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Mutation Analysis of the *ATP7B* Gene in 43 Vietnamese Wilson's Disease Patients

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***Abstract*—**Wilson's disease (WD) is an autosomal recessive disorder of the copper metabolism, which is caused by a mutation in the copper-transporting P-type ATPase (*ATP7B*). The mechanism of this disease is the failure of hepatic excretion of copper to bile, and leads to copper deposits in the liver and other organs. The *ATP7B* gene is located on the long arm of chromosome 13 (13q14.3). This study aimed to investigate the gene mutation in the Vietnamese patients with WD, and make a presymptomatic diagnosis for their familial members. Forty-three WD patients and their 65 siblings were identified as having *ATP7B* gene mutations. Genomic DNA was extracted from peripheral blood samples; 21 exons and exon-intron boundaries of the *ATP7B* gene were analyzed by direct sequencing. We recognized four mutations ([R723=; H724Tfs*34], V1042Cfs*79, D1027H, and IVS6+3A>G) in the sum of 20 detectable mutations, accounting for 87.2% of the total. Mutation S105* was determined to have a high rate (32.6%) in this study. The hotspot regions of *ATP7B* were found at exons 2, 16, and 8, and intron 14, in 39.6 %, 11.6 %, 9.3%, and 7 % of patients, respectively. Among nine homozygote/compound heterozygote siblings of the patients with WD, three individuals were determined as asymptomatic by screening mutations of the probands. They would begin treatment after diagnosis. In conclusion, 20 different mutations were detected in 43 WD patients. Of this number, four novel mutations were explored, including [R723=; H724Tfs*34], V1042Cfs*79, D1027H, and IVS6+3A>G. The mutation S105* is the most prevalent and has been considered as a biomarker that can be used in a rapid detection assay for diagnosis of WD patients. Exons 2, 8, and 16, and intron 14 should be screened initially for WD patients in Vietnam. Based on risk profile for WD, genetic testing for presymptomatic patients is also useful in diagnosis and treatment.

Keywords*—ATP7B* gene, mutation detection, presymptomatic diagnosis, Vietnamese Wilson's disease.

I. Introduction

WD is an inherited and treatable liver disorder. It is a rare autosomal recessive disorder of copper metabolism, which is characterized by hepatic and neurological disease [1], [3]. WD is estimated to affect approximately one in 30,000 individuals globally. It can present clinically as liver disease in its early stages (acute hepatitis, cirrhosis, fatty liver…), and later as a progressive neurological disorder (movement disorder, seizures, rigid dystonia…) or a psychiatric illness (depression, psychosis, neurosis…). The WD gene, *ATP7B*, codes for a copper transporting P-type ATPase, which is expressed mainly in hepatocytes and functions in the transmembrane transport of copper. Absent or reduced function of *ATP7B* protein leads to decrease hepatocellular excretion of copper into the bile [1], [5], [9].

The *ATP7B* gene, which consists of 21 exons, is approximately 100kb of genomic span. The 4.3kb open reading frame encodes a 1,465 amino acid protein with features characteristic of the P-type ATPase [4]. To date, there are >500 distinct mutations reported [8], [25]. The mutation spectrum of *ATP7B* gene is dissimilar between ethnic groups. Although missense mutations are the most frequent, deletions, insertions, nonsense, and splice site mutations all occur [1], [24]. The mutations are distributed across the *ATP7B* gene, so as a result, the distribution of *ATP7B* genotypes is complex and most patients are compound heterozygote, having two different mutations from each parent [2], [3]. Some mutations appear to be population-specific, while others are common among various populations [6], [17]. Mutation H1069Q, has been reported to be the most prevalent in Europe (30-40% of WD mutations), but R778L has been reported to have high allele frequency (14-49%) in East Asia [10], [24].

WD was one of the first liver diseases for which effective pharmacological treatment was identified [1], [2]. The diagnosis of WD is determined by clinical manifestations combined with laboratory findings. However, these tests may give false positive results or false negative results, and these can lead to severe complications or lost opportunities for treatment. Traditional methods cannot identify a carrier or presymptomatic patient; these are most often detected by family screening [8], [19]. Genetic analysis for *ATP7B* can be valuable to confirm a diagnosis of WD, especially when presentation is unusual [26]. This prevents the early-onset of WD [3], [12].

For the above-mentioned reasons, this research on *ATP7B* mutation in Vietnamese people will be meaningful in Vietnam for initial screening. This research can help design appropriate screening strategies, as well as identify common mutations, and save costs for patients in disease treatment and prevention, prenatal diagnosis, and living standard improvement.

The goal of this study is to investigate characteristics of *ATP7B* mutation in Vietnamese WD patients, and develop presymptomatic diagnosis for their family members.

II. Patient and Method

A. Patient

Peripheral blood was collected from 43 WD patients—19 females and 24 males, their ages ranging from three years to 26 years old. 65 siblings of patients were also performed in DNA analysis. Genomic DNA was extracted by Qiagen DNA blood mini kit (QIAamp DNA Blood Mini preparation kits, Germany). The diagnosis score of WD was based on all

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available tests was proposed by the Working Party at the 8th International Meeting on Wilson's disease, Leipzig 2001 [8].

B. Method

Twenty-one exons and their intron-flanking primers in the *ATP7B* gene were successfully polymerase chain reaction (PCR) amplified for 43 WD patients by using 24 primer pairs (four primer pairs for exon 2, one primer pair each for others). Their siblings were detected mutations of probands. The reaction mixture of a final volume of 25 µl contained 10X PCR buffer (Invitrogen, USA), 20 mM magnesium chloride, 10 µM dNTPs, 10 µM of each primer, 5U Taq DNA polymerase (Invitrogen, USA), and 50 ng DNA temple. PCR amplification was performed in ABI GeneAmp PCR system 9700. The size and quantity of PCR products were verified by electrophoresis in 1% agarose gel.

Direct sequencing with the both forward and reverse strands was performed with a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) after purification with a Bigdye X.terminator purification kit (Applied Biosystems, USA). The sequence analyses were carried out with an ABI PRISM 3130 Genetic Analyzer machine (Applied Biosystems, USA). Gene analysis used SeqScape 2.5 software, Chromas software, or equivalent software, comparing obtained *ATP7B* gene sequence with the standard sequence on Gene Bank NT_024524.

C. Ethics in Research

Informed consent was obtained from the child's parent or guardian prior to the collection of blood samples. This process included the preparation of an information sheet for the parents or guardian of the involved child, identifying any potential risks, as well as informing them that the sample is for research purposes only. A trained counselor from the Human Genetics Department discussed this information with the parent/guardian prior to signing of the consent form.

TABLE I
CLINICAL SYMPTOMS AND MUTATIONS IDENTIFIED IN 43 VIETNAMESE WD PATIENTS

Type	Mutation	Nucleotide change	Exon	Mutant allelic frequency (n) (%)
Nonsense	S105*	c.314C>A	2	28 (32,6)
Frameshift	V176Sfs*28	c.525dupA	2	6 (7)
	V1042Cfs*79	c.3124delG	14	1 (1,2)
	[R723=; H724Tfs*34]	c.[2169A>G; 2169_2181del]	8	1 (1.2)
Missense	M769Hfs*26	c.204dupC	8	2 (2,3)
	R778L	c.2333G>T	8	3 (3,5)
	R765G	c.2294A>G	8	2 (2,3)
	T850I	c.2549C>T	10	4 (4,7)
	P992L	c.2975C>T	13	3 (3,5)
	K1010T	c.3029 A>C	13	1 (1,2)
	D1027H	c.3081 G>C	14	1 (1,2)
	P1052L	c.3155C>T	14	1 (1,2)
	I1148T	c.3443T>C	16	7 (8,1)
	E1173K	c.3517G>A	16	3 (3,5)
	P1273G	c.3818 C>A	18	1 (1,2)
	G1281D	c.3842 G>A	18	1 (1,2)
	P1273Q	c.3818C>A	18	1 (1,2)
	L1371P	c.4112T.>C	20	3 (3,6)
Splice site	**IVS6+3A>G**	c.1946+3A>G	Int6	1 (1,2)
	IVS14-2A>G	c.3244-2A>G	Int14	6 (7%)
Total				75 (87,2)

Bold lettering: novel mutation.

III. RESULT

A total of 20 different mutations (missense mutations, n= 12; small insertions n=3; nonsense mutations, n=1; splice site mutations, n=3) were detected in this study, which accounted for 87.2% of WD mutations (Table I), suggesting that the other unknown mutation might be located on the outside of exon and flanking regions, such as a promoter and deep intronic areas [21].

TABLE II
PHENOTYPE AND GENOTYPE OF FOUR NOVEL MUTATIONS

Patient	Genotype	Age/sex (years)	Age of onset (years)	Phenotype	K-F ring	s-Cp (g/L)	u-Cu/day (mg/d)
1	**[R723=;H724Tfs*34]**/S105*	13/M	13	H	-	0.01	0.81
2	**V1042Cfs*79**/IVS14-2A>G	14/F	12	H	-	0.03	0.32
3	**D1027H**/IVS14-2A>G	8/M	8	H	-	0.04	0.89
4	V176Sfs*28/**IVS6+3A>G**	9/FM	9	H	-	0.03	0.45

Bold lettering: novel mutation; s (Serum); s-Cp (serum ceruloplasmin; normal range > 0.2 g/L); u-Cu/day (Urinary copper; normal range < 0.1mg/day); K-F (Kayser-Fleischer) [8].

Notably, novel mutations ([R723=; H724Thrfs*34], V1042Cfs*79, D1027H, and IVS6+3A>G) were explored, as the compound heterozygotes (Table II and Fig. 2). V1042Cfs*79 and D1027H occurred as a compound heterozygote with the mutation IVS14-2A>G. The novel mutation [R723=;H724Tfs*34], a new frameshift mutation marked by a 13-base-pair (bp) deletion, was determined as a compound heterozygote with S105*. IVS6+3A>G was found as a compound heterozygote with V176Sfs*28.

Of the 43 patients, *ATP7B* mutations were identified in both alleles in 38 patients (homozygous; n=13, compound heterozygote; n=25); and in only one allele in three patients (n=3).

Most of the mutations in the study occurred in exon 2 (39.6%), exon 16 (11.6%), exon 8 (9.3%), and intron 14 (7%) (Table I). The most frequent WD mutation was S105*, often found in homozygous patients, present in 32.6% of the 86 alleles studied.

TABLE III
LABORATORY TESTS AND DNA ANALYSIS OF 3 ASYMPTOMATIC SIBLINGS OF WD PATIENTS

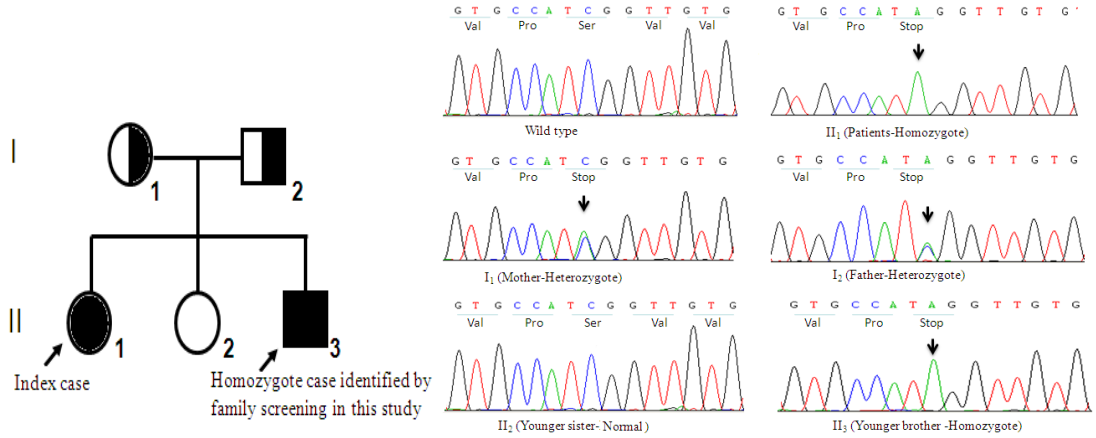
Sibling		1	2	3
Age/sex (year)	Normal range	3/F	7/F	12/F
Bilirubin -Direct (μmol/L)	0.5-6.8	0.75	0.21	0.43
Bilirubin-Total (μmol/L)	3.4-17	2.87	4.01	5.46
AST (u/L)	<40	37.19	43.29	33.01
ALT (u/L)	<40	32.46	60.01	54.44
GGT (u/L)	<40	20.46	40.44	87.16
PT%	>70%	121.00	94.50	87.00
Albumin (g/L)	35-50	29.00	38.94	43.39
Total protein (g/L)	60-80	57.80	65.42	71.39
Cp (g/L)	>0.2	0.05	0.01	0.04
u- Cu/day (mg)	<0.1	< 0.25	0.13	0.21
s-Cu (u/L)	12-28	2.7	2.26	2.57
ATP7B mutation		S105*/S105*	S105*/I1148T	P992L/P992L

AST (Alanin Amino Transferase); ALT (Aspartate Amino Transferase); GGT (Gamma Glutamyl Transferase); PT (Prothrombin); Cp (Ceruloplasmin; normal range > 0.2 g/L); u-Cu (Urinary copper); s-Cu (Serum copper/day).

Based on the index mutation in WD patients, we screened for these mutations in their siblings. Among the 65 siblings tested, we identified nine homozygote or compound heterozygote and 41 heterozygote individuals. Of the nine siblings who had mutations in both alleles, three people who were asymptomatic would begin treatment after DNA diagnosis (Table IV). Laboratory findings of these individuals were collected (Table III and Fig. 1); the remaining were already suffering from WD, with three now deceased from fulminant hepatic disease.

TABLE IV
DNA ANALYSIS OF 65 SIBLINGS OF WD PATIENTS

	Homozygote/Compound heterozygote	Heterozygote	Normal
Suffered from WD	6		
Asymptomatic	3		
Total	9	41	15



(a) (b)

Fig. 1 Pedigree and sequence analysis of a homozygous patient. *a*. Pedigree: Affected males and females are indicated with filled squares and circles, respectively; heterozygote individuals are indicated with a half-filled circle or half-filled square. The asymptomatic younger brother of the WD patient was detected by family screening; *b*. Sequence analysis of mutation S105*: The patient (II_1) and his brother (II_3) were homozygous, his parents (I_1, I_2) were heterozygous, his younger sister (II_2) was normal

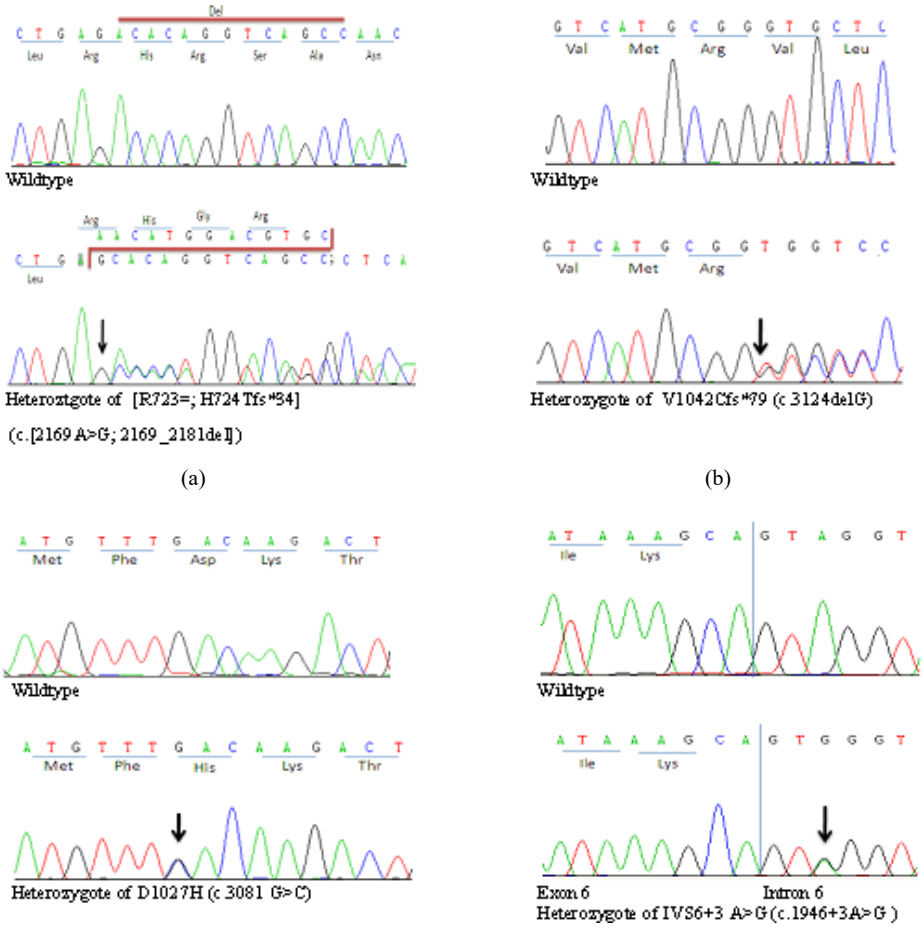


Fig. 2 Sequence analysis of the four novel mutations of the *ATP7B* gene. *a.* A 13-bp (ACACAGGTCAGCC) deletion (accompanied by a changed base pair [A>G at nucleotide 2169, a polymorphism]) from nucleotide 2169_2181 (codon 724) of exon 8 in patient 1. The frame shift results in a truncated protein by introduced a new stop codon at position 758; *b.* A one-bp (G) deletion at nucleotide 3124 (codon 1042) of exon 14 from patient 2. The in-frame deletion results in introducing a new stop codon (codon 1121); *c.* D1027H (c.3081G>C) from patient 3; *d.* IVS6+3A>G (c.1946+3A>G) from patient 4

IV. Discussion

Analyses of the *ATP7B* gene were studied on 86 Vietnamese WD chromosomes, and 20 different mutations were identified. The detection rate is high (87.2%). This is similar to the Chinese rate (83.8-94.7%), American rate (84%) and is measurably higher than that reported in Korea (75%) or Taiwan (65.52%) [7], [13]-[15], [17]. The reason may be that research is small-scale—we will continue to collect more samples in the future. Most of the *ATP7B* mutations in the 43 Vietnamese WD patients tested were found in compound heterozygous mutations and mutation S205* is very common, which accounted for about 32.6% in the total. This phenomenon has been described in several international publications before this study [2], [10], [21].

The result revealed that there were four novel mutations out of the 20 overall mutations. Two novel mutations ([R723=; H724Tfs*34], V1042Cfs*79) were identified to shift the reading frame for truncating abnormal protein; other novel mutations, D1027H and IVS6+3A>G are missense mutations and splice site mutations—which are in need of further protein functional analysis [22]. All of these novel mutations were found in hepatic patients. IVS6+3A>G and D1027H were found in a nine-year-old girl and an 11-year-old boy, who both showed signs of liver disease. Val1042Cysfs*79, which is an insertion mutation, was found in a 15-year-old girl with all the hepatic symptoms, including chronic disease and a face covered with blackheads. [R723=; H724Tfs*34] is a 13-bp deletion that was found in a 13-year-old boy who presented clinical liver disease. We sequenced their parents' DNA, and found that all novel mutations were inherited from their parents.

The mutation spectrum of *ATP7B* in the Vietnamese population markedly differs from other East Asian populations, such as the Taiwanese, where mutations often occur in exons 8, 12, 13, 14, 16, and 18; and the Chinese, where mutations are most present in exons 8, 12, 13, and 16

[13], [15], [17]. In this study, exon 2 (39.6%) is the most sensitive region. Exon 8, intron 14, and exon 16 are also considered as hotspot regions, with decidedly less of the distribution (9.3%, 7%, and 11.6 %, respectively). These exons should be accorded high priority for genetic testing in Vietnam. This is a very curious thing, because Vietnam has proximity to countries where the frequency of mutation R778L in exon 8 is overwhelmingly frequent, present in approximately 30-40% of Chinese patients, 43.1% of Taiwanese patients, and 39.2% of Korean patients [14], [16], [17]. However, the mutation of the *ATP7B* gene is very diverse and population-specific even within East Asia [10], [14], [17]. In China, the most common mutations are R778L (31.9-37.7%), P992L (11.2%) and T935M (10%); in Hong Kong, most common are R778L (17.3%), P992L (13.4%), and T1178A (8.7%); in Korea, most prolific are R778L, A874V, L1083F, and D1270S (55.4%); are most prevalent [12], [15], [24]. But in Japan, mutation 2871delC (15.9%), 1708-5T>G (11. 0%), and R778L (13.4%) are the highest prevalent [23]. Mutation S105* in exon 2 was very common in Vietnamese patients with WD, but the most widespread Asian mutation, R778L in exon 8, was detected in only three alleles. This is quite unique among WD findings in Asia, and could be the characteristic feature of *ATP7B* mutation in the Vietnamese population. The second most frequent mutation was I1148T in exon 16 in the study, which accounted for 8.1% of the alleles studied; this was followed by V176Sfs*28 (7%) and IVS14-2A>G (7%). The second most common mutation, I1148T, has a similar rate of occurrence in Chinese WD patients, but the most common mutation in the study is very different from those reported through Asia [21], [24].

The occurrence of homozygous mutation S105* phenotype was found in more severe hepatic and neurologic patients also in this study. In the early stages, patients show hepatic symptoms; later, patients start showing neurological symptoms in significant and progressive ways. They subsequently present edema, weak limbs, trembling, difficulty speaking, dystonia, and paralysis. On the other hand, one patient with a homozygous missense mutation, I1148T, also had severe symptoms and died at 26 from acute liver failure. So, early detection mutations for WD patients would prevent manifestations and develop to neurological disease [8], [15]. In some reports, the literature revealed that R778L was found in patients with hepatic manifestations; that A874V homozygote phenotype showed a hepato-neurologic type with a relatively late onset [12]; or that homozygotes of H1069Q mutation were associated with a neurologic type of late onset [18]. In contrast, other studies showed that homozygotes for c.2871delC, one of the most frequent Japanese mutations, or for R778L, did not show a correlation with their phenotypes [23]. There are correlations between genotype and phenotype in WD [12]. This is the first report on Vietnamese patients with WD, so further cases should be accumulated to better elucidate the phenotype-genotype correlation of mutation S105* in Vietnamese WD patients.

Currently, almost all Vietnamese WD patients suffer late symptoms—when the disease is serious—because of an infrequent habit of visiting doctors. At this time the situation can be heavily varied, and result in confusion with other diseases. In this situation, family screening is very important in diagnosis for siblings of WD patients. Once homozygous or compound heterozygous mutations in *ATP7B* have been established in the probands, detected mutations can be screened for in their family members, thus allowing early treatment before the onset of complications [8], [26]. Three siblings of patients in this study were found to have mutations in both alleles, although they have not had any clinical-abnormality. They can be treated with zinc therapy [1], [6], [19]. To date, these individuals have been healthy and have had medical examinations every three months. Biochemical tests can only be used in diagnosing WD patients who have presented manifestations; they cannot distinguish individuals who are asymptomatic or presymptomatic, especially in the case of heterozygote carriers [11], [17], [20], [25]. Besides, some variants which have been identified as non-disease-causing (e.g. V456L and K832R) can have a compound effect when occurring in combination with other heterozygote mutations, thereby causing disease. And thus, the heterozygote person can be put at a disadvantage when they have such variants [3]. Furthermore, the age of onset of WD varies from three to 70, so the diagnosis and treatment is easily missed [4]. Genetic analysis for *ATP7B* mutations can certainly diagnose siblings of index cases with known mutations and differential diagnosis of any young patient with unexplained liver disease [8], [9], [15], [26]. The chance of a sibling being a homozygote or compound heterozygote individual—and therefore developing clinical disease—is 25%. The possibility that third-degree relatives, such as cousins, share the same allele is 12.5% [8], [25]. Family screening of patients with WD is the best method of prognosis, and indicator for further treatment, for those suffering from WD [25]. This can help to prevent serious complications of WD, and is an effective and safe treatment [6]. The discovery of the *ATP7B* gene has opened up a new molecular diagnostic approach in genetic pre-marriage counseling and prenatal diagnosis, and could form the basis of genetic therapy in the future [2], [11], [24].

V. Conclusion

By performing the study on 43 WD patients, 20 different mutations were identified, with four novel mutations—[R723=; H724Thrfs*34], V1042Cfs*79, D1027H, and IVS6+3A>G. The most common Asian mutation, R778L, was quite rare in the study, whereas the S105* mutation was very popular. The findings' highest diagnostic importance for patients and their family members is in prognosis and the prevention of morbidity and mortality. Identifying the characteristic types of mutations in Vietnamese WD patients will facilitate faster and more effective genetic diagnoses of WD in the Vietnamese population.

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