Are the new genetic tools for diagnosis of Wilson disease helpful in clinical practice?

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Summary

The diagnosis of Wilson disease is not always easy. For many patients, a combination of tests reflecting disturbed copper metabolism may be needed. Testing for *ATP7B* variants has become part of the routine diagnostic approach. The methods of genetic testing include analysis of the 21 coding exons and intronic flanking sequences, in which exons with recurrent variants would be prioritised depending on the mutation frequency in the local population. If sequencing the entire *ATP7B* gene cannot identify 2 variants and the suspicion for Wilson disease is high, after reviewing the clinical data, WES (whole-exome sequencing) or WGS (whole-genome sequencing) could be applied. A workflow based on the type and number of *ATP7B* variants responsible for Wilson disease is proposed. Genetic testing is indicated for confirmation of diagnosis, family screening, and screening of newborns and infants and in unclear cases suspected of suffering from Wilson disease. However, genetic testing is not a routine screening test for Wilson disease. If no additional variants can be identified, it can be assumed that other hereditary disorders may mimic Wilson disease (congenital disorders of glycosylation, MEDNIK syndrome, idiopathic or primary copper toxicoses).

Introduction

Wilson disease is an autosomal recessive disorder caused by *ATP7B* gene variants. The dysfunction of the ATP7B protein leads to impaired biliary excretion of copper and thereby to copper accumulation in the liver and extrahepatic tissues. The clinical picture is highly variable with hepatic and neurologic disease as leading forms of presentation. The disease may become symptomatic at any age[1] or possibly may never become symptomatic. Diagnosis of Wilson disease is not always easy. It is beyond the scope of this review to discuss details of the clinical diagnosis of Wilson disease (for review[2]). Diagnosis is straightforward in patients with neurologic Wilson disease, ceruloplasmin is low and Kayser–Fleischer rings are present in 95% of cases. If this is the case, no further diagnostic tests are needed. In patients presenting with liver disease neither normal plasma ceruloplasmin levels nor the absence of Kayser–Fleischer rings exclude Wilson disease.[3] If the suspicion of Wilson disease is high, further tests are needed, including measurement of hepatic copper content and 24 h urinary copper excretion. Since becoming available, testing for *ATP7B* variants has become part of the routine diagnostic approach.

For many patients, a combination of tests reflecting disturbed copper metabolism may be needed. No single test is specific, so a range of tests have to be applied. A diagnostic sum score rated -1 to 2 for each available test and clinical symptoms was proposed by the Working Party at the 8th International Meeting on Wilson disease, Leipzig 2001 and provides good diagnostic accuracy.[4] The Leipzig score also includes the results of genetic testing, with the emphasis that only disease-causing variants should be considered. Diagnosis of Wilson disease is highly likely with a sum score of 4 or greater and becomes very unlikely with a score of 2 or lower. Therefore, this score was recommended for diagnosis of Wilson disease in the most recent EASL (European Association for the Study of the Liver) clinical practice guidelines.[5,6]

Role of genetic testing in clinical practice

Before sending a sample for genetic testing, informed consent must be obtained and all international and national laws should be followed.

Methods

Wilson disease is a well-established monogenic disorder caused by biallelic variants in *ATP7B*, which is why genetic testing can comprise several phases. First, the 21 coding exons and 'exon/intron boundaries' are genotyped. Exons with recurrent variants are prioritised depending on the variant frequency in the local population (Fig. 1, Fig. 2).[7,8] Thereby point variants, small deletions, and insertions in the nucleotide sequence can be

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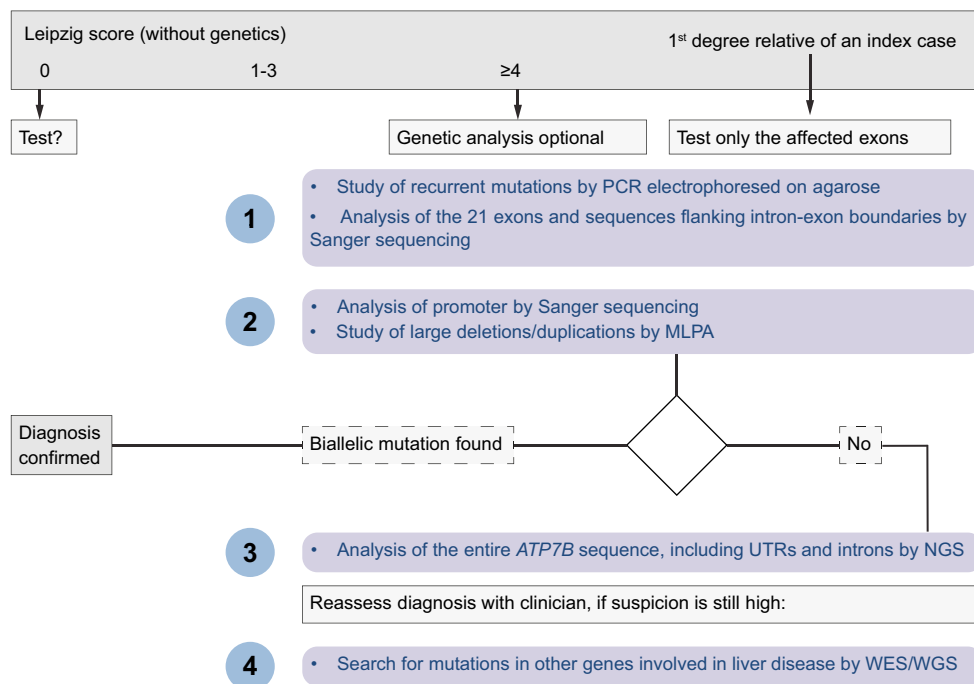E-mail address: peter. ferenci@meduniwien.ac.at (P. Ferenci).

visualised. A variety of alternative methods can be used to detect common variants. The simplest is to detect them by PCR and restriction analysis, multiplex PCR or PCR-MassArray screening,[9–11] enabling detection of disease-causing variants in up to 80% of patients of particular ethnic background. The proposed workflow (Fig. 2) is based on the type and number of *ATP7B* variants responsible for Wilson disease. To date, 907 different deleterious variants are included in the HGMD Professional v. 2018.2, and 878 changes (96.8%) could be detected by analysis of the coding exons and intronic sequences (Table 1).[12]

If genotyping fails to identify variants in the 21 codified exons and flanking intronic sequences of *ATP7B* using Sanger sequencing or indirect methods, further analysis by multiplex ligation-dependent probe amplification (MLPA) enables the investigation of promoter region, study of large deletions/duplications, changes in copy number, and/or sequencing of the whole *ATP7B* gene using a custom target enrichment library. MLPA is a well-established technique to detect large deletions and duplications by screening for the loss or gain of target sequences, allowing for the identification of variants undetectable by Sanger sequencing. Meanwhile, a custom target enrichment library allows the capture of selected sequences, not just exons, and therefore makes it possible to analyse the whole sequence of a gene introns and untranslated regions (UTRs). Twenty-three gross distinct deletions and 5 variants in the promoter (from the ATG initiation codon to 600 base pairs upstream)[12]

Key points

- Wilson disease is a well-established monogenic disorder caused by biallelic variants in *ATP7B*.

- To date, 907 different deleterious variants are known; 96.8% of them can be detected by analysis of the coding exons and intronic sequences.

- Genetic testing is indicated for confirmation of diagnosis, family screening, and in unclear cases suspected to suffer from Wilson disease.

- Variant analysis is not a screening test for Wilson disease. It should only be requested if there is a clinical suspicion of Wilson disease.

- If no additional variants can be identified, it can be assumed that other hereditary disorders may mimic Wilson disease.

have been described. In the 5′-UTR, 2 further changes have been identified, c.-1449A>G (rs77505745) and c.-1279C>T (rs111871296) that increase and decrease *ATP7B* expression, respectively,[13] although both of them were considered benign polymorphisms based on their high frequency (>1%) in control populations. Regarding intronic variants, the deep intronic variant c.2865+467A>G was observed in a patient in compound heterozygosity with c.482T>C (p.I161T).[14] The analysis of c.2865+467A>G by transcription analysis and by minigene assay suggested that this change may not affect proper splicing.

It is surprising that in many clinical series, 1–27% of affected individuals have only 1 detected *ATP7B* variant.[1,15–17] This is in part due to incomplete genetic analysis focusing mainly on

Fig. 1. **Geographic distribution of Wilson disease variants (based on[8]).**

Fig. 2. Flowchart for genetic analysis of Wilson disease. A molecular diagnosis is achieved in most of patients after 1st step. MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; UTR, untranslated region; WES, whole-exome sequencing; WGS, whole-genome sequencing.

Table 1. Types of variants in Wilson disease.[12]

Type	Number	Frequency
Regulatory	5	0.55%
Splicing site	71	7.83%
Missense/Nonsense	568	62.62%
Small deletions	154	16.98%
Small insertions	74	8.16%
Small indels	11	1.21%
Gross deletions	23	2.54%
Complex variant	1	0.11%

coding regions. However, even in the most complete study of *ATP7B* variants in Wilson disease (including promoter and even, UTRs) the detection rate was around 77–98%,[1,13] and a few cases had 1 or no *ATP7B* variant.

The missing variants could be caused by additional mechanisms involved in atypical patterns of inheritance,[18] such as uniparental disomy (person receives 2 copies of a chromosome, or of part of a chromosome, from 1 parent and no copy from the other parent), or structural genomic variants. To our knowledge, 2 cases of Wilson disease are known to be caused by segmental paternal or maternal isodisomy (both copies of a chromosome pair being inherited either from the biological mother or the father) giving rise to autozygosity (alleles or chromosomal segments of DNA that are identical as a result of inheritance through consanguineous mating) of a disease-causing variant in *ATP7B*. The most complex rearrangement known in *ATP7B* implies the deletion of 23 base pairs and insertion of 7 base pairs (c.2986_3008del23insTATGTGG).[19]

If the sequence of the entire *ATP7B* gene does not enable identification of 2 variants and the suspicion for Wilson disease is high (review the clinical data), whole-exome sequencing (WES) or whole-genome sequencing (WGS) could be applied, since other disorders can mimic the clinical picture of Wilson disease. Thus, WES was associated with a success rate of 25% in genomic analysis of idiopathic liver disease.[20] For rare disorders in children, the diagnostic rate using WES of only the proband is ∼28%, and increases to ∼40% when parents are also sequenced (trio-based WES); trio-based WGS improves the diagnostic yield slightly (∼42%), and is the most demanding and expensive approach.[21]

Next generation sequencing (NGS) is the catch-all term used to describe a number of different modern sequencing technologies. These technologies allow for sequencing of DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, revolutionising the study of genomics and molecular biology. In any case, in clinical practice and research, NGS-based approaches are increasingly used as they become more cost effective. However, NGS requires sophisticated statistical and computational tools to evaluate the data obtained. In fact, NGS is the preferred strategy for molecular diagnosis in some populations, even for Wilson disease that presents a high diagnostic rate just analysing the coding regions of *ATP7B*.[19]

Variants in Wilson disease

At present, The Human Gene Variant Database lists 907 sequence variants of *ATP7B*.[12,22] Finding 2 alleles carrying a known *ATP7B* variant (*in trans*) is generally sufficient to make the diagnosis of Wilson disease. However, different *ATP7B* variants have different effects on protein expression levels, as well as catalytic and transport activity.[23] Functional studies are rare and prediction of the severity of a particular variant is difficult.[24]

When should genetic analysis be performed in Wilson disease

Variant analysis is not a screening test for Wilson disease. It should only be requested if there is a clinical suspicion of Wilson

disease (*i.e.* evidence of altered copper metabolism, unexplained liver disease or extrapyramidal symptoms).

Confirmation of diagnosis

In a patient with a clear-cut clinical diagnosis of Wilson disease, variant analysis is not necessary but may provide information to guide the screening of relatives. Knowing the variant status is not currently needed for therapeutic decision making. However, the variant status may be required with new technologies like gene repair or gene therapy.

Family screening

Asymptomatic siblings of an index patient have to be screened by determination of the *ATP7B* gene variants. This requires the identification of an index patient with the unquestionable diagnosis of Wilson disease within the family. If the sibling carries both alleles with the disease-causing variants of the index patient, no further diagnostic tests are necessary. Heterozygosity for only 1 variant or absence of variants at all excludes Wilson disease in siblings.

Wilson disease may be transmitted from affected but asymptomatic parents[25–30] to their offspring. Thus, systematic family-wide screening (not just 1st degree relatives) is recommended despite the autosomal recessive nature of transmission.

Screening of newborns and infants

Early diagnosis of Wilson disease may prevent patients ever becoming symptomatic. Since Wilson disease rarely becomes symptomatic in the first 3 years of life, early identification of Wilson disease of newborns (unrelated to a patient with Wilson disease) will allow regular surveillance by paediatricians and timely initiation of treatment. So far, measuring holoceruloplasmin or testing by TaqMan single-nucleotide polymorphism assay for the 6 most common variants in Korea yielded insufficient evidence that this approach is useful.[31–33] Molecular neonatal screening in Sardinia, an island with a high proportion of genetic diseases due to consanguinity reported a Wilson disease prevalence of 1:2,707.[34] In the Korean and Sardinian neonatal investigations, like in the French[16] and UK[15] population screening studies, the estimated allele and carrier frequencies based on DNA screening were higher than those based on clinical ascertainment. Unfortunately, no data were reported on whether any of the neonates with a genetic diagnosis of Wilson disease ever became sick.

With any screening study the question of disease penetrance arises. A recent article by Sandahl *et al.*[35] reviewed all published studies on Wilson disease frequency and concluded that available population-based genetic studies in Caucasian populations indicate a higher prevalence than clinical estimates, raising the question of whether the penetrance is actually 100% as generally assumed. The problem common to all of these studies is that they found only 2 disease-causing variants but did not examine them clinically. A further limitation is that Wilson disease may become symptomatic even at very old age (my oldest patient was 79 years old at diagnosis) and it was suggested ATP7B variants may be involved in Alzheimer's disease.[36] Thus, based on current knowledge, one must assume a 100% penetrance and start treatment once Wilson disease is diagnosed.

Another useful approach may be an immuno-SRM[37] (selected reaction monitoring) platform to quantify *ATP7B* in dried blood spots in the picomolar range. This assay readily (positive predictive value: 0.9) distinguishes affected cases from normal controls.[38,39] One theoretical problem of testing asymptomatic individuals is the limited knowledge on disease penetrance of Wilson disease. Asymptomatic carriers of biallelic causative variants in *ATP7B* have been described.[40–42]

Variant testing in unclear cases

Again, genetic testing is not a screening test for Wilson disease. There should be a clear suspicion that the patient may suffer from Wilson disease; for example, a patient with slightly elevated aminotransferases and a borderline decreased ceruloplasmin level or a patient with extrapyramidal symptoms and abnormal liver tests. In most cases the patient may just be a true heterozygote or no evidence for Wilson disease will be found. The most demanding cases are those with significant pathology with only 1 detectable variant. A clear distinction from heterozygotes is challenging. True heterozygosity can be confirmed by analysis of both parents. In a large study from Poland, the clinical usefulness of WES was unquestionable, but WES results were negative and inconclusive in 3 and 27 patients with Wilson disease, respectively. These negative and inconclusive results likely reflect challenges in distinguishing structural variation of the DNA sequence, including duplication and heterozygous deletions.[43]

Epigenetics refers to the regulatory mechanisms that may alter gene expression without altering the genome. Genomic imprinting is a form of non-Mendelian inheritance. It is when the phenotype of the offspring depends on the source of the chromosome containing the imprinted gene whether it is from the female or from the male parent. Epigenetic factors can cause genomic imprinting. An imprinted gene is one in which the DNA is methylated. When methylated, gene expression is suppressed. Methylated genes are acquired at fertilization. The new gene imprints would consequently affect the phenotype of its progeny. Whole-genome bisulfite sequencing analyses in human liver samples identified 969 hypermethylated and 871 hypomethylated differentially methylated regions (DMRs), including 18 regions with genome-wide significance.[44] Wilson disease-specific liver DMRs were associated with genes enriched for functions in folate and lipid metabolism and acute inflammatory response and could differentiate early from advanced fibrosis in patients with Wilson disease. DMRs detected in blood differentiated patients with Wilson disease patients from healthy and disease controls, and distinguished between patients with hepatic and neurologic Wilson disease manifestations.

Genetic disorders which may mimic Wilson disease

Even after an exhaustive genetic analysis of *ATP7B,* patients with a clinical diagnosis like Wilson disease but no *ATP7B* variants have been reported.[1,14,45] If genotyping fails to identify variants, it can be assumed that other hereditary disorders may mimic a Wilson disease-like picture. Before continuing further genetic testing, the available data should be discussed by the involved clinicians and geneticists. Unravelling the genes involved in a Wilson-like disease is relevant, since early diagnosis is essential if the disease can be treated. The genetic tools based on NGS allow us to be more efficient in genetic diagnosis.

Some congenital disorders of glycosylation (CDG), such as the deficiencies caused by variants in *CCDC115* (MIM 616828) or in

TMEM199 (MIM 616829), can display a clinical presentation resembling Wilson disease. CDGs are a heterogeneous family comprising more than 100 congenital disorders characterised by impaired glycosylation of proteins and lipids. Clinical outcomes of CDGs are extremely diverse and range from neuromuscular disorders, such as a congenital myasthenia or a congenital muscular dystrophy, to the Dowling-Degos disease (MIM 179850), a skin disorder. The CDG caused by a defective *CCDC115* gene has a storage-disorder-like phenotype with hepatosplenomegaly, elevated aminotransferases and alkaline phosphatase in combination with psychomotor disability, hypercholesterolemia, and hypotonia.[46] Patients with an initial diagnosis of Wilson disease but with an ultimate diagnosis of CCDC115-CDG have been reported by Girard *et al.*[47] and by Sánchez-Monteagudo *et al.*[14] based mainly on hypoceruloplasminemia or hypertransaminasemia, respectively. TMEM199 is a CCDC115 interacting protein and patients suffering from TMEM199-CDG present with a clinical picture similar to Wilson disease[47]: elevated aminotransferases and alkaline phosphatase, hypercholesterolemia, hypoceruloplasminemia and hepatic steatosis. Vajro *et al.*[48] reported on 2 siblings who displayed a striking similar presentation to Wilson disease with abnormal laboratory data and mildly increased liver copper content; the genetic study ruled out Wilson disease and revealed a *TMEM199* deficiency, which emphasises the power of genetics to delineate complex clinical pictures.

Progressive familial intrahepatic cholestasis (PFIC) is a heterogeneous group of liver disorders with at least 5 forms (PFIC1-5). They are caused by a defective transport of bile acids, and are clinically characterised by early onset cholestasis that progresses to hepatic fibrosis, cirrhosis, and end-stage liver disease before adulthood. Patients affected by PFIC type 3 (PFIC3; MIM 602347) due to variants in *ABCB4*, show a cholestatic liver disorder with liver histological features including elevated hepatic copper and increased urine copper excretion, which is indicative of Wilson disease.[49,50] This striking resemblance may lead to misdiagnosis and inappropriate treatment.

MEDNIK (mental retardation, enteropathy, deafness, neuropathy, ichthyosis, and keratodermia; MIM 609313) syndrome is a severe neurocutaneous disorder with multisystem involvement that combines clinical and biochemical signs of both Menkes disease (MIM 309400) and Wilson disease.[51,52] MEDNIK shares some of the neurological, cutaneous and skeletal symptoms, and low plasma copper and ceruloplasmin, with Menkes disease; while hepatic copper accumulation along with increased urinary copper excretion, and mild T2 hyperintensity of bilateral caudate and putamen on brain MRI are consistent with Wilson disease. The first manifestations of MEDNIK can occur from birth to 1 year of age.[53] MEDNIK is due to defects in the *AP1S1* gene, which encodes a protein that regulates the intracellular copper machinery; mediated by ATP7A and ATP7B, the defective copper ATPases in Menkes disease and Wilson disease, respectively.[53]

Finally, idiopathic or primary copper toxicoses (ICT), like Indian childhood cirrhosis (ICC) are rare diseases whose molecular bases remain poorly understood.[54,55] ICT is characterised by elevated hepatic copper concentrations, Mallory-Denk body formation, severe liver disease and normoceruloplasminemia. The main hallmarks are different from those of Wilson disease, but nonetheless, patients are often misdiagnosed, based mainly on hepatic disease and high urinary copper excretion.[56] Despite being rare, ICT should be considered as a differential diagnosis in affected children with liver disease of unknown aetiology.

Other factors modifying Wilson disease

Modifier genes comprise the other genes that may interact with the disease-involved gene and potentially contribute to the clinical outcome. Several possible modifier genes have been described for Wilson disease, but validation of these reports is lacking (for review[2,57]).

Environmental factors (diet, exercise, stress, toxins and others) may alter the epigenome and may play a role in Wilson disease.[58] The possible role of such factors has been investigated in animal models of Wilson disease.[59]

Moreover, the importance of diet on the clinical outcome is established according to studies revealing that alterations of the intestinal microbiome may contribute to the pathogenesis of a variety of diseases including Wilson disease.[60] Bacterial metabolites like the siderophore yersiniabactin,[61] a polypeptide-polyketide produced by infectious *Yersinia* species and uropathogenic *E coli*[26] or methanobactin[62] produced *by Methylosinus trichosporium* OB3b27 may influence/modulate dietary copper absorption directly by binding copper.

Interpretation of genetic findings in Wilson disease

The availability of new technology does not necessarily mean that disease-causing variants can be found in all patients.[63] Sequencing results or WES reports, which contain information on genetic variants, require clinical correlation. The classification and interpretation of these variants (as pathogenic, likely pathogenic, or variant of uncertain significance [VUS]) reflects the current state of scientific understanding. It should be kept in mind that a VUS corresponds to a genetic alteration that current information is insufficient to determine pathogenicity; therefore, a VUS should not be used in clinical decision making.[64] Finding an undescribed sequence variation of *ATP7B* is not necessarily sufficient to establish the diagnosis of Wilson disease. Asymptomatic patients with 2 unknown *ATP7B* variants (homozygous or compound-heterozygotes) should undergo complete diagnostic work-up including liver biopsy and imaging of the central nervous system.[65] Synonymous sequence variants are usually neglected. We found p.Phe764 = (c.2292C>T, rs372979339) in 11 patients with Wilson disease, who all carried a well-known second variant too. The allele frequency of c.2292C>T is >650-fold higher in a Wilson disease cohort than in public databases. *In silico* prediction algorithms and *in vitro* studies show that c.2292C>T is pathogenic and causes Wilson disease by skipping of exon 8, resulting in an alternative *ATP7B* transcript that is likely inactive as a copper transporter.[66] The prediction by tools like SIFT (sorting intolerant from tolerant), CADD (combined annotation dependent depletion), Polyphen 2 (polymorphism phenotyping) or MutationTaster is not always correct and should be used only as a guide and with caution. These tools are based on different algorithms which cannot be directly compared.

In summary, genetic testing has become an important part of the evaluation of patients with suspected Wilson disease. However, it is not a screening test in patients without any clear evidence for Wilson disease. Interpretation of results of variant testing must be discussed with the referring clinician. In rare cases Wilson disease-like diseases can be detected by NGS, including CDGs, MEDNIK syndrome and ICTs.

Abbreviations

CDG, congenital disorders of glycosylation; DMR, differentially methylated regions; ICC, Indian childhood cirrhosis; ICT, idiopathic or primary copper toxicosis; MLPA, multiplex ligation-dependent probe amplification; NGS, next-generation sequencing; PFIC, progressive familial intrahepatic cholestasis; WES, whole-exome sequencing; WGS, whole-genome sequencing.

Conflict of interest

PF is member of the advisory boards of Alexion, Vivet Therapeutics and Univar; CE has nothing to disclose.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Both authors contributed equally to the production of this manuscript.

Supplementary data

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