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Original Article

Genetic Analysis of Wilson Disease in South China: Hotspots and One Novel Mutation in *ATP7B*

Z **Qiao**, G **Zhou**, M **Jiang**

Abstract

Objectives: Wilson disease (WD), also known as hepato-lenticular degeneration, is an autosomal recessive inherited disorder of copper metabolism. The gene responsible for WD is located on chromosome 13 at 13q14.3, which encodes copper transporter P-type ATPase, namely *ATP7B*. Mutation of *ATP7B* is a genetic signal of highly risk suffering from WD. Here, we aim to analyse the hotspot of *ATP7B* mutations in WD patients from South China using Sanger sequencing. ***Methods:*** In this study, 50 healthy individuals and 32 identified WD patients were enrolled, who manifested with abnormal hepatic function including increased Alanine Transaminase (ALT) or Aspartate Transaminase (AST), and/or dyskinesia. The genomic locus of *ATP7B* of these patients were amplified by polymerase chain reaction (PCR), then sequenced via Sanger sequencing. ***Results:*** Genetic variations were found in all patients, including 17 mutations and nine SNPs, one of which (c.1757T>A) is novel. The most frequent mutations were P.778R>L (22.5%), P.770L>L (17.5%), and P.935T>M (10.0%). These mutations were mainly clustered in the exon 5, 8, 12, and 13 of *ATP7B*. ***Conclusions:*** Based on Sanger sequencing of *ATP7B* in this study, p.778R>L mutation clustered in the CU channel transmembrane domain consisted over 50% of *ATP7B* mutations, suggesting hotspots in South China and supplying a suitable strategy focusing on this hotspot for *ATP7B* screening in South China WD patients. The newly identified mutation, c.1757T>A, is deleterious based on an array of predictions and highly conserved upon comparison among different species. Furthermore, the c.1757T>A is classified as likely pathogenic variant according to American College of Medical Genetics (ACMG) guidelines (2015 Edition).

Key words *ATP7B*; Mutation hotspot; Sanger sequencing; Wilson disease

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Introduction

Wilson disease (WD), also known as hepatolenticular degeneration that was first reported by Kinnear Wilson in 1912,[1] is an autosomal recessive inherited disorder of copper metabolism. WD is characterised by impaired synthesis of copper chaperonin leading to lower ceruloplasmin concentration,[2] resulting in copper excessive accumulation in organs like liver, brain, cornea, and kidney, thus, leading to a series of complex clinical manifestations, such as acute or chronic hepatitis, liver cirrhosis, hepatic failure, dyskinesia, neuro-psychiatric symptoms, and Kayser-Fleischer corneal ring (K-F ring).[3-6]

WD is caused by mutations in the *ATP7B* gene, discovered in 1993, which encodes the copper-specific

transporting P-type ATPase and the gene is located on the chromosome 13 at 13q14. The *ATP7B* is consisted of 21 exons and 20 introns, nearly 7.5 kb in size, encoding a 1465 amino acid-protein that comprises of six copper-binding domains (exons 2-5), eight transmembrane domains of copper channel (exons 6-8, 12-13,19-20), and the ATP-binding domain (exons 10-11,14-18).[7,8]

The widely accepted incidence of WD is about 1/30 000 worldwide, which is higher in China and Japan (about 1/10 000),[9,10] with an estimated carrier frequency of 1/90. This incidence rate was at least partly based on assumption and calculation, and has been questioned.

To date, over 500 mutations have been found in the *ATP7B* gene, and distribution of the mutations differs among various ethnic groups.[11,12] The mutations are scattered throughout within *ATP7B* gene, however some hotspots were reported to be varied in different populations. For example, the first hotspot in Europeans and North Americans was identified in exon 14, p.His1069Gln.[13,14] In some Asian countries, such as Korean and Japan, the first hotspot lies in exon eight, p. Arg778Leu.[15,16] More than 100 of these mutations were identified in Chinese populations, characterised by a few hotspot mutations and a variety of rare mutations, with a high genetic heterogeneity and a large variation in prevalence according to the geographic distribution and ethnic background.[17] Previous studies had shown that hotspot mutations for some Chinese populations mainly clustered in exons eight with p. Arg778Leu.[18,19] In this study, Sanger sequencing was used to identify the mutation spectrum in South Chinese population to investigate hotspot mutation that could facilitate diagnosis confirmation genetically.

Materials and Methods

Patients

A total of 32 identified WD patients were analyzed, ranging from 2 to 30 years old, consisted of 71.5% males and 28.5% females. All WD patients were diagnosed and treated at the second affiliated hospital of Wenzhou Medical University among the year of 2010-2017. Diagnosis of WD was based on clinical symptoms, including hepatic dysfunction, and/or typical neurological symptoms, or the presence of Kayser-Fleischer ring, and biochemical parameters, such as low serum ceruloplasmin (<0.2 g/l) and high level of urinary copper (>100 μg/24h).[9] Fifty healthy local individuals were enrolled as controls. All patients were provided written informed consent in regarding with the Declaration of Helsinki and ratified by the Ethics Committee of Wenzhou Medical University.

DNA Extraction

We used a salt precipitation and extraction method to extract genomic DNA from peripheral blood samples. In brief, two ml venous blood was extracted into ethylenediaminetetraacetic acid (EDTA) sample tube and the genomic DNA was extracted from peripheral blood leukocytes using the standard phenol/chloroform extraction protocol.

PCR and Sanger Sequencing

All the exons and intron/exon boundaries of *ATP7B genes* were amplified by polymerase chain reaction (PCR), using a set of twenty-five primer pairs designed before.[20] PCR was performed using GoTaq Green Master Mix (Promega) with 100 ng of genomic DNA in a mix containing 10 pmol of each primer, 12.5 μl of 2×GoTaq Green Master mix in a total volume of 25 μl. The thermocycle programme consisted of an initial denaturation at 94ºC for five min, followed by 35 cycles at 94ºC for 30 s, 56ºC for 30 s and 72ºC for 30 s, with a final extension at 72ºC for five min. The size and quantity of PCR products were verified by electrophoresis in 2% (w/v) agarose gel. Then the PCR products were sequenced on an ABI PRISM 3730 DNA Sequencer.

Prediction of the Virulence of Gene Mutation

Three bioinformatics software that contains polymorphism phenotyping (PolyPhen)-2 (http://genetics.bwh.harvard.edu/pph2/), sorting intolerant from tolerant (SIFT) (http://sift.jcvi.org/) and Taster Mutation (http://www.mutationtaster.org/) were used to predict the virulence of mutations in *ATP7B*.

Results

Identification of Genetic Variants

All of the exons and intron/exon boundaries of *ATP7B* genes were amplified by PCR and sequenced for mutation. A total of 26 genetic variations, including 17 mutations (12 missense mutations, two synonymous mutations, two splicing mutations and one deletion) and nine SNPs, were identified (Tables 1 and 2). All the mutations were heterozygous. Among those mutations, we found one missense mutation (c.1757T>A) was novel (Figure 1) after a search in a series of databases, including the 1000

Table 1 Analysis of *ATP7B* mutations and prediction of the functional effects of the mutation

NT change	AA change	Location	Mutation type	Functional region	PROVEAN	PolyPhen-2 Prediction score		Allele count	Allele Freq %
c.1708-1G>C		IVS5-1	Splicing	MBD6	NA	NA	NA	1	1.56
c.1708-5G>C		IVS5-5	Splicing	MBD6	NA	NA	NA	1	1.56
c.3799delG		Exon18	Deletion	ATP hinge	NA	NA	NA	1	1.56
c.1351G>A	p.451G>S	Exon3	Missense	MBD5	Neutral	Benign	0.004	1	1.56
c.1757T>A*	p.586L>H	Exon5	Missense	MBD6	Deleterious	Probably damaging	1.000	1	1.56
c.1857C>A	p.619I>I	Exon5	Synonymous	MBD6	MBD6	NA	NA	2	3.12
c.2294A>G	p.765D>G	Exon8	Missense	Tm4	Deleterious	Probably damaging	1.000	1	1.56
c.2306T>C	p.769M>T	Exon8	Missense	Tm4	Deleterious	Probably damaging	1.000	1	1.56
c.2310C>G	p.770L>L	Exon8	Synonymous	NA	NA	NA	NA	7	10.92
c.2333G>T	P.778R>L	Exon8	Missense	Tm4	Deleterious	Probably damaging	1.000	9	14.04
c.2621C>T	p.874A>V	Exon11	Missense	ATPase	Deleterious	Possible damaging	0.832	1	1.56
c.2755C>G	p.919R>G	Exon12	Missense	Tm5	Deleterious	Probably damaging	1.000	1	1.56
c.2804C>T	p.935T>M	Exon12	Missense	Tm5	Deleterious	Possible damaging	0.832	4	6.24
c.2828G>A	p.943G>D	Exon12	Missense	Tm5	Deleterious	Possible damaging	0.832	1	1.56
c.2930C>T	p.977T>M	Exon13	Missense	Ch/Tm6	Deleterious	Probably damaging	1.000	2	3.12
c.2975C>T	p.992P>L	Exon13	Missense	Ch/Tm6	Deleterious	Probably damaging	1.000	4	6.24
c.3960G>C	p.1320R>S	Exon19	Missense	Tm7	Deleterious	Probably damaging	1.000	1	1.56

Abbreviations: AA, amino acid; ATPase, copper-(or silver)-translocating P-type ATPase segment; MBD, mental-binding domain; Ch, ion channel region; Tm, transmembrane segment.

*Represent newly discovered mutations.

Genome, dbSNPs (v130), Wilson disease Mutation Database, and HapMap. No mutations were found in the healthy control group.

Prediction of Functional Virulence Caused by *ATP7B* Mutations

We combined PolyPhen-2, SIFT, and Taster Mutation to predict the effects of the missense mutations on ATP7B function. Results showed that all of the missense mutations were deleterious except c.1351G>A, which was calculated as a benign one. Two synonymous mutations (c.1857C>A and c.2310C>G) were considered as polymorphic variations. The deletion mutation, c.3799delG, would lead to frame shift or PTC-further downstream change, which was predicted to be deleterious according the Taster Mutation prediction. The splicing mutations, c.1708-1G>C, first reported by Thomas,[13] was predicted as a 'pathogenic variant'. The splicing mutation, c.1708-5T>G, first reported by Norikazu Shimizu,[20] was predicted as 'variants with uncertain significance'. Particularly, the novel missense mutation, c.1757T>A, a heterozygous mutation in exon five, was identified in this study as a disease-causing mutation predicted by all three software. This mutation would lead to the replacement of a highly conserved Leucine with a Histidine at the 586 amino acid position (p. L586H) (Figure 2), which is deleterious for the last copper-binding domain.

Table 2 Information of *ATP7B* SNPs

SNP	RS-ID	Location	1000G Freq
c.-123_-119dupCGCCG	rs148013251	5'UTR	C=0.3704
c.1543-93A>C	rs3742288	Intron2	G=0.3776
c.1707-53A>C	rs2147363	Intron3	T=0.3882
c.2448-25G>A	rs9526811	Intron9	T=0.3069
c.3903+6C>T	rs2282057	Intron18	G=0.4982
c.1216T>G	rs1801243	Exon2	C=0.3762
c.1366G>C	rs1801244	Exon3	G=0.3770
c.2495A>G	rs1061472	Exon10	C=0.4976
c.2855G>A	rs732774	Exon12	T=0.4690

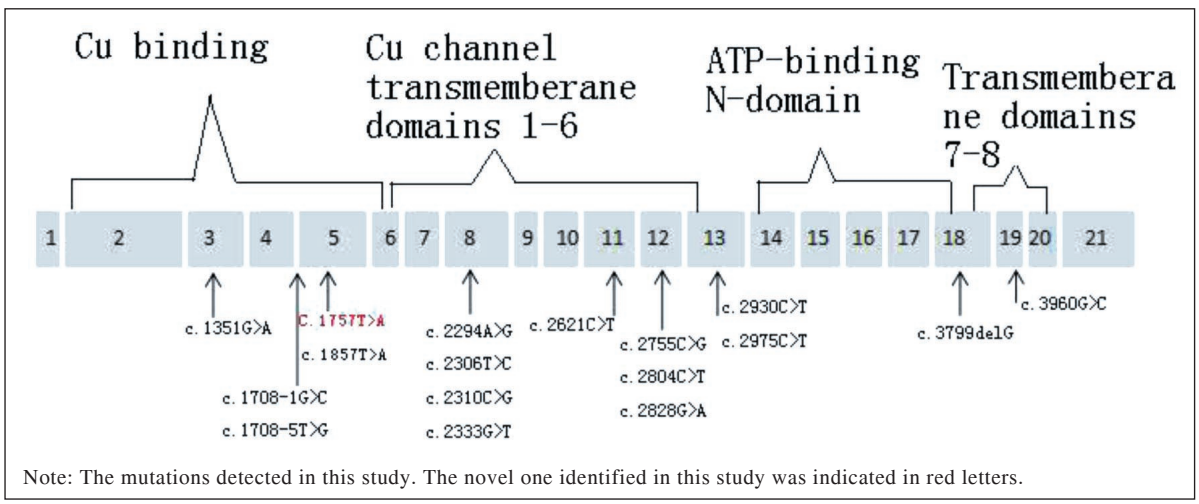
Discussion

WD is a life threatening autosomal recessive disorder, caused by abnormal copper metabolism. However, early diagnosis and effective therapy can prevent disease progression and minimise injuries to the organs, including liver, brain, cornea.[21,22] To date, the diagnosis of WD is mainly based on typical clinical manifestations and laboratory findings, including decreasing concentration of ceruloplasmin. Nevertheless, the clinical manifestations of WD are extraordinarily diverse and atypical, so that establishing timely diagnosis based on clinical manifestations is not easy, especially in adolescent patients.[21,23] As to the ceruloplasmin, which is commonly used as a serum marker for WD, is a kind of positive acute phase reactant in nature. In the case of acute inflammation, the ceruloplasmin concentration would increase, which might sometime be mislead when the WD patients undergoing infection. On the other hand, the concentration of ceruloplasmin will be declined in chronic liver disease, which will also be troublesome in diagnosing WD patients along with chronic liver disease. Therefore, genetic confirmation of WD diagnosis is necessary, which would be facilitated with hotspot in the *ATP7B*.

In this study, 16 known mutations and one novel *ATP7B* mutation (p. L586H) were found in those patients from South China. The mutations were mainly clustered in exons 8 (25%), 12 (18.7%), 4 (12.5%), 5 (12.5%), and 13 (12.5%), the same as previous results from North China.[19,24] However, P.778A>L was not detected in this study, though this mutation was recognised as the most frequent mutation in Chinese population.[24] Meanwhile, 50% (8/16) of the mutations happened to CU channel transmembrane domain, indicating a hotspot region for genetic screening of WD diagnosis in South China.

Interestingly, the mutation c.2310C>G and c.2333G>T as a unit, comprised 18.7% of the mutations in these WD patients, indicating these two mutations might act on *ATP7B* in the manner of cis-action (Table 3). In this study, p.778R>L showed the highest allele frequency (Table 1), indicating a hotspot for genetic confirmation of WD.

The newly identified mutation p.586L>H, not found in the 50 healthy individuals meaning <1% allele frequency, is a missense mutation existing in exon five, affecting the



Note: The mutations detected in this study. The novel one identified in this study was indicated in red letters.

Figure 1 Mutations in the different domains of the *ATP7B* gene.

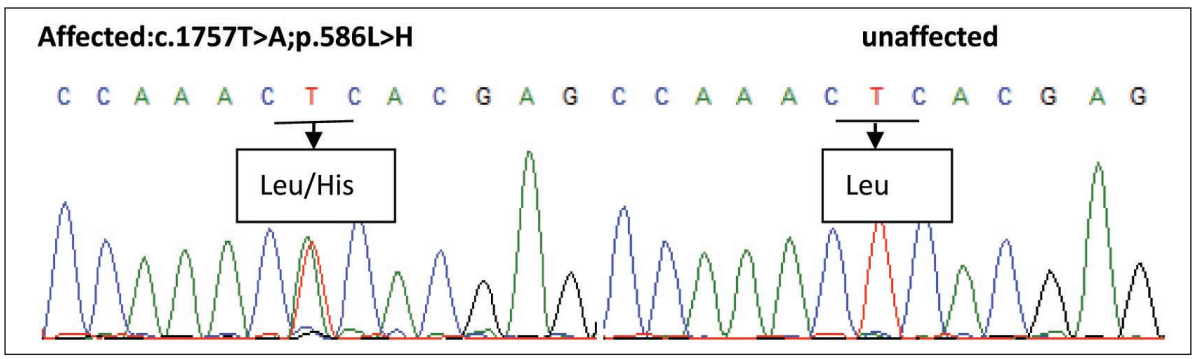


Figure 2 Sequencing diagram of the new mutation.

Table 3 Informations for patients with WD

No	Gender	Age (year)	CER (mg/dl)	CU (umol/l)	K-Fring	Manifestation	Mutations classification	ACMG of the variants	Literatures
1	Male	5	8.8	6.8	None	Liver ribs 3.0 cm	c.1757T>A	PM1	This study
							c.2310C>G	BA1	Ref.8
							c.2333G>T	PM1	Ref.7
2	Female	13	9.7	8.9	Yes	Liver ribs 2.0 cm	c.1857C>A	BP7	Ref.11
3	Female	3	8.0	7.6	Yes	Liver ribs 2.0 cm	c.2310C>G	BA1	Ref.8
							c.2333G>T	PM1	Ref.7
							c.3799delG	PM4	Ref.8
4	Male	2	2.6	8.9	None	Liver ribs 1.0 cm	c.1857C>A	BP7	Ref.11
5	Female	7	6.8	8.4	Yes	Liver ribs 4.0 cm	c.2306T>C	PM1	Ref.7
6	Male	12	8.8	8.9	None	Liver ribs 2.0 cm	c.2306T>C	PM1	Ref.7
							c.2310C>G	BA1	Ref.8
							c.2333G>T	PM1	Ref.7
7	Male	3	4.8	8.0	None	Liver Not touched	c.2755C>G	PM1, PM4	Ref.8
8	Female	16	4.5	10.0	None	Liver Not touched	c.2828G>A	PM1, PM4	Ref.7
9	Male	8	7.2	11.3	Yes	Liver cirrhosis	c.2294A>G	PM1, PM4	Ref.8
10	Male	11	<2.0	6.1	Yes	Liver ribs 1.0 cm	c.2333G>T	PM1	Ref.7
11	Male	4	3.3	14.9	None	Liver ribs 1.0 cm	c.2975C>T	PM4	Ref.8
12	Male	16	2.4	11.0	None	Liver ribs 2.0 cm	c.1857C>A	BP7	Ref.11
13	Female	15	<2.0	8.8	None	Liver ribs 1.5 cm	c.2804C>T	PM1	Ref.8
							c.2310C>G	BA1	Ref.8
							c.2333G>T	PM1	Ref.7
14	Male	5	4.0	10.0	None	Liver ribs 2.0 cm	c.3960G>C	PM4	Ref.11
							c.2294A>G	PM1, PM4	Ref.8
15	Male	19	<2.0	10.1	Yes	Liver Not touched	c.2310C>G	BA1	Ref.8
						Limb tremor	c.2333G>T	PM1	Ref.7
						Erectile dysfunction			
16	Male	28	4.1	14.3	None	Liver cirrhosis	c.1351G>A	BP4	Ref.11
							c.2930C>T	PM4	Ref.8
							c.2975C>T	PM4	Ref.8
17	Male	26	2.2	11.0	None	Liver Not touched	c.2310C>G	BA1	Ref.8
							c.2333G>T	PM1	Ref.7
18	Male	2	2.6	8.9	None	Liver ribs 1.0 cm	c.2828G>A	PM1, PM4	Ref.7
19	Female	3	4.1	11.6	None	Liver Not touched	c.2804C>T	PM1	Ref.8
							c.2975C>T	PM4	Ref.8
20	Female	4	9.7	15.0	None	Liver ribs 1.0 cm	c.2310C>G	BA1	Ref.8
							c.2333G>T	PM1	Ref.7
21	Male	21	3.6	6.8	None	Liver ribs 1.0 cm	c.2930C>T	PM4	Ref.11
							c.2975C>T	PM4	Ref.8
22	Male	10	8.0	10.1	None	Liver ribs 1.0 cm	c.3960G>C	PM4	Ref.11
23	Male	6	5.8	9.7	None	Liver Not touched	c.1708-3G>C	BP4	Ref.8
24	Male	30	4.0	11.5	Yes	Liver ribs 1.5 cm	c.2621C>T	PM4	Ref.8
							c.2333G>T	PM1	Ref.7
25	Male	9	<2.0	10.1	None	Liver Not touched	c.2804C>T	PM1	Ref.8
							c.-123_-119dupCGCCG	PM4	Ref.7
26	Male	2	4.0	8.1	None	Liver Not touched	c.-123_-119dupCGCCG	PM4	Ref.7
27	Female	3	7.6	10.1	None	Liver Not touched	c.2755C>G	PM1, PM4	Ref.11
28	Male	5	14.0	6.8	None	Liver Not touched	c.1751G>A	PM1	Ref.7
29	Female	8	13.5	8.8	None	Liver Not touched	c.2621C>T	PM4	Ref.8
30	Male	9	4.1	9.0	Yes	Liver ribs 1.0 cm	c.1708-5G>C	BP4	Ref.8
						Liver cirrhosis	c.-123_-119dupCGCCG	PM4	Ref.7
						Haematuria			
31	Male	14	3.0	7.9	Yes	Liver ribs 1.5 cm	c.2294A>G	PM1, PM4	Ref.8
						Ataxia			
						Aphasia			
32	Male	11	3.8	8.9	None	Liver ribs 2.0 cm	c.2306T>C	PM1	Ref.7

Reference range: CER, 19-67mg/dl; CU level, 11.8-39.3 umol/l.
According to ACMG, PM1-PM6 refers to moderate evidence for pathogenicity; BA1 refers to alone evidence for benign impact; BP1-BP7 refers to supporting evidence of benign impact.
Ref. refers to Reference attached at the end of the paper.

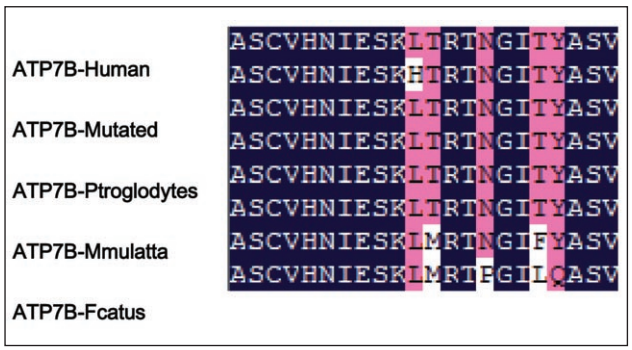


Figure 3 Multiple-sequence alignment in *ATP7B* from different species revealed that codon 586 was located within a highly conserved region.

CU binding domain, which was predicted as deleterious. The 586 amino acid is a highly conserved region when compared between human and other species, indicating an indispensable impact on *ATP7B* function (Figure 3).

In summary, we recommend that sequencing of the CU channel binding domain of *ATP7B* in genetic screening for WD patients, which cover over 50% in south China. And the mutation p.778R>L might also serve as a hotspot for genetic confirmation of WD. The newly identified mutation, p.586L>H, is an addition for the genetic mutation base of WD.

Declaration of Interests

The authors declare no competing financial interests.

Acknowledgments

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