Original article

Identification of one novel and nine recurrent mutations of the *ATP7B* gene in 11 children with Wilson disease

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Background: **Wilson disease (WND), also called hepatolenticular degeneration, is an autosomal recessive genetic disorder in which copper abnormally accumulates in several organs. WND arises from the defective *ATP7B* gene, which encodes a copper transporting P-type ATPase.**

Methods: **The molecular defects in 11 unrelated Chinese WND patients aged from 3 to 12 years were investigated. The diagnosis of these patients was based on typical clinical symptoms and laboratory testing results. All 21 exons and exon-intron boundaries of the *ATP7B* gene were amplified by polymerase chain reaction from the genomic DNA of the patients and then analyzed by direct sequencing. One hundred healthy subjects served as controls to exclude gene polymorphism.**

Results: **In one novel (c.3605 C>G) and nine recurrent mutations of *ATP7B* identified, there were eight missense mutations, one splice-site mutation, and one nonsense mutation. The novel c.3605 C>G mutation resulted in the substitution of alanine by glycine at amino acid position 1202 (p.Ala1202Gly). The most frequent *ATP7B* mutation was c.2333 G>T (p.Arg778Leu), followed by c.2975 C>T (p.Pro992Leu), which accounted for 63.6% of the WND mutated alleles.**

Conclusions: **The novel c.3605 C>G mutation in *ATP7B* is one of the molecular mechanisms of WND.**

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Key words: ***ATP7B*; gene; mutation; Wilson disease**

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Introduction

Wilson disease (WND; OMIM#277900), is an autosomal recessive disorder of copper metabolism. The prevalence of WND is estimated at one per 30 000 in most populations, with an approximate carrier frequency of one in 90. The prevalence is as high as one in 10 000 in China, Japan, and Sardinia.[1] Wilson disease is caused by homozygous and compound heterozygous mutations in the *ATP7B* gene, which is the only gene known to be associated with Wilson disease. The *ATP7B* gene is located on the chromosome 13 (13q14.3) and codes a P-type ATPase (cation transport enzyme), which transports copper into bile and incorporates it into ceruloplasmin. The protein contains six copper-binding domains, eight putative transmembrane domains, one ATP-binding domain, and one phosphorylation domain.[2,3] Defective protein due to *ATP7B* mutations would impair the copper excretion pathway and result in abnormal accumulation of copper and an elevated concentration of nonceruloplasmin copper in plasma. So far, more than 500 *ATP7B* gene mutations associated with Wilson disease have been identified.[4]

Wilson disease is clinically characterized by excessive copper deposition in various organs, particularly the liver, kidney, brain, and cornea. Consequently, individuals with WND can manifest any combination of hepatic, neurological, or psychiatric disturbances with varying degrees of severity.[5] Currently, diagnosis of Wilson disease mainly depends upon clinical presentations and biochemical testing. Patients with hepatic symptoms tend to attract medical attention early, while the diagnosis of those with neurological or other symptoms tends to be delayed. Some patients are identified only because their relatives have been diagnosed with WND.[4] Laboratory findings, such as levels of ceruloplasmin and the amount of copper excreted in urine over a 24-hour period, are

often used in conjunction with clinical presentations to diagnose WND. In addition, molecular genetic analysis has also been used clinically.

In the current study, we investigated *ATP7B* gene mutations in 11 unrelated Chinese children with WND. One novel mutation, namely c.3605 C>G, and nine recurrent mutations were identified.

Methods

Patients

Eleven patients with WND (seven male and four female patients, aged from 3 to 12 years) from Shanghai Children's Medical Center (SCMC) were studied. They were diagnosed on the basis of clinical symptoms [neurologic evaluation, corneal Kayser-Fleischer rings (K-F rings), and liver function testing] and biochemical indicators, including low serum ceruloplasmin (<0.2 g/L enzymatic method), and/or increased urinary copper excretion (>60 μg copper/24 hours). Informed consent was obtained from the parents of all patients or their guardians, and the study was approved by the Ethical Committee of SCMC.

DNA extraction

Peripheral venous blood from the patients was collected in tubes containing 0.109 mol/L sodium citrate (9:1 v/v). Genomic DNA was extracted from the whole blood by using Genomic DNA Purification Kit (QIAGEN GmbH, Hilden, Germany).

Genetic analysis

All 21 exons of the *ATP7B* gene and their exon-intron boundaries were amplified by polymerase chain reaction (PCR). Primers (sequences available on request) were designed through the online primer design software Primer 3 according to the published *ATP7B* sequence (GenBank accession number: NG_008806). PCR was carried out for each fragment in a total volume of 25 μL containing about 100 ng of genomic DNA, 1×PCR buffer, 2 mmol $MgCl_2$, 0.2 mmol dNTPs, 0.2 μmol of each primer, and 1U Taq DNA polymerase (Takara, Dalian, China). The PCR products were amplified for 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 50 seconds with an Eppendorf Mastercycler® Pro thermal cycler (Eppendorf, Hamburg, German). PCR products were subjected to DNA sequencing via the ABI 3130XL sequencer (Applied BioSystems, Foster City, CA, US). Blood DNA samples from 100 normal unrelated human subjects were screened as controls.

Results

Phenotype analysis

The serum ceruloplasmin concentration ranged from 0.02 g/L to 0.25 g/L in the patients. The lowest level of 24-hour urinary copper excretion detected was 71 μg/24 h, which was above the upper limit of the reference interval. The highest level of 24-hour urinary copper excretion reached 791 μg/24 h. The organs affected and the severity of the disease varied among the patients. The six youngest patients (patients 1-6) presented with abnormally high alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels but lacked specific clinical symptoms. The ALT and AST levels

Table 1. Laboratory statistics of 11 patients with Wilson disease

Patient	Gender	Age (y)	Clinical symptoms			Laboratory findings			
			Liver disease	Neurologic/psychiatric disturbances	K-F ring	ALT (IU/L)	AST (IU/L)	Serum CP (g/L)	Urine Cu (μg/24 h)
1	F	3	-	-	-	71↑	81↑	0.12↓	71↑
2	F	5	-	-	-	116↑	78↑	0.04↓	791↑
3	F	3	-	-	-	158↑	93↑	0.10↓	350↑
4	M	4	-	-	-	265↑	130↑	0.15↓	257↑
5	M	3	-	-	-	228↑	151↑	0.15↓	110↑
6	F	7	-	-	-	183↑	142↑	0.08↓	225↑
7	M	9	-	-	-	137↑	106↑	0.16↓	ND
8	M	11	-	-	-	162↑	78↑	0.02↓	532↑
9	M	12	-	Rigid dystonia; movement disorders	+	<40	<46	0.19↓	272↑
10	M	12	Hepato-splenomegaly	-	+	<40	<46	0.25	384↑
11	M	12	-	Dysarthria; rigid dystonia	+	<40	<46	<0.02↓	142↑

"-": negative; "+": positive. ND: no data. The reference interval for ALT is 10-40 IU/L, for AST is 15-46 IU/L, for serum CP is 0.2-0.6 g/L, and for urine Cu is 0-60 μg/24 h. The serum CP of patient 10 was within the normal range. F: female; M: male; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CP: ceruloplasmin.

Table 2. *ATP7B* genotype of 11 patients with Wilson disease

Patient	Genotype	Mutation	Nucleotide sequence	Codon change	Exon/Intron	Mutation type
1	Compound heterozygote	c.2975 C>T	CCC>CTC	p.Pro992Leu	13	Missense
		*c.3605 C>G	GCT>GGT	p.Ala1202Gly	17	Missense
2	Homozygote	c.2333 G>T	CGG>CTG	p.Arg778Leu	8	Missense
3	Homozygote	c.2333 G>T	CGG>CTG	p.Arg778Leu	8	Missense
4	Compound heterozygote	c.2333 G>T	CGG>CTG	p.Arg778Leu	8	Missense
		c.2755 C>G	CGG>GGG	p.Arg919Gly	12	Missense
5	Compound heterozygote	c.2975 C>T	CCC>CTC	p.Pro992Leu	13	Missense
		c.1708-1G>C	-	-	Intron 4	Splice-site
6	Homozygote	c.2333 G>T	CGG>CTG	p.Arg778Leu	8	Missense
7	Compound heterozygote	c.2333 G>T	CGG>CTG	p.Arg778Leu	8	Missense
		c.2975 C>T	CCC>CTC	p.Pro992Leu	13	Missense
8	Compound heterozygote	c.2333 G>T	CGG>CTG	p.Arg778Leu	8	Missense
		c.3517 G>A	GAG>AAG	p.Glu1173Lys	16	Missense
9	Homozygote	c.2333 G>T	CGG>CTG	p.Arg778Leu	8	Missense
10	Compound heterozygote	c.994 G>T	GAA>TAA	p.Glu332X	2	Nonsense
		c.4003 G>C	GGG>CGG	p.Gly1335Arg	19	Missense
11	Compound heterozygote	c.2621 C>T	GCG>GTG	p.Ala874Val	11	Missense
		c.3443 T>C	ATT>ACT	p.Ile1148Thr	16	Missense

*: novel mutation.

of older patients (patients 9-11) were normal but their clinical symptoms involved K-F rings, neurological and psychiatric issues, and hepatic problems (Table 1).

The diagnosis of only 3 of the 11 patients (patients 9-11) was based on their clinical symptoms and the new guideline approved by the American Association for the Study of Liver Diseases (AASLD).[6] Others required liver biopsy, molecular testing, or both to confirm the diagnosis. In all patients, their parents or guardians refused liver biopsy but agreed to perform molecular genetic testing.

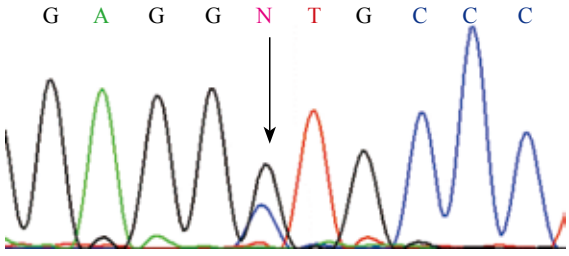

Fig. A novel c.3605 C>G mutation in exon 17 of *ATP7B* by direct DNA sequencing. Patient 1 had a compound heterozygous mutation including one novel c.3605 C>G mutation (indicated with an arrow). This novel transition, which was identified in exon 17, results in the substitution of alanine by glycine in the well-conserved area.

Genotype analysis

In the 11 patients, we observed 10 different mutations, including eight missense mutations, one splice-site mutation, and one nonsense mutation (Table 2). Homozygous mutations were observed in 4 patients, and compound heterozygotes with different mutations on each chromosome in 7. All the 4 homozygous mutations were c.2333 G>T (p.Arg778Leu) in exon 8 of the *ATP7B* gene, which was the mutation most frequently seen in this study, followed by p.Pro992Leu. The p.Glu332X mutation, identified earlier this year in the Chinese population, was also found in our study.[7]

In our series, patient 1 had a compound heterozygous mutation that included one novel mutation (c.3605 C>G) and one known mutation (c.2975 C>T) (Fig.). The novel c.3605 C>G transition would lead to the substitution of alanine by glycine at amino acid position 1202 (p.Ala1202Gly) in the ATP binding region of *ATP7B*. None of the 100 normal controls had the c.3605 C>G mutation, indicating that this is not a polymorphism. Pedigree analysis results showed that the new mutation was inherited from the mother of the patient. Bioinformatic tool SIFT was used to evaluate the possible impact of the novel mutation on the function of *ATP7B* protein (http://sift.bii.a-star.edu.sg/). The results showed that this newly identified missense mutation may affect protein function (data not shown).

Discussion

WND typically begins with an asymptomatic period, during which copper is thought to accumulate in the liver, causing subclinical hepatitis and to develop neuropsychiatric symptoms. In our study, children often lacked typical symptoms and only 3 patients presented with K-F rings, and one of them had hepatic problems. Younger patients without specific clinical

manifestations initially attracted the attention of physicians because of their high ALT levels only, which were detected during physical examination for admission to kindergarten or nursery school. Since child health checkups are popular in China, the rate of WND diagnosis in children has been increasing. Because early diagnosis can help to improve the outcome of the patient, clinical manifestations of children with abnormal biochemical results are inadequate to diagnose Wilson disease as demonstrated in our study. Our findings suggest that molecular genetic testing involving analysis of the sequence of the *ATP7B* gene may be of clinical importance.

The *ATP7B* gene is the only gene known to be associated with Wilson disease. More than 500 *ATP7B* mutations have so far been identified.[4] WND patients in different populations seem to be due to a small number of population-specific mutations. The p.His1069Gln mutation is the one that can be found frequently and exclusively in the Caucasian population originating from the central or eastern Europe, and is responsible for more than 35% of the cases.[8] This mutation seems to predict late and mainly neurological problems.[4,9,10] In contrast, p.Arg778Leu is the most common mutation in the Asian population, but it has not been found in European patients.[11-13] Individuals with p.Arg778Leu mutation, especially those who are homozygous, tend to demonstrate more hepatic manifestations at younger age and higher serum ALT levels at diagnosis.[7] Studies in Chinese patients showed that mutations in exons 8, 12, 13, and 16 accounted for more than 70% of all cases of WND.[7,14-18] These four exons are considered as hot spots and should be given priority when genetic testing is performed.

In our study, mutations p.Arg778Leu and p.Pro992Leu, which occur in exons 8 and 13, respectively, were found to be responsible for approximately 63% of mutated alleles. This finding is consistent with the published data.[7] Also, there was a novel missense mutation, which may impair the P-type ATPase function. The mutation is p.Ala1202Gly mutation located on exon 17 and involved in the ATP-binding domain. According to SIFT, the substitution of alanine by glycine in the well-conserved region may disturb the ATP binding process, affecting normal copper metabolism. This novel variant was not detected in the 100 controls we examined nor in the database of the 1000 genome project (www.1000genomes.org).

Disease-causing mutations are not always identified in patients who have been diagnosed with WND.[4,19] This is possibly due to the fact that mutations may be present in non-coding regulatory elements of the *ATP7B* gene or that the sequencing may have missed the mutations. The high cost of genetic test, the difficulty in interpreting the results, and the lack of a standard operative protocol all restrict its use in clinical settings.[20] However, early diagnosis and treatment can effectively prevent the progression of the disease and improve the quality of life. As commercial molecular genetic test for *ATP7B* mutations is widely adopted, it will be incorporated into the diagnostic algorithm to provide a more timely diagnosis of WND.

In conclusion, diagnosis of WND in children without obvious symptoms should be based on combined test of liver function, ceruloplasmin/urinary copper, and genetic test. The novel mutation that we identified should be considered as part of the mutation spectrum of WND.

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Contributors: Geng J and Wang J contributed equally to this work. Geng J and Wang J developed the protocol, wrote the main body of the article, and were responsible for data analysis and interpretation of results. Liu XQ performed all physical examinations, and provided advice on medical aspects. Yao RE was responsible for data collection and data analysis. Fu QH was responsible for interpretation of results and scientific editing of the manuscript. All authors have read and approved the content of the manuscript.

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