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Research Paper

Spectrum and Classification of *ATP7B* Variants in a Large Cohort of Chinese Patients with Wilson's Disease Guides Genetic Diagnosis

Yi Dong,[1*] Wang Ni,[1*] Wan-Jin Chen,[2*] Bo Wan,[3*] Gui-Xian Zhao,[4] Zhu-Qing Shi,[5] Yue Zhang,[4] Ning Wang,[2] Long Yu,[3] Jian-Feng Xu,[5] and Zhi-Ying Wu[1]✉

1. Department of Neurology and Research Center of Neurology in Second Affiliated Hospital, and the Collaborative Innovation Center for Brain Science, Zhejiang University School of Medicine, Hangzhou, China;
2. Department of Neurology and Institute of Neurology, First Affiliated Hospital, Fujian Medical University, Fuzhou, China;
3. State Key Laboratory of Genetic Engineering, Institutes of Biomedical Sciences, School of Life Science, Fudan University, Shanghai, China;
4. Department of Neurology and Institute of Neurology, Huashan Hospital, Shanghai Medical College, Fudan University, Shanghai, China;
5. Center for Genomic Translation and Health Intervention, School of Public Health, Fudan University, Shanghai, China.

*These authors contributed equally to this work.

✉ Corresponding author: Prof. Zhi-Ying Wu, Department of Neurology and Research Center of Neurology in Second Affiliated Hospital, and the Collaborative Innovation Center for Brain Science, Zhejiang University School of Medicine, 88 Jiefang Rd, Hangzhou, 310009, China. E-mail: zhiyingwu@zju.edu.cn.

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Abstract

Background: Wilson's disease (WD) is an autosomal recessive disorder of copper metabolism caused by *ATP7B* pathogenic mutations. The symptoms of WD can be effectively prevented if the affected individuals are identified and intervened early. However, clinical utility of this molecular analysis is challenging due to hundreds of variants with various clinical effects in the gene. Here, we aim to describe the spectrum of *ATP7B* variants and assess their clinical effects in the Han Chinese population.

Methods: The *ATP7B* gene was directly sequenced in 632 unrelated WD patients and 503 unrelated healthy individuals. The effects of identified variants were classified according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines. Different frequency of variants observed in both cases and controls were tested using Chi-square or Fisher's exact tests.

Results: We detected 161 non-synonymous variants in these 632 WD patients, 58 of which were novel. Among these variants, 78, 64, 8, 4, and 7 were classified as 'pathogenic variants', 'likely pathogenic variants', 'variants with uncertain significance', 'likely benign variants', and 'benign variants', respectively. Ninety percent (569/632) of these WD patients can be genetically diagnosed with two or more 'pathogenic' or 'likely pathogenic' variants. The 14 most common disease-causing variants were found at least once in 94% (537/569) of genetically diagnosed patients.

Conclusions: These data expand the spectrum of *ATP7B* variants and facilitate effective screening for *ATP7B* variants for early diagnosis of WD and development of individualized treatment regimens.

Key words: Wilson's disease; ATP7B; variants classification.

Introduction

Wilson's disease (WD, OMIM #277900), or progressive hepatolenticular degeneration, is an autosomal recessive disease caused by pathogenic mutations within the *ATP7B* gene. [1-3]*ATP7B* encodes a P-type ATPase that is involved in the transport of copper into the plasma protein ceruloplasmin (Cp) and in the ex-

cretion of copper from the liver. ATP7B protein malfunction leads to massive accumulation of copper in the liver, brain, and other tissues. The accumulation of copper in these areas can present a wide spectrum of symptoms, including liver cirrhosis; neuronal degeneration of the brain, particularly in the basal ganglia; Kayser-Fleischer (K-F) rings at the corneal limbus; and kidney damage.[4] The incidence of WD is estimated to be 1 in 30,000 individuals, and the heterozygous carrier rate is about 1 in 90 among many ethnic groups.[5] However, a recent study identified the genetic prevalence of WD as 1:7,026 in the United Kingdom by sequencing *ATP7B* in 1000 control subjects,[6] much higher than the typically reported prevalence of WD of 1:30,000. In addition, WD is thought to be more frequent in Asian populations. The presumed prevalence of WD is approximately 1 in 3,000 in the Korean population[7] and the expected frequency of WD is 1 in 5,400 in the Hong Kong Han Chinese population.[8]

WD is among a limited number of inherited diseases for which symptoms can be prevented if the affected individuals can be identified and intervened early. However, accurate and early clinical diagnoses are often difficult to make due to the wide array of phenotypic variations.[9] While genetic analysis is feasible, its clinical utility has been limited due to more than 700 *ATP7B* reported variants with various clinical effects (https://portal.biobase-international.com/hgmd/pro/gene.php?gene=ATP7B, Human Gene Mutation Database Professional, access date: 20 October, 2015). Therefore, better understanding of the spectrum of *ATP7B* variants and the clinical effects of these variants are necessary for clinical application.

Here, we reported variants identified from a study that directly sequenced the *ATP7B* gene in 632 consecutively treated WD patients at three Chinese academic medical centers between 2004 and 2015, and 503 unrelated phenotypically normal individuals. The clinical effects of identified variants were classified according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines.

Subjects and Methods

Participants

Consecutive patients who sought for diagnosis and treatment of WD between January 15, 2004 and April 30, 2015 at the Departments of Neurology at three leading Chinese academic medical centers were recruited, including Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou; First Affiliated Hospital, Fujian Medical University, Fuzhou; Huashan Hospital, Fudan University, Shanghai. Each patient was examined by at least two senior neurologists, including medical history review and physical exams. The patients were clinically diagnosed with WD according to the diagnostic criteria described previously.[4] Blood samples of 632 unrelated patients (346 males and 286 females, aged 9 to 56) who have average onset age of 18.5 ± 0.6 ranging from 3 to 48 years and 503 unrelated phenotypically normal controls with no known family history of WD were included in the study. Family history were detected in 16 out of 632 WD patients. All participants are of Han Chinese descent. The probands, or their legal guardians, provided informed consents. This study was approved by three institutional review boards.

Direct DNA Sequencing for Mutation Analysis of ATP7B

Genomic DNA was isolated from peripheral blood lymphocytes using a DNA isolation kit (Qiagen Inc, Valencia, CA). All 21 exons and their flanking regions as well as the 5′-untranslated region (UTR) and promoter regions of the *ATP7B* gene were sequenced. Exon 2, which is 1,234 base pair (bp) in length, was covered by three overlapping PCR fragments. The primer sequences, annealing temperatures, and sizes of the PCR products of exons 1, 2, 4, 16, and 18 were listed in **Table S1**. Information related to the remaining primers has been described previously.[10] PCR amplification and direct sequencing were performed as previously reported.[11] Obtained sequences were compared, and nucleotide changes were numbered according to their position in *ATP7B* mRNA (NM_000053). Novel variants were further confirmed by sequencing both the forward and reverse strands. The sequence variants were interpreted and classified according to ACMG Standards and Guidelines.[12]

Statistical Analysis

Different frequency of variants observed in both WD patients and controls was tested using Chi-square test or the Fisher's exact test (for variants with the expected number of subjects below 5). The Bonferroni correction was used to declare statistical significance.

Results

Variants identified in the ATP7B Gene

A total of 173 variants in the coding region and the adjacent splice sites of the *ATP7B* gene were identified among 632 unrelated WD patients and 503 controls, including 161 non-synonymous and 12 synonymous variants. Among the 161 non-synonymous variants, 150 were detected only in WD patients and 11 were detected in both WD patients and normal controls. To assess link of the latter group of variants with WD, we compared allele frequency for 10 of these 11 variants between WD patients and controls

(one of the 11 variants, p.R778L, was excluded because it is a well-established pathogenic variant[13]). Three variants (p.I390V, p.T935M, and p.V1106I) were considerably more common in WD patients than controls and the differences reached a statistical significance level (p<0.005) after adjusting for 10 multiple tests (0.05/10) (**Table 1**). The estimated odds ratios (ORs) of these three variants greater than 10 (p.I390V, OR=11.26; p.T935M, OR=77.04; p.V1106I, OR=10.44). For the remaining 7 variants, the allele frequency was similar between cases and controls (p>0.005), with ORs close to 1.00. Homozygotes in controls were found for 5 of these 7 variants (**Table 1**).

Among 12 synonymous variants, 5 were novel, including c.2145c>T (p.Y715Y), c.3243G>A (p.E1081E), c.3861C>G (p.G1287G), c.4014T>A (p.I1338I), c.4194c>T (p.S1398S). The remaining variants have been previously identified (http://asia.ensembl.org/Homo_sapiens/Transcript/Sequence_cDNA?db=core;g=ENSG00000123191;r=13:51932673-52011494;t=ENST00000242839, access date: 20, October, 2015), including c.747G>A (p.L249L, rs554554415), c.1620c>T (p.L540L,rs145798966), c.2292c>T (p.F764F, rs372979339), c.2310C>G (p.L770L, rs398123136), c.2973G>A (p.T991T, rs1801246), c.3009G>A (p.A1003A, rs1801247) and c.4251A>G (p.T1417T, rs546721020).

Classification of Variants

Among 161 non-synonymous variants, 7 were similar between cases and controls (**Table 1**), thus classified as benign variants according to the ACMG Standards and Guidelines.[12] The remaining 154 non-synonymous variants were only observed in WD patients or significantly more frequent in WD patients (**Table 2**). They are distributed throughout the *ATP7B* gene exons 1 to 21 (**Figure 1**). Among these 154 non-synonymous variants, 18 (11.7%) are nonsense variants, 25 (16.2%) are small deletions or insertions, 11 (7.1%) are splice site variants, and 100 (64.9%) are missense variants. According to the ACMG Standards and Guidelines,[12] 18 nonsense variants can be classified as 'pathogenic variants'. Among 25 small deletions/insertions, three shift variants including c.2316_2317insCTCTTTGTG (p.V772insLFV), c.2790_2792del(p.I930del) and c.4005_4006insTTATAATGGGTTGCG (p.G1335insLXWVA) can only be classified as 'likely pathogenic variants' due to its in-frame characteristics, other 22 ones can be classified as 'pathogenic variants' as well. For the 11 splice site mutations, six (c.51+1g>a, c.1543+1g>t, c.1708-1g>c, c.1870-2a>g, c.2356-1g>c, and c.3557-2a>g) are predicted to result in exon skipping and lead to the production of a defective protein, therefore can also be classified as 'pathogenic variants'. For the other five splice site variants (c.1543+4a>g, c.1708-5t>g, c.1946+5g>a, c.2447+5g>t, and c.3903+5g>a), because additional functional analysis is required to assess their impact on RNA and protein, they can be classified as 'variants with uncertain significance' at this stage. For the 100 missense variants (**Table 3**), 32, 61, 4, and 3 can be classified as 'pathogenic variants', 'likely pathogenic variants'', 'likely benign variants'', and 'variants with uncertain significance', respectively, based on results of SIFT, PolyPhen-2, 1000 Genomes Project, Exome Aggregation Consortium and the ACMG Standards and Guidelines. In total, among 161 non-synonymous variants, 78 are classified as 'pathogenic variants', 64 as 'likely pathogenic variants', 8 as 'variants with uncertain significance', 4 as 'likely benign variants' and 7 as 'benign variants'. Therefore, 142 variants could be considered as potential disease-causing variants ('pathogenic variants' and 'likely pathogenic variants') at this stage.

Table 1. The Allele Frequency of 10 Non-Synonymous Variants in 632 WD Patients and 503 Controls.

Variants	Controls				WD Patients				Probabilities	OR(95%CI)
	Wildtype	Heterozygote	Homozygote	Allele Frequency	Wildtype	Heterozygote	Homozygote	Allele Frequency		
p.I390V	502	1	0	0.001	619	12	1	0.011	0.003*	11.256(1.478-85.743)
p.S406A	146	223	134	0.488	153	271	208	0.544	0.044	1.249(1.058-1.474)
p.L456V	150	240	113	0.463	157	304	171	0.511	0.085	1.211(1.026-1.430)
p.R832K	339	59	105	0.267	441	47	144	0.265	0.045	0.988(0.819-1.192)
p.I929V	488	15	0	0.015	624	8	0	0.006	0.042	0.421(0.178-0.997)
p.T935M	502	1	0	0.001	543	88	1	0.071	1.40E-22*	77.044(10.716-553.910)
p.R952K	164	261	78	0.415	206	335	91	0.409	0.862	0.978(0.826-1.157)
p.V1106I	502	1	0	0.001	619	13	0	0.010	0.0049*	10.444(1.364-79.969)
p.V1140A	195	240	68	0.374	258	286	88	0.366	0.706	0.965(0.813-1.146)
p.V1297I	498	5	0	0.005	628	4	0	0.003	0.520	0.636(0.17-2.373)

*Based on Fisher's Exact test due to small observed number of variants.

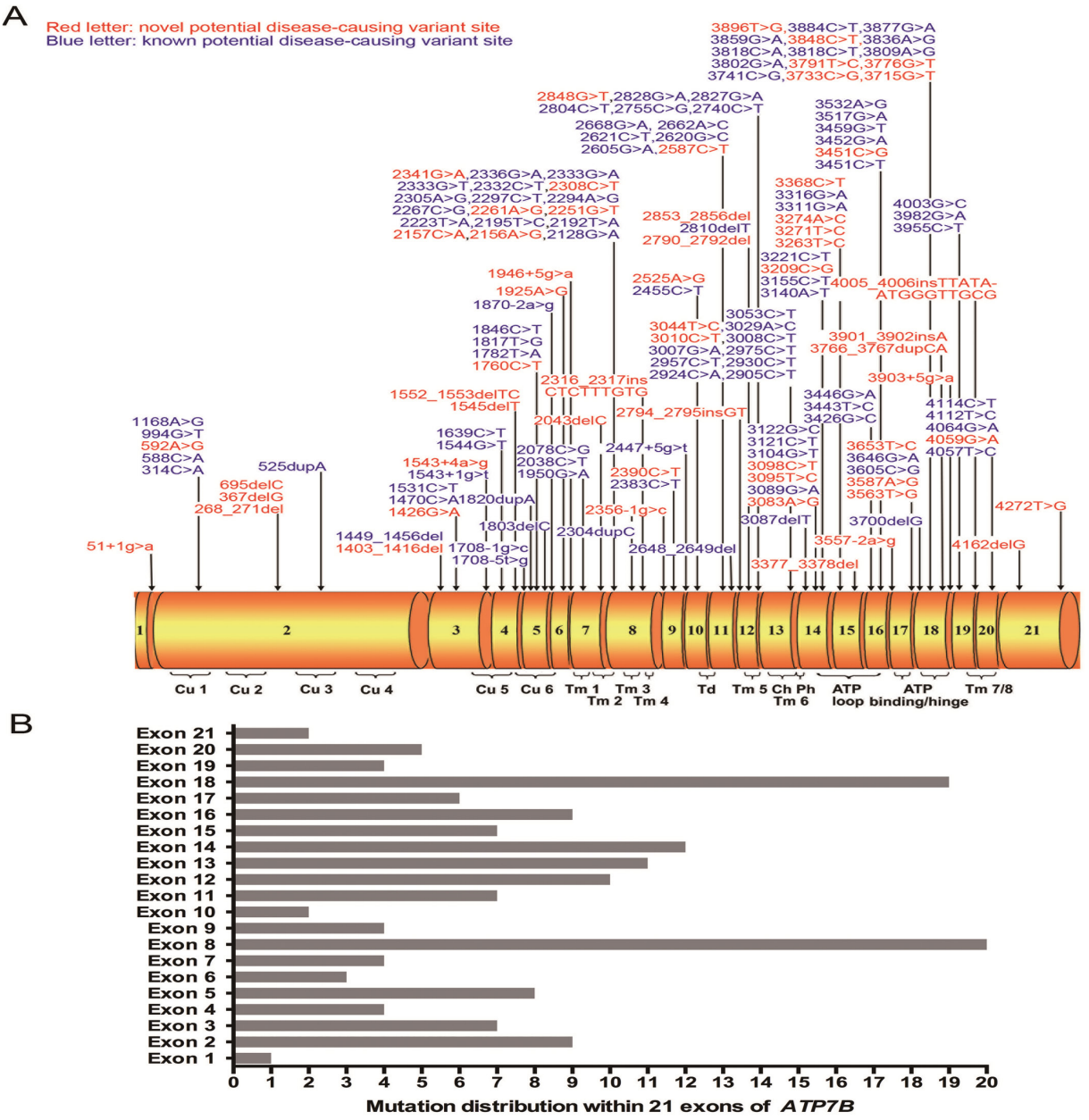

Figure 1: (A) Schematic representation of the *ATP7B* gene with 21 exons. All here identified variants in WD patients with Chinese Han descent are visualized in the corresponding *ATP7B* protein regions. Fifty-eight novel variants are indicated in red characters. Cu 1~6, six copper binding domains; Tm 1~8, eight transmembrane domains; Td, a transduction domain converting the ATP hydrolysis energy to the impetus of transporting copper cation; Ch, the transmembrane cation channel; Ph, the phosphorylation domain; ATP loop/binding/hinge, the ATP binding domain. (B) Distribution of *ATP7B* variants is shown within 21 exons of *ATP7B* gene.

Table 2. One Hundred and Fifty-Four Non-Synonymous Variants were Only Observed in WD Patients or Significantly More Frequent in WD Patients.

Mutation analysis			Domain	Frequency of MU (%)	No. of patients	
Nucleotide mutation	Protein alteration	Exon			MU/MU	WT/MU
c.51+1g>a	Na	1	before Cu1	0.08	0	1
c.268_271del	p.K90FfsX10	2	Cu2	0.08	0	1
c.314C>A	p.S105X	2	Cu2	0.08	0	1
c.367delG	p. A123PfsX30	2	Cu2	0.08	0	1
c.525dupA	p.V176SfsX28	2	Cu2	1.50	1	17
c.588C>A	p.D196E	2	Cu2	0.40	0	5
c.592A>G	p.R198G	2	Cu2	0.08	0	1
c.695delC	p.P232QfsX30	2	Cu3	0.08	0	1
c.994G>T	p.E332X	2	Cu3/Cu4	0.55	0	7
c.1168A>G	p.I390V	2	Cu3/Cu4	1.11	1	12

Mutation analysis			Domain	Frequency of MU (%)	No. of patients	
Nucleotide mutation	Protein alteration	Exon			MU/MU	WT/MU
c.1403_1416del	p.A468GfsX33	3	Cu4/Cu5	0.08	0	1
c.1426G>A	p.A476T	3	Cu4/Cu5	0.08	0	1
c.1449_1456del	p.R483SfsX20	3	Cu5	0.16	0	2
c.1470C>A	p.C490X	3	Cu5	0.24	0	3
c.1531C>T	p.Q511X	3	Cu5	2.37	2	26
c.1543+1g>t	Na	3	Cu5	0.47	0	6
c.1543+4a>g	Na	3	Cu5	0.08	0	1
c.1544G>T	p.G515V	4	Cu5	0.08	0	1
c.1545delT	p.G515GfsX9	4	Cu5	0.08	0	1
c.1552_1553delTC	p.S518RfsX15	4	Cu5	0.08	0	1
c.1639C>T	p.Q547X	4	Cu5	0.08	0	1
c.1708-5t>g	Na	5	Cu6	1.03	1	11
c.1708-1g>c	Na	5	Cu6	1.50	2	15
c.1760C>T	p.T587M	5	Cu6	0.08	0	1
c.1782T>A	p.Y594X	5	Cu6	0.08	0	1
c.1803delC	p.S602AfsX46	5	Cu6	0.08	0	1
c.1817T>G	p.V606G	5	Cu6	0.16	0	2
c.1820dupA	p.F608VfsX2	5	Cu6	0.16	0	2
c.1846C>T	p.R616Y	5	Cu6	0.08	0	1
c.1870-2a>g	na	6	Cu6	0.08	0	1
c.1925A>G	p.D642G	6	Cu6	0.08	0	1
c.1946+5g>a	na	6	Cu6/TM1	0.08	0	1
c.1950G>A	p.W650Term	7	Cu6/TM1	0.08	0	1
c.2038C>T	p.Q680X	7	Cu6/TM1	0.08	0	1
c.2043delC	p.S681SfsX15	7	TM2	0.08	0	1
c.2078C>G	p.S693C	7	TM2	0.16	0	2
c.2128G>A	p.G710S	8	TM2/TM3	0.08	0	1
c.2156A>G	p.Y719C	8	TM2/TM3	0.08	0	1
c.2157C>A	p.Y719X	8	TM2/TM3	0.24	0	3
c.2192T>A	p.V731E	8	TM3	0.08	1	0
c.2195T>C	p.L732P	8	TM3	0.08	0	1
c.2223T>A	p.Y741X	8	TM3	0.08	0	1
c.2251G>T	p.A751S	8	TM3	0.08	0	1
c.2261A>G	p.E754G	8	TM3/TM4	0.08	0	1
c.2267C>G	p.A756G	8	TM3/TM4	0.08	0	1
c.2294A>G	p.D765G	8	TM4	0.24	0	3
c.2297C>T	p.T766M	8	TM4	0.16	0	2
c.2304dupC	p.M769HfsX26	8	TM4	0.87	0	11
c.2305A>G	p.M769V	8	TM4	0.08	0	1
c.2308C>T	p.L770F	8	TM4	0.08	0	1
c.2316_2317ins CTCTTTGTG	p.V772insLFV	8	TM4	0.08	0	1
c.2332C>T	p.R778W	8	TM4	0.16	0	2
c.2333G>T	p.R778L	8	TM4	29.67	64	247
c.2333G>A	p.R778Q	8	TM4	1.98	3	19
c.2336G>A	p.W779X	8	TM4	0.08	0	1
c.2341G>A	p.E781K	8	TM4	0.08	0	1
c.2356-1g>c	na	9	TM4/Td	0.08	0	1
c.2383C>T	p.L795F	9	TM4/Td	0.08	0	1
c.2390C>T	p.S797F	9	TM4/Td	0.08	0	1
c.2447+5g>t	Na	9	TM4/Td	0.08	0	1
c.2455C>T	p.Q819X	10	TM4/Td	0.08	0	1
c.2525A>G	p.D842G	10	Td	0.08	0	1
c.2587C>T	p.P863S	11	Td	0.08	0	1
c.2605G>A	p.G869R	11	Td	0.16	0	2
c.2620G>C	p.A874P	11	Td/TM5	0.08	0	1
c.2621C>T	p.A874V	11	Td/TM5	3.56	3	39
c.2648_2649del	p.V883AfsX3	11	Td/TM5	0.08	0	1
c.2662A>C	p.T888P	11	Td/TM5	0.32	0	4
c.2668G>A	p.V890M	11	Td/TM5	0.16	0	2
c.2740C>T	p.Q914X	12	Td/TM5	0.08	0	1
c.2755C>G	p.R919G	12	Td/TM5	1.98	0	25
c.2790_2792del	p.I930del	12	TM5	0.47	0	6
c.2794_2795insGT	p.S932CfsX4	12	TM5	0.08	0	1
c.2804C>T	p.T935M	12	TM5	7.12	1	88
c.2810delT	p.V937GfsX5	12	TM5	0.63	0	8
c.2827G>A	p.G943S	12	TM5	0.55	1	5
c.2828G>A	p.G943D	12	TM5	2.21	1	26
c.2848G>T	p.V950F	12	TM5/TM6	0.08	0	1

Mutation analysis			Domain	Frequency of MU (%)	No. of patients	
Nucleotide mutation	Protein alteration	Exon			MU/MU	WT/MU
c.2853_2856del	p.Q951HfsX15	12	TM5/TM6	0.08	0	1
c.2905C>T	p.R969W	13	TM6	0.08	0	1
c.2924C>A	p.S975Y	13	TM6	0.79	0	10
c.2930C>T	p.T977M	13	TM6	0.08	0	1
c.2957C>T	p.S986F	13	TM6	0.08	0	1
c.2975C>T	p.P992L	13	TM6/Ph	14.56	26	132
c.3007G>A	p.A1003T	13	TM6/Ph	0.16	0	2
c.3008C>T	p.A1003V	13	TM6/Ph	0.08	0	1
c.3010C>T	p.Q1004X	13	TM6/Ph	0.08	0	1
c.3029A>C	p.K1010T	13	TM6/Ph	0.16	0	2
c.3044T>C	p.L1015P	13	TM6/Ph	0.08	0	1
c.3053C>T	p.A1018V	13	TM6/Ph	0.24	0	3
c.3083A>G	p.K1028R	14	Ph	0.08	0	1
c.3087delT	p.G1030AfsX91	14	Ph	0.08	0	1
c.3089G>A	p.G1030D	14	Ph	0.40	0	5
c.3095T>C	p.I1032T	14	Ph	0.08	0	1
c.3098C>T	p.T1033I	14	Ph	0.08	0	1
c.3104G>T	p.G1035V	14	Ph	0.08	0	1
c.3122G>C	p.R1041P	14	ATP loop	0.16	1	0
c.3121C>T	p.R1041W	14	ATP loop	0.08	0	1
c.3140A>T	p.D1047V	14	ATP loop	0.24	1	1
c.3155C>T	p.P1052L	14	ATP loop	0.16	0	2
c.3209C>G	p.P1070R	14	ATP loop	0.08	0	1
c.3221C>T	p.A1074V	14	ATP loop	0.08	0	1
c.3263T>C	p.L1088S	15	ATP loop	0.32	0	4
c.3271T>C	p.C1091R	15	ATP loop	0.08	0	1
c.3274A>C	p.T1092P	15	ATP loop	0.08	1	0
c.3311G>A	p.C1104Y	15	ATP loop	0.08	0	1
c.3316G>A	p.V1106I	15	ATP loop	1.03	0	13
c.3368C>T	p.P1123L	15	ATP loop	0.08	0	1
c.3377_3378delAC	p.H1126PfsX3	15	ATP loop	0.08	0	1
c.3426G>C	p.Q1142H	16	ATP loop	1.58	1	18
c.3443T>C	p.I1148T	16	ATP loop	3.32	1	40
c.3446G>A	p.G1149E	16	ATP loop	0.08	0	1
c.3451C>T	p.R1151C	16	ATP loop	0.08	0	1
c.3451C>G	p.R1151G	16	ATP loop	0.08	0	1
c.3452G>A	p.R1151H	16	ATP loop	0.16	0	2
c.3459G>T	p.W1153C	16	ATP loop	0.08	0	1
c.3517G>A	p.E1173K	16	ATP loop	0.79	1	8
c.3532A>G	p.T1178A	16	ATP loop	0.16	0	2
c.3557-2a>g	na	17	ATP loop	0.08	0	1
c.3563T>G	p.L1188R	17	ATP loop	0.08	0	1
c.3587A>G	p.D1196G	17	ATP loop	0.08	0	1
c.3605C>G	p.A1202G	17	ATP loop	0.08	0	1
c.3646G>A	p.V1216M	17	ATP bind	1.98	2	21
c.3653T>C	p.L1218P	17	ATP bind	0.08	0	1
c.3700delG	p.V1234LfsX96	18	ATP bind	0.32	0	4
c.3715G>T	p.V1239F	18	ATP bind	0.24	0	3
c.3733C>G	p.P1245A	18	ATP bind	0.08	0	1
c.3741C>G	p.H1247Q	18	ATP bind	0.08	0	1
c.3766_3767dupCA	p.Q1256PfsX75	18	ATP bind	0.08	0	1
c.3776G>T	p.G1259V	18	ATP hinge	0.08	1	1
c.3791T>C	p.M1264T	18	ATP hinge	0.08	0	1
c.3802G>A	p.G1268R	18	ATP hinge	0.08	0	1
c.3809A>G	p.N1270S	18	ATP hinge	2.22	0	28
c.3818C>T	p.P1273L	18	ATP hinge	0.08	0	1
c.3818C>A	p.P1273Q	18	ATP hinge	0.16	0	2
c.3836A>G	p.D1279G	18	ATP hinge	0.24	0	3
c.3848C>T	p.A1283V	18	ATP hinge	0.08	0	1
c.3859G>A	p.G1287S	18	ATP hinge	0.08	0	1
c.3877G>A	p.E1293K	18	ATP hinge	0.08	0	1
c.3884C>T	p.A1295V	18	ATPhinge/TM7	0.40	0	5
c.3896T>G	p.L1299R	18	ATP hinge/TM7	0.08	0	1
c.3901_3902insA	p.R1301KfsX3	18	ATP hinge/TM7	0.08	0	1
c.3903+5g>a	na	18	ATP hinge/TM7	0.08	0	1
c.3955C>T	p.R1319X	19	ATP hinge/TM7	0.24	0	3
c.3982G>A	p.A1328T	19	TM7	0.16	0	2
c.4003G>C	p.G1335R	19	TM7	0.24	0	3
c.4005_4006insTTATAATGGGTTGCG	p.G1335insLXWVA	19	TM7	0.16	0	2

Mutation analysis			Domain	Frequency of MU (%)	No. of patients	
Nucleotide mutation	Protein alteration	Exon			MU/MU	WT/MU
c.4057T>C	p.W1353R	20	TM8	0.16	0	2
c.4059G>A	p.W1353X	20	TM8	0.08	0	1
c.4064G>A	p.G1355D	20	TM8	0.08	0	1
c.4112T>C	p.L1371P	20	TM8	0.40	0	5
c.4114C>T	p.Q1372X	20	TM8	0.63	0	8
c.4162delG	p.A1388RfsX5	21	after TM8	0.08	0	1
c.4272T>G	p.Y1424X	21	after TM8	0.08	0	1

MU: mutant; WT: wild type; Cu: copper binding domain; Td: transduction domain converting energy from ATP hydrolysis to cation transportation; Tm: transmembrane domain; Ch: ion channel; Ph: phosphorylation loop.

Table 3. Classified mutations within 100 missense mutations.

Nucleotide mutation	Protein alteration	Exon	Sift		PolyPhen 2		1000g	ExAc	Classification	Evidence of pathogenicity
			Score	Prediction	Score	Prediction				
c.588C>A	p.D196E	2	1	Tolerated	0.924	Probably damaging	0	5	US	4*PP
c.592A>G	p.R198G	2	0.05	Damaging	0.994	Probably damaging	0	0	LP	1*PM,4*PP
c.1168A>G	p.I390V	2	0.57	Tolerated	0.001	Benign	0	2	LB	1*BS,1*BP
c.1426G>A	p.A476T	3	0.63	Tolerated	0.503	Possibly damaging	2	53	US	1*BS
c.1544G>T	p.G515V	4	0	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.1760C>T	p.T587M	5	0.19	Tolerated	0.099	Benign	0	0	LB	1*BS,1*BP
c.1817T>G	p.V606G	5	0	Damaging	1	Probably damaging	0	0	LP	1*PM,5*PP
c.1846C>T	p.R616Y	5	0	Damaging	1	Probably damaging	0	4	LP	2*PM,5*PP
c.1925A>G	p.D642G	6	0.01	Damaging	1	Probably damaging	0	0	LP	1*PM,4*PP
c.2078C>G	p.S693C	7	0.01	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.2128G>A	p.G710S	8	0.02	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.2156A>G	p.Y719C	8	0	Damaging	0.991	Probably damaging	0	0	LP	2*PM,4*PP
c.2192T>A	p.V731E	8	0	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.2195T>C	p.L732P	8	0	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.2251G>T	p.A751S	8	0.16	Tolerated	0.999	Probably damaging	0	0	LP	2*PM,3*PP
c.2261A>G	p.E754G	8	0.34	Tolerated	0.999	Probably damaging	0	0	LP	2*PM,3*PP
c.2267C>G	p.A756G	8	0.21	Tolerated	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.2294A>G	p.D765G	8	0	Damaging	1	Probably damaging	0	0	LP	3*PM,5*PP
c.2297C>T	p.T766M	8	0	Damaging	1	Probably damaging	0	1	P	1*PS,2*PM,5*PP
c.2305A>G	p.M769V	8	0	Damaging	1	Probably damaging	0	8	LP	3*PM,5*PP
c.2308C>T	p.L770F	8	0	Damaging	1	Probably damaging	0	0	LP	2*PM,4*PP
c.2332C>T	p.R778W	8	0	Damaging	1	Probably damaging	0	5	P	2*PS,3*PM
c.2333G>A	p.R778Q	8	0	Damaging	1	Probably damaging	0	5	P	2*PS,3*PM
c.2333G>T	p.R778L	8	0	Damaging	1	Probably damaging	0	5	P	2*PS,3*PM
c.2341G>A	p.E781K	8	0	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,4*PP
c.2383C>T	p.L795F	9	0	Damaging	1	Probably damaging	0	4	P	1*PS,3*PM
c.2390C>T	p.S797F	9	0	Damaging	1	Probably damaging	0	0	LP	1*PM,4*PP
c.2525A>G	p.D842G	10	0	Damaging	1	Probably damaging	0	0	LP	1*PM,4*PP
c.2587C>T	p.P863S	11	0	Damaging	1	Probably damaging	0	0	LP	1*PM,4*PP
c.2605G>A	p.G869R	11	0	Damaging	1	Probably damaging	5	82	LP	2*PM,5*PP
c.2620G>C	p.A874P	11	0.01	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.2621C>T	p.A874V	11	0.01	Damaging	1	Probably damaging	0	9	LP	2*PM,5*PP
c.2662A>C	p.T888P	11	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.2668G>A	p.V890M	11	0.01	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.2755C>G	p.R919G	12	0.01	Damaging	0.988	Probably damaging	0	2	P	1*PS,2*PM,5*PP
c.2804C>T	p.T935M	12	0	Damaging	1	Probably damaging	0	21	P	1*PS,1*PM,5*PP
c.2827G>A	p.G943S	12	0.16	Tolerated	1	Probably damaging	0	2	P	1*PS,1*PM,4*PP
c.2828G>A	p.G943D	12	0	Damaging	1	Probably damaging	0	2	P	1*PS,2*PM,5*PP
c.2848G>T	p.V950F	12	0.01	Damaging	0.994	Probably damaging	0	0	LP	2*PM,4*PP
c.2905C>T	p.R969W	13	0.02	Damaging	1	Probably damaging	0	5	LP	2*PM,5*PP
c.2924C>A	p.S975Y	13	0.01	Damaging	1	Probably damaging	0	1	LP	2*PM,5*PP
c.2930C>T	p.T977M	13	0	Damaging	1	Probably damaging	0	10	P	1*PS,2*PM,5*PP
c.2957C>T	p.S986F	13	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.2975C>T	p.P992L	13	0	Damaging	1	Probably damaging	0	5	P	2*PS,3*PM
c.3007G>A	p.A1003T	13	0	Damaging	1	Probably damaging	1	2	LP	2*PM,5*PP
c.3008C>T	p.A1003V	13	0	Damaging	1	Probably damaging	0	7	LP	2*PM,5*PP
c.3029A>C	p.K1010T	13	0	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.3044T>C	p.L1015P	13	0	Damaging	1	Probably damaging	0	0	LP	2*PM,4*PP
c.3053C>T	p.A1018V	13	0.07	Tolerated	1	Probably damaging	0	3	LP	2*PM,5*PP
c.3083A>G	p.K1028R	14	0.02	Damaging	0.998	Probably damaging	0	0	LP	2*PM,4*PP
c.3089G>A	p.G1030D	14	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.3095T>C	p.I1032T	14	0	Damaging	0.998	Probably damaging	0	0	LP	2*PM,4*PP
c.3098C>T	p.T1033I	14	0.01	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,4*PP

Nucleotide mutation	Protein alteration	Exon	Sift		PolyPhen 2		1000g	ExAc	Classification	Evidence of pathogenicity
			Score	Prediction	Score	Prediction				
c.3104G>T	p.G1035V	14	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.3121C>T	p.R1041W	14	0	Damaging	1	Probably damaging	0	5	LP	2*PM,5*PP
c.3122G>C	p.R1041P	14	0.01	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.3140A>T	p.D1047V	14	0.13	Tolerated	0.997	Probably damaging	0	0	LP	2*PM,4*PP
c.3155C>T	p.P1052L	14	0.06	Tolerated	0.998	Probably damaging	0	1	LP	2*PM,4*PP
c.3209C>G	p.P1070R	14	0	Damaging	1	Probably damaging	0	0	LP	2*PM,4*PP
c.3221C>T	p.A1074V	14	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.3263T>C	p.L1088S	15	0.2	Tolerated	1	Probably damaging	0	0	LP	1*PS,1*PM,3*PP
c.3271T>C	p.C1091R	15	0	Damaging	0.96	Probably damaging	0	0	LP	1*PM,4*PP
c.3274A>C	p.T1092P	15	0.11	Tolerated	0.832	Possibly damaging	0	0	US	1*PM,3*PP
c.3311G>A	p.C1104Y	15	0	Damaging	1	Probably damaging	0	1	P	1*PS,2*PM,5*PP
c.3316G>A	p.V1106I	15	0.15	Tolerated	0.984	Probably damaging	2	16	P	1*PS,2*PM,4*PP
c.3368C>T	p.P1123L	15	0.31	Tolerated	0.025	Benign	0	25	LB	1*BS,1*BP
c.3426G>C	p.Q1142H	16	0.16	Tolerated	0.007	Benign	0	3	LB	1*BS,1*BP
c.3443T>C	p.I1148T	16	0	Damaging	0.999	Probably damaging	0	5	LP	1*PM,5*PP
c.3446G>A	p.G1149E	16	0	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.3451C>G	p.R1151G	16	0	Damaging	1	Probably damaging	0	0	P	1*PS,1*PM,4*PP
c.3451C>T	p.R1151C	16	0	Damaging	1	Probably damaging	0	5	P	1*PS,1*PM,4*PP
c.3452G>A	p.R1151H	16	0.01	Damaging	1	Probably damaging	0	2	LP	3*PM,5*PP
c.3459G>T	p.W1153C	16	0	Damaging	1	Probably damaging	0	0	LP	3*PM,5*PP
c.3517G>A	p.E1173K	16	0	Damaging	1	Probably damaging	0	1	LP	3*PM,5*PP
c.3532A>G	p.T1178A	16	0	Damaging	0.988	Probably damaging	0	0	LP	1*PM,5*PP
c.3563T>G	p.L1188R	17	0	Damaging	0.998	Probably damaging	0	0	LP	1*PM,4*PP
c.3587A>G	p.D1196G	17	0	Damaging	1	Probably damaging	0	0	LP	1*PM,4*PP
c.3605C>G	p.A1202G	17	0	Damaging	1	Probably damaging	0	0	LP	1*PM,4*PP
c.3646G>A	p.V1216M	17	0	Damaging	1	Probably damaging	0	5	LP	1*PM,4*PP
c.3653T>C	p.L1218P	17	0	Damaging	0.999	Probably damaging	0	0	LP	1*PM,4*PP
c.3715G>T	p.V1239F	18	0	Damaging	0.998	Probably damaging	0	2	P	1*PS,1*PM,4*PP
c.3733C>G	p.P1245A	18	0	Damaging	1	Probably damaging	0	0	LP	1*PM,4*PP
c.3741C>G	p.H1247Q	18	0.17	Tolerated	1	Probably damaging	0	1	LP	1*PM,4*PP
c.3776G>T	p.G1259V	18	0	Damaging	0.997	Probably damaging	0	0	LP	1*PM,4*PP
c.3791T>C	p.M1264T	18	0	Damaging	0.998	Probably damaging	0	0	LP	1*PM,4*PP
c.3802G>A	p.G1268R	18	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.3809A>G	p.N1270S	18	0	Damaging	1	Probably damaging	0	18	P	1*PS,2*PM,5*PP
c.3818C>A	p.P1273Q	18	0	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,5*PP
c.3818C>T	p.P1273L	18	0	Damaging	1	Probably damaging	0	4	P	1*PS,2*PM,5*PP
c.3836A>G	p.D1279G	18	0.01	Damaging	1	Probably damaging	0	2	LP	2*PM,5*PP
c.3848C>T	p.A1283V	18	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.3859G>A	p.G1287S	18	0	Damaging	1	Probably damaging	0	3	P	1*PS,2*PM,5*PP
c.3877G>A	p.E1293K	18	0	Damaging	1	Probably damaging	0	1	LP	2*PM,5*PP
c.3884C>T	p.A1295V	18	0	Damaging	1	Probably damaging	0	0	P	1*PS,2*PM,4*PP
c.3896T>G	p.L1299R	18	0	Damaging	0.999	Probably damaging	0	0	P	1*PS,2*PM,4*PP
c.3982G>A	p.A1328T	19	0	Damaging	1	Probably damaging	0	0	LP	1*PM,5*PP
c.4003G>C	p.G1335R	19	0	Damaging	1	Probably damaging	0	0	LP	1*PM,5*PP
c.4057T>C	p.W1353R	20	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.4064G>A	p.G1355D	20	0	Damaging	1	Probably damaging	0	0	LP	2*PM,5*PP
c.4112T>C	p.L1371P	20	0.01	Damaging	1	Probably damaging	0	0	LP	1*PM,5*PP

PolyPhen 2/ SIFT: Software prediction programs used for sequence variant effect explanation; 1000g: 1000 Genomes Project; ExAc: Exome Aggregation Consortium.

Novel Variants

Among the 161 non-synonymous variants, 58 are novel and the chromatograms of these variants were illustrated in **Figures 2A, 2B, 2C and 2D**, grouped by the type of variation. None of which was found in the 503 normal individuals. Of these 58 novel variants, 16 are small deletions or insertions, 4 are nonsense variants, 6 are splice site variants, and 32 are missense variants. The other 103 variants have been recorded in the professional version of HGMD (Human Gene Mutation Database Professional, access date: 20 October, 2015).

Among the 16 small deletions or insertions, 13 alter the reading frame of *ATP7B* and result in production of a truncated dysfunctional protein. They are c.268_271del (p.K90FfsX10), c.367delG (p.A123PfsX30), c.695delC (p.P232QfsX30), c.1403_1416del (p.A468GfsX33), c.1545delT (p.G515GfsX9), c.1552_1553delTC (p.S518RfsX15), c.2043delC (p.S681SfsX15), c.2794_2795insGT (p.S932CfsX4), c.2853_2856delGAGA (p.Q951HfsX15), c.3377_3378delAC (p.H1126PfsX3), c.3766_3767dupCA (p.Q1256PfsX75), c.3901_3902insA (p.R1301KfsX3) and c.4162delG (p.A1388RfsX5). The other 3 small deletions or insertions, c.2316_2317insCTCTTTGTG (p.V772insLFV),c.2790_2792delCAT (p.I930del) and c.4005_4006insTTATAATGGGTTGCG (p.G1335insLXWVA) do not change the reading frame of *ATP7B*, but cause pro-

longed (p.V772insLFV and p.G1335insLXWVA) or shortened (p.I930del) dysfunctional ATP7B protein. Similarly, the 4 nonsense variants could result in production of a shortened, dysfunctional protein.

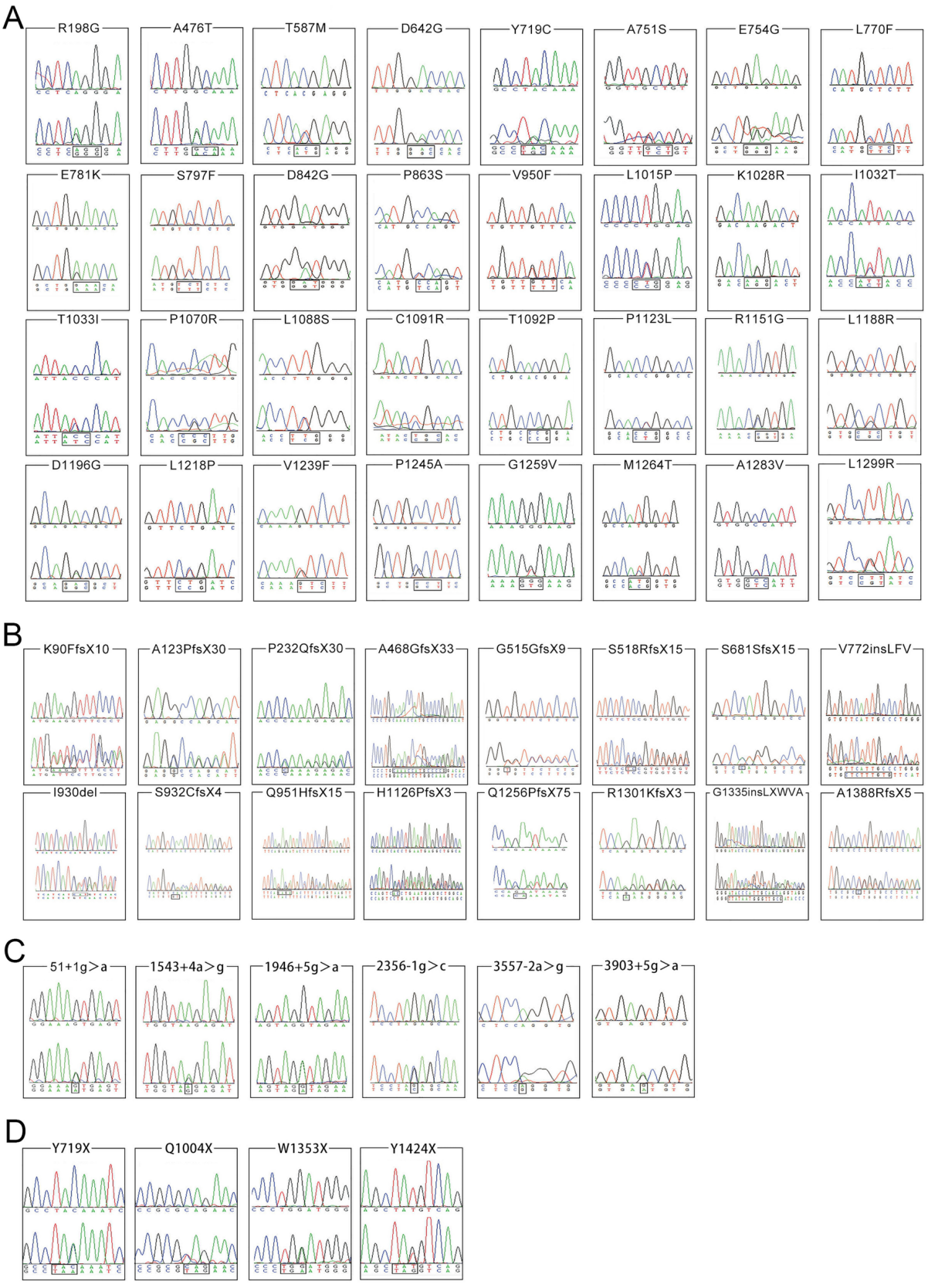

Figure 2: Chromatograms of 58 novel *ATP7B* variants identified in the present study. The lower chromatogram in each frame represents the variant, while the upper one represents the normal sequence. The c.4162delG variant is shown in reverse sequence, while the other 57 variants are illustrated in forward sequence. 2A, 2B, 2C and 2D respectively illustrates 32 missense changes, 16 small deletions or insertions, 6 splicing site variants as well as 4 nonsense variants.

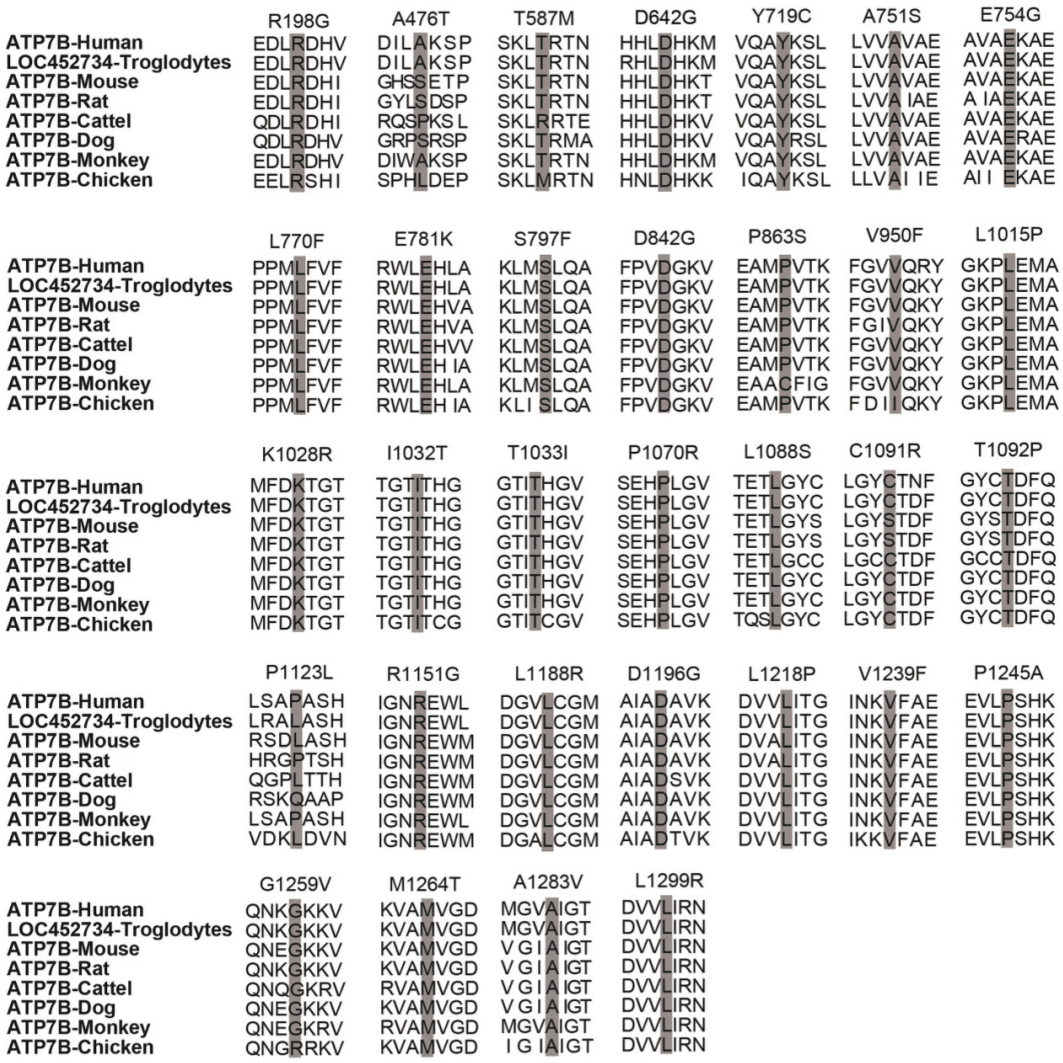

Figure 3: Homology comparisons of ATP7B protein sequences. The highlighted zones respectively indicate 32 novel missense variant sites among 8 species.

For six splice site variants, three (c.51+1g>a, c.2356-1g>c and c.3557-2a>g) are classified as 'pathogenic variants', the other three (c.1543+4a>g, c.1946+5g>a, 3903+5g>a) are 'variants with uncertain significance'. In addition, among the 32 novel missense variants, 2 (p.T587M, p.P1123L) are classified as 'likely benign variants', 2 (p.A476T, p.T1092P) as 'variants with uncertain significance', 5 (p.E781K, p.T1033I, p.R1151G, p.V1239F, p.L1299R) as 'pathogenic variants' and the other 23 as 'likely pathogenic variants' (**Table 3**). A homology search of the ATP7B protein in different species demonstrated that 32 missense variants occur within highly conserved regions of ATP7B protein (**Figure 3**). In total, among 58 novel variants, 25 are classified as 'pathogenic variants', 26 as 'likely pathogenic variants', 5 as 'variants with uncertain significance' and 2 as 'likely benign variants'.

Most Common Variants

Among the 632 WD patients, 569 patients (90%) were identified with homozygous or compound heterozygous potential disease-causing variants, 58 patients (9%) with one heterozygous variant and other 5 patients (1%) did not have any potential disease-causing variant. Therefore, 90% (569/632) of patients can be genetically diagnosed with WD. Among the 142 potential disease-causing variants including 78 'pathogenic variants' and 64 'likely pathogenic variants', 14 were relatively common in the WD patient cohort, each with an allelic frequency 1% or higher. These 14 most common disease-causing variants were found in 94% (537/569) of genetically diagnosed WD patients with two or more 'pathogenic' or 'likely pathogenic' variants. The allelic frequencies and numbers of patients with each of these variant are presented in **Table 4**. Notably, the three most prevalent variants, p.R778L, p.P992L and p.T935M, were

detected at least once in 78% (445/569) of genetically diagnosed WD patients. The allelic frequencies of p.R778L, p.P992L and p.T935M are 0.319, 0.155 and 0.077, respectively; all of which are 'pathogenic variants'.

Patients with More than Two Variants

Six patients carried three disease-causing variants and their 3-variant genotypes are described in **Figure 4**. As two patients had the same genotype, there are 5 unique 3-variant genotypes. Four of these 3-variant genotype include at least one 'likely pathogenic' variant, while one genotype (T935M+G943D+V1106I) consists of three 'pathogenic' variants.

Table 4. Common disease-causing variants within *ATP7B* among 569 WD patients.

Mutation	Domain affected	Number of patients			Allelic Frequencies	Classification
		WW	WM	MM		
p.R778L	TM4	270	235	64	0.319	Pathogenic
p.P992L	TM6/Ph	419	124	26	0.155	Pathogenic
p.T935M	TM5	482	86	1	0.077	Pathogenic
p.A874V	Td/TM5	529	37	3	0.038	Likely pathogenic
p.I1148T	ATP loop	530	38	1	0.035	Likely pathogenic
p.Q511X	Cu5	541	26	2	0.026	Pathogenic
p.G943D	TM5	543	25	1	0.024	Pathogenic
p.N1270S	ATP hinge	544	25	0	0.022	Pathogenic
p.R778Q	TM4	548	18	3	0.021	Pathogenic
p.R919G	Td/TM5	545	24	0	0.021	Pathogenic
p.V1216M	ATP bind	547	20	2	0.021	Likely pathogenic
p.V176SfsX28	Cu2	551	17	1	0.017	Pathogenic
c.1708-1g>c	Cu6	553	14	2	0.016	Pathogenic
p.V1106I	ATP loop	556	13	0	0.011	Pathogenic

WW: neither of chromosome carries mutation; WM: one chromosome carries mutation;
MM: both chromosomes carry mutations.

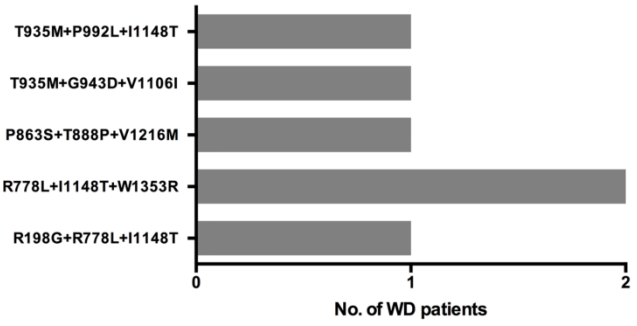

Figure 4: Three distinct ATP7B disease-causing variants co-occur in 6 WD patients.

Discussion

In the present study, the prevalence of *ATP7B* variants was systematically investigated in the largest Chinese WD cohort to date, with 632 patients and 503 normal controls. One hundred and sixty-one non-synonymous variants within the *ATP7B* gene were found in WD patients, including 58 novel variants. This study catalogs *ATP7B* variants in Han Chinese WD patients and expands the spectrum of *ATP7B* variants.

Another major contribution of the study is the classification of *ATP7B* variants. Based on the type of alterations, their predicted impact, and frequency between WD cases and controls, these 161 non-synonymous variants are classified as 'pathogenic variants' (N=78), 'likely pathogenic variants' (N=64), 'variants with uncertain significance' (N=8), 'likely benign variants' (N=4), and 'benign variants' (N=7). The observation that 90% (569/632) of these WD patients had two or more 'pathogenic' or 'likely pathogenic' variants demonstrates the clinical utility of the catalog and classification of *ATP7B* variants.

The 14 most common disease-causing variants were found at least once in 94% (537/569) of genetically diagnosed patients. Notably, the three most prevalent pathogenic variants, p.R778L, p.P992L and p.T935M, were detected in 78% (445/569) of the patients. These results demonstrate the feasibility of developing a rapid and cost-effective genetic test such as multiplex allele-specific PCR to screen for WD.

We found 6 unrelated WD patients (1.1%) carrying three disease-causing variants, and which was reported previously in Caucasian populations.[6] This observation is important because it suggests that two disease-causing variants may reside in a chromosome. The implication is that if an individual carries two disease-causing variants but his/her clinical features do not support the diagnosis of WD, it is necessary to test these variants in parents to identify the specific location of them. If the two variants reside in a chromosome, the individual is a heterozygous carrier and cannot be genetically diagnosed. If two variants reside in different chromosomes, then a genetic diagnosis of

WD can be made.

According to ACMG Standards and Guidelines,[12] we classified 4 variants as 'likely benign' variants, one of which (p.Q1142H) was previously considered as a pathogenic variant.[14] Tsai et al. found that Q1142 was located at the ATP pocket of ATP7B protein, and the amino acid replacement of the site would directly disrupt ATP7B function. However, the amino acid change in p.Q1142H was predicted to be tolerated by SIFT (score: 0.16) and benign by PolyPhen-2 (score: 0) software programs, this variant should be considered as 'likely benign'. This finding is important because the variant is relatively frequent and found in 19 WD patients (18 heterozygous and one homozygous). Caution should be made in interpreting the carrier of the Q1142 variant to avoid misdiagnosis and unnecessary treatment.

Sixty-three clinically diagnosed WD patients cannot be genetically diagnosed with WD because they were detected to carry only one disease-causing variant or none. Several factors may induce the observation. First, a subset of these 63 patients may have been misdiagnosed. For example, these patients may have been included in the patient cohort on the basis of false positive K-F rings. Second, large hemizygous deletions may occur in a subset of these patients and these alterations are difficult to be detected using the Sanger sequencing method. In fact, a hemizygous large deletion spanning the exon 20 and adjacent introns has previously been reported.[15] Other methods such as multiplex ligation-dependent probe amplification (MLPA) may be used to detect large deletions. Third, other genetic alterations outside the *ATP7B* coding region and adjacent splice sites as well as other cellular factors associated with WD may contribute to the clinical development of WD in these patients.[16]

In conclusion, the current study considerably expands the spectrum of *ATP7B* variants and provides classification of their clinical effects. These results improve the genetic diagnosis of suspected WD patients and facilitate genetic screening for WD among asymptomatic children in the general Chinese population.

Abbreviations

Cp: ceruloplasmin; K-F: Kayser-Fleischer; ACMG: American College of Medical Genetics and Genomics; HGMD: Human Gene Mutation Database; MLPA: multiplex ligation-dependent probe amplification.

Supplementary Material

Supplementary Table S1.
http://www.thno.org/v06p0638s1.pdf

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Conflicts of Interest

All authors reported no biomedical financial interests or potential conflicts of interest.

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