Brief communication (Original)

Mutations of *ATP7B* gene in two Thai siblings with Wilson disease

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Background: Wilson disease (WD) is an autosomal recessive disorder of copper metabolism caused by mutations in *ATP7B* gene.

Objective: Report the clinical data and mutation analysis of two Thai siblings suspected of WD.

Subject and methods: A 13-year-old boy who presented with cirrhosis, arthralgia, hypoalbuminemia, and coagulopathy, and his 11-year-old sister who was asymptomatic but had hepatomegaly with elevation of transaminases, were studied. Mutation analysis of *ATP7B* gene and mRNA analysis was performed in both patients and their parents.

Results: Investigations were consistent with WD, and their liver diseases improved after standard treatment for WD. DNA analyses in these two patients revealed two novel mutations, which were a deletion of the first 2bp of exon 6 (c.1870_1871delGA), and a single base substitution from A to G at nucleotide 4075 (c.4075A>G) in the exon 20 (p.M1359V). PCR-restriction digestion with *NcoI* restriction enzyme was employed as the second method for confirmation of the c.4075A>G mutation and for rapid screening in 100 chromosomes from unrelated healthy controls, and this variant was not present in the controls. The c.1870_1871delGA deletion caused a frameshift effect, which results in a premature stop codon (p.E624fsX753), and the p.M1359V mutation is a substitution of methionine with valine, which may have effects upon its orientation and interaction with other adjacent amino acids.

Conclusion: Two novel mutations of *ATP7B* gene were identified in two Thai siblings with WD.

Keywords: *ATP7B* gene, cirrhosis, liver disease, Wilson disease

Wilson disease (WD) is a rare autosomal recessive disorder of copper metabolism with an average prevalence of approximately 30 affected individuals per million population [1]. Clinical presentations include liver diseases, progressive neurologic disorders, and psychiatric illness [2]. Most children who suffer from WD usually present with liver manifestations [3]. The spectrum of hepatic involvement varies from mild degree of acute hepatitis to decompensated cirrhosis or acute liver failure [3]. Some children are asymptomatic with hepatomegaly and elevation of transaminases found incidentally.

WD is caused by mutation of *ATP7B* gene on chromosome 13q14.3, which encodes a 60 kDa copper-transporting P-type ATPase [4]. The *ATP7B* protein is an important transporter of copper out of the cells. It has six copper binding domains and eight transmembrane domains. Its mutation causes decreased biliary excretion of copper and defective hepatocyte incorporation of copper into ceruloplasmin, resulting in an accumulation of copper in the liver, brain, and cornea [5]. The *ATP7B* gene is composed of 21 exons with coding region of 4.3 Kb. The majority of *ATP7B* mutations are located in the transmembrane regions of the protein. These mutations include insertion, deletion, splice site, and point mutations. There are at least 300 distinct mutations identified [6]. Herein, we report two Thai siblings with WD and novel mutations of *ATP7B* gene.

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Case report

A 13-year-old boy presented with generalized edema, ascites, arthralgia, and anemia. Before being referred to our hospital, he was previously diagnosed with possible systemic lupus erythematosus and nephritis. On physical examination, he had mild pallor without jaundice, puffy eyelids, edema of both legs, and ascites without hepatosplenomegaly,

Initial laboratory tests revealed Hb 8.9 g/dL, Hct 25%, red blood cells were normochromic and normocytic with anisocytosis, poikilocytosis and few polychromasia, white blood cells 10,600/mm³, platelets 112,000/mm³, albumin 15.2 (43-53) g/L, total protein 56.3 (64-82) g/L. Liver function tests are shown in **Table 1**. Coagulogram revealed partial thromboplastin time (PTT) 40.8 (24-34) sec, prothrombin time (PT) 29.3 (10-13) sec, INR 2.48, thrombin time (TT) 12.9 (10-13.5) seconds. Anti-HBs was positive, and anti-HCV was negative. Urinalysis revealed no proteinuria, no white blood cells, and red blood cells. WD was suspected due to the evidence of hemolysis and decreased hepatic synthetic functions. Further evaluations revealed serum ceruloplasmin of 8.24 (20-45) mg/dL, 24-hour urine copper 180 (below 100)

µg, and positive Kayser-Fleischer (KF) rings. Abdominal ultrasonography showed coarse parenchymal echogenicity and nodular surface of the liver associated with hypertrophy of lateral segment of left lobe, suggesting liver cirrhosis, with moderate amount of ascites. Percutaneous liver biopsy was performed and histology showed features of cirrhosis and active regeneration. Orcein stain showed copper deposit in the hepatocytes. However, the quantitative study of hepatic copper was unavailable in our center.

Family history revealed that both parents were clinically healthy and non-consanguineous couple from Northeastern Thailand. The patient had an 11-year old asymptomatic sister who was subsequently screened for WD. She had hepatomegaly without jaundice and absence of KF rings. Her liver function tests were abnormal (**Table 1**). Serum ceruloplasmin was 8.21 mg/dL and 24-hour urine copper was 109.2 µg. After the diagnosis of WD, both patients were started on D-penicillamine, zinc, and vitamin B6. After treatment, hepatic synthetic functions of the patient became normal at six months and liver biochemistries of the patient and his sister were normal at 12 months and four months, respectively (see **Table 1**).

Table 1. Laboratory parameters of the two siblings before and after treatment.

Parameters (normal values)	Patient						Sister					
	Pre Rx	1 m	2 m	4 m	6 m	12 m	Pre Rx	1 m	2 mo	4 m	6 m	12 m
Hb (12-16 g/dL)	8.9	10.6	10.2	11.5	12.3	14.0	12.4	12.0	11.4	11.9	12.2	12.2
Hct (36-48%)	25.2	31.8	31.1	32.8	37.4	42.1	36.6	37.1	35.3	36.5	37.2	37.0
AP (50-360 U/L)	328	1091	696	573	617	490	366	376	316	354	340	331
AST (15-37 U/L)	72	78	74	56	55	34	229	64	41	39	31	32
ALT (30-65 U/L)	57	75	68	64	71	51	396	179	77	57	48	44
GGT (15-85 U/L)	84	69	54	75	83	40	118	82	53	43	32	26
Total protein (64-82 g/L)	56.3	71.8	65.6	69.3	85.2	81.4	77.3	78.5	80.5	76.8	84.9	83.1
Albumin (43-53 g/L)	15.2	25.2	27.9	33.9	44.0	43.1	42.6	44.4	42.2	43.2	46	47.5
Total bi (0.2-1.2 mg/dL)	1.5	1.3	1.3	0.7	0.8	0.9	0.4	0.7	0.4	0.4	0.4	0.5
Direct bi (0.1-0.5 mg/dL)	0.7	0.5	0.4	0.3	0.2	0.2	0.1	0.3	0.2	0.2	0.2	0.2

Rx: treatment, m: month, Hb: hemoglobin, Hct: hematocrit, AP: alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma glutamyl transferase, bi: bilirubin.

Genetic analysis

Genetic analysis was performed after obtaining written informed consent, following Institutional Review Board approval. Isolation of genomic DNA and PCR was performed following established protocols. All coding exons 1-21 and part of 5' UTR of the *ATP7B* gene were amplified and sequenced with intron-flanking primers. Program PRIMER 3 was used for primer design (http://www.Frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; Primer sequences are available as per request). Protocols for mRNA isolation, first stranded cDNA synthesis, and RT-PCR were as described elsewhere [7]. GenBank reference sequences were NT_024524, NM_000053.2, and NP_000044.

DNA analyses in the patients and their parents revealed two different mutations that were a paternally inherited deletion of the first 2bp of exon 6 (c.1870_1871delGA) (**Fig. 1**), and a maternally

inherited single base substitution from A to G at nucleotide 4075 (c.4075A>G) in exon 20 (p.M1359V). For the c.4075A>G variant, PCR-restriction digestion with *NcoI* restriction enzyme was employed as the second method for confirmation of the mutation and for rapid screening in 100 chromosomes from unrelated healthy controls. This variant was not present in the control individuals, suggesting highly probable pathogenic alleles (**Fig. 2A**).

In addition, an insertion of 5bp, CGCCG, in 5' UTR, five non-synonymous polymorphisms, and two intronic single nucleotide polymorphisms (SNPs) were identified (**Table 2**). PCR and size fractionation by using primer pair ATP7B_E1 and ATP7B_E1R-s was employed to screen the CGCCG insertion in 53 controls, and revealed 15 homozygous and 31 heterozygous individuals with insertion allele (see **Fig. 2B**).

Fig 1. cDNA analysis for 1870_1871delGA (p.E624fsX753) mutation. Sequencing of PCR fragments derived from using exonic primers in exons 4 and 7. **A)** Normal sequence from control individual. **B)** Patient's cDNA sequence revealing GA deletion without missplicing, leading to frameshift mutation at codon 624.

Fig. 2 Screening for p.M1359V mutation and -117ins CGCCG allele in controls. **A)** PCR-*Nco*I restriction digest for p.M1359V. The wild type (M) allele was cut with *Nco*I, yielding 194 and 171bp alleles, whereas the mutant (V) allele abolished the restriction site, leaving only uncut product 365bp allele. Numbers represents individuals as follows. 923: patient, 929: sister, 930: father, 931: mother, N39: normal control. **B)** PCR-size fractionation of 5bp insertion polymorphism (-117ins CGCCG). PCR with primer pair ATP7B_E1 and ATP7B_E1R-s normally yielded 155bp fragments verified on 2.5% agarose gel electrophoresis. The -117ins CGCCG allele (upper band 160bp) was separated clearly from the non-insertion allele (lower band 155bp) as shown in individuals N34, N36, N42, which were confirmed by direct sequencing. Homozygous -117ins CGCCG (N35, N43, and N48), and the homozygous non-insertion band (N47) show little shift compared to the lower bands seen in heterozygous individuals (confirmed by sequencing).

Discussion

The patient in this report manifested with cirrhosis and impaired hepatic synthetic functions, which are common characteristics of WD. Arthralgia has been a known manifestation in WD, yet not a common feature [4]. Arthralgia was described in 7% in a series of 283 Japanese patients with WD [8], and 20% in the series of 21 Thai adult patients with WD [9].

Diagnosis of WD can be made readily if the patient has clinical triad of hepatic disease, neurologic involvement, and the presence of KF rings, the deposition of copper in Descemet's membrane of the cornea. Diagnosis of WD can also be established if the patient has KF ring and serum ceruloplasmin below 20 mg/dL and 24-hour urine copper over 100 μ g [2]. Therefore, the diagnosis of WD in this patient was undoubted. However, about half of the WD patients

with liver manifestations have no KF rings, making the diagnosis more difficult.

Liver histology is non-diagnostic for WD and findings are ranging from mild steatosis, acute hepatitis, chronic hepatitis, fibrosis, and cirrhosis [10]. The most reliable test for the diagnosis is hepatic copper concentration (≥ 250 μ g/g dry weight). Although this test is the most specific for WD, the availability of the test is limited. Therefore, it is recommended to be performed in cases where the diagnosis is not straightforward [2]. Finally, mutation analysis can be helpful if the results of investigations are inconclusive, for example, the level of liver copper of 50-250 mcg/g dry weight. However, genetic testing has some limitations for the diagnosis of WD due to a wide variety of mutations.

When WD is confirmed in the individual, the first-degree relatives must be screened for WD after the age of three or four years unless hepatomegaly or abnormal aminotransferase are found earlier [2]. Evaluation of the siblings includes history and physical examinations, liver function tests, complete blood count, serum ceruloplasmin, and 24-hour urine copper [2]. Haplotype analysis or specific testing of known mutation can be used for family screening [2]. In this report, investigations in an asymptomatic sister were consistent with WD.

To our knowledge, genetic studies of WD in Thai population have not been described. The c.1870_1871delGA deletion is clearly predicted to affect the function of ATP7B due to its frameshift effect, which results in a premature stop codon at position 752, eliminating N-terminal half of the protein (full length peptide 1456 residues) starting from

the 3rd transmembrane domain (TM3) [6]. The p.M1359V mutation is believed pathogenic because of its cosegregation with the disease and its absence in the controls. The p.M1359V mutation residing in the TM8 of the ATP7B peptide is a substitution of a neutral, very hydrophobic, polar residue (methionine) with a hydrophobic, aliphatic, non-polar residue (valine), which may have effects upon its orientation and interaction with other adjacent amino acids. To our knowledge, both mutations are novel. However, a mutation at codon 1359, p.M1359I has been reported once in a British patient [11]. The methionine residue at this position is highly conserved among ATP7B peptides across various species: mouse (NM_007511.2), rat (NM_012511.2), cow (XM_596258.3), horse (XM_001488450.1), pig (XM_00192535.1), dog (NM_001025267.1), and chick (XM_417073.2) (GenBank: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 2. Genetic variations identified in this study.

Exon/IVS	Nucleotide change	Peptide change	Pathogenicity	Reference	Comment
5'UTR	-117insCGCCG	none	Benign	5bp insertion based on GenBank Sequence, but normal sequence based on Cullen et al. [12]; Nanji et al. [14]	patient, sister: hom; both parents: het
2	c.1216T>G	p.S406A	SNP rs# 1801243	Wu et al. [18]; GenBank	patient: hom; father: het
3	c.1366G>C	p.V456L	SNP rs# 1801244	Wu et al. [18]; GenBank	patient: hom; father: het
6	c.1870_1871delGA	p.E624fsX753	Pathogenic	Present study	patient, sister, father: het; mother, 50 controls: wild type
10	c.2495A>G	p.K832R	SNP rs# 1061472	Wu et al. [18]; Liu et al. [19]; GenBank	patient: hom; father: het
12	c.2855G>A	p.R952K	SNP rs# 732774	Wu et al. [18]; GenBank	patient, sister: hom; both parents, a normal control: het
16	c.3419T>C	p.V1140A	SNP rs# 1801249	Wu et al. [18]; GenBank	patient: hom; father: het
IVS18	c.3903+6c>t (or IVS18+6c>t)	none	Benign	Davies et al. [11]	patient, sister, both parents: hom
IVS19	c.4021 (or IVS19+50g>c)	none	Benign	Davies et al. [11]	patient, sister: hom; both parents: het
20	c.4075A>G	p.M1359V	Pathogenic	Present study	patient, sister, mother: het; father, 50 controls: wild type

Hom: homozygous, het: heterozygous, IVS: intervening sequence.

Mutation analysis of the 5' UTR (nucleotide -1 to -335, where +1 is the translational initiation site) and the promoter region of *ATP7B* gene have not been commonly characterized. However, of those available data, it was found that mutations in the regulatory region of *ATP7B* were infrequent in Caucasian populations, except that a 15bp deletion between nucleotides -424 and -441 in the promoter region being the most common mutation in Sardinian WD patients [12,13]. Among 82 unrelated WD patients of white European ancestry, a 5bp deletion (-132delAGCCG) in 5' UTR was identified as a common polymorphism, both in patients and controls with allele frequency of 0.31 and 0.42, respectively. This polymorphism has not been described in Asian populations, and was not found in the present cases. Based on GenBank sequence database, we identified a 5bp insertion (-117insCGCCG) in the patients and controls with allele frequency of 0.58 in the controls. This insertion is described as normal sequence reported by others [12, 14]. In fact, this could represent common polymorphism in some populations including Thai and Japanese populations [14]. It remains to be studied whether or not this 5' UTR polymorphism has effect on the expression of *ATP7B* gene and its protein. In addition, the relatively common mutation (R5778) in East Asian populations [15] was not identified in our cases.

All WD patients require lifelong pharmacological therapy. Initial treatment with chelating agents, D-penicillamine, or trientine, is the first choice for symptomatic patients [2]. Zinc, which interferes with copper uptake in gastrointestinal tract [16], can be used in addition to chelating agents in severe cases or used as monotherapy for pre-symptomatic patients and for maintenance therapy in the long-term [2, 17]. Liver transplantation is indicated for those present with acute liver failure and decompensated cirrhosis unresponsive to medical therapy. Both patients in the report had marked improvement in liver functions soon after treatment.

Conclusion

Two novel mutations of *ATP7B* gene were identified in two Thai siblings with WD. WD should be suspected in children who present with unexplained liver diseases.

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