Genetics and Molecular Research

Direct sequencing of mutations in the copper-transporting P-type adenosine triphosphate (*ATP7B*) gene for diagnosis and pathogenesis of Wilson's disease

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Genet. Mol. Res. 15 (3): gmr.15038746
Received April 28, 2016
Accepted June 10, 2016
Published September 23, 2016
DOI http://dx.doi.org/10.4238/gmr.15038746

ABSTRACT. Copper-transporting P-type adenosine triphosphatase (*ATP7B*) has been identified as the pathogenic gene in hepatolenticular degeneration, or Wilson's disease (WD). The aim of this study was to explore the correlation between genetic mutations and the clinical profile of WD, and to discuss the value of mutation examination in its diagnosis for providing a scientific basis for the development of a method to examine genetic mutations. Sixty-eight Chinese Han patients with WD and 20 controls were included in this study. The *ATP7B* gene in DNA extracted from patient blood samples was amplified by PCR and sequenced. These sequences were compared against corresponding gene sequences obtained from healthy controls to statistically analyze the genetic mutations. Five of the nineteen mutations in *ATP7B*

were newly detected mutations; moreover, 8 of these mutations were polymorphic (2 were newly identified). The Arg778Leu and Pro992Leu mutations in exons 8 and 13 were detected at the highest mutation frequencies of 25.74 and 16.91%, respectively. The frequencies of all other mutations were below 5%. However, the clinical manifestations of WD did not differ significantly in patients with the Arg778Leu and Pro992Leu mutations. Therefore, these mutations were considered as hotspot mutations in Chinese WD patients. However, we observed no significant correlation between these genetic types and the clinical symptoms of WD. The correlation between the mutation genotype and disease phenotype remains to be elucidated. In conclusion, the highly sensitive and specific direct DNA sequencing method can be used to screen for the causative genes of WD.

Key words: Genetic mutation; ATP7B; Wilson's disease; Impaired copper metabolism

INTRODUCTION

Hepatolenticular degeneration (HLD), an autosomal recessive disease resulting in impaired copper metabolism, has different clinical manifestations, because of the differences in the copper ion deposition load and retention time in the organs (Chen et al., 2011; Ding et al., 2014). Patients with Wilson's disease (WD) present hepatic and neurological symptoms, as well as digestive symptoms (in some cases), during the early stages of the disease. Further, WD causes damages to the circulatory system and the kidneys, as well as changes in the skin in some patients. Therefore, this disease can be easily misdiagnosed (Liu et al., 2014). WD has high disability rate and mortality rate; however, it is one of the few curable congenital inherited diseases. WD can be controlled if diagnosed and treated at an early stage (Alam et al., 2014).

The copper-transporting P-type adenosine triphosphate gene *ATP7B*, located in the autosomal chromosome 13 at position q14.3, has been confirmed to play a pathogenic role in WD. *ATP7B* is a 78,821-bp long gene with 21 exons and 20 introns (Zhang and Liu, 2008; Lu et al., 2014). ATP7B, a member of the P-type ATPase family, has three functional regions: six conserved GMTCXXC sequences at the N-terminal region (copper-binding domain) that can bind to a copper ion; a P-type ATPase domain (P-ATPase); and trans-membrane domains that influence the passage of copper ion pumps (Forbes and Cox, 2000). Mutations in *ATP7B* are responsible for disorders in the synthesis of ceruloplasmin and copper excretion blockage in the bile; hence, excess copper cannot be discharged and is deposited *in vivo*, which manifests clinically as cirrhosis, neurological symptoms, intacs, or urine copper elevation, among others (Petrukhin et al., 1994). More than 550 mutations have been reported in *ATP7B* so far, some of which are hotspot mutations with obvious ethnic and regional differences (Forbes et al., 2014). Ferenci (2006) identified 23 mutations in *ATP7B* in 2006, and was the first to classify the H1069Q and G1266K mutations in exons 14 and 18 as hotspot mutations in European WD patients, accounting for 28 and 10% of the total number of patients, respectively.

Preliminary Chinese studies, which concerns WD, have shown that the missense mutation Arg778Leu, located in exon 8 of *ATP7B*, is the common mutation type. However, a consensus is yet to be reached regarding the patterns and frequencies of other mutations

in *ATP7B*, which hinders the development of gene-based clinical diagnostic and treatment methods in the Chinese population (specifically) to some extent (Gu et al., 2003). Therefore, in this study, we have analyzed all exonic regions in *ATP7B* obtained from 68 WD patients (diagnosed between June 2011 and March 2013) for mutations.

MATERIAL AND METHODS

General data

Sixty-eight patients with WD (35 males and 33 females) were recruited from the First Affiliated Hospital of Zhengzhou University between June 2011 and March 2013. The patients were unrelated Han Chinese individuals diagnosed with WD based on a diagnostic score and the following criteria: 1) positive manifestation of the Kayser-Fleischer ring (K-F ring) in the cornea, extrapyramidal symptoms, ceruloplasmin (CER) level in the serum <0.1 g/L, or 24-h urinary copper excretion level >100 μg (or urine copper level >5-fold lower than that in normal patients following penicillamine provocation) was denoted by a diagnostic score of 2 points (per symptom); 2) positive manifestation of Coombs-negative hemolytic anemia, 24-h urinary copper excretion levels ranging from 40-100 μg, positive copper staining in the liver tissue, or 0.1-0.2 g/L CER in the serum, was denoted by a score of 1 point (each); and each homozygous or compound heterozygous mutation in the exon region of *ATP7B* (as determined by gene sequencing) was given a score of 4 points, whereas a single site heterozygous mutation was given a score of 1 point. Patients with hepatitis, renal diseases, and other systemic diseases were excluded from this study.

The control group comprised 20 unrelated healthy (as determined from an epidemiological survey) individuals without any neuro-genetic or mental disorders or a family history of nervous system disorders.

Ethical approval was obtained for this study from the Ethics Committee of our institution; signed informed consent forms were obtained from all patients. The patients and controls were subjected to an epidemiological survey. Subsequently, peripheral venous blood was obtained from all subjects for whole genome extraction and preserved at -20°C until use.

Genomic DNA extraction and amplification

Genomic DNA was extracted from the peripheral blood samples using the PureLink® Genomic DNA Mini kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer protocols. The DNA samples were amplified using the Thomas method (Thomas et al., 1995). The polymerase chain reaction (PCR) amplification consisted of the following steps: pre-denaturation at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 1 min; and final elongation steps at 72°C for 7 min and at 4°C for 5 min. The PCR products were stored at 4°C.

DNA sequencing

The amplified DNA products were sequenced at Sangon Biotechnology (Shanghai, China), and the sequenced DNA were compared to human normal *ATP7B* using the Basic Local Alignment Search Tool. Samples showing abnormal results were subjected to bidirectional sequencing, and the mutation was characterized.

Statistical analysis

The obtained data were statistically analyzed using SPSS v.20.0 (IBM, Armonk, NY, USA). The composition ratio of enumeration data was determined by the following tests: the chi-square test was used to analyze data with actual frequency >5 and the Fisher test was used to analyze data with actual frequency ranging 0-5. P values <0.05 indicated statistically significant differences.

RESULTS

Sequencing of *ATP7B* in patients with WD

The 21 exonic regions of *ATP7B* extracted from WD patients were sequenced and compared against the corresponding sequences from healthy controls. This yielded 19 pathogenic mutations, 16 of which were missense mutations. Of the remaining mutations, 1 was a nonsense mutation and the remaining 2 were frameshift mutations (Table 1). A comparison of these mutations with those uploaded on the HLD gene mutation database (http://uofa-medical-genetics.og/wilson/index.php) revealed five novel mutations and eight polymorphic mutations (of which 2 had not been reported previously) (Table 2).

Table 1. Mutations detected in the copper-transporting P-type adenosine triphosphatase (*ATP7B*) gene.

Exon or intron	Mutation site	Mutation type	Amino acid	Mutation rate (%)
Exon2	523InsA	Insert	frameshift	1.47 (2/136)
Exon3	C1531T	Nonsense mutation	Gln511Term	1.47 (2/136)
Exon7 (new)	T2075C	Missense mutation	Leu692Pro	0.73 (1/136)
Exon8	G2333T	Missense mutation	Arg778Leu	25.74 (35/136)
Exon8 (new)	C2333A	Missense mutation	Leu745Ile	0.73 (1/136)
Exon8	A2305G	Missense mutation	Met769Val	0.73 (1/136)
Exon8	2299InsC	Insert	frameshift	0.73 (1/136)
Exon10 (new)	C2549A	Missense mutation	Thr850Ile	0.73 (1/136)
Exon12	G2828A	Missense mutation	Gly943Asp	1.47 (2/136)
Exon12	C2755G	Missense mutation	Arg919Gly	2.94 (4/136)
Exon13	C2924A	Missense mutation	Ser975Tyr	2.94 (4/136)
Exon13	C2930T	Missense mutation	Thr977Met	0.73 (1/136)
Exon13	G2906A	Missense mutation	Arg969Gln	0.73 (1/136)
Exon13	C2975T	Missense mutation	Pro992Leu	16.91 (23/136)
Exon14 (new)	C3209G	Missense mutation	Pro1070Arp	2.94 (4/136)
Exon18	A3809G	Missense mutation	Asn1270Ser	0.73 (1/136)
Exon18 (new)	T3824C	Missense mutation	Leu1275Ser	0.73 (1/136)
Exon18	G3859A	Missense mutation	Gly1287Ser	1.47 (2/136)
Exon19	A3982G	Missense mutation	Ala1328Thr	0.73 (1/136)

Table 2. Polymorphic forms of the copper-transporting P-type adenosine triphosphatase (*ATP7B*) gene.

Exon or intron	Nucleotide change	Amino acid change	Polymorphism (N)/Total number
Exon1	A9G	Glu3Glu	2/68
Exon2	G1216T	Ala406Ser	50/68
Exon2	G1216T	Ala406Ser	33/68
Exon2	A1168G	Ile390Val	1/68
Exon3	C1366G	Leu456Val	2/68
Exon8	C2310G	Leu770Leu	4/68
Exon13 (new)	T2913A	Ala971Ala	43/68
Exon18 (new)	G3798T	Gly1266Gly	2/68

Sequencing graphics

Figures 1-5 show the sequenced novel mutations in *ATP7B*. The arrows indicate the mutation sites.

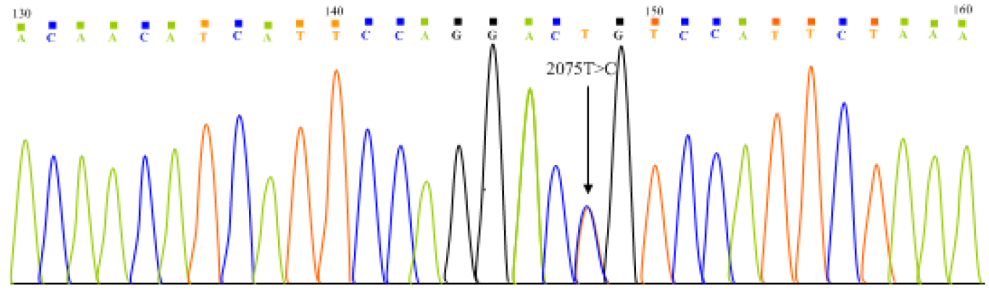

Figure 1. Sequencing of the Leu692Pro mutation in exon 7. The T to C substitution at base 2075 (from CTG to CGG) in the *ATP7B* DNA resulted in an L-leucine (Leu) to L-proline (Pro) substitution in amino acid 692 in the resultant protein.

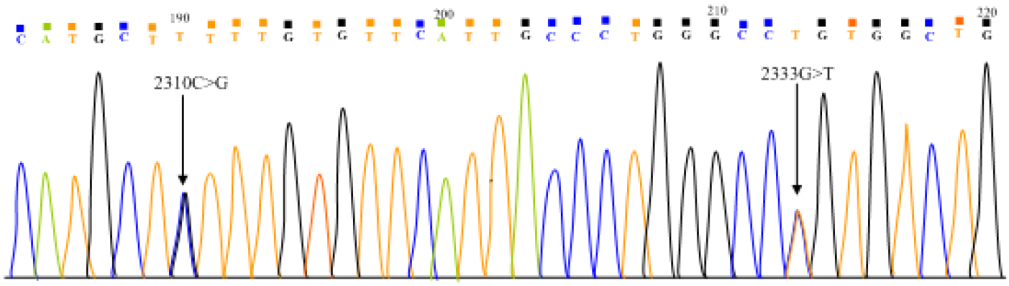

Figure 2. Sequencing of the Leu745Ile mutation in exon 7. The C to G and G to T substitutions at bases 2310 and 2333 (from CTC to CTG and CGG to CTG, respectively) in the *ATP7B* DNA resulted in an L-leucine (Leu) at amino acid 770 and an L-arginine (Arg) to L-leucine (Leu) substitution at amino acid 778, respectively.

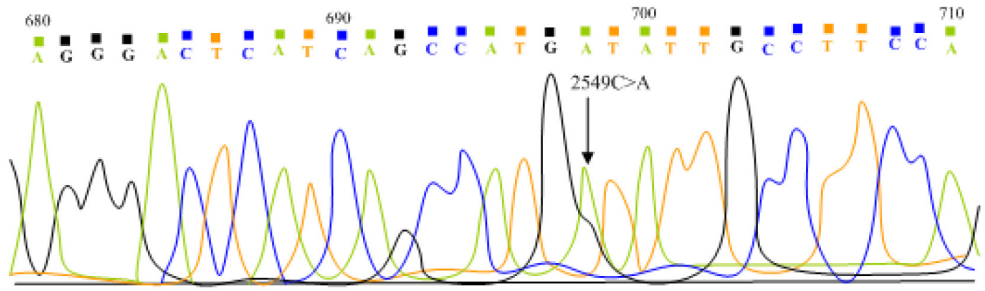

Figure 3. Sequencing of the Thr850Ile mutation in exon 10. The C to A substitution at base 2549 (from ACC to ATC) in the *ATP7B* DNA resulted in an L-threonine (Thr) to L-isoleucine (Ile) substitution at amino acid 850 in the resultant protein.

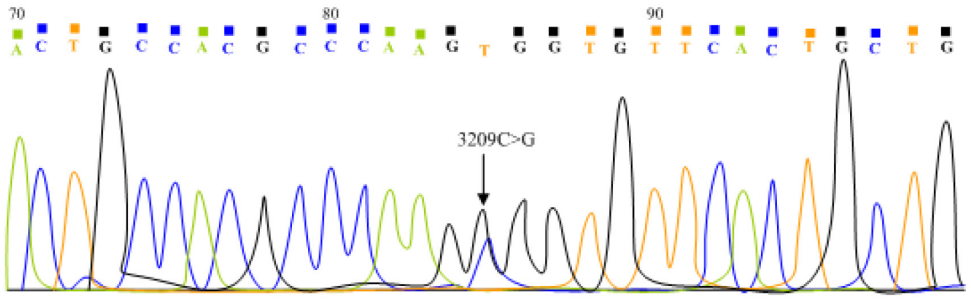

Figure 4. Sequencing of the Pro1070Arp mutation in exon 14. The C to G substitution at base 3209 (from CCC to CGC) in the *ATP7B* DNA resulted in an L-proline (Pro) to L-arginine (Arg) substitution at amino acid 1070 in the resultant protein.

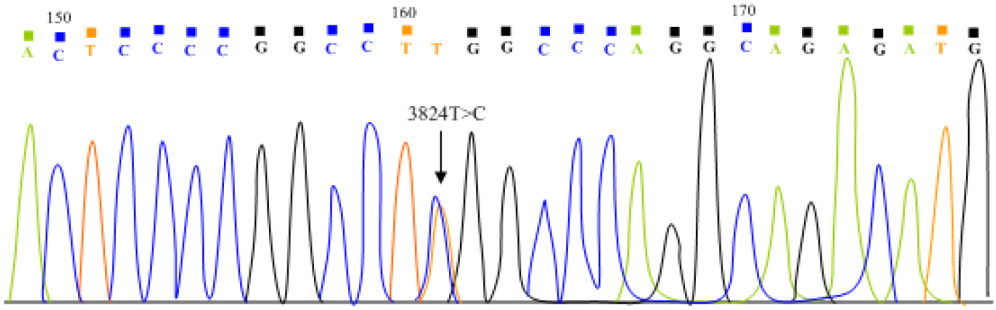

Figure 5. Sequencing of the Leu1275Ser mutation in exon 18. The T to C substitution at base 3824 (from TTG to TCG) in the *ATP7B* DNA resulted in an L-leucine (Leu) to L-serine (Ser) substitution at amino acid 1275 in the resultant protein.

Genotype-phenotype relation

The Arg778Leu (2333G > T) and Pro992Leu (2975C > T) mutations at exons 8 and 13 were observed at the highest frequencies in this study. The other mutations were discretely dispersed. Therefore, these two major mutation sites were selected as the genotype index to study the relationship between high frequency gene mutations and clinical manifestations of WD.

WD patients (26/68) were pre-symptomatic, with no clinical manifestations; therefore, these patients were not included in the genotype-phenotype analysis. These results showed that the Arg778Leu and Pro992Leu mutations in *ATP7B* were not correlated with the clinical type of WD (P > 0.05; Tables 3 and 4).

Table 3. Relation between the *ATP7B* Arg778Leu mutation and clinical type of hepatolenticular degeneration.

Mutation type	Hepatic type	Nerve type	Hepatic nerve type	P value
Arg778Leu mutation	11	3	0	0.06
No Arg778Leu mutation	17	7	2	

Table 4. Relationship between the *ATP7B* Pro992Leu mutation and clinical type of hepatolenticular degeneration.

Mutation types	Hepatic type	Nerve type	Hepatic nerve type	P value
Pro992Leu mutation	6	1	0	0.15
No Pro992Leu mutation	24	7	2	

DISCUSSION

WD, an autosomal recessive disorder widely distributed in humans, is primarily characterized by changes in the copper metabolism pathway, leading to excess copper deposition in the liver, brain, kidneys, and eyes, which in turn induces severe pathophysiological changes in the body (Zong and Kong, 2015). This disease can affect humans at all ages; however, it is predominantly seen in people aged 5-35 years. The major clinical manifestations of WD include liver cirrhosis, extrapyramidal symptoms, K-F ring formation, and acute hemolysis (Burkhead et al., 2011). Gollan and Gollan (1998) reported that the incidence rates of WD in live birth infants and the total population are 1 in 30,000 and 15-30 in a million, respectively, whereas the carrier rate of the pathogenic gene ranged from 0.3 to 0.7%. They also estimated the age of onset to be in the range of 7-12 years. However, Mak et al. (2008) reported a WD incidence rate of 1/5400 in people belonging to the Han ethnicity, which was much higher than that of the occidental population. However, as it is a curable hereditary disease, a majority of the patients show good prognosis if diagnosed at an early stage (pre-symptomatic diagnosis) and provided with timely treatment. Therefore, considerable efforts are being focused on developing a gene-based diagnostic method for the diagnosis of pre-symptomatic WD (Park et al., 2007).

In this study, the mutations in *ATP7B* from 68 Chinese WD patients were identified. Several type of mutations (missense mutation, nonsense mutation, insertion, etc.), whose dispersal characteristics formed a broad-spectrum feature, were identified in *ATP7B*. These characteristics were consistent with those from previous studies conducted in China and abroad (Cheng et al., 2009). The Arg778Leu (2333G > T) and Pro992Leu (2975C > T) mutations at exons 8 and 13 were observed at mutation frequencies of 25.74 and 16.91%, respectively, in the population (Table 1), and were considered as the first and second mutation hotspots in this study. Mutations in all other exons were observed at a frequency of 5% or less. These results indicated that the Arg778Leu and Pro992Leu mutations in *ATP7B* were chiefly responsible for WD disease in Chinese patients; Pro992Leu mutation may be another high frequency mutation site in Chinese HLD patients.

ATP7B mutations in 68 WD patients were identified and analyzed by DNA sequencing; the positive detection rate of DNA sequencing was 91.18%, indicating the high sensitivity of this method (for detecting mutations in the gene). However, 6 patients showed no mutations; this could be attributed to various reasons, such as limitations of the currently used gene-based diagnostic technology, localization of the mutation in the intron or promoter region, or misdiagnosis owing to non-obvious or nonspecific clinical manifestations in the patients. However, 5 new pathogenic mutations were identified in patients with WD. The identification of these pathogenic mutations is of great significance, as they enriched the gene database of HLD and expanded on the existing knowledge on functional mutations in the causative genes of HLD. This identification also provides a theoretical basis for future research on the pathogenic mechanism of this disease.

A number of studies conducted in China and abroad have attempted to genotype *ATP7B* and characterize the clinical types of WD; however, a consensus has not been reached so far (Wu et al., 2003; Liu et al., 2004). Gupta et al. (2003) reported no specific correlation between genotyping and the clinical types of WD patients in India, theorizing that the same genetic type may have different clinical manifestations in different patients with WD. Kucinskas et al. (2008) reported that the His1069Gln functional mutation responsible for liver damage is a hotspot mutation in Lithuanian WD patients. Studies conducted in China regarding the correlation between the genotype and clinical manifestations of WD primarily centered on the Arg778Leu mutation in *ATP7B* (Huang et al., 2000; Wu et al., 2001; Li et al., 2008). Huang et al. (2000) detected no obvious correlation between the Arg778Leu mutation and the clinical indices of WD, such as the gender, blood copper level, and primary symptoms, in 67 patients. However, Li et al. (2008) reported a close association between the Arg778Leu mutation and liver damage in WD patients. In this study, we found no obvious correlation between the Arg778Leu mutation and clinical manifestations of WD. This could be attributed to the high genetic heterogeneity of WD [WD can be caused by various heterozygous (primarily) gene mutations; moreover, a single patient can present mutations at several exon sites, which cannot be statistically analyzed effectively]; lack of obvious specificity of the primary symptoms of WD, allowing for easy misdiagnosis (which in turn would lead to failure of genetic analysis); and the effect of the environment and other factors on the clinical manifestations of WD.

Study limitations

HLD is an inherited autosomal-recessive disease with low global incidence. Therefore, studies with large sample sizes are few in China and abroad. The patients included in this study were recruited from a single hospital. Therefore, the correlation between mutation hotspots and the clinical phenotype of WD remains to be explored with a larger sample size.

CONCLUSION

In conclusion, direct sequencing is a highly sensitive and specific method that can be applied to screen for mutations in *ATP7B* related to WD. The results of this study must be validated by further studies with a larger sample size and consistent study standard, using a more accurate method for genotyping and clinical typing.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by grants provided by the Foundation and Frontier Technology Research Plan of Henan for research on the relationship between the ATP7B mutation and Wilson's disease (Project #22300410259) and the Scientific Problem Tackling Plan of Zhengzhou for the clinical study of mesenchymal stem cells in treating hepatic fibrosis in patients with Wilson's disease (Project #121PPTGG494-1).

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