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Original Article

Mutational analysis of ATP7B in Chinese Wilson disease patients

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Abstract: Wilson Disease (WD) is an inborn error of copper metabolism inherited in an autosomal recessive manner caused by the mutations in the P-type ATPase gene (ATP7B). In this study, we screen and detect the mutations of the ATP7B gene in unrelated Chinese WD patients. A total of 68 individuals from ten provinces of China with WD were recruited. Of them, 43 were males and 25 were females, and their onset ages were from 1 to 48 years with a median onset age of 22.2 years. All the exons and exon/intron boundaries of ATP7B gene of the patients were sequenced and aligned to the referred ATP7B gene sequence. The results suggested that 66 of the 68 patents carried with at least one mutation and 48 different mutations were identified including 34 missense, one synonymous, two nonsense, two splicing, and nine frameshift mutations (five insertion and four deletion). Among these mutations, c.2333G>T, c.2310C>G, c.2975C>T, and c.3443T>C were the most prevalent mutants and c.2310C>G always linked with c.2333G>T. The eighth, 11th, and 18th exons carried more mutations (6/48, 5/48, and 5/48, respectively) than others. After comparing with the mutations reported previously, 22 out of the 48 mutations were identified as novel mutations. A popular algorithm, Polyphen-2, was used to predict the effects of the amino-acid substitution due to the mutations on the structure and function of ATP7B function and the predicted results indicated that all the missense mutations were unfavorable except c.121A>G and c.748G>A. Phenotype/genotype correlation analysis suggested that the patients with c.2975C>T or c.3809A>G often presented WD features before 12 years old while the patients with c.3443T>C almost presented WD after 12 years old. This is the first time to identify the common mutations contributing to early onset age in Chinese WD patients. Our study will broaden our knowledge about ATP7B mutations in WD patients.

Keywords: ATP7B, Chinese, mutational analysis, Wilson disease

Introduction

Wilson Disease (WD, MIM#277900) is an inborn error of copper metabolism inherited in an autosomal recessive manner caused by the mutations in the P-type ATPase gene (ATP7B) [1]. It is firstly reported by Dr. Samuel Alexander Kinnier Wilson in the year of 1912 and characterized by excess accumulation of intracellular hepatic copper and hepatic and neurologic abnormalities [2]. The frequency of WD disease was 1/30000 to 1/100000 worldwide while the carrier frequency reaches a high value of 1/90 [3-5]. In addition, a higher incidence is observed in Han population in China [5-7]. The disease is diagnosed on the basis of typical symptoms and conventional biochemical indicators, which include low serum concentration of ceruloplasmin and elevated excretion of urinary copper [8]. The prognosis of the WD patients is very poor and an early diagnosis and therapy is verified to help the patients to prevent lifelong neurological disability and liver cirrhosis and improve life quality remarkably [9-11].

ATP7B gene is located at 13q14.3 (about 80 kb) and consisted of 21 exons and 20 introns encoding a protein containing 1465 amino acids [12]. It plays a great role in transforming apoceruloplasmin into ceruloplasmin and excreting copper into biliary canaliculi. Defects of ATP7B will reduce the blood ceruloplasmin and affect hepatocytes. And also many other

organs including brain, eyes, and kidney will be involved [13, 14].

Mutation screening has revealed a larger number of genetic mutations in ATP7B gene in WD patients. In this study, we performed a mutational analysis of ATP7B by exon deep sequencing in 68 subjects in China Han population with WD and identified 48 mutations, of which 22 mutations have never been reported previously.

Materials and methods

Subjects

A total of 68 individuals from ten provinces of China with WD were recruited in the present study. Of them, 43 were males and 25 were females with the mean age of 24.0 (5-61) years and mean onset age of 22.2 (1-48) years. All the patients were informed and signed for agreement. The diagnostics were performed according to the following criteria: (1) liver or brain failure symptoms; (2) presence of K-F ring in the cornea by slit-lamp examination; (3) reduced serum ceruloplasmin (<0.20 g/l) and/or elevated 24-hour urinary copper excretion (>1.6 μmol/24 h) and/or hepatic copper content >250 mg per g of dry weight [15].

DNA extraction and sequencing

The peripheral blood leukocyte of the patients was collected and genomic DNAs were extracted with QIAamp blood kits (Qiagen, Hilden, Gemany) based on the manufacture's instructions. All of 21 exons of ATP7B and their ± 10 bp regions were analyzed by next sequencing.

Identification of mutations and prediction of deleterious variants

The sequencing results were aligned to referred ATP7B sequence (NM_000053.3) to figure out the mutations. The sequencing data from local 854 health controls were used to identify the polymorphisms. Allele and patient frequency of each mutation was also calculated. A novel mutant was identified when a genetic variant met all the following criteria: (1) no records in dbSNP database; (2) no reports in PubMed literatures. Then, *in silico* analysis tool PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) was used to predict the putative effects of each mutation on the structure and function of ATP7B protein.

Results

Clinical feathers and laboratory examinations of the included WD patients

We included 68 WD patients in the present study, of which 43 (63.2%) were males and 25 (36.8%) were females. Their onset age ranged from 1 to 48 years with the mean onset age of 22.2 years. The presence of K-F rings of 65 patients was examined and 50 (76.9%) of them were verified. Then serum ceruloplasmin and 24 h-urinary copper concentrations of the patients were determined. 67 of 68 patients (98.5%) owned low serum ceruloplasmin (<0.20 g/l) and 54 of 62 (87.1%) patients had high values of 24 h-urinary copper (>1.6 μmol/24 h). Further, the hepatic copper content of 19 patients was examined and that of 18 (94.7%) patients were >250 μg per g of dry weight. Based on the clinical analysis and laboratory examination, the phenotypes of the 68 WD patients were clarified into hepatic (32, 47.1%), hepatic and neurological (23, 33.8%), neurological (8, 11.8%), presymptomatic or no symptoms (5, 7.4%).

Identification of ATP7B genetic variants in Chinese patients with WD

The exons of ATP7B and 10 bps upstream and downstream of the exons of 68 Chinese WD patients were examined. All the sequencing results were aligned to the referred ATP7B gene sequence (NM_000053.3) and compared with the data from 854 health controls from China. Finally, 58 genetic variations including 48 mutations and eight SNPs were identified (**Tables 1** and **3**).

Among the 48 different mutations, 34 were missense, one was synonymous, two were nonsense, two were splicing, and nine were frameshift (five insertion and four deletion). The mutations were distributed in exon 1, 2, 5, 7, 8, 10-19, and 20 and intron 4 and 13. Exon 8 (6/48) was showed to more frequent to suffer the mutations, followed by exon 11 (5/48), exon 18 (5/48), exon 13 (4/48), and exon 16 (4/48) suggesting these exons might be more susceptible to Wilson disease in Chinese Han population, consisted with the previous studies [6, 16, 17]. 11 mutations were identified to locate at the transmembrane segments encoding region, nine at copper-(or silver)-translocating P-type ATPase segment region, six at haloacid deha-

Table 1. Information of ATP7B mutations and prediction of the functional effects of the mutations

NT change	Location	Functional Region	AA change	Polyphen-2		Mutation Type	Patient count	Patient Freq %	Allele count	Allele Freq %	Novelty
				Prediction	Score						
c.121A>G	Exon 1 15021707309		p.Asn41Asp	BENIGN	0.008	Missense	1	1.47	1	0.74	Novel
c.748G>A	Exon 2		p.Gly250Arg	BENIGN	0.002	Missense	1	1.47	1	0.74	Novel
c.898_902 delAAGTA	Exon 2	MBD3	p.Lys300Ter	NA	NA	Nonsense	1	1.47	1	0.74	Novel
EX2 DEL	Exon 2			NA	NA	Deletion	2	2.94	2	1.47	Novel
c.1708-1G>C	Intron 4			NA	NA	Splicing	2	2.94	2	1.47	Novel
c.1745_1746 delTA	Exon 5	MBD6		NA	NA	Deletion	1	1.47	1	0.74	Novel
c.1994T>G	Exon 7	TMS1	p.Met665Arg	PROBABLY DAMAGING	0.995	Missense	1	1.47	1	0.74	Novel
c.2012_2013 insATAT	Exon 7	TMS1		NA	NA	Insertion	1	1.47	1	0.74	Novel
c.2128G>A	Exon 8	TMS2	p.Gly710Ser	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.2231C>T	Exon 8	TMS3	p.Ser744Phe	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.2304_2305 insC	Exon 8	TMS4		NA	NA	Insertion	4	5.88	4	2.94	Novel
c.2308C>T	Exon 8	TMS4	p.Leu770Phe	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	Novel
c.2310C>G	Exon 8	TMS4	p.Leu770Leu	NA	NA	Synonymous	24	35.29	28	20.59	
c.2333G>T	Exon 8	TMS4	p.Arg778Leu	PROBABLY DAMAGING	1.000	Missense	31	45.59	35	25.74	
c.2506G>A	Exon 10	ATPase	p.Gly836Arg	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	Novel
c.2549C>T	Exon 10	ATPase	p.Thr850Ile	BENIGN	0.037	Missense	1	1.47	1	0.74	
c.2561A>G	Exon 10	ATPase	p.Glu854Gly	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	Novel
c.2593_2594 insGTCA	Exon 11	ATPase		NA	NA	Insertion	1	1.47	1	0.74	Novel
c.2605G>A	Exon 11	ATPase	p.Gly869Arg	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.2620G>C	Exon 11	ATPase	p.Ala874Pro	PROBABLY DAMAGING	1.000	Missense	1	1.47	2	1.47	
c.2621C>T	Exon 11	ATPase	p.Ala874Val	PROBABLY DAMAGING	1.000	Missense	6	8.82	6	4.41	
c.2662A>C	Exon 11	ATPase	p.Thr888Pro	PROBABLY DAMAGING	0.998	Missense	3	4.41	3	2.21	
c.2755C>G	Exon 12	ATPase	p.Arg919Gly	POSSIBLY DAMAGING	0.832	Missense	3	4.41	3	2.21	
c.2804C>T	Exon 12	TMS5	p.Thr935Met	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.2828G>A	Exon 12		p.Gly943Asp	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.2924C>A	Exon 13	TMS6	p.Ser975Tyr	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.2975C>T	Exon 13	TMS6	p.Pro992Leu	PROBABLY DAMAGING	1.000	Missense	10	14.71	11	8.09	
c.3007G>A	Exon 13	ATPase	p.Ala1003Thr	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.3056A>C	Exon 13		p.His1019Pro	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	Novel
c.3061-3C>A	Intron 13			NA	NA	Splicing	1	1.47	1	0.74	Novel
c.3140A>T	Exon 14		p.Asp1047Val	POSSIBLY DAMAGING	0.927	Missense	1	1.47	1	0.74	
c.3316G>A	Exon 15		p.Val1106Ile	POSSIBLY DAMAGING	0.863	Missense	5	7.35	5	3.68	
c.3377_3378 delAC	Exon 15			NA	NA	Deletion	2	2.94	2	1.47	Novel
c.3443T>C	Exon 16	ATP bind	p.Ile1148Thr	PROBABLY DAMAGING	0.999	Missense	9	13.24	10	7.35	
c.3445G>A	Exon 16	ATP bind	p.Gly1149Arg	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.3451C>T	Exon 16	ATP bind	p.Arg1151Cys	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.3452G>A	Exon 16	ATP bind	p.Arg1151His	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	

c.3584C>T	Exon 17	HAD	p.Ala1195Val	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	Novel
c.3677C>T	Exon 17	HAD	p.Thr1226Ile	PROBABLY DAMAGING	0.999	Missense	1	1.47	1	0.74	Novel
c.3679G>C	Exon 17	HAD	p.Ala1227Pro	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	Novel
c.3700delG	Exon 18	HAD		NA	NA	Deletion	1	1.47	1	0.74	Novel
c.3809A>G	Exon 18	HAD	p.Asn1270Ser	PROBABLY DAMAGING	1.000	Missense	4	5.88	4	2.94	
c.3843_3844 insT	Exon 18	HAD		NA	NA	Insertion	1	1.47	1	0.74	Novel
c.3884C>T	Exon 18		p.Ala1295Val	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.3901_3902 insA	Exon 18			NA	NA	Insertion	1	1.47	1	0.74	Novel
c.3960G>C	Exon 19		p.Arg1320Ser	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.3982G>A	Exon 19	TMS7	p.Ala1328Thr	PROBABLY DAMAGING	1.000	Missense	1	1.47	1	0.74	
c.4114C>T	Exon 20		p.Gln1372Ter	NA	NA	Nonsense	1	1.47	1	0.74	

Abbreviations: AA, amino acid; ATPase, copper-(or silver)-translocating P-type ATPase segment; ATP bind, ATP binding segment; HAD, haloacid dehalogenase-like hydrolases segment; MBD, mental-binding domain; NT, nucleotide; TMS, transmembrane segment.

Table 2. Linkage analysis of the mutations

EX2 Del	2												
c.1708-1G>C	0	2											
c.2304_2305 insC	0	0	4										
c.2310C>G	0	1	1	28									
c.2333G>T	1	1	1	28	35								
c.2621C>T	0	0	0	1	3	6							
c.2662A>C	0	0	0	0	0	0	3						
c.2755C>G	0	0	0	0	1	1	0	3					
c.2975C>T	0	0	1	0	0	0	0	1	10				
c.3316G>A	0	0	0	0	0	0	1	0	0	5			
c.3377_3378 delAC	0	0	0	0	0	1	0	0	0	0	2		
c.3443T>C	0	0	1	2	2	0	2	0	0	2	0	9	
c.3809A>G	0	0	1	1	2	0	0	0	0	0	0	0	4
	EX2 Del	c.1708-1G>C	c.2304_2305 insC	c.2310C>G	c.2333G>T	c.2621C>T	c.2662A>C	c.2755C>G	c.2975C>T	c.3316G>A	c.3377_3378 delAC	c.3443T>C	c.3809A>G

Table 3. Information of ATP7B SNPs

SNP	RS-ID	dbSNP freq	Hapmap freq	1000 G freq	Patient freq	Control freq
p.Lys832Arg	rs1061472	0.5	0.507	0.4753	0.6119	0.4940
p.Val1297Ile	rs148399850	0.012	0	0.0101	0.0448	0.0386
p.Ser406Ala	rs1801243	0.489	0.482	0.4414	0.6716	0.5024
p.Val456Leu	rs1801244	0.486	0.416	0.4423	0.6418	0.4952
p.Ala1003Ala	rs1801247	0.055	0	0.0568	0.0149	0.0193
p.Val1140Ala	rs1801249	0.498	0.5	0.4652	0.5970	0.4964
c.3093+6C>T	rs2282057	0.5	0	0.4753	0.5970	0.4976
p.Arg952Lys	rs7322774	0.499	0.504	0.4725	0.5970	0.4964

logenase-like hydrolases segment region, two at mental-binding region, and five at the ATP binding region. Then the allele and patient frequencies of every mutation were calculated. The top four allele frequencies were 35/136 (c.2333G>T), 28/136 (c.2310C>G), 11/136 (c.2975C>T), and 10/136 (c.3443T>C). And their corresponding patient frequencies were 31/68, 24/68, 10/68, and 9/68. After comparing with the previous reports, 22 out of the 48 mutations were identified to be novel and they were made up of all the splicing (2) and frameshift (9) mutations, one nonsense mutation, and 10 missense mutations.

Predicting the functional effects of mutations

We applied a popular algorithm, PolyPhen-2, to predict the effects of the mutations on ATP7B function. Polyphen-2 can predict the possible impact of an amino-acid substitution on the structure and function of a human protein by using straightforward physical and comparative considerations [17]. The predict results indicated that all the missense mutations were unfavorable except c.121A>G and c.748G>A, both of which were novel mutations (**Table 1**). And also two nonsense mutations, c.898_902 delAAGTA and c.4114C>T identified in our study should be most deleterious.

Identification of the linker mutations and identification of the SNPs

As shown in **Table 2**, the paired emerging of the mutations with an allele count of no less than two was analyzed to investigate the potential linkage between mutations. The resulted demonstrated that a pair of mutations, c.2310C>G and c.2333G>T, was most closely correlated. Then the SNPs of the patients were analyzed, as shown in **Table 3**, a total of eight SNPs were also identified and were compared with the frequencies in NCBI dsSNPs, Hapmap, 1000 genomes, and health controls.

Correlation between genotype and phenotype of each WD patient

66 out of the 68 WD patients (97.1%) harbored at least one mutation. As shown in **Table 4**, 19 out of the 68 patients (24.6%) harbored three mutations, 38 patients (60.6%) harbored two mutations, and 9 patients (14.8%) harbored one mutation. The c.2333G>T linked with c.2310C>G mutations exist in all patients who harbored three mutations except one and exist in 21 patients who had two mutations. Eight patients were found to harbor homozygous mutations. Four of the homozygous mutations were c.2333G>T and c.2310C>G homozygote. The genotype of each patient and their corresponding detailed phenotypes were also showed in **Table 4**. Furthermore, to investigate the correlation between genotype and phenotype of the patients, the synonymous mutation c.2310C>G were excluded and we established a genotype/phenotype matrix by plotting the onset ages of each patient on the ATP7B mutation background and grouping by phenotypes. The ATP7B mutation backgrounds of individual patient were classified by mutation type and numbers, such as Missense (M), Missense/Missense (M/M), Missense/Splicing (M/S), and Missense/Insertion (M/I). As shown in **Figure 1A**, for the patients with heterozygous mutations, the mutation types and distribution were different in different phenotype groups. The top three mutation types were M/M (15/27), M/I (6/27) in H group and M/M (11/20), M/D (3/20), and M/N (2/20) in H and N group. And only the number of M/M was over two in N group and Presymptomic/no symptoms group due to the limited amount of patients. For the 8 patients with homozygous mutations, 7 patients carried with only unique missense mutation while one patient carried with unique splicing mutation (**Figure 1B**). The patient frequency with onset age ≤ 12 years old in H group and N group were bigger than that in H and N group and Presymptomic/no symptoms group in regardless of the heterozygous and homozy-

Table 4. Phenotypes and genotypes of the WD patients

No.	Sex	Onset age	NT change	Mutation type	Phenotype	Presense of K-F ring	Serum ceruloplamin (g/L)	24h-urinary copper (μmol)	Liver copper content (μg/g)
WD001	female	11	c.2975C>T; c.2561A>G	heterozygous	H	+	0.07	8.63	1,434.00
WD002	male	41	c.3818C>A; c.3316G>A	heterozygous	N	+	0.09	5.98	NA
WD003	male	29	c.2975C>T	heterozygous	H	-	0.14	0.53	348.00
WD004	male	34	c.2333G>T; c.3982G>A	heterozygous	presymptomic or no symptoms	+	0.09	5.44	400.00
WD005	female	19	c.3901_3902 insA; c.2333G>T; c.2310C>G	heterozygous	H and N	+	0.02	4.88	NA
WD006	male	38	c.2333G>T; c.2310C>G	heterozygous	H	-	0.09	2.45	1,504.00
WD007	female	28	c.2975C>T; c.2755C>G	heterozygous	H	+	0.11	5.70	NA
WD008	male	29	c.2333G>T; c.2310C>G	heterozygous	N	+	0.03	4.50	NA
WD009	male	18	c.3443T>C; c.2662A>C	heterozygous	H and N	+	0.03	3.28	NA
WD010	female	29	c.2333G>T; c.2549C>T	heterozygous	H and N	+	0.14	2.33	NA
WD011	female	12	c.2975C>T; c.3445G>A; c.748G>A	heterozygous	H and N	+	0.04	3.09	1,331.00
WD012	male	9	c.3443T>C; c.1745_1746 delTA	heterozygous	N	+	0.03	2.50	295.80
WD013	male	38	c.2621C>T	heterozygous	H and N	+	0.03	6.15	NA
WD014	male	41	c.1708-1G>C; c.3960G>C	heterozygous	H and N	+	0.15	2.33	NA
WD015	male	21	c.2333G>T; c.3451C>T	heterozygous	H and N	-	0.02	2.72	764.00
WD016	male	27	c.2620G>C	homozygous	N	+	0.02	9.92	NA
WD017	male	10	c.2975C>T; c.3843_3844 insT	heterozygous	H	+	0.12	5.17	NA
WD018	female	23	c.2333G>T; c.2506G>A; c.2310C>G	heterozygous	H	+	0.02	3.53	1,109.00
WD019	male	24	c.3443T>C; c.3316G>A	heterozygous	presymptomic or no symptoms	-	0.06	1.47	1,110.70
WD020	female	27	c.3443T>C; c.3316G>A	heterozygous	H	-	0.07	4.11	956.00
WD021	female	25	c.3809A>G; c.2804C>T	heterozygous	H	+	0.38	5.13	NA
WD022	male	31	c.2333G>T; c.2310C>G; c.2231C>T	heterozygous	H	+	0.08	59.18	NA
WD023	female	14	c.3884C>T	heterozygous	H	+	0.05	4.73	NA
WD024	female	5	c.3061-3C>A	homozygous	H	+	0.03	2.34	NA
WD025	female	5	c.2333G>T; c.3679G>C; c.2310C>G	heterozygous	H	-	0.02	1.25	NA
WD026	female	32	c.2333G>T; c.2310C>G; c.2621C>T	heterozygous	H and N	+	0.03	5.86	NA
WD027	male	8	c.2304_2305 insC; c.3809A>G	heterozygous	N	+	0.04	1.92	421.00
WD028	male	11	c.2975C>T; c.2304_2305 insC	heterozygous	H	+	0.07	4.70	NA
WD029	male	12	c.2333G>T; c.2924C>A; c.2310C>G	heterozygous	H and N	+	0.07	5.53	NA
WD030	male	28	c.2333G>T; c.3443T>C; c.2310C>G	heterozygous	N	+	0.03	1.28	NA
WD031	male	18	c.2333G>T; c.2310C>G; c.121A>G	heterozygous	H	-	0.06	2.13	950.00
WD032	male	4	c.3809A>G; c.2333G>T; c.2310C>G	heterozygous	presymptomic or no symptoms	+	0.04	5.95	395.00
WD033	female	23	c.2333G>T; c.2310C>G	homozygous	H and N	+	0.04	1.42	1,161.00
WD034	male	21	c.2333G>T; c.2310C>G	homozygous	presymptomic or no symptoms	+	0.03	6.55	NA
WD035	female	37	c.3140A>T; c.2333G>T	heterozygous	H and N	+	0.03	3.15	NA
WD036	male	25	c.3700 delG; c.1994T>G	heterozygous	H and N	+	0.02	NA	NA
WD037	female	12	c.3584C>T; c.2333G>T; c.2310C>G	heterozygous	H	+	0.20	20.51	NA
WD038	male	4	c.2975C>T; c.2662A>C	heterozygous	H	-	0.03	1.31	NA

WD039	male	36	c.3377_3378 delAC	heterozygous	presymptomic or no symptoms	NA	0.19	NA	NA
WD040	male	42	c.2593_2594insGTCA; c.3316G>A	heterozygous	H	+	0.03	1.97	NA
WD041	female	14	EX2 DEL; c.3056A>C	heterozygous	H and N	+	0.05	55.08	NA
WD042	female	8	c.2333G>T; c.2012_2013 insATAT	heterozygous	H	+	0.04	4.19	NA
WD043	male	24	c.898_902 delAAGTA; c.2308C>T	heterozygous	H and N	+	0.09	12.19	NA
WD044	male	8	c.2975C>T; c.2828G>A	heterozygous	N	+	0.05	NA	NA
WD045	male	21	c.3007G>A; c.3677C>T	heterozygous	H	-	0.12	2.53	1,080.00
WD046	male	10	c.2333G>T; c.2310 C>G	heterozygous	H	-	0.02	3.92	1,030.30
WD047	female	24	c.2333G>T; c.2310C>G	homozygous	H	+	0.02	2.08	NA
WD048	male	18	c2333G>T; c3452G>A; c.2310C>G	heterozygous	H	-	0.02	4.10	NA
WD049	male	30	c.2975C>T; c.2605G>A	heterozygous	H	-	0.06	2.81	908.00
WD050	male	13	c.3443T>C; c.2662A>C	heterozygous	H and N	+	0.12	8.61	NA
WD051	male	22	c.2333G>T; c.2304_2305 insC; c.2310C>G	heterozygous	H	+	0.03	5.39	NA
WD052	female	48	c.2621C>T; c.3377_3378 delAC	heterozygous	H and N	+	NA	14.45	NA
WD053	male	1	c.2333G>T; c.1708-1G>C; c.2310C>G	heterozygous	N	+	0.05	NA	111.00
WD054	female	8	c.2975C>T	homozygous	H and N	+	0.09	8.95	NA
WD055	male	24	c.4114C>T; c.2128G>A	heterozygous	H and N	+	0.03	5.26	NA
WD056	female	24	c.3443T>C	homozygous	H	+	0.03	5.47	NA
WD057	male	7	c.2333G>T; c.3809A>G	heterozygous	H	+	0.04	7.08	NA
WD058	female	23	c.2333G>T; c.3443T>C; c.2310C>G	heterozygous	H and N	+	0.07	5.41	1,540.00
WD059	male	19	c.2333G>T; c.2310 C>G	homozygous	H and N	+	0.04	5.39	NA
WD060	male	13	c.3443T>C; c.2304_2305 insC	heterozygous	H	+	0.03	5.47	NA
WD061	male	24	c.3316G>A	heterozygous	H	-	0.07	13.92	NA
WD062	male	35	c.2333G>T; c.2310C>G; c.2621C>T	heterozygous	H and N	+	0.02	14.26	NA
WD063	female	40	c.2333G>T; c.2310C>G; c.2621C>T	heterozygous	H and N	-	0.03	NA	NA
WD064	male	27	c.2755C>G; c.2333G>T; c.2310C>G	heterozygous	H and N	NA	0.05	NA	NA
WD065	male	8	c.2333G>T; EX2 DEL	heterozygous	H	+	0.02	33.69	NA
WD066	female	41	c.2621C>T; c.2755C>G	heterozygous	H	NA	0.07	1.95	NA
WD067	male	34	-	-	H	-	0.12	0.13	NA
WD068	female	42	-	-	H	+	0.17	0.03	NA

Abbreviations: H, hepatic; H and N, hepatic and neurological; N, neurological.

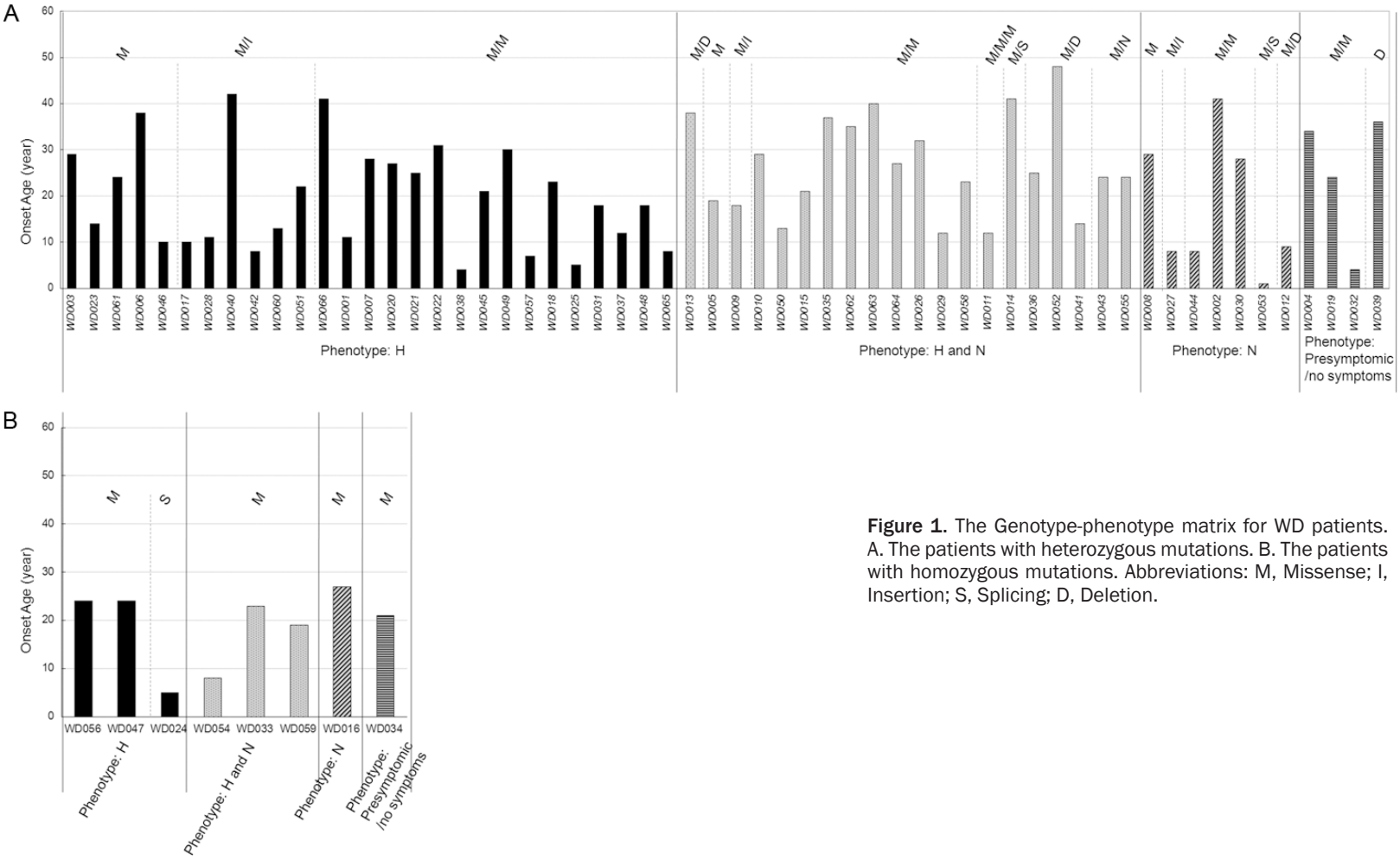

Figure 1. The Genotype-phenotype matrix for WD patients. A. The patients with heterozygous mutations. B. The patients with homozygous mutations. Abbreviations: M, Missense; I, Insertion; S, Splicing; D, Deletion.

Table 5. Information of ATP7B mutations in WD patients with onset age less than 12 years old

Mutation	Location	Function region	Mutation type	No. of Total patient with the mutation	No. of ≤ 12 years patient with the mutation
c.1708-1G>C	Intron 4		Splicing	2	1
c.1745_1746 delTA	Exon 5	MBD6	Deletion	1	1
c.2012_2013insATAT	Exon 7	TMS1	Insertion	1	1
c.2304_2305 insC	Exon 8	TMS4	Insertion	4	2
c.2333G>T	Exon 8	TMS4	Missense	31	9
c.2561A>G	Exon 10	ATPase	Missense	1	1
c.2662A>C	Exon 11	ATPase	Missense	3	1
c.2828G>A	Exon 12		Missense	1	1
c.2924C>A	Exon 13	TMS6	Missense	1	1
c.2975C>T	Exon 13	TMS6	Missense	10	7
c.3061-3C>A	Intron 13		Splicing	1	1
c.3443T>C	Exon 16	ATP bind	Missense	9	1
c.3445G>A	Exon 16	ATP bind	Missense	1	1
c.3584C>T	Exon 17	HAD	Missense	1	1
c.3679G>C	Exon 17	HAD	Missense	1	1
c.3809A>G	Exon 18	HAD	Missense	4	3
c.3843_3844insT	Exon 18	HAD	Insertion	1	1
c.748G>A	Exon 2		Missense	1	1
EX2 DEL	Exon 2		Deletion	2	1

gous conditions although the difference was not significant (**Figure 1**). And the mutation c.2333G>T were most prevalent in four groups while the other top prevalent mutations were not consistent. c.2975C>T was prevalent in H group, c.2621C>T was prevalent in H and N group, and c.3443T>C was prevalent in N group. The different mutation types and distribution in four groups might result in the difference of onset age.

To further study the correlation between onset age and ATP7B mutation background, the mutations in patients with onset age less than 12 years were analyzed. As shown in **Table 5**, a total of 19 mutations were identified and most the mutations located in TMS and HAD regions. Out of the mutations, 13 were missense mutations, three were insertion mutations, two were splicing mutations, and two were deletion mutation. The patients with c.2975C>T or c.3809A>G mostly presented WD features before 12 years old while the patients with c.3443T>C almost presented WD features after 12 years old. On the other hand, the patients with c.2333G>T, the prevalent mutations in Chinese WD patients, might have no little contribution on the onset age that the patients frequency with c.2333G>T with onset age ≤ 12 years old (9/31) was similar with that in total patients (19/66).

Discussion

In the present study, we performed mutational analysis of 68 WD patients from China. Finally, 48 mutations were identified, of which 22 mutations were novel. And the rate of mutation detection was 97.1% (66/68).

Up to now, more than 500 mutations have been reported in many different populations and the frequency and type of mutations are closely related with the geographic areas and ethnicities. For example, the most prevalent mutation in Europe is H1069Q missense mutation of exon 14, which is associated with neurological manifestation with allele frequency of 30%-65% of allele [18-20]. c.441_427del is present in 61.5% of patients in Sardinia [21], mutation M645R is frequent in Spain with a high value over 55% [22], and R778L in exon 8 associating with hepatic presentation is most frequent in China Han population with an allele frequency of 12-39% [6, 23-25]. In this study, the allele frequency of R778L is 25.7% and the mutation is present in 45.6% patients. And we did not found the mutations H1069Q, c.441_427del, and M645R. Another most prevalent mutation

in the study was p.P992L with an allele frequency of 8.1%, similar with the result in a previous north China WD patient cohort study (6.1%) [17]. Consistent with the results in other studies performed in Chinese population, we also found that the mutation p.L770L, was completely linked with the most prevalent mutation p.R778L [6, 17]. Although p.L770L was a synonymous mutation, it was rare in the normal population (only five of the 854 healthy controls in the present study were identified to harbor this mutation) while appeared frequently in WD patients (24/68) and seemed to be a polymorphism in European population. All the exons and exon/intron boundaries of ATP7B gene were analyzed in our study and the rate of mutation detection is 97.1%, more than that in the previous reports. It also means that almost all the WD patients own pathological mutations in exons and exon/intro boundaries of ATP7B and tell us that a deep sequencing of the ATP7B exons and flanking regions other than the whole length will be economical and relative accuracy to help diagnose WD. The failure of detecting any mutations in the two patient might be because that some unknown mutations have not been detected due to the they may locate on the outside of the exons and flanking regions, such as the promoter, introns or other DNA control regions [26, 27].

Although there are a number of studies analyzing the mutations of ATP7B in WD patients around the world, the detailed and specific correlation between phenotypes and mutation in WD patients is not well known. In the present study, we established a phenotype/genotype matrix by plotting onset age on ATP7B mutation types according to phenotype groups and we found the mutation types and distribution in different groups were different. Further analysis suggested that the mutations c.2975C>T or c.3809A>G contributed to early onset age in WD patients. This is the first time to identify the common mutations in the Chinese WD patients with an early onset age of less than 12 years old. A further multicenter study with a larger scale of WD patients needs to be performed to clarify the conclusions.

Conclusions

In this study, we perform mutational analysis of the ATP7B gene in 68 unrelated Chinese WD patients. The results suggested that mutations are present in 66 of the 68 patents and a total of 48 different mutations are identified, of which 22 mutations have never been reported previously. Among these mutations, c.2333G>T, c.2310C>G, c.2975C>T, and c.3443T>C are the most prevalent mutants. Phenotype/genotype correlation analysis suggests that the patients with c.2975C>T or c.3809A>G often present WD features before 12 years old while the patients with c.3443T>C almost present WD after 12 years old. Our study will broaden our knowledge about ATP7B mutations and their associations with phenotypes in WD patients in China.

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Disclosure of conflict of interest

None.

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