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ORIGINAL ARTICLE

Mutational analysis of *ATP7B* gene in Egyptian children with Wilson disease: 12 novel mutations

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Abstract The aim of this work was to study the mutations within *ATP7B* in Egyptian children with Wilson disease and to evaluate any potential correlation between genotype and phenotype in this cohort. The study consisted of 48 children with Wilson disease from 32 independent families. The 21 exons of the *ATP7B* gene were amplified in a thermal cycler. Direct sequencing of the amplified polymerase chain reaction (PCR) products was performed by cycle sequencing using fluorescent dye terminators in an automatic ABI sequencer. Thirty-one different mutations in 96 chromosomes were detected (19 missense, three nonsense, seven frameshift deletions, and two splice-site mutations). Of these, 12 mutations have not been previously reported. The p.N1270S, p.C703Y, IVS18-2A > G, p.R1319X, c.2304-2305insC, and p.H1069Q were present in 7.8%, 6.2%, 6.2%, 6.2%, 4.7%, and 4.7%, respectively, of studied chromosomes in independent families. One patient was homozygous for both p.N1270S and p.T1434M mutations. Frameshift and nonsense mutations were found in 50% of patients with disease onset ≤8 years compared with only 26% in patients with onset >8 years. Despite mutation heterogeneity in Egyptian children, genotype–phenotype correlation analysis seems to be promising in this population, as many patients carry homozygous mutations, a situation that mandates a larger-scale population screening to identify the carrier rate in this community.

Keywords *ATP7B* · Mutation · Wilson disease · Egypt · Genetics

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Introduction

Wilson disease (WD) is an autosomal recessive disorder of copper transport characterized by decreased hepatobiliary copper excretion and reduced copper incorporation into ceruloplasmin (Danks 1989). *ATP7B*, the gene mutated in WD, consists of 21 exons and encodes a 1,465 amino acid protein representing a copper transporting P-type adenosine triphosphatase (ATPase) (Thomas et al. 1995).

Mutation screening in WD patients has led to the detection of at least 300 disease-specific mutations (University of Alberta Department of Medical Genetics 2002). The published data suggest that some mutations appear to be population specific, whereas others are common to many populations. The spectrum of mutations and their clinical consequences has not previously been studied in the Egyptian population. In addition, mutational studies in pediatric WD are still limited in the literature.

The aim of this work was to study mutations within *ATP7B* in Egyptian children with WD and to evaluate any

potential correlation between genotype and phenotype in this Egyptian cohort.

Patients and methods

The study included 48 children (28 boys and 20 girls) from 32 independent families with WD who presented to Yassin Abdelghaffar Charity Center for Liver Disease and Research (a major tertiary referral center for liver diseases in Egypt) and the hepatology clinic, Children's Hospital, Ain Shams University. Children referred from different parts of Egypt (37.5% of families from lower Egypt, 33.3% from upper Egypt, 20.8% from Cairo and Giza, and 8.3% from Sinaa). For each family, full medical history of all members including those with the disease was recorded and included family pedigree analysis. Screening of suspected family members was performed by clinical examination, ceruloplasmin oxidase activity in serum, and liver function tests, and was confirmed by mutational analysis.

The diagnosis of WD was established in the presence of at least two out of three of the following criteria: low ceruloplasmin level <20 mg/dl or the presence of Kayser–Fleischer rings by slit-lamp examination and/or hepatic copper content of 250 μg/g dry weight liver tissue in the presence of hepatic or neurological manifestations consistent with WD (Sternlieb 1990). Liver involvement was ascertained based on clinical evaluation, liver function tests, ultrasonography, and liver biopsy when necessary. Neuropsychiatric involvement was based on clinical evaluation and brain magnetic resonance imaging (MRI) in individual cases.

ATPB mutation analysis

Genomic DNA was extracted from whole venous blood collected in ethylenediaminetetraacetate (EDTA) using standard procedures from the 48 patients and 113 unrelated healthy Egyptian subjects. The 21 exons of the WD gene were amplified in a thermal cycler (Biometra T3 Thermocycler, Göttingen, Germany) as described elsewhere (Waldenström et al. 1996). Direct sequencing of the amplified polymerase chain reaction (PCR) products was performed by cycle sequencing using fluorescent dye terminators in an automatic sequencer (Applied Biosystems, Darmstadt, Germany). Mutations were quoted according to the guidelines from http://www.HGVS.org/mutnomen/ using the reference sequence with the GenBank accession number NM_00053.1. The nucleotide +1 is the A of the ATG-translation initiation codon. The ATG-translation initiation codon is also the first codon.

RFLP analysis and allele-specific PCR

The novel mutations modifying endonuclease restriction sites were studied by specific restriction fragment length polymorphism (RFLP) analysis. The novel mutations that did not modify any known restriction sites were analyzed by allele-specific PCR (Table 1).

Results

Sequencing of the *ATP7B* gene revealed 31 different mutations in 96 chromosomes (19 missense, three nonsense, seven frameshift deletions, and two splice-site mutations). Of these, 12 mutations had not been previously reported (novel mutations). Novel mutations included five missense, two nonsense, four frameshift deletions, and one splice-site mutation (Table 1). They were confirmed using RFLP analysis or allele-specific oligohybridization analysis. None of the mutations were found in the 226 chromosomes of 113 healthy subjects.

Consanguinity was present in 75% of families. Affected family members shared the same genotype. In some instances, the same genotype was shared by more than one family (Table 2). One patient was homozygous for both p.N1270S and p.T1434M mutations.

From the 32 families studied, 32 children (index or symptomatic patients) presented with clinical manifestations suggestive of WD, and 16 sibs were diagnosed by screening family members of index patients. Whereas the index patients presented with hepatic or/and neurological symptoms, all screened children had only hepatic manifestations (Table 3). Index patients characteristics in relation to mutations are described in Table 4. In the first group (children who where <8 years of age when they had their symptoms), most patients were boys, and all had hepatic symptoms only with no neurological manifestations, which was statistically significant (p value 0.03). Frameshift and nonsense mutations (indicative of severe disease) were the responsible mutation in 50% of patients compared with 26% in children who presented at >8 years of age.

The diversity of mutations limited our genotype–phenotype association analysis; however, several observations were noted: frameshift mutations affected more than one third of children with hepatic phenotype, and in 46% of these cases, the child presented with subacute or acute hepatic failure. Splice-site mutation seems to result in severe disease, as patients carrying this type of mutation either presented with decompensated cirrhosis (two patients) or they later develop neurological manifestations (three patients). Frameshift mutations were significantly

Table 1 Mutations detected in children with Wilson disease in this study

Mutation	No. children	Exon	Type	Domain	Second assay
c.507delA[a]	2	2	Deletion/frameshift	Cu2	*Pst I*
p.E396X[a]	6	2	Nonsense	Cu4	*Hpy188 I mismatch*
c.330delA[a]	4	2	Deletion/frameshift	Cu1	*Bsg I*
p.L549P[a]	2	4	Missense	Cu5	*Sma I*
IVS4 + 5G > A[a]	4	4	Splice?	?	*Rsa I*
p.G591D	1	5	Missense	Cu 6	
p.D642H	1	6	Missense	Tm1	
p.C703Y	4	7	Missense	Tm2	*Rsa I*
c.2304_2305insC	3	8	Insertion/frameshift	Tm4	
p.D765N	2	8	Missense	Tm4	
c.2532delA	4	10	Deletion/frameshift	Td	
p.N878K[a]	1	11	Missense	Td	ASO
p.G998D[a]	2	13	Missense	Ch/Tm6	*Sdu I*
p.T977M	4	13	Missense	Ch/Tm6	
c.2997_2998insC	2	13	Insertion/frameshift	ATP loop	
p.H1069Q	7	14	Missense	ATP loop	
c.3373_3377delAGTCAinsTCT[a]	8	15	Deletion/insertion/ fs	ATP loop	*Bsr I*
p.I1148T	1	16	Missense	ATP loop	
p.H1207R	1	17	Missense	ATP loop	*Apa I*
p.T1220M	1	17	Missense	ATP binding	
IVS18-2A > G	6	18	Splice	Tm7/8	
p.N1270S	5	18	Missense	ATP hinge	
p.P1273L	6	18	Missense	ATP hinge	
c.3731delT[a]	2	18	Deletion/frameshift	ATP hinge	ASO
p.P1273Q	1	18	Missense	ATP hinge	*Msp I*
p.N1332D[a]	2	19	Missense	Tm7	*Nla III mismatch*
p.R1319X	6	19	Nonsense	Tm7	
p.G1341R[a]	4	19	Missense	Tm7	*HpyCH4 IV*
p.G1341D	2	20	Missense	Tm7	
p.T1434M	2	21	Missense	3′COOH	
p.W1410X[a]	2	21	Nonsense	3′COOH	*Hph I*

[a] Novel mutations detected in this study

Table 2 Mutations detected more than once in children with Wilson disease in this study

Mutation	No. of chromosomes carrying the mutation			Number of families carrying the mutation	Percentage of studied chromosomes in independent families (%)
	Homozygous state	Heterozygous state	Total		
p.N1270S	4	1	5	3	7.8
p.C703Y	2	2	4	3	6.2
IVS18-2A > G	6	–	6	2	6.2
p.R1319X	6	–	6	2	6.2
c.2304-2305insC	2	1	3	2	4.7
p.H1069Q	6	1	7	2	4.7

higher in patients with hepatic phenotype, whereas splice-site mutations were significantly higher in patients with neurological phenotype (Tables 5, 6).

There was a tendency of mutations in the transmembrane domains and ATP loop to result in early onset of disease (≤8 years). Also, mutations of the ATP loop

Table 3 Clinical criteria of the studied group

No.	Patient initials	Gender	Onset age	Index/FM	Symptoms	Treatment	Prognosis
1	MR	M	9.5	Index	H*	Z + A + E	Improved
2	FH	M	7.5	Index	H*	Z + A + E	Improved
3	SH	F	10	FM	H	Z + A + E	Improved
4	AS	M	10	Index	H* + N	Z + A + E	Improved
5	SA	M	12	Index	H + N	Z + A + E	Died
6	MT	F	14.5	Index	H	Z + A + E	Improved
7	MA	M	12	Index	H* + N	Z + A + E	Improved
8	SM	F	10.7	Index	H + N	Z + A + E	Improved
9	AE	F	10	FM	H	Z + A + E	Improved
10	AA	M	13	Index	H + N	Z + A + E	Improved
11	LA	F	10	FM	H	Z + A + E	Improved
12	MA	F	8	FM	H	Z + A + E	Improved
13	NI	F	11	FM	H	Z + A + E	Improved
14	AI	M	10	Index	H	Z + A + E	Improved
15	EE	F	8	FM	H	Z + A + E	Improved
16	MS	M	11	Index	H* + N	Z + A + E	Improved
17	AG	M	14	Index	H	Z + A + E	Died
18	KK	F	15	Index	H*	Z + A + E	Died
19	AK	F	6	FM	H	Z + A + E	Improved
20	SH	F	18	FM	H + N	Z + A + E	Worsened
21	AH	M	14	FM	H	Z + A + E	Stationary
22	SB	F	8	Index	H	Z + A + E	Improved
23	MB	M	6	Index	H	Z + A + E	Died
24	AG	M	6	Index	H*	Z + A + E	Improved
25	IS	M	9	Index	H*	Z + A + E	Improved
26	OH	M	15	Index	H*	Z + A + E	Improved
27	IA	M	13	Index	H*	Z + A + E	Died
28	RR	F	12	Index	H*	Z + A + E	Died
29	WZ	M	7	FM	H	Z + A + E	Improved
30	MF	M	11	Index	H + N	Z + A + E	Improved
31	EA	M	9	FM	H	Z + A + E	Improved
32	DY	F	10	Index	H* + N	Z + A + E	Worsened
33	AY	F	17	FM	H	Z + A + E	Worsened
34	LS	F	7	Index	H*	Z + A + E	Improved
35	AG	F	13	Index	H* + N	Z + A + E	Improved
36	EH	M	12	Index	N	Z + A + E	Worsened
37	SH	F	14	FM	H	Z + A + E	Improved
38	AH	M	8	FM	H	Z + A + E	Improved
39	WD	M	8	Index	H*	Z + A + E	Died
40	KD	M	10	Index	N	Z + A + E	Improved
41	AA	F	9	Index	H*	Z + A + E	Improved
42	SS	M	8	Index	H*	Z + A + E	Died
43	MS	M	7	Index	H*	Z + A + E	Died
44	AS	M	4	FM	H	Z + E	Improved
45	HE	F	12	FM	H	Z + A + E	Improved
46	MH	M	9.5	Index	H*	Z + A + E	Improved
47	AR	M	11	Index	Asymptomatic	Z + E	Improved

Table 3 continued

No.	Patient initials	Gender	Onset age	Index/FM	Symptoms	Treatment	Prognosis
48	MS	M	8	Index	H	Z + A + E	improved

M male, *F* female, *FM* family member, *A* asymptomatic, *H* hepatic, *N* neurological, *H** decompensated cirrhosis, *Z* zinc, *A* artamine, *E* vitamin E

Asymptomatic: patients with accidental discovery of increased liver enzymes ± hepatomegaly, hepatic manifestations included decompensated cirrhosis, acute hepatitis, chronic hepatitis, fulminant liver cell failure. Neurological manifestations included tremors, dysarthria, dystonia, and migraine

Table 4 Index patients' characteristics in relation to mutations detected

	Disease onset ≤8 years	Disease onset >8 years	*P* value
Number	8	23	
Gender			
Male	6	16	0.77
Female	2	7	
Disease phenotype			
Hepatic	8 (100%)	11 (47.8%)	0.03
Neurological	0 (0.0%)	1 (4.3%)	
Both	0 (0.0%)	11 (47.8%)	
Type of mutation			
Missense	3 (37.5%)	13 (56.5%)	0.37
Nonsense	2 (25.0%)	1 (4.3%)	
Frameshift	2 (25.0%)	5 (21.7%)	
Splice site	1 (12.5%)	4 (17.4%)	

Table 5 Comparison of mutation type in relation to Wilson disease phenotype

	Disease phenotype		*P* value
	Hepatic	Neurological ± hepatic	
Number	36	12	
Mean age of onset	9.86 ± 2.188	9.86 ± 3.091	
Gender			
Male	20	8	0.69
Female	16	4	
Type of mutation			
Missense	35 (48.6%)	12 (50.0%)	0.56
Nonsense	10 (13.9%)	4 (16.7%)	0.71
Frameshift	23 (31.9%)	2 (8.3%)	0.01
Splice site	4 (5.5%)	6 (25.0%)	0.014

Hepatic manifestations included decompensated cirrhosis, acute hepatitis, chronic hepatitis, and fulminant liver cell failure. Neurological manifestations included tremors, dysarthria, dystonia, and migraine

resulted in hepatic symptoms with absence of neurological manifestations, whereas ATP hinge mutations resulted in hepatic failure in half of patients, and transmembrane and copper-binding domain mutations were associated with neurological manifestations (Tables 7, 8).

Table 6 Type of mutation detected in Wilson disease children with liver cell failure

	Liver cell failure
Number	19
Mean age of onset	9.98 ± 3.25
Gender	
Male	13
Female	6
Type of mutation	
Missense	16 (42.1%)
Nonsense	4 (10.5%)
Frameshift	12 (31.5%)
Splice site	6 (15.9%)

Discussion

To date, at least 300 different mutations have been identified in WD chromosomes. Some mutations appear to be population specific [e.g., p.H1069Q in populations of European origin (Tanzi et al. 1993); p.R778L in Asian populations (Thomas et al. 1995)]. Unlike the European and Chinese populations, we found no single predominant mutation in Egyptian children. However, p.N1270S mutation was present in 7.8%; p.C703Y, IVS18-2A > G, and p.R1319X were each present in 6.2%; and p.H1069Q and c.2304-2305insC in 4.7% of studied chromosomes in independent families. A probable explanation of this diversity is that the Egyptian population is very heterogeneous with respect to ethnicity, and it may also indicate a high carrier rate for WD in this community.

In this study, children with disease onset <8 years of age were mostly boys, which may be explained by the cultural philosophy of better care for boys. Mean age of appearance of neurological symptoms did not differ from that of hepatic symptoms (9.8 compared with 10.9 years); furthermore, onset of neurological symptoms was much earlier than that reported in the literature (Machado et al. 2006).

Table 7 *ATP7B* mutation site in relation to disease phenotype

	ATP loop	ATP hinge	Cu-binding domain	Transmembrane domain
No. of patients (families)	9 (4)	7 (5)	3 (3)	21 (17)
Median age of onset (range)	10.2 (7–14.5)	11.9 (8–15)	11.5 (9.5–11)	8.5 (4–14.5)
Patients $\leq$ 8 years (%)	22.2	14.3	0.0	33.3
Liver (%)	100	83.3	66	71.4
Liver and neurological (%)	0	14.3	33.3	28.6
Liver cell failure (%)	11.1	50	33.3	38.1

Cu copper, *ATP* adenosine triphosphate

Table 8 Polymorphisms detected in this study

Exon	Mutation				Prevalence (%)
	Nucleotide	Amino acid	Type	Sequence	
Common polymorphisms					
2	c.1216T >< G	p.Ser406Ala	Missense	TCT >< GCT	12.5
3	c.1366G > C	p.Val456Leu	Missense	GTG > CTG	12.5
10	c.2495A >< G	p.Arg832Lys	Missense	AGG >< AAG	26.5
12	c.2855A >< G	p.Lys952Arg	Missense	AAA > AGA	7.8
12	c.2866–13G > C	IVS12-13G > C	No change	tctgtcc > tctctcc	6.25
13	c.3009G > A	p.Ala1003Ala	No change	GCG > GCA	15.6
13	c.3045G > A	p.Leu1015Leu	No change	CTG > CTA	3.1
16	c.3419C >< T	p.Alal1140Val	Missense	GCC >< GTC	40.6
18	c.3903 + 6T >< C	IVS18 + 6T > C	No change	gagtgtg > gagcgtg	42.2
Rare polymorphisms					
14	c.3105C > T	p.Ala1035Val	Missense	GGC > GGT	1.6
15	c.3366A > G	p.Ala1122Ala	No change	GCA > GCG	1.6
19	c.3985C > T	p.Leu1329Leu	No change	CTG > TTG	1.6

Exchange nucleotides are underlined

Genotype–phenotype correlation analysis was limited in this study due to diversity of the mutations detected, but it was found that frameshift and nonsense mutations (a possible predictor of severe phenotype) were found in 50% of children with age of onset <8 years compared with only 26% of patients with age of onset >10 years. It was also observed that frameshift mutations affected more than one third of children with hepatic phenotype, and in about 46% of these cases, the child presented with hepatic failure. On the other hand, splice-site mutation resulted in hepatic failure, and children having this mutation all developed neurological manifestations. Also, when the mutation affected the ATP loop, it resulted in early presentation of disease and hepatic symptoms with absence of neurological manifestations, whereas ATP hinge mutations resulted in hepatic failure in half the patients, and transmembrane mutations resulted in the appearance of neurological manifestations. All these observations require larger-scale patient analysis to confirm their significance.

It should be noted that because all patients were referred from a pediatric hepatology clinic, a sample bias might have occurred. It was of interest to note that even in the same family member with the same genotype, children had different phenotypes. In one family carrying the homozygous p.E396X mutation, the index patient had neurological manifestation only at the age of 18 years (onset was 15 years), his younger sister had both hepatic and neurological manifestation at the age of 17, and the youngest brother had only hepatic manifestations until the writing of this study (20 years). In another family (homozygous IVS18-2A > G mutation), the index patient presented with fulminant hepatic failure at the age of 8 years, whereas his brother had only neurological manifestations, which began at the age of 10 years and continued without hepatic manifestations till this writing (15 years). This suggests that other factors (outside the *ATPB7* gene or environmental factors) affect the disease phenotype (Takeshita et al. 2002).

As Egypt is considered an Arab country in the Mediterranean region, we compared this study with other studies performed in Mediterranean or Arab populations. In the study of Loudianos et al. (1999) on Mediterranean patients

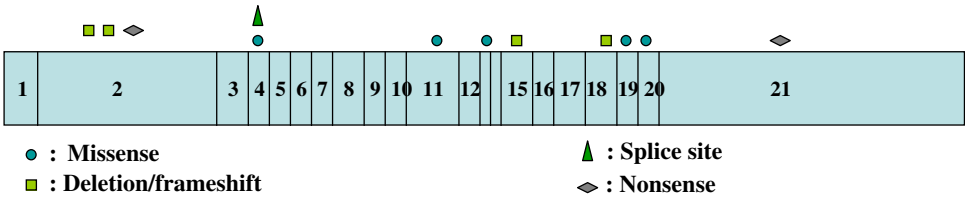

Fig. 1 Distribution of new mutations on the *ATP7B* gene

with WD, ten out of 19 of the novel mutations found were localized on exons 14, 16, and 19, which belong to the group of exons previously found to be the site of many frequent, as well as rare, WD-causing mutations. The genetic analysis of 56 Saudi patients with WD revealed that 50% of them had mutations in three exons (8, 19, and 21) of the *ATP7B* gene. Mutations in exon 19 and 21 were unique for Saudi patients (Takeshita et al. 2002). A novel deletion mutation, c.4193delC, in exon 21 of the *ATP7B* gene mutation, appears to be unique to Saudi patients and is found frequently in this ethnic group (Al Jumah et al. 2004; Majumdar et al. 2000, 2003). This is in contrast to our study, in which the novel mutations detected were randomly distributed all over the *ATP7B* gene, including exons (2, 4, 5, 7, 11, 13, 15, 17–21; Fig. 1).

Again, the majority of mutations detected in this study were found in the homozygous state (42 patients from 26 independent families), whereas in the study of Loudianos et al. (1999), most mutations detected were in the compound heterozygous state, and in only four cases, homozygosity was present. This may be explained by the high percentage of consanguinity in our study (75%) compared with consanguinity in the Egyptian population (32–35%).

Although homozygous mutations were found in 42 patients from 26 independent families, six of those patients from six different families were the result of nonconsanguineous mating. Also heterozygous mutations were found in six patients, two were the result of consanguineous mating. This unexpected finding, together with the finding of one patient homozygous for both p.N1270S and p.T1434M mutations, necessitates a larger-scale population study for the carrier frequency and the origins of these mutations in different families in this community.

Conclusion

The mutational spectrum of *ATP7B* among Egyptian children presenting with WD is very heterogenous, which mandates a larger-scale population screening to identify the carrier rate in this community. Frameshift and nonsense mutations may result in earlier presentation of the disease at childhood. Despite mutation heterogeneity in Egyptian patients, genotype–phenotype correlation analysis seems to be promising in this population, as many patients carry homozygous mutations.

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