


ORIGINAL ARTICLE

The dilemma to diagnose Wilson disease by genetic testing alone

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Abstract

Background: Wilson disease (WD) is an autosomal recessive disorder of hepatic copper excretion. About sixty per cent of patients present with liver disease. WD is considered a fatal disease if undiagnosed and/or untreated but recent data indicate that disease penetrance may not be 100%.

Materials and Methods: All patients underwent liver biopsy as part of the diagnostic workup. Genetic testing for ATP7B was performed by Sanger sequencing.

Results: We report on a large family with multiple affected siblings. The first patient (male, 31 years) underwent orthotopic liver transplantation (OLT) because of fulminant WD. He was homozygous for p.G710A. One asymptomatic brother (37 years) had the same mutation. He is doing well on chelation therapy. Fifteen years later, a second-degree sibling (female, 16 years) presented with fulminant WD and underwent OLT. She was compound heterozygote (p.G710A/p.G710S). Further family screening revealed a third mutation (p.V536A) in a female (21 years) and male (16 years) compound-heterozygote sibling (p.G710A/p.V536A). In both, serum ceruloplasmin and 24-hour urinary copper excretion were normal. Liver biopsy showed normal histology and a quantitative hepatic copper content within the normal range or only slightly elevated (19 and 75 µg/g dry weight, respectively). No decoppering treatment was initiated so far.

Conclusion: Genetic testing alone is not always sufficient to diagnose WD in asymptomatic patients, and human mutation databases should be used with caution. Even patients carrying two disease-causing mutations do not necessarily have demonstrable alteration of copper metabolism. Asymptomatic siblings diagnosed by genetic screening require further testing before initiating treatment.

KEYWORDS

family screening, genetic testing, incomplete penetrance, Leipzig score, liver biopsy

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1 | INTRODUCTION

Wilson disease (WD) is an autosomal recessive disorder of copper transport with tissue accumulation of copper especially in the liver and other organs^{1,2} due to mutations of the *ATP7B* gene. *ATP7B* encodes for a copper binding P-type ATPase in the trans-Golgi network in hepatocytes,³ which is involved in copper transport into secretory pathways and incorporation of copper into apo-ceruloplasmin.⁴ Clinical presentation is broad and encompasses liver disease, neuropsychiatric symptoms and ophthalmoscopic findings (eg Kayser-Fleischer corneal rings).⁵ Diagnosis is based on the presence of these clinical symptoms and laboratory findings (ceruloplasmin, 24 hours urinary copper excretion, hepatic copper content). Since the discovery of *ATP7B* gene mutations genetics became an additional useful tool. The results of these findings are summarized in the Leipzig score.⁶ Finding two disease-causing mutations alone establishes the diagnosis of WD.

There is increasing evidence that WD may be more common than previously suggested by a population-based approach estimating the incidence to be at least 1 in 30 000-50 000.⁷ Reports on the prevalence of WD are inconsistent depending on the region of the world and the methods used to diagnose or screen for WD. Mass screening studies using determination of ceruloplasmin in urine⁸ or blood⁹ indicated a substantially higher prevalence (1-2 in 3000). By sequencing the entire coding region and adjacent splice sites of *ATP7B* in 1000 control subjects in the UK, the frequency of carriers with two *pathogenic ATP7B* mutations was estimated to be of 1 in 7026.¹⁰ Furthermore, another study¹¹ in France showed a high heterozygous carrier frequency of *ATP7B* yielding a prevalence of 1:31 subjects. The authors explained this discrepancy partly due to the clinical variability of WD with incomplete penetrance and existence of modifier genes. If these observations can be replicated, it is possible that many patients with WD will have any or only very subtle symptoms and will be detected by genetic screening alone. Whole exome sequencing will soon become an affordable option to screen for rare diseases.¹² If two mutations are detected in an asymptomatic individual, the question will arise whether the genetic finding can be translated to the presence of a disease.

We herewith present two asymptomatic patients detected by family screening of an Austrian family with three symptomatic patients with WD. Both were compound heterozygotes but had no evidence of impaired copper metabolism.

2 | METHODS

2.1 | Patients

All subjects were identified by genetic family screening. Index patients (n = 2) and asymptomatic siblings (n = 3)

were included in this study. Besides, heterozygous carriers (n = 13) and subjects without mutations in *ATP7B* (n = 2) were tested by family genetic testing.

2.2 | Genetic testing for *ATP7B*

Genetic testing of the family for mutations in *ATP7B* was performed as part of the diagnostic workup. The methods are described elsewhere.¹³

2.3 | Laboratory assessment

All subjects underwent laboratory assessment at onset of symptoms or at genetic. Subjects were tested for complete blood count, transaminases (aspartate aminotransferase [AST], alanine aminotransferase [ALT]), liver function tests (prothrombin time [PT], international normalized ratio [INR], bilirubin, albumin), serum copper and ceruloplasmin levels. All tests were performed according to the manufacturer's instructions.

2.4 | Liver biopsy and histological assessment

Liver histology was assessed in all subjects with two mutations in *ATP7B*. In the first index patient (patient-ID: II/5), liver biopsy was obtained via transjugular access due to severe coagulopathy. In the second index patient (patient-ID: III/1), liver histology was performed in the explant liver after liver transplantation. All other patients underwent percutaneous liver biopsy by Menghini technique. Biopsies were routinely processed (formalin-fixed and paraffin-embedded) and stained with haematoxylin/eosin and chromatrope aniline blue for assessment for fibrosis, steatosis and inflammation. Furthermore, rhodamine staining was used to identify copper deposits in liver biopsies.

Hepatic copper content (in $\mu\text{g/g}$ dry weight) was measured by flame atomic absorption spectroscopy according to Kingston and Jassie.¹⁴ A cut-off of $< 50 \mu\text{g/g}$ dry weight was considered as normal.

3 | RESULTS

3.1 | The first index patient

This 31 years old patient (patient-ID: II/5 in Table 1) was admitted to hospital in 2002 for jaundice and acute hepatic failure. He neither had Kayser-Fleischer corneal rings nor neurologic symptoms. A routine blood test taken 15 years earlier showed slightly increased liver transaminases but no further diagnostic measures were taken. The patient deteriorated and was successfully transplanted a few days later. By genetic testing, he was homozygous for p.G710A (c.2129G > C) in exon 8 in *ATP7B*.

TABLE 1 Genetic and clinical characteristics of patients with Wilson disease

Patient-ID	II/5	II/1	III/1	III/4	III/5
Sex	Male	Male	Female	Female	Male
Age at diagnosis	31	37	16	21	15
<i>ATP7B</i> genotype	p.G710A/p.G710A	p.G710A/p.G710A	p.G710A/p.G710S	p.G710A/p.V536A	p.G710A/p.V536A
Liver histology	Cirrhosis	Steatohepatitis, fibrosis	Cirrhosis, intracellular cholestasis	Normal	Normal
Rhodanine staining	Positive	Negative	Positive	Negative	Negative
Hepatic copper content (µg/g)	2060	219	nd	19	75
ALT (U/L)	34	153	26	27	13
AST (U/L)	75	53	175	22	17
γGT (U/L)	198	42	174	17	13
Alkaline phosphatase (U/L)	47	78	46	51	218
Prothrombin time (%)	37	88	42	100	86
INR	2.2	1.0	1.9	1.0	1.1
Bilirubin (mg/dL)	7.9	1.42	13.0	0.4	0.2
Albumin (g/L)	30.5	51.8	29.2	42.0	50.0
Platelets (G/L)	60	216	340	258	297
Ceruloplasmin (mg/dL)	21.7	28.0	14.0	40.0	24.5
Serum-Cu (µg/dL)	252	118	107	161	94
Urine-Cu (µg/24 h)	1181.2	nd	nd	16.0	12.0
Haemolysis	No	No	Yes	No	No
Kayser-Fleischer rings	No	No	No	No	No
Neurological symptoms	No	No	No	No	No
Leipzig score	8	5	7	4	5

3.2 | Family screening

As expected, both parents were heterozygous carriers of p.G710A. The older asymptomatic brother (37 years, patient-ID: II/1 in Table 1) was also homozygous for p.G710A. Liver biopsy showed diffuse macrovesicular steatosis (up to 80% of hepatocytes affected) and bridging fibrosis. Hepatic copper content was 219 µg/g dry weight. He is now doing well on chelation therapy. Two further siblings (one male, one female) were heterozygous carriers, none of them had any signs of liver disease.

3.3 | The second index patient

In 2017, a second-degree niece (16 years; patient-ID: III/1 in Table 1) of the first index patient was hospitalized for acute onset of jaundice and malaise. Ceruloplasmin level was 14.0 mg/dL, she had no Kayser-Fleischer rings. She rapidly

deteriorated and was successfully liver transplanted a few days later. Histological workup showed liver cirrhosis with chronic inflammation and profound intracellular cholestasis; rhodanine staining was positive and showed intracytoplasmic copper accumulation in around 20% of hepatocytes. Genetic sequencing of *ATP7B* identified two different mutations in exon 8: p.G710A and p.G710S (c.2128G > A). Since transplantation she is doing well.

3.4 | Further family screening

As expected, the father—a first-degree cousin of the first index patient—carried p.G710A in exon 8. His wife was heterozygous for p.G710S. One sister was a heterozygous p.G710A carrier, while no mutation was found in the other sister. Surprisingly, a third mutation in exon 4 (p.V536A, c.1607T > C) was found in the grandmother and two aunts of the patient; all three were heterozygous carriers. Another

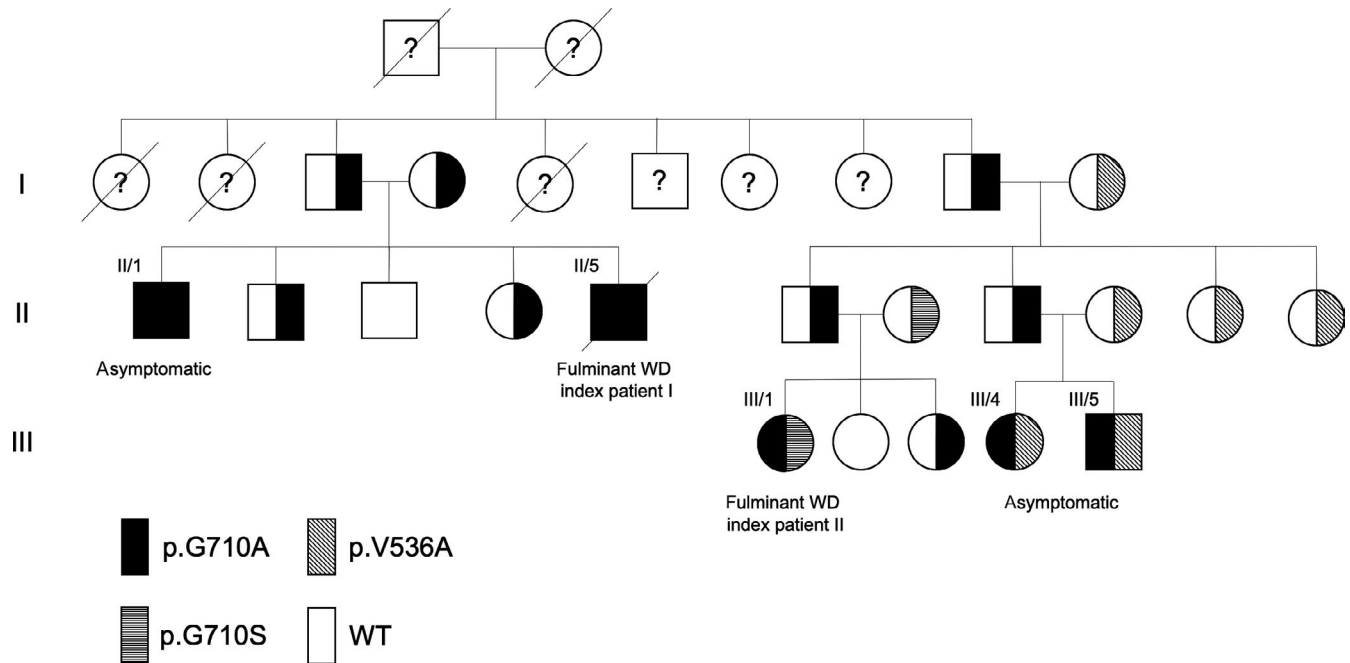


FIGURE 1 Pedigree of the family (? = unknown, WT = wild type)

uncle was a heterozygous carrier for p.G710A and his wife (unrelated to the family but from the same small village) was heterozygous for p.V536A. Both of their children—a 21-year-old female and a 15-year-old male (patient-ID: III/4 and III/5 in Table 1)—were compound-heterozygous (p.G710A/p.V536A). Both had normal transaminases and liver synthesis. Ceruloplasmin levels (40.0 and 24.5 mg/dL, respectively) and urinary copper excretion (16.0 and 12.0 $\mu\text{g}/\text{day}$, respectively) were in normal range. No Kayser-Fleischer corneal rings or neurological symptoms were observed. Liver biopsy showed no pathological findings, hepatic copper content was 19 and 75 $\mu\text{g}/\text{g}$ dry weight, respectively (upper limit of normal: 50 $\mu\text{g}/\text{g}$ dry weight). Until today, no chelation therapy was started so far and both patients are under close monitoring.

4 | DISCUSSION

The variable clinical penetrance and genetic heterogeneity of WD are poorly understood and pose significant challenges for diagnosis, disease prognosis and genetic counselling. This large Caucasian family illustrates the poor genotype to phenotype correlation and the variable disease penetrance of WD even in siblings with identical *ATP7B* genotype. Despite homozygosity for disease-associated *ATP7B* mutations in two brothers disease presentation can range from asymptomatic without hepatic copper storage to fulminant hepatic failure and cirrhosis. The presence of three different *ATP7B* mutations within a single pedigree (see Figure 1) in this Austrian family with multiple patients with Wilson disease is also unique. All

identified mutations have been described previously and are considered disease-associated.¹⁵⁻¹⁷ Surprisingly, two compound heterozygotes (p.G710A/p.V536A) had no evidence for disturbed copper metabolism. Both were asymptomatic and had no evidence of liver disease.

Mutations in *ATP7B* have different effects on protein expression levels, as well as catalytic and transport activity. Functional studies are rare and prediction of the severity of a particular mutation is difficult.¹⁸ Detailed characterization of *ATP7B* mutations may contribute to genotype-phenotype correlations in WD.¹⁹ p.G710A is a disease-causing missense mutation in transmembrane region 2 which may affect copper transport. The key question is whether p.V536A is a disease-causing mutation, as it was considered in the study by Davies et al¹⁵ previously. The REVEL score for this mutation of 0.555 suggests that the mutation is likely disease causing.²⁰ There are further tools to assess the functional consequence of a particular mutation. We calculated these scores for the p.V536A mutation none of which suggests pathogenicity (SIFT [0.07], PolyPhen [0.012], CADD [17], MetaLR [0.341] and Mutation Assessor [0.232]). Although functional studies on p.V536A are lacking, structural information on this mutation shows that the affected residue is located within the copper binding domain 4, where changes in hydrophobicity and size are predicted to affect the protein function. Previous clinical genetic studies have also shown that this mutation segregated with the disease.¹⁵ The limited value of in silico scores in classifying *ATP7B* mutations is further highlighted by the observation that none of the afore mentioned scores (SIFT [0.42], PolyPhen [0], CADD [21], REVEL [0.477], MetaLR [0.239] and Mutation Assessor [0.008]) classifies the

most common mutation in Spanish WD patients, p.M645R²¹ (c.1934T > G), as pathogenic. In our own databank,¹³ we have 16 patients with p.M645R; 14 with symptomatic WD; and two asymptomatic siblings identified by family screening. Fourteen were compound-heterozygote; in two patients, the second mutation was not identified yet. All presented with liver disease, only one patient had Kayser-Fleischer corneal rings, two had cirrhosis. Hepatic copper content was increased in all subjects, ranging from 250 to 1970 µg/g dry weight. These observations clearly indicate that p.M645R is indeed a disease-causing mutation.

In the largest cohort of patients with WD published so far, age and sex but not *ATP7B* genotype influenced the clinical phenotype.¹³ Environmental, epigenetic and other genetic factors than *ATP7B* seem to play a pivotal role in the clinical manifestation and course of patients with WD. Some of these factors have been identified within the past years,^{22–24} but their overall contribution deserves for further clarification. The true penetrance of specific *ATP7B* mutations and genotypes is unknown and it is unclear if environmental cofactors or genetic modifiers determine disease expression and severity.

The complex control of copper homeostasis is only partially understood. Copper transfer to the secretory pathway in hepatocytes is a sophisticated process orchestrated by both soluble and membrane proteins. Emerging data suggest existence of additional important players and regulatory pathways that may utilize metabolic feedback loops.²⁵ Bacterial metabolites like the siderophore yersiniabactin, a polypeptide-polyketide produced by infectious *Yersinia* species and uropathogenic *E coli*²⁶ or methanobactin produced by *Methylosinus trichosporium* OB3b²⁷ may influence/modulate dietary copper absorption directly by binding copper. Besides, some nutrients like curcumin also bind copper.²⁸ Nevertheless, the impact of the intestinal microbiome on WD has not been explored yet.

Although patients III/4 and III/5 had two mutations in *ATP7B* and therefore a Leipzig score of at least 4 sufficient to make the diagnosis of WD, no decoppering treatment was initiated, as they had no symptoms and no evidence of liver pathology or hepatic copper accumulation. Both patients are under regular clinical monitoring. As long as they remain asymptomatic or do not show laboratory evidence of copper accumulation, no treatment will be initiated. As chelation therapy is a lifelong treatment with diverse side effects, the benefit for patients without any disease manifestation may be negligible or even harmful.

There were a few similar cases reported. An adult asymptomatic patient with WD (*ATP7B*: c.441_427del/c.19_20delCA) identified by family screening with normal liver function tests, steatosis and mildly elevated hepatic copper content declined medical treatment and is still asymptomatic on regular clinical monitoring.²⁹ Similarly, another patient (homozygous for p.H1069Q) refused treatment and remained

asymptomatic for almost 30 years of follow-up.³⁰ Finally, the asymptomatic sister of a young boy with severe neurological and hepatic damage (both homozygous for p.H1069Q) refused treatment and did not become symptomatic even 50 years after diagnosis.³¹ In contrast to the asymptomatic patients in the current study and in the report by Loudianos et al²⁹ the latter two cases—although clinically asymptomatic—presented with impaired copper metabolism or even elevated transaminases suggesting the need of a decoppering treatment.

Reduced penetrance is a widespread phenomenon in human genetics³² and may become a major problem if individuals will order commercial whole exome sequencing. Variable expression causes different exhibition of disease severity in different individuals, even among members of the same family. In some cases, patients harbouring a disease-causing mutation remain completely asymptomatic throughout their whole life—a genetic phenomenon known as incomplete penetrance, which predominantly occurs in autosomal dominantly inherited disorders. However, reduced penetrance can also occur in autosomal recessive disorders where the same mutation can have different phenotypic effects, depending at least in part upon the second disease allele present.³²

In conclusion, genetic testing alone is not enough for clinical and therapeutic decision-making in asymptomatic patients with WD. Furthermore, human mutation databases as well as in silico mutation testing must be used with caution, as they are insufficient to predict pathogenicity of a certain mutation in every case. We suggest that such asymptomatic patients with two *ATP7B* mutations (homozygous or compound-heterozygotes) require a complete diagnostic workup including measurement of hepatic copper content, having the highest sensitivity and specificity for diagnosis of WD³³ and imaging of the central nervous system. The decision for treatment in patients with variants of unknown significance in *ATP7B* should then be based on an individual basis. Nevertheless, asymptomatic WD patients without treatment should be monitored regularly. The clinical and therapeutic management of asymptomatic patients with WD needs to be clarified in future clinical studies.

CONFLICT OF INTEREST

All authors have no financial disclosures concerning this study to report.

AUTHORS' CONTRIBUTIONS

AFS wrote the manuscript and collected the data. AE, MG and HH acquired data and critically revised the manuscript for important intellectual content. HZ critically revised the manuscript for important intellectual content. PF conceived and designed the study and critically revised the manuscript for important intellectual content.

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