Spectrum of ATP7B Gene Mutations in Pakistani Wilson Disease Patients: A Novel Mutation Is Associated with Severe Hepatic and Neurological Complication

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Abstract
Wilson disease (WND) is an autosomal recessive disorder caused by mutation in ATP7B gene that impairs copper metabolism. ATP7B is involved in the transport of copper into the plasma protein ceruloplasmin and copper excretion out of the liver. Defects in ATP7B lead to excess of copper in various organs primarily in liver. The diagnosis of WND is more complex due to variations in its biochemical and clinical features and the broad range of disease onset. The objective of the present study was to establish molecular analysis system for screening of Wilson disease in Pakistani population. Three mutations were identified; with one being a novel mutation never reported before.

Keywords: ATP7B, Neurological Disorder, Ceruloplasmin, Wilson Disease, Pakistani population

1. Introduction
Wilson disease (WND) is an autosomal recessive disorder of copper metabolism described by Wilson in 1912. Patients with WND usually suffer from hepatic, neurologic and psychiatric complications. Most frequently findings associated with WND are low serum ceruloplasmin level, high concentrations of copper in liver and the presence of Kayser–Fleischer (KF) ring (Sternlieb, 1980, 1990). The worldwide prevalence of WND was reported to be 1 in 30,000 with a carrier ratio of 1 in 90 (Scheinberg and Sternlieb, 1984). The symptoms appear between ages 5 to 35 (Schoen and Sternlieb, 1990) but it can vary from 2-years old to 72 years (Ala et al., 2005; Beyersdorff et al., 2006; Wilson et al., 2000). It is generally believed that WND is caused by defect in ATP7B gene located on q14.3 band of chromosome 13 which was cloned in 1993 (Bull et al., 1993; Yamaguchi et al., 1993; Tanzi et al., 1993). ATP7B gene consists of six copper binding domains, eight transmembrane domains and ATP loop that transports copper into bile. ATP7B is a copper-transporting P-type ATPase involved in transporting copper into the secretory pathway for incorporation into apoceruloplasmin and excretion of copper into the bile (Bartee et al., 2007; Ferenci, 2004; Hellman et al., 2002). Mutation in ATP7B gene can impair the protein function leading to accumulation of copper in liver, kidney and cornea. The diagnosis of WND is much complex due to variations in biochemical and clinical features and broad range of disease onset. Therefore, molecular diagnosis plays a pivotal role in pre-symptomatic diagnosis of WND and effected individuals can get treatment in time to prevent further progression of disease.
This study was performed to screen patients with WND through molecular genetic testing for ATP7B gene mutation and establish a molecular diagnostic system for detection of pre-symptomatic WND. This may serve as a very useful approach for early introduction of therapeutic intervention in order to check the progression of the disease.

2. Materials and Methods

This study was approved by the ethical committee of National University of Sciences and Technology. Informed consent was obtained from 11 WND patients included in this study. There were (5) males and (6) females with the mean age 6.3. ± 1.35 (hepatic manifestation), 15±5.6 (hepatic & neurological complications) and 19-6eras (neurological manifestation). Each of these patients had a score of at least 3 according to a scoring system based on clinical and biochemical parameters. The patients were diagnosed on the basis of clinical features such as presence of hepatic disturbance, typical neurological symptoms, low serum ceruloplasmin concentration, high urinary copper level and presence of the KF ring. The molecular genetic analyses were also performed for detection of mutation in the ATP7B gene. The WND patients and their family members were screened through molecular genetic testing. The healthy individuals were included as control for the confirmation of mutation. Genomic DNA was extracted from peripheral blood of patients as well their family members and healthy individuals by standard phenol/chloroform extraction method. The exons 2, 3, 8, 13, 14 of ATP7B gene were used for PCR and direct sequencing (forward and reverse strands) on CEQ8000 Genetic Analyzer (Beckman Coulter). The mutations in WND patients were confirmed in repeated experiments and through comparison with their parents and controls.

3. Results

A total of 11 WND patients were clinically examined. All patients had shown variations in clinical features and biochemical analysis. Eight patients had a hepatic manifestation confirmed through ultrasonography and liver biopsy, 1 showed severe neurological symptom and 2 had both neurological and hepatic complications (Table 1 & 2). All patients were receiving chelation and oral zinc treatment. The mutation analysis had confirmed the defect in entire ATP7B gene in three patients. Three variants were identified at exon 2 & 3 with one novel never reported before. The insertion (c.815-816insT) at exon 2 was resulted in premature protein truncation with creation of stop codon 10bp downstream (Figure-1d). This novel frameshift mutation might have produced functionless protein. This patient-1 was presented with severe hepatic & neurological complications of WND. The patient was died within six month after disease onset. Her sister was earlier died of WND. The patient’s parents were found normal in both biochemical and genetic analysis. The sibling was found negative in KF ring and urinary copper analysis but ceruloplasmin level was at border range and under investigation through mutation analysis. This case shows the importance of molecular genetic testing for diagnosis of Wilson disease.

The non-sense mutation Cys271X (Figure-1e) in exon 2 was previously reported (Hao et al., 1998, Gupta et al., 2005). The patient having this non-sense mutation had severe neurological symptoms like dystonia, dysarthria and arthralgia. Behavioral and psychiatric changes were also developed in later stage of treatment. This nonsense mutation was resulted in the premature truncation of protein at amino acid residue 271. The c.G1366C (Figure-1f) resulted in valine transition to leucine on exon 3 was detected as nonpathogenic variation as described previously (Gupta et al., 2007).

4. Discussion

Early diagnosis of WND disease is very crucial to prevent its progression. Late onset of Wilson disease is also creating hinders in proper diagnose and treatment. We have identified several cases where late onset of disease caused a sudden death of patient (personal communication). Investigation of genotype–phenotype correlations in WND is impeding by a variety of factors. The frequency of most mutations is low and initial symptoms of WND may be nonspecific and will not be easily recognized, resulting in a considerable diagnostic delay and imprecise clinical data (Caca et al., 2001). Therefore molecular genetic testing is a powerful tool for pre-symptomatic diagnose and proper treatment of this disease. Our clinical data has confirmed these findings. In our study, two patients were presented with severe hepatic symptoms but KF ring was absent. Similarly, one patient had neurological type of Wilson disease but developed KF ring at the age of 20 years.

The two frameshift mutations have resulted in premature protein truncation and expected as disease causing mutations. The fulminant hepatic failure and early symptoms in WND are related to nonsense or frameshift mutations that encode a truncated ATP7B protein having significant functional loss and being responsible for the seriousness of the disease (Okada et al., 2000). There are several factors involved in the pathogenesis of WND (Schilsky, 2005). Both insertion and non-sense mutations found here were diseases causing affected copper domain 3. The insertion of T at nucleotide position 816 although does not change the amino acid, but resulted in premature termination.

All patients examined in this study had strong hepatic and neurological symptoms as compare to control. We have concentrated specifically on exon 8 & 14. The occurrence of most common mutation H1069Q (Olivarez et al., 2001) and R778L (Mak et al., 2008) are reported on these exons. The frequencies of R778L mutation have been reported 14.6% in Japanese patients (Nanji et al., 1997), 37.5% in Korean patients (Kim et al., 2000), 30% Chinese patients (Fan...
et al., 2002). Both mutations were not detected in our patients. The onset of WND in neurological and hepatic patient was same as reported earlier that hepatic disorders are major symptoms during childhood and neuropathy becomes evident during adolescence (Shimizu and Nakazono, 1999; Ferenci, 2001; Roberts and Schilsky, 2003).

Our report is the first local study to elucidate the genotype of ATP7B gene in Pakistani WND patients. The detection of either novel or common mutations will help to assess their impact on disease severity and functional characterization will open the way to understand the mechanisms of protein dysfunction for particular mutation of ATP7B. This study will help in screening heterozygote carrier to prevent progression of disease in a family or population.

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References


Table 1. Serum ceruloplasmin levels and Urinary copper level of WND patients (p1-p11). The patients were presented with hepatic, neurological or with both complications

<table>
<thead>
<tr>
<th>Patients</th>
<th>Ceruloplasmin (mg/dl)</th>
<th>Urinary Copper ug/24hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1</td>
<td>15</td>
<td>1200</td>
</tr>
<tr>
<td>p2</td>
<td>10</td>
<td>1800</td>
</tr>
<tr>
<td>p3</td>
<td>12</td>
<td>700</td>
</tr>
<tr>
<td>p4</td>
<td>9.7</td>
<td>800</td>
</tr>
<tr>
<td>p5</td>
<td>18</td>
<td>2000</td>
</tr>
<tr>
<td>p6</td>
<td>16</td>
<td>1800</td>
</tr>
<tr>
<td>p7</td>
<td>20</td>
<td>2100</td>
</tr>
<tr>
<td>p8</td>
<td>6.8</td>
<td>1796</td>
</tr>
<tr>
<td>p9</td>
<td>15</td>
<td>1600</td>
</tr>
<tr>
<td>p10</td>
<td>20</td>
<td>1000</td>
</tr>
<tr>
<td>p11</td>
<td>20</td>
<td>500</td>
</tr>
<tr>
<td>Controls (30)</td>
<td>35 (average)</td>
<td>80 (average)</td>
</tr>
<tr>
<td>Normal</td>
<td>&lt;20mg/dl*</td>
<td>&gt;100μg/24h *</td>
</tr>
</tbody>
</table>

*(Sternlieb 1990)*
Table 2. Hematological Analysis in Wilson disease patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Hb</th>
<th>Total Bilirubin</th>
<th>AST</th>
<th>ALT</th>
<th>ALP</th>
<th>Serum Albumin</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
<td>7.9g/dl</td>
<td>4.4mg/dl</td>
<td>215U/L</td>
<td>30U/L</td>
<td>304U/L</td>
<td>2.4g/dl</td>
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<tr>
<td>P2</td>
<td>10.5g/dl</td>
<td>4.5mg/dl</td>
<td>160 U/L</td>
<td>91 U/L</td>
<td>2264 U/L</td>
<td>2g/dl</td>
</tr>
<tr>
<td>P3</td>
<td>8.7 g/dl</td>
<td>30 mg/dl</td>
<td>200 U/L</td>
<td>40 U/L</td>
<td>350 U/L</td>
<td>1.7g/dl</td>
</tr>
<tr>
<td>P4</td>
<td>6.7 g/dl</td>
<td>26.8 mg/dl</td>
<td>945 U/L</td>
<td>160 U/L</td>
<td>150 U/L</td>
<td>2g/dl</td>
</tr>
<tr>
<td>P5</td>
<td>5.5g/dl</td>
<td>26mg/dl</td>
<td>200U/L</td>
<td>100 U/L</td>
<td>300 U/L</td>
<td>2.3g/dl</td>
</tr>
<tr>
<td>P6</td>
<td>10.5g/dl</td>
<td>2mg/dl</td>
<td>40U/L</td>
<td>45 U/L</td>
<td>95 U/L</td>
<td>4.4g/dl</td>
</tr>
<tr>
<td>P7</td>
<td>4.5 g/dl</td>
<td>1.9 mg/dl</td>
<td>215 U/L</td>
<td>20 U/L</td>
<td>300 U/L</td>
<td>2g/dl</td>
</tr>
<tr>
<td>P8</td>
<td>8.9 g/dl</td>
<td>1.8 mg/dl</td>
<td>1400 U/L</td>
<td>40 U/L</td>
<td>569 U/L</td>
<td>2.3g/dl</td>
</tr>
<tr>
<td>P9</td>
<td>5.2 g/dl</td>
<td>2.9 mg/dl</td>
<td>50U/L</td>
<td>122 U/L</td>
<td>272 U/L</td>
<td>2.2g/dl</td>
</tr>
<tr>
<td>P10</td>
<td>5 g/dl</td>
<td>30 mg/dl</td>
<td>450 U/L</td>
<td>200 U/L</td>
<td>350 U/L</td>
<td>2.2g/dl</td>
</tr>
<tr>
<td>P11</td>
<td>10.2 g/dl</td>
<td>2.4 mg/dl</td>
<td>85 U/L</td>
<td>85 U/L</td>
<td>312 U/L</td>
<td>2.5g/dl</td>
</tr>
<tr>
<td>Normal</td>
<td>13–17 g/dl</td>
<td>0.3–1.2 mg/dl</td>
<td>7–45 U/L</td>
<td>7–45 U/L</td>
<td>98–279 U/L</td>
<td>3.5-5.5g/dl</td>
</tr>
</tbody>
</table>

Note. Hb, hemoglobin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase;
Figure 1. **a-c:** The DNA sequence of controls. **d-f,** The DNA sequence of the patients shows an insertion of T, substitution of C>A, G>C (as indicated by an arrow). The DNA sequencing of patients and controls was performed with forward and reverse primers for the confirmation of sequence change.