Am. J. Hum. Genet. 57:1318–1324, 1995

Molecular Pathology and Haplotype Analysis of Wilson Disease in Mediterranean Populations

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Summary

We analyzed mutations and defined the chromosomal haplotype in 127 patients of Mediterranean descent who were affected by Wilson disease (WD), 39 Sardinians, 49 Italians, 33 Turks, and 6 Albanians. Haplotypes were derived by use of the microsatellite markers D13S301, D13S296, D13S297, and D13S298, which are linked to the WD locus. There were five common haplotypes in Sardinians, three in Italians, and two in Turks, which accounted for 85%, 32%, and 30% of the WD chromosomes, respectively. We identified 16 novel mutations: 8 frameshifts, 7 missense mutations, and 1 splicing defect. In addition, we detected the previously described mutations: 2302insC, 3404delC, Arg1320ter, Gly944-Ser, and His1070Gin. Of the new mutations detected, two, the 1515insT on haplotype I and 2464delC on haplotype XVI, accounted for 6% and 13%, respectively, of the mutations in WD chromosomes in the Sardinian population. Mutations H1070Q, 2302insC, and 2533delA represented 13%, 8%, and 8%, respectively, of the mutations in WD chromosomes in other Mediterranean populations. The remaining mutations were rare and limited to one or two patients from different populations. Thus, WD results from some frequent mutations and many rare defects.

Received April 10, 1995; accepted for publication September 13, 1995.

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0002-9297/95/5706-0008$02.00

Introduction

Wilson disease (WD) is an autosomal recessive disorder of copper transport that is characterized by a reduced incorporation of copper into ceruloplasmin and by decreased biliary excretion. This leads to copper accumulation in the liver and, consequently, to progressive liver damage. Subsequent overflow of copper determines its accumulation in other organs, mainly in the brain, kidneys, and cornea (Danks 1989). The worldwide prevalence of the disease is estimated in the order of 30 per 1 million, with a gene frequency of 0.56 % and a carrier frequency of 1 in 90 (Scheiberg and Sternlieb 1984). Higher prevalence of WD seems to exist in Sardinia, where ~10–12 new cases per year are identified. Treatment is based on the removal of copper excess by chelating agents such as penicillamine, trientine (Scheinberg et al. 1987), or tetrathiomolybdate (Walshe 1986; Brewer et al. 1994) or by blocking the intestinal copper absorption with zinc salts (Hoogenraad et al. 1983).

The WD locus was assigned to chromosome 13 by the tight linkage with red-blood-cell enzyme esterase D locus (Frydman et al. 1985). Subsequent linkage analysis detected the disease locus in a genomic region limited by DNA markers loci D13S31 and D13S59 (Bowcock et al. 1987; Stewart et al. 1993). Linkage disequilibrium mapping and haplotype analysis with microsatellite markers spanning the WD region refined the position of the disease locus (Bull and Cox 1993; Kooy et al. 1993; Petrukhin et al. 1993; Thomas et al. 1993, 1994; Bowcock et al. 1994). The WD gene has been subsequently cloned on the basis of homology with the Menkes disease gene by using YACs mapping on 13q14.3 (Bull et al. 1993) or from a brain cDNA library during a search for genes encoding metal binding proteins (Tanzi et al.

1993). The WD gene contains 22 exons spanning a DNA region of ~100 Kb (Petrukhin et al. 1994; Thomas et al. 1995).

The protein product belongs to the group of cation-transporting P-type ATPase (ATP7B) and shows a high degree of homology to the Menkes disease protein (ATP7A) (Chelly et al. 1993; Vulpe et al. 1993) and to bacterial heavy metal ion–transporting ATPase (Silver and Walderhaug 1992; Odermatt et al. 1993). More recently, the exon-intron boundaries of the WD gene have been characterized, and tissue-specific alternative splicing has been detected, (Petrukhin et al. 1994). Mutation screening in WD patients led so far to detect 25 specific disease-causing mutations, some of which appear to be population specific and some common to many populations (Tanzi et al. 1993; Bull et al. 1993; Thomas et al. 1995). This study reports the results of haplotype analysis and mutation screening carried out in patients of Mediterranean descent with WD.

Subjects, Material, and Methods

Subjects

This study includes 127 unrelated WD patients, of whom 39 were Sardinian, 49 continental Italian, 33 Turkish, and 6 Albanian. In all cases, the parents were available for linkage studies. Diagnosis of WD was based on low ceruloplasmin and copper serum level, high urinary copper elimination, and high hepatic copper content.

Haplotype Analysis

Haplotypes were derived by using four microsatellite markers (D13S301, D13S296, D13S297, and D13S298) flanking the WD locus. These markers have been found, in a previously published study (Petrukhin et al. 1993) and in this study, to be linked to the WD gene. The D13S301 marker is proximal and <40 Kb from the WD gene (Petrukhin et al. 1993; Thomas et al. 1995). The remaining markers are distal to the WD gene, being D13S296, the closest, and D13S298, the farthest, within a region of ~1 Mbp (Petrukhin et al. 1993). The amplification of these markers was carried out by using pairs of specific primers according to the method of Petrukhin et al. (1993). The analysis was performed by PCR in 25 μl total volume containing 25 ng of genomic DNA, 12 pmol of each primer, 1 pmol of one of the primers radioactively labeled by the activity of T4 Kinase with λ[32P]ATP, 0.5 U *Taq* polymerase, 50 mM KCL, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, and 0.01% gelatin. One-twentieth of the reaction was loaded on a sequencing gel (6% polyacrilamide, 8 M urea, 100 mM Tris Borate, and 10 mM EDTA). After electrophoresis at 70 W, the gel was dried on Whatman 3M paper and exposed to X-ray films (X-Omat; Kodak).

Detection of Mutation

Each individual exon was amplified by using primers complementary to the DNA sequences flanking the exon-intron boundaries, which were defined by the direct sequencing of overlapping P1 and cosmid clones containing the WD gene (data not shown). The "*Long* PCR" (Cheng et al. 1994) led us to determine the size of introns that resulted that were identical in both P1 clone and genomic DNA. Incidentally, the size of intron 2, according to our estimate, is much higher than previous estimate (4 vs. 0.9 Kb) (Petrukhin et al. 1994; Thomas et al. 1995). This was confirmed on 10 genomic DNA samples and both P1 and cosmid clones. "*Long* PCR" was carried out using a commercial kit (XL PCR; Perkin-Elmer) according to the manufacturer's protocol.

Mutation screening was performed on two-to-four WD chromosomes carrying each specific haplotype by using two different strategies. In Sardinian patients carrying the two most common haplotypes, the exons from 1 to 21 and their flanking regions were sequenced. In the remaining Sardinian patients and in all the other Mediterranean patients, we have carried out SSCP analysis of amplified exons from 1 to 21 of the coding region, followed by direct sequencing of shifted exons.

After mutation identification, all DNA samples with the same haplotype were tested for the specific mutation by either Dot Blot analysis or SSCP. The PCR conditions are as described for the analysis of the microsatellite markers.

For SSCP analysis, 2 μl of the PCR product was mixed with 8 μl of a solution containing 95% formamide, 20 mM EDTA, 0.05% xilene cyanol, 1 N NaOH. The samples were denatured at 95°C, cooled on ice, and then applied on a 6% polyacrylamide gel containing 10% glycerol, 90 mM Tris Borate, and 10 mM EDTA. Electrophoresis was performed with a sequencing apparatus (model S2; Gibco-BRL, Life Technologies) at a constant temperature of 20°C and at constant power of 50 W for 7 h. After electrophoresis, the gels were either silver-stained (Silver Stain Plus; Bio-Rad) (Berry and Samuel 1982; Goldman and Merril 1982) or dried and exposed to X-ray film in case ^{32}P-labeled PCR product had been used. In most of the cases, heterozygosity for a mutation/polymorphism, suggested by the presence of abnormal single-stranded conformers, was confirmed by the detection of heteroduplex in the lower part of the gel. Sequence analysis of each shifted exon was performed by the dideoxy chain-termination method of Sanger by using the Sequenase version 2.0 polymerase (USB).

Statistical Analysis

In all populations examined, the differences in the overall distribution of the alleles among normal and WD chromosomes were evaluated by a χ^2 test with a $2 \times K$ contingency table, where K is the marker allele number

Table 1

Haplotypes of Wilson Disease Chromosomes

	Loci D13S				Population[a]						
					S		C		T		A
Haplotype	301	296	297	298	WD (78)	N (53)	WD (98)	N (76)	WD (66)	N (43)	WD (12)
I	2	5	4	3	5	...	...	...	...	...	...
II	3	6	6	5	...	...	2	...	1	...	1
III	3	10	6	3	...	...	...	...	3	1	1
IV	4	10	4	3	...	...	3	2	1	...	...
V	5	5	3	3	4	1	...	1	...	...	...
VI	5	5	4	3	...	...	3	2	...	...	...
VII	5	5	6	3	...	...	4	5	...	...	...
VIII	5	9	4	3	...	...	12	2	11	...	...
IX	5	10	3	3	43	2	7	1	1	4	1
X	5	10	4	3	5	2	10	7	9	...	...
XI	6	10	3	3	...	...	1	3	1	...	...
XII	6	10	4	3	...	...	2	4	2	3	...
XIII	7	10	4	3	...	...	3	5	...	...	...
XIV	8	6	6	3	...	...	...	...	4	...	...
XV	9	10	4	5	...	...	10	1	3	1	1
XVI	10	5	7	7	10	1	...	...	...	...	...
Chromosome[b] (%)					85	12	58	43	54	21	33

NOTE.—We characterized the haplotypes in all the WD and normal chromosomes investigated, the number of which is reported in parentheses. In the table are reported only those haplotypes found in more than one individual within the same or different populations.

[a] Populations investigated are: S, Sardinian, C, continental Italian; T, Turkish, and A, Albanian. Part of the results of the Sardinian population were described elsewhere (Petrukhin et al. 1993). Alleles of each locus are numbered according to Petrukhin et al. (1993).

[b] Chromosome = number of haplotype-associated chromosomes showed in the table as percentage of the total number of chromosomes studied.

present in the sample. Significant association of the allele with the largest positive deviation between the observed and the expected frequency under no association hypothesis was tested with a 2×2 table by a one-side χ^2 test, corrected for multiple testing by using Buonferroni's correction (by multiplying the P-value by the number of alleles observed in that marker). For each population studied, the normal chromosomes, used to compare the marker allele frequency with that of the WD chromosomes, were derived by segregation analysis of the WD families with the four microsatellites.

Results

Linkage Disequilibrium and Haplotype Analysis

Linkage disequilibrium was detected at D13S301 ($P \geq .01$), D13S296 ($P \geq .0001$), D13S297 ($P \geq .01$), and D13S298 ($P \geq .001$) loci in the Sardinian population and only at the D13S298 locus in the Turkish one. No recombination between these markers and the WD locus was observed. Haplotypes, derived from the study of these four microsatellites, were characterized in all the normal and WD chromosomes (table 1). In the Sardinian population, we detected 16 different haplotypes associated with WD chromosomes. Of these, a single haplotype, IX, is very common, being observed in 55% of the WD chromosomes. In this population other relatively frequent haplotypes were XVI, X, I, and V, which were detected in 13%, 6%, 6%, and 5% of the chromosomes, respectively. On the whole, these five haplotypes account for 85% of the WD chromosomes. In WD patients of continental Italian and Turkish origin, we found 46 and 41 different haplotypes, respectively. In both these populations, two haplotypes, VIII and X, were common and accounted for 22% and 30% of the WD chromosomes, respectively. In Italians, we found also a third common haplotype, haplotype XV, which was observed in 10% of the chromosomes.

Mutation Screening

WD mutations.—We have identified 21 mutations, of which 16 are novel and 5 have been described elsewhere (Tanzi et al. 1993; Thomas et al. 1995) (table 2). The mutations already reported are 3043delC, 2302insC, Arg1320ter, His1070Gln, and Gly944Ser. Of the novel mutations, eight are frameshifts, one is a splice-site alteration, and seven are missense mutations. An example of mutation analysis is shown in figure 1.

Table 2

Mutations Detected in Wilson Disease Chromosomes

Mutations	N[a]	Exon	Domain	Haplotype	Groups
Frameshift:					
214delAT	1	2	Cu 1	...	Sardinian
1515insT	5	3	Cu 5	I	Sardinian
1785delT	1	5	Cu 6	...	Continental Italian
1886delAT	1	6	Tm 1	VI	Turkish
2302insC[b]	14	8	Tm 4	X, II, various	Sardinian, continental Italian, Turkish, Albanian
2464delC	10	10	Td	XVI	Sardinian
2533delA	14	10	Td	XV	Continental Italian, Turkish, Albanian
3403delC[b]	1	15	ATP loop	...	Continental Italian
3085delAGA→G	2	14	Ph	Rare	Continental Italian
3855del24	1	18	ATP	...	Sardinian
Splicing:					
3907-2A→G	1	19	...	...	Continental Italian
Nonsense:					
Arg1320ter[b]	2	19	ATP Tm 7	Various	Continental Italian
Missense:					
Gly627Ala	6	6	Tm 1	Various	Sardinian, continental Italian, Turkish
Asp766Asn	3	8	Tm 4	Various	Continental Italian
Arg779Gly	4	8	Tm 4	X, III	Turkish
Ileu858Thr	2	10	Td	X	Continental Italian, Turkish
Gly944Ser[b]	1	12	Tm 5	...	Continental Italian
Arg970Gln	4	13	Tm 6	Rare	Continental Italian, Turkish
Glu1065Lys	2	14	Tm 7	Rare	Turkish
His1070Gln[b]	23	14	SEHPL	VIII	Continental Italian, Turkish, Albanian
Asn1271Ser	3	18	ATP	Various	Continental Italian, Turkish

[a] Number of chromosomes in which the specific mutation has been detected.
[b] Mutations described elsewhere; see text.

The frameshift mutation alters the reading frame and most likely disrupts the function of the WD gene resulting either in the absence of the protein product or in the production of a shortened functionless protein. The splicing mutation 3907-2A→G lies within the consensus sequences of a splice-donor site. According to the other gene system, this mutation may lead to exon skipping or to the use of alternative splice sites that result in premature termination of translation. This leads to the production of a shortened protein product lacking the terminal end, which most likely modifies the protein conformation or adversely affects its stability.

Of the novel missense mutations, five reside in the transmembrane regions 1, 4, 6, and 7. All these mutations are nonconservative and changing an acidic with a neutral polar residue (Asp766Asn), a basic with a neutral polar residue (Arg779Gly, Arg 970Gln), an acidic with a basic residue (Glu1065Lys), or a neutral polar with a neutral hydrophobic residue (Gly627Ala). These mutations most likely disrupt the structure of a transmembrane domain. The Ileu858Thr mutation resides in a phosphatase domain. The mutation is not conservative and changes a neutral and hydrophobic amino acid with a polar residue. Furthermore, the isoleucine at position 858 is a part of the ITGEA sequence that is highly conserved in both Menkes disease and WD proteins.

To assess the possibility that missense substitutions might be polymorphisms, we systematically tested for their presence in a control population of the same origin. None of these mutations was seen among 100 normal chromosomes analyzed.

DNA polymorphisms.—We have identified 13 sequence changes in the WD gene that do not modify the amino acid sequence of the protein product or result in nonconservative changes in nonessential residues of the protein or are detected in normal chromosomes of the same population or in chromosomes with defined disease-causing mutation (table 3). These variants, of which four have been described elsewhere (Tanzi et al. 1993; Thomas et al. 1995), have been considered "polymorphisms."

Haplotype association and population distribution of WD mutations.—The large majority of the patients investigated are compound heterozygotes for the mutations detected in this study and an unknown mutation. Homozygotes were detected only for the following mutations: 1515insT, 2464delC, Arg970Gln, Glu1065Lys, 3085delAGA→G.

Mutations were usually linked to a single specific haplotype. However, several mutations, including 2302insC, Arg1320ter, Gly627Ala, Asp766Asn, Arg779Gly, and Asn1271Ser, were found in different haplotypes.

A few mutations are common, being detected in more than five chromosomes. The common mutations are 1515insT and 2464delC in Sardinians, in which they account for 6% and 13% of the WD chromosomes, and 2302insC, 2533delA, and His1070Gln in the other Mediterranean populations, in which their frequency is 8%, 8%, and 13%, respectively, and the Gly627Ala, which has been detected in Sardinians, Turkish, and continental Italian populations.

Discussion

In this study, we have characterized the mutations and defined the respective haplotype association in a large number of patients of Mediterranean descent affected by WD. Haplotype analysis showed a relative homogeneity among the Sardinian population compared to the continental Italian and Turkish ones. In the Sardinian population, we have indeed detected 16 haplotypes, of which 1 is very common (IX) and 4 are relatively frequent (XVI, I, X, and V), accounting for 55%, 13%, 6%, 6%, and 5% of the WD chromosomes, respectively. By contrast, in Turkish and continental Italian WD patients, we characterized 46 and 41 haplotypes, respectively. Among them, we detected two and three common haplotypes, respectively, representing 30% and 32% of the WD chromosomes in these two populations. In spite of the large haplotype heterogeneity in the Turkish population, the number of WD patients homozygous for different haplotypes was much higher than expected (28% vs. 1%; $P < .05$). This may reflect the high consanguinity in the Turkish population. When one assumes the existence at the WD locus of an association between haplotypes and specific mutations, as detected in other genes, these data suggest the presence, in Sardinians, of five common mutations, of which one is largely prevalent, and a limited number of rare mutations. In continental Italian and Turkish patients, WD seems to be more heterogeneous at the molecular level. Most likely, in these populations the disorder results from two or three relatively common mutations and a large number of more rare mutants.

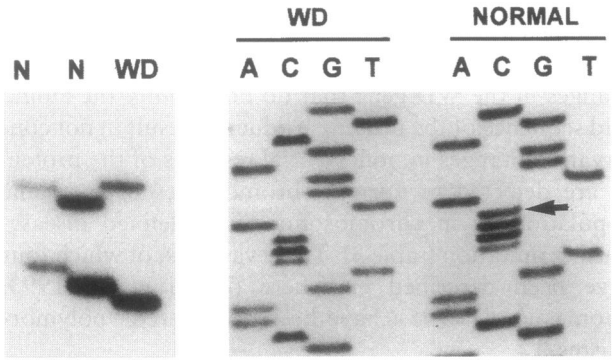

Figure 1 Detection of the WD mutation 2464delC. *Left,* SSCP analysis of exon 10. From left to right, normal subject (N) homozygous for lysine at position 833; normal subject (N) homozygous for arginine at position 833 (Lys833Arg is a common polymorphism); WD patient (WD) homozygous for 2464delC. Two SSCP conformers can be detected in our experimental conditions. *Right,* Sequence of exon 10 from the WD patient and a normal control. Arrow in the normal sequence indicates cytosine, which is deleted in the WD sample.

Table 3

DNA Polymorphisms Detected in Wilson Disease Chromosomes

Polymorphisms	Exon	Domain
−74A→C	1	...
−26insCGCCG	1	...
Ala407Ser[a]	2	Cu 4
Leu457Val	3	Cu 4/Cu 5
2447-25G→A	9	...
Lys833Arg	10	Td
Arg953Lys[a]	12	Tm 5
Thr992Thr	13	Ch/Tm 6
Ala1004Ala	13	Ch/Tm 6
Leu1016Leu	13	Ph
Val1098Val	15	Tm 7
Ala1141Val[a]	16	ATP loop
3906+6T→C[a]	19	...

[a] Polymorphisms described elsewhere; see text.

The relative homogeneity of WD in Sardinians is most likely related to the relative isolation of this population. In Sardinians, a similar homogeneity has been detected in other genetic disorders; for instance in the β-globin gene defects, in which a single mutation (nonsense at codon 39) accounts for >95% of the β-thalassemia chromosomes (Rosatelli et al. 1992).

Linkage disequilibrium among the microsatellites studied and the WD locus was evident for all four microsatellites in the Sardinian population and only for D13S298 in the Turkish population. These data indicate again the relative homogeneity of the Sardinian sample.

The mutation analysis detected 21 different mutations, of which 16 are novel and 5 have been described elsewhere in WD patients mostly from northern or eastern European populations (Tanzi et al. 1993; Thomas et al. 1995). The novel mutations include eight frameshift, one splice alteration, and seven missense mutations. The frameshift and splicing mutations most likely led to the production of a functionless protein. Evidence for the morbidity of the missense mutations came from several sources: their relative high incidence among WD patients, the failure to observe these muta-

tions in control chromosomes, the nonconservative nature of the substitution, and the occurrence of mutations at evolutionary conserved residues in regions of documented functional importance.

For the B779G mutation, the conclusion in favor of a disease-causing defect is supported by the recent description of another amino acid substitution, B779L, at the same position in a patient of Chinese descent (Thomas et al. 1995). These findings indicate the critical role of Arginine at position 779 for the function of Tm 4.

In the Sardinian population we have so far detected two common mutations 1515insT linked to haplotype I and 2464delC associated with haplotype XVI, which represent 6% and 13%, respectively, of the mutations in WD chromosomes. Direct sequencing of the coding sequence and restriction-enzyme analysis of the "*Long* PCR" products of the WD gene, however, failed to detect the mutation in those WD chromosomes associated to the most common haplotype IX. This mutation, which most likely derives from a single event occurring in haplotype IX, may reside either in the promoter region or within introns or in a remote DNA region controlling the function of the WD gene. Studies are in progress to define this common mutation. In Sardinia, we have so far defined the 24% of the mutations of the WD chromosomes screened.

In the other Mediterranean populations, including continental Italians, Turks, and Albanians, we have identified three common mutations, His1070Gln, 2302insC, and 2533delA. The H1070Q mutation has been previously detected in populations of eastern and northern European ancestry, where it accounts for ~30% of the WD chromosomes (Petrukhin et al. 1993). In all these populations, as well in the Mediterranean one, the H1070Q mutation is associated with an identical haplotype (VIII). These findings indicate that the H1070Q mutation is most likely the most common molecular defect of the WD gene and suggest that it arose as a single and very ancient mutational event. The 2302insC, which was found in all the Mediterranean populations investigated in this study, has been elsewhere described in two patients, one of Italian and one of British origin (Thomas et al. 1995). The 2302insC resides in several haplotypes, including X, II, and other rare ones, indicating most likely a multiple origin of the mutation. The cytosine insertion occurred in a stretch of six cytosine, which could be a hotspot for mutation. The third common mutation detected in this study in Mediterranean population is the 2533delA mutation, which is present in all cases on haplotype XV, suggesting it originated as a single mutational event. The 2533delA mutation is most likely a Mediterranean-specific mutation. The other mutations detected in this study from data so far available appear to be less common and are most likely limited to a single or a few populations.

From the data collected so far, WD seems to result from a limited number of frequent mutations, both common and population specific, and from a large number of rare mutations. The characterization of the mutations in 101 (40%) of 254 chromosomes may lead in combination with linkage analysis to improve our capability of genetic counseling in WD families of Mediterranean origin.

Acknowledgments

We are indebted to Y. W. Kan for the continuous advice and generous support. We thank Colin Colins for the P1 clones, Associazione Baschirotto and Società Italiana di Gastroenterologia Pediatrica (SIGEP) for providing WD families, and Francesca Fodde for technical support in writing the manuscript. This research was supported by grants from Telethon Italy No. E 129 and Assesorato Igiene e Sanità, Regione Sardegna, L.R. No. 11, 30.04.1990.

References

Berry MJ, Samuel CE (1982) Detection of subnanogram amounts of RNA in polyacrylamide gels in presence and absence of protein by staining with silver. Anal Biochem 124:180–184

Bowcock AM, Farrer LA, Cavalli-Sforza LL, Hebert JM, Kidd KK, Frydman M, Bonne-Tamir B (1987) Mapping the Wilson disease locus to a cluster of linked polymorphic markers on chromosome 13. Am J Hum Genet 41:27–35

Bowcock AM, Thomforde J, Weissenbach J, Bonne-Tamir B, St George-Hyslop P, Giagheddu M, Cavalli-Sforza LL, et al (1994) Refining the position of Wilson disease by linkage disequilibrium with polymorphic microsatellites. Am J Hum Genet 54:79–87

Brewer GJ, Dick DR, Johnson V, Wang Y, Yuzbasiyan-Gurkan V, Klutn K, Fink JK, et al (1994) Treatment of Wilson's disease with ammonium tetrathiomolybdate. Arch Neurol 51:545–554

Bull PC, Cox DW (1993) Long range restriction mapping of 13q14.3 focused on the Wilson disease region. Genomics 16:593–598

Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DW (1993) The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. Nat Genet 5:327–337

Chelly J, Tumer Z, Tonnesen T, Petterson A, Ishikawa-Brush Y, Tommerup N, Horn N, et al (1993) Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. Nat Genet 3:14–19

Cheng S, Fockler C, Barnes WM, Higuchi R (1994) Effective amplification of long targets from cloned inserts and human genomic DNA. Proc Natl Acad Sci USA 91:5695–5699

Danks DM (1989) Disorders of copper transport. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, 6th ed, vol 1, McGraw-Hill, New York, pp 1416–1422

Frydman M, Bonne-Tamir B, Farrer LA, Conneally PM, Ma-

gazanik A, Ashbel S, Goldwitch Z (1985) Assignment of the gene for Wilson disease to chromosome 13: linkage to esterase D locus. Proc Natl Acad Sci USA 82:1819–1821

Goldman D, Merril CR (1982) Silver staining of DNA in polyacrylamide gels: linearity and effect of fragment size. Electrophoresis 3:24–26

Hoogenraad TU, Van Den Hammer CJA (1983) Three years of continuous oral zinc therapy in 4 patients with Wilson's disease. Acta Neurol Scand 67:356

Kooy RF, Van der Veen AY, Verlind E, Houwen RH, Scheffer H, Buys CH (1993) Physical localisation of the chromosomal marker D13S31 places the Wilson's disease at the junction of bands q14.3 and q21.1 of chromosome 13. Hum Genet 91:504–506

Odermatt A, Suter H, Krapf R, Solioz M (1993) Primary structure of two P-Type ATPase involved in copper homeostasis in Enterococcus hirae. J Biol Chem 268:12775–12779

Petrukhin K, Fischer SG, Pirastu M, Tanzi RE, Chernov I, Devoto M, Brzustowicz LM, et al (1993) Mapping, cloning and genetic characterization of the region containing the Wilson disease gene. Nat Genet 5:338–343

Petrukhin K, Lutsenko S, Chernov I, Ross MB, Kaplan JH, Gilliam TC (1994) Characterization of the Wilson disease gene encoding a P-type copper transporting ATPase: genomic organization, alternative splicing, and structure/function predictions. Hum Mol Genet 3:1647–1656

Rosatelli MC, Dozy A, Faà V, Meloni A, Sardu R, Saba L, Kan YW, et al (1992) Molecular characterization of β-thalassemia in the Sardinian population. Am J Hum Genet 50:422–426

Sanger F, Micklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA. 74:5463–5467

Scheinberg I, Jaffe M, Sternlieb I (1987) The use of trientine in preventing the effects of interrupting penicillamine therapy in Wilson's disease. N Engl J Med 317:209–213

Scheinberg I, Sternlieb I (1984) Wilson's disease. Vol 23 in: Lloyd H, Smith J (eds) Major problems in internal medicine. WB Saunders, Philadelphia

Silver S, Walderhaug M (1992) Gene regulation of plasmid and chromosome determined inorganic ion transport in bacteria. Microbiol Rev 56:195–228

Stewart EA, White A, Tomfohrde J, Osborne-Lawrence S, Prestridge L, Bonne-Tamir B, Scheinberg IH, et al (1993) Polymorphic microsatellites and Wilson disease. Am J Hum Genet 53:864–873

Tanzi RE, Petrukhin K, Chernov I, Pellequer JL, Wasco W, Ross B, Romano DM, et al (1993) The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nat Genet 5:344–350

Thomas GR, Bull PC, Roberts EA, Walshe JM, Cox DW (1994) Haplotype studies in Wilson disease. Am J Hum Genet 54:71–78

Thomas GR, Forbes JR, Roberts EA, Walshe JM, Cox DW (1995) The Wilson disease gene: spectrum of mutations and their consequences. Nat Genet 9:210–217

Thomas GR, Roberts EA, Rosales TO, Moroz SP, Lambert MA, Wong LT, Cox DW (1993) Allelic association and linkage studies in Wilson disease. Hum Mol Genet 2:1401–1405

Vulpe C, Levinson B, Whitney S, Packman S, Gitschier J (1993) Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nat Genet 3:7–13

Walshe JM (1986) Tetrathiomolybdate as an anticopper agent in man. In: Sheinberg IH, Walshe JM (eds) Orphan disease and orphan drugs. Manchester University Press, Manchester, pp 76–85