Molecular Genetics and Metabolism Reports 36 (2023) 100984

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism Reports

journal homepage: www.elsevier.com/locate/ymgmr

Clinical, biochemical and molecular characterization of Wilson's disease in Moroccan patients

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ARTICLE INFO

Keywords:
Wilson disease
Molecular genetic diagnosis
Mutations
ATP7B gene

ABSTRACT

Background: Wilson Disease (WD) is an autosomal recessive inherited metabolic disease caused by mutations in the *ATP7B* gene. WD is characterized by heterogeneous clinical presentations expressed by hepatic and neuro-psychiatric phenotypes. The disease is difficult to diagnose, and misdiagnosed cases are commonly seen.
Methods: In this study, the presented symptoms of WD, the biochemical parameters as well as its natural history are described based on cases collected in Mohammed VI Hospital University of Marrakech (Morocco). We screened and sequenced 21 exons of *ATP7B* gene from 12 WD patients that confirmed through biochemical diagnosis.
Results: Mutational assessment of the *ATP7B* gene showed six homozygous mutations in 12 individuals however, 2 patients had no evidence of any mutation in promoter and exonic regions. All mutations are pathogenic and most were missense mutations. c.2507G > A (p.G836E), c.3694A > C (p.T1232P) and c.3310 T > C (p.C1104R) that were identified in 4 patients. The other mutations were a non-sense mutation (c.865C > T (p.C1104R)) detected in 2 patients, a splice mutation (c.51 + 4A > T) detected in 2 patients and a frameshift mutation (c.1746 dup (p.E583Rfs*25) detected in 2 patients.
Conclusion: Our study is the first molecular analysis in Moroccan patients with Wilson's disease, the *ATP7B* mutational spectrum in the Moroccan population is diverse and still unexplored.

1. Introduction

Wilson disease (WD; MIM 277900) is a rare autosomal recessive inborn error of the copper metabolism, which is caused by a homozygous or compound heterozygous mutations in the copper-transporting gene (*ATP7B*), localized on the short arm of chromosome 13 [1]. Through ATP-dependent mechanisms, the ATP7B protein regulates transmembrane copper export and the maturation of ceruloplasmin, which is involved in the transport of copper in the blood through the transgolgiate network [2]. An absent or a reduced function of the ATP7B protein leads to a decreased hepatocellular excretion of copper into bile resulting in injuries mainly to the liver, brain and kidneys [3].

Patients with WD are typically characterized by a wide range of clinical features, including hepatic, neurologic and psychiatric disorders

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https://doi.org/10.1016/j.ymgmr.2023.100984
Received 23 March 2023; Received in revised form 23 May 2023; Accepted 30 May 2023

[4,5]. The phenotype is often different between patients with the same genotype, even in the same family [6]. This metabolic disease is a multisystem disorder that can mimic any internal disease [7]. The WD had an onset age between 10 years and 20 years, although some cases have been reported at a very younger [8] and older age [9]. With this variability and overlap in the age at the onset of symptoms, the diversity of phenotypic manifestations and the multisystemic involvement of WD make the diagnosis very complicated. The diagnosis of WD is based on a combination of clinical, biochemical, histological, and genetic findings, since no individual laboratory marker indicating copper accumulation is 100% sensitive and specific for the disease [10]. Typically, the diagnosis of WD mainly depends upon clinical manifestations and conventional biochemical indicators, including elevated 24-h urinary copper, low serum ceruloplasmin and increased hepatic copper content. Thus, genetic analysis of the *ATP7B* gene is considered as the gold standard for diagnosis of Wilson disease, but genetic heterogeneity makes molecular diagnosis difficult, as well as the clinical presentation, which varies greatly from one patient to another, even with the same genotype [11]. Further research on the regional distribution of mutations and their correlation with the ethnic background can help to establish a good diagnosis of WD [12].

Through this work, we aim to emphasize the importance of biochemical markers for the orientation of diagnosis and to identify molecular genetic characteristics and their correlations with clinical phenotypes in a cohort of Moroccan patients with WD. This will enlarge the spectrum of *ATP7B* mutations previously established and will contribute to the understanding of the genotypic and phenotypic profiles of WD in Moroccan patients.

2. Patients and methods

2.1. Patients characteristics

All patients referred to our laboratory for WD diagnosis during a period from september 2018 to october 2021 were included in this study. From 104 patients, 30 cases were confirmed biochemically to have WD. For significant constraints, such as the loss to follow-up or death, the molecular diagnosis was limited only to 12 patients. The most common clinical features were cholestatic jaundice, hepatocellular insufficiency, hepatosplenomegaly, ocular involvement and progressive neuroregression with behavioral problems.

This study was conducted in compliance with the recommendations of the Declaration of Helsinki and Good Clinical Practice (GCP) Guidelines. The study was approved by the Independent Ethics Committee of Marrakech Hospital University, and consent was obtained from patients and/or their parents or guardians.

2.2. Biochemical analysis

2.2.1. Determination of serum ceruloplasmin oxidase activity

Assay procedure for measuring serum ceruloplasmin oxidase activity with o- dianisidine as substrate was performed according to the method described by Schosinsky et al. [13]. The ceruloplasmin activity in I.U./L was expressed in terms of substrate consumed.

2.2.2. Measurement of the concentration of copper in urine and serum samples

The serum and urine levels of copper were measured by atomic absorption pectrophotometry (Shimadzu 6800/6650) according to procedures mentioned by Sinha and Gabrieli [14] and Spector et al. [15]. All measurements were performed in triplicate.

2.3. Genetic analysis

Twelve patients diagnosed with WD based on clinical examination and biochemical tests (low serum ceruloplasmin level, increased 24-h urinary copper excretion and low copper serum level were included in the genetic analysis. Only probands were investigated as parents' samples were not available.

Genetic study of *ATP7B* was performed as previously described by Sánchez-Monteagudo (11). Genomic DNA was isolated from peripheral blood samples using Wizard Genomic DNA Purification Kit (Promega, WI, USA). Mutational screening in *ATP7B* gene was achieved by PCR amplification of the 21 exons including their intronic flanking sequences. Analysis of the promoter region (1438 bp of 5′ upstream sequence) and detection of large deletions and duplications by MLPA (Multiplex Ligation-dependent Probe Amplification) assay (SALSA MLPA P098 WD probemix, MRC-Holland, Amsterdam, Netherlands) were performed in cases with only one or no mutations identified in coding and adjacent splice site regions. PCR products were subjected to direct sequencing on an ABI Prism 3130XL analyzer (Applied Biosystems, CA, USA).

Sequences were aligned to a reference sequence (NCBI accession NM_000053.4/Ensembl accession ENST00000242839.10) and inspected to detect variants. To investigate novelty of identified variants, different databases were accessed: gnomAD version 2.1.1 to retrieve allele frequencies and HGMD® Professional 2022.1 to confirm if the variant was already described in the literature. Pathogenicity of missense variants was evaluated by the in silico predictors SIFT, PROVEAN and Polyphen-2. Splice prediction tools NNSplice, NetGene2 and SpliceAI were assessed to investigate possible alterations caused by variants in splice site regions.

3. Results

3.1. Clinical and biochemical characteristics

All patients recruited in this study fulfilled the clinical diagnostic criteria for WD with a score of >4 in the scoring system developed at the 8th International Meeting on WD, Leipzig 2001 (EASL, 2012) [16].

The clinical and biochemical characteristics of the 12 patients are presented in Table 1, (while data relating to 30 patients are represented as supplementary data) . Seven out of 12 patients were from consanguineous marriages. Four cases had hepatic subtype presenting with active clinical hepatic symptoms (jaundice, anorexia, nausea, coagulopathy, ascites, etc.) diagnosed before the age of 10 years, while 2 cases had neurological subtype with neurological features (dystonia, tremor, gait abnormality, swallowing difficulty, dysarthria, salivation, mental illness, etc.) diagnosed at 13 and 18 years. Both hepatic and neurological or behavioral abnormalities disorders were detected in 6 cases showing a predominance of hepatic manifestations. Furthermore, within this cohort, 3 cases had one sibling affected by WD and showed different clinical expressions (Table 1).

The biochemical diagnosis (Table 1) revealed an impairment of the copper balance. We observed low levels of ceruloplasmin and serum copper and high levels of 24 h urinary copper.

4. Genetic analysis

Mutational assessment of the *ATP7B* gene showed that 10 patients were detected with homozygous mutations (Table 2, Fig. 1); however, two patients (P4 and P6) had no evidence of any mutation in promoter and exonic regions of ATB7B gene.

Most mutations detected were missense mutations (p.G836E, p.T1232P and p.C1104R) which were identified in 4 patients (P1, P7, P8 and P10). The other types of mutation were one non-sense mutation (p.Q289X) (detected in P2 and P12), one splice mutation (c.51 + 4A > T) (detected in P3 and P11) and one frameshift mutation (p.E583Rfs*25) (detected in P5 and P9).

Table 1
Clinical data of patients with WD at diagnosis.

ID Patient	Sex	Consanguinity	Age at diagnosis (years)	KF-Rings	Hepatic presentations	Neuropsychiatric presentations	Ceruloplasmin (U/L) NV (62–140)	Serum copper (μg/dl) NV (65–180)	24 h copper (μg/24 h) NV (<60)	AST (IU/L) NV (0–38)	ALT (IU/L) NV (0–41)
P1*	M	Yes (1st degree)	10	No	Cholestatic icterus, slight abdominal distension	-	11.25	31	950	151	176
P2	F	Yes (1st degree)	13	No	-	Disturbance of stability and equilibrium, extremity tremor, dysarthria, dysphagia	8	63	430	ND	ND
P3$	F	Yes (3 rd degree)	10	No	Abdominal distension	-	10	36.7	800	36	77
P4	F	No	10	ND	Behavioral disturbances: panic and anxiety episodes)	Oedemato-ascitic syndrome, cholestatic icterus, cirrhosis	20	56	400	ND	ND
P5♣	M	No	13	Yes	Mild hepatic cytolysis	Dysarthria, tremor, aphasia, chorea	5.2	68	924	ND	ND
P6	F	No	10	No	Cholestatic icterus, homogeneous hepatomegaly, hepatocellular insufficiency, PT:18%	Neuropsychic disturbances with hallucinations	8.75	43.4	288	1500	1800
P7	F	Yes (3 rd degree)	7	No	Cholestatic icterus and pruritus, rigid hepatomegaly with sharp edge, ascites and collateral venous circulation, hepatocellular insufficiency, PT:15%, hemolytic anemia, cirhosis	-	8	23	790	ND	ND
P8	F	No	18	Yes	-	Grabatary with extrapyramidal syndrome	6	63	1034	ND	ND
P9	M	Yes (1st degree)	9	Yes	Hepatocellular Insufficiency, PT: 25%, slight cytolysis	Neurological impairment established at the age of 12: dysarthria and tremor	6.9	15.32	1020	ND	ND
P10	M	Yes (2 nd degree)	8	No	- Cholestatic icterus, ascites and collateral venous circulation, hepatocellular insufficiency, PT: 20%, no hepatomegaly	-	6.2	11.8	200	ND	ND
P11	M	No	11	No	Cirhosis, cholestatic icterus, generalized edematous syndrome, hepatocellular insufficiency, PT 20%, no hepatomegaly	Dysarthria, extremity tremor	5.6	34.2	300	ND	ND
P12**	M	Yes (1st degree)	17	Yes	Mild hepatic cytolysis	Dysarthria, tremor and depression	4	6.81	722	ND	ND

AST: aspartate aminotransferase; ALT: Alanine aminotransferase; ND: No determined, PT: prothrombin time, ND: No determined, *: death in sibling with cirrhosis, **: 2 siblings died with the same symptomatology; $: Sibling with neurological symptom, ♣: Asymptomatic sibling -: no symptoms, PT: prothrombin time, ND: No determined, *: 2 siblings died with the same symptomatology; –: no symptoms, F:female, M: male.

5. Discussion

WD is considered as a clinically and genetically heterogeneous disease [17]. The diagnosis of WD is made based on clinical symptoms and biochemical tests. However, biochemical markers can be misleading, making the diagnosis of WD difficult. Therefore, genetic testing has become the method of choice for accurate diagnosis [18,19]. This was also the fact in our data set; moreover, overlapping between the different symptoms was very common. According to our data, we recorded a predominance of hepatic alterations, hepatomegaly and cholestasis jaundice were the most frequent presentations and the leading cause of hospitalization and death in patients with WD. These data are similar to that described in other case series [20–22]. The age of onset is generally in infancy or in early childhood. Our patients' age (confirmed with WD) ranged from 7 to 17 years for both sex. Other study, with a large patient samples, showed that the age of onset in WD is considerably wider with a range from 2 to 62 years [23].

To our knowledge, this is the first report on genetic diagnosis of WD in a Moroccan cohort. We identified six different homozygous mutations of *ATP7B* gene in ten patients, while in two patients, no mutation was

Table 2
Mutations detected in *ATP7B* gene.

Nucleotide change	Amino acid change	Exon	Type	Chromosomal position	Region of protein	rs	MAF*	Patients ID	Previous studies that reported these mutations[a]
c.2507G > A	p.G836E	10	Missense	13–52,524,476-C-T	A-domain	rs773809011	0.000004007	P1	[28]
c.865C > T	p.Q289X	2	Nonsense	13–51,974,355-G-A	Cu3	rs121907999	0.00002822	P2, P12	[41] [42] [50] [43]
c.51 + 4A > T	At the 5' UTR, affecting a splicing site	1	Splice site	13–52,585,419-T-A		rs369488210	0.00001314	P3, P11	[33] [35] [45] [44] [34] [51] [46]
c.1746dup	p.E583Rfs*25	5	Frame shift	13–52,539,130-C-CT	MBD6	rs753962912	0.000006574	P5, P9	[48] [49]
c.3694A > C	p.T1232P	17	Missense	13–52,513,192-T-G	ATP binding domain	rs568009639	0.000004007	P7, P10	[32] [33] [35] [52]
c.3310 T > C	p.C1104R	15	Missense	13–51,942,488-A-G	ATP loop	NA	NA	P8	[37]

* MAF: minor allele frequency, according gnomAD v2.1.2; NA: not annotated; rs: rs number.

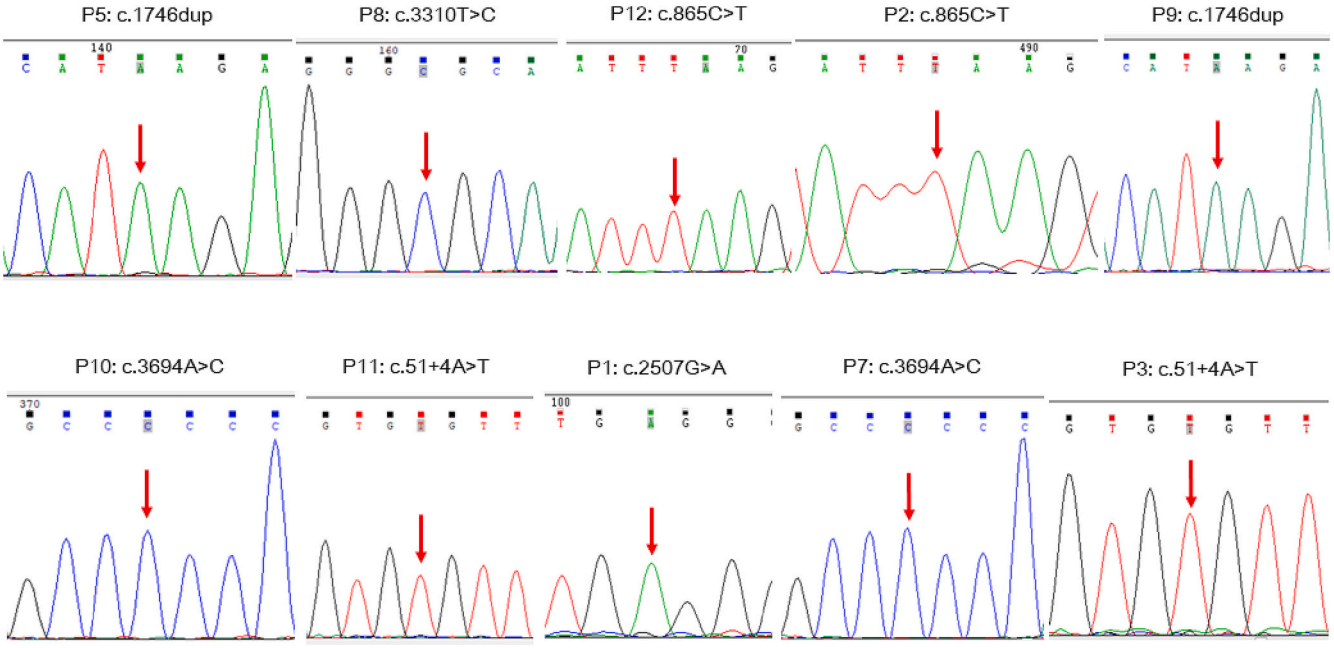

Fig. 1. The electropherograms show the identified mutations confirmed by Sanger sequencing in each index case. Red arrows point to the mutated nucleotide. The patient and the observed deleterious variant are indicated at the top of each image.

detected in promoter and exonic regions. Both individuals had WD according to clinical and laboratory findings (Table 1). In different populations, individuals with no mutations have been reported in different proportions [24]. Failure to detect any mutations may be explained by: a) unknown mutations that may be located on the outside of the exons and flanking regions, such as the promoter, introns, or other DNA control regions [25,26], thus highlighting the limitations in genetic testing for WD diagnosis, b) other hereditary disorders may mimic Wilson disease (congenital disorders of glycosylation, MEDNIK syndrome, idiopathic or primary copper toxicoses) [27].

One interesting missense mutation is the p.G836E. It can be considered probably as a rare mutation in worldwide population, Since only one case was previously reported in Italy in a patient of Moroccan origin [28]. This mutation occurs in exons 10 and substitutes a neutral polar residue with an acidic one in the A-domain. Some studies have suggested that A-domain regulates the release of the ion at the luminal site by triggering movements of transmembrane helices [29,30] The TGE motif in the A-domain is responsible for dephosphorylation by removing of γ-phosphate from the DKTGT motif in P-domain [31]. The alteration of the A-domain structure can impair the optimal protein activity by affecting the communication between functional domains (A- and P-domain). The p.G836E mutation was detected in the patient P1 who was diagnosed at 10 years with hepatic phenotype and without neurological involvement. This patient had low ceruloplasmin level (11.25 U/L), and high level of urinary copper (950 μg/24 h).

The missense mutation p.T1232P was detected in two patients (P7,

P10). It's a substitution in exon 17 involving the ATP loop of the protein. Both patients were diagnosed at a young age (7 and 8 years) and both showing similar hepatic symptoms. This mutation was found previously in Columbia [32], Brazil [33,34] and Spain, [35]. Advanced protein functional studies indicate that this missense mutation is expected to disrupt protein function [36].

The third missense mutation detected was C1104R. This mutation was previously described in a single case in China [37]. Another mutation in the same position was previously annotated p.C1104F [38]. Functional studies for p.C1104F revealed lower protein expression, probably because of proteasome degradation [39]. The patient with C1104R mutation (P8) presented severe neurological phenotype without identifiable liver damage and the age of symptoms onset was at 18 years.

We detected one nonsense mutation (p.Q289X). It occurs in exon 2 and could result in the production of highly unstable mRNA that is rapidly degraded via a pathway known as nonsense-mediated mRNA decay [40]. This mutation was detected in two patients (P2, P12) and are associated with the more severe neurological subtype of WD. This mutation was detected in several worldwide population (Sweden [41], Greece [42], Bulgaria [43] and France [44].

The c.51 + 4 A > T mutation was detected in two patients (P3, P11) presenting severe hepatic phenotype. It resides in the consensus sequence of the donor splice site of intron 1. The first patient P3 is a 10-year-old girl presented with severe liver disease without neurological signs, with low ceruloplasmin, high urinary copper excretion and absence of KF, she had a sibling, diagnosed with WD, exhibiting severe neurological phenotype. The second patient P11, an 11 years old boy had presented with a cholestasis jaundice, a hepatocellular insufficiency associated with neuropsychic disorders. Unfortunately, this patient died due to hepatic cirrhosis. This mutation was detected previously in Brazil [33]; Spain [35]. Italy [45], France [44] and Venzuela [46]. The consensus sequence splice-site mutations result in disease by interfering with the production of the normal protein [47], One functional study demonstrated that the c.51 + 4A > T mutation resulted in aberrant splicing in individuals who were homozygous for the variant but not in compound heterozygotes [47].

The *ATP7B* c.1746dup; p.Glu583ArgfsTer25 mutation is reported in the literature as a rare mutation [48], with compound heterozygous and homozygous in individuals affected with WD [49]. This variant causes a frameshift by inserting a single nucleotide, so it is predicted to result in a truncated protein or mRNA subject to nonsense-mediated decay. This mutation was detected in two patients (P5, P9) who presented neuropsychiatric disorders with KF rings, considerably low ceruloplasmin values (5.2 and 6.9 mg/dL) and high level of urinary copper (940 μg/24 h, 1020 μg/24 h).

In conclusion, this study revealed the clinical (for 30 patients, supplementary data) and genetic pattern of Moroccan cases and confirmed the pathogenic genotypes observed in 12 patients. The *ATP7B* mutational spectrum in the Moroccan population is diverse and still unexplored. Large-scale studies involving a large cohort and high-throughput techniques such as next-generation sequencing may reveal the disease burden in Morocco.

Author contributions

All authors contributed significantly to this work: N. FDIL, K. LAFHAL and A. HAKMAOUI devised the research plan and wrote the manuscript. K. LAFHAL, Es. SABIR, A. HAKMAOUI, M. HAMMOUD, A. AIMRANE, S. NAJEH, I. ASSIRI, A. BERRACHID, C. AIT BOUJEMAA, and F. AZIZ were responsible for performing the Biochemical analysis. The genetic testing was performed by I. BOYKO, A. SÁNCHEZ-MONTEAGUDO and C. ESPINÓS. N. FDIL, N. ABOUSSAIR and A. HAKMAOUI polished the manuscript. N. IMAD, FZ. EL HANAFI, A. LALAOUI, H. AAMRI, I. AIT SAB, and A. BOURRAHOUAT, were responsible for analysis interpretations, patient's collection. All authors support the publication of the manuscript.

Declaration of Competing Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data availability

No data was used for the research described in the article.

Acknowledgments

The authors would like to thank all patients, and their families for their contributions and cooperation. We thank the Spanish Foundation Per Amor a l'Art (FPAA) for their support of the genetic analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2023.100984.

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