Diagnostic Accuracy of Serum Ceruloplasmin in Wilson Disease: Determination of Sensitivity and Specificity by ROC Curve Analysis among ATP7B-Genotyped Subjects

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BACKGROUND: A serum ceruloplasmin concentration below 0.20 g/L is conventionally considered as one of the major diagnostic criteria for Wilson disease. This decision threshold has not been fully validated for its diagnostic characteristics, however. In this study, we evaluated various decision thresholds of serum ceruloplasmin concentration in the diagnosis of Wilson disease based on genotype-verified Wilson disease patients, carriers, and normal individuals.

METHODS: Serum ceruloplasmin concentration was measured by a nephelometric method in 57 Wilson disease patients and 71 family members (49 heterozygotes and 22 wild-type homozygotes), a validation group of 25 subjects clinically suspected of Wilson disease, and 690 normal individuals. We performed ROC analysis using Analyze-it software and confirmed the genotypes by direct DNA sequencing of ATP7B.

RESULTS: Serum ceruloplasmin concentrations <0.20, 0.14, and 0.10 g/L showed positive predictive values of 48.3%, 100%, and 100%, respectively, and negative predictive values of 98.7%, 97.1%, and 91.9%. In the validation group, a serum ceruloplasmin threshold of 0.14 g/L rendered 100% sensitivity and specificity. Forty of 690 healthy subjects had serum ceruloplasmin concentrations <0.20 g/L; however, these 40 individuals had normal genotypes by DNA sequencing, and none of the 40 had ceruloplasmin concentrations <0.14 g/L.

CONCLUSIONS: The diagnostic accuracy for Wilson disease using a serum ceruloplasmin concentration of 0.14 g/L as the local decision threshold was better than that using a threshold of 0.20 g/L. We suggest that laboratories providing ceruloplasmin assays determine decision thresholds based on local populations.

Wilson disease (MIM # 277900), an autosomal recessive disorder of copper transport, is characterized by progressive neurological deterioration and hepatic damage that can be catastrophic if untreated. Timely diagnosis and early treatment are crucial to prevent permanent damage and to avert disease progression. The worldwide prevalence of Wilson disease (WD)3 was reported to be 1:30 000 from data established more than 20 years ago by Scheinberg and Sternlieb (1). The disease frequency is highly variable, and WD is seen more frequently in Sardinia, China, Japan, and other Asian populations (2–9). Underdiagnosis of WD is highly likely, especially during the asymptomatic phase and in patients with atypical presentations.

Conventionally, diagnosis of WD is based on at least 2 of the following findings: typical neurological symptoms, presence of Kayser-Fleischer (KF) rings by slit lamp examination, and low serum ceruloplasmin concentration. Interestingly, hepatic dysfunction is not one of the criteria. A diagnostic scoring system has been developed that includes 7 parameters (neurological symptoms, KF rings, Coombs-negative hemolytic anemia, serum ceruloplasmin concentration, urinary copper excretion, liver copper measurement, and genetic mutations) (10). Other biochemical markers have been proposed such as serum free copper concentration (11) and copper:ceruloplasmin ratio (12), but these appear to be less reliable. False-positive and -negative rates are high among the traditional markers. For examples, up to 50% patients presenting with hepatic

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Nonstandard abbreviations: WD, Wilson disease; KF, Kayser-Fleischer.
Liver copper in quantities >25 μmol/day after penicillamine challenge was found to be useful in confirming symptomatic WD patients with active liver disease (sensitivity 92%) but unreliable for excluding WD in asymptomatic siblings (sensitivity 46%) (15). Liver copper in quantities >250 μg/g dry weight without cholestasis is considered to be the diagnostic gold standard, with a reported sensitivity of 86.3% and specificity of 98.3% (16), but liver copper less than this threshold does not allow WD to be confidently ruled out. Moreover, the distribution of copper within the liver is heterogeneous, and hepatic copper content may vary up to 500-fold (17). Genetic analysis of ATP7B is the most decisive tool and is considered the only reliable test for family screening of asymptomatic siblings. Comprehensive diagnostic approaches with limited mutation screening have been proposed for populations with common mutations (18–22). More than 340 mutations have been reported so far (http://www.medicalgenetics.med.ualberta.ca/wilson/index.php), and the spectrum is population specific (22).

Serum ceruloplasmin is one of the biochemical markers for the diagnosis of WD. A concentration below the lower reference limit (conventionally taken as 0.20 g/L) is considered diagnostic of WD (1, 10, 23). A lower cutoff of <0.10 g/L has been proposed in the WD scoring system (10); however, such values have not been fully characterized in terms of sensitivity and specificity. In fact, the lower reference limit varies with many factors, e.g., different analytical methods, standardization, and the age of testing subjects. Our current study investigated the diagnostic value of serum ceruloplasmin concentration for WD patients and family screening among genotypically confirmed subjects.

Materials and Methods

SUBJECTS
We recruited 57 WD patients (ages 4–50 years) from 42 unrelated families. The diagnosis of WD was established by a WD score of 4 or more (10). Serum ceruloplasmin concentrations were measured at the time of diagnosis and before the initiation of chelation therapy. We also recruited 71 family members from these families. The validation group comprised 25 patients suspected of having WD who presented with hepatic dysfunction and/or neurological deficits. The reference interval of ceruloplasmin was established using data from 690 healthy Chinese residents of Hong Kong (ages 18–60 years) in whom there was no clinical evidence of WD. Informed consent was obtained from all the participants, and the local ethics committee approved the study.

MEASUREMENT OF SERUM CERULOPLASMIN
We measured serum ceruloplasmin concentration by use of the IMMAGE Immunochemistry System (Beckman Coulter); specimens collected before May 2, 2002, were measured by use of the Array instrument (Beckman Coulter). Both analyzers employed a nephelometric assay calibrated against the same primary standard CRM470 (RPPHS 91/0619) for measurement of ceruloplasmin. Correlation data between the 2 instruments provided by the manufacturer showed a correlation coefficient of 0.995, slope 0.996, and intercept of −0.02 g/L (Beckman Coulter IMMAGE Immunochemistry chemistry information sheet for CER, Ceruloplasmin, 988627 AE, November 2007). All the samples were handled as routine specimens according to the manufacturer’s recommendations.

ANALYSIS OF ATP7B
We extracted genomic DNA from peripheral blood samples using a QIAamp Blood Kit (Qiagen). In all WD patients, the coding exons 1–21 and the flanking introns of the ATP7B gene were amplified by PCR. Family members were screened only for the mutations found in their respective probands. Exons 2, 3, 8, 10, 11, 12, 13, 16, and 18 covered more than 80% of mutations in our study population (24). We screened healthy subjects with ceruloplasmin concentrations <0.20 g/L and all subjects in the validation group for the aforementioned exons. Once a heterozygous mutation was found, all the remaining coding exons were sequenced. We carefully designed the PCR primers to avoid allele dropout, and thermal conditions were as described (25). PCR products were purified by Microspin S-300 HR columns (GE Healthcare) and sequenced using their amplification primers and BigDye–Deoxy terminator cycle sequencing reagents, according to the manufacturer’s instructions (Applied Biosystems). We purified products of the sequencing reactions using AutoSeq G-50 columns (GE Healthcare), separated purified sequencing fragments by capillary electrophoresis, and detected fragments by laser-induced fluorescence on an ABI Prism 3100 genetic analyzer.

ROC CURVE ANALYSIS
We performed all statistical analyses using Analyze-it Clinical Laboratory version 2.07 software (26). We determined the sensitivity, specificity, and predictive values of serum ceruloplasmin concentration in predicting WD at different cutoff values by the construction of a ROC curve (sensitivity plotted against 1 − specificity at different concentrations of ceruloplasmin) includ-
ing the WD patients, their family members, the validation group, and 40 subjects with serum ceruloplasmin concentrations <0.20 g/L from the reference interval group.

Results

We found disease-causing mutations in all WD patients except for 2 patients in whom only 1 mutation was found after sequencing all the coding exons of ATP7B and about 1 kb of each 5’ and 3’ untranslated region including the promoter region. Among 71 family members, 49 were heterozygous for their family mutations and 22 were wild-type homozygotes. The ranges of serum ceruloplasmin concentrations for the 49 heterozygotes and 22 wild-type homozygotes were 0.15–0.48 g/L (mean 0.24; SD 0.06) and 0.16–0.40 g/L (mean 0.29; SD 0.06), respectively.

We performed ROC curve analysis with use of data from 57 WD patients, 71 family members, 25 patients of the validation group, and 40 subjects with serum ceruloplasmin concentrations <0.20 g/L from the reference interval group. The ROC curve analysis (Fig. 1) suggested that the most useful cutoff value of serum ceruloplasmin concentration was 0.14 g/L, where the sum of sensitivity (93%) and specificity (100%) was the highest, and the numbers of false results were minimized among all the calculated values with 0 false positives and 4 false negatives. The positive and negative predictive values were 100% and 97.1%, respectively. The conventional cutoff of 0.20 g/L gave a sensitivity of 98.2% and a specificity of 55.9%, with 60 false positives and 1 false negative. The positive and negative predictive values were 100% and 91.9%, respectively. The area under the ROC curve was 0.99 (SE 0.008; 95% CI 0.97–1.01; P < 0.0001).

FALSE NEGATIVES

Four false-negative diagnoses resulted when using a ceruloplasmin threshold of 0.14 g/L; all 4 of these false-negative patients had WD scores ≥4 despite having serum ceruloplasmin concentrations ≥0.14 g/L (Table 1). All had KF rings and other abnormal parameters suggestive of impaired copper metabolism (increased 24-h urinary copper excretion >1.0 μmol/day and/or hepatic copper content >250 μg/g dry weight). All presented with acute liver failure; of note, their serum ceruloplasmin concentrations were measured during acute-phase reactions.

Fig. 1. ROC curve of serum ceruloplasmin concentration for the diagnosis of WD.

The area under the curve was 0.99 (SE 0.008; 95% CI 0.97–1.01; P < 0.0001). The curve was constructed using the data of 57 WD patients, 71 family members (49 heterozygotes and 22 wild-type homozygotes), 25 patients of the validation group, and 40 subjects with serum ceruloplasmin concentrations <0.20 g/L from the reference interval group. Status of all subjects was confirmed by genotyping.

FALSE POSITIVES

There were no false-positive diagnoses using a ceruloplasmin concentration threshold of 0.14 g/L, whereas a threshold of 0.20 g/L led to 60 false positives. Fourteen of the 60 false positives were heterozygotes (with serum ceruloplasmin concentration range 0.15–0.19 g/L, median 0.18 g/L), and 46 were wild-type homozygotes (ceruloplasmin concentration range 0.14–0.19 g/L, median 0.18 g/L). In 10, we repeated testing of ceruloplasmin, slit lamp examination, 24-h urinary collection for copper excretion, and/or liver ultrasonography. Four had liver biopsy for copper staining and/or copper measurement, but the results of all of these examinations were unremarkable. Five had been treated with chelation therapy (penicillamine and/or zinc) for >3 years.

REFERENCE INTERVAL GROUP

Forty of 690 healthy adults showed serum ceruloplasmin concentrations <0.20 g/L (range 0.16–0.19 g/L). None of these individuals had concentrations <0.14 g/L. Molecular analysis of the 40 subjects revealed normal DNA sequences. The central 95% reference interval
for ceruloplasmin was 0.18–0.39 g/L (mean 0.27; SD 0.06). Fig. 2 depicts the serum ceruloplasmin concentrations of all studied subjects.

**Discussion**

We derived an ROC curve for serum ceruloplasmin concentration in the diagnosis of WD from 193 genetically confirmed subjects (57 WD patients, 71 family members, 25 patients in the validation group, and 40 subjects from the reference interval group). Using 0.20 g/L as the diagnostic threshold, the positive and negative predictive values were 48.3% and 98.7%, respectively. Ceruloplasmin concentrations ≥0.14 g/L gave the best positive and negative predictive values —100% and 97.1%, respectively. Ceruloplasmin concentrations <0.14 g/L were not diagnostic —93% and 97.1%, respectively. The negative predictive values of serum ceruloplasmin concentration were 93% and 97.1%, respectively. The negative predictive values were 83% and 97.1%, respectively. The negative predictive values were 83% and 97.1%, respectively. The negative predictive values were 83% and 97.1%, respectively.

**Table 1. Clinical and laboratory data of 4 false negatives using serum ceruloplasmin concentration <0.14 g/L as diagnostic cutoff.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at onset, years</th>
<th>Presentation</th>
<th>KF rings</th>
<th>Serum ceruloplasmin concentration, g/L</th>
<th>Ceruloplasmin oxidase activity, μmol/min/L</th>
<th>Basal urinary copper, μmol/day</th>
<th>Liver copper, μg/g</th>
<th>WD score</th>
<th>Mutation analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>14</td>
<td>Acute liver failure</td>
<td>Yes</td>
<td>0.14</td>
<td>ND</td>
<td>ND</td>
<td>1462</td>
<td>5</td>
<td>p.G943D and p.I1148T</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>21</td>
<td>Acute liver failure</td>
<td>Yes</td>
<td>0.15</td>
<td>24.5</td>
<td>30</td>
<td>ND</td>
<td>5</td>
<td>p.P1273Q and 1 unknown</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>50</td>
<td>Acute liver failure</td>
<td>Yes</td>
<td>0.16</td>
<td>ND</td>
<td>13</td>
<td>ND</td>
<td>5</td>
<td>p.R778L and p.T935 mol/L</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>27</td>
<td>Acute liver failure</td>
<td>Yes</td>
<td>0.21</td>
<td>36.7</td>
<td>97.4</td>
<td>ND</td>
<td>4</td>
<td>p.V176SfsX28 and c.1708-5T&gt;G (IVS4-5T&gt;G)</td>
</tr>
</tbody>
</table>

*Reference interval of ceruloplasmin oxidase activity is 62–108 μmol/min/L. ND, not done.
nary copper excretion (15) and liver copper quantification (16) tests. If genetic analysis is available, it will provide the highest diagnostic accuracy for family screening.

Application of the WD scoring system includes a full clinical workup, the invasive procedure of liver biopsy, and labor-intensive mutational screening. The scoring system is more reliable when all 7 parameters have been fully assessed. Nonetheless, such information may be difficult to obtain in asymptomatic subjects and patients with atypical presentations. It also is not suited for screening purposes.

None of the 690 healthy subjects we examined had serum ceruloplasmin concentrations <0.14 g/L. This may have implications for population screening of WD to minimize false positives. Although 4 of our WD patients had ceruloplasmin ≥0.14 g/L, they all presented with acute liver failure, and their serum ceruloplasmin concentrations were measured during the acute-phase reaction. Ceruloplasmin is a positive acute-phase reactant, and this can account for an apparently normal ceruloplasmin concentration in WD patients with fulminant hepatic failure (27).

Ceruloplasmin can be measured by immunochemical and enzymatic activity methods, expressed in mass units and in activity units, respectively. Because the former measures both active holoceruloplasmin and inactive apoceruloplasmin (which has a half-life in the circulation of only a few hours), immunochemical methods may give apparently normal results in individuals with WD. Therefore, it has been suggested that assay of ceruloplasmin oxidase activity is a better indicator of WD than mass immunoassay (28–30). We measured the ceruloplasmin oxidase activities of 2 of our WD patients whose ceruloplasmin concentrations were 0.15 and 0.21 g/L and found that the ceruloplasmin oxidase activities were, respectively, 24.5 and 36.7 μmol/min/L (reference interval 62–108), thus, confirming the potential value of ceruloplasmin oxidase activity measurements.

On the other hand, low concentrations of ceruloplasmin can be observed in non-WD patients in decompensated liver failure whose synthetic liver function is greatly reduced. Ceruloplasmin deficiency also has been observed in other pathological conditions, including Menkes disease, protein calorie malnutrition, nephrotic syndrome, protein-losing enteropathy, acquired copper deficiency, and hereditary aceruloplasminemia (31). Patients with the last condition can resemble those with WD in having an undetectable serum ceruloplasmin concentration and similar neurological symptoms.

$ATP7B$ protein has dual roles: copper transport, which is reflected by serum ceruloplasmin concentrations, and cellular trafficking. Some mutations inter-
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...fering with the trafficking of \(ATP7B\) within the cell do not lead to low serum ceruloplasmin concentrations. Thus, our results might not be applicable to WD patients with such mutations who present with normal ceruloplasmin concentrations. The correlation between serum ceruloplasmin concentration and \(ATP7B\) genotypes is controversial. Merle et al. (32) found no significant correlation, whereas Kumar et al. (33) reported that serum ceruloplasmin concentrations were significantly lower in homozygous WD patients than in compound heterozygotes. Patients in the same family tend to have similar concentrations of serum ceruloplasmin.

In general, the reference interval of serum ceruloplasmin concentration is between 0.20 and 0.40 g/L in normal adults. A concentration less than the lower reference limit (0.20 g/L) has been considered as the conventional diagnostic cutoff for WD (1, 10, 23). However, the lower reference limit can vary with different measurement methods, standardization, and the age of subjects. Serum ceruloplasmin concentration is age dependent (34). It is lower in normal neonates and rises to adult concentrations by the age of 6 months. It increases further to a maximum concentration between 2 and 3 years old, and then falls slowly until the teenage years, when it reaches the adult concentrations. In addition, females who are pregnant or on estrogens have higher concentrations. Therefore, a single cutoff value may not be applicable for all age groups (35). The youngest age suitable for use of serum ceruloplasmin measurement in the diagnosis of WD is 3 years (4).

Despite the use of primary protein reference material CRM 470/RPPHS, method-dependent variation of ceruloplasmin measurement is still significant, and the use of CRM 470 alone cannot achieve a universal reference interval for ceruloplasmin (36–38). If the concept of a lower reference limit is applied, only the method-dependent lower reference limit should be used. It cannot be overemphasized that 0.20 g/L is not a universal clinical decision value. For example, a concentration of 0.22 g/L is recommended by the manufacturer instead of 0.20 g/L when serum ceruloplasmin concentration is measured by Beckman IMMAGE (manufacturer insert, 2007). However, we recommend that each laboratory establish the reference interval based on its population. For Hong Kong Chinese, we found that the lower reference limit of serum ceruloplasmin concentration measured by Beckman IMMAGE was 0.18 g/L. The positive and negative predictive values were 70.9% and 99.1%, respectively. This still gave 23 false positives and 1 false negative (Table 2). In our opinion, the use of the lower reference limit for the diagnosis of WD should be discouraged, and each laboratory should develop cutoff values of serum ceruloplasmin for the diagnosis of WD based on the local population.

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