

Mutation analysis in patients of Mediterranean descent with Wilson disease: identification of 19 novel mutations

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Revised version received
18 May 1999
Accepted for publication
7 June 1999

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Abstract

In this study, we report further results of mutation analysis of the *ATP7B* gene in Wilson disease (WD) patients of Mediterranean origin. A total of 136 WD chromosomes, 73 of which were of Italian, 43 of Turkish, 18 of Sardinian, and two of Spanish origin, were analysed and the mutation characterised in 84.5% of them. We found 50 different mutations of which 19 are novel, including three nonsense, one frameshift, and 15 missense mutations. The mutations detected were rare and mostly found in the compound heterozygous state together with other mutations and only rarely in homozygosity. Most of these mutations lie in the transmembrane and ATP binding loop regions. These data expand our knowledge of both the structure-function relationships of the WD protein and the molecular pathology of WD, thus improving our capability of prevention and genetic counselling.

(J Med Genet 1999;36:833-836)

Keywords: Wilson disease; mutation; *ATP7B*; compound heterozygote

Wilson disease (WD) is an autosomal recessive disorder of copper transport, characterised by decreased biliary copper excretion and reduced copper incorporation into caeruloplasmin.¹ The world wide 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 a copper transporting P type ATPase (*ATP7B*)³⁻⁶ with a high homology to the Menkes disease gene product (*ATP7A*).⁷⁻¹¹ To date, more than a hundred disease causing mutations have been defined,^{3-4 12-23} most of them in populations of Mediterranean origin.^{14 15 20 22 23}

In this paper, we present further results of an ongoing project on the delineation of the spectrum of mutations in the WD gene in patients of Mediterranean origin.

Materials and methods

The study includes a new group of 66 unrelated WD patients and four parents of patients for whom a DNA sample was not available, a total of 136 WD chromosomes. Of these chromosomes, 73 were of Italian, 43 of

Turkish, 18 of Sardinian, and two of Spanish origin. The diagnosis of WD was based on low caeruloplasmin and copper serum levels, high urinary copper excretion, and high hepatic copper content.

DNA extraction and PCR were carried out by standard methods. Mutation detection was performed by single strand conformation polymorphism analysis of the amplified 21 exons and the known 5' UTR region of the *ATP7B* gene, followed by direct sequencing of the shifted exons as described in previous papers.^{22 23} Mutation analysis was carried out according to the following strategy. Firstly, we analysed exons 6, 8, 10, 12, 13, 14, 16, and 19 where most of the disease causing mutations lie in our sample.^{14 15 22 23} In those chromosomes in which the mutation was not detected or in those found to have a new missense mutation, we carried out mutation analysis in the remaining exons.

Results

DNA analysis of the WD gene using SSCP and direct sequencing of the shifted bands enabled us to characterise 84.5% of the WD chromosomes analysed and to identify 50 mutations of which 19 were novel. Of these mutations, three are nonsense, one is a frameshift, and 15 are missense (fig 1). The nonsense mutations Gln110ter, Tyr741ter, and Glu1142ter occur in exons 2, 8, and 16 respectively and are expected to result in absence of the protein product or the production of a shortened functionless protein. The frameshift mutation resulting from the insertion of a guanine at nucleotide position 267 creates a downstream stop codon at amino acid position 162, thus acting with the same mechanism as described for the nonsense mutations.

Of the missense mutations, four occurred in transmembrane regions. The Gly711Arg mutation, which substitutes a neutral polar for a basic residue, occurs in Tm2. The Ala1003Val substitution replaces a residue that is highly conserved in Ch/Tm6. Both mutations occurred in positions where another putative disease causing mutation had been reported.^{15 22 23}

The Leu1327Val substitution, which replaces a large hydrophobic with a smaller residue, resides in Tm7; Ser1363Phe, which substitutes a neutral polar for a hydrophobic residue containing an aromatic side group, occurs in Tm8. Both residues involved in Leu1327Val and Ser1363Phe are highly con-

Table 1 Novel mutations and polymorphisms detected in the Wilson disease gene

Mutation	Nucleotide	Chr	Exon	Domain	Origin	% †
*267insG /Ala874Val	CATGGAA	1	2	Cu1	Turkish	1
*Gln110ter Homozygous	CAA→TAA	2	2	Cu1	Turkish	2
*Gly711Arg/Arg1041Trp	GGG→AGG	1	8	Tm2	Italian	0.4
*Tyr741ter/His1069Gln	TAT→TAA	1	8	Tm3	Italian	0.4
*Ala1003Val/1707+3insT	GCG→GTG	1	13	Ch/Tm6	Turkish	1
*Arg1041Pro/Unknown	CGG→CCG	1	14	ATPloop	Italian	0.4
*Gly1061Glu/Pro840Leu Gly1061Glu/1782delT	GGG→GAG	2	14	ATPloop	Turkish	3
*Ala1063Val/1744delAT	GCG→GTG	1	14	ATPloop	Italian	0.4
*Glu1068Gly/His1069Gln	GAA→GGA	1	14	SEHPL	Italian	0.4
*Cys1104Phe Homozygous Cys1104Phe/Met1169Thr	TGC→TTC	3	15	ATPloop	Turkish	3
*Gln1142ter/Arg969Gln	CAG→TAG	1	16	ATPloop	Italian	
*Arg1151His/Gly1089Glu	CGT→CAT	1	16	ATPloop	Turkish	1
*Met1169Thr/Cys1104Phe	ATG→ACG	1	16	ATPloop	Turkish	1
*Glu1173Lys/2530delA Glu1173Lys/Gly710Ser	GAG→AGG	2	16	ATPloop	Italian	0.9
*Asp1222Val/Leu1327Val	GAC→GTC	2	17	ATPbinding	Turkish	2
*Val1262Phe Homozygous Val1262Phe/Arg969Gln	GTC→TTC	3	18	ATPloop	Italian	1.3
*Leu1327Val/Asp1222Val	CTA→GTA	1	19	Tm7	Turkish	1
*Ser1363Phe Homozygous	TCT→TTT	2	20	Tm8	Turkish	2
*Thr1434Met/Unknown	ACG→ATG	1	21	3'-COOH	Italian	0.4
<i>Polymorphisms</i>						
Arg1054Arg	AGG→CGG	1	14	ATPloop	Turkish	
His1207Arg	CAC→CGC	3	17	ATPloop	Turkish, Italian	
Asp1215Asp	GAC→GAT	1	17	ATPloop	Italian	
3699+27 T→C		1	17	ATPloop	Italian	
Val1297Ile	GTC→ATC	1	18	ATPloop	Turkish	
Ser1370Ser	TCC→TCT	1	20	Tm8	Turkish	
Lys1437Lys	AAG→AAA	1	21	3'-COOH	Italian	

*In the genotype we have reported the novel mutation on the left and the known mutation detected in the opposite chromosome on the right.

†The frequency of the new mutation in the specific population is reported (our unpublished data).

served and are localised in a protein region that is also highly conserved.²⁴ Previous studies have shown that the corresponding region in the non-heavy metal transporting P type ATPases contain a number of residues that are critical for cation binding and translocation.²⁵ These mutations would probably affect the ion transporting capabilities of the protein.

Eleven missense mutations occur in the ATP binding loop; four of them lie between the phosphorylation site and the SEHPL motif. Arg1041Pro replaces a basic residue with a hydrophobic residue having a completely different structure. Two mutations, namely the Gly1061Glu substitution that replaces a neutral polar with an acidic residue, and Ala1063Val, replacing a small with a larger hydrophobic residue, lie close to the SEHPL motif. Ala1063Val was previously considered to be a neutral polymorphism without pathological significance. However, it was the only nucleotide change found on the chromosomes of the family analysed in our study. Furthermore, the mutation replaces a residue conserved in Menkes disease as well as in murine homologues of WD and Menkes proteins. To the best of our knowledge, Glu1068Gly is the second mutation occurring in the SEHPL

motif after His1069Gln, and replaces an acidic with a neutral polar residue in a motif that is highly conserved in the heavy metal ATPases.

The remaining six of the new missense mutations occurred between the SEHPL motif and the next Tm7 protein region. The Cys1104Phe mutation, lying in exon 15, replaces a neutral polar with a hydrophobic residue. Three mutations, namely Arg1151His that replaces a large basic with a basic but structurally different residue because of an aromatic ring in the side chain, Met1169Thr replacing a hydrophobic with a neutral polar amino acid, and the Glu1173Lys substitution that changes an acidic to a basic residue reside in exon 16 and are expected to change the tertiary structure of the protein. Asp1222Val affects the TGDN site which is considered to be the ATP binding site. It is the second mutation affecting this residue after Asp1222Tyr which was previously described by others.¹⁹

A Val1262Phe substitution replaced a highly conserved residue close to the GDGVND-SPAL hinge region, the flexible loop connecting the ATP binding domain with the transmembrane portion of the molecule. Finally, Thr1434Met changed a neutral polar to a large

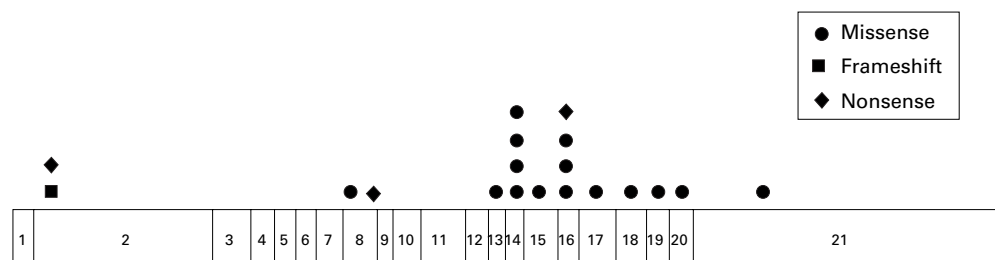


Figure 1 Schematic representation of the new mutations detected as distributed on the WD gene exons.

Table 2 Most frequent mutations in Mediterraneans*

Mutation	No of chromosomes	%
Continental Italians		
His1069Gln	39	17.5
2530delA	20	9
2299insC	13	6
Arg969Gln	10	4.5
Arg1319ter	9	4
Gly626Ala	7	3.1
Sardinians		
2461delC	13	8.5
Val1146Met	12	7.9
Turkish		
Thr1220Met	10	10

*Mutations detected in more than five chromosomes.

hydrophobic residue in the 3'-COOH region of the protein.

In order to exclude the possibility that missense mutations might be common polymorphisms, we tested a control population of the same origin as the patients carrying each specific mutation. None of these mutations was found among the 100 chromosomes analysed.

In addition to putative disease causing mutations, we identified seven novel sequence changes that we considered to be neutral polymorphisms not affecting the gene function. Three were found in normal chromosomes (Arg1054Arg, His1207Arg, Ser1370Ser), while the rest were detected in chromosomes with a defined Wilson disease causing mutation.

Discussion

In this paper, we report the molecular analysis of the WD gene in a further group of patients of Mediterranean origin affected with WD, including Italians, Sardinians, Turks, and Spaniards. Using SSCP analysis followed by direct sequencing, we characterised 84.5% of the WD chromosomes analysed and detected 50 mutations of which 19 are novel. Of the novel mutations, three were nonsense, one frameshift, and the rest were missense mutations. Evidence of the morbid effect of the missense mutations comes from their presence only on WD chromosomes, from failure to detect them in normal chromosomes of the same origin, and from their non-conservative nature or their occurrence in evolutionarily conserved regions of evident functional importance. Obviously only functional assays can clarify definitively the nature of the missense mutations.

The detected missense mutations mostly occur in DNA exons that encode for transmembrane regions as well as for the large ATP binding loop. Four of the novel missense mutations, Gly711Arg, Ala1003Val, Arg1041Pro, and Asp1222Val, involve residues affected by previously reported mutations.^{15 19 22} Furthermore, most of the mutations reported in this paper are located in regions where adjacent residues have been reported to be involved in WD patients.^{12 17 19 20} These data confirm the critical role of the transmembrane regions as well as the ATP binding loop in the protein structure, organisation, and function. Most of the detected mutations were found to be present in only one chromosome. This finding further suggests that WD in the Mediterranean

population results from a limited number of relatively frequent mutations and from a large number of rare mutations. It is interesting to note that 10 of the novel mutations are localised in 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. This finding confirms the validity of our approach in the mutation screening process.

The large majority of the mutations detected in this study were found in the compound heterozygous state together with another known or unknown defect and in only four cases were they in homozygosity (table 1). This makes it difficult to carry out a genotype-phenotype correlation analysis. Most patients presented with hepatic symptoms from 5 to 23 years of age and only four with neurological manifestations at 10-20 years. Of the homozygotes, those homozygous for Gln110ter and Ser1363Phe presented from 10 to 12 years with neurological symptoms, whereas homozygotes for Cys1104Phe and Val1262Phe presented at 9 to 10 years of age with hepatic symptoms.

By SSCP analysis we characterised 84.5% of the chromosomes analysed, again suggesting that SSCP is a simple, efficient method for mutation screening of a large number of samples in a large gene. Failure to detect the remaining mutations might be related to the limitation of SSCP analysis or to the presence of mutations outside exons and the flanking regions, that is, the promoter, introns, or other DNA control regions.

By adding the results of this study, to date we have been able to define the mutation in 82% of the WD chromosomes from continental Italians, 31% from Sardinians, and 82.6% from Turks. Considering arbitrarily as frequent mutations those occurring in more than five chromosomes, we found six more common mutations in continental Italians, two in Sardinians, and only one in Turks (table 2).

The data reported in this paper further expand our knowledge both on the structure-function relationships of the WD protein and on the molecular pathology of WD, thus improving our capability of prevention and genetic counselling.

We thank Associazione Baschiroto and Società Italiana di Gastroenterologia Pediatrica (SIGEP) for providing the WD families. This work was supported by grants from Assessorato Igiene e Sanità Regione Sardegna-LR No 11, 30.04.1990, Programma di Educazione Sanitaria DGR 1380/98 - 60% to A Cao.

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