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ORIGINAL PAPER

Haplotype and mutation analysis in Greek patients with Wilson disease

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In this study, we report the results of haplotype and mutation analysis of the *ATP7B* gene in Wilson disease (WD) patients of Greek origin. We have analysed 25 WD families and two single patients and characterised 94% of the WD chromosomes investigated. We have found 12 different molecular defects (three frameshifts, two splice site, two nonsense, five missense mutations), four of which are novel. Five of the mutations are widely prevalent accounting for 74% of the WD chromosomes analysed. These results may enable preclinical diagnosis in the large majority of WD patients of Greek descent, thereby improving genetic counselling and disease management.

Keywords: Wilson disease; haplotype analysis; ATP7B mutations; compound heterozygote; Greek populations

Introduction

Wilson disease (WD) is an autosomal recessive disorder of copper transport, characterised by decreased biliary copper excretion and reduced copper incorporation into ceruloplasmin.[1] The worldwide incidence is in the order of 30 per million, with a gene frequency of 0.56% and a carrier frequency of 1 in 90.[2] The WD gene, which maps on chromosome 13q14.3, has been cloned and found to encode for a copper-transporting P-type ATPase (*ATP7B*)[3–5] with high homology to the Menkes disease gene product (*ATP7A*).[6–9] The characterisation of the WD gene exon-intron boundaries has recently made it possible to carry out mutation analysis in Wilson disease patients. Sixty-six disease causing mutations have to date been defined,[3,4,10–15] 33 of them in Mediterranean-origin populations.[11,12] In this paper, we present results on the delineation of the spectrum of mutations at the WD gene in patients of Greek origin.

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Subjects and Methods

This study includes 23 unrelated WD families, and two single patients originating from different regions of Greece (106 individuals). In the majority of cases, parents were available for haplotype analysis. All patients presented with hepatic manifestations at an age in the range 3–16 years.

Diagnosis of WD was based on low ceruloplasmin and copper serum levels, high urinary copper elimination, and high hepatic copper content.

DNA extraction and PCR were carried out by standard methods. Haplotype analysis was carried out using four microsatellite markers (D13S301, D13S296, D13S297, D13S298) flanking the WD locus.[16] Mutation detection was performed by SSCP analysis followed by direct sequencing of the shifted exons according to Figus *et al*[11] and Loudianos *et al*.[12] Each individual exon of the WD gene was amplified by using primers complementary to the DNA sequences flanking the exon–intron boundaries. Exon 2 was amplified in six overlapping fragments. DNA amplification was carried out in 50 μl total volume containing 50–100 ng of genomic DNA, 25 pmoles of each primer 1U Taq polymerase, 50 mM KCL, 10 mM Tris-HCI pH 8.3, 1.5 mM $MgcI_2$, and 0.01% gelatin. The conditions were denaturation at 94°C for 30 s, 20 s of annealing at 50–64°C and 1 min of extension at 72°C for 30 cycles with a 7 min at 72°C final extension. Primers, sequences and annealing temperatures are reported in Table 1. For SSCP analysis 2 μl of amplified product were mixed with 5 μl of a solution containing 95% formamide, 0.05% bromophenol blue, 0.05% xylen cyanol and 10 mM NAOH. The samples were denatured at 95°C, cooled on ice and then applied on an MDE TM (JD Baker Inc) gel containing 10% glycerol, 90 mM Tris Borate and 10 mM EDTA. Electrophoresis was performed with a sequencing apparatus at a constant power of 8 W for 16 hours. After electrophoresis, gels were silver stained.[11] Sequence analysis of the shifted exons was carried out on both DNA strands.[12]

Results

Haplotype analysis

The analysis of 44 WD chromosomes using four microsatellite markers (D13S301-D13S296-D13S297-D13S298) led to the definition of 15 different haplotypes at the WD locus (Table 2), which suggests allelic heterogeneity of WD in the Greek population. The WD chromosome haplotypes were very rare among normal chromosomes. Since microsatellite markers were informative in all families we have been able to predict the genotype at the WD locus by haplotype analysis in all the siblings of our patients.

Mutation Screening

By SSCP and direct sequencing analysis of the shifted exons, we identified 12 different mutations (Table 3), which account for 94% of the WD chromosomes analysed. The molecular defects defined include three frameshift, two splice site, two nonsense and five missense mutations. Four mutations are novel, while the remaining have already been described elsewhere. Five mutations are relatively frequent and account for 74% of WD chromosomes analysed; the remaining eight are rare since they have been found in only one or two chromosomes.

To assess whether the novel missense Ile1148Thr, Ter1466Arg and the 1707 + 3insT mutation might be polymorphisms, we tested a control population of Greek origin for their presence by SSCP or by dot-blot analysis. None of these mutations were detected among 100 normal chromosomes analysed.

In addition to these mutations, we also found 13 sequence polymorphisms (Table 4), two of which are new, while the remaining 11 have already been described in other Mediterranean populations.[11]

Haplotype Association of WD Mutations

Most of the mutations detected (7/12) were linked to a single haplotype; only two mutations were found on more than one haplotype (Table 3). In these cases it is very likely that the two haplotypes containing the same mutation have a common origin and derive by recombination or mutation of the CA repeat.

Genotype of the WD Locus

Of the mutations detected in our study, six were found in the compound heterozygous state with a known or unknown mutation, one in homozygosity, and five in homozygosity as well as compound heterozygosity.

Discussion

In this paper, we report the results of the molecular analysis of the *ATP7B* gene in 35 WD patients of Greek origin. We detected 15 different WD haplotypes, which suggests allelic heterogeneity of WD in Greek WD patients. Haplotypes 5-9-4-3, 7-5-7-3 and 9-6-6-6 are the most common since they have been detected in 8, 8 and 6 chromosomes respectively.

Mutation analysis led to the characterisation of 94% of the Wilson disease chromosomes analysed. Twelve different mutations (Table 3) were detected four of which are novel, while the remaining eight have already been described elsewhere.[4,10,11,14] The most common mutation is His1069Gln (26%), which is the most frequent worldwide, accounting for 30% of the WD chromosomes in eastern and northern European populations,[4,10,17] and for 17% in Italian (our unpublished data). This mutation was mostly found linked to the haplotype 5-9-4-3 as in other Mediterranean WD patients,[11] but in three cases it was detected linked to haplotypes 4-9-4-3, 5-5.5-4-3 and 7-9-4-3. This heterogeneity can be explained by assuming a recombination, or most likely a mutation of the CA repeat. The

Table 1 Primers, sequences, annealing temperatures

Exon	*Amplimers*	*Annealing temperature*
1	5′TTCCCGGACCCCTGTTTGCT 3′[a]	62°C
	5′AATCCTCCTGGTGGGAGTGAG 3′[a]	
2a	5′AGAAGCTGGGATGTTGTAGAAAATATTAGG 3′[a]	58°C
	5′GAAATCCTGTCCTCAATGGAC 3′	
2b	5′TGTGAAGTCCATTGAGGACAG 3′	58°C
	5′AGGCTGCCTTTCCTTCTGCA 3′	
2c	5′AGCATTGCAGAAGGAAAGGCAG 3′	64°C
	5′CAGGCTTAAGGGAGCCACTT 3′	
2d	5′GTGGCTCCCTTAAGCCTGGGAC 3′	62°C
	5′TGCCTCGATAGCCCTCTGCAGA 3′	
2e	5′TGCCCAAGTAAAGTATGACCCT 3′[b]	58°C
	5′TGCATGTGCCCTGGACCTG 3′	
2f	5′CGAGAGAACCAGGTCCAGGGC 3′	63°C
	5′CCTATACCACCATCCAGGAG 3′	
3	5′GCCCTGAAACCTCTTGTTCTG 3′[a]	58°C
	5′CTACTGATAAACACAGTTGCTGGG 3′[a]	
4	5′TGTGCAACCTAGAGGCCCTG 3′	58°C
	5′CCGTTACGCACCCACAGTA 3′	
5	5′CTGGACTGGCTTTCACAGGC 3′	55°C
	5′TTCCATGGGAAAAGTTGAAGAATT 3′[a]	
6	5′GAAAAGTGCTTTCTGCCAATGC 3′	58°C
	5′GCCCAGGTAGAGGAAGGGAC 3′	
7	5′TGTAATCCAGGTGACAAGCAG 3′	58°C
	5′CACAGCATGGAAGGGAGAG 3′[b]	
8	5′CTACTTGCTGGCAGCCTTCACTG 3′	62°C
	5′GGAGCAGCTCTTTTCTGAACCTG 5′	
9	5′TTTCGATAGCTCTCATTTCACA 3′[b]	55°C
	5′TGCCCACACTCACAAGGTC 3′[b]	
10	5′CAGCTGGCCTAGAACCTGAC 3′	60°C
	5′TATCCTCCTGAGGGAACATG 3′	
11	5′GGGCTGAGCAAGTGACAGTTG 3′	58°C
	5′TGTCTGATTTCCCAGAACTCT 3′	
12	5′TCATAGGTTGTAATTTCCCATG 3′	53°C
	5′CAGGATCAATGTCAGTAGATTAT 3′	
13	5′ATTGAACTCTCAACCTGCCTCT 3′	58°C
	5′TAAGGATGGGAAAAGCCGTGCT 3′	
14	5′AGTTCTGCCTCAGGAGTGTGAC 3′	58°C
	5′CAGCTACCAGAGAAGGACATGG 3′	
15	5′TTGGCTTACAGTTTCCTCTTCC 3′	58°C
	5′TACGTCCATCACGGTGCTGTGC 3′	
16	5′GTTCACAGTGAAATTGGACC 3′	50°C
	5′ACTGTATTTCTGAGAGACGG 3′	
17	5′AACATTGCAAGTGTGGTATC 3′	50°C
	5′CTTTGTCTCTAACTGCTATTAT 3′	
18	5′GTAACTTGAGGTTTCTGCTG 3′	53°C
	5′AGCAAATCATTCTGATGGAG 3′	
19	5′TGCAGCCAGGCTGTGGGTGCT 3′	60°C
	5′TGGGGCGCAGCTGGAGCAGAGT 3′	
20	5′GACCTAGGTGTGAGTGCGAGTT 3′[a]	58°C
	5′CAAGTTCCACTGTGCTAAGC 3′	
21	5′CTAGAATGGCTCAGATGCTGTTG 3′	61°C
	5′GCTTGTGGTGAGTGGAGGCA 3′	

[a]Reported in Petrukhin *et al*.[9]
[b]Reported in Thomas *et al*.[10]

Table 2 Haplotypes of WD and normal chromosomes

Haplotype, by marker locus				*No (%) of chromosomes*	
D13S301	D13S296	D13S297	D13S298	*WD (n=44)*	*Normal (n=35)*
3	11	6	3	1 (2.2)	
4	9	4	3	2 (4.4)	
5	5	7	7	2 (4.4)	1 (2.8)
5	5.5	4	3	1 (2.2)	
5	9	4	3	8 (18)	
5	10	4	3	2 (4.4)	3 (8.5)
5	11	4	3	5 (11.3)	1 (2.8)
6	5	7	–	1 (2.2)	
6	11	4	3	2 (4.4)	
7	5	7	3	8 (18)	
7	9	4	3	1 (2.2)	
7	10	4	–	1 (2.2)	2 (5.6)
8	4	7	7	3 (6.8)	1 (2.8)
9	6	6	6	6 (13.6)	
10	9	4	5	2 (4.4)	

Arg969Gln mutation that was found in nine of the WD chromosomes analysed, is the second most common (18%) and was linked to the haplotype 7-5-7-3, as described in WD patients of Italian and Turkish origin[11] (and our unpublished data). Two nonsense mutations, ie Gln289ter and Leu936ter, were both present in six

Table 3 Mutations detected in WD chromosomes

Mutations	*No. Ch.r*	*Exon*	*Domain*	*Haplotype*	*%*
Frameshift:					
845delT	2	2	Cu3	5 5 7 7	4
2299insG[a]	2	8	Tm4	ND[e]	4
2530delA[b]	2	10	Td	9 10 4 5	4
Splicing:					
1707+3insT	1	4	–	5 10 4 3	2
3904-2A-G[b]	1	19	–	5 11 4 3	2
Nonsense:					
Gln289ter[c]	6	2	Cu3	6 11 4 3 5 11 4 3	12
Leu936ter[a]	6	12	Tm5	9 6 6 6	12
Missense:					
Arg778Gly[b]	1	8	Tm4	3 11 6 3	2
Arg969Gln[a]	9	13	Tm6	7 5 7 3	18
His1069Gln[d]	13	14	SEHPL	4 9 4 3 5 5 5 4 3 5 9 4 3 7 9 4 3	26
Ile1148Thr	3	16	ATP loop	8 4 77	6
Ter1466Arg	1	21	–	5 10 4 3	2

[a]Mutations described by Thomas *et al*[10].
[b]Mutations described by Figus *et al*[11].
[c]Mutation described by Waldenstrom *et al*[14].
[d]Mutation described by Tanzi *et al*[4].
[e]Not detected.

Table 4 DNA polymorphisms in Wilson disease and normal chromosomes

Polymorphism	*Exon*	*Sequence*	*Domain*
–75 A–> C[a,b]			
–26insCGCCG[a,b]			
Ala407Ser[a,b]	2	GCT–> TCT	Cu 4
2451–25G–> A[a,b]	10		
Lys833Arg[a,b]	10	AAG–> AGG	Td
Arg953Lys[a,b]	12	AGA–> AAA	Tm 5
Thr992Thr[a,b]	13	ACG–> ACA	Ch/Tm 6
Ala1004Ala[a,b]	13	GCG–> GCA	Ch/Tm 6
Leu1016Leu[a,b]	13	CTG–> CTA	Ph
Ala1141Val[a,b]	16	GCC–> GTC	ATPloop
Ile1237Ile[c]	18	ATC–> ATT	ATPloop
Ala1296Ala[c]	18	GCC–> GCT	ATPloop
3906+6 T–> C[a]	18		ATPloop

[a]Described in Figus *et al* 1993[14].
[b]Found in both normal and in chromosomes with defined mutation.
[c]Found in chromosomes with defined mutation.

WD chromosomes (12%). Gln289ter has already been described in WD patients of Swedish descent.[14] In our cases this mutation was found linked to haplotypes 5-11-4-3 and 6-11-4-3. The Leu936ter mutation has been described in patients of Greek and Saudi Arabian origin[10] and is linked in our sample to haplotype 9-6-6-6. In addition four rare mutations, ie 2299insC, 2530 delA, Arg778GIy and 3904-2A- > G,[10,11] were detected in 2, 2, 1 and 1 chromosomes respectively.

Of the new mutations, the 845delT found in the homozygous state, creates a frameshift and a downstream stop codon at position 283, most likely resulting in the absence of a protein product or in a shortened functionless protein lacking several essential functional regions. The 1707 + 3insT mutation is an insertion in the donor splice site consensus sequence, and most probably alters the efficiency of normal splicing as occurs in other gene systems.[18] Two new missense mutations have also been detected. Ile1148Thr replaces a non-polar residue with a smaller neutral polar one which is conserved also in the human Menkes protein and in the *atp7a* and *atp7b* murine homolog proteins. This mutation occurs in the ATP loop, which forms a specific secondary structure, and is expected to impair the function of the WD protein by altering the secondary structure of the ATP loop. The Ter1466Arg substitution replaces the stop codon in the 3' region of the *ATP7B* with an arginine, thus most likely adding an aminoacidic residue in the 3' carboxyl terminus of the *ATP7B*, and causing the WD phenotype probably by affecting the stability of WD protein. To rule out the possibility that the new missense mutations and the 1707 + 3insT substitution might be simple polymorphisms, we tested

a control population of the same origin for their presence. None were found positive, indicating that these molecular lesions are probably disease-causing mutations.

Eight of the mutations found by us were also present in other Mediterranean populations.[11] Four, ie His1069Gln, Arg969Gln, 2530delA, and Arg778Gly, were linked to the same haplotype in all the populations tested,[11] (and our unpublished data) suggesting a common origin.

In addition to these mutations, we also found 13 sequence polymorphisms (Table 4), two of which are new, while the remaining eleven have already been described in other Mediterranean populations. These sequence changes were considered simple polymorphisms not affecting the function of the gene because they do not modify the aminoacid sequence of the protein product or are detected in normal chromosomes or in chromosomes with defined disease-causing mutation.

In this study, we have characterised the mutations in the large majority (94%) of the WD chromosomes in patients of Greek origin, and found that five mutations are widely prevalent, accounting for 74% of the chromosomes analysed. These results may enable preclinical diagnosis in the large majority of WD patients of Greek descent, thereby improving genetic counselling and disease management.

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