Clinical value of molecular testing in patients with Wilson disease

Valoarea clinică a testelor moleculare la pacienţii cu boala Wilson

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Abstract

Wilson's disease is an autosomal recessive disorder characterized by excess hepatic copper accumulation and impaired biliary copper excretion. Over 300 mutations of ATP7B gene have been reported in this disorder, 60% are homozygotes, 30% compound heterozygotes, while 37-63% of the Caucasian patients have the H1069Q mutation. We report an extended family with an affected homozygous member and four heterozygotes showing H1069Q mutation. The diagnosis was suspected on the bases of the presence of Kayser-Fleischer rings in the cornea and the presence of a low serum ceruloplasmin level. Neurological involvement was assessed by clinical examination, while liver involvement was assessed by liver function tests and ultrasonography. The patient with the homozygote genotype presented only neurological manifestations, without the alteration of the hepatic function. By contrast with other studies on patients with H1069Q mutation, our patient showed a degree of concordance between genotype and phenotype, having only neurological symptoms. It is possible that common epigenetic or environmental factors within this family strengthened the genotype phenotype concordance not seen in other families. An important note is that patients with Wilson's disease presenting predominantly neurological symptoms have a later onset and a longer delay until definitive diagnosis and poorer outcome than patients with hepatic symptoms. Wilson's disease should be considered in the differential diagnosis for patients with neurological manifestations, as the disorder benefits of specific treatment with chelating agents and zinc salts, which will improve the clinical status.

Key words: *Wilson disease, ATP7B gene mutation, molecular analysis*

Rezumat

Boala Wilson este o afecţiune recesiv autosomală caracterizată prin acumulare hepatică în exces a cuprului şi excreţie biliară insuficientă. Au fost raportate peste 300 mutaţii ale genei ATP7B care determină această afecţiune, 60% dintre bolnavi fiind homozigoţi, 30% heterozigoţi compuşi, iar 37-63% dintre pacienţii caucazieni prezentând mutaţia H1069Q. Prezentăm o familie extinsă cu un membru afectat, homozigot şi patru

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heterozigoți care prezintă mutația H1069Q. Diagnosticul a fost suspicionat în prezența inelului Kayser-Fleischer şi a unui nivel mai redus al ceruloplasminei serice. Implicarea neurologică a fost evaluată prin examen clinic, în timp ce implicarea hepatică a fost evaluată prin teste funcționale hepatice şi ecografie. Pacienta la care s-a detectat genotipul homozigot a prezentat numai manifestări neurologice, fără alterarea funcției hepatice. Comparativ cu alte studii ale pacienților cu mutație H1069Q, cazul nostru are un grad de concordanță între genotip şi fenotip, având doar simptome neurologice. Este posibil ca factori epigenetici sau de mediu în această familie să fi contribuit la concordanța fenotip-genotip, neobservată la alte familii. Important este faptul că pacienții cu boala Wilson care prezintă predominant simptome neurologice au un debut mai tardiv şi o perioadă mai lungă până la stabilirea diagnosticului definitiv, precum şi prognostic mai nefavorabil decât pacienții cu simptome hepatice. Boala Wilson trebuie luată în considerare în cadrul diagnosticului diferențial pentru pacienții cu manifestări neurologice, afecțiunea beneficiind de tratament specific, cu agenți chelatori şi săruri de zinc, care va îmbunătăți statusul clinic.

Cuvinte cheie: *boala Wilson, mutația genei ATP7B, analiza moleculară*

Introduction

Wilson's disease is a rare autosomal recessive disorder of copper metabolism, with a prevalence estimated to be between 1 in 5,000 and 1 in 30,000, but the frequency of heterozygotes is 1 in 90 individuals. Wilson's disease is present in most populations, especially in those in which consanguineous marriage is common. It is caused by a defective incorporation of copper into ceruloplasmin, leading to progressive accumulation of copper in different tissues and decreased biliary copper excretion. Initially the early accumulation of copper in the liver is associated with steatosis, but if the liver injury is acute, copper may be released in the blood stream and cause hemolytic anemia. Copper may also accumulate in the brain and cause neuropsychological deterioration (1, 2).

The disease usually affects young patients, the mean age being between 8 and 20 years, but elder cases were also described (3). Hepatic and neurological symptoms can be highly variable (4). Liver is affected in half of the patients, and thus individuals with recurrent hepatic disease or unexplained neurological symptoms should be investigated for Wilson's disease. Neurological and psychiatric manifestations are the presenting features in about 50% of cases and may occur without liver disease. They present in different ways, such as tremors, spasticity, rigidity, chorea, slowness of speech or unsteady gait. Neurological symptoms often resemble those seen in Parkinson's disease (5). Psychiatric disturbances occur in many patients and include different forms of psychosis and neuroses. The Kayser-Fleischer ring denotes neurologic impairment and consists of copper deposition in the cornea (6, 7). It is estimated that patients with Wilson's disease that have predominantly neuropsychiatric symptoms manifest them later, have a longer time delay from the onset until definitive diagnosis and have a poorer outcome than patients with hepatic symptoms. Biochemically, the disorder is characterized by low ceruloplasmin and total serum copper levels, increased 24-hour urinary copper excretion and high hepatic copper content (8, 9).

Wilson's disease is caused by mutations of the *ATP7B* gene, which encodes an important transporter of copper with dual synthetic and excretory roles (1, 10). If the mutation has been identified in a patient, family members can be tested for that specific mutation. To detect mutations in the entire gene, gene sequencing can be performed (11, 12). The diagnosis of the disease is based on the combination of clinical, biochemical and genetic tests, which provide a powerful and reliable tool for the diagnosis.

The available treatments are chelating agents and zinc salts. It is appropriate to start treatment as soon as possible, as symptomatic recovery can be achieved if intervention is early (13, 14). It is estimated that after initiating the

treatment, 76.1% of the patients had a stable or improved course of the disease, otherwise death may not be prevented or recovery will be only partial (15, 16).

Material and Method

A 42 year old woman was referred to the Medical Genetics Department for genetic counseling, under the suspicion of Wilson's disease. She presented neurologic manifestations dysarthria, Parkinsonian symptoms such as rigidity and bradykinesia, polyneuropathy, arthropathy and also Kaiser-Fleischer ring. The onset of the disorder was at 16 years and initial symptoms were weakness, general fatigue and impaired mobility. She also had primary amenorrhea and received different regimens of hormonal replacement therapy. The family history revealed that she had two brothers, one of them, who also had neurological involvement, deceased due to an accident. The other otherwise healthy brother is married to an unrelated woman and has two sons of 15 and 16 years. She also has a healthy sister married to an unrelated man and has a 6 year old son.

Molecular tests were performed in the Department of Biochemistry, University of Medicine and Pharmacy, Timisoara. DNA analysis was performed for all family members.

H1069Q mutation in the *ATP7B* gene was identified by Real Time PCR with melting curve analysis. Genomic DNA was extracted from whole blood collected in EDTA using the QIAmp DNA Blood Kit (Qiagen, Hilden, Germany). The ATP7B H1069Q ToolSet™ (Genes-4U by Ratiogen AG, Düsseldorf, Germany) for LightCycler 1.5 instrument (Roche Diagnostics) was used for detection of the H→Q mutation at aminoacidic position 1069 in the ATP7B protein.

PCR reactions were carried out in 10 µl final volume containing 20 ng DNA. The primers (mutation detection and anchor probe), $MgCl_2$ and LightCycler DNA Master Hybridization Probes (Roche Diagnostics) were used at a concentration according to manufacturer protocol. The PCR program required F2 filter (640 nm) and it consisted of an initial denaturation step at 95 °C for 60 s, followed by a 45-cycle program (denaturation at 95°C for 5 s, annealing at 55°C for 10 s with the reading of the fluorescence; acquisition mode: single and elongation at 72°C for 10 s. The melting curve analysis program included three steps: denaturation at 95°C for 30 s, renaturation at 40°C for 60 s and subsequent melting that consists of a continuous fluorescent reading (chanel F2) from 40 to 85°C at the temperature transition rate of 0.1°C per s. The melting curve analysis showed a single melting maximum [-(dF2/F1)/dT] of 68 °C for homozygote HH1069 DNA and 61°C for homozygote 1069QQ. In the case of heterozygote H1069Q DNA, the two melting maxima were present.

Results

Laboratory testing revealed that serum ceruloplasmin level was 20 mg/dl (normal values 20-80 mg/dl) not only for the proband, but also for her sister and living brother.

Molecular testing was performed for the proband, her brother, sister and their spouses and children. *ATP7B* gene mutation analysis of the proband's blood sample revealed H1069Q point mutation in *ATP7B* gene. After analyzing the patient's genotype it was established as homozygous for H1069Q mutation (*Figure 1*). DNA of the family members was tested for the presence of this mutation and two carriers were identified (*Figure 2*).

ATP7B gene mutation analysis of the proband's brother blood sample (II.1) revealed a H1069Q point mutation in one *ATP7B* gene, a heterozygous genotype for H1069Q. *ATP7B* gene mutation analysis of blood samples of his wife and two children did not reveal the presence of the point mutation H1069Q in *ATP7B* genes. After analyzing these last genotypes, they were established as homozygous normal (wildtype) (*Figure 3*).

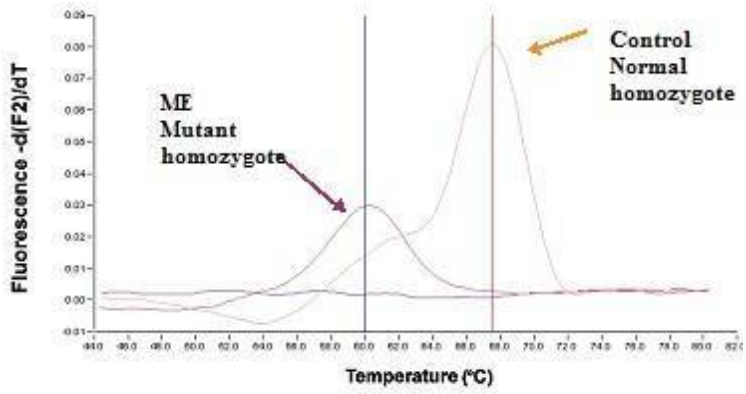

Figure 1. Melting curve analysis for point mutation H1069Q revealing the presence of the homozygous status of the mutation in the proband

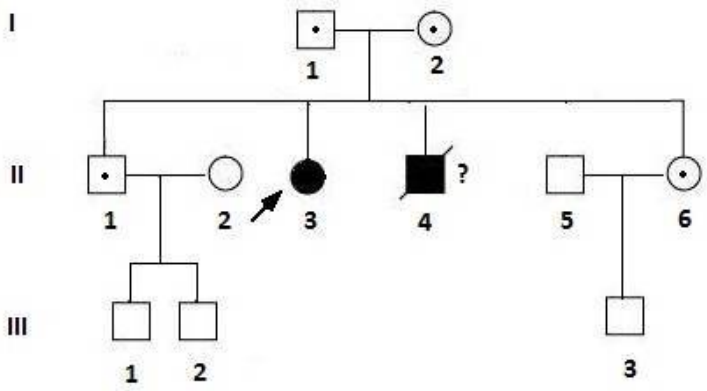

Figure 2. Pedigree of the investigated kindred. The proband, aged 42 years, (arrow) was shown to be a homozygote for the point mutation H1069Q. One brother, deceased at 29 years, also had clinical manifestations. The other brother and her sister were shown to be heterozygotes for the same point mutation, but their descendents do not carry the mutation.

ATP7B gene mutation analysis of the proband's sister (II.6) revealed a H1069Q point mutation in one *ATP7B* allele, a heterozygous genotype for H1069Q. Blood samples from her husband and their son were also analyzed. *ATP7B* gene mutation analysis did not reveal the presence of the mutation in their DNA, thus, the genotypes were established as homozygous normal (wildtype) (*Figure 4*).

Discussion

In 1912, Wilson first described a familial disorder associated with neurologic symptoms and cirrhosis, afterwards this was called Wilson's disease. It is a rare inherited disorder of copper transport, leading to excess storage of copper in liver, brain, kidney and cornea. Copper is incorporated into enzymes that play different roles in the regulation of iron metabolism, formation of connective tissue, energy production and function of the nervous system. It is estimated that about 95% of the copper found in the blood is bound to ceruloplasmin.

Cases with liver involvement typically have symptoms starting in early childhood, those with brain involvement may have the onset of neurologic or psychiatric symptoms in their teens or early twenties, but the age range for both types can vary from about three years old to more than fifty. The diagnosis is based on the classic triad consisting of Kayser-Fleischer ring, low serum ceruloplasmin and increased amounts of liver and urinary copper (17).

In our patient, neurologic manifestations occurred at 16 years of age. In literature cases with such manifestations are also described. Thus, Jung et al. (18) reported a 17 year-old affected boy, who presented with polyneuropathy, his initial symptoms being intermittent paresthesia and weakness in hands and feet. Other authors (19) reported a 13-year-old boy with leukoencephalopathy that occurred early in the disease course. A significant phenotypic variation was described. In a study on patients mainly from Austria, neurological involvement was significantly more common than hepatic mani-

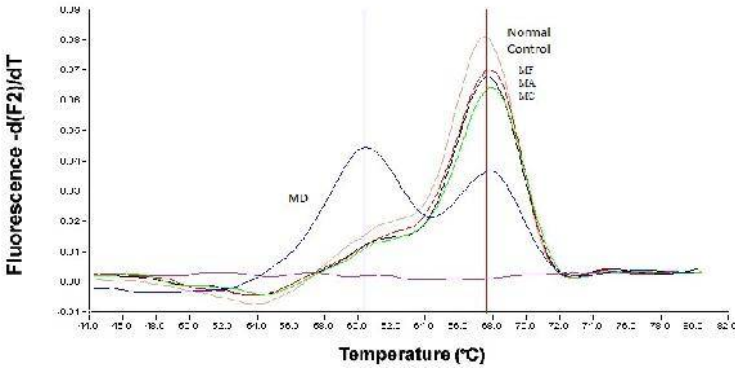

Figure 3. Melting curve analysis for point mutation H1069Q in one family, revealing the presence of a heterozygote and 3 healthy individuals

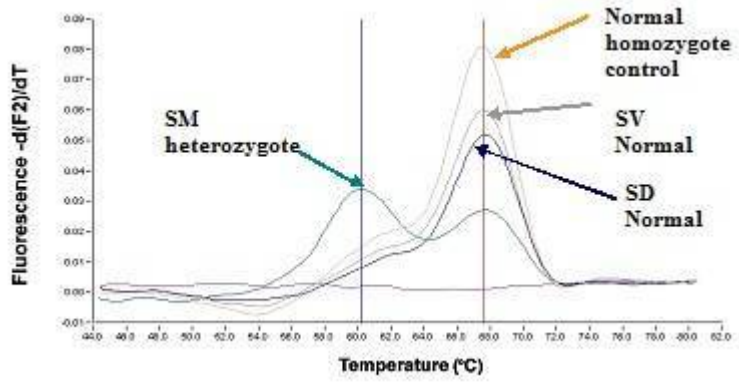

Figure 4. Melting curve analysis for point mutation H1069Q revealing the presence of one carrier of the mutation and two healthy subjects

festations in homozygotes that had H1069Q mutation, compared to H1069Q compound heterozygotes or H1069Q negative patients (20). Genotype-phenotype studies could not explain yet the underlying complex mechanism of copper-mediated cellular network involved in regulation of cell function.

Ferenci proposed a scoring system for the disease that takes into account clinical, biochemical and histological features, each having allocated a score and the total score indicated the possibility of a person to have Wilson's disease (21). Assessment of patients should include medical and family history, physical examination, liver function tests, full blood count, serum copper and ceruloplasmin and 24-h urinary copper excretion. Liver biopsy quantitative copper concentrations are considered the best biochemical evidence for Wilson's disease.

The disorder cannot be diagnosed on the basis of a specific test, as symptoms are often nonspecific. Different tests can be performed for affected individuals or their families. Testing objectives are establishing the correct diagnosis, differential diagnosis, evaluating the severity of the disorder, distinguishing between cases with the disease and those who carry the mutant gene and monitoring the effectiveness of treatment. Testing may be also performed to identify presymptomatic or carrier family members or for prenatal diagnosis (22).

Biochemical tests evaluate ceruloplasmin, which is usually decreased, but about 5% of affected patients with neurological symptoms and up to 40% of those with hepatic symptoms have normal ceruloplasmin levels. Total serum copper is usually decreased and free serum copper has increased values. 24-hour urine copper is also usually increased.

Disease diagnosis is now based on molecular testing (23). The gene being known to be associated with the disease, *ATP7B* gene, is located on chromosome 13q 14.3, contains 21 exons and encodes a protein, a copper-transporting P-type ATPase, which is needed to attach copper to the developing ceruloplasmin molecule, but also to excrete copper into the bile, eliminating excess copper from the body. The encoded protein has 14 domains: 6 copper binding, 4 transmembrane, 1 phosphatase, 1 transduction, 1 phosphorylation, and 1 adenosine triphosphate (ATP)

binding. Mutations in this gene were detected in 90% of patients. It is estimated that about 24.6% of all mutations involve the ATP-binding domain. There are currently known about 40 normal variants of the *ATP7B* gene and over 400 different mutations of *ATP7B* gene that have been associated with the disease (1, 24). These may be insertions, deletions, splicing or point mutations. Frame shift and nonsense mutations that lead to the formation of a truncation protein usually cause a severe form of the disease (25). The prevalence of the mutations varies throughout the world. In most ethnic groups, usually one of these mutations is prevalent, the others are quite rare. The disorder has an autosomal recessive inheritance. Affected individuals may have two copies of the same genetic mutation or two different mutations. Compound heterozygotes are frequently found, causing difficulties in assessing genotype–phenotype correlations.

The most common mutation in Caucasians is H1069Q, mutation that was also detected in our patient. It is estimated that it has a frequency of up to 72% among affected cases, but it is less frequent in the Mediterranean area and almost absent in the Asian patients (26). This mutation was associated with a mildly disturbed copper metabolism and late onset of the disease. Even if the gene mutation is present some of the cases never develop liver disease. The molecular basis of phenotypic variation in patients that have the same mutation of *ATP7B* gene is not clear yet. It may be due to environmental factors such as different copper intake and/or modulation by different modifier genes such as *ATOX1* and *COMMD1*, which might play a role in the sensing or trafficking function of the encoded protein. In Sardinians a 15-nucleotide deletion seems to be frequent and a missense mutation (Arg778Leu) was found among Mongoloids. In India, more than 50% of the cases have one of the seven frequent haplotypes, mutations of *ATP7B* gene being found in about 37% of the patients (10). Thus, knowing the prevalent mutation in a given population is helpful in achieving rapid mutational screening. Otherwise, identification of unknown mutations in *ATP7B* gene, which has a large size with 21 expressed exons, may be very challenging (27).

Molecular testing allows detection of mutations, but does not predict the severity of the disorder, which can vary significantly, even within the same family. Clinical value of molecular analysis is represented by establishment of the diagnosis, allowing early detection, determining the risk for family members, presimptomatic or carrier identification and prenatal diagnosis.

The aim of the treatment is to remove the excess copper and to prevent its reaccumulation. Therapy is based on copper chelators (28, 29). Liver transplantation can be used for complete reversal of the metabolic abnormality (30). Gene therapy may represent the future option for treating Wilson disease (31).

Conclusions

Cases with Wilson disease may have a negative family history as it is an autosomal recessive disorder, but also there might have been mild subclinical expressions in other family members. Molecular analyses that identify mutations in the *ATP7B* gene allow unequivocal diagnosis in affected symptomatic and nonsymptomatic individuals. Genetic counseling will reveal the importance of biochemical and genetic testing of other family members, who might be carriers of the mutation. Molecular testing is a reliable, simple and cost-effective method, which allows detection of the most common mutation H1069Q.

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