

## Original Article

# The Same Haplotype for Two Unrelated Wilson Disease Patients with New ATP7B Mutation

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## Abstract

**Background:** Wilson disease is a rare autosomal recessive disorder of copper metabolism caused by mutation in the *ATP7B* gene. The combination of markers (such as SNPs) on a single chromosome can be used to understand the structure of haplotype in the human genome, in which provide notable information on the origin of the mutation in human genetic disorders. The purpose of this study was to determine a haplotype analysis of two unrelated Wilson disease patients with the same missense mutation, c.2335T>G (g.58164 T>G) in exon 8.

**Methods:** DNA was prepared from two patients with the c.2335T>G mutation, their first-degree relatives, and 50 selected homozygous individuals from consanguineous marriage for eight SNPs around this particular *ATP7B* mutation. PCR was performed for SNPs of exons 4 (g.47964 C>T), 5 (g.51482G>A), 6 (g.54622A>G), 7 (g.56255G>A), 9 (g.59042G>T), 11 (g.66363G>A), 13 (g.70004 G>C), and 14 (g.72244 A>G), which are located in upstream and downstream of this mutation. Then, restriction fragment length polymorphism (RFLP) for these eight SNPs was designed and performed using eight different restriction enzymes.

**Results:** Eight different haplotypes were found in the present study and the patients with the same missense mutation had the same haplotype. The most prevalent haplotype in 100 normal studied *ATP7B* alleles was the same as reference haplotype (C G A G T G G G A) for *ATP7B* gene (NG\_008806.1).

**Conclusion:** As these two geographically separated families with the same mutation had the same haplotype, we concluded that this mutation possibly had the same origin in this population.

**Keywords:** ATP7B, c.2335T>G, haplotype, Wilson disease

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## Introduction

Wilson disease (WD) (OMIM 277900) is a rare autosomal recessive disorder of copper metabolism caused by different mutations in *ATP7B* gene.<sup>1,2</sup> This gene codes a copper transporting p-type ATPase that is essential for biliary copper excretion. So far, more than 500 mutations and several single nucleotide polymorphisms (SNPs) have been reported in the *ATP7B* gene (Wilson disease database mentioned at the university of Alberta).<sup>3-7</sup> The combination of markers (such as SNPs) on a single chromosome can be used to understand the structure of haplotype in the human genome, in which provide notable information on the origin of the mutation in human genetic disorders. In addition, SNPs markers have been used in linkage analysis, recombination, and disease associations.<sup>8-12</sup>

One of the most common approaches implemented for DNA typing is restriction fragment length polymorphism (RFLP) analysis. Using restriction endonucleases, polymorphic DNA digests and separates by gel electrophoresis, which can give different banding patterns from different individuals. This pattern is heritable and

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results from the presence or absence of the enzyme restriction site at the certain loci.

Several SNPs located at *ATP7B* gene have been reported as common SNPs in different populations. Several studies in Iranian population have shown that rare disorder such as Wilson disease was due to the high rate of consanguinity.<sup>13,14</sup> Haplotype analysis of the mutations will provide necessary information for convenient screening of carrier individuals in a particular population. In our previous study, we identified a novel mutation in two unrelated WD patients. In order to determine whether these two WD patients with the same mutation, c.2335T>G (g.58164 T>G),<sup>13</sup> have the same origin; this study aims to investigate a RFLP haplotype analysis of these patients and 50 unrelated individuals. The present study is the first investigation towards haplotype analysis of a particular mutation in *ATP7B* gene using RFLP analysis.

## Material and Methods

In the present study, eight single nucleotide polymorphisms (SNPs) located at exons 4 (g.47964 C>T, MAF: < 0.01), 5 (g.51482G>A, MAF: 0.01), 6 (g.54622A>G, MAF: < 0.01), 7 (g.56255G>A, MAF:NA), 9 (g.59042G>T, MAF: < 0.01), 11 (g.66363G>A, MAF: < 0.01), 13 (g.70004G>C, MAF: 0.10), and 14 (g.72244 A>G, MAF:<0.01) (Figure 1) were selected at upstream and downstream of the c.2335T>G (g.58164 T>G) missense mutation which is located in exon 8 of the *ATP7B* gene. This haplotype track was around 24kb in size. In addition, this investigation was performed for 50 selected homozygous individu-

als and first-degree relatives of the patients. In order to select a homozygous individual for these particular SNPs, we investigated the families with consanguineous marriage, who are homozygous for these SNPs. These SNPs were obtained from NCBI dbSNP and Wilson disease database at the University of Alberta.

All normal individuals and the patients gave informed consent before undergoing DNA test for haplotype analysis based on the requirements of the ethics committee in Shiraz University of Medical Sciences. Whole blood samples were collected in EDTA tube and stored at -20°C until use. Genomic DNA was extracted from the whole blood samples using a Cinnapure DNA kit (Cinnagen, Iran). Nine primer pairs were used to amplify these nine exons (Table 1). The PCR amplification was conducted using Eppendorf Master Cycler gradient (Germany) in a 50- $\mu$ l final reaction volume according to Cinnagen Taq polymerase protocol. The PCR product was then purified by ethanol precipitation followed by enzyme digestion according to the manufacturer's instructions (all information about RFLP haplotype are given in Table 1). The 15  $\mu$ l enzyme digestion reaction consisted of 10  $\mu$ l of the purified PCR product, 2  $\mu$ l diluted enzyme (Vivantis, Malaysia), 1.5  $\mu$ l buffer V (Vivantis), and 1.5 distilled water. The reaction was then visualized on 2% agarose gel containing ethidium bromide.

## Results

We have used single nucleotide polymorphism (SNP) genotyping around a novel mutation to investigate the possibility of the same origin for this mutation of *ATP7B* gene in two separated families.

In 100 chromosomes from the 50 unrelated homozygous individuals for the studied SNPs, eight different haplotypes were identified. RFLP haplotype analysis of the unrelated patients with c.2335 T>G missense mutation showed the same RFLP haplotype (CGAGGGGGA) (Table 2). In order to detect the presence of the mutation in carrier family members of the patients 1 and 2, NciI

restriction enzyme was used. Following enzyme digestion, 360bp, 271bp, and 89bp bands were separated from heterozygous carrier individuals. The 271bp and 89bp bands were also separated from affected homozygous individuals. A 360bp band was detected in wild type individuals following enzyme digestion.

The most prevalent haplotype (number 1 in Table 2) was same as reference haplotype (C G A G T G G G A) for *ATP7B* gene (NG\_008806.1). This common haplotype was identified in 58 chromosomes (Table 2).

Three different haplotypes were identified each in two chromosomes from three different individuals. These three haplotypes had the lowest frequency in normal population. Minor allele frequency (MAF) for each SNP in our population were as follow: g.47964 C>T (MAF: 0), g.51482G>A (MAF: 0.02), g.54622A>G (MAF: 0), g.56255G>A (MAF: 0.02), g.59042G>T (MAF: 0.04), g.66363G>A (MAF: 0), g.70004G>C (MAF: 0.06), and g.72244 A>G, (MAF: 0.02).

## Discussion

*ATP7B* gene c.2335T>G (p.W779Q) mutation was first reported by our group as a new mutation in two far separated families with Wilson disease in Southern Iranian population.<sup>13</sup>

Due to the presence of this novel mutation in the separated families, we sought to investigate whether c.2335T>G originated from a single mutational event. Therefore, we applied RFLP haplotype around this mutation to determine whether there were any segments shared between patients with c.2335T>G mutation that are not present in those without the mutation or controls. We reasoned that if c.2335T>G mutation had a single ancestral origin; patients would have the same chromosomal segments. In order to support our hypothesis, haplotype analysis was performed by the PCR-RFLP method using eight SNPs around this mutation.

The estimated prevalence of Wilson disease is the range of 1 in 30000 to 1 in 100000.<sup>2</sup> To date, more than 500 mutation in *ATP7B*

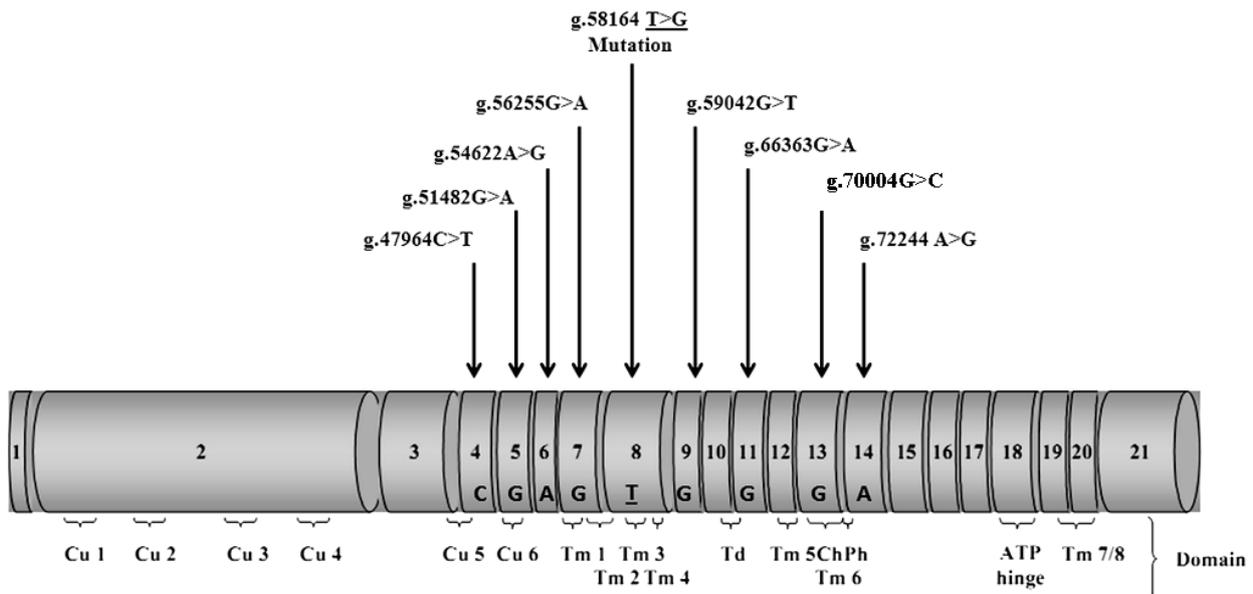
**Table 1.** PCR primers and restriction enzymes used in this study

Exon	Primer pair	Restriction enzyme	PCR product (bp)	RFLP (-)	RFLP (+)
4	F: TCG GTT ATA TTG ACT GTG R: TAA TCA CAA AGA TGG ATG	Taq I	314	150 + 164	314
5	F: ACT GGC TTT CAC AGG CTT R: TCT CAT TTT TCT TCA CTG ATT AT	SfaN I	298	128 + 170	298
6	F: TCC AAA GCT GAA AAG TGC R: GGT AGA GGA AGG GAC TTA GA	HhaI	220	220	92 + 128
7	F: TGT AAT CCA GGT GAC AAG CAG R: CAC AGC ATG GAA GGG AGA G	BAN II	276	141 + 135	276
8	F: GTG TCG CTC ATT GAA CTC TC R: TTC AGA GGA AGT GAG ATT TG	NciI	360	360	271 + 89
9	F: GGT TTG GAC AGG TCT GCT TTC R: CCT GGA ATA CTA ATC ATC TAC ACC	NdeI	390	390	209 + 181
11	F: GTG ACC GAA TGA GTG GC R: TTT CCC AGA ACT CTT CAC A	Hpa II	510	313 + 197	510
13	F: GAA ATG TCC TTA TGT GAT T R: AGT AAA CAG ATA CTA CTT TCA TC	Tth111 I	332	62 + 270	332
14	F: AGG TTG GGT GAA GTT CTG CC R: GGA CAT GGT GAG GAA TAA AAG AGC	Nco I	334	127 + 207	334

**Table 2.** Haplotype analysis results

Exons →	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9	Exon 11	Exon 13	Exon 14	Number of haplotype
Reference haplotype→	C	G	A	G	T	G	G	G	A	
Number of individuals with specific haplotype										
29	+	+	+	+	+	+	+	+	+	1
1	+	-	+	+	+	+	+	+	+	2
12	+	+	+	-	+	+	+	+	+	3
2	+	+	+	+	+	-	+	+	+	4
4	+	+	+	+	+	-	+	-	+	5
1	+	+	+	+	+	+	+	-	+	6
1	+	+	+	+	+	+	+	-	-	7
Patient 1	+	+	+	+	-	+	+	+	+	8
Patient 2	+	+	+	+	-	+	+	+	+	8
Father of patient 1	+	+	+	+	-/+	+	+	+	+	8
Mother of patient 1	+	+	+	+	-/+	+	+	+	+	8
Father of patient 2	+	+	+	+	-/+	+	+	+	+	8
Mother of patient 2	+	+	+	+	-/+	+	+	+	+	8

(-): mutated RFLP, (+): wild type RFLP



**Figure 1.** Exons and polymorphisms which were used in haplotype analysis. The reference haplotype was as follow: C G A G T G G A.

have been identified (Wilson disease database mentioned at the University of Alberta). While in the Iranian population prevalence of Wilson disease has not been reported, several mutations and SNPs have been identified in *ATP7B* gene.<sup>14</sup> Some genotype-phenotype correlation has been established for different mutations, such as hepatic manifestations with R778L mutation<sup>15,16</sup> and neurological presentations with H1069Q mutation.<sup>17</sup> Patients with the novel c.2335T>G mutation were characterized by neurological manifestation.<sup>13</sup>

Here, we performed haplotype analysis in 100 alleles from normal homozygous individuals in our population and also two patients with their parents.

RFLP haplotype analysis indicated a prevalent haplotype in our

population, which is the same segment as the patients' except the mutation. The results of the haplotype analysis on the 100 alleles suggest that this mutant allele has a single origin. Haplotyping of 50 normal unrelated homozygous individuals using a total of the eight SNPs at up and downstream of this mutation, revealed that 58 out of 100 alleles carried the common haplotype [g.47964 C, g.51482G, g.54622A, g.56255G, g.58164T, g.59042G, g.66363G, g.70004G, g.72244 A], and two patients with the same novel mutation had the same haplotype.

Using this data if we accept that these two separated families with the same mutation have the same origin, therefore it can be concluded that this mutation would be common in the south of Iran and should be considered in mutation analysis of *ATP7B* gene.

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**Conflicts of interest:** The authors report no conflicts of interest.

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