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RAPID COMMUNICATION

Wilson disease: Identification of two novel mutations and clinical correlation in Eastern Chinese patients

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Abstract

AIM: To study mutations in the P-type ATPase (ATP7B) gene responsible for Wilson disease (WD) in the Eastern Chinese population, and the possible correlation of specific mutations with clinical characteristics.

METHODS: Mutations of the ATP7B gene were sought by means of direct sequencing in 50 Eastern Chinese WD patients of Han ethnic origin.

RESULTS: Two novel mutations, Asp96Gly and Asp196Glu, were first identified. We also compared the characterization of mutations in ATP7B with the clinical findings, and a significant correlation with hepatic manifestations between patients carrying the Arg778Leu mutation and those without was found.

CONCLUSION: Gene sequencing analysis was shown to have a high detection rate and accuracy. It may become the first priority in screening of WD patients.

Key words: Wilson disease; ATP7B gene; Mutations; Polymorphisms

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INTRODUCTION

Wilson disease (WD, hepatolenticular degeneration) is an autosomal recessive disorder with an incidence of 1 in 35 000 to 100 000 live births[1-3]. It is characterized by pathological copper accumulation in different tissues, especially in the liver and brain. As a result of defective putative copper-transporting ATPase in the liver, copper remains in the liver and causes hepatic dysfunction. The clinical presentation of WD consists of hypoceruloplasminemia, presence of Kayser-Fleischer (KF) rings, and hepatic, psychiatric and/or neurological disturbance[4]. Treatment of WD has progressed from chelation therapy using D-penicillamine and trientine to the more recent use of zinc, and finally to liver transplantation for fulminant presentation[5]. Timely diagnosis and treatment may protect patients from severe organ damage. The diagnosis of WD is determined by clinical presentation and laboratory testing for KF rings, hepatic injury and low serum ceruloplasmin. However, some asymptomatic patients do not receive effective treatment before irreversible injury is present. Genetic diagnosis may detect presymptomatic patients, in whom initiation of prophylactic therapy can effectively prevent the otherwise inevitable hepatic and neurological injury[6,7].

Mutations in the P-type ATPase (ATP7B, MIM#277900) gene are responsible for WD. The ATP7B gene, which was identified in 1993, is located on chromosome 13q14.3, which spans a genomic region of ~80 kb. It comprises 21 exons and encodes for a P-type copper-transporting ATPase[8-10]. Direct mutation analysis has been performed in many WD patients and > 200 different ATP7B mutations have been detected[11-13]. H1069Q is the most common type of mutation, with an allelic frequency of 10%-40% in European patients[14-16]. However, Arg778Leu in exon 8 is the most frequently observed mutational type in Chinese, Japanese and Korean patients[17-22]. It has been reported that the Arg778Leu mutation may be correlated with hepatic manifestations in Chinese patients[12]. However, the mutations in Chinese patients with WD, and the possible correlation of specific mutations with clinical characteristics, have not been addressed.

In the present study, mutations of the ATP7B gene were sought by means of direct sequencing in 50 Eastern Chinese WD patients of Han ethnic origin, who comprise 99% of the population of mainland China. We also

compared the characterization of mutations in ATP7B with the clinical findings, and report the preliminary results of the genotype/phenotype correlation.

MATERIALS AND METHODS

Subjects

A total of 50 unrelated Han ethnic subjects (24 female, 26 male) in mainland Eastern China were diagnosed as being affected by WD. They were mainly from Zheijang, Anhui and Fujian Provinces. They were recruited when they came to the hospital for seeking assistance. The diagnosis was based on the presence of hepatic disturbance, typical neurological symptoms, KF rings, biochemical tests (low serum concentrations of ceruloplasmin and copper, and high urinary and hepatic copper content). Each of these patients had a score of at least 3 according to a scoring system based on clinical and biochemical parameters[23]. DNA samples from 100 healthy Chinese individuals (50 female, 50 male) were screened to determine whether the missense/splicing mutations identified in this study were present in the normal population. The healthy subjects were mainly students in our teaching hospitals. Informed consent was obtained from all patients or their parents before inclusion in the study.

PCR and DNA sequencing

Anticoagulated blood samples were obtained from the WD patients. Genomic DNA was isolated from peripheral blood lymphocytes by a DNA extractor kit (Qiagen, Germany). All 21 exons of ATP7B were amplified by polymerase chain reaction (PCR). PCR was performed in a 50-μL reaction volume containing 800 ng genomic DNA, 2.5 U Taq polymerase (Takara, Japan), 12.5 pmol each primer, 3 mmol/L $MgCl_2$, 200 μmol/L each dNTP, and 1 × PCR Buffer (Takara, Japan). The PCR reaction conditions were as follows: 15 min initial activation step at 94℃; 35 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s and extension at 72℃ for 120 s; and a final extension at 72℃ for 10 min. PCR products were visualized on 2% agarose gel containing ethidium bromide, and subsequently purified using QIAEXⅡ (Qiagen, Germany). Direct sequencing was performed using an ABI 377 fluorescent sequencer (Applied Biosystems, Foster City, CA, USA).

Analysis of genotype–phenotype correlations

We analyzed the correlation between Arg778Leu genotype and WD phenotype, including age of onset and initial symptoms. Data were analyzed using SPSS 13.0. Age of onset of symptoms was compared using Student's *t* test. Categorical variables were compared between groups by the χ^2 test or Fisher's exact test. Differences were considered to be significant at the $P < 0.05$ level.

RESULTS

Mutation analysis

DNA from the 50 patients with WD was screened for mutations. Exons 1-21 were PCR-amplified and the products were sequenced bidirectionally. Mutations in ATP7B were found in at least one of the alleles in all the patients with WD, distributed throughout the gene. In eight patients, only one allelic change was detected, which suggests that the other one might have been located in the introns or regulatory regions. We identified five different mutations (Table 1). Two different mutations found in exons 8 and 13 account for about 77% of all 100 WD alleles studied, thereby indicating that these exons are important regions for detecting mutations in Eastern Chinese patients with WD. The most frequent mutation, Arg778Leu, was found in 32 (64%) patients, in at least one allele. The frequency of the other most prevalent mutation Pro992Leu was 27%. In the copper-binding domain, we found two novel mutations: one non-conservative, which replaced a highly conserved acidic amino acid with a small non-polar amino acid (Asp96Gly); and the other conservative (Asp196Glu), but lying at a highly conserved position in Menkes disease and other copper-transporting ATPases. The two novel mutations were both missense mutations, and the nucleotide changes were not found in the 100 normal individuals. The novel features of these mutations were identified according to the database that is maintained by the University of Alberta (http://www.medicalgenetics.med.ualberta.ca/wilson/index.php).

DNA polymorphism

Seven DNA sequence polymorphisms were identified (Table 2). DNA sequence polymorphisms were those nucleotide changes that either did not modify the amino acid sequence of the polypeptide, resulted in conservative changes in the amino acid residues, or that were found in normal chromosomes or chromosomes with known disease-causing mutant alleles. In order to clarify these nucleotide and amino acid sequence changes were mutations or polymorphisms, we analyzed 100 normal individuals with direct sequencing of their PCR products. The nucleotide changes present in those 100 normal individuals were considered to be polymorphisms, and those which were not present, were considered to be mutations.

Genotype-phenotype correlations

A total of 50 patients with WD were analyzed. The mean age of patients at disease manifestation was 8.9 ± 1.9 years (median, 8 years; range, 4-13 years). Thirty patients (60%) had a primary hepatic manifestation, 13 showed a primary neurological manifestation, and five had combined hepatic and neurological manifestations. Two patients (4%) identified by family screening had no symptoms; they were classified as asymptomatic, and were not included in analysis of genotype-phenotype correlations.

We identified 18 homozygotes and 14 heterozygotes for Arg778Leu. In the 32 patients carrying Arg778Leu, 25 (78.1%) had hepatic manifestations (age of onset, 8.1 ± 1.7 years). There were a total of 18 patients (36%) without the Arg778Leu mutation, 10 (55.6%) with hepatic manifestations (age of onset, 9.2 ± 2.3 years). The Arg778Leu homozygous patients were not significantly younger at the time of symptom onset (8.5 ± 2.1 years), compared with compound heterozygotes (7.9 ± 1.8

Table 1 Mutations identified in WD chromosomes

Mutation	Nucleotide change	Exon	Predicted effect	Frequency of WD alleles (%)	Frequency of WD alleles in Caucasian patients (%)
Asp96Gly	287A>G	2	Disrupts Cu 1	8	novel
Asp196Glu	588C>A	2	Disrupts Cu 2	2	novel
Arg778Leu[1]	2333G>T	8	Disrupts TM4	50	2
Pro992Leu[2]	2975C>T	13	Disrupts Ch/TM6	27	2
Val1216Met[3]	3646G>A	17	Disrupts ATP binding	3	< 1

[1]Mutation previously described[5]. [2]Mutation previously described[1]. [3]Mutation previously described[1].

Table 2 Polymorphism identified at the ATP7B locus

Polymorphism	Nucleotide change	Exon	Frequency (%)	
			Healthy individuals (n = 100)	Patients (n = 50)
-75 A > C[1]		5′UTR	38	10
-123 del CGCCG[1]		5′UTR	10	25
S406A[1]	1216 T>G	2	35	30
V456L[1]	1366 G>C	3	36	22
L770L[2]	2310 C>G	8	2	50
K832R[2]	2495 G>A	10	27	30
S1166S[3]	3498 C>T	16	19	20

[1]Polymorphism previously described[12]. [2]Polymorphism previously described[12]. [3]Polymorphism previously described[13].

years). However, the number of Eastern Chinese patients with hepatic manifestations among those carrying the Arg778Leu mutation was significantly greater than that among those without the mutation ($\chi^2 = 5.26$, $P < 0.05$).

DISCUSSION

Mutation analysis is important in the early diagnosis of patients with a family history of WD, as well as in prenatal diagnosis. Here, we report a group of 50 subjects affected with WD, analyzed by direct sequencing of the entire coding sequence of ATP7B gene. We found mutations in 50 patients. The detection rate of mutation was 92%. Seventy-seven percent of the mutations detected were lying in exons 8 and 13. According to this study, we recommend screening of exons 8 and 13 by sequence analysis. Mutations in exons 14 and 18, which have been found to have a high frequency among Caucasian patients[24-27], were not detected in our study. As reported previously[12], Arg778Leu was the most common WD chromosomal mutation detected in the present study (50%). However, Arg778Gln was not detected in our study, which is similar to the results of previous research in Shanghai[12]. The unusual high frequency of codon 778 mutation may have been due to sampling disequilibrium or to the presence of a founder effect among the Eastern Chinese population. Pro992Leu was the second most frequent allele, with a frequency of 27%. The above-mentioned two mutations accounted for ~80% of all mutations detected in our study. In eight patients, only one allelic change was detected, which suggests that the other change might be located in the introns or regulatory regions. Incomplete assessment of intronic and regulatory sequences may have accounted for the second unidentified mutation.

We could not find any significant difference between Arg778Leu homozygosity and heterozygosity with regard to the mean age of onset of symptoms, although Wu *et al*[7] have reported that the average age of onset in 18 Chinese homozygotes was significantly lower than that in 11 Chinese compound heterozygotes for Arg778Leu. However, the number of patients with hepatic manifestations among those carrying Arg778Leu mutation was significantly greater than that among patients without the mutation. Caca *et al*[28] have reported that symptoms in most His1069Gln homozygotes started between 16 and 25 years of age. In our study, symptoms in most Arg778Leu homozygotes (15/18) started before 10 years of age, which suggests that the age of onset in Arg778Leu homozygotes in Eastern Chinese patients is earlier than that in His1069Gln homozygotes in East German patients. His1069Gln is the most common WD mutation found in the European population[29-31], but it was not detected in our study in Eastern Chinese patients.

Molecular diagnosis of pre-symptomatic WD patients is important in the control of disease progression and treatment. Mutation detection in WD is challenging because of the presence of a large number of mutations in a 4.4-kb coding region in 21 exons spread over 80 kb of genomic DNA. The mutation detection rate among the WD chromosome was > 90% in our study. This gene sequencing analysis method was shown to have a high detection rate and accuracy. It may become the first priority in the screening of WD patients.

COMMENTS

Background

Direct mutation analysis has been performed in many WD patients, and more than 200 different ATP7B mutations have been detected.

Research frontiers

The mutations in the Chinese population with WD, and the possible correlation of specific mutations with clinical characteristics have not been investigated. In the present study, mutations of the ATP7B gene were sought by means of direct sequencing in 50 Eastern Chinese patients of Han ethnic origin with WD.

Innovations and breakthroughs

Two novel mutations were identified in our normal individuals. The novel features of the mutations were identified according to a database that is maintained by the University of Alberta.

Peer review

It is an interesting manuscript. The first concern is on the study population. The

subjects were from mainland Eastern China, which covers a large area. More specific information on subject source, which clinic they attended, and conditions under which they were recruited would be helpful.

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