RESEARCH

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

✍Yulia Clark[1], Caridad Ruenes[2], Elsa F García[2], Teresa Collazo[1], Hilda Roblejo[1], Estela Morales[1], Zoe Robaina[1]

[1] Laboratorio de Biología Molecular, Centro Nacional de Genética Médica, CNGM
Ave. 31 Esq. 146 No. 3102, Reparto Cubanacán, Playa, CP 11400, La Habana, Cuba
[2] Gastroenterology Institute
Calle 25 No. 503 entre H e I, Vedado, Havana, Cuba
✍yuliacf@cug.co.cu

ABSTRACT

Wilson's disease is characterized by the accumulation of copper mainly in the liver. It is transmitted with an autosomal recessive inheritance pattern. The molecular cause is mutations in the atp7b gene, which is found on chromosome 13. Several polymorphisms in the atp7b gene have been reported in the literature. To identify the T991T polymorphism in Cuban patients clinically diagnosed with Wilson's disease, a descriptive study was conducted at the Centro Nacional de Genética Médica during the 2008-2012 period including 100 patients. For the amplification of the fragment of interest, the Polymerase Chain Reaction (PCR) technique was used and the PCR-amplified DNA product of exon 13 from the atp7b gene was analyzed by Single Stranded Conformational Polymorphism (SSCP) technique to identify the conformational shifts. The presence of the T991T polymorphism was identified by sequencing the analyzed DNA fragment. Two different conformational shift were detected: a, corresponding to the normal gene alleles variant; and b, corresponding to the T991T polymorphism in heterozygous state. The allelic frequency of the T991T polymorphism in the 100 Cuban patients clinically diagnosed with Wilson's disease was found to be 9.5 %. These findings provide a characterization of this Wilson's disease polymorphism in the Cuban population and it will make possible to conduct molecular studies by indirect methods.

Keywords: Wilson's disease, T991T polymorphism, SSCP, sequencing, atp7b gene

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RESUMEN

Estudio del polimorfismo T991T en pacientes cubanos con diagnóstico clínico de la enfermedad de Wilson. La enfermedad de Wilson se caracteriza por la acumulación de cobre, fundamentalmente en el hígado y se transmite hereditariamente con un patrón de herencia autosómico recesivo. La causa molecular que la provoca son las mutaciones en el gen atp7b, que se encuentra en el cromosoma 13. Se han informado en la literatura diversos polimorfismos en el gen *atp7b*, uno de ellos el T991T, aunque es necesario estudiar su frecuencia entre los pacientes cubanos clínicamente diagnosticados con la enfermedad. Por tales razones, en este estudio se identificó el polimorfismo T991T en 100 pacientes cubanos diagnosticados clínicamente con la Enfermedad de Wilson. Se realizó un estudio descriptivo en el Centro Nacional de Genética Médica (CNGM) de Cuba durante el período 2008-2012. Para la amplificación del fragmento de interés en el ADN de los pacientes, se utilizó la técnica de Reacción en Cadena de la Polimerasa (PCR) y los productos amplificación del exón 13 del gen atp7b fueron analizados mediante técnica de Polimorfismo Conformacional de Simple Cadena (SSCP) para identificar los cambios conformacionales, cuya presencia fue identificada por secuenciación de ADN. Se detectaron dos cambios conformacionales: a, correspondiente a la variante alélica normal; y b, perteneciente al polimorfismo T991T en estado heterocigótico. La frecuencia alélica del polimorfismo T991T en los 100 pacientes cubanos diagnosticados clínicamente con la Enfermedad de Wilson fue de 9.5 %. Estos hallazgos proporcionan una caracterización de la frecuencia del polimorfismo T991T y posibilitarán la realización de otros estudios moleculares por métodos indirectos.

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

*I*ntroduction

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.

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Notably, the study of the mutational spectrum and the identification of polymorphisms in the *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 *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.

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 *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 ethylenediaminetetraacetic 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 GAT AA-3' and (Reverse) 5'-CTG AGG GAA CAT GAA ACA A-3', 1 mM dNTPs (Boehringer), 10× PCR Buffer, 15 mM of $MgCl_2$, 1 U of Taq polymerase (Amplicen), in a`final volume of 25 µL [7].

The technique was developed in the MJ Research PTC-200 Thermal Cycler (GMI, USA). Initial DNA denaturation was performed at 94 °C for 4 min. Amplification included 35 cycles of denaturation/hybridization/extension: 94 °C for 20 s, 58 °C for 30 s and 72 °C for 25 s, respectively, with a final extension at 72 °C for 10 min. Amplification of the desired fragment (260 bp) was checked by gel electrophoresis.

Later, the SSCP electrophoresis was performed. Then, 3.5 µL were mixed with a blue bromophenol stop solution (0.05 %, 10 mM NaOH, 95 % formamide, 20 mM EDTA) and 1 µL of the amplified product in a final volume of 7 µL. For the implementation of the SSCP technique, the sample was denatured at 96 °C for 6 min and rapidly placed on ice to avoid renaturation. Then, PCR-amplified DNA products were loaded onto a commercial acrylamide gel (GeneGel Excel 12.5/24 Kit; Sigma-Aldrich, USA), with the Genephor system. Run conditions were: 500 V, 15 W, 8 °C and 3 h of run time. DNA was visualized by using the silver staining method, following the commercial kit instructions (PlusOne DNA Silver Staining kit; Amersham Biosciences, USA).

Subsequently, the pattern altered by SSCP was sequenced following the amplification of exon 13 of the *atp7b* gene. The amplified product was purified using the commercial kit QIAquick PCR Purification (Qiagen, Germany), following the manufacturer's instructions. Also, 4 µL of the purified product were mixed with reagents from a commercial Thermo Sequence Cy5 Dye Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech, USA), following the manufacturer's instructions. The oligonucleotide (direct sense) was used to amplify exon 13. Initial DNA denaturation was performed at 94 °C for 30 denaturation cycles (94 °C for 30 s, hybridization at 58 °C for 30 s and extension for 72 °C for 45 s. A final extension step was done at 72 °C for 3 min.

For the electrophoretic run, the semi-automatic sequencer ALFexpress II (Amersham Pharmacia Biotech) was used. The results were analyzed by computer software and compared to the reference DNA sequence of the *atp7b* gene (GenBank: NM000053) using the BLAST (Basic Local Alignment Search Tool) accessible at: http://www.ncbi.nlm.nih.gov/BLAST), to determine the degree of similarity of the DNA or protein sequence with the sequences present in the databases.

Results

Two conformational shifts were identified: a, normal variant of the *atp7b* gene sequence, and b, or 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).

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

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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].

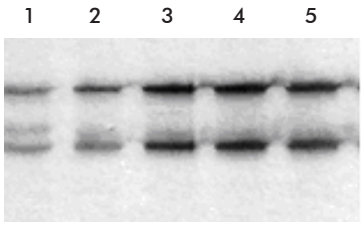

Figure 1. Visualization of electrophoresis resulting from the SSCP technique of exon 13 of the *atp7b* gene in four Cuban patients with Wilson's disease in a 12.5 % acrylamide gel. Lane 1: conformational shift called b. Lanes 2-4: conformational shift called a. Lane 5: conformational shift called a, negative control.

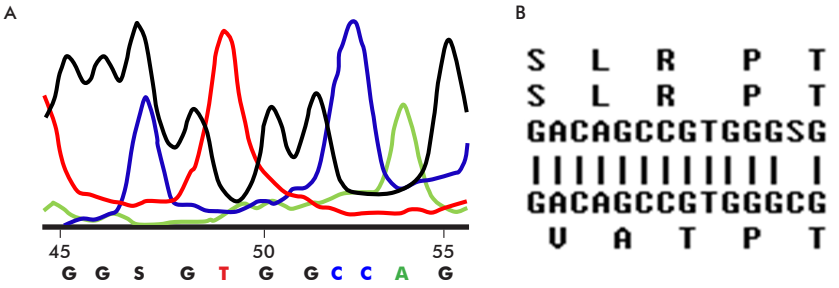

Figure 2. Detection of T991T polymorphism by DNA sequencing. A) Electrophoregram of the sample exhibiting conformational shift b. The presence of letter S is observed, presence of T991T polymorphism in heterozygous state. B) Fragment of the analysis of the Blast sequence alignment program.

Table. Allelic frequencies of T991T polymorphism in different human populations

Country	Allele frequency	Reference
Cuba	9.5 % (19/200 alleles)	This study
Canada	5.4 % (22/408 alleles)	[8]
Iran	5 % (7/140 alleles)	[9]
Turkey	Not informed	[10, 11]
United Kindgom	2.7 % (11/408 alleles)	[17]
Spain	Not informed	[7]
Croatia	Not informed	[14]
India	Not informed	[15]
Venezuela	Not informed	[16]

Cuban western provinces were the most representative having patients with T991T polymorphism, with Habana at the top with 36.8 %, followed by Pinar del Río (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

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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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