RESEARCH ARTICLE

Genetic analysis of 55 Northern Vietnamese patients with Wilson disease: 7 novel mutations in *ATP7B*

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Running title: *ATP7B* gene mutations in Vietnamese WD patients

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Abstract

Wilson disease(WD) is an autosomal recessive disorder of copper metabolism. The gene responsible for WDwas discovered in 1993 and is located on chromosome 13 at13q14.3. It encodes a copper specific transporting P-type ATPase. Early diagnosis can ameliorate treatment outcome and decrease the rate of disability or even mortality.We used Sanger sequencing to identifymutation hotspots in 55 Northern Vietnamese with a clinical diagnosis of WD. Mutations were screened and detected by direct DNA sequencing. A total of 26 different *ATP7B*gene mutations were identified, including seven novel mutations (five nonsenseand twomissense mutations). The most frequent mutations werep.Ser105Ter (24.55%), p.Arg778Leu (5.45%), p.Thr850Ile (4.55%).Mutation detection rate in exon 2 was 34.55% and ranked first, followed by exon 8 with 16.36%,and exon 18 with 10.91% each, thus exons2, 8and 18 are the mutation hotspotsfor Northern Vietnamese WD patients.These findings were different from previous studies in Asia. Our research established a suitable strategy for *ATP7B* gene testing in Northern Vietnamese WD patients.

Keywords: *ATP7B*, Wilson disease, mutation hotspot,pSer105Ter, Vietnam

Introduction

Progressive hepatolenticular degeneration also known as Wilson disease (WD; OMIM #277900) was first defined as a syndrome in 1912. It is a rare autosomal recessive genetic disorder of copper metabolism in which excessive amounts accumulate in the body, particularly in the liver, brain, and eyes (Kayser-Fleischer ring in the cornea).Biochemical indicators for the disease include low serum concentrations of ceruloplasmin (<20 g/l) and elevated excretion of urinary copper (>100 µg 24-hour urinary copper) (Sternlieb *et al.* 1990).Early-onset presentations in infancy and late disease onset manifestations in adults older than 70 years of age are now well recognized(Figus *et al.* 1995; Vajro *et al.* 2013).

WD is caused by mutations in the *ATP7B*gene discovered in 1993 that encodesa copper specific transporting P-type ATPaseand the gene islocated on chromosome13 at 13q14 (Bull *et al.* 1993; Tanzi *et al.* 1993).

The widely cited prevalence figure of 1 in 30000 for WD with a heterozygous carrier frequency of 1 in 90 was estimated in 1984 and thus predates the identification of *ATP7B* as the causative gene. This prevalence estimate was at least partly based on assumptions, and has been questioned (Bull *et al.* 1993).More recent data frompopulation screeningof WD in the UKsuggested a potentiallyhigher rate of *ATP7B*heterozygote mutation carriers, predicting 1 in 7021 prevalence in the UK population (Coffey *et al.* 2013).Results from biochemicaland genetic prevalence studies suggest that WD might be much more common than previously estimated and may vary by population(Terada *et al.* 1998; Duc *et al.* 1998).Early diagnosis can ameliorate treatment outcome and decrease the rate of disability and mortality (Bull *et al.* 1993; Tanzi *et al.* 1993).

The *ATP7B* gene consists of 21 exons and 20 introns. It is approximately 7.5kb in size and it encodes a 1465 amino acid protein that consists of six copper binding site (exon 2-5), eight transmembrane domains of copper channel (exon 6-8, 12-13, 19-20) and the ATP-binding domain (exon10-11, 14-18). The copper transportation is provided by converting energy of ATP hydrolysis in the ATP-binding domain (Terada *et al.* 1998).

Up to date, more than 500 mutations in the *ATP7B*gene have been identified,as detailed in the database of the University of Alberta, Canada(www.wilsondisease.med.ualberta.ca/database.asp). Mutations in *ATP7B* are scattered in the whole gene, but some hotspots have been reported varying in different populations. The mutation hotspotsin Europeans and North Americanswereidentified in exon 14, p.His1069Gln (Duc *et al.* 1998; Kucinskas *et al.* 2008; Riordan *et al.* 2001; Thomas *et al.* 1995).In some Asian countries, such as China, Korea, and Taiwan the hotspot lies in exon 8 with the p.Arg778Leumutation (Diao *et al.* 2014; Li *et al.* 2013; Liu *et al.* 2004; Wei *et al.* 2014; Yoo *et al.* 2002; Wan et al. 2006).Identification of mutation hotspotsmay reduce the timeof genetic testprocessing significantly. However, in Vietnam, nosuch studyhas previously been conducted. In this paper, we used Sanger sequencing to identify the mutation spectrum in Northern Vietnamese WD patients to investigate whether any mutation hotspotexist that would facilitate diagnosis confirmation genetically.

Materials and methods

Patients

55 WD patients from unrelated families in Northern Vietnam were enrolled in this study. All WD patients were diagnosed and treated at the National Pediatrics Hospitalin Hanoi from

2010 to 2015. Diagnosis of WD was based on many clinical symptoms and signs, including acute or chronic liver failure and/or typical neurological symptoms, or the presence of Kayser-Fleischer ring, and biochemical parameters, such as low serum ceruloplasmin (<0.2g/l) and high level of urinary copper (>100 μg/24h) (Roberts et al., 2008).40healthy Northern Vietnamese individuals were enrolled as controls. Informed consent was obtained from all patient families for molecular analysis and this study was approved by the ethical committees of Hanoi Medical University (IRB00003121 Hanoi Med U IRB, Hanoi Vietnam).

DNA extraction

Genomic DNA samples were extracted from peripheral blood collected in EDTA-coated tubes using Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin, USA) following the manufacturer's recommendations.

PCR

The full length gene was amplified by using primer pairs for exons 1-21 (IDT, Coralville, Iowa, USA). PCR was performed using GoTaq Green Master Mix (Promega, Madison, Wisconsin, USA) with 100ng of genomic DNA in a mix containing 10pmol of each primer, 12.5μl of 2x GoTaq Green Master Mix in a total volume of 25μl. The thermo cycle program consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The size and quantity of PCR products were verified by electrophoresis in 2%(w/v) agarose gel.

DNA sequencing

PCR products were directly sequenced using an Advant 3100 automated sequencer (Applied Biosystems Inc., Foster City, California, USA). Sequences were aligned and inspected using a reference sequence from GenBank (NM_0000.53). DNA sequencing was used to detect variations of the entire coding region of *ATP7B* gene from the 55patientsand 40 healthy controls.

Results

We investigated 55 patients with WD and detected 26 different *ATP7B* gene mutations, including 17 missense, six nonsense mutations, two frameshift deletions and oneframeshift insertion. Of these, seven mutations have not been previously reported (novel mutations) that included two missense(p.Asp1027His and p.Asn1270Asp) and five nonsense mutations(p.Glu45Ter, p.Met119Ter, p.Lys867Ter, p.Glu905Terand p.Leu1159Ter)(table 1). All of novelmissense mutations weretested for the possibilities being pathogenic in nature using Alamut Visual version 2.7 (Interactive Biosoftware, Rouen, France) and were not found in 80 alleles of 40 healthy controls.Additionally, five novel variants were detected (p.Glu583Gln, p.Pro1098Gln, p.Gly1099Asp and p.Gly1213Asp, p.Cys980Ser) where in silico analysis was either predicted to be tolerated, partly based on the conservation of amino acid residues or in one case (p.Cys980Ser) predicted to be benign by PolyPhen-2 (table 2). The result of sequencing and distribution of the novel mutations and variants were shown in figure 1. Four different mutations were identified on exon 2,8 or 18, three on exon 14 or 16, two on exon5 or 11 and one on exon10, 12, 13 or 20;whereas, no mutationwas found on exon 1, 3, 4, 6, 7, 9,15, 17, 19, 21 and in the promoter region.In exon 2, four mutations were found in 19 patients (one patient withp.Glu45Ter and p.Ser105Ter), with a detection rate of 34.55% (19/55) and ranked first;two other most frequent exon mutations,exon 8 detected in 9 patients, exon 18 detected in 6 patients,accounted for16.36% (9/55) and10.91% (6/55),respectivelly. In all, p.Ser105Ter is the

most frequent mutation in this study. It was detected in 18 cases with nine homozygous, eight compound heterozygous and one single heterozygous, at detection rate of 32.73% (18/55) and allele frequency of 24.55% (27/110).p.Arg778Leu, the common mutation in Chinese population stayed in exon 8 (Li *et al.* 2013; Wei *et al.* 2014), was found infour compound and two single heterozygous cases, accounted for 10.9% (6/55) of cases and 5.45% (6/110) of studied alleles, became the second most frequent mutation. Following, p.Thr850Ile, reveled infourcompound and one singleheterozygous cases, contributed for 9.1% of cases and 4.55% of studied alleles.Interestingly, we found a novel mutationp.Glu905Ter on exon 11, which were prevalent inVietnamese population. Homozygous mutation, p.Glu905Ter, was identified in two patients accounted for cases and allele frequency of 3.64%. We found that, exon 2, 8, and 18 were thus recognized as hotspots for WD mutation detection in this study.The total mutation detection rate on these three exons was 52.73% (29/55). The most frequent mutations are p.Ser105Ter (24.55%), p.Arg778Leu (5.45%)and p.Thr850Ile (4.55%).

In addition to the mutations, six single nucleotide polymorphisms (SNPs) were identified and details of them were shown in table 3. These base substitutions were defined as polymorphisms because they predicted as polymorphisms by Alamut Visual version 2.7 (Interactive Biosoftware, Rouen, France), or existed in healthy controls and were demonstrated previously (Gupta *et al.* 2007; Gu *et al.* 2003; Haas *et al.* 1999; Wan *et al.* 2006).

Discussion

Copper is anessentialscomponent of many enzymes such as lysyl oxidase, superoxide dismutase, dopamine-β-hydroxylase and cytochrome C oxidase. These copper-dependent enzymes are needed for diverse process of oxidase metabolism including respiration, free-radical

detoxification, neurotransmitter synthesis, maturation of connective tissue and iron uptake (Linder *et al.* 1996; Yuan *et al.* 1995). However, copper is only required in trace amount;accumulation of copper can damage plasma membranes, peroxisomes, mitochondria, microtubules, enzymes and even DNA (Duc *et al.* 1998).Typical presentations of WD include neuropsychiatric and hepatic dysfunction, whereas a typical presentation isextremely variable. Diagnosis relies typically on a high clinical suspicion, typical neurological symptoms, presence of Kayser-Fleischer rings, and reduced serum ceruloplasmin concentration. The conventional value of <0.20 g/l is not a universal diagnostic value. Age of the subjects and analytical variations should be considered when interpreting these levels. Patients with inconclusive findings require further investigations including 24 hours urinary free-copper excretion, penicillamine challenge test, livercopper measurement, and more recently detection of gene mutations. Direct molecular diagnosis remains the most decisive test.

Early diagnosis and treatment of WD are associated with better outcome. *ATP7B* gene testing has been proved as a suitable method for prenatal diagnosis and neonatal screening (Roberts *et al.* 2008).At present, more than 500 mutations in the*ATP7B* gene have been listed in the WD mutation database. In this study, we identified26 different mutations including seven novel mutations in 55 WD patients from Northern Vietnam. Mutations exist as compound heterozygous,homozygous and single heterozygousforms. The mutation detection rate of exon 2 was 34.55% and ranked first, followed by exon 8 with 16.36% and exons 18 with 10.91%, we recognized exon 2, 8, and 18, which can cover 52.73% of mutations as the hotspots for Northern Vietnamese WD patients.Our result was different from previous studies in Asian populations. Most mutations located on exons 8, 12, 13 and 16 in Northern Chinese covering 60.5-74% (Liu *et al.* 2004; Wu *et al.* 2001); on exons 8, 11 and 18 in Korean covering 59.8-71.4% (Park *et al.*

2007; Yoo *et al.* 2002); andon exons 5, 8, 12, 13 and 18 in Japanese with coverage 59.8-71.4% (Okada *et al.* 2000; Shimizu *et al.* 1999)mutations. Thus, these three exons 2, 8 and 18 should be screened first in our upcoming *ATP7B* genetic testing. We could not find any trace of mutations in exon 1, 3, 4, 6, 7, 9, 15, 17, 19, 21 and promoter region in this series. Maybe in these regions, there are large deletion and duplication mutations that are not detectable by sequencing, stayed as heterozygous, which should be examined and detected by other methodsuch as MLPA, particularly when a single heterozygous mutation has been detected at sequencing. This is a limitation of our study, which had limited funding. Our study provides for the first time, the mutation spectrum in WD in a Vietnamese population.

The p.Ser105Ter mutation on exon 2 was most common in our cohort, accounting for24.55% of diagnosed alleles. The second most frequent mutationswerep.Arg778Leuon exon 8 with 5.45%,following the mutations p.Thr850Ile on exon 10 accounted for4.55% of studied alleles. These findings were different from the results of previous studies, in other continents, as well as in related regions in Asia. The p.His1069Gln is the most common mutation in European and North American populations (Duc *et al.* 1998; Kucinskas *et al.* 2008; Riordan *et al.* 2001; Thomas *et al.* 1995), and the common mutation in Indian population, the p.Cys271Ter (Aggarwal *et al.* 2013; Mukherjee *et al.* 2014), but we could not detect any case in our patients. p.Arg778Leu was recognized as the most frequent mutation in Chinese (Diao *et al.* 2014; Li *et al.* 2013; Liu *et al.* 2004; Wei *et al.* 2014), Korean (Park *et al.* 2007; Yoo *et al.* 2002) and Taiwanese (Wan *et al.* 2006)populations. This mutation was the second ranked in our cohort, but the allele frequency (5.45%)is much lower thanthose of other previous studies in Asia(Liu *et al.*: 74%; Li*et al.*: 21.5%; Yoo*et al.*:37.9 %). Also, other relatively common mutations in these studies, such as p.Pro992Leu, p.Thr935Met or p.Ala874Val,were not found in our study. On the

other hand, the most common mutation in our cohort, p.Ser105Ter, was found only in a few cases in the Chinese population (Liu *et al.* 2004; Mak *et al.* 2008). Whereas, we detected novel mutationwhich was prevalent in this study as p.Glu905Ter (3.64%).In addition, five novel variants were detected in our study where in silico analysis was either predicted to be tolerated, partly based on the conservation of amino acid residues or in one case (p.Cys980Ser) predicted to be benign by PolyPhen-2. Although none of these novel variants were present in the 1000 Genomes nor the ExAC databases. These variants can at best be presently considered as possibly pathogenic and might be relatively common in Vietnamese population but this remains to be proven in the future.

In summary, we have revealedthe mutation spectrum of the *ATP7B* gene in Northern Vietnamese WD patients with sevennovel mutations identified.Ourstudy provided an additional data for understanding mutation patterns in the *ATP7B* gene worldwide. Direct sequencing has proved a sensitive, specific, and relatively low invasive method and it is increasingly used for *ATP7B* gene testing for early diagnosis confirmation and prenatal diagnosis of WD. Similar analysis in Vietnamese patients with WD from other regions of Vietnam is warranted to provide a better assessment of the mutation spectrum of this disorder in Vietnam. We recommend screening exons 2, 8 and 18, which can cover 52.73% of mutations. This finding is expected to reduce time and costs of mutation screening significantly.

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Table 1. Distribution and frequency of mutationsdetected in *ATP7B* gene

Nucleotide change	Amino acid change	Homo-zygous	Compound hetero-zygous	Single hetero-zygous	Exon	Allele frequency (%)
*c.132G>T	p.Glu45Ter	0	1	0	2	0.91
c.314C>A	p.Ser105Ter	9	8	1	2	24.55
*c.354-356 ATG>TAA	p.Met119Ter	0	1	0	2	0.91
c.525dupA	p.Val176Ser-fsX28	0	1	0	2	0.91
c.1771G>A	p.Gly591Ser	0	1	0	5	0.91
c.1810G>C	p.Ala604Pro	0	1	0	5	0.91
c.2160delA	p.Lys720AsnfsX3	0	1	0	8	0.91
c.2297C>T	p.Thr766Met	0	0	1	8	0.91
c.2305A>G	p.Met769Val	0	1	0	8	0.91
c.2333G>T	p.Arg778Leu	0	4	2	8	5.45
c.2549C>T	p.Thr850Ile	0	4	1	10	4.55
*c.2599A>T	p.Lys867Ter	0	0	1	11	0.91
*c.2712_2713insT	p.Glu905Ter	2	0	0	11	3.64
c.2828G>A	p.Gly943Asp	0	1	0	12	0.91
c.2954G>A	p.Cys985Tyr	0	0	1	13	0.91
*c.3079G>C	p.Asp1027His	0	1	0	14	0.91
c.3098C>T	p.Thr1033Ile	0	2	0	14	1.82
c.3155C>T	p.Pro1052Leu	0	1	0	14	0.91
c.3443T>C	p.Ile1148Thr	0	1	0	16	0.91
*c.3476T>G	p.Leu1159Ter	0	2	0	16	1.82
c.3526G>A	p.Gly1176Arg	0	1	0	16	0.91
c.3794_3803del	p.Val1265Gly-fsX62	0	1	0	18	0.91
*c.3808A>G	p.Asn1270Asp	0	1	0	18	0.91

c.3818C>A	p.Pro1273Gln	0	2	1	18	2.73
c.3841G>T	p.Gly1281Cys	0	1	0	18	0.91
c.4112T>C	p.Leu1371Pro	0	2	0	20	1.82

(*) Asterisks were indicated for novel mutations.

Table 2. In silico analysis to determine if the novel variants identified in the study are mutations

Nucleotide change	**Amino acid change**	**Exon**	**Align-GVGD**	**SIFT**	**Mutation-Taster**	**PolyPhen-2**
c.1606G>A	p.Val536Ile	4	Class C0	Tolerated	Polymorphism	BENIGN with a score of 0.001
c.1606G>C	p.Val536Leu	4	Class C0	Tolerated	Polymorphism	BENIGN with a score of 0.001
c.1747G>C	p.Glu583Gln	5	Class C0	Tolerated	Disease causing	Probably damaging with a score of 0.996
c.2939G>C	p.Cys980Ser	13	Class C0	Tolerated	Disease causing	BENIGN with a score of 0.100
c.3079G>C	p.Asp1027His	14	Class C65	Deleterious	Disease causing	Probably damaging with a score of 0.999
c.3293C>A	p.Pro1098Gln	15	Class C0	Tolerated	Disease causing	Probably damaging with a score of 0.995
c.3296G>A	p.Gly1099Asp	15	Class C0	Tolerated	Disease causing	Probably damaging with a score of 0.998
c.3638G>A	p.Gly1213Asp	17	Class C0	Tolerated	Disease causing	Probably damaging with a score of 0.999
c.3808A>G	p.Asn1270Asp	18	Class C0	Deleterious	Disease causing	Probably damaging with a score

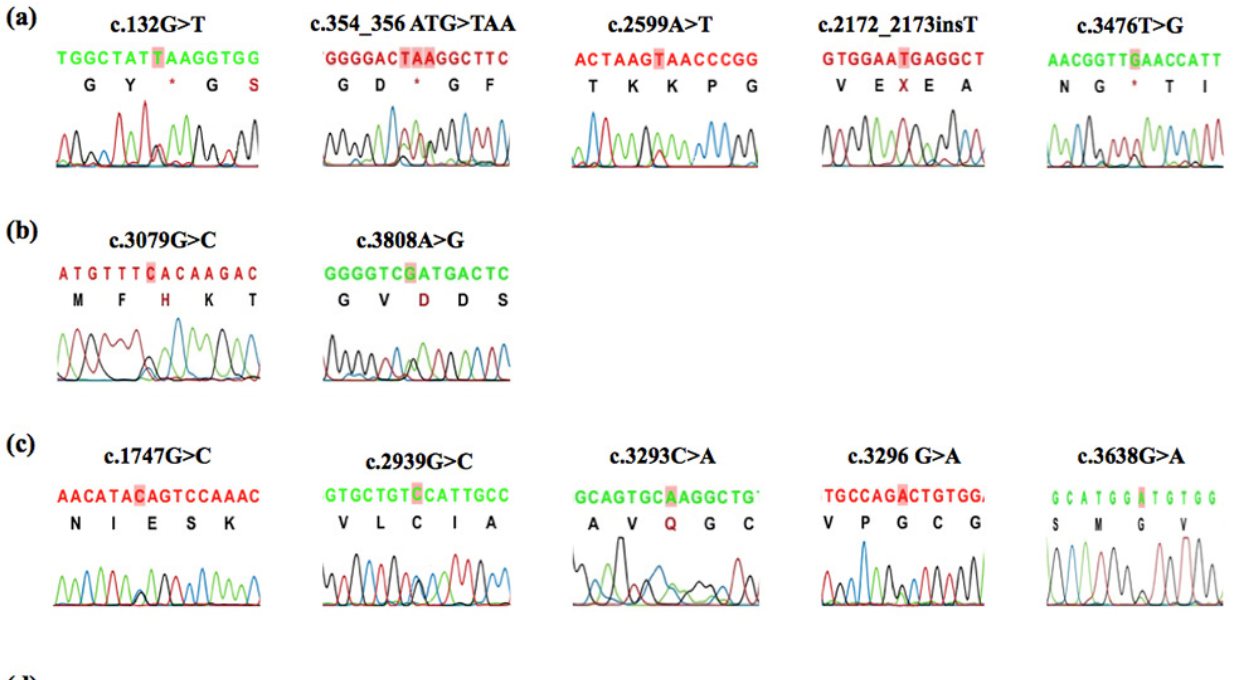
of 1.000

Table 3. Distribution and frequency of SNPs in *ATP7B* gene

Nucleotide change	Polymorphisms	Homozygous	Heterozygous	Exon	Allele frequency %
c.1366G>C	p.Val456Leu	6	16	3	25.45%
c.2495A>G	p.Lys832Arg	7	7	10	19.09%
c.2855G>A	p.Arg952Lys	2	1	12	4.55%
c.3419C>T	p.Ala1140Val	2	5	16	8.18%
c.1606G>A	p.Val536Ile	0	5	4	4.55%
c.1606G>C	p.Val536Leu	0	1	4	0.91%

Figure legend

Figure 1. The sequencing results and distribution of novel mutations and variants in *ATB7B* gene. DNA sequences were shown with highlight letters indicating for the substitution nucleotides of nonsense (a), missense mutations (b), and variants (c), respectively. Illustration for distribution of novel mutations and variants in *ATB7B* gene (d).


(a)
c.132G>T
TGGCTATTAAGGTGG
G Y * G S
c.354_356 ATG>TAA
GGGGACTAAGGCTTC
G D * G F
c.2599A>T
ACTAAGTAACCCGG
T K K P G
c.2172_2173insT
GTGGAATGAGGCT
V E X E A
c.3476T>G
AACGGTTGAACCATT
N G * T I
(b)
c.3079G>C
ATGTTTCACAAGAC
M F H K T
c.3808A>G
GGGGTCGATGACTC
G V D D S
(c)
c.1747G>C
AACATACAGTCCAAAC
N I E S K
c.2939G>C
GTGCTGTCCATTGCC
V L C I A
c.3293C>A
GCAGTGCAAGGCTG
A V Q G C
c.3296 G>A
TGCCAGACTGTGG
V P G C G
c.3638G>A
S M G V
(d)
1 2 3 4 5 6 7 8 9 10 11 12 15 16 17 18 19 20 21
▲ Nonsense mutation
● Missense mutation
■ Variant