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Mutation spectrum of *ATP7B* gene in pediatric patients with Wilson disease in Vietnam

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ABSTRACT

Background: Wilson disease (WD) is caused by mutations in the copper-transporting P-type adenosine triphosphatase encoded by the *ATP7B* gene. In this study, we screened and identified the *ATP7B* mutations among unrelated Vietnamese pediatric patients.

Methods: One-hundred-thirteen pediatric patients with clinically diagnosed WD were recruited. DNA samples were extracted from peripheral blood. Mutations in the *ATP7B* gene were identified by Sanger sequencing.

Results: Approximately 98% of the clinically diagnosed WD patients carried *ATP7B* mutations. A total of 35 different *ATP7B* variants were detected, including five novel mutations (L658P, L792P, T977K, IVS4 + 1G > A and IVS20 + 4A > G). Remarkably, this study revealed that S105* was the most prevalent variant (32.27%), followed by L1371P (9.09%), I1148T (7.27%), R778L (6.36%), T850I (5.45%), V176Sfs*28 and IVS14-2A > G (4.55%). Most *ATP7B* mutations were located in the exon 2 (37.73%), exon 16 (10.00%), exon 8 (9.55%), exon 20 (9.09%), exon 10 and exon 18 (5.45%), exon 14 (5.00%), exon 13 and intron 14 (4.55%). We developed a streamlined procedure to quickly characterize mutations in the *ATP7B* gene in the Vietnamese children, starting with sequencing exon 2 and subsequently to exons 8,10,13-16,18, and 20 to allow quick diagnosis of clinically suspected patients.

Conclusion: The mutational spectrum and hotspots of *ATP7B* gene in the Vietnamese population were fairly different from other East Asian populations. A streamlined procedure was developed to screen exon 2 in *ATP7B* gene among suspected WD patients to reduce genetically diagnostic cost, to facilitate early detection and intervention in countries with limited resources.

1. Introduction

Wilson disease (WD) is a rare inherited metabolic disease caused by mutations in the *ATP7B* gene [6,59]. The prevalence of WD is estimated roughly 01 in 30,000 live births with an estimate of 01 in 90 persons as mutational carriers [12,46,47]. However, the number of pathogenic *ATP7B* variant carriers are under-reported and are equally distributed in all ethnic groups worldwide [33]. Wilson disease is caused by the defective copper-transporting protein P-type adenosine triphosphatase (ATPase) encoded by the *ATP7B* gene on chromosome 13 (13q14.3); The *ATP7B* gene is approximately 80 kb in size, with 21 exons, 20 introns, and an open reading frame of 4.3 kb [6,59]. Pathologically, the failure of hepatic excretion of copper into the bile results in excessive copper accumulation in the brain, liver, and other organs [48]. Patients with WD may have disease manifestations as early in infancy or later on in adulthood; The clinical manifestations are commonly covert, and they will be overt until progressive hepatic failure and/or neurologic dysfunctions [31,62]. The Kayser-Fleischer ring is a crucial ophthalmologic feature of WD; however, this specific sign is only presented in a limited proportion of WD patients [7,17]. Other clinical characteristics of WD

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including skeletal-muscular, renal, cardiac and endocrine disorders were also reported [28,45,58,66]. Most importantly, WD diagnosis is commonly delayed due to the absence of reliable diagnostic tests [64]. In addition, serum ceruloplasmin levels do not change in parallel with the hepatic and neurologic disorders [29,43].

More than 800 variants associated with WD were reported and located across the whole *ATP7B* gene [14,56]. The *ATP7B* mutation spectrum is highly specific to each population. Specifically, H1069Q mutant is the most frequently seen in European, North American and Mediterranean residents with carrier prevalence ranging from 10% to 40%, while R778L is the most common variant in several Asian populations including China, South Korea, Taiwan, and Japan with prevalence varying from 20% to 44% [27,50,70]. Nevertheless, other *ATP7B* variants are unique and predominant in specific populations. For example, the G1267R (38%), 44/−427del on 5'UTR (Untranslated region-UTR) and 246delC in Sardinia [35]; mutations in exon 8 (6.8%), 3400delC (3%) and P969E (1.6%) in some European countries [16]; or A1003T and P969E in Turkey [54].

Although *ATP7B* mutations is studying and reporting worldwide, there is still a lack of information in certain Asian countries [19]. Despite several previous genetic studies about *ATP7B* mutations in Vietnam, most are case reports and case series with relatively small sample sizes [39,60,61]. Therefore, a comprehensive spectrum of *ATP7B* mutations in Vietnamese pediatric patients with WD is crucial for early detection and prompting appropriate intervention strategies.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Institutional Review Board of the National Children's Hospital, Hanoi, Vietnam. The approval number was IRB00011976 (IRB/2019–32). All study participants and/or legal guardians completely understood the study objectives, provided their written informed consents giving permissions for anonymous uses of their genomic data for the study. This study was performed in compliance with the Good Clinical Practice and Helsinki Declaration.

2.2. Study design and participants

This was a single-center, cross-sectional analytic study conducted from January 2018 to December 2020 at the National Children's Hospital, Hanoi, Vietnam. Study participants were recruited using a consecutive sampling method. A total of 113 unrelated pediatric patients with Wilson disease were enrolled in the study. Eligible criteria included age range from 3 to 18 years old, diagnosis of Wilson disease based on the Leipzig 2001 criteria [14] with score ≥ 4. Exclusion criteria include chronic viral hepatitis, autoimmune hepatitis and other metabolic liver diseases.

2.3. Study definitions [33,37,55]

Asymptomatic Wilson disease was defined as the patients who did not have any symptoms and were diagnosed during family screening or routine check-up.

Acute hepatic failure Wilson disease was defined as the acute onset of liver failure by criteria of the Pediatric Acute Liver Failure Study Group: children with no evidence of chronic liver disease; biochemical evidence of acute liver injury; or liver-based coagulopathy with prothrombin time (PT) $\geq$ 15 s or international normalized ratio (INR) $\geq$ 1.5 that was not corrected by vitamin K administration in the presence of hepatic encephalopathy; or as PT $\geq$ 20 s or INR $\geq$ 2.0, regardless of the presence or absence of clinical encephalopathy.

Chronic liver disease WD was defined as the clinical and laboratory presentations of chronic liver failure, portal hypertension, or cirrhosis without evidence of other liver diseases.

Neuropsychiatric with chronic liver disease WD was defined as presentations of both neuropsychiatric symptoms and clinical and laboratory presentations of chronic liver disease. Neuropsychiatric symptoms included dysphagia, excessive salivation, incoordination, behavior changes, resting and intention tremors, dystonia, mask-like face.

Neurological and psychiatric WD was defined as solely neurological or psychiatric manifestations without any clinical and laboratory presentations of liver disease.

2.4. Mutational analysis

A total of 2 ml of peripheral blood from each patient was collected in K2 EDTA-coated tubes and stored at −20 °C until sequencing analysis. Genomic DNA was extracted by the QIAamp DNA Blood Mini kit (Qiagen, Germany). Polymerase chain reaction (PCR) was used to amplify 25 exons and exon-intron flanking regions of the *ATP7B* gene, using 21 specific primer pairs (Invitrogen, United States). Primer sequences are presented in Supplementary Table S3. PCR products were purified and directly sequenced on ABI-3130 (Applied Biosystems, Foster City, USA) by Sanger sequencing. Sequencing results were analyzed by ChromasPro, Seqscape software v2.5, and compared with the reference sequence NG_008806.1 of *ATP7B* gene from GenBank.

3. Results

3.1. Baseline characteristics of study participants

Among a total of 113 pediatric patients with Wilson disease enrolled from 22 provinces in Vietnam, 112 participants were Kinh ethnicity and one patient originated from Tay ethnic minority. Geographical distribution of 113 pediatric patients in different provinces of Vietnam was mapped in Supplementary Fig. S1. The mean patient age at disease onset was 11.5 years old. There were 62 (55%) male and 51 (45%) female patients. None of the patients in this study was blood-related. Family history of WD was noted in 14 (12.4%) patients.

3.2. Clinical presentations of pediatric patients with Wilson's disease in Vietnam

The clinical and laboratory characteristics of all participants are presented in Table 1A and Table 1B, and Supplementary Table S1. On the whole, patients with Wilson disease have diverse presentations. The most common hepatic signs were jaundice, hepatomegaly and ascites.

Table 1A
Clinical presentations of vietnamese pediatric patients with Wilson's disease. Number of patients in each category $n = 113$, except for Kayser- Fleischer rings ($n = 78$) and typical brain symptoms ($n = 96$).

Clinical presentations	Number of incidents	Percentage
Jaundice	21	18.6%
Hepatomegaly	24	21.2%
Splenomegaly	14	12.3%
Neuropsychiatric symptoms[&]	18	15.9%
Encephalopathy	11	9.7%
Ascites	10	8.8%
Hepatosteatosis in ultrasonography	12	10.6%
Kayer- Fleischer ring	11/78[*]	14.2%
Typical brain Wilson in MRI	18/96[**]	15.9%
Other symptoms[&&]	9	7.9%
Family history with Wilson disease	14	12.4%

[&] Neuropsychiatric symptoms: Dysphagia, excessive salivation, incoordination (handwriting deterioration), behavior changes, resting and intention tremors, dystonia, mask-like face, etc.

[&&] Other symptoms as renal tubular dysfunction; proteinemia, arthropathy, cardiomyopathy, etc.

[*] Only 78 (not all 113) patients were checked Kayer- Fleischer ring.

[**] Only 96 (not all 113) patients who were checked for brain MRI.

Table 1B
Laboratory findings at the time of diagnosis of Vietnamese Pediatric Patients with Wilson's disease. Number of patients in each category $n = 113$.

Laboratory finding	Normal range	Patients' range	Mean ± SD*
Hemoglobin (g/dL)	11–14.3	(8.7–14.2)	11.5 ± 3.8
Platelet (G/l)	140–440	(45–257)	205 ± 138
White blood cell (G/l)	5.2–9.7	(1.9–8.5)	5.6 ± 3.2
INR	0.8–1.2	(0.9–3.8)	1.2 ± 0.8
AST (U/l)	12–32	(15.1–318.1)	85.7 ± 76.2
ALT (U/l)	<35	(5.4–219)	118.6 ± 84.6
GGT (U/l)	5–15	(11.6–489.7)	128.4 ± 109.6
Total bilirubin (μmol/l)	3–14	(15.9–458.3)	87.5 ± 49.6
Direct bilirubin (μmol/l)	0.7–4.2	(7.9–219.5)	38.2 ± 35.6
Albumin serum (g/l)	35–48	(22.4–46.8)	39.5 ± 19.6
Serum ceruloplasmin (g/l)	0.15–0.37	(0.01–0.15)	0.08 ± 0.05
Urine copper in 24 h (mg)	<0.05	(0.54–2.5)	1.2 ± 0.9

Note. g, gram; G,10^9 units.
Abbreviations: AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; GGT, Gamma glutamyl transferase; MRI, magnetic resonance imaging; INR, international normalized ratio.
* SD: standard deviation.

Neuropsychiatric symptoms and encephalopathy were also remarkably seen. Most importantly, Kayser-Fleischer rings as a specific sign of Wilson disease were only observed in approximately one-fifth of patients. The magnetic resonance imaging (MRI) revealed Wilson-associated brain lesions in roughly 16% of patients, some representative MRI images are presented in Supplementary Fig. S2. Other WD presentations such as renal tubular dysfunction, proteinemia, arthropathy and cardiomyopathy were also noticeable (Table 1A). Laboratory results revealed quite normal full blood counts, slightly elevated transaminases [mean aspartate aminotransferase (AST), 85.7 UI/L and mean alanine aminotransferase (ALT), 118.6 UI/L]; mean gamma-glutamyl transferase (GGT), 128.4 UI/L; mean total bilirubin, 85.7 μmol/L with predominantly direct bilirubin, 38.2 μmol/L. Liver function tests were stable with mean serum albumin, 39.5 g/L and international normalized ratio (INR), 1.2. Most significantly, serum ceruloplasmin was low with mean level of 0.08 g/L. In contrast, there was a substantial increase in copper measurement in 24-h urine collection with mean level of 1.2 mg (Table 1B). (See Fig. 1.)

3.3. The spectrum of ATP7B mutations among 113 pediatric patients with Wilson disease

Table 2 showed the sequencing results of all 21 exons of *ATP7B* gene from all WD pediatric patients. Among 113 study samples, pathogenic or likely pathogenic mutations were identified in 110/113 (97.79%) samples. There was a total of 73 different types of genotypes which were classified as homozygous variants (35/113, 30.97%), compound heterozygous variants (75/113, 66.37%), heterozygous variants (1/113, 0.88%) and no mutation (2/113, 1.77%). There was a total of 35 pathogenic or likely pathogenic variants identified and categorized into 1 nonsense mutation, 6 frameshift mutations, 21 missense mutations, and 7 splice site mutations. The allele frequencies of the 35 *ATP7B* mutations are presented in Table 2. Remarkably, S105* was the predominant variant in pediatric patients with Wilson disease in Vietnam with allele frequency of 32.27%. Other common variants were L1371P, I1148T, R778L, T850I, V176Sfs*28, and IVS14-2A > G with allele frequency ranging from 4.55% to 9.09%. Conversely, the least observed variants included H251Afs*19, R723S / H724Tfs*34, V1042Cfs*79, R148W, L658P, L792P, T935M, T977K, F1026Y, N1270S, G1281D, IVS4-1G > C, IVS12-2A > G and IVS18-2A > G with the allele frequencies of 0.45%. Most importantly, this study identified five novel *ATP7B* mutations, which were L658P, L792P, T977K, IVS4 + 1G > A and IVS20 + 4A > G (Table 2).

3.4. Five novel ATP7B variants were detected in Vietnamese pediatric patients with Wilson disease

We identified five novel *ATP7B* mutations in our patient population. These newly-detected variants consisted of three missense mutations (L658P, L792P, and T977K) and two splice site mutations (IVS4 + 1G > A and IVS20 + 4A > G) of which electropherograms were presented in Fig. 2. These five novel mutations were detected in a total of six patients: five patients with clinical presentations of chronic liver disease WD and one with neuropsychiatric WD symptoms. Interestingly, these patients presented neither jaundice nor encephalopathy at the time of WD diagnosis. Their genotypes and phenotypes, clinical manifestations and laboratory test results, are shown in Table 3. Interestingly, we identified one patient (Patient 2) who is homozygous for the novel mutation IVS4 + 1G > A. This patient had chronic liver disease. No Kayser-Fleischer rings or brain lesion was observed. This patient had normal blood tests except for high AST and ALT, low serum ceruloplasmin and high 24 h copper in urine. Patient 4, unlike other, is the only patient with neuropsychiatric WD instead of chronic liver disease. This patient carries the novel mutation T977K, and is also the only one to have Kayser-Fleischer rings. Blood test results of this patient were quite normal with only a minor increase in total bilirubin and direct bilirubin. AST, ALT, and ALP were in normal range indicated quite normal liver function. However, this patient still had low serum ceruloplasmin and high 24 h copper in urine which are typical for WD patient.

3.5. Pathogenicity of five novel ATP7B mutations in Vietnamese pediatric patients with Wilson disease

To examine the pathogenicity of newly-detected *ATP7B* variants, we performed functional prediction for each mutation using Polyphen-2 [1], Provean [10], MutationTaster [51], and SIFT analytic tools [38]. In these *in silico* analyses, all novel *ATP7B* mutations were classified as deleterious disease-causing mutations (Supplementary Table S2). Polyphen-2, Provean and SIFT packages predicted the three newly-detected *ATP7B* substitution mutations as "probably damaging", "damaging" and "deleterious", while MutationTaster predicted the L658P, L792P, T977K to be "disease-causing" with the predictive scores of 1.000, 0.997 and 1.000, respectively. For the splice-site mutations (IVS4 + 1G > A and IVS20 + 4A > G), we used MaxEntScan [68] to predict the functional impact, and the results showed that the predictive score of the mutant sequence was much lower than the value of the standard sequence (NG_008806.1). This indicated that they might be pathogenic variants.

3.6. ATP7B mutational hotspots among Vietnamese pediatric patients with Wilson disease

Our study found that most of our identified mutations belong to nine exons and four introns in *ATP7B* gene as shown in Fig. 2, we consider them mutational hotspots. Most remarkably, exon 2 was predominantly seen with the highest mutation frequency of 37.73%, followed by exon 16 (10%), exon 8 (9.55%), exon 20 (9.09%), exons 10 and 18 (5.45%), exons 13 and 14 (4.55%) (Fig. 2). Conversely, exon 11 had the lowest frequency (3.18%). We observed no mutations in other exons of *ATP7B* gene.

3.7. Development of a procedure to characterize mutations of ATP7B gene in pediatric patients with Wilson disease

In our study, mutations in exon 2 was seen the most often, followed by exons 16, 8, 20, 10, 13 and 14. These hotspot regions were distinct from other populations. For example, exon 8, 12, 13, and 16 were identified as the mutation hotspots in China [21,67], while they are 8, 11, 15 and 18 in Korean; 5, 8, 12, 13, and 18 in Japanese [40,42]. In Thailand, our neighbor country, mutation hotspots were identified as

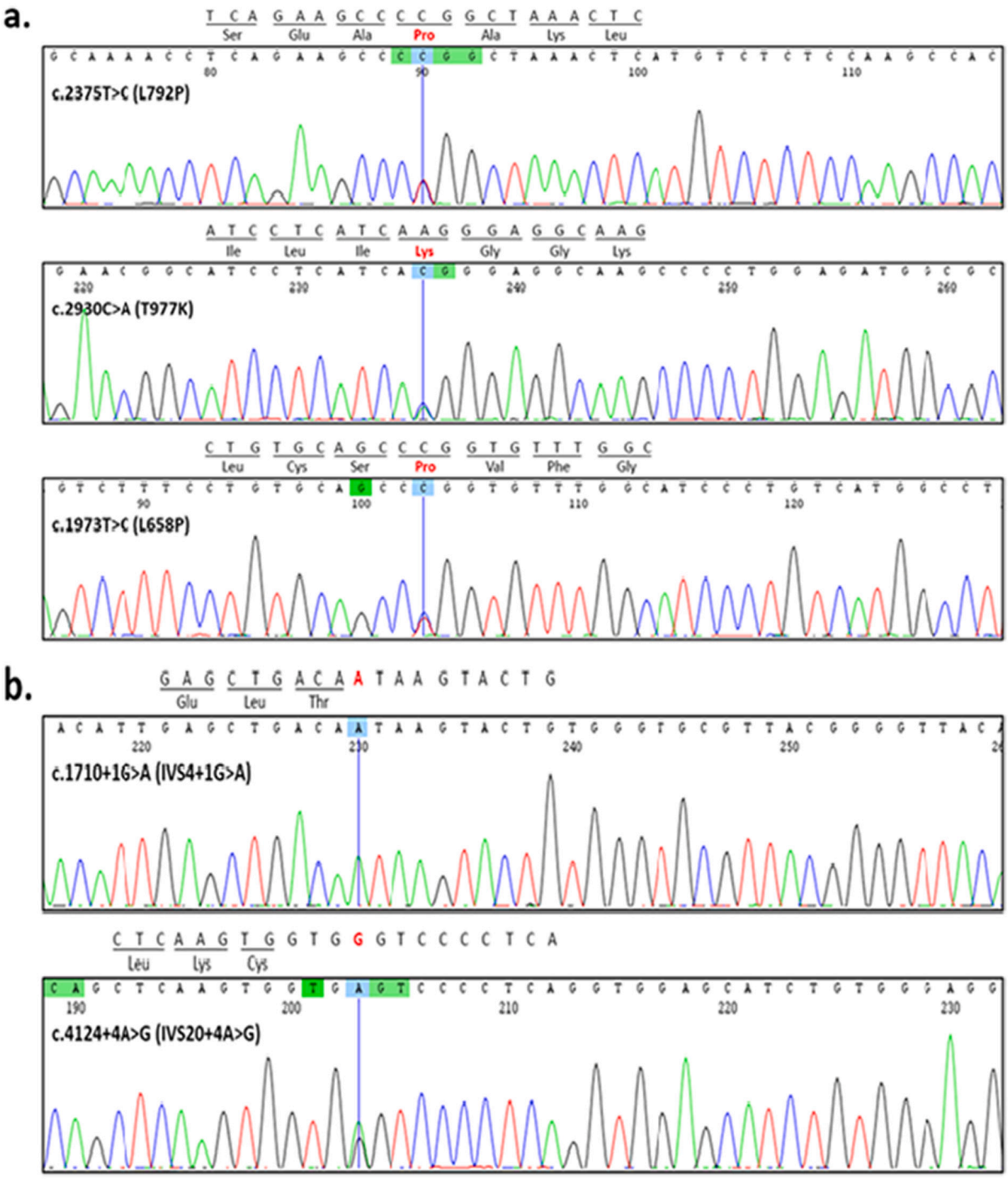

Fig. 1. Electropherograms of five novel *ATP7B* mutations identified in vietnamese pediatric patients with Wilson disease. (a) Three novel missense mutations: L792P (converting Leucine into Proline at position 792), T977K (transforming Threonine into Lysine at the position 977) and L658P (converting Leucine into Proline at position 658). (b) Two novel splice site mutations: IVS4 + 1G > A and IVS20 + 4A > G.

exon 14 and 20 [41]. Therefore, we have developed a streamlined procedure to quickly characterize mutations in the *ATP7B* gene in the Vietnamese children. Instead of sequencing the whole gene, we start with sequencing exon 2 to find pathogenic mutations of WD. If none is found, we continue to sequence exons 8,10,13-16,18, and 20. At the last step, we would sequence the remaining exon to identify mutations (Fig. 3). This streamline allows quick diagnosis of clinically suspected patients, and at the same time minimize the use of resources which are very limited in the country.

4. Discussions

The prevalence of *ATP7B* mutations in our study was 97.35%, which was much higher than previous studies, which reported variants detection in only 80% of all WD patients with the clinically confirmed diagnosis [8,49]. However, our detection rate quite similar to other studies which reported 98% in the United Kingdom [11] or 97.1% in China [23]. There have been reports of sibling with the same genotype regarding mutation in *ATP7B* genes but different clinical manifestations of WD [30,57]. Takeshita et al. reported two families in which sibling

Table 2
Pathogenic and likely pathogenic mutations in the *ATP7B* gene identified in the study cohort of Vietnam.

No.	Variants		Exon/ Intron	Type	Protein region	Number of allele	Mutant allelic frequency (%) (n = 221)	Variant reference (Reference in http://www.hgmd.cf.ac.uk/ac/validate.php)
	Amino acid change	mRNA change						
1	S105*	c.314C > A	2	nonsense	Cu 1	70	32.27	CM003916
2	V176Sfs*28	c.525_526dupA	2	frame	Cu 2	10	4.55	Tsai et al., 1998
3	M769Hfs*26	c.2304dupC	8	shift	TM 4	3	1.36	rs780558532
4	*H251Afs*19*	*c.750dupG*	2		Cu 2/3	1	0.45	[25]
5	*Gly869Glufs*4*	*c.2604del*	11		Td	3	1.36	[25]
6	*R723S; H724Tfs*34*	*c.[2169A > C; 2170_2182del]*	8		TM 3	1	0.45	Huong et al., 2017
7	*V1042Cfs*79*	*c.3124delG*	14		ATP loop	1	0.45	Huong et al., 2017
8	R148W	c.442C > T	2	missense	Cu 1	1	0.45	CM133424
9	***L658P***	*c.1973 T > C*	*7*			*1*	*0.45*	***Novel mutation***
10	R778L	c.2333G > T	8		TM 4	14	6.36	CM960124
11	D765G	c.2294A > G	8		TM 4	3	1.36	CM032847
12	***L792P***	*c.2375 T > C*	***9***			*1*	*0.45*	***Novel mutation***
13	T850I	c.2549C > T	10		Td	12	5.45	CM111988
14	*L902P*	c.2705 T > C	11		Td/TM 5	4	1.82	Phuc et al., 2017
15	T935M	c.2804C > T	13			1	0.45	CM004606
16	***T977K***	*c.2930C > A*	*13*			*1*	*0.45*	***Novel mutation***
17	P992L	c.2975C > T	13		Ch/TM 6	6	2.73	CM970142
18	K1010T	c.3029A > C	13		Ch/TM 6	2	0.91	CM016058
19	*F1026Y*	c.3077 T > A	14		Ph	1	0.45	Huong et al.
20	*D1027H*	c.3079 G > C	14		Ph	9	3.18	Phuc et al., 2017
21	P1052L	c.3155C > T	14		Ph	3	1.36	CM992599
22	I1148T	c.3443 T > C	16		ATP loop	16	7.27	CM990276
23	E1173K	c.3517G > A	16		ATP loop	6	2.73	CM993112
24	P1245S	c.3733C > T	18		ATP hinge	2	0.91	[21]
25	N1270S	c.3809A > G	18		ATP hinge	1	0.45	CM930060
26	P1273Q	c.3818C > A	18		ATP hinge	8	3.64	CM061645
27	G1281D	c.3842G > A	18		ATP hinge	1	0.45	CM074058
28	L1371P	c.4112 T > C	20		TM 8	20	9.09	HM971560
29	***IVS4 + 1G > A***	***c.1707 + 1G > A***	*Intron 4*	splice site	*splicing*	*2*	*0.91*	***Novel mutation***
30	IVS4-1G > C	c.1708-1G > C	Intron 4		splicing	1	0.45	CS951351
31	*IVS6 + 3A > G*	*c.1946 + 3A > G*	Intron 6		splicing	4	1.36	Huong et al.
32	IVS12-2A > G	c.2866-2A > G	Intron 12		splicing	1	0.45	CS136099
33	IVS14-2A > G	c.3244-2A > G	Intron 14		splicing	10	4.55	CS117986
34	IVS18-2A > G	c.3904-2A > G	Intron 18		splicing	1	0.45	CS952024
35	***IVS20 + 4A > G***	***c.4124 + 4A > G***	*Intron 20*		*splicing*	*2*	*0.91*	***Novel mutation***
Total						220	97.35	

Notes. Italic letters, mutations were firstly reported in Vietnamese population; Bold and italic letters, novel mutations reported in this study

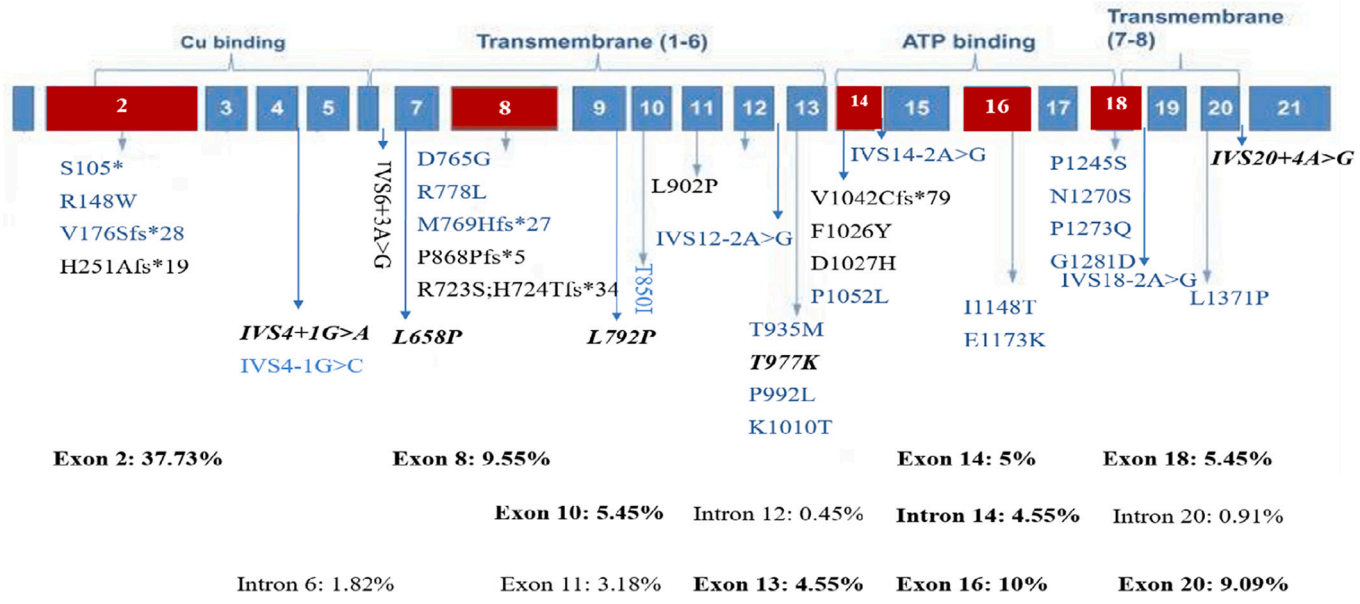

Fig. 2. Map of *ATP7B* mutation hotspots in vietnamese pediatric patients with Wilson disease in this study. Blue letters: previously reported mutations. Black letters: variants reported in Vietnamese WD. Italic black bold letters: novel mutations. Black and bold Exons or Introns: hotspot regions in *ATP7B* gene.

Table 3
Phenotypic manifestations in WD patients with the novel *ATP7B* variants. Bold letters present novel *ATP7B* variants; (+) denotes presence of signs or symptoms, and (−) denotes absence of signs or symptoms.

Clinical characteristics	Normal range (For laboratory tests)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (years)	Not applied	8	12	15	18	12	18
Gender		Female	Male	Male	Female	Female	Male
Genotypes		**IVS20 + 4A > G**/ V176Sfs*28	**IVS4 + 1G > A**/ **IVS4 + 1G > A**	S105*/**L792P**	L1371P/**T977K**	S105*/**L658P**	**IVS20 + 4A > G**/S105*
Phenotypes		Chronic liver disease WD	Chronic liver disease WD	Chronic liver disease WD	Neuropsychiatric WD	Chronic liver disease WD	Chronic liver disease WD
Family history with WD		+	−	−	−	−	−
Hepatomegaly		−	−	−	−	−	−
Splenomegaly		−	−	+	−	−	+
Ascites		−	−	−	−	+	+
Kayser-Fleischer rings		−	−	−	+	−	−
Neuropsychiatric symptoms		−	−	−	+	−	−
Other WD-related symptoms †		−	−	+ §	+ ¶	−	+ ¶
Hepatosteatosis on sonography		−	−	+	−	−	−
Typical Wilson brain lesions on MRI		+	−	−	+	−	−
Hemoglobin level, g/dL	11–14.3	13.8	13.0	13.4	12.7	8.4	10.3
White blood cell count, g/L	5.2–9.7	9.2	9.1	5.2	10	12.1	2.7
Platelet count, (g/l)	140–440	349	472	113	328	239	23
Total serum protein, g/L	57–80	71.4	76.2	56.5	62.1	54.2	46.5
Serum albumin, g/L	35–48	46	43	41.2	42.2	25.5	17
Total bilirubin, μmol/L	3–14	17.5	12.8	24.7	22.9	21.4	19.8
Direct bilirubin, μmol/L	0.7–4.2	2.8	1.8	6.5	5.5	3.7	6.1
AST, UI/L	12–32	86.2	224.2	69.8	23.6	135.5	48.7
ALT, UI/L	<35	142.3	476.1	33.4	11.5	18.0	29.9
ALP, UI/L	74–390	138	162	205	194	185	175
Serum ceruloplasmin, g/L	0.15–0.37	0.015	0.033	0.063	0.062	0.084	0.029
24 h copper in urine, gram	<0.05	0.63	1.08	0.42	0.8	0.28	1.6
Ure, mmol/L	2.6–7.3	3.6	5.3	4.1	4.2	6.5	2.75
Creatinine, μmol/L	40–71	55.2	50.9	59.2	60.5	71.1	45.39
INR	0.8–1.2	1.22	0.95	1.15	1.2	1.05	0.95

Notes. g, gram; G,10^9 units. Abbreviations: AST, Aspartate aminotransferase (normal, < 37); ALT, Alanine aminotransferase (normal, < 40); ALP, Alkaline phosphatase; MRI, magnetic resonance imaging; INR, international normalized ratio (normal <1.2). † Other WD relevant symptoms include § arthropathy, ¶ proteinemia.

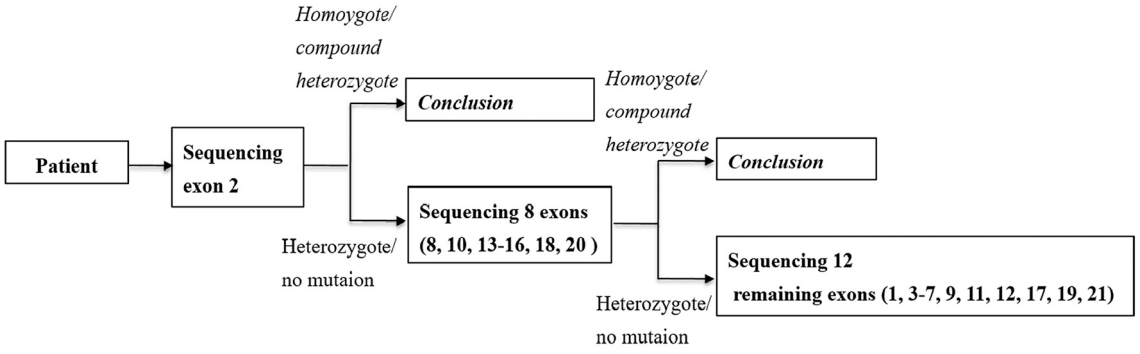

Fig. 3. Development of a procedure to characterize mutations of *ATP7B* gene in vietnamese pediatric patients with wilson disease. The procedure comprises three main stages: step 1, sequencing exon 2; step 2, sequencing further eight exons (exons 8, 10, 13, 14, 15, 16, 18, 20) and the intron 14; step 3, sequencing the remaining regions of *ATP7B* gene.

had different WD clinical phenotypes and age of onsets, which indicated possible inferences by other genetic variations. Kegley et al. reported a case of monozygotic twins, one needed liver transplant while the other only had mild liver disease. Medical histories of these twins showed that a few years prior to one needed liver transplant, the one needed liver transplant suffered from bulimia nervosa, while the other suffered from anorexia nervosa. The authors suggested that the periods of malnutrition in the twin with anorexia nervosa might affected copper homeostasis (limit copper overloading incidents) thus prolong the WD progression. However, we designed our study to include only one pediatric patient from each family; none of the patients in this study is blood-related. Thus, we did not look at correlation between similar genotypes to phenotypes between siblings. In term of the influences of age and sex to WD disease manifestations, Ferenci et al. reported a positive correlation between female patients and the likelihood of having hepatic presentation, while it is a positive correlation between male and neurologic presentation [18]. In future study, we could look at the correlation of age and sex with clinical phenotypes in our Vietnamese patients.

Most of our identified variants were missense mutations located on the transmembrane region of the *ATP7B* protein, metal binding domains, and ATP binding site [8,23,36,69]. Many mutations presented in the heterozygous forms, leading to the fact that most patients are in compound heterozygotes, thus making genotype and phenotype correlations very difficult [8]. WD patients who only had one heterozygous mutation or no mutation at all in the *ATP7B* gene might still have rare mutations in the promoter regions [22].

In this study, five novel variants including L658P, L792P, T977K, IVS4 + 1G > A, and IVS20 + 4A > G were identified, which had not been reported elsewhere. These mutations contributed to previously reported variants unique among the Vietnamese WD patients, including H251Afs*19, P868Pfs*5, [R723S; H724Tfs*34], V1042Cfs*79, F1026Y, IVS6 + 3A > G, L902P, D1027H [25,39,60,61]. All of them were predicted to be disease-causing by *in-silico* analyses. Variants L658P, L792P, T977K were discovered in the C-terminal segment of ATP7B protein, which spans residues 643 to 1377 of full length 1465 amino acid sequence. *ATP7B* gene encodes the copper-transporting ATPase which has eight transmembrane segments (TMS) which are responsible for translocation of copper across membrane [50]. The L658P, L792P and T977K mutations occurred in the TMS, in which L658P in TMS1 and L792P in TMS3[36]. TMS1 is situated in the immediate vicinity of the predicted copper release site where it may regulate the rate of copper release from the transport sites [44]. L792P mutation locates near R778L mutation that appears to play an important role in maintaining protein folding and/or restricting protein motions [69]. Variant T977K on TMS6 which contains the conserved copper-binding motif CPC [50], was also in the vicinity of phosphorylation domain (P-domain) [69][27]. TMS6 of *ATP7B* gene contains a conserved proline residue found in all P-type ATPases. This conserved proline residue flanked by two cysteine residues to form CPC motif, which has been revealed to be essential for copper interaction and transportation across the membrane [2,15,32]. Two patients with L658P and L792P mutations were diagnosed upon chronic hepatic disease WD. This finding is in total agreement with the conclusion that the majority of pathogenic mutations are located in the transmembrane domains in pre-symptomatic patients or those with hepatic symptoms [27]. The IVS4 + 1G > A and IVS20 + 4A > G mutations may cause abnormal exon skipping [21] or eliminated adjacent exons [50].

Five out of six patients who carried novel mutation in this study had the clinical phenotype of only chronic liver disease WD, one patient had neuropsychiatric WD. Interestingly, we identified one patient who is homozygous for the novel mutation IVS4 + 1G > A. This patient had hallmarks of WD such as chronic liver disease, very low serum ceruloplasmin, and very high 24 h copper in urine. Further study on this particular mutation could reveal the role of intron 4 junction toward function of the copper-transporting ATPase ATP7B. The most common mutation identified was a nonsense mutation, S105* in exon 2, with the frequency of 32.27% which is higher than the frequencies of previously reported variants in Vietnam and significantly different from that found in other Asian countries [8,60,70]. Other common mutations were L1371P (9.09%) in exon 20, I1148T (7.27%) in exon 16, and R778L, V176Sfs*28, T850I, IVS14-2A > G (4.55%–6.36%). Other remaining mutations were detected in only a few patients, indicating low frequencies in the Vietnamese population. This spectrum of mutations is distinct from those reported in East-Asia countries with the most widespread mutation being R778L [8,42], in which R778L, P992L, and T935M (Dong et al., 2016) or R778L, P992L, A874C (Li et al.) in China; R778L, P992 and T1178A in Hong Kong; R778L, A874V, and N1270S (Yoo et al., 2002; [42]) or R778L, A874V, and N1270S, L1083F, L838Ffs*35 (Seo et al., 2018) in Korea; 2874delC, and R778L [52] or c.1708-5 T > G, 2874delC, and R778L in Japan [8,40] and R778L, R778Q, P992L, G943D in Taiwan [8,27]. In our cohort of Vietnamese WD patients, S105* was detected in 70 alleles while R778L was merely detected in 14 alleles. The change of serine to stop codon (*) at position 105 in the polypeptide chain of *ATP7B* protein could affect the copper metal-binding site 1 (MBD1) in *ATP7B* where very few diseases causing mutations have been reported [69]. This region played a key role in the reception of copper from ATOX1 *via* protein-protein interaction [63]. MBD1–3 was shown to interact with each other to form a dynamic domain [20,24]. Mutation in MBD1 will disrupt MBD1–3 interactions, and interfere with proper *ATP7B* trafficking and activity regulation [44]. Some mutations in MBDs not only result in dysfunctional *ATP7B* but also protein degradation [4]. Besides, protein-truncating nonsense mutations and frameshift mutations could cause decay of mRNA or a severely truncated protein, resulting in absent, mislocation or diminished levels of protein [3,26]. It is therefore expected that most patients with Wilson disease have absent or significantly reduced levels of *ATP7B* [27]. Thus, S105* is considered a severe mutation and may be associated with early-onset and severe clinical manifestation in children with WD, causing of non-functional *ATP7B* [37,44,53]. The highest prevalence of S105* showed that it may be the most common mutation in Vietnamese patients with WD, and this strongly indicated that the mutational spectrum of *ATP7B* is indeed highly population-specific [8,9,16]. Moreover, the spectrum could be unique to the pediatric patients since some prevalent mutations in our pediatric patients were either rare or not previously reported in other Vietnamese adult cohorts with WD and *vice versa*.

The mutations in this study were mainly distributed in exons 2, 8, 10, 13, 14, 16, 18, 20 and intron 14, with the higher prevalence of mutation observed in exons 2, 8, 16, 20. This suggested that these mutation in these exons are more popular among Vietnamese patients with Wilson disease. Among them, exon 2 has the highest mutation frequency, and it should be prioritized to sequence in patients suspected Wilson disease for quick diagnosis, particularly in cases of hepatic failure disease. This study showed that the hotspot regions in the Vietnamese population were wider than in previous studies [25,60,61]. Pham et al. examined 55 patients in Northern Vietnam and identified exons 2, 8 and 18 in the *ATP7B* gene [60]. These mutant regions which were found and previously reported in France, but it was dissimilar from other reports in the world [5,8,70]. Exon 2 is only considered the hotspot region in southern India and Taiwan [8,34]. Three exons 2, 8, 14 have the highest mutational proportions in the United Kingdom [11]. Otherwise, exons 8, 13, 14 have higher frequencies in France than other countries [5]. However, the hotspots of *ATP7B* variants in the Vietnamese population markedly differed from other East Asian populations, for instance in the Taiwanese where *ATP7B* mutations commonly occur in exons 8, 11, 12, 13, 16, 17, 18 [65] or exons 8, 12, 16, 18 (Tsai et al., 1998). In China, the hotspots are located in the exons 8, 13, 12, 16 [34] or 8, 11, 13, 16, 18 ([23];) or 8, 12, 16 [67]; in Korea, these are exons 8, 11 and 18 [42]; in Japan, these are exons 8, 13 [40,52]; in Northeast India, exons 2, 8, 13, 14, 15; Iran, exon 8, 14 [13] and Thailand, exons 14 and 20 [41]. Our study revealed hotspot regions distinct from other studies which indicate differences in populations, phenotypes and the age of onset of WD patients.

5. Conclusions

The mutational spectrum of the *ATP7B* gene in Vietnamese pediatric patients with Wilson disease is both diverse and distinct from other Asian populations. This once again confirms that mutational “hotspots” in *ATP7B* gene reported considerably vary by geographic regions. Based on these results, this study developed a streamlined procedure to quickly characterize mutations in the *ATP7B* gene in the Vietnamese children with WD. This procedure plays a vital role in diagnosis, and it will make genetic analysis for clinically suspected patients quicker, in order to detect disease earlier and restrict complications of the WD disease. In addition, novel and pathogenic variants reported in this study will be significantly added to the archive of *ATP7B* gene bank. The common mutations and hotspot regions of *ATP7B* gene in Vietnamese pediatric patients are also very helpful for screening asymptomatic siblings of WD patients.

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The authors have nothing to disclose.

Declaration of Competing Interest

We declare that there is no conflict of interest.

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Appendix A. Supplementary data

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