Study of T991T polymorphism in Cuban patients with clinical diagnosis of Wilson’s disease

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Resumen: En pacientes cubanos con diagnóstico clínico de la Enfermedad de Wilson, el polimorfismo T991T se identificó en el ADN de 100 pacientes, de los cuales 9.5% fueron portadores del polimorfismo. Estos hallazgos proporcionan una caracterización de la frecuencia del polimorfismo T991T en la población cubana y posibilitarán la realización de otros estudios moleculares por métodos indirectos.

Palabras clave: Enfermedad de Wilson, polimorfismo T991T, SSCP, secuenciación, gen atp7b

Introduction

Wilson’s disease (WD, MIM 277900) is an inherited disorder that has an autosomal recessive inheritance pattern. It is considered one of the rare diseases described at international level, characterized by the accumulation of copper mainly in the liver, brain and cornea and of complex clinical diagnosis [1]. The clinical manifestations are classified as: hepatic, neurological, psychiatric, mixed, renal, etc. The damage to the liver generates symptoms from hepatitis with unexplained cause to decompensated cirrhosis, including even fulminant hepatitis. Patients may be affected at brain level; the manifestations can range from tremors to the presence of Parkinson’s Disease. In spite these manifestations; Wilson’s disease is a treatable genetic disorder, with effective diagnosis required to identify the disease and avoid those irreversible alterations that can lead to death in the pediatric age if not attended properly.

The disease is caused by mutations in the atp7b gene (MIM 606882), with 21 exons and more than 500 mutations reported [1, 2]. Over 139 polymorphisms have been identified so far, which are distributed throughout the atp7b gene and in the introns, with exons 2, 8 and 16 found to be the most polymorphic. Particularly in exon 13, several polymorphisms have been identified [3], and one of them is the T991T polymorphism.

Resumen de la enfermedad de Wilson (WD, MIM 277900) es un trastorno hereditario que se caracteriza por un patrón autósómico recesivo. Es una de las enfermedades más raras, caracterizada por una acumulación de cobre principalmente en el hígado, el cerebro y la córnea y de complejo diagnóstico clínico [1]. Las manifestaciones clínicas se clasifican como: hepáticas, neurológicas, psiquiátricas, mixtas, renales, etc. El daño al hígado genera síntomas de hepatitis sin explicación conocida a cirrosis descompensada, incluyendo incluso hepatitis fulminantes. Los pacientes pueden verse afectados a nivel cerebral; las manifestaciones pueden variar desde temblores hasta la presencia de enfermedad de Parkinson. Incluso aunque estas manifestaciones; enfermedad de Wilson es un trastorno genético tratable, con un diagnóstico efectivo requerido para identificar la enfermedad y evitar aquellos alteraciones irreversibles que pueden conllevar a la muerte en la edad pediátrica si no son adecuadamente atendidas.

La enfermedad es causada por mutaciones en el gen atp7b (MIM 606882), con 21 exones y más de 500 mutaciones informadas [1, 2]. Desde 139 polimorfismos han sido identificados hasta la fecha, que se encuentran distribuidos a lo largo del gen atp7b y en los intrones, con exones 2, 8 y 16 encontrados como los más polimórficos. Particularmente en el exón 13, varios polimorfismos han sido identificados [3], y uno de ellos es el polimorfismo T991T.
Notably, the study of the mutational spectrum and the identification of polymorphisms in the \textit{atp7b} gene require the use of an adequate cleavage technology. One of the most commonly used techniques for this purpose is the single-strand conformation polymorphism (SSCP) [4], which needs to be combined with adequate DNA extraction procedures [5]. This allows the analysis of the polymorphisms in the \textit{atp7b} gene, which is necessary for the construction of haplotypes and diagnosis by indirect methods.

Specifically in Wilson’s disease, single nucleotide polymorphism (SNP) characterization has emerged as a leading diagnosis procedure, and one of the most studied SNP in patients with Wilson’s disease as reported in the scientific literature worldwide is T991T. However, the analysis of its distribution frequency among Cuban patients was still unaccomplished. Therefore, this work was aimed to identify the conformational shift in exon 13 and to detect T991T polymorphism in the gene of Cuban patients with clinical diagnosis of the Wilson’s disease. Based on the obtained data, SNP identification will constitute a molecular tool for proper genetic counseling for the affected individuals and their families.

\textbf{Materials and methods}

A descriptive study was carried out at the Centro Nacional de Genética Médica during the 2008-2012 period, which included 100 patients (40 women and 60 men) with clinical diagnosis of WD, who attended the consulting room of the Instituto Nacional de Gastroenterología. Fifty subjects were included as negative controls, who do not suffer from Wilson’s disease. All subjects who participated in this research gave their consent to participate in the research, in accordance with the ethical principles of the Declaration of Helsinki [6].

The variables analyzed were: allelic frequency of the T991T polymorphism, conformational shift a for the normal variant and conformational shift b for the presence of T991T polymorphism in heterozygous state and clinical manifestations (hepatic, neurological and mixed). A multidisciplinary team (gastroenterologists, geneticists, neurologists and biochemists) performed the evaluation of the clinical manifestations, following the diagnostic criteria of the disease.

The exon 13 of the \textit{atp7b} gene was selected for the detection of conformational shifts and the identification of the T991T polymorphism. Blood samples were taken from all patients and the DNA was extracted by the saline precipitation method from 5 to 10 mL of peripheral blood with ethylendiaminetetraacetic acid (EDTA) (56 mg/mL).

The conditions for the amplification of exon 13 by the Polymerase Chain Reaction (PCR) technique were: 100ng of ADN, 5 pmoles/mL of each oligonucleotide of exon 13: (Forward) 5'-AGT CGC CAT GTA AGT AA-3' and (Reverse) 5'-CTG AGG GAA CAT GAT AA-3' and mixed). A multidisciplinary team (gastroenterologists, geneticists, neurologists and biochemists) performed the evaluation of the clinical manifestations, following the diagnostic criteria of the disease.

The age at diagnosis of the disease in patients with the T991T polymorphism is 19.8 ± 8.1 (mean ± SD).

\textbf{Results}

Two conformational shifts were identified: a, normal variant of the \textit{atp7b} gene sequence, and b, the presence of T991T polymorphism in heterozygous state, by using the SSCP technique (Figure 1).

The samples showing conformational shift b were sequenced for the corresponding verification of polymorphism or mutation (Figure 2). As a result, the T991T polymorphism was identified in heterozygous in 19 patients (19\%).

Patients with T991T polymorphism are distributed in six provinces and in the special municipality of Isla de la Juventud, as follows: Pinar del Río (4 patients), La Habana (7 patients), Artemisa (2 patients), Matanzas (1 patient), Ciego de Ávila (2 patients), Guantánamo (1 patient) and the special municipality of Isla de la Juventud (2 patients).

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and 30 ± 13.4 (mean ± SD) for those who carried this polymorphism.

T991T polymorphism is a consequence of a cytosine shift by guanine, which does not cause the threonine amino acid change at position 991 of the ATP7B protein in the sixth transmembrane segment, and therefore, it is a silent change. It does not affect the function of the protein and has been identified in several populations with a frequency above 1 % [7-11]. The allelic frequency in the 100 Cuban patients studied was 9.5 %.

The main clinical manifestations in patients with T991T polymorphism were hepatic (63.2 %), among which were jaundice, splenomegaly, ascites, hepatomegaly, liver cirrhosis, among others. Mixed manifestations (hepatic and neurologic) were identified in 31.6 % of patients with the T991T polymorphism. Additionally, a heterozygous patient carrying the T991T polymorphism was identified to also have the L708P [12] and N41S [13] mutations.

**Discussion**

More than 139 polymorphisms have been reported in the ATP7b gene in patients with Wilson’s disease [2]. In Cuba, the detection of ATP7b gene polymorphisms began in 2008 at the National center for Medical Genetics (CNGM). A step prior to the search of mutations and polymorphisms in this ATP7b gene is the detection of conformational shifts. The presence of T991T polymorphism has been reported in several countries; however, there are reports in which they do not show allelic frequency [14-16].

The detection of T991T polymorphism was carried out by the SSCP screening technique and the gold-standard sequencing technique made it possible to detect T991T polymorphism in Cuban patients with Wilson’s disease. These studies were confirmed with the use of positive controls. The allelic frequency of T991T polymorphism in 100 Cuban patients with clinical diagnosis of Wilson’s disease was the highest detected as compared to data from a number of countries, so that in our population T991T polymorphism behave as an informative molecular marker of the disease (Table). Therefore, it is a good candidate for the construction of haplotypes in families where at least one patient is diagnosed with Wilson’s disease. It would also allow making associations of this polymorphism with point mutations detected in the ATP7b gene [16]. This polymorphism has been identified in several populations, in countries such as India, Canada, the United Kingdom and Iran.

A few molecular studies on the ATP7b gene have been describe in the Americas. In patients with clinical diagnosis of Wilson’s disease in different states of the United States, Puerto Rico and Venezuela, T991T polymorphism was identified, although its allelic frequency was not reported [16, 17].

The age at diagnosis of the patients having T991T polymorphism is much lower than in the patients who do not have it, so it seems that the presence of the polymorphism influences in some way on the age of diagnosis of the disease. Nevertheless, the sample needs to be extended to corroborate this assertion. Moreover, it should be taken into account that factors such as the environment, disease modifying genes (ApoE, Murr 1) and epigenetic factors influence on the phenotype of patients with Wilson’s disease [18].

Cuban western provinces were the most representative having patients with T991T polymorphism, with Habana at the top with 36.8 %, followed by Pinar del Rio (21 %). The least represented provinces were Matanzas and Guantánamo (5.3 % each). It is necessary to consider the remoteness of the Eastern provinces from the capital, which can influence on the low percentage of these patients in the analyzed sample.

Detection of mutations in the ATP7b gene in patients with T991T polymorphism is important to analyze the associations between polymorphisms and mutations. The identification of T991T polymorphism will allow molecular diagnosis by indirect methods. In addition, it may be used, together with other molecular markers such as: K832R [19], L456V [16], V1140A [16], to construct the haplotype and make associations between the haplotypes and the identified mutations. The detection of mutations in the ATP7b gene in four Cuban patients with Wilson’s disease in a 12.5 % acrylamide gel. Lane 1: conformational shift called a. Lane 2-4: conformational shift called b. Lane 5: conformational shift called a, negative control.
of this polymorphism in Cuban patients will be a molecular tool for Genetic Counseling, and whether this polymorphism influences on the age of diagnosis of the disease or not, requires further investigation.

Conclusions

T991T polymorphism detection is available in Cuban patients with clinical diagnosis of Wilson’s disease for the National Network of Medical Genetics and the National Institute of Gastroenterology, which would become a molecular tool for diagnosis. This is the first study in Cuba reporting on associations between mutations and polymorphisms in the ATP7B gene in patients with Wilson’s disease.

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