Original Article

Common Polymorphisms of ATP7B Gene as a Good Marker in Linkage Analysis in Wilson Disease Patients from Southern Iran

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Abstract

Background: Wilson disease (WD) is caused by numerous pathogenic mutations of the ATP7B gene. There are several mutation screening methods that can be used for the diagnosis and carrier detection of WD, however such methods are costly and time-consuming. Therefore, other diagnostic methods should be used for urgent situations such as prenatal diagnosis. **Objective:** To report common polymorphisms of ATP7B gene in WD patients from southern Iran to be use in linkage analysis in the WD affected families. **Material and methods:** Genomic DNA was extracted from 30 patients and PCR was carried out for ATP7B gene. DHPLC was then performed and PCR products with abnormal peak profiles were subjected to direct DNA sequencing. **Result:** Several patients showed abnormal peak profiles in DHPLC analysis and subsequent sequencing results demonstrated that some polymorphisms were more common in southern Iran. Those were c.1216T>G (exon 2), c.1366C>G (exon 3), c.3419 T>C (exon 16), c.3903 + 6C>T (intron 18) and c.4021+50G>C (intron 19). **Conclusion:** These common polymorphisms can be used by linkage analysis for the prenatal diagnosis and carrier detection in affected families with Wilson disease.

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Introduction

Wilson disease (WD) (OMIM 277900) is an autosomal recessive disorder due to pathogenic mutations of the ATP7B gene. The diagnosis of WD is based on decreased serum ceruloplasmin concentration, an elevated 24h urinary copper excretion, clinical findings such as hepatic and neurological manifestations, and the presence of Kayser-Fleischer ring (1-6).

The biochemical tests can be useful as a primary screening; however, several mutation detection methods such as Denaturing High Performance Liquid Chromatography (DHPLC) (7) analysis followed by direct DNA sequencing should be used to confirm the diagnosis and carrier identification of WD. DHPLC is one of the most sensitive and reliable methods to screen variations (8-10). This method detects DNA variations on the basis of formation of hetero- and homoduplexes between amplified DNA sequences. In order to screen mutations using this method, it is not necessary to know the exact location and nature of the mutation. Identification of variants in DNA sequences by this method is linked to the alterations in retention time and peak profiles (8, 11-14).

ATP7B gene containing 21 exon codes for a copper-transporting p-type 2 ATPase protein, which is essential for biliary copper excretion and copper incorporation into ceruloplasmin (15-19). The absence of ATP7B protein causes accumulation of copper in several main organs (20).

Over 500 mutations (database maintained at the University of Alberta:

www.wilsondisease.med.ualberta.ca/search3.asp) have been identified in ATP7B gene till date, meanwhile performing mutation analysis as a diagnostic method for WD is often expensive and difficult.

In Iranian population, a few disease-associated mutations have been identified, while the common mutations of ATP7B gene have not been reported yet (21). The majority of ATP7B mutations occur within several transmembrane (TM) domains and ATP-binding loop of ATP7B protein (22-25).

A few variations depending on population are more common, (2, 26-29). Given the above, the purpose of this study was to report common polymorphisms of ATP7B gene in WD patients in southern Iran to be use in linkage analysis in the affected families.

Materials and Methods

30 patients were analyzed in this study. All WD patients were diagnosed by a group of gastroenterology specialists in the gastroenterology division at Namazi hospital based on decreased serum ceruloplasmin concentration (<20 mg/dl), an elevated 24h urinary copper excretion (>100 µg/day), an elevated liver copper concentration (>250 µg/g dry weight) and clinical findings. All patients were consented before undergoing DNA test for ATP7B mutation analysis and the process followed the ethical committee guidelines at Shiraz University of Medical Sciences. Three milliliter (ml) whole blood samples from the patients were collected into EDTA tubes (Vacutainer EDTA K3 Tube). Genomic DNA was then extracted from peripheral blood lymphocytes using Cinnapure DNA extraction kits. The concentration of DNA samples were measured by NanoDrop (ND1000, USA) and then were stored at -20°C until use.

The previously reported oligonucleotide primers for PCR amplification of all selected exons (30) and their exon-intron boundaries of ATP7B gene were synthesized by Bioneer Inc. (Korea). The PCR reaction was carried out in 50µl total volume containing 1 µl of each primer (20 pmol/µl), 5 µl DNA template (50-200ng), 5 µl ViBufferA (Vivantis), 0.5 µl dNTPs (10 mM), 1.5 µl MgCl2 (50mM, Vivantis), 0.2 µl Pfu DNA Polymerase (5 U/µl, Vivantis), 35.8 µl dH2O. PCR was done using the Eppendorf Mastercycler gradient (Germany) according to the Pfu DNA polymerase protocol: first denaturation step at 95°C for 2 min, second denaturation step at 94°C for 20 sec, annealing temperature step for 30 sec, extension step 72°C for 50 sec and then the second denaturation step to extension step was repeated for 35 cycles, finally the thermocycler was programmed to a further extension step at 72°C for 7 min and was held at 4°C.

The PCR products were analyzed through a 2.0% agarose gel (Cinnagen, Iran) stained with ethidium bromide and visualized by UV detector. A 100-bp ladder (Vivantis,) was employed as the size marker.

DHPLC was then performed using the WAVE™ DNA Fragment Analysis System (Transgenomic Inc., USA) to detect variations in eight exons and their exon-intron boundaries of ATP7B gene. All samples were heteroduplexed before analysis on the WAVE® System. In order to generate hetero- and homoduplexes, an equal amount of the PCR products from the patients and the wild type were

mixed. Then, heteroduplex formation was performed by Eppendorf Mastercycler gradiant using the following program: heating at 95ºC for 5 min, 95°C for 22 sec and re-annealing by decreasing 1°C every 22 sec until the temperature reached 25ºC (Fig.1) (30-31).

A wild type DNA confirmed by DNA sequencing was also included which showed a wild type pattern on DHPLC analysis. The Low and High Range Mutation Standards were used as the instrument controls. These standards were considered at the beginning of each "run" to make sure the system was working properly and was valid for use. It was important to observe chromatograms within the given normal ranges for both mutation standards.

Navigator™ software was used to predict elution temperature of analyzed sequences. Using the Navigator™ software (Transgenomic), optimal DHPLC conditions for elution of the PCR products were calculated based on sequences of the PCR amplicons.

Eight to ten µl of the unpurified PCR products (concentration between 165 and 400ng/µl) were injected automatically to a preheated column and eluted with a linear acetonitrile gradient at a flow rate of 0.9 ml/min.

The analysis of DHPLC results from patient's samples was carried out by comparing them with the normal peak profile on the analysis page of Navigator™ software.

Samples with abnormal DHPLC peak patterns were subjected to DNA sequencing using both forward and reverse primers (Bioneer, Korea).

Results

DHPLC analysis of exon 16, 18-19 of ATP7B gene was performed in 30 patients, while for exon 2 and 3 direct DNA sequencing was only performed. Several patients showed abnormal DHPLC peak profiles (Fig.2 and 3).

Sequencing data from samples with abnormal DHPLC peak profile were blasted with reference sequence using NCBI BLAST: http://blast.ncbi.nlm.nih.gov/Blast.)
and several patients showed polymorphisms in the analyzed exons. Here, we were able to identify several common SNPs in WD patients in southern Iran. Results are outlined in Table 1.

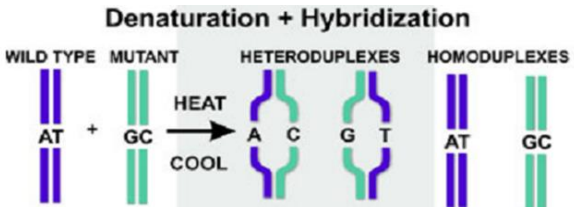

Figure 1. *Heteroduplex formation. When a DNA sequence containing mutant alleles is mixed with a wild-type DNA, heated to 95°C, and then slowly cooled, two homoduplex and two heteroduplex species is expected to form (31).*

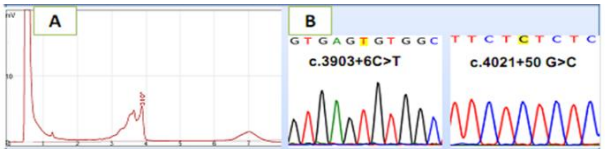

Figure 2. *DHPLC and direct DNA sequencing results. A: DHPLC peak profile of exon 18-19, B: Sequencing chromatogram for two SNPs of exon 18-19 with high frequency in the south of Iran.*

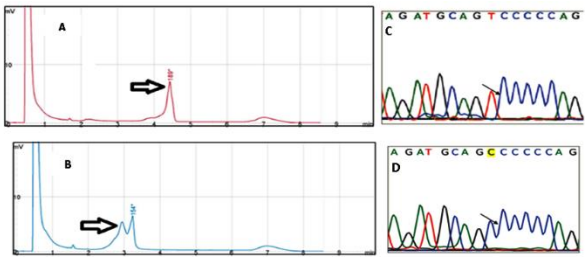

Figure 3. *DHPLC and direct DNA sequencing results for SNP of exon 16 with high frequency in the south of Iran. A: DHPLC peak profile for normal DNA, B: DHPLC peak profile for Sample with c.3419 T>C SNP, C: DNA sequencing chromatogram of normal DNA, D: DNA sequencing chromatogram of sample with the SNP.*

Discussion:

Table 1. Common SNPs identified in the analyzed exons of ATP7B gene in patients with Wilson disease. [1]Hom: Homozygote, [2]Het: Heterozygote, [3]Minor Allele Frequency

The Numbers of homo and heterozygote	Variant name (nucleotide)	Variant type	Amino acid change	Exon/ Intron	[3]MAF In our population	Reported MAF	Area of protein
5 Het[1] and 12 Hom[2]	c.1216T>G (rs1801243)	Substitution	p.Ser406Ala	Exon 2	0.48	0.37	Cu4
21 Hom	c.1366C>G (rs1801244)	Substitution	p.Leu456Val	Exon 3	0.7	0.37	bet Cu4/Cu5
20 Hom	c.3419 T>C (rs1801249)	Substitution	p.Val1140Ala	Exon 16	0.66	0.45	ATP loop
5 Het and 10 Hom	c.3903 + 6C> T (rs2282057)	Substitution	-	Intron 18	0.41	0.49	Bet-ATP hinge/ TM7
5 Het and 10 Hom	c.4021+50G >C (rs9535795)	Substitution	-	Intron 19	0.41	0.46	TM7

ATP7B gene, which is mutated in WD (OMIM 277900), consists of 21 exons and encodes a copper-transporting p-type 2 ATPase protein containing 1465 amino acids. In this large gene, more than 500 variants have been identified in exons, introns, and intron-exons boundaries, so mutation screening for this gene is costly and time-consuming (2, 32). On the other hand, using a fast diagnostic method is necessary in some situations such as prenatal diagnosis. In addition, using mutation analysis for prenatal diagnosis has some other limitations such as allelic dropout that occurs as the result of preferential amplification of one of alleles in heterozygous individuals. Therefore, application of other genetic diagnostic methods is essential for the diagnosis WD.

SNP analysis has been applied as an efficient method for identification of gene location in genetic diseases, determination of paternity, calculation the rick of genetic diseases, and mapping of genome (33-36).

SNP-based diagnosis should be considered when genetic diseases are caused by mutations in genes containing numerous exons. Application of linkage analysis by SNP markers allows accurate diagnosis of potential carriers and WD homozygotes (37-38). In addition, linkage analysis by SNP markers, which are common in the population, is a useful method for prenatal diagnosis. In fact, using the combination of linkage analysis and mutation screening for prenatal diagnosis can reduce the risk of allelic dropout and gives a more reliable diagnosis.

In this study we identified five common polymorphisms in ATP7B gene in different unrelated WD patients from southern Iran. These SNPs showed high frequency in the south of Iran and can be used in linkage analysis. Therefore, linkage analysis by such SNPs which are common among Iranian population suggests an effective method in order to improve prenatal diagnosis and carrier detection of WD. In addition, finding more polymorphisms in ATP7B gene as common-polymorphisms among Iranian population should be considered in future investigations since the accuracy and reliability of a genetic linkage test relies on the number of selective markers.

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No financial interests was disclosed.

Authors' Contribution

MF designed the project, contributed to the interpretation of the data, and the revision of the manuscript. FA, HD, NV and ZF performed the experiments, contributed to the interpretation of the data and the revision of the manuscript. MHI, MH, SMD, and MM diagnosed and critically followed-up the patients.

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