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Original Research Article

Mutation Profile of Exon 14 and Exon 21 of ATP7B Gene in Patients with Wilson Disease

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Abstract

Wilson disease (WD) is an autosomal recessive disorder in ATP7B gene which encodes a copper-transporting ATPase. This protein involves in the transport of copper into the plasma protein ceruloplasmin and in excretion of copper from the liver. Large numbers of mutations in ATP7B gene were found to have a role in the pathogenesis of WD. The variation in the clinical and biochemical features of the disease renders the diagnosis difficult task. This study aimed to determine the mutations in exon14 and 21 of ATP7B gene in Iraqi patients with WD to be used for molecular diagnosis.A total of 35 patients with WD and other 10 apparently healthy individuals were recruited for this study. Blood sample was obtained from each subject from which DNA was extracted and exon 14 and 21 were amplified with amplification refractory mutation system (ARMS) and conventional polymerase chain reaction using specific primers. Direct sequencing was used to find out the mutations in these exons.Five novel mutations and one single nucleotide polymorphism (homozygous or heterozygous) were recorded, among which 3 silent (c.3133C>T, c.4194T>C and c.4302G>A), 1 missense (c.3181G>C) and 2 splice site mutations (IVS21+40delA and IVS21+23G>A). The heterozygous mutation c.3133C>T was the most prevalent one among WD patients (40%).
For diagnostic purpose, these results strongly suggested the heterozygous mutation c.3133C>T could be exploited in combination with the other high prevalent mutations.

Key Words: Exon 14, Exon 21, ATP7B gene, Wilson Disease

طفرات الاكسونات 14 و21 في جين ATP7B في المرضى المصابين بمرض ولسن

الخلاصة

مرض ولسن هو إضطراب جيني متنح في جين ATP7B الذي يشفر لانزيم ATPase الناقل للنحاس . يشترك هذا الانزيم في نقل النحاس من خلايا الكبد الى بروتين السريولوبلازمين في بلازما الدم . وجد ان هناك دورا لعدد كبير من الطفرات في جين ATP7B في امراضية مرض ولسن . أن التباين في المظاهر السريرية والكيموحيوية يجعل من تشخيص المرض مهمة صعبة . هدفت الدراسة الى تحديد الطفرات في الاكسونات 14 و 21 في جين ATP7B في المرضى العراقيين المصابين بمرض ولسن من اجل استخدام هذه الطفرات في التشخيص الجزيئي للمرض . استخدم 35 مريضا بالاضافة الى 10 أفراد أصحاء ظاهريا كمجموعة سيطرة . جمعت عينات دم من كل مشترك واستخلص الحامض النووي DNA وتمت مضاعفة الاكسونات 14 و21 بطريقة ARMS-PCR باستخدام بادئات خاصة. كما تم اجراء اختبار تتابع القواعد لنواتج تفاعل سلسلة البلمرة.سجلت خمس طفرات جديدة وتغاير جيني واحد (متماثلة او متغايرة الزيجة) من بينها ثلاث طفرات صامتة(c.3133C>T و c.4194T>C و c.4302G>A) وطفرة تحول الحامض الاميني (c.3181G>C) وطفرتان في موقع التجديل (IVS21+23G>A IVS21+40delA) وكانت الطفرة المتغايرة الزيجة (c.3133C>T) اكثر الطفرات شيوعا بين المرضى المصابين بمرض ولسن (40%). تشير نتائج هذه الدراسة الى ان الطفرة المتغايرة الزيجة (c.3133C>T) يمكن ان تستغل مع توليفة من الطفرات الاكثر شيوعا في عملية التشخيص الجزيئي للمرض.

الكلمات المفتاحية : اكسون 14 ، اكسون 21 ، جين ATP7B ، مرض ولسن.

Introduction

So far, more than 500 mutations and several single nucleotide polymorphisms (SNPs) have been identified in the *ATP7B* gene (RefSeq Gene: NG_008806.1) which encodes for a copper transporting P-type ATPase [1].Of these mutations, as many as 380 have been reported to have a role in the pathogenesis of WD [2].It seems that prevalence of these mutations differs according to races and geographical regions [3]. In most cases, the burden disturbance resulting from these mutations lies in a disorder in copper transporting with a consequence of a toxic accumulation of this metal in different body organs [4]. Of these, liver, brain, kidney and cornea are especially affected. Accordingly, patients with WD frequently suffer from hepatic, neurologic, renal and ocular complications.

ATB7B gene has 21 exons with 14 domains and ATP loop. Six of these domains are copper binding motifs at the N-terminal segment, and the other eight are transmembranous domains. In addition, there is a phosphorylation site with a conserved aspartate residue [5]. Mutations in *ATB7B* gene can impair the protein function with eventual accumulation of copper. Treatment is available and is effective especially when given at early stages of the disease[6], but the difficulties lie in the diagnosis especially of early cases and carriers. That is because of the wide variation in biochemical and clinical features and the broad range of disease onset. Recently, molecular diagnosis gradually substitutes the conventional methods for confirmation of this disease. Many kinds of molecular techniques have been used for this task. Full molecular-genetic test is a very satisfactory method, but it takes several months, aside from the high cost which makes it impractical method particularly in laboratories with limited facilities. Allele-specific probes is a rapid and very helpful method. It can identify a mutation in a certain piece of gene. One disadvantage of this method is that it can only be done if this mutation occurs in a high frequency in the population. Practically, none of the known mutations exceeded the frequency of 38% in certain population[7], and different populations have different kinds of mutations. Therefore many probes are needed each of which detects specific mutation.

With the development of DNA-based diagnostic methods such as that which can identify many mutations in a particular segment of DNA, the task becomes less complicated. All we have to do is to find narrow limits of mutations that are predominant to deal with. To start such investigation, it is reasonable to find out the most common mutations in the neighboring countries and the world. From some previous studies, the exon 14 and exon 21 were found to have predominant mutations associated with the more than half percent of WD cases [8]. Therefore, the present study aimed to determine the mutations in these two exons among Iraqi patients with WD.

Materials and Methods

The current study involved 35 family-unrelated patients with WD (20 males and 15 females, age range 3-16 years, average 11.68 years) who were attending Al-Imamain Al-Kadhumain Medical City and Baghdad Medical City during the period from November 2014 to July 2015. The diagnosis of WD was based on clinical features, low serum levels of ceruloplasmin and copper and high urinary copper elimination (Thomas et al., 1995). Another 10 age-matched family unrelated apparently individuals (8 males and 2 females) were recruited to represent control group.

All patients and control or their parents were given written informed consent before undergoing DNA test according to ethic committee in College of Medicine/ Al-Nahrain University.

Three ml of the peripheral venous blood from each subject were collected in EDTA tubes and stored at -20ºC until be used. DNA was extracted from these samples using ready kit (AccuPrep® Genomic DNA Extraction kit/ Bioneer/ Korea) according to the manufacturer's manual.

Gene Amplification and Genotyping

Initially, two methods of genotyping were intended to be used: direct sequencing for amplification of exon 14 and ARMS for amplification of exon 21. Two sets of primers were used which were F: AGG TTG GGT GAA GTT CTG CC and R: GGA CAT GGT GAG GAA TAA AAG AGC for exon 14, and wild F:ATGAAGCCCCTGACGGCATCTC,mut antF:ATGAAGCCCCTGACGGCATCTA and Common R: GCTTGTGGTGAGTGGAGGAAGTCCCTG for exon 21. A ready 50 µl PCR master mix (Bioneer/Korea) was used for preparing the PCR reaction. Template DNA (10 ng) from each sample and primers (5 ng from each) were added to each master mix tube. The mixture then put in shaker and spinner for 10 cycles for better mixing. Then, the mastermix tubes were transferred to the thermocycler (Hybaid/ UK) which is programmed with the specific protocols according to the gene to be amplified.

For exon 14, the cycling conditions involved an initial denaturation for 5 min at 95 ºC, followed by 30 cycles of denaturation at 95 ºC for 30 sec , annealing at 58 ºC for 40 sec, extension at 72 ºC for 1 min, followed by final extension at 72 ºC for 7 min with an expected fragment length of 334 bp. For Exon 21, the cycling conditions were an initial denaturation for 3 min at 94 ºC, followed by 35 cycles of denaturation at 94 ºC for 30 sec , annealing at 64 ºC for 45 sec, extension at 72 ºC for 1 min, followed by final extension at 72 ºC for 7 min with an expected fragment length of 277bp.

The presence of an amplicon was documented by gel electrophoresis using 2% agarose gel. Ten µL aliquot of PCR product from each PCR tube was mixed with 2 µL loading dye and loaded into the wells of the gel. After 1 hour of electrophoresis, the gel was stained with ethidium bromide (Biobasic/Canada) (0.5 g/mL) for 20 min and examined using U. V. transilluminator with camera. The amplified products were determined by comparison with a commercial 1000 bp ladder (Kappa Biosystem/USA). PCR products were sequenced by Microgen Laboratories/ Korea. The sequenced DNA was edited using Chromas Pro v1.5 (Technelysium Pty Ltd) and aligned to the published human genomic database using BLAST function from pubmed. DNA mutation numbering was based on c.DNA.

Results

Characteristics of the Patients

The clinical profiles of the affected individuals are summarized in table 1.

Table 1: Characteristic features of WD patients

Index	Value (mean±SD)	Normal range
ALT	164.56±43.12 (IU/L)	7-55 IU/L
AST	139.52±39.22 (IU/L)	8-48IU/L
ALP	191.08±61.98 (IU/L)	45-115IU/L
Serum bilirubin	3.9±1.19 (mg/dl)	0.3-1.9mg/dl
Serum ceruloplasmin	30.3±14.39 (mg/dl)	20-35mg /dl
Serum copper	82.18± 42.33(µg/dl)	32-147 µg/dl
Urinary copper	122.26± 37.09/ 24 h	> 60 µg/24h
Presence of Kayser-Fleicher ring	14 (20%)	

ALT:alanine transaminase; AST: aspartate transaminase; ALP: alkaline phosphatase; IU: international unit; SD: standard deviation

ARMS-PCR

Only the wild type allele (allele C) gave positive results for amplification as shown in figure 1. Therefore, ARMS-PCR products with positive amplification were used for sequencing.

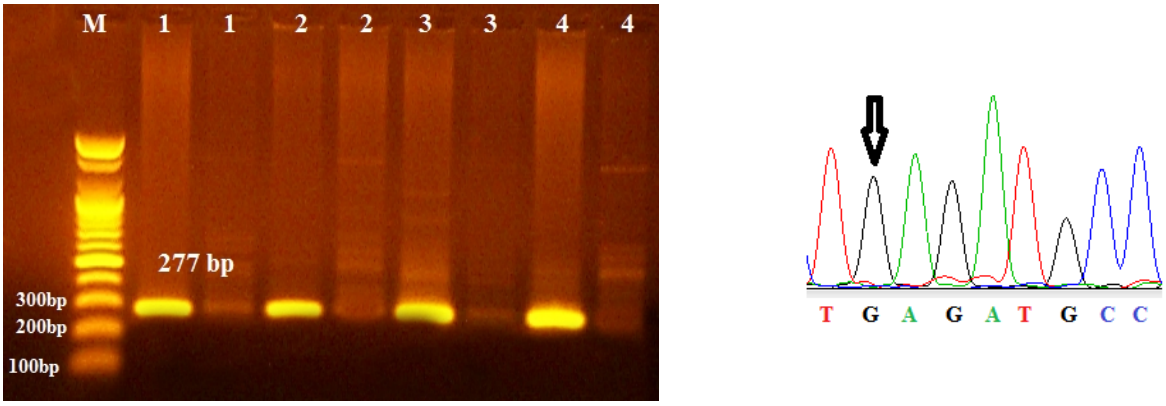

Figure 1: Left side figure: Amplification refractory mutation system (ARMS) for detection of the mutation 4193delC using the normal forward primer and mutant forward primer. Lane M: DNA marker, lanes 1, 2, 3 and 4 DNA from patients with WD which shows a positive result for the normal allele (left side for each pair) and a negative result for the deleted allele (right side of each pair). Right side figure: sequencing of the target locus which shows the non-deletion of C nucleotide (reverse strand).

Mutation Profile

A total of 70 chromosomes belong to 35 WD patients and other 20 chromosomes belong to healthy individuals have been examined for mutations in the exon 14 and exon 21 of *ATP7B* gene. Five different mutations have been recorded, all of which are novel mutation and have not been recoded previously according to University of Alberta database (available at http://www.wilsondisease.med.ualberta.ca) . These mutations were c.3133C>T, c.4194T>C, c.4302G>A, IVS21+40delA and IVS21+23G>A. In addition , the study revealed one SNP (G3181C) which was recorded only in WD patients (table 2).

Table 2: Characteristics of the mutations and the affected domain of *ATP7B* gene in WD patients

Mutation	Nucleotide change	Type	Exon	No of alleles (%)	Affected protein domain
Gly1060Arg[1]	c.3181G>C	Missense	14	7 (10%)	ATP loop
Leu1044Leu	c.3133C>T	Silent	14	14 (20%)	ATP loop
Ser1398Ser	c.4194T>C	Silent	21	70 (100%)	TM7[2]
Thr1434Thr	c.4302G>A	Silent	21	2 (2.86%)	ATP hinge
IVS21+40delA	Deletion A	Splice site	Intron	14 (20%)	Splice site
IVS21+23G>A	G>A	Splice site	Intron	2 (2.86%)	Splice site

[1]Single nucleotide polymorphism
[2]TM: transmembranous domain

For the exon14, only one mutation (c.3133C>T) was recorded 14 (20%) in heterozygous form (figure 2). The other genetic disorder in this exon is the SNP G3181Cwhich occurred in 7 chromosome (10%). It appeared in both heterozygous and homozygous (Figure 3).

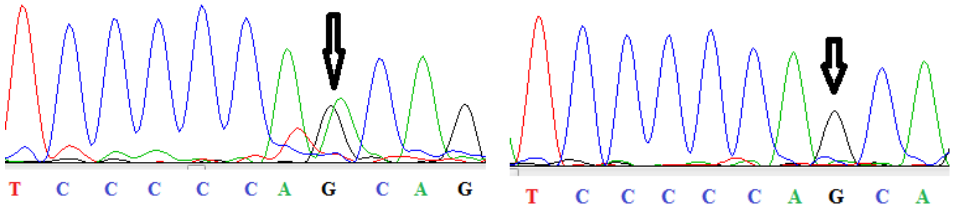

Figure 2: Electropherogram pattern of the mutation c.3133C>Tin exon 14 (reverse strand) showing: A: heterozygous mutant allele and B: wild type allele.

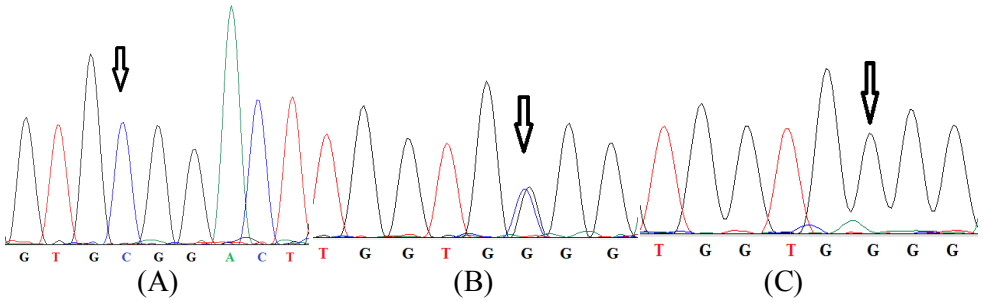

Figure 3: Electropherogram pattern of SNPc.3181G>C in exon 14 (forward strand) showing: A: homozygous mutant allele, B: heterozygous mutant allele and C: wild type allele.

Similarly, two mutations were recorded in exon21 which were c.4194T>C and c.4302G>A. Interestingly, the mutation c.4194T>C was recorded as homozygous in all studied chromosome in both patients and control, while the mutation c.4302G>A was recorded in only one patient (two chromosomes) (figure 4). The other two mutations were in intron 20 which were IVS21+40delA and IVS21+23G>A and recorded only in WD patients.

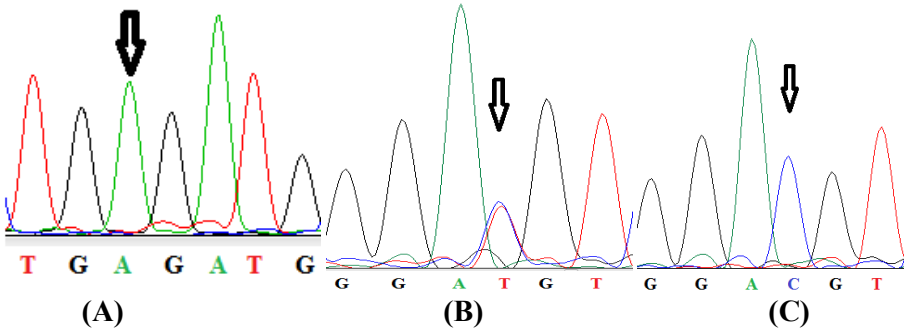

Figure 4: Electropherogram pattern of mutations in exon 21**.** A: the c.4194T>C mutation (forward strand)**,** B: heterozygous allele of c.4302G>A mutation and C: wild type allele of c.4302G>A (reverse strand).

Discussion

Since WD is treatable, early detection of presymptomatic patients is critical to prevent irreversible damage [9]. Rapid diagnostic investigation for WD does not only ensure the early management of this disease but also allows suitable treatment of non-wilsonian liver diseases once copper toxicosis ruled out. The variable clinical and biochemical manifestation render the diagnosis of WD difficult task. Despite most biochemical test involved in table (1) had values beyond the normal range, unfortunately most of them have

little diagnostic. In fact, almost all the diseases that affect the liver can raise the value of these tests. Both of serum ceruloplasmin and serum copper were within normal range. Nevertheless, one cannot ignore the diagnostic value of Urinary copper /24h, but beside the problem of incomplete urine collection, this test is not specific for WD. Patients with different liver diseases including autoimmune hepatitis, chronic active liver disease and acute hepatic failure of any origin may have basal 24-hours copper excretion of 100-200 µg/24 hours [10].On the other hand determination of hepatic copper content by means of liver biopsy is the most available sensitive and accurate available test for the disease. However, elevated copper content of hepatocytes is not pathognomonic for WD; it occurs in liver diseases such as primary biliary cirrhosis,biliary atresia, extrahepatic biliary obstruction, primary sclerosing cholangitis, autoimmune (chronicactive) hepatitis, and others[11]. Moreover, the invasiveness of biopsy and the risk of complications from the procedure are major arguments for its use in all patients suspected with WD [12]. Therefore, intensive research to find out the suitable molecular diagnosis is very necessary requirement.

The current study based on the presumption that mutations in ATP7B gene prevalent in neighboring countries are also present in Iraqi patients with WD. Accordingly, two exons (14 and 21) were chosen to be investigated. However, the results of the study revealed entirely different set of mutations. In Saudi Arabia, the mutation 4193delC is prevalent in 53.3% of Saudi patients with WD [8], while none of our patients had such mutation. Instead, all patients and control had T>C substitution at 4194 position (exactly after one base after 4193delC). In many other countries including Iran and Lebanon, the mutation H1069Q at the exon 14 was frequently recorded [13,14]. In contrast, none of Iraqi patients with WD involved in current study had this mutation. It seems that each population has its mutational profile of the ATP7B gene although there is some basic outline for distribution of mutations. For example, H1069Q mutation is more prevalent in WD patients of European origin such as those from Italy, Romania, and Sweden [15], whereas the R778L mutation is the more common in East Asia [6]. None of these two mutations have been found in India where 17 other mutations have been identified [16].

Gromadzka *et al*. [17]stated that the most common mutations in ATP7B gene are missense mutations. Other forms such as fram shift, nonsense, silent and splice site mutations were also frequently recorded. Although it restricted for two exons, the current study revealed that the half of the mutations are silent ones. Despite they have no importance role in the disease; these mutations can be exploited in diagnosis purpose. Practically, the heterozygous mutationc.3133C>T is the most prevalent one in this study and affected 14 from 35 patients (40%). As the study aimed to find a limited range of mutations which can be used for molecular diagnosis of the disease, finding of other two or three mutations with prevalent like that of this mutation will greatly facilitate the task.

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Competing Interests

The authors have declared that no competing interest exists.

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