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Clinical and genetic analysis of pediatric patients with Wilson disease

LIVER

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Original Article

ABSTRACT

Background/Aims: Wilson disease (WD, MIM# 277900) is an autosomal recessive disorder of copper transport resulting from the defective function of a copper transporting P-type ATPase. Detecting mutations and single nucleotide polymorphisms (SNPs) of the ATP7B gene in Turkish pediatric WD patients (n=32) and controls (n=52) is the aim of this research.

Materials and Methods: For screening mutations and SNPs of the ATP7B gene, sequencing was performed.

Results: Mutations were determined in the ATP7B gene in 23 out of the 32 pediatric patients. The mutation detection rate in the ATP7B gene of the pediatric Turkish WD patients was 71.875%. Fifteen different mutations were determined in the ATP7B gene. These mutations were distributed throughout the ATP7B gene and were as follows: 2 deletion, 1 insertion, 3 nonsense, and 9 missense mutations. Four of these, including c.3111delC (1 deletion) and c.2363C>T, c.3733C>A, and c.3451C>T (3 missense) mutations, were detected in the Turkish WD patients. Eleven polymorphisms were detected in both groups. Among these, c.3727G>A (SNP) was reported in the Wilson Disease Mutation Database by our group. Nine out of the thirty-two pediatric Turkish WD patients had no mutations in the ATP7B gene.

Conclusion: To find the cause of WD in pediatric patients who have no mutation in ATP7B, additional research is necessary.

Keywords: Mutation, Wilson disease, SNP, ATP7B

INTRODUCTION

Cu is a trace element required for the survival of all organisms because it is an essential cofactor in many enzymatic pathways and numerous cellular processes. The amount of copper in cells and tissues must be regulated. The importance of maintaining copper homeostasis is demonstrated by the existence of the following two well-characterized hereditary disorders in humans: Wilson disease due to excessive Cu load and Menkes disease due to Cu deficiency (1). Wilson disease (WD, MIM# 277900) is an autosomal recessive disorder of copper transport resulting from the defective function of the ATP7B gene (2).

ATP7B maps on 13q14.3-q21 contain 21 exons and encode 1464 amino acids that consist of six copper binding sites, eight transmembrane domains, and the ATP-binding domain (3).

The ATP7B gene is large and encodes Cu-translocating ATPase primarily expressed in the liver. ATP7B resides in the trans-Golgi membrane compartment and mainly loads Cu on newly synthesized apoceruloplasmin (4).

Over 520 mutations have been identified in the ATP7B gene according to the Wilson Disease Mutation Database (5). The diversity of the mutations makes genetic testing difficult in different exons. Most mutations are found in only single patients, and they are rare.

Wilson disease symptoms mostly do not develop until the age of three years, although the failure to excrete

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biliary copper is present from birth and rarely becomes evident before the age of five years. Unfortunately, symptoms at any age are frequently non-specific. Most patients present with liver disease, whereas after the age of 18 years, neuropsychiatric symptoms are more common (6).

During childhood, the presenting symptoms of WD can be sudden behavioral changes, inability to perform activities that need good hand-eye coordination, modifications in handwriting as micrographia, and worsening in school performances. In approximately 10–25% of WD patients, a psychiatric disturbance is the initial clinical presentation, even before the appearance of any movement disorder (7). The diagnosis of WD is rarely made during the period in which psychiatric symptoms predominate. Although neuropsychiatric symptoms are considered secondary to liver damage, neurologic and psychiatric manifestations without hepatic involvement have also been described in children (7).

Ophthalmic findings include Kayser–Fleischer (KF) rings and sunflower cataracts. Both findings are reversible with medical therapy or after liver transplantation. It should be pointed out that KF rings, although very specific for WD, are rarely described in early pediatric ages and in WD children presenting with clinically asymptomatic hypertransaminasemia (8), being more frequent (up to about 50% of cases) in adolescents and young adults with more severe liver disease and/or with neurologic symptoms (9).

Detecting mutations and SNPs of the ATP7B gene in pediatric WD patients and controls is the aim of this research.

MATERIALS AND METHODS

Clinical findings

Thirty-two pediatric Wilson disease patients from 28 unrelated families were included in this research. The patients were diagnosed as having WD according to laboratory and clinical findings (abnormal liver function, neurological abnormalities, presence of KF rings, low serum ceruloplasmin levels, and increased urinary Cu excretion) (10,11). A liver biopsy was conducted for patients with normal coagulation tests. D-penicillamine and zinc treatments were started for the patients with the diagnosis of WD. Patients having fulminant WD were evaluated for liver transplantation. Family screening was performed considering the aspects of the clinical and laboratory findings for the available family members of the patients with WD.

To evaluate the possibility that the new mutations identified in our research may be single nucleotide polymorphisms (SNPs), we tested 104 chromosomes (52 DNAs as controls) from the patients who had no family history and no clinical

Table 1. Distribution and frequency of mutations detected throughout the entire coding region of ATP7B gene

Exon	Nucleotide change	Nucleotide sequence	Codon change	Area of Protein	Type	Mutation	Frequency (%)	SIFT Score[a]	Predictions of Functional Affect with SIFT
3	c.1369C>T	CAG-TAG	p.G457X	Cu4/Cu5	nonsense	known	6.25	-	-
8	c.2128G>A	GGT - AGT	p.G710S	TM 2	missense	known	6.25	0.54	tolerated
8	c.2332C>T	CGG - TGG	p.R778W	TM 4	missense	known	3.125	0.00	affect protein function
8	c.2293G>A	GAC - AAC	p.D765N	TM 4	missense	known	3.125	0.01	affect protein function
8	c.2298-2299insC	ACGCCCCCCCA	p.P767R-fsX28	TM 4	frameshift	known	1.5625	-	-
9	c.2363C>T	ACC - ATC	p.T788I	TM 4	missense	NOVEL*	9.375	0.00	affect protein function
10	c.2532delA	GAAAGTC	p.V845S-fsX28	Td	frameshift	known	1.5625	-	-
11	c.2621C>T	GCG-GTG	p.A874V	Td/TM5	missense	known	6.25	0.01	affect protein function
12	c.2807T>A	TTG - TAG	p.L936X	TM5	nonsense	known	3.125	-	-
13	c.2906G> A	CGG-CAG	p.R969Q	Ch/TM6	missense	known	3.125	0.24	tolerated
14	c.3111delC	TCCCCAGGG	p.R1038G-fsX83	ATP loop	frameshift	NOVEL*	1.5625	-	-
14	c.3207C>A	CAC - CAA	p.H1069Q	ATP loop	missense	known	15.625	0.00	affect protein function
16	c.3451C>T	CGT -TGT	p.R1151C	ATP loop	missense	NOVEL*	1.5625	0.00	affect protein function
18	c.3733C>A	CCT -ACT	p.P1245T	ATP hinge	missense	NOVEL*	3.125	0.00	affect protein function
19	c.3955C>T	CGA-TGA	p.A1319X	ATP hinge/TM7	nonsense	known	6.25	-	-

Notes: Genebank accession NM_000053.
The first nucleotide of ATG translation codon is considered nt +1.
*Not detected on 104 normal chromosome and first found at Turkish Wilson disease patients
[a]Functional classification of missense variants along with SIFT scores. Variants are predicted to be deleterious with score of <0.05 with SIFT
TM: trans Membrane; Td: trans Membrane Domain; Ch: channel; Ins: insertion; del: deletio

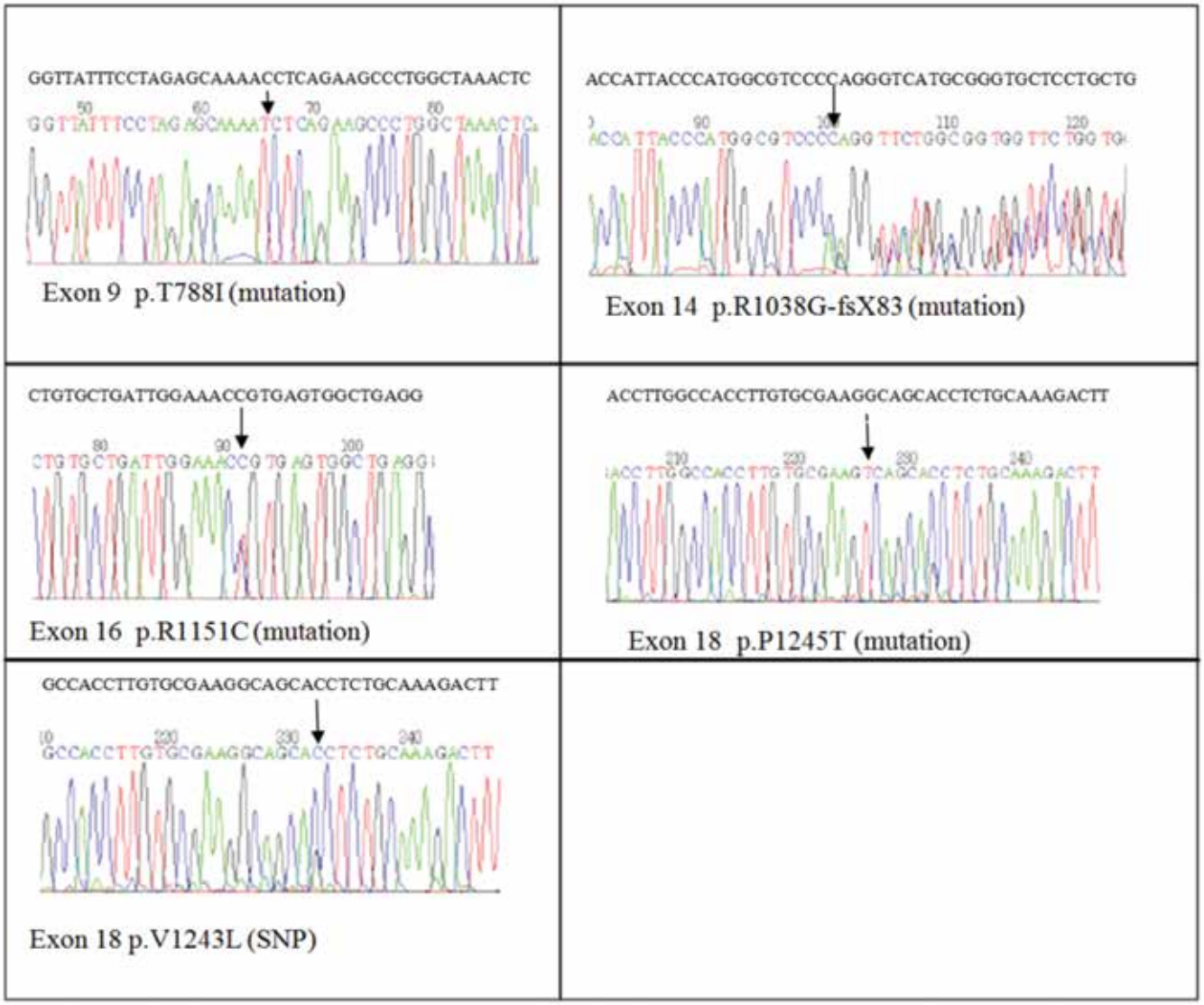

Figure 1. Sequencing results of four novel mutations and one novel SNP of the ATP7B gene for Turkish pediatric Wilson disease patients.

symptoms of WD. Informed consent was taken from all the patients and controls who participated in this research. The Ethics Committee of Dokuz Eylül University approved the study protocol.

DNA extraction

DNA was isolated from blood using a blood kit according to the manufacturer's instructions (Macherey-Nagel Nucleospin blood kit; Duren, Germany).

Polymerase chain reaction

The entire ATP7B gene was amplified using intronic primers (12,13) (Primers; MWG Biotech, Ebersberg, Germany). Polymerase chain reaction (PCR) was performed with DNA (100 ng) and was amplified in a mix in a total volume of 25 mL (2.5 mM MgCl2, 10 mM dNTP mix, 10 pmol of each primer, 1 U of Taq polymerase in the buffer; MBI Fermentas, Opelstrasse, Germany). Amplification was performed according to our previous study (14).

DNA sequencing

To detect mutations and SNPs in the ATP7B gene of the 32 pediatric patients with WD and 52 controls, direct sequencing was performed (3730xl automated DNA sequencer; Macrogen Inc., Seoul, South Korea). Both strands of shifted exons and splice sites within the first two amino acids of introns were sequenced, aligned, and inspected to identify nucleotide variations using a reference sequence from GenBank (accession number NM_00053.1).The first nucleotide of the ATG translation codon is considered as nucleotide +1.

The computational predictive analysis of missense variants was performed using the Sorting Intolerant From Tolerant algorithm (SIFT version 5.2.2, Genome Institute of Singapore, Singapore).

Table 2. Distribution of single nucleotide polymorphisms detected in the entire coding region of the ATP7B gene

Exon/intron	Nucleotide change	Nucleotide sequence	Codon change	Area of Protein	Type	SNP
2	c.1216T>G	TCT-GCT	p.S406A	Cu4	Missense	Known
3	c.1366G>C	GTG-CTG	p.V446L	Cu4/Cu5	Missense	Known
10	c.2495A>G	AAG-AGG	p.K832R	TM 4/Td	Missense	Known
12	c.2855G>A	AGA-AAA	p.R952K	TM5	Missense	Known
13	c.2973G>A	ACG-ACA	p.T991T	Ch/TM6	Silent	Known
13	c.3009G>A	GCG-GCA	p.A1003A	Ch/TM6	Silent	Known
13	c.3045G>A	CTG-CTA	p.L1015L	Ph	Silent	Known
16	c.3419T>C	GTC-GCC	p.A1140V	ATPloop	Missense	Known
17	c.3620A>G	CAC-CGC	p.H1207R	ATPloop	Missense	Known
18	c.3727G>C	GTG-CTG	p.V1243L	ATPhinge	Missense	NOVEL*
intron18	c.3903+6C>T	GAGCG-GAGTG	-	-	-	Known

Notes: GenBank accession NM_000053.
The first nucleotide of the ATG translation codon is considered nt +1.
*This novel SNP has been found first by us.
SNP: single nucleotide polymorphism; TM: transmembrane; Td: transmembrane domain; Ch: channel; Ph: phosphorylation

RESULTS

Mutations were detected in 23 out of the 32 pediatric patients. The mutation detection rate of the pediatric Turkish WD patients in the ATP7B gene was 71.875%. Fifteen different mutations were detected in the ATP7B gene. These mutations were distributed throughout the APT7B gene and included nonsense, missense, and frameshift mutations. The distribution of mutations was 9 missense, 3 nonsense, and 2 deletion mutations, and 1 insertion mutation (Table 1). Four of these including c.3451C>T, c.2363C>T, c.3733C>A (3 missense), and c.3111delC (1 deletion) mutations were first detected in the Turkish WD patients (Figure 1). None of these were detected in the controls. The computational predictive analysis of the missense variants by SIFT is shown in Table 1. Eleven SNPs were found (Table 2). Of these, one, c.3903+6C>T, was found in the intron, and ten of them, c.1366G>C, c.1216T>G, c.2495A>G, c.2973G>A, c.2855G>A, c.3009G>A, c.3419T>C, c.3045G>A, c.3727G>A, and c.3620A>G, were detected in exons. Among these, c.3727G>A was submitted to the Wilson Disease Mutation Database by our group. The clinical data of patients who have mutations in the ATP7B gene are shown in Table 3, whereas the data of patients who have no mutations in the ATP7B gene are in shown in Table 4.

DISCUSSION

The mutation analysis of the copper transporting P-type ATPase in the Turkish pediatric WD patients yielded a mutation detection rate of 71.875%. Mutations were found in 23 out of the 32 patients. Three missense (c.2363C>T, c.3733C>A, and c.3451C>T) mutations and a deletion (c.3111delC) mutation were first detected in the Turkish WD patients (14). Among these, c.3727G>A was submitted to the Wilson Disease Mutation Database by our group (Figure 1). c.3207C>A was most frequently detected in pediatric WD patients at a rate of 15.625% of the studied alleles. These mutations are also common in Poland 72%, Bulgaria 58.8%, Slovakia and Czech Republic 57%, Benelux 48.1%, Yugoslavia 48.9%, Germany 47.9%, Hungary 47%, Sweden 38%, Austria 34.1%, and the United Kingdom 17% (15).

The computational predictive analysis for the nine missense variants was detected by SIFT. According to the SIFT score, seven variants (p.D765N, p.R778W, p.T788I, p.A874W, p.H1069Q, p.R1151C, and p.P1245T) were predicted to affect protein function and two (p.G710S and p.R969Q) were predicted as tolerated (Table 1).

Nine out of the thirty-two WD patients had no mutations in the ATP7B gene. These nine individuals had WD according to the clinical and laboratory findings (Table 4). In different populations, individuals with no mutations have been reported in different proportions (16-20). Failure to detect any mutations may be explained by unknown mutations that may be located on the outside of the exons and flanking regions, such as the promoter, introns, or other DNA control regions (16,19). To find out the cause of WD in pediatric pa-

Table 3. ATP7B genotype and clinical and laboratory findings of Turkish pediatric patients with Wilson disease

			Mutation		Affected organ			Laboratory finding	
Patient	Age of onset	Gender	Nucleotide Change	Exon	Liver	Brain (Neurological abnormalities)	Eye (Kayser–Fleischer ring)	Serum ceruloplasmin (mg/dL)	Urinary copper (µg/day)
1.*	12	Female	c.2621C>T	11	+	-	+	3.1	1600
2.	15	Female	c.2532delA/c.3451C>T[b]	10 / 16	+	+	+	3.1	1340
4.*	7	Male	c.2128G>A	8	+	-	-	12.0	1260
6.*	12	Male	c.2621C>T/c.3207C>A	11 / 14	+	-	+	5.0	1380
6s	6	Female	c.2621C>T/c.3207C>A	11/14	+	-	-	ND	ND
7.	6	Male	c.2807 T>A	12	+	-	+	5.0	1280
9.	9	Male	c.2363 C>T[b]	9	+	+	+	15.0	796
9s1	5	Female	c.2363 C>T[b]	9	+	-	-	ND	ND
9s2	4	Female	c.2363 C>T[b]	9	+	-	-	ND	ND
10.[a]	12	Female	c.1369 C>T	3	+	+	+	4.0	466
12.[a,c]	8	Male	c.3955 C>T	19	+	+	-	5.0	1590
12s1	4	Female	c.3955 C>T	19	+	-	-	ND	ND
14[a]	4,5	Male	c.3207C>A	14	+	-	+	22.0	220
18	7	Male	c.2332C>G/ c.3207C>A	8/14	+	-	+	6.0	1200
20.	5	Male	c.2332C>T/c.3111delC[b]	8 / 14	+	-	-	5.0	1900
21	11	Female	c.2906C>A	13	+	-	-	6.22	1100
22	10	Male	c2298-2299insC/c.3207C>A	8/14	+	-	+	5.34	1250
24	13	Male	c.3207C>A	14	+	+	+	6.9	135
29.[a]	12	Female	c.2293 G>A	8	+	-	-	4.3	1060
33	9	Male	c.1369C>T	3	+	+	+	23.0	622
42[a]	12	Female	c.3207C>A	14	+	-	-	11.0	4017
43	14	Female	c.2128G>A	8	+	+	-	5.0	880
49.[a]	10	Male	c.3733 C>A[b]	18	+	+	-	4.0	886

Notes: Genebank accession NM_000053.
The first nucleotide of the ATG translation codon is considered nt +1.
Serum ceruloplasmin (CP) level was measured by the immunoturbidimetric test.
Normal values for serum CP are 20–80 mg/dL.
Normal values for urine Cu level are less than 35 µg/day.
[a]Liver biopsy was performed and Cu level was found >250 µg/g.
[b]Novel mutations detected by us.
[c]Patient died.
*Liver transplantation was performed.
ND: no data; ins: insertion; del: deletion

tients who have no mutation in the ATP7B gene, additional research is necessary.

The limitation of this study was the small number of children in the patient group; we need a large number of patients to analyze the exact mutation rate in Turkish WD population. The mutation analysis was conducted on 28 pediatric patients who came from the same pool of 46 patients who were studied before. Also, the same 52 people were used in this study as the control group (14). The clinical assessment of pediatric WD patients is conducted in various centers in Turkey. However, the complete diagnosis, clinical follow-up, genetic laboratory analysis, and assessment of the data were made by our group for the first time in Turkey. Mutations that were detected by centers in the Mediterranean region in the Wilson Disease Mutation Database were also found in our study; in addition to these, we found novel mutations.

The mutation analysis of the ATP7B gene in the Turkish pediatric WD patients has an identification rate of 71.875% in this study. In total, 23 out of the 32 pediatric patients had mutations in the ATP7B gene, whereas 9 out of the 32 patients had

Table 4. Clinical and laboratory findings of nine Turkish pediatric patients with Wilson disease who have no detected mutations on the ATP7B gene coding regions

			Affected organ			Laboratory finding	
Patient	**Age of onset**	**Gender**	**Liver**	**Brain (Neurological abnormalities)**	**Eye (Kayser–Fleischer Ring)**	**Serum ceruloplasmin (mg/dL)**	**Urinary copper (µg/day)**
3.	10	Female	+	+	+	4.0	245
5.	7	Male	+	-	-	9.1	154
8.[a]	7	Male	+	-	+	14.0	76
11.[a]	6	Female	+	+	-	11.0	80
16.[a]	10	Male	+	-	+	5.0	211
17	6	Male	+	-	+	6.0	1245
27.[a]	11	Male	+	+	+	14.0	121
30.[a]	11	Male	+	-	-	14.0	200
38.[a]	6	Female	+	-	-	18.0	489

Notes: Genebank accession NM_000053.
The first nucleotide of the ATG translation codon is considered nt +1.
Serum ceruloplasmin (CP) level was measured by the immunoturbidimetric test.
Normal values for serum CP are 20–80 mg/dL.
Normal values for urine Cu level are less than 35 µg/day.
[a]Liver biopsy was conducted and Cu level was found >250 µg/g.

no mutations in the exons of the ATP7B gene. To find out the cause of WD in pediatric patients who have no mutation in the ATP7B gene, additional research is necessary.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Dokuz Eylül University.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author contributions: Concept - Ö.Ş.P.; Design - Ö.Ş.P., S.A.A., O.T.; Supervision - Ö.Ş.P., O.T.; Resource - Ö.Ş.P., O.T.; Materials - Ö.Ş.P., S.A.A.; Data Collection &/or Processing - Ö.Ş.P., S.A.A.; Analysis &/or Interpretation - Ö.Ş.P., S.A.A., O.T.; Literature Search - Ö.Ş.P., S.A.A., O.T.; Writing - Ö.Ş.P., S.A.A.; Critical Reviews - Ö.Ş.P., S.A.A.

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