

# ***Compound heterozygote mutations in ATP7B gene in an Iranian family with Wilson disease***

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## **Abstract:**

Wilson disease (WD) is an autosomal recessive disorder of copper transport and is characterized by excessive accumulation of cellular copper in liver and other tissues because of impaired biliary copper excretion and disturbed incorporation of copper into Ceruloplasmin. Hepatic failure and neuronal degeneration are the major symptoms of WD. Mutations in the *ATP7B* gene are the major cause of WD.

In this study we have screened one pedigree with several suspected members by amplifying the coding regions including exon-intron boundaries with PCR and sequencing. The c.1924G>C and c.3809A>G mutations were identified in affected members as compound heterozygote state. These mutations segregated with the disease in the family and they were absent in a cohort of 100 Iranian ethnicity matched healthy controls. Additionally no homozygote state has been reported for these two variants in the public databases. In-silico predicting tools consider these two variants as damaging. So this study introduces the combination of c.1924G>C and c.3809A>G variants as the cause for WD.

## INTRODUCTION

Wilson disease is a copper metabolism disorder causing injuries in several tissues like eye cornea, brain and Liver [1] which was first introduced by a British neurologist S. A. K. Wilson as progressive lenticular degeneration [2].

The lenticular degeneration is a condition in eye cornea which now will be referred as Kayser-Fleischer ring [3] and this special trait is hallmark of Wilson's disease (WD). The disease incidence is 1 in 35,000-100,000 in live births [4]. WD has autosomal recessive (AR) mode of inheritance and occurs due to the deficiency in one of the ATPase  $\text{Cu}^{2+}$  cellular pumps called ATP7B which is coded by *ATP7B* gene. This gene is located on chromosome 13q14.3 with 78826 bp length and consists of 21 exons [5].

*ATP7B* gene is coding ATP7B protein which is an acronym for; ATPase activity, 7 distinct domain and B class for second P-type ATPase copper binding pump.

This molecule is interacting with several other different molecules such as ATOX1, ATP7A, CTR1 and DMT1 depending on the cell type [3].

WD is a disease with extensive clinical heterogeneity and largely non-specific symptoms.

The age of onset varies from age 8 to 50. WD patients usually are diagnosed with some forms of hepatic dysfunction in their early teens result in decreased biliary excretion and elevated excretion of urinary copper [6]. Small proportions of them (approximately 20%) develop bone and joint disorders. The patients can be divided to three major groups based on their disease outcomes: those displaying hepatic symptoms, those displaying neurological symptoms and finally those displaying both hepatic and neurological symptoms [7]. Also in patient with mainly neurologic outcomes Serum Pentraxin3 is elevated [8]. The last stage of the disease involves accumulation of copper in organs such as brain, kidney and cornea [9].

Therapeutic activities against this disease mainly consist of using chelating agents like Penicillamine and Trientine to reduce the amounts of accumulated copper in the body [10].

More than 400 [11] different mutations consist of deletions, insertions, duplications and substitutions which can cause missense, nonsense, frame-shift, splice site mutation and *Alu* Exonization in *ATP7B* gene transcription have been reported which they led to relatively different clinical features [12] [3][13][14].

The rate of these mutations varies in different subpopulations [15]. The majority of WD mutations (~ 60%) are missense type [16] and most commonest point mutation in WD is His1069Glu [6], which is presented in about 38% of all the WD patients genome from North American, Russian and Swedish samples [17].

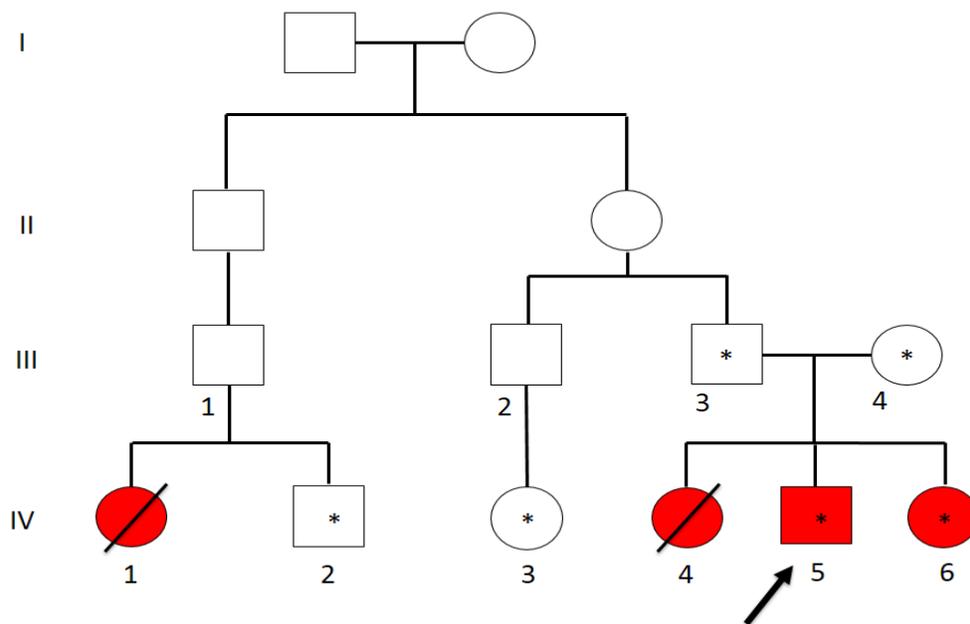
## Material and Methods

### Case presentation

The pedigree studied here was consisting of 4 affected members with similar clinical features out of them only two were alive at the time of this report (Figure 1).

All participants, or their legal guardian, provided written and informed consent. The institutional review boards of Tarbiat Modares and Mazandaran University reviewed the project. The affected individuals underwent examination at the Medical Genetics Department, DeNA laboratory, Tehran.

Patients showed psychiatric and neurological symptoms in various severities. These symptoms included ataxia, tremor, dystonia, dysarthria in speech and weak mental state. Seruloplasmin range was elevated in patients and Kayser-Fleischer ring also were present in all of them.



**Figure 1:** Pedigree of an Iranian family with Wilson Disease. Red boxes denote affected individuals. Boxes with (\*) indicates individuals that were analyzed by Sanger sequencing.

### Genetic analysis

DNA was extracted from peripheral blood of the patients (IV:5 and IV:6), their parents (III:3 and III:4) and two healthy members (IV:2 and IV:3), using standard protocols.

Table 1 depicts the sequence of primers used for amplifying the whole coding region and exon-intron boundaries of the *ATP7B*. Primers were designed using Primer 3 software (version 0.4.0). Primers specificity was checked by in-silico-PCR and blat tools of UCSC genome browser and lack of SNPs in the genomic region corresponding to the 3' ends of the primers was checked by looking through dbSNP database.

PCR analysis was carried out in a total volume of 25 µl containing 0.5 µl of each forward and reverse primers (10 Pmol), 10 µl of PCR Master mix MgCl<sub>2</sub> 1.5 mM and 1 µl DNA (about 100 ng). The reaction was adjusted to the total volume of 25 µl by ddH<sub>2</sub>O.

The PCR was performed using an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 58 for 30 s, and elongation at 72 °C for 30 s. PCR products were examined by 1 % agarose gel electrophoresis for the presence and sizes of amplicons.

Consequently, DNA sequencing of the PCR products was performed on 3130 ABI capillary electrophoresis using forward and reverse primers for each amplicons. Sequencing chromatograms were analyzed by using CodonCode aligner software.

The allele frequencies of the identified variants in *ATP7B* gene were determined by using the following public databases: dbSNP Common 144 (NCBI), 1K Genome project phase 3 ([www.1000genomes.org](http://www.1000genomes.org)), Exome Aggregation Consortium version 0.3 (ExAC) and the Iranian Genome Project (<https://irangenes.com/data-2/>).

In silico prediction of the pathogenicity of the identified variants were performed using the following software: SIFT (<http://sift.icvi.org/>), PolyPhen (<http://genetics.bwh.harvard.edu/pph2/>), Mutation Taster (<http://www.mutationtaster.org/>) and PROVEAN human genome variants ([http://provean.icvi.org/genome\\_submit\\_2.php](http://provean.icvi.org/genome_submit_2.php)). Finally conservation of the region harboring the mutations was surveyed by comparing these regions of the genome in the Human, Dog, Rhesus, Mouse, Elephant, Chicken and some other vertebrates in UCSC and ConSurf databases[20].

<b>Table1:</b> Primer sequences used for PCR amplification of all exons and exon-intron boundaries of <i>ATP7B</i> gene			
Exon 1	F5'-CCGGTCCCAATGAAGG-3' R5'-TTTTCTCCACGCCAAG-3'	Exon 11	F5'-GATGGCTGTTCATGTTCC-3' R5'-CTGATTTCCAGAACTCTTAC-3'
First part of Exon 2	F5'-CAGAGAAGCTGGGATGTTGTAG-3' R5'-GATAAAGGTCTCTTGGGTTAGTG-3'	Exon 12	F5'-GTAATTGCGGGTCTATAAATG-3' R5'-TAATAGAACTGCAGAAGGAGAG-3'
Second part of exon 2	F5'-TTTGAAGCTGCCATCAAGAG-3' R5'-GACACAAAGAGAAAAGGAGACAAG-3'	Exon 13	F5'-CCTCTGACTCTGCTGTTTC-3' R5'-TTGGTCAAGTTACCTAATCTCCTC-3'
Exon 3	F5'-GACAATGAACCCTACCAAG-3' R5'-ACTGAGAAGTCTATCCAAAAAGG-3'	Exon 14	F5'-ATCTGTATTGTGGTCAGTGAGTTG-3' R5'-TAGGAGAGAAGGACATGGTGAG-3'

Exon 4	F5'-GGGAAGATGTGTTTCTTTGTTTC-3' R5'-CACCGTCTTTAATTCTGTGTTTC-3'	Exon 15	F5'-GCTTACAGTTTCTTCTCTCTC-3' R5'-AATTTAGACGCACCCAAGAAC-3'
Exon 5	F5'-CTGTTGCCATCTGCTTCAC-3' R5'-CTCATCTTTCTTTACCCATTAC-3'	Exon 16	F5'-CAAATACCTGAGTGCTTCTAATCC-3' R5'-GGAAGGCTTTTGTGTTCTTC-3'
Exon 6	F5'-TCTACTGAGGCACTTTTAGATTAC-3' R5'-CTGTTTCAGAGGGTTACATTAC-3'	Exon 17	F5'-TTCTGCAGGAAAAGACGAAG-3' R5'-ATCCAGCAAGGGAGAAAGAG-3'
Exon 7	F5'-GCAGGTCTAAACTGTGTCCTC-3' R5'-GGTGATCCAGTTGTTGCTTC-3'	Exon 18	F5'-ATGTGAAGCAGGAGAGTAGGG-3' R5'-AGCAAATCATTCTGATGGAGAG-3'
Exon 8	F5'-GACTGTGCACAAAGCTAGAGG-3' R5'-CTAAACATGGTGTTCCAGAGGAAG-3'	Exon 19	F5'-ACTGTGTGCTCCTCTCCATC-3' R5'-GTCAAAGAGCCATTCTTTCC-3'
Exon 9	F5'-CAGTGGGAAGACTGATGTTTG-3' R5'-GTTCTCTGTGAAGTTCCCTTG-3'	Exon 20	F5'-GAGCTCGCCCTGAAATG-3' R5'-TGTCCAGGTGAATGAATG-3'
Exon 10	F5'-CATTCTACCACAGAAGTTGCTTC-3' R5'-TTGACATCTGAGCCTCTTCC-3'	Exon 21	F5'-CTCAGATGCTGTTGCGTTTC-3' R5'-TCACAGCAGTCATCTAAATACTC-3'

In order to check the allele frequencies of c.1924G>C and c.3809A>G variants in 100 healthy ethnicity matched controls tetraplex ARMS-PCR were employed. The primers and size of expected bands in each reactions are depicted in table-2.

<b>Table2:</b> ARMS primer sequences used for checking c.1924G>C and c.3809A>G variants in healthy populations controls			
	Primer name	Sequence	Expected size of product fraction
c.1924G>C	EX6F_inn	CCCAACGCTCATCACTTAG	<b>173bp</b>
	EX6R_inn	GCTTTATTTCCATCTTGTGGAG	<b>237bp</b>
	EX6F_out	CTATTGGGTAAAGAAGTTGTAAGCAG	<b>369bp</b>
	EX6R_out	ATTACAAGGGTAAAGGCAGCTAAT	
c.3809A>G	EX18F_in	GTGGGGGATGGGGTCTG	<b>289bp</b>
	EX18R_in	CAAGGCCGGGGAGTCCT	<b>196bp</b>
	EX18Fout	GTAAGTTGAGGTTTCTGCTGCTAT	<b>451bp</b>
	EX18Rout	AGGTTATAAATCAGTGCCAGGAC	

## Results:

### Analysis of mutations

In sample IV5 who was the proband of this family sequencing of all exons and exon-intron boundaries of *ATP7B* gene revealed seven alterations which they are presented in Table-2. Among these variants, only the allele frequencies of c.1924G>C and c.3809A>G variants were less than 0.01 in 1K genome project and other public databases. The rest of variants were

common and had allele frequencies more than 0.01. For example there were no homozygote cases in ExAC database for these two variants and the heterozygote rate was also very low (0.0001491 and 8.281e-06 respectively). Therefore only these two variants were considered for further investigations.

**Table3:** list of all variants found in *ATP7B* gene by sequencing of all the exons and exon-intron boundaries in sample IV:5

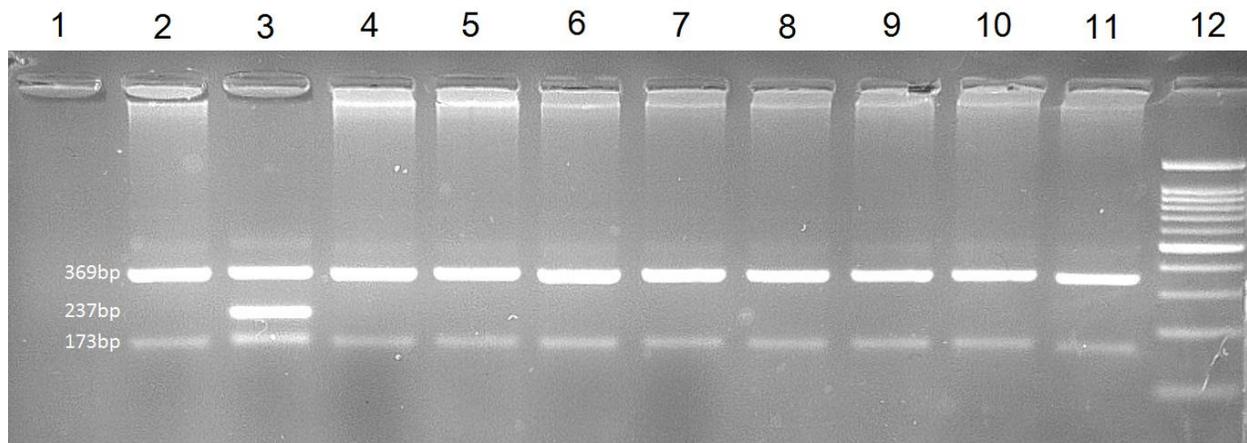
Nucleic Acid Alteration	Amino Acid Alteration	Location of Gene	Zygoty	Chr. location	rs ID	1000-genome freq
c.1924G>C	p.Asp642His	EX06	Het	chr13:52535995	rs72552285	0
c.3809A>G	p.Asn1270Ser	EX18	Het	chr13:52511706	rs121907990	0
c.3903+6C>T	-	IN18	Het	chr13:52511606	rs2282057	0.4753
c.3419T>C	p.Val1140Ala	EX16	Het	chr13:52515354	rs1801249	0.4652
c.3009G>A	p.Ala1003Ala	EX13	Het	chr13:52520471	rs1801247	0.0568
c.2855G>A	p.Arg952Lys	EX12	Het	chr13:52523808	rs732774	0.4725
c.2495A>G	p.Lys832Arg	EX10	Het	chr13:52524488	rs1061472	0.4753

The c.1924G>C variant is inside the exon 6 of *ATP7B* gene at the position of chr13:52,535,995 and is a missense point mutation (5'GAC->CAC3') which results in substituting Aspartic acid (D) codon to Histidine (H) aa at the residue of 642 in the translated protein sequence. This variant has been reported in dbSNP as rs2552285.

The c.3809A>G variant is affecting the exon 18 of *ATP7B* gene at the position of chr13:52,511,706 (5'AAT->AGT3') causing Asparagine (N) aa codon change to serine (S) at the residue of 1270 in protein level. This variant has been reported also in dbSNP as rs121907990

The segregation of these two variants in the family was checked by Sanger sequencing on all available samples (figure 2 and 3). Segregation study indicated that the c.3809A>G; p.Asn1270Ser and c.1924G>C; p.Asp642His mutations in *ATP7B* gene are co-segregating with the disease in this family. Affected members were compound heterozygote for these mutations and each of the parents carried only one of the defected alleles.

100 healthy controls were checked for both variants in exons 6 and 18 using ARMS method and acquired results showed they were negative.



**Figure 2:** Picture of 2% Agarose gel electrophoresis of ARMS test products for Exon 6 variant (c.1924G>C)

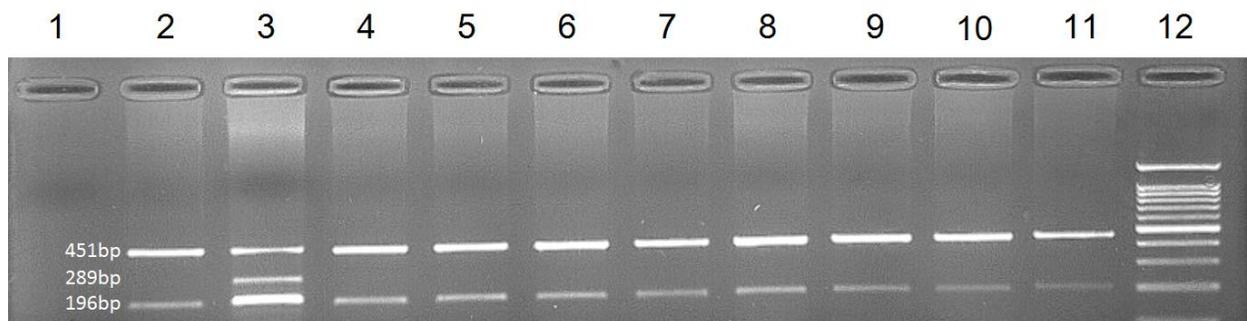
Well 1: Negative PCR test control (NTC)

Well 2: normal case homozygote for GG

Well 3: IV: 5 member in the family whom was heterozygote for c.1924G>C (GC)

Well 4-11: eight healthy members

Well 12: 100kb ladder



**Figure 2:** Picture of 2% Agarose gel electrophoresis of ARMS test products for Exon 18 variant (c.3809A>G)

Well 1: Negative PCR test control (NTC)

Well 2: normal case homozygote for AA

Well 3: IV: 5 member in the family whom was heterozygote for c.3809A>G (AG)

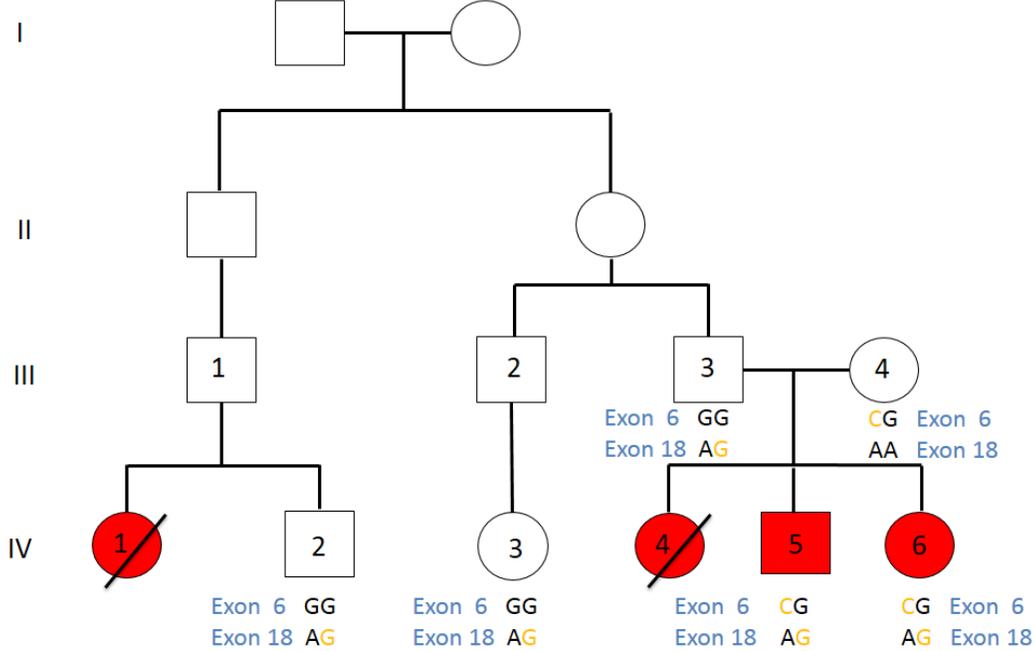
Well 4-11: eight healthy members

Well 12: 100kb ladder

In silico analysis of these two variants by Mutation Taster, SIFT, Polyphen2 and PROVEAN software predicted that these two variants are pathogenic and they have damaging effects on the protein function.

Additionally multiple alignment of the region of these two mutations in several species such as Human, Dog, Rhesus, Mouse, Elephant and Chicken in UCSC and ConSurf tool showed that they

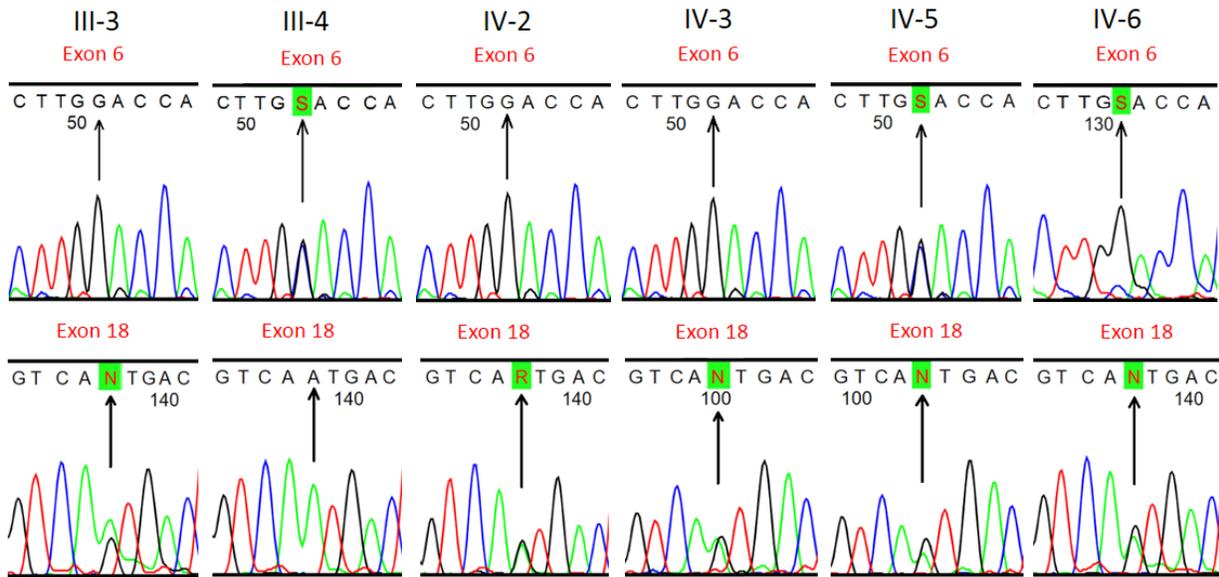
are highly conserved arguing that these residues play an important role in the function of this protein (figure 3).



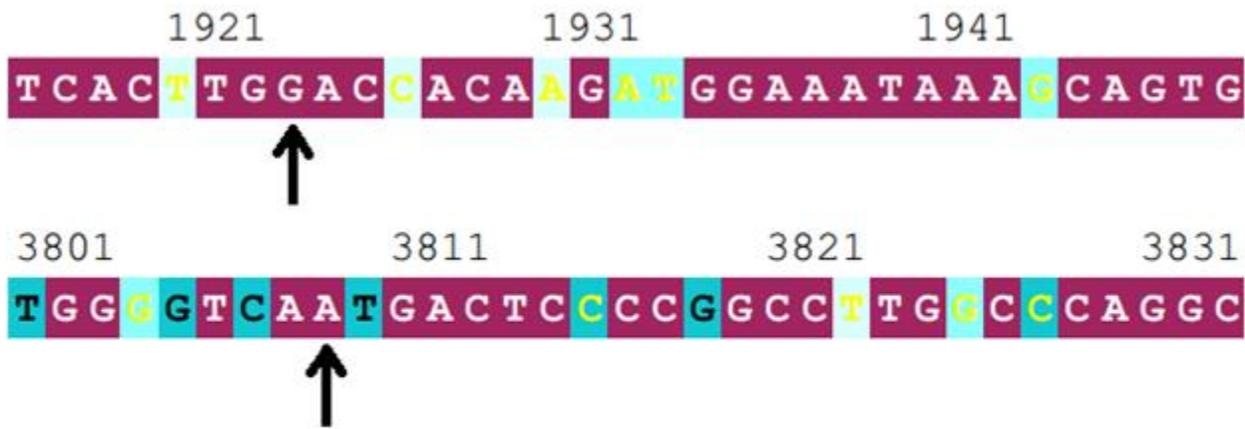
**Figure 4:** Genotypes of the c.3809A>G; and c.1924G>C mutations in *ATP7B* gene in studied healthy and affected individuals of the pedigree.

Assembly : GRCh37.p13

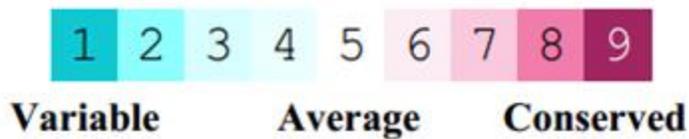
5' GGAGTCATTGACCCC 3' Positive Strand  
 chr13:52,511,698 Exon 18  
 3' CCTCAGTAACTGGGG 5' Negative Strand  
 Chr13:52,511,714.....chr13:52,535,986 Exon 6  
 5' CTTGTGGTCCAAGTG 3'  
 chr13:52,536,002 Exon 6  
 3' GAACACCAGGTTCAC 5'



**Figure 5:** Sanger sequencing traces showing the c.1924G>C; p.Asp642His mutation in exon 6 (upper row) and c.3809A>G; p.Asn1270Ser mutation in exon 18 of the ATP7B (lower row). The segregation of these two mutations as compound heterozygote has been confirmed in 6 available DNA samples (two affected and four unaffected individuals) from this family.



**The conservation scale:**



**Figure 6:** The conservation scores for the amino acids at the region of p.Asn1270Ser and p.Asp642His mutations in *ATP7B* gene calculated by ConSurf tool. ConSurf estimates the evolutionary conservation of amino acid residues in a peptide based on the phylogenetic relations between homologous sequences as well as amino acid’s structural and functional importance.

**Discussion:**

After analyzing all affected and healthy members available in this pedigree, compound heterozygote pattern of inheritance in this family was confirmed which is not considered to be the common phenomenon for WD mutations in Iranian population [21].

In this study we examined mutations in the coding regions of *ATP7B* gene in an Iranian family which result in identification of 2 mutations. A cohort of 100 ethnicity matched healthy controls turned out to be negative for these two mutations as well as the 1K genome and ExAC databases.

In-silico analysis of the two mentioned mutations with ConSurf software showed high conservation of these mutations residues among the vertebrate species.

The first mutation (p.Asp642His) found in this pedigree is located in the cytoplasmic region at 64 aa after the last copper binding domain. This mutation transforms a negatively charged residue (asp) to a positively charged one (His). Presumably this mutation by affecting the domain affinity to copper or the folding structure in the cytoplasmic region decreases the

stability of this domain and this can lead to abnormal localization of the protein within cytoplasm and impairment of protein function.

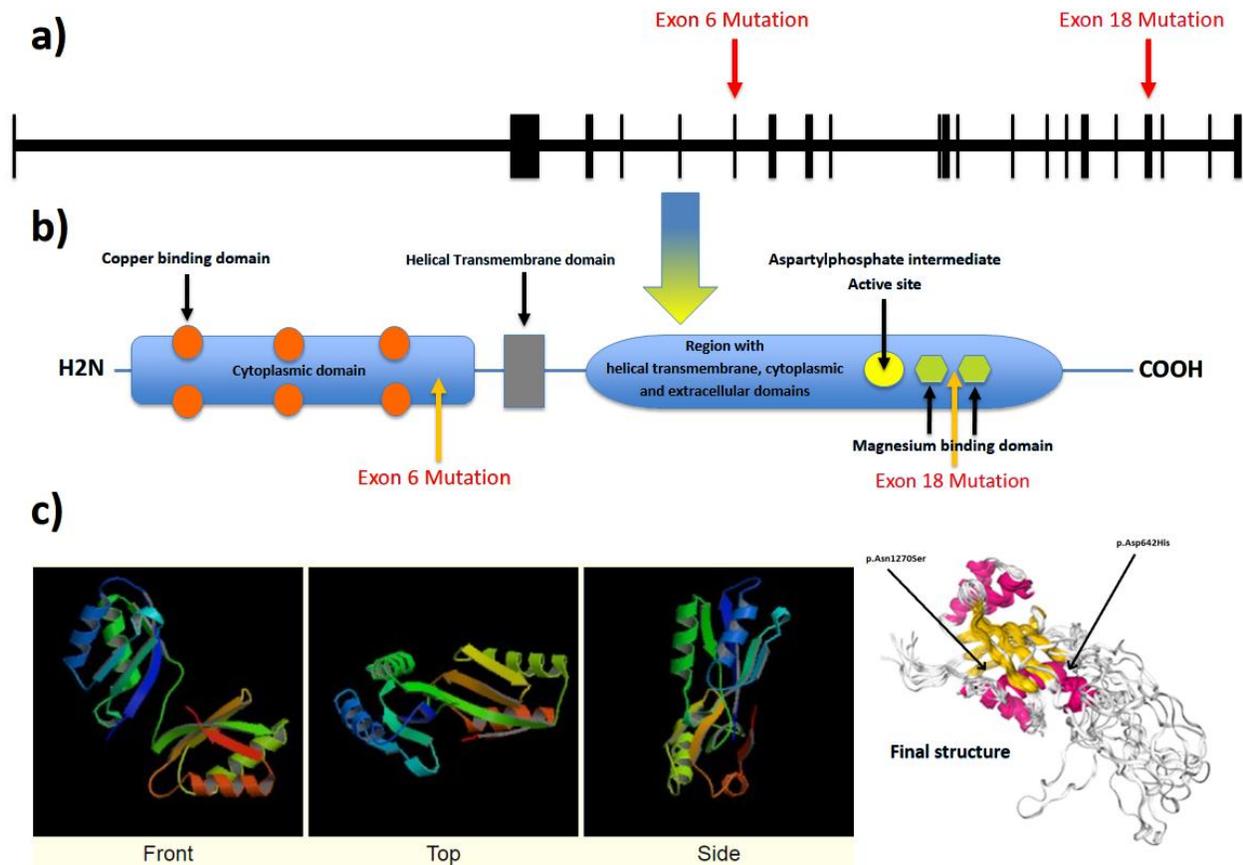
Consequently the second mutation (p.Asn1270Ser) also affects the cytoplasmic region of this protein at one aa before the second magnesium binding domain region of ATP7B protein (1271 residue), transforming a nonionic polar aa (Asn) to another nonionic polar one with the different side chain (Ser). Presumably this mutation affects on the magnesium binding affinity and domain structure of the protein.

WD mutation frequency and types are different among different populations. For example c.2333G>T in exon 8 is the commonest mutation in Korean population [22]. The c.3207C>A mutation (H1069Q) in exon 14 is the commonest mutation in Turkish and Iranian population which is also shown to be a common mutation in European population [6] [21]. The c.3207C>A has been shown to be associated with late onset neurological conditions in European countries whereas in the one cohort study in Iranian population has been observed in patients whom they all had hepatic symptoms and were 5-40 years old [21]. Exon 18 is reported as hotspot exon in Western countries while the hot spot exons reported in China were 8 and 12. In addition some population like Egyptian has shown very heterogeneous allele frequencies for WD mutations in their population respect to their ethnicities [23].

The attractive dimension in diseases like WD -that the defective gene in the disease encodes a protein with several distinct domains and any of them play a certain role in protein function and phenotype characteristic- is that: differences in the type of mutation and its place on protein structure, may result in different symptoms and clinical outcomes, due to the location of mutation in protein structure and severity of the mutation.

It has been reported that mutations in the transmembrane domain and ATP loop result in early onset of disease (>8 years), furthermore ATP loop mutations tend to cause hepatic symptoms with absence of neurological symptoms. Mutations in ATP hinge result in hepatic failure and transmembrane and copper binding mutations are associated with neurological manifestations. Finally frame shift mutations have been reported more in patients with early hepatic manifestation whereas splice site mutations have been reported in patients with neurological phenotypes [23].

It seems the missense mutations on cytoplasmic region result in hepatic impairment with late onset of neurological manifestations like what was experienced by affected members in the family studied here, but these observations need large scale analysis in bigger cohorts to confirm the genotype-phenotype correlation.



**Figure 7:** schematic presentation of ATP7B gene structure in DNA (a) and protein levels (b). Location of the c.3809A>G; p.Asn1270Ser and c.1924G>C; p.Asp642His mutations in ATP7B gene are depicted according to their relevant domains. (C). 3 D structure of ATP7B and the coordination of mutations are depicted.

In this study we showed that even in a population like Iran with high rate of inbreeding still it is possible to find recessive disorders like WD as a result of compound heterozygote mutations in a pedigree with no recent consanguinity. Therefore the heterozygote mutations should be also considered both in affected and carrier members of high risk families.

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