Neurological Symptoms, Genotype-Phenotype Correlations and Ethnic-specific Differences in Bulgarian Patients With Wilson Disease

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Wilson disease (WD) is an autosomal recessive disorder of copper metabolism presenting with a variety of symptoms, commonly as liver and/or neuropsychiatric disease.1 The neurological symptoms are various, but the most frequent are dysarthria, tremor, rigidity, or dystonia.2,3 WD is caused by mutations of ATP7B coding for P-type copper transporting ATPase. More than 500 ATP7B mutations have been described.4 The genotype-phenotype correlations are complicated because of the rarity of some mutations and the large number of compound heterozygotes. The most frequent mutation for Northern, Central, and Eastern Europe is p.H1069Q with allele frequency of 43.5%.5

Some previous reports demonstrated that patients who carried 2 protein-truncating mutations had an earlier disease onset and lower ceruloplasmin levels when compared with patients who carried 2 missense mutations.8,14 A study of 58 Italian children with WD failed to demonstrate differences in respect to age of onset between patients with nonsense/frameshift mutations and patients with missense mutations. However, the former had significantly lower levels of ceruloplasmin and serum copper compared with the latter.15 A recently published study of 59 German WD patients showed that truncating ATP7B mutations are associated with lower ceruloplasmin serum oxidase levels and an earlier age of onset compared with missense mutations.16

The aim of our study was to characterize the neurological symptoms in Bulgarian WD patients, to investigate genotype-phenotype correlations, and to test whether there are differences in phenotype between patients of different ethnic origin.

PATIENTS AND METHODS

A total of 126 Bulgarian patients with WD from 106 unrelated families were included in the study. For all of the patients detailed information from all available family members for constructing the pedigrees was obtained not only to detect additional affected individuals but also to exclude or confirm relation of the families, which is especially important for Roma families. The patients were recruited from clinics of neurology (61 patients), gastroenterology (26 patients), and pediatrics (36 patients) of University hospitals located in 3 Bulgarian cities, representing all cases of WD patients in the study hospitals. Thirty-seven patients are Roma, 7 are of Turkish origin, and the remaining are Bulgarians. Although
the Roma and the patients of Turkish descent were born in Bulgaria and have a Bulgarian citizenship, they are not ethnic Bulgarians. At the time of the most recent examination the patients were aged between 10 and 71 years (mean age 28.6±12.05 y). Fifty-nine patients are female and 67 are male. Seventy-one patients had neurological or neuropsychiatric presentation, while 52 had hepatic manifestation and 3 were presymptomatic having been identified during family screening. Detailed history was obtained from all patients and from available medical records. Physical and neurological examination, laboratory investigations of copper metabolism, slit-lamp examination, abdominal ultrasound, and magnetic resonance imaging or computed tomography of the brain were performed. Molecular genetic testing using single-strand conformation polymorphism analysis followed by direct sequencing was performed in all patients. The diagnosis was based on the clinical symptoms, laboratory data of abnormal copper metabolism, detection of Kayser-Fleischer rings (KF Ring), if present, and identification of the disease-causing ATP7B mutations. We used the following variables to assess genotype-phenotype correlations: age at onset, mode of presentation, development of neurological or hepatic signs during the course of the disease, KF Ring, levels of serum ceruloplasmin and copper before treatment, basal 24-hour urinary copper excretion, neurological form (parkinsonian, pseudo-sclerotic, or dystonic-choletic). We compared the patients who carried some frequent mutations with the remaining patients and with patients homozygous for p.H1069Q. Then we divided the patients in 3 groups: homozygous and compound heterozygous for p.H1069Q and noncarriers of p.H1069Q. Finally, we classified the patients according to the type of mutations: patients with 2 missense mutations or 1 missense and 1 protein-truncating mutation or 2 protein-truncating mutations. SPSS, version 17.0, descriptive statistics, Pearson correlation, Kruskal-Wallis, Mann-Whitney U and t tests were used for statistical analysis of the data. The level of statistical significance was set at \( P < 0.05. \)

Written informed consent has been obtained from all patients or parents/guardians. The study was approved by the ethics committee of University Hospital “Alexandrovskaya.”

RESULTS

Neurological Signs

Eighty-two patients demonstrated neurological signs at the beginning or during the course of disease representing 66.7% of all symptomatic patients. The neurological signs are diverse (Fig. 1). Tremor was the most frequent sign found in >85% of the patients. It is predominantly intention or postural. Resting tremor was also observed, less frequently though. The highly characteristic “wing-beating” tremor was found in 22 patients. The second most commonly observed sign was dystonia. It was found in a total of 62 out of 82 patients and was very severe in 7 patients, who were almost unable to speak. Rigidity was found in <40% of the patients; bradykinesia, hypomimia, and parkinsonian gait were less frequently observed, in 25.61% and 20.73% of the patients, respectively. Dysphagia was found in 28 patients and was accompanied by drooling in 22. Pyramidal signs were not an uncommon finding and were recorded in 30% of the patients. Cerebellar disturbances other than tremor and dystarthritis were also observed: dysmetria, dysdiadochokinesia, ataxic gait, and nystagmus. Surprisingly, dystonia was not frequently found in our cohort, as it was detected in only 10 patients (12%). Chorea, spasmus mobilis (a tonic spasm occurring in extremities on attempted movement), choreoathetosis, myoclonic jerks, and athetosis were rarely observed. Interestingly, an 18-year-old patient presented with acute onset of right-sided hemiballismus. Five of the patients were wheelchair-bound because of pronounced rigidity and contractures (3 patients) or severe ataxia (2 patients).

Epileptiform seizures were observed in 2 patients. One patient developed convulsive status epilepticus following initiation of D-penicillamine treatment.

Six patients reported sleep disturbances, 5 had insomnia, and 1 declared hypersomnia.

Tremor was the most frequent initial neurological sign found in 50 out of 76 patients (65.8%). Ten out of 76 patients (13.2%) had dystonia as a presenting sign. Five out of 76 patients (6.6%) presented with dysphagia and dysphonia at the first consultation. Three juvenile patients had declined school performance, while 2 adults demonstrated personality changes at disease onset.

Psychiatric signs were observed in 15 patients and were the initial signs in 8. Schizophrenia-like psychosis occurred in 6 patients and was the initial presentation in 3. Depression occurred in 7 patients and was the presenting sign in 2.

Slit-lamp examination before treatment was performed in 101 patients. KF Ring was found in 80 patients, 65 of them (81.25%) had neurological manifestation.

Genetic Results

Molecular genetic testing identified disease-causing ATP7B mutations in 123 patients.

Although the diagnosis of WD was highly likely in 3 patients, with scores of 5 and 6 according to the scoring system developed at the 8th International Meeting on WD,17 no mutations by sequencing of all 21 exons of ATP7B were found. Molecular genetic testing was not completed in 2 patients and they remained with 1 ATP7B mutation.

We identified a total of 27 different mutations of ATP7B (Fig. 2).

H1069Q is the most frequent mutation in the Bulgarian population. Ninety-six out of 123 patients (78%) carry H1069Q on at least 1 allele and 60 are homozygous for it. With the exception of 3 patients from 2 unrelated families, who are compound heterozygous for p.H1069Q, the remaining 34 Roma patients are homozygous for p.H1069Q.

Genotype-Phenotype Correlations

A total of 117 patients were included in the study of genotype-phenotype correlations. The presymptomatic patients and patients with 1 or no ATP7B mutations were not included in this statistical analysis.

Our results showed no significant difference in respect to age of onset between patients who carried or did not carry p.H1069Q. The mean age at onset of patients carrying p.H1069Q homozygously or heterozygously was 19.17 ± 8.4 years and of patients without p.H1069Q was 19.04 ± 9.3 years. The patients who carried p.R616Q either homozygously or heterozygously had significantly later age of onset than the remaining patients (27.5 ± 8.2 vs. 18.34 ± 8.2 y, \( P < 0.001 \)) and compared with the patients homozygous for p.H1069Q (27.5 ± 8.2 vs. 17.8 ± 6.5 y, \( P < 0.001 \)).

No significant difference was found between patients homozygous for p.H1069Q, patients heterozygous for p.H1069Q, and patients without p.H1069Q in respect to neurological presentation. In contrast, patients who carried p.R616Q on at least 1 allele had significantly more frequent neurological manifestation than patients homozygous for p.H1069Q in the Roma and all other patients. The patients homozygous for p.H1069Q significantly more frequently presented with hepatic signs than...
patients heterozygous for p.H1069Q and patients without p.H1069Q.

No significant difference was observed with respect to serum ceruloplasmin level and basal 24-hour urinary copper excretion (before treatment) between patients homozygous or heterozygous for p.H1069Q and patients without p.H1069Q. Patients who carried p.R616Q either homozygously or heterozygously had higher ceruloplasmin level than patients homozygous for p.H1069Q (0.24 ± 0.1 vs. 0.12 ± 0.06 g/L, \( P = 0 \)) and all other patients (0.24 ± 0.1 vs. 0.1 ± 0.06 g/L, \( P = 0 \)).

The patients homozygous for p.H1069Q had significantly less frequently KF Ring than patients heterozygous for p.H1069Q and patients without p.H1069Q. All patients who carried p.R616Q on at least 1 allele had KF Ring.

A positive statistically significant correlation was observed between parkinsonian signs and p.A874V mutation. Three patients with neurological signs had this mutation; 1 of them was homozygous for p.A874V, whereas the others carry the mutation heterozygously (p.A874V/p.H1069Q and p.A874V/p.Q1277X). These 3 patients demonstrated predominantly parkinsonian signs and extremely severe rigidity leading to contractions in 2 of them. The patient homozygous for p.A874V showed severe rigidity in all limbs and contraction of the left elbow. On the basis of these findings it can be speculated that p.A874V is associated with severe rigidity, however, more patients homozygous for p.A874V are necessary to support this observation.

We designated nonsense and frameshift mutations predicted to cause production of truncated protein as “severe.” A total of 82 patients carried 2 missense mutations, 7 patients carried 2 “severe” mutations, and 21 patients carried 1 missense and 1 “severe” mutation. The patients who carried 2 “severe” mutations had significantly earlier disease onset than patients carrying 2 missense mutations, 13 ± 5.1 versus 20 ± 8.9.

![FIGURE 1. Observed neurological signs.](image1)

![FIGURE 2. Mutations of ATP7B identified in Bulgarian Wilson disease patients. The exons are presented as numbered boxes connected with lines representing introns. The location of mutations is marked by arrows. The newly identified mutations are in italic.](image2)
years (P = 0.02). No significant difference between the 3 groups was observed with respect to mode of presentation.

The patients who carried 2 “severe” mutations had significantly lower ceruloplasmin levels than patients who carried 2 missense mutations (0.06 ± 0.02 vs. 0.12 ± 0.07 g/L, P = 0).

No significant difference was found between the 3 groups with respect to basal urinary 24-hour copper excretion and presence of KF Ring.

**Mutation p.R616Q**

Interestingly, mutation p.R616Q was associated with the pseudosclerotic form of WD. Out of 123 patients, 9 carried p.R616Q on 1 allele and 2 additional patients were homozygous for p.R616Q. Ten of these patients presented with late onset of neurological and/or psychiatric symptoms (Table 1). The neurological signs were predominantly or exclusively cerebellar in all but patient 9 (Table 1). Patient 9 developed schizophrenia-like psychosis 4 years after the onset of tremor. She was treated with a variety of neuroleptics and developed parkinsonism 2 years later.

The level of serum ceruloplasmin was normal in 4 out of 6 patients in whom it was measured. Slit-lamp examination detected KF Ring in all 10 examined patients.

**Ethnic-specific Differences in WD**

We compared Bulgarian and Roma symptomatic WD patients homozygous for p.H1069Q (24 and 32 patients, respectively). Our data showed that Roma patients presented with symptoms at a significantly younger age than Bulgarian patients (15.85 ± 6.6 vs. 20.5 ± 5.4 years, respectively; P = 0.02). Bulgarian patients had significantly more frequent neurological presentations, whereas Roma patients had more frequently a hepatic manifestation. In addition, Roma patients significantly more rarely developed neurological signs during the course of the disease compared with Bulgarian patients homozygous for p.H1069Q (P = 0).

No significant differences were observed between the 2 groups in respect to serum ceruloplasmin level, basal 24-hour urinary copper excretion, and presence of KF Ring.

**DISCUSSION**

Neurological signs are commonly found in WD. Tremor and dysarthria were most frequently encountered in our patients. Unlike previously reported studies, Parkinsonian and pyramidal signs were less frequently observed. In contrast, tremor and pyramidal signs were more commonly found in our patients than the reported frequencies in Brazil, India, and Iran. The rarely observed dystonia in our cohort can be attributed to the relatively small number of children with neurological manifestation included in the study (50 of the patients had disease onset under the age 18 years and 14 of them demonstrated neurological signs). Alternatively, different ATP7B mutations are likely to influence the neurological type of presentation, as we found an association of some mutations with parkinsonism or pseudosclerosis. The genetic background of WD is quite different in Bulgarian population compared with this of Brazilian and Indian population.

Psychiatric signs were observed in 12% of the patients and sometimes are the initial presentation of the disorder. In one of our patients the disease presented with schizophrenia-like psychosis 14 years before the development of tremor. Thus, psychiatrists can be the first medical specialists to diagnose WD.

KF Rings, the hallmark of the disease, were found in 81% of the patients with neurological manifestation, suggesting that the absence of KF Ring in a neurological presentation should not preclude the diagnosis.

Similar to other countries from Eastern and Central Europe, p.H1069Q mutation is the most frequent mutation in the Bulgarian population. Our data show that the p.H1069Q mutation is not associated with later onset and neurological manifestation unlike some previously published studies. In contrast, the Bulgarian patients homozygous for p.H1069Q had significantly more frequent hepatic presentation. One study to date described an association of p.H1069Q with hepatic manifestation, but the number of patients was only 13.

Our study demonstrated that the missense mutation p.R616Q is associated with a milder phenotype—later onset of neurological signs and higher levels of ceruloplasmin.

Here, we report for the first time the association of p.R616Q with the pseudosclerotic type of WD. Only 1 patient who carried p.R616Q had parkinsonism in addition to pseudosclerosis which should not be easily attributed to WD, as parkinsonian features developed 2 years after administration of neuroleptics. Eight patients have p.R616Q in combination with different missense, frameshift, or splice-site mutations, which are likely to influence the phenotype. However, all these patients may be classified as having the pseudosclerotic type of WD. Moreover, the 2 patients homozygous for p.R616Q demonstrated only tremor and dysarthria. In practical terms, we recommend screening for p.R616Q (after exclusion of the frequent for the respective geographical area ATP7B mutations) in WD patients presenting with pseudosclerosis.

In contrast to p.R616Q, the mutation p.A874V is likely to cause parkinsonism in WD, but more patients are needed to confirm this observation.

Mutations predicted to cause premature termination of translation and production of truncated protein lead to more severe phenotype, and an earlier disease onset and lower ceruloplasmin levels. These data are in line with previously published studies. Absence of a full-length gene product leads to complete disruption of ATP7B function thus conferring a more severe phenotype.

In contrast to Bulgarian patients, the genetic basis of WD in Roma patients is quite homogeneous. All Roma patients carry p.H1069Q suggesting that screening for this mutation is a cost-effective approach for the molecular diagnosis in Roma patients. This is very important for the family screening, which should be applied not only to siblings but also to children of the proband given the higher p.H1069Q carrier frequency in some Roma subisolates making possible pseudodominant inheritance.

The Roma represent a young founder population of common Indian descent. Studies of Roma have made a major contribution to Mendelian genetics through novel private diseases and private mutations causing disorders of wide ethnic distribution. However, the common WD p.H1069Q mutation has been imported into the Roma population by admixture, similar to cystic fibrosis ΔF508 and has expanded with population growth. In support of this, p.H1069Q has not been detected in Indian WD patients and haplotype studies indicate that p.R616Q is associated most commonly with the D13S314/D13S316 haplotype, that is the most common haplotype observed in the ethnic Bulgarians. In contrast to Bulgarian patients homozygous for p.H1069Q, Roma patients had an earlier disease onset and hepatic manifestation. Given the identical ATP7B genotype such a difference in clinical presentation can be explained by additional genetic and environmental factors that modify the phenotype. Mutation or polymorphism in a yet unidentified modifier gene that has
<table>
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<th>Patient</th>
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<th>Liver Cirrhosis</th>
<th>Scanning Dysarthria</th>
<th>Dysmetria/Intention Tremor/Dysdiadochokinesis</th>
<th>Postural Tremor</th>
<th>Ataxic Gait</th>
<th>Other Neurological or Psychiatric Symptoms</th>
<th>Kayser-Fleischer Ring†</th>
<th>Ceruloplasmin g/L‡</th>
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*Detection of KF ring at the age of 37 years on routine ophthalmological examination.
†Before treatment.
— indicates no; +, yes; CT, computed tomography; MRI, magnetic resonance imaging; ND, no data; T2WI, T2 weighted image.
spread by founder effect and genetic drift in the Roma population could predispose Roma patients to earlier onset and hepatic presentation of WD. To our knowledge, this is the first study to describe ethnic-specific differences in WD.

The extreme heterogeneity in WD can be partially explained by the different ATP7B mutations, for example, protein-truncating mutations lead to earlier onset and more severe phenotype. The different missense mutations result in a variety of defects in the molecular function of ATP7B thus leading to phenotypic heterogeneity. A promising approach in investigating genotype-phenotype correlations will be the comparison of patients according to the functional effects of the disease-causing mutations, as previously suggested.27

In contrast, the marked differences observed among siblings in our cohort (the siblings in 9 out of 18 families demonstrated a similar age at onset and mode of presentation) coupled with the above-mentioned ethnicity-specific differences point to the implication of factors other than ATP7B mutations that shape the phenotype. The thorough characterization of all molecular mechanisms participating in copper transport functions of ATP7B and interacting partners for ATP7B is very important for understanding the molecular pathogenesis of WD27 and will provide a number of candidate modifier genes of the WD phenotype.

ACKNOWLEDGMENT

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