Identification of two novel mutations in the *ATP7B* gene that cause Wilson's disease

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Background: **Wilson's disease is an autosomal recessive disorder characterized by liver disease and/or neurologic deficits due to copper accumulation and is caused by pathogenic mutations in the *ATP7B* gene.**

Methods: **Two unrelated Chinese patients born to nonconsanguineous parents who were diagnosed with early-onset Wilson's disease. DNA sequencing and bioinformation analysis were conducted.**

Results: **We have identified four mutations in two family trios, of which two were novel, namely, c. 3028A>G (p. K1010E) and c.3992T>G (p.Y1331X), in each patient.**

Conclusions: **Gene testing is playing an important role in diagnosis of Wilson's disease. The early-onset of Wilson's disease is apparently not associated with P-ATPase domain in the ATP7B protein. Our findings further widen the spectrum of mutations involving the *ATP7B* gene.**

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Introduction

Wilson's Disease (WD; OMIM #277900), also known as hepatolenticular degeneration, is an autosomal recessive disorder characterized by liver disease and/or neurologic deficits due to copper accumulation. The global prevalence rate of WD is 1/30 000, and its carrier frequency is 1/90.[1] The onset of WD is typically during adolescence and the second decade of life, but can also begin later than age 40.[2] One hallmark feature of WD is Kayser-Fleischer (K-F) rings, and the most common neurologic manifestations include parkinsonism, ataxia, rigid dystonia, psychiatric symptoms, and a series of complex hepatic symptoms like acute fulminant hepatic failure, hepatomegaly, chronic hepatitis and hepatic dysfunction.[3]

WD is caused by pathogenic mutations in the *ATP7B*, which has been mapped to chromosome 13q14.3.[4] Approximately 790 disease-causing mutations have been identified in this gene, which include missense, nonsense, small deletions, small insertions and splicing variants (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=*ATP7B*). Most patients are compound heterozygotes for sequence variants in the *ATP7B* gene.[5]

The ATP7B protein plays an important role in maintaining cellular copper levels. Copper metabolism defect leads to deficient incorporation of copper into ceruloplasmin and decrease biliary copper excretion. The toxic accumulation of copper thus damages various organs such as liver, kidneys, eyes and the basal ganglia of the brain, particularly the lenticular nucleus. Copper imbalance also can lead as a pathogenetic pathway toward Alzheimer's disease.[6]

In this study, we conducted a molecular analysis of two unrelated pediatric WD cases from Lanzhou City, China and identified two novel mutations in the *ATP7B* gene.

Methods

Patients and clinical features

The Ethics Committee of The Second Hospital of Lanzhou University approved the present work, and the parents

of the two WD patients provided their informed consent to participate in the investigation. The parents of the two probands were apparently normal and nonconsanguineous. The two probands were designated as WD-F1 and WD-F2, respectively (Fig. 1A). Both patients presented neurologic manifestations such as tremor, slurred speech, mild dystonia, hepatic symptoms like hepatomegaly, chronic hepatitis and K-F rings at age 8 (Table). No special clinical manifestations were shown in the two patients.

Mutation analysis

DNA was isolated from whole venous blood using the standard sodium dodecyl sulfate-proteinase K-phenol/chloroform method. Genetic analysis of the *ATP7B* of the two patients was performed by polymerase chain reaction (PCR) amplification of all 21 coding exons and its adjacent splice junctions using intronic primer pairs. PCR conditions were set as previously reported.[7] PCR products were sequenced using a 3500 Dx automatic sequencer (Thermo Fisher Scientific, Massachusetts, USA). To screen for mutations in WD-F1 from the parents and 100 control individuals, genomic DNA was amplified using primers as follows. E13F: 5'-AACCCAAGTTCGTCACGTTG-3' and E13R: 5'-TATGACTGGTGGCTACTCTG-3', followed by restriction endonuclease analysis with the TaqI restriction enzyme (TaKaRa Biotech, Dalian, China). For WD-F2, mutations were detected by comparing the proband's findings with the PCR results of the normal parents using primers as follows, E18-19F: 5'-TCTGCTGCTATCTGATACCT-3' and E18-19R:

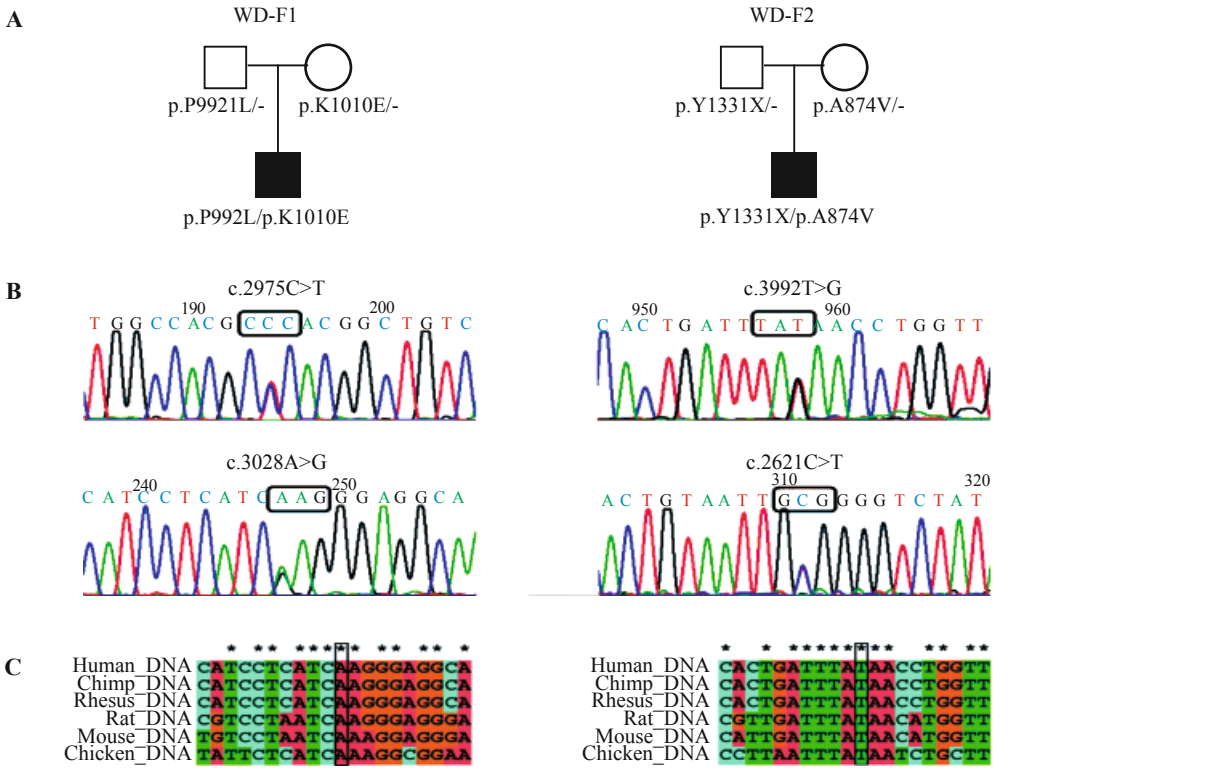

Fig. 1. Pedigrees and disease mutations. **A:** Wilson's disease (WD)-F1 is heterozygous for p.P992L and p.K1010E in exon 13. WD-F2 is heterozygous for p.Y1331X and p.A874V in exons 19 and 11, respectively; **B:** DNA sequencing results of patients WD-F1 and WD-F2; **C:** Description of the novel mutations in patients WD-F1 and WD-F2 based on multiple sequence alignment with those of various species. The black outline in the alignment shows the position of the mutation.

Table. Clinical characteristics of two Wilson's disease (WD) patients described in details

Patients	Sex	Onset age (y)	Family history	Kayser Fleischer ring	Neuropsychiatric symptoms	Liver function	Serum copper (g/L) (normal: >0.7-1 g/L)	Serum ceruloplasmin (g/L) (normal: >0.2 g/L)	24-h urinary copper (g/d) (normal: <0.1 g/d)	Cranial magnetic resonance imaging	Outcome
WD-F1	Male	8	No	Positive	Tremor slurred speech mild dystonia	Abnormal	0.1	0.03	0.63	Abnormal	Alive
WD-F2	Male	8	No	Positive	Tremor slurred speech mild dystonia	Abnormal	0.2	0.04	Absent	Absent	Dead

5'-AGCCACTCACTAACCCCAGC-3'. Sequence alignment and mutation detection were performed by using the clustalx1.83 software. The predicted effect of the mutations in the protein coding region were assessed by polymorphism phenotyping-2 (http://genetics.bwh.harvard.edu/pph2/), sorting intolerant from tolerant (SIFT; http://sift.jcvi.org/), protein analysis through evolutionary relationships (Panther; http://www.pantherdb.org/tools/csnpScoreForm.jsp), predictor of human deleterious single nucleotide polymorphisms (PhD-SNPs; http://snps.uib.es/phd-snp/phd-snp.html) and Combined Pdeleterious (cPdel) calculate.[8,9] Multiple mutation databases were performed by Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk/ac/all.php), Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/) and 1000 Genomes Project (http://browser.1000genomes.org/index.html).

Results

In WD-F1, direct sequencing of the *ATP7B* gene revealed a compound heterozygous mutation in exon 13, namely c.2975C>T and c.3028A>G (NM_000053) (Fig. 1B). The mutations c.2975 C>T and 3028A>G lead to amino acid substitutions p.P992L and p.K1010E (NP_000044.2), respectively. c.2975C>T has been described previously,[10] while c.3028A>G was found to be a novel missense mutation. The patient's father and mother were heterozygous for p.P992L and p.K1010E, and did not show any clinical manifestations. The mutation p.K1010E was not detected in the healthy individuals by restriction analysis. Sequence alignment using the clustal×1.83 software suggested that the amino acid residues located in 1010 is evolutionarily highly conserved and p.K1010E variation might be a pathogenic mutation (Fig. 1C). Through prediction of the functional effect of p.K1010E mutation, the cP_{del} score is 0.99. The result shows mutation p.K1010E may alter the structure of *ATP7B* gene and contribute to the disease.

WD-F2 was determined to be heterozygous for c.2621C>T and c.3992T>G (NM_000053) (Fig. 1B) in exons 11 and 19, respectively. The mutations c.2621C>T and c.3992T>G, lead to amino acid substitutions p.A874V and p.Y1331X (NP_000044.2), respectively. c.2621C>T has been described previously,[11] while c.3992T>G was found to be a novel nonsense mutation. The patient's father and mother harbored both p.A874V and p.Y1331X variants; however, they did not present any clinical symptoms. Using the clustalx1.83 software, we confirmed that the novel mutation p.Y1331X is a pathogenic mutation (Fig. 1C).

These two novel mutations were not identified in the HGMD, ExAC and 1000 Genomes Project.

Discussion

In the present two trio families, each proband was diagnosed with WD based on clinical characteristics and K-F rings. The age of disease onset of the two patients was 8 years. By sequencing the *ATP7B* gene, we identified heterozygous mutations in each patient. These results further demonstrated that WD was caused by mutations in the *ATP7B* gene.

The *ATP7B* gene encodes a protein that consists of six heavy metal-associated domains (HMA), four membrane-spanning domains, an ATPase domain (also known as E1-E2 ATPases), and a haloacid dehalogenase-like hydrolases domain (HAD). HMA is a conserved domain of nearly 30 amino acid residues that transport or detoxify heavy metals. P-ATPases transport ions and phospholipids across the membrane by using ATP hydrolysis.[12] HAD domain includes a L-2-haloacid dehalogenase, epoxide hydrolases, and phosphatases (Fig. 2). The ATP7B protein functions as a monomer that exports copper from cells such as that involving the efflux of copper from hepatocytes and into the bile.

In WD-F1, the variants c.2975C>T (p.P992L) and c.3028A>G (p.K1010E) were detected in exon 13. The variant c.3028A>G (p.K1010E) is a novel missense mutation. The mutation p.P992L is located in the sixth helical domain of the transmembrane region, whereas

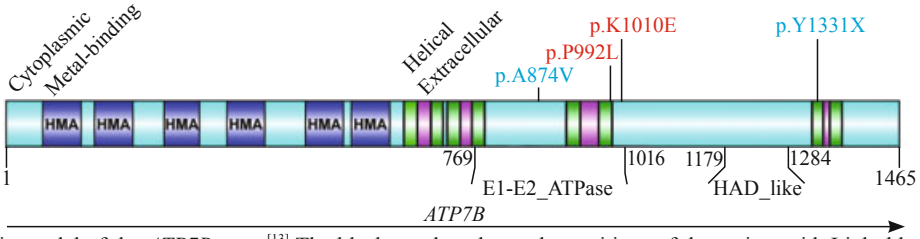

Fig. 2. Structure domain model of the *ATP7B* gene.[13] The black number shows the positions of the amino acid. Light blue: amino acid residues located in the cytoplasmic domain; Green: amino acid residues located in the helical region; Purple: amino acid residues in the extracellular domain. HMA: heavy metal-associated domain, which transports or detoxifies heavy metals; E1-E2_ATPase: transports ions and phospholipids across the membrane via ATP hydrolysis. HAD_like: epoxide hydrolases and phosphatases. The mutations in WD-F1 are shown in red, whereas those of WD-F2 are indicated in blue.

p.K1010E is located in the cytoplasmic region of the polypeptide. These two loci belong to a P-ATPase domain. These two mutations are predicted to affect protein function, thereby leading to WD. WD-F2 was heterozygous for c.2621C>T (p.A874V) and c.3992T>G (p.Y1331X) in exons 11 and 19, respectively. The mutation p.A874V is located in P-ATPase domain, possibly influencing enzyme activity. The mutation p.Y1331X is located in the seventh helical domain of the transmembrane region, and is predicted to result in protein truncation. These findings suggest that these two mutations were involved in the pathogenesis of WD in patient of WD-F2.

The onset of WD could be highly variable. We analyzed two 8-year-old patients and identified three mutations that were located in the P-ATPase domain (region spanning amino acids 769 to 1016), which is the most important region of the ATP7B protein. Therefore, it is essential to determine whether the onset of WD is relevant to the P-ATPase domain. Merle et al[14] demonstrated that patients with two severe mutations such as frameshift/nonsense/splice site mutations manifested their disease at an early age (9-13 years), whereas patients with two missense mutations showed a later onset (14-27 years). The most severe mutations involved the P-ATPase domain, which included p.Y741X, p.W779X, p.L722-fs, p.P767-fs, c.778dupC, p.K844K-fs, c.930-935del and c.1708-1G>A. However, other pathogenic mutations outside the P-ATPase region have also been previously reported, which include p.V1217-L1218del, p.I1330I-fs, c.3402delC and c.3843insT.[14] In a previous report, 27 of 46 late-onset cases (>40 years) showed homozygous mutations for p.H1069Q.[2] Patients with variation in age of on set also have the same homozygous p.H1069Q mutation.[7] This locus is not located in the P-ATPase domain. On the other hand, patients who are homozygous for mutation p.R778L, which is located in the P-ATPases domain, have late-onset WD (15 or 18 years). Therefore, one hypothesis is that the mutation locus might be not be associated with the age of onset of WD, and that other factors may modify this specific disease parameter.

The spectrum of mutations for WD is highly heterogeneous. Furthermore, various mutations in the *ATP7B* gene have been reported in different countries and regions. The prevalent mutations in Chinese populations are p.R778L and p.P992L,[15] whereas the mutation p.R778L is common in Asian populations such as Korean (37%)[16] and Japanese (13.4%).[17] The p.H1069Q mutation is the prevalent mutation in German (63%),[18] whereas the M645R mutation is particularly prevalent in Spanish patients (27%).[19]

ATP7B mutation sites are also very complex. According to HGMD, various types of mutations may occur at one locus such as p.S693P, p.S693Y and p.S693C; p.G710S, p.G710R, p.G710A and p.G710V. The mutations p.K1010T and p.K1010R have been previously identified.[20,21] In the present, we identified a novel mutation, p.K1010E, and the involved mutation site was similar to that described in previous reports, although of a distinct mutation type. There are too many different mutations and most patients are compound heterozygotes mutations. So, it is difficult to establish the relationship between the genotype and phenotype. WD is a lethal disorder when not appropriately treated; therefore, mutation analysis is an important diagnostic approach for this disease. Because of its wide mutation spectrum and complex mutation types, we should analyze the entire *ATP7B* gene of a WD patient instead of screening for hot mutations only.

In conclusion, gene testing is palying an important role in diagnosis of WD. We have identified four mutations in two Han Chinese family trios, of which two were novel, namely, c.3028A>G (p.K1010E) and c.3992T>G (p.Y1331X). The early onset of WD is apparently not associated with P-ATPase domain in the ATP7B protein. Other factors may modify the onset of WD.

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Ethical approval: The Ethics Committee of The Second Hospital of Lanzhou University approved the present study (A2015-045). Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor of this journal.

Competing interest: No benefits in any form have been received or will be received from any commercial party related directly or indirectly to the subject of this article.

Contributors: Zhu HW, Jin QY, Zhao LT, Zhu JR, Yan J, Yu TY and Ding JX contributed to conception and design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. Tao ZB provided samples of Wilson's disease in family 2 and patient's clinical information, and supplied control individual samples. Su G provided samples of Wilson's disease in family 1 and patient's clinical information, and analyzed and interpreted the data. Li YM contributed to critical revision of the manuscript for important intellectual content, and approved the final version of the manuscript.

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