplified specific gene.

Direct DNA sequencing was conducted in forward and reverse directions using an automated sequencer. The accuracy of fluorescent PCR analysis of exon 8 of *ATP7B* was confirmed by restriction enzyme digestion of *MspI* (Promega, Madison, Wis) according to nucleotide sequence analysis.

sequence detection system, indicating that there were no mutations at exon 8 in this group. In 106 patients with WD, 5 mutant homozygotes (5%) and 32 heterozygotes (30%) of exon 8 were found by fluorescent PCR analysis (**Figure 1**). We examined 55 siblings of 10 families with WD; 13 siblings (24%) were carriers and the others were normal (**Figure 1A, C**). Direct DNA sequencing for 1 case of homozygous, 2 cases of heterozygous, and 1 normal sample showed 2 different mutations—a missense (2273G→T) and a nonsense (2250C→G)—together in these abnormal DNA samples. Sequence analysis of exon 8 in homozygotes showed a 2273G→T mutation and a 2250C→G mutation together in forward and reverse sequencing. There were 2273N and 2250N mutations in one heterozygote and two 2273G→T and 2250C→G mutations in another heterozygote. The data suggest that the heterozygote is a partial homozygote (eg, a duplex zygote of homozygote combined with a heterozygote of gene mutation). No mutations of 2273G→T and 2250C→G were found in any controls (**Figure 2**).

To verify the accuracy of fluorescent analysis, all the PCR products of exon 8 of *ATP7B* were digested by the restrictive enzyme *MspI*. Of 106 patients, 5 were homozygotes and 32 were heterozygotes. The results of analysis by restriction digestion were the same as those using fluorescent PCR analysis (**Table 2**).

Clinically, patients with WD who had homozygous or heterozygous 2273G→T and 2250C→G mutations showed no significant difference from the other 69 patients with a normal sequence of exon 8 in terms of onset age, primary symptoms, and laboratory findings.

### COMMENT

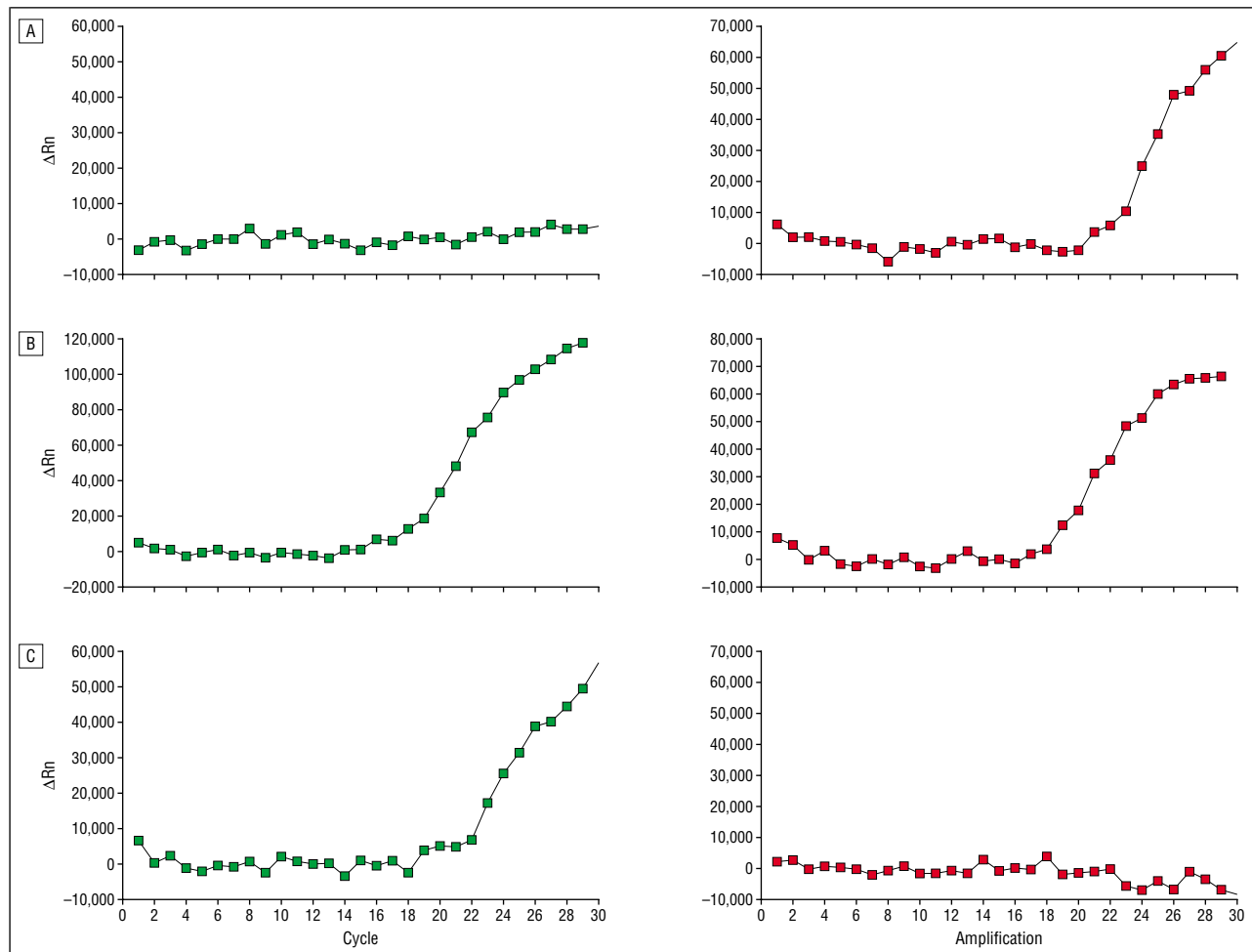
A previous study<sup>13</sup> showed that Arg778Leu might be a hotspot of mutations in the Chinese population by PCR-single-stranded conformational polymorphism analysis in 21 exons of the *ATP7B* gene. But the study was based on a small number of Chinese patients with WD, and the mu-

**Table 1. Summary of Clinical and Laboratory Data in 106 Patients With Wilson Disease, 55 Heterozygous Carriers, and 55 Control Subjects\***

Group	Family History, No.	K-F Ring, No.	Liver Symptoms, No.	Neurological Symptoms, No.	Level, Mean $\pm$ SD		
					Serum Cu, $\mu\text{g/dL}^\dagger$	Serum CP, mg/dL	Urine Cu, $\mu\text{g/24 h}$
Patients	35	92	87	97	55 $\pm$ 30	3.6 $\pm$ 2.3	645.0 $\pm$ 159.2
Siblings	10	NA	NA	NA	100 $\pm$ 16	22.4 $\pm$ 3.4	32.7 $\pm$ 9.2
Controls	NA	NA	NA	NA	115 $\pm$ 33	35.3 $\pm$ 7.1	18.3 $\pm$ 8.7

\*K-F indicates Kayser-Fleischer; Cu, copper; CP, ceruloplasmin; and NA, not applicable.

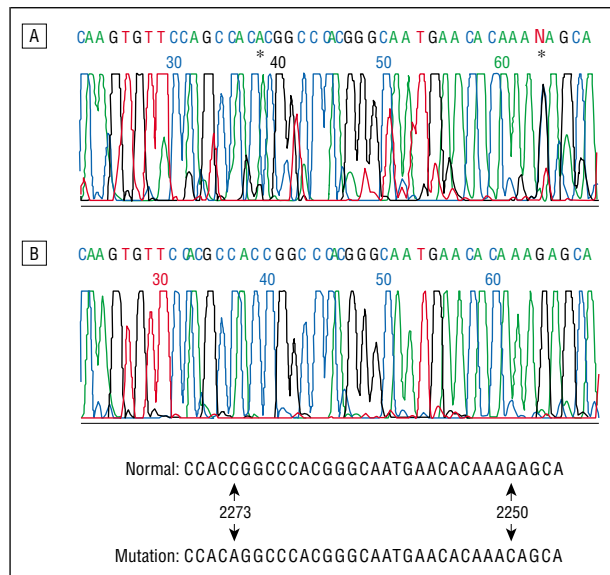
$^\dagger$ To convert serum Cu from micrograms per deciliter to micromoles per liter, multiply micrograms per deciliter by 0.1574.



**Figure 1.** Fluorescent polymerase chain reaction analysis of exon 8 of the *ATP7B* gene in patients with Wilson disease (homozygotes [A] and heterozygotes [B]) and controls (C). The red line indicates increased numbers of the mutation at exon 8 of the *ATP7B* gene; green line, increased numbers of the normal gene.

tant frequency of exon 8 of the *ATP7B* gene was less than that in other studies reported in China.<sup>15-17</sup> Xu et al<sup>13</sup> also did not find other mutations of exon 7, exon 12, and exon 16 of the *ATP7B* gene in the previous study. Obviously, these mutations were rare in Chinese patients.<sup>15-17</sup> In this study, we examined the exon 8 mutations instead of screening all 21 exons of *ATP7B* by fluorescent PCR. We found that 2 different mutations, 2273G→T (missense) and 2250C→G (nonsense), exist together in exon 8 of the *ATP7B* gene in Chinese patients with WD. Clinical analysis in patients with WD who had 2273G→T and 2250C→G did not find a correlation of the genotype with phenotype compared with

normal exon 8 in patients with WD. The frequency of 2273G→T and 2250C→G mutations in 106 independent Chinese patients examined is 35%, which is higher than that reported in Taiwan,<sup>12</sup> Japan,<sup>10,19</sup> Korea,<sup>8</sup> and China.<sup>15-18</sup> The 2273G→T and 2250C→G mutations have not been reported in whites; a high frequency of mutations present in exons 14 and 18 represented 38% of the mutations in patients of European origin,<sup>7,20,21</sup> which indicates a different genetic background in whites and Chinese. Exon 8 is located in the transmembrane domain (Tm4) of the *ATP7B* gene.<sup>4-6</sup> The amino acid changes codon 778 because of the mutation at exon 8, which may disrupt



**Figure 2.** The 2273G→T (missense) and 2250C→G (nonsense) mutations at exon 8 of the *ATP7B* gene. The sequence of polymerase chain reaction products shows compound heterozygotes (A) and controls (B).

**Table 2. Detection of the Arg778Leu (2273G→T) Mutation in Chinese Patients With Wilson Disease and in Controls by Fluorescent Polymerase Chain Reaction Analysis**

Group	Homozygotes, No. (%)	Heterozygotes, No. (%)	Total, No.
Patients	5 (5)	32 (30)	106
Siblings	0	13 (24)	55
Controls	0	0	55

the appropriate anchorage of the transporter in the membrane. The change from a basic (Arg) to a neutral (Leu) amino acid found in this study predicts a dramatic change in the primary and secondary structure of this protein, which could culminate in impaired copper transport.<sup>22,23</sup>

This is the first study of fluorescent PCR analysis used in the clinic setting to screen the gene mutations of patients with WD. It is a 2-temperature PCR cycle that enhances the speed and overall sensitivity of the amplification procedure.<sup>24</sup> Because the fluorescent PCR assay was applied for the selective amplification of a characteristic sequence within the exon 8 fragment of *ATP7B*, this technique—designed for the direct mutation detection of exon 8 of the *ATP7B* gene—has the advantage of being efficient, sensitive, cost effective, and applicable to large-scale screening for the *ATP7B* gene mutation. Screening for WD in the patient's family is important for early diagnosis and treatment to improve the patient's prognosis. Using this method, we identified 13 siblings as WD gene carriers among 55 siblings from 10 Chinese WD pedigrees; the accuracy of this method is proved by the results of restriction enzyme *MspI* analysis and by DNA direct sequencing.

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Corresponding author: Wei-dong Le, MD, PhD, Room NB205, Department of Neurology, Baylor College of Medicine, 6501 Fannin St, Houston, TX 77030 (e-mail: Weidongl@bcm.tmc.edu).

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