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ORIGINAL ARTICLE

Molecular analysis of 53 Chinese families with Wilson's disease: Six novel mutations identified

Zhongyan Xiao[1,2] | **Yuan Yang**[3,1] | **Hui Huang**[1] | **Haiyan Tang**[1] | **Liqun Liu**[4] | **Jianguang Tang**[5] | **Xiaoliu Shi**[1]

[1]Department of Medical Genetics, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China

[2]Department of Gastroenterology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China

[3]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), ICU, Peking University Cancer Hospital & Institute, Beijing, 100142, People's Republic of China

[4]Department of Pediatrics, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China

[5]Department of Neurology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China

Correspondence
Xiaoliu Shi, Department of Medical Genetics, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China.
Email: shixl6@csu.edu.cn

Abstract

Background: Wilson's disease (WD) is a rare autosomal recessive inherited disorder that is induced by defects of the *ATP7B* gene and characterized by damage to the liver and nervous system caused by aberrant copper metabolism. The identification of pathogenic mutations on two homologous chromosomes has become the gold standard for the diagnosis of WD.

Methods: Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) were combined to establish a genetic diagnosis for patients from 53 unrelated Chinese WD families.

Results: Biallelic mutations were detected by Sanger sequencing in 50 of the probands, while single heterozygous mutations were detected in the remaining three probands. A total of 45 diverse pathogenic mutations were detected, and 6 previously unreported mutations were involved. Five asymptomatic patients were screened from 85 family members of 38 probands participating in the study.

Conclusion: This study contributes to the enlargement of the mutational spectrum of the *ATP7B* gene among the population of China and highlights the significance of genetic testing for asymptomatic patients.

KEYWORDS

ATP7B, mutation, Wilson's disease

1 | INTRODUCTION

Wilson's disease (WD, OMIM#277900) is an autosomal recessive inherited disease that is characterized by liver cirrhosis and brain degenerative diseases dominated by basal ganglia damage caused by a copper metabolism disorder. In 1912, Kinnier Wilson first described the clinical and genetic characteristics of a group of WD patients in some detail (Compston, 2009). *ATP7B* (OMIM#606882) is the only disease-causing gene of WD that consists of 21 exons and 20 introns (approximately 80 kb), and it is located at 13q14.3 (Bull et al., 1993). The P-type ATPase is encoded by *ATP7B* gene and plays an important role in the transformation of apoceruloplasmin into holoceruloplasmin and the excretion of excess copper in bile (Petrukhin et al., 1994).

A prevalence of 1/100,000–1/30,000 and a carrier frequency of 1/90 based on previous clinical studies has been recognized and cited extensively (Ala et al., 2007). A higher

prevalence is observed in Asian countries than in Western countries. From 2010 to 2012, Chinese scholars conducted two epidemiological surveys based on clinical studies in three counties of the Anhui Province. In all, 153,370 people were investigated, and the estimated prevalence of WD was 1/17,000 (Hu et al., 2011; Wenbin et al., 2012). With the identification of the *ATP7B* gene and the development of technology, genetic testing has gradually become a conventional detection method and a useful tool in epidemiological investigations. In recent years, several epidemiological surveys based on *ATP7B* gene sequencing have been respectively conducted in the United Kingdom, France, South Korea, and Hong Kong. Based on these surveys, the estimates of the carrier frequency of disease-causing mutations were raised to approximately 1/40 (Coffey et al., 2013; Collet et al., 2018; Jang et al., 2017; Mak et al., 2008), which was higher than the previous estimation. A cause of this tremendous difference may be the incomplete penetrance (Sandahl et al., 2020), which needs to be further studied.

The age at onset of WD patients was mainly concentrated between the ages of 5 and 35 years. However, patients aged 8 months to 72 years have also been reported in previous studies (Abuduxikuer et al., 2015; Ala et al., 2005). The initial symptoms of WD patients can be hepatic manifestations (40%–50%), neurological manifestations (30–40%) or Psychiatric manifestations (10%–25%) (Cleymaet et al., 2019). In most sufferers, the slow onset of the disease is associated with gradual aggravation. The state of the illness could fluctuate during the course of disease. A few patients have acute onset, and stroke-like onset can even appear (Pan et al., 2018).

As a treatable genetic disease, early diagnosis and decoppering chelation are of great significance (Członkowska et al., 2018). Nevertheless, the disease has strong clinical heterogeneity and sometimes defies diagnosis by clinical experience alone. Genetic testing has become an increasingly important method of early, rapid, and accurate diagnosis. In 2001, participants at the International Liver Congress in Leipzig, Germany developed a diagnostic scoring system for WD ("8th International Conference on Wilson Disease and Menkes Disease. Leipzig, Germany, April 16–18, 2001. Abstracts," 2001), in which the identification of two allelic mutations within the *ATP7B* gene were deemed to be the gold standard for a definite diagnosis.

2 | MATERIALS

2.1 | Editorial policies and ethical considerations

The contents of the research conformed to the moral code of the Declaration of Helsinki. Authorization was obtained from the Review Board of the Second Xiangya Hospital of Central South University. Informed consent was acquired from the participants or their guardians after adequate explanation.

2.2 | Object

Fifty-three unrelated Chinese WD patients (23 males and 30 females) were enrolled in study and are numbered 1–53. All the enrolled patients went to the Second Xiangya Hospital of Central South University and were diagnosed according to the Leipzig scoring system from 2015 to 2020, and some of them had been diagnosed before at other hospitals. In addition, a total of 85 members from 38 of these families were recruited for the study.

3 | Experimental methods

3.1 | Sample collection and DNA extraction

Peripheral blood from probands and their relatives was drawn. Genomic DNA was extracted (QIAGEN, Germany) and stored at - 20 ℃.

3.2 | Genetic testing and result interpretation

Primers were designed through the Primer3 online website (https://primer3.ut.ee/) and verified by primer-blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast). Carte blanche was given to Tsingke Biotech (Hunan, China) for the synthesis of primers (Table S1). Polymerase chain reaction (PCR) was performed to amplify promoter region, all 21 exons and their flanking introns of *ATP7B* gene of the probands (RefSeq: NM_000053.4). The products were verified by agarose gel electrophoresis and then delivered to Biosune Biotech for sequencing by an ABI 3730XL Genetic Analyzer (ABI). To explore the distribution of each mutation in the family and identify potential patients, we screened the corresponding mutation sites in 85 willing individuals, including the probands' parents, siblings and the next generation, from 38 families. The prediction of the damaging effects of detected mutations was accomplished by bioinformatic analysis websites such as Sift, Polyphen2, Human Splicing Finder, and Mutation Taster. Comprehensive assessments of the clinical effects of all the mutations were performed according to the ACMG guidelines (Richards et al., 2015). To screen for potential intragenic deletions/duplications of the *ATP7B* gene, MLPA was performed in the probands with only a single heterozygous mutation detected through Sanger sequencing.

The implementation of MLPA was supported by Primbio Genes Biotech.

4 | RESULTS

By direct sequencing, we detected 45 different *ATP7B* gene mutations that could be evaluated as "pathogenic" or "likely pathogenic" according to the ACMG guidelines, including 26 missense mutations, 5 splicing mutations, 4 nonsense mutations, 1 in-frame deletion, 8 frameshift mutations, and 1 synonymous mutation. Among them, six mutations, including three missense mutations (NM_000053.4: c.3215G>T, c.3265G>A, c.3542T>A) and three frameshift mutations (NM_000053.4: c.631_632delAA, c.2887_2887delC, c.2932_2933insG) (Figure 1a–f), have not been reported before. MLPA was performed in three of the probands (No.46, 52 and 53) in whom only one mutation was detected but no large intragenic deletions/duplications of *ATP7B* gene were found (Figure S1).

Most of the probands involved in this study were compound heterozygotes (38/53), while the others were homozygotes (12/53) and heterozygotes (3/53) (Table S2). The distribution and allele frequency of all the mutations are shown in Figure 1g and Table 1, respectively. Most of the mutations occurred in exons 8, 11, 12, 13, and 16, accounting for 67.0% (69/103) of all. The most frequent mutations were NM_000053.4: c.2333G>T, p.Arg778Leu (allele frequency: 22.64%) and NM_000053.4: c.2975C>T, p.Pro992Leu (allele frequency: 13.21%), which was in accordance with a previous report (Li et al., 2011). A total of 85 family members participating in this study consisted of five asymptomatic patients, 74 heterozygous carriers and 6 normal individuals. All the five asymptomatic patients were siblings of probands from families 8, 11, 12, 15, and 29, respectively.

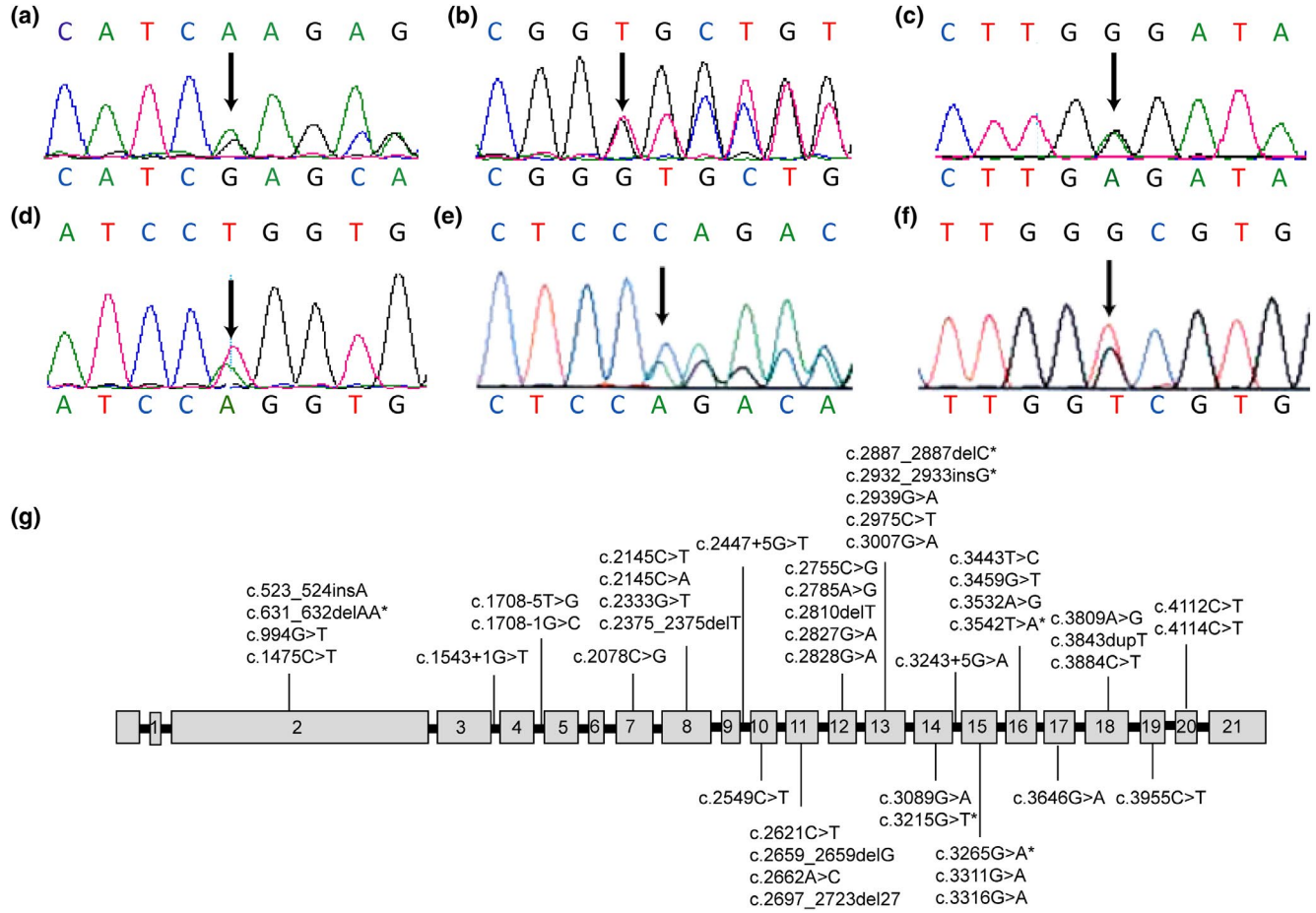

FIGURE 1 Mutation spectrum of the *ATP7B* gene (NM_000053.4) in this study. (a)-(e) Six novel mutations detected in this study which were indicated by arrows. The lower nucleotide sequence is the abnormal sequence and the upper one is the normal. (a) c.631_632delAA (b) c.2932_2933insG (c) c.3265G>A (d) c.3542T>A (e) c.2887_2887delC (f) c.3215G>T (g) The distribution of 45 mutations on the *ATP7B* gene; novel mutations are noted with a * behind

TABLE 1 The 45 pathogenic/likely pathogenic mutations of *ATP7B* gene (NM_000053.4) identified in the 53 probands

Nucleotide change	Amino acid change	Location	Mutation type	Area of protein	Allele frequency (%)	Minor allele frequency (%)		Classification according to ACMG
						1000G	ExAC	
c.523_524insA	p. Val176Serfs*28	Exon2	Frameshift	Cu2	1.89	—	0.0025	Pathogenic (PVS1+PM2+PP3+PP5)
c.631_632delAA[a]	p.Lys211Glufs*12	Exon2	Frameshift	Cu2/Cu3	0.94	—	—	Pathogenic (PVS1+PM2+PP3+PP5)
c.994G>T	p.Glu332*	Exon2	Nonsense	Cu3/Cu4	0.94	—	—	Pathogenic (PVS1+PM2+PP3+PP5)
c.1475T>C	p.Leu492Ser	Exon2	Missense	Cu5	1.89	—	—	Likely pathogenic (PS3+PM3+PP3+PP5)
c.1543+1G>T		Intron2	Splicing	Cu5	0.94	—	—	Pathogenic (PVS1+PM2+PP3+PP5)
c.1708-5T>G		Intron4	Splicing	Cu6	0.94	—	0.0008	Likely pathogenic (PM2+PM3+PP3+PP5)
c.1708-1G>C		Intron4	Splicing	Cu6	1.89	0.02	0.003	Pathogenic (PVS1+PM2+PP3+PP5)
c.2078C>G	p.Ser693Cys	Exon7	Missense	TM2	0.94	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.2145C>A	p.Tyr715*	Exon8	Nonsense	TM2/TM3	0.94	—	—	Pathogenic (PVS1+PM2+PP3+PP5)
c.2145C>T	p.Tyr715=	Exon8	Synonymous	TM2/TM3	0.94	—	0.01325	Likely pathogenic (PM2+PM3+PP3+PP5)
c.2333G>T	p.Arg778Leu	Exon8	Missense	TM4	22.64	—	0.016	Pathogenic (PS4+PM1+PM2+PP3+PP5)
2375_2375delT	p.Leu792Argfs*15	Exon9	Frameshift	TM4/Td	0.94	—	—	Pathogenic (PVS1+PM2+PP3)
c.2447+5G>T		Intron9	Splicing	TM4/Td	0.94	—	—	Pathogenic (PVS1+PM2+PP3)
c.2549C>T	p.Thr850Ile	Exon10	Missense	Td	2.83	—	0.0008	Likely pathogenic (PM2+PM3+PP3+PP5)
c.2621C>T	p.Ala874Val	Exon11	Missense	Td/TM5	4.72	—	0.007	Likely pathogenic (PM2+PM3+PP3+PP5)
c.2659_2659delG	p.Ala887Leufs*14	Exon11	Frameshift	Td/TM5	0.94	—	-	Pathogenic (PVS1+PM2+PP3+PP4)
c.2662A>C	p.Thr888Pro	Exon11	Missense	Td/TM5	0.94	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.2697_2723del27	p.Ile889_Gln907del	Exon11	In-frame deletion	Td/TM5	0.94	—	0.0017	Likely pathogenic (PM2+PM3+PP3+PP5)

(Continues)

TABLE 1 (Continued)

Nucleotide change	Amino acid change	Location	Mutation type	Area of protein	Allele frequency (%)	Minor allele frequency (%)		Classification according to ACMG
						1000G	ExAC	
c.2755C>G	p.Arg919Gly	Exon12	Missense	Td/TM5	3.77	—	0.0017	Likely pathogenic (PM1+PM2+PP3+PP5)
c.2785A>G	p.Ile929Val	Exon12	Missense	TM5	0.94	—	0.09	Likely pathogenic (PM1+PM2+PP3+PP5)
c.2810delT	p.Val937Glyfs*5	Exon12	Frameshift	TM5	0.94	—	—	Pathogenic (PVS1+PM2+PP3+PP5)
c.2827G>A	p.Gly943Ser	Exon12	Missense	TM5	0.94	—	0.0017	Likely pathogenic (PM2+PM3+PP3+PP5)
c.2828G>A	p.Gly943Asp	Exon12	Missense	TM5	0.94	—	0.02	Likely pathogenic (PM2+PM3+PP3+PP4)
c.2887_2887delC[a]	p.Gln963Argfs*4	Exon13	Frameshift	TM5/TM6	0.94	—	—	Pathogenic (PVS1+PM2+PP3+PP5)
c.2932_2933insG[a]	p.Val978Glyfs*50	Exon13	Frameshift	TM6	0.94	—	—	Pathogenic (PS4+PM2+PP3)
c.2939G>A	p.Cys980Tyr	Exon13	Missense	TM6	0.94	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.2975C>T	p.Pro992Leu	Exon13	Missense	TM6/Ph	13.21	—	0.004	Pathogenic (PS4+PM2+PM3+PP3+PP5)
c.3007G>A	p.Ala1003Thr	Exon13	Missense	TM6/Ph	0.94	0.02	0.002	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3089G>A	p.Gly1030Asp	Exon14	Missense	Ph	0.94	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3215G>T[a]	p.p.Gly1072Val	Exon14	Missense	ATP loop	0.94	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3243+5G>A		Intron14	Splicing	ATP loop	0.94	—	0.037	Likely pathogenic (PM2+PM5+PP3+PP5)
c.3265G>A[a]	p.Gly1089Arg	Exon15	Missense	ATP loop	0.94	—	—	Likely pathogenic (PM2+PM5+PP3+PP5)
c.3311G>A	p.Cys1104Tyr	Exon15	Missense	ATP loop	0.94	—	0.0083	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3316G>A	p.Val1106Ile	Exon15	Missense	ATP loop	1.89	0.04	0.013	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3443T>C	p.Ile1148Thr	Exon16	Missense	ATP loop	3.77	—	0.004	Likely pathogenic (PM2+PM3+PP3+PP5)

(Continues)

TABLE 1 (Continued)

Nucleotide change	Amino acid change	Location	Mutation type	Area of protein	Allele frequency (%)	Minor allele frequency (%)		Classification according to ACMG
						1000G	ExAC	
c.3459G>T	p.Trp1153Cys	Exon16	Missense	ATP loop	0.94	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3532A>G	p.Thr1178Ala	Exon16	Missense	ATP loop	2.83	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3542T>A[a]	p.Leu1181Gln	Exon16	Missense	ATP loop	0.94	—	—	Likely pathogenic (PM2+PM3+PP3+PP4)
c.3646G>A	p.Val1216Met	Exon17	Missense	ATP bind	1.89	—	0.004	Likely pathogenic (PM1+PM2+PP3+PP5)
c.3809A>G	p.Asn1270Ser	Exon18	Missense	ATP hinge	1.89	—	0.014	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3843dupT	p.Val1282Cysfs*22	Exon18	Frameshift	ATP hinge	0.94	—	—	Pathogenic (PS4+PM2+PP3)
c.3884C>T	p.Ala1295Val	Exon18	Missense	ATP hinge/TM7	0.94	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.3955C>T	p.Arg1319*	Exon19	Nonsense	ATP hinge/TM7	1.89	—	0.004	Pathogenic (PVS1+PM2+PP3)
c.4112C>T	p.lle1371Pro	Exon20	Missense	TM8	1.89	—	—	Likely pathogenic (PM2+PM3+PP3+PP5)
c.4114C>T	p.Gln1372*	Exon20	Nonsense	TM8	0.94	—	0.0008	Pathogenic (PVS1+PM2+PP3+PP5)

Mutation nomenclature (RefSeq:NM_000053.4).

Abbreviations: [a], Novel mutation; ATP bind, ATP bind domain; ATP hinge, ATP hinge domain; ATP loop, ATP loop domain; Cu, copper-binding domain; Ph, phosphorylation; Td, transduction domain converting energy from ATP hydrolysis to cation transportation; TM, transmembrane domain.

5 | DISCUSSION

Previous studies have suggested that two allelic variants of the *ATP7B* gene can be identified in approximately 80%–98% of WD patients by direct sequencing (Coffey et al., 2013; Dong et al., 2016; Schmidt, 2009). Gene-targeted deletion/duplication analysis can be used as a supplement for a few of the patients whose genetic diagnosis cannot be successfully established by direct sequencing (Bost et al., 2012; Chen et al., 2019); although, large *ATP7B* deletion/duplication has been proven to be rare in practice (Todorov et al., 2016). At present, the number of *ATP7B* gene pathogenic mutations included in the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/index.php) has exceeded 800. Most of them are missense mutations but others include nonsense mutations, small insertions/deletions, splicing mutations, etc. The broad mutation spectrum of this gene consists of a few common mutations and a large number of rare mutations.

There was great genetic diversity in the mutation spectrum of WD patients from several provinces of mainland China in this study. Forty-five pathogenic/likely pathogenic mutations were found from 106 chromosomes, including six novel mutations previously unreported. NM_000053.4: c.631_632delAA, c.2887_2887delC, and c.2932_2933insG are three novel mutations that lead to the early termination of amino acid coding and have not been included in the Exome Aggregation Consortium database and 1000 Genomes Project database thus far. They are predicted to be pathogenic by bioinformatics software analysis. Therefore, we classified them as "pathogenic mutations" (PVS1+PM2+PP3). The other three novel missense mutations, NM_000053.4: c.3215G>T, c.3542T>A, and c.3265G>A, are carried at a low frequency in the general population according to the Exome Aggregation Consortium database and 1000 Genomes Project database. Bioinformatics software prediction supported a disease-causing effect of these mutations on the gene. Probands with these three mutations were all confirmed to be compound heterozygous by parents' samples. In summary, we classified them as "likely pathogenic mutations" (PM2+PM3+PP3+PP4).

Although synonymous mutations could be detected by exome sequencing, it is still a vexing issue to evaluate the pathogenicity of a large number of synonymous mutations. In patient 26 of this cohort, we found a heterozygous synonymous mutation (NM_000053.4: c.2145C>T, P.Y715=) in addition to a common mutation NM_000053.4: c.2333G>T. The patient presented with recurrent ascites and edema of both lower extremities. Imaging methods confirmed the existence of cirrhosis. The copper metabolism test showed a significant decrease in ceruloplasmin (53.3 g/L) and an abnormal increase in 24-h urine copper (316 μg/24 h). Slit lamp examination confirmed the existence of a K-F ring. The clinical diagnosis of WD is clear. The synonymous mutation has been included in the ClinVar database, but the pathogenicity is still controversial. It is predicted to be a deleterious mutation with bioinformatics software analysis. It is carried at a low frequency in the general population according to the Exome Aggregation Consortium database (0.01325%) and 1000 Genomes Project database (not included). Nonsense mutation at the same site leading to disease have been reported before (Abuduxikuer & Wang, 2014). Only the synonymous mutation was detected in the sample of her son, confirming that the two mutations constitute a compound heterozygous relationship. These evidences support that the mutation is "likely pathogenic" according to the rules of the ACMG guidelines (PM2+PM3+PP3+PP4). To further clarify its pathogenicity, reverse transcription-polymerase chain reaction (RT-PCR) detection using RNA from peripheral leukocytes was performed. However, we could still not determine whether it affected the splicing of mRNA by gel electropherosis due to the existence of the dominant transcript with skipping of exon 8 (RefSeq: NM_001330578.2) (Loudianos et al., 2002).

Two allelic mutations were identified by direct sequencing in 50 patients (94%), and a single heterozygous mutation was identified in the remaining three patients (6%). Thus, there was a mutation detection rate of 97% (103/106). MLPA was supplemented for the three probands but the results were still negative, suggesting the rarity of large intragenic deletions/duplications. We consider that the other potential mutations might exist in the deep intron region and affect mRNA splicing, which needs further exploration at the RNA level. Finally, five asymptomatic siblings were screened out in this study, which illustrated that genetic testing can help patients with presymptomatic diagnosis and timely treatment.

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We owe a debt of gratitude to all subjects involved in this research.

CONFLICTS OF INTEREST

Authors vowed that there is no collide of interest.

ETHICS STATEMENT

Authorization was obtained from the Ethics Committee of the Second Xiangya Hospital of Central South University. The reference number was S046 in 2014.

AUTHOR'S CONTRIBUTIONS

Zhongyan Xiao was involved in family recruitment, DNA extraction, analysis and collation of data, and manuscript drafting. Yuan Yang was involved in family recruitment, DNA extraction and genetic analysis of data. Hui Huang carried out blood sampling and genetic analysis of data. Haiyan Tang carried out blood sampling and genetic analysis of data.

Liqun Liu have contributed to family recruitment and clinical analysis. Jianguang Tang have contributed to family recruitment and clinical analysis. Xiaoliu Shi supervised the whole study, helped in data analysis, edited, and refined the manuscript. All authors have read and approved the refined manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Zhongyan Xiao https://orcid.org/0000-0003-3089-9697
Haiyan Tang https://orcid.org/0000-0002-9029-8431

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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