Am. J. Hum. Genet. 61:317–328, 1997

Identification and Analysis of Mutations in the Wilson Disease Gene (*ATP7B*): Population Frequencies, Genotype-Phenotype Correlation, and Functional Analyses

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Summary

Wilson disease (WD) is an autosomal recessive disorder characterized by toxic accumulation of copper in the liver and subsequently in the brain and other organs. On the basis of sequence homology to known genes, the WD gene (*ATP7B*) appears to be a copper-transporting P-type ATPase. A search for *ATP7B* mutations in WD patients from five population samples, including 109 North American patients, revealed 27 distinct mutations, 18 of which are novel. A composite of published findings shows missense mutations in all exons—except in exons 1–5, which encode the six copper-binding motifs, and in exon 21, which spans the carboxy-terminus and the poly(A) tail. Over one-half of all WD mutations occur only rarely in any population sample. A splice-site mutation in exon 12 accounts for 3% of the WD mutations in our sample and produces an in-frame, 39-bp insertion in mRNA of patients homozygous, but not heterozygous, for the mutation. The most common WD mutation (His1069Glu) was represented in ~38% of all the WD chromosomes from the North American, Russian, and Swedish samples. In several population cohorts, this mutation deviated from Hardy-Weinberg equilibrium, with an overrepresentation of homozygotes. We did not find a significant correlation between His1069Glu homozygosity and several clinical indices, including age of onset, clinical manifestation, ceruloplasmin activity, hepatic copper levels, and the presence of Kayser-Fleischer rings. Finally, lymphoblast cell lines from individuals homozygous for His1069Glu and 4 other mutations all demonstrated significantly decreased copper-stimulated ATPase activity.

Received April 8, 1997; accepted for publication June 2, 1997.

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0002-9297/97/6102-0010$02.00

Introduction

Copper is an essential trace element, acting as a cofactor for a number of proteins, including cytochrome *c* oxidase, dopamine β hydroxylase, superoxide dismutase, peptide α amidating enzyme, lysyl oxidase, tyrosinase, and ceruloplasmin. Copper-dependent enzymes are required for diverse processes of oxidative metabolism, including respiration, free-radical detoxification, neurotransmitter synthesis, and maturation of connective tissue, and also for iron uptake (Yuan et al. 1995). The importance of copper homeostasis can be seen in the devastating effects of heritable human disorders that disrupt the normal processes of copper metabolism. In Menkes disease (MNK) and occipital horn syndrome (OHS), reduced copper levels in most tissues diminish the activity of numerous copper-containing proteins, producing symptoms such as neurological degeneration and connective-tissue defects. In contrast, Wilson disease (WD) and Indian childhood cirrhosis arise from the toxic accumulation of copper primarily in the liver. Copper-dependent enzyme activities remain basically unaffected, with the notable exception of ceruloplasmin, which is dramatically reduced in the majority of WD patients. Copper toxicity results in tissue and organ damage, particularly in the liver and brain (Petrukhin and Gilliam 1994), presumably owing to the generation of metal dependent oxyradicals and to metal ion antagonism (copper competing with other metal ions for the same biochemical sites) (Jungmann et al. 1993).

The recent molecular cloning of highly homologous genes, which when defective cause MNK (Chelley et al. 1993; Mercer et al. 1993; Vulpe et al. 1993) and WD (Bull et al. 1993; Petrukhin et al. 1993; Tanzi et al. 1993), has provided new insight into the mechanisms of copper transport in humans, as well as new molecular tools for continued investigation of normal and abnormal copper metabolism. Both genes encode a P-type (transducing) ATPase distinguished by six metal-binding

sites and by signature features (a unique transmembrane [Tm] pattern and two unique amino acid motifs, CPC and SEHPL) that constitute a new subgroup of heavy metal–binding ATPases. The MNK gene (*ATP7A*) is expressed in all tissues except the adult liver (Vulpe et al. 1993). Disruption of protein function apparently leads to trapping of copper in the intestinal mucosa, kidney, and connective tissues, accompanied by the failure to distribute copper to other tissues (Vulpe et al. 1993). The WD gene (*ATP7B*) is expressed most abundantly in the liver, where disruption of protein function reduces or prevents both the elimination of excess copper from the hepatocyte into bile and the loading of apoceruloplasmin with copper to form ceruloplasmin.

We report a preliminary characterization of the WD gene that includes the identification of 27 disease mutations, 18 of which have not been described elsewhere. Mutation frequencies were determined, by reverse dot-blot hybridization (RDBH) or by detailed haplotype analysis, in five geographically diverse clinical samples. Several mutations were analyzed in greater detail, including the most common WD mutation, His1069Glu (which disrupts the canonical SEHPL motif), a relatively rare splice-site mutation, and several missense mutations that lead to reduced copper-stimulated ATPase activity in lymphocytes of WD patients.

Subjects, Material, and Methods

Subjects

This study was approved by the appropriate institutional review board (IRB 2094), and appropriate informed consent was obtained from the human subjects. The Russian cohort, drawn from 18 unrelated families, consisted of 66 individuals and was comprised of 24 WD patients, 36 parents, and 6 unaffected siblings. The American cohort, drawn from 119 unrelated families, consisted of 317 individuals and was comprised of 128 WD patients, 138 parents, and 51 unaffected siblings. The families in the American sample were from 13 states in the U. S. or from Puerto Rico (7 families), Greece (1 family), or India (1 family). In 13 of the 128 families, both parents were Jewish. The Sicilian sample included 11 WD patients, 12 parents, two paternal grandparents, and 10 unaffected siblings, from a total of 9 unrelated families. The Swedish sample included 26 affected individuals from 22 unrelated families. These families recently have been described elsewhere (Waldenström et al. 1996). The Costa Rican cohort was comprised of 15 unrelated families, 4 of which included more than one affected sibling, for a total of 19 WD patients, 24 parents, 11 sibs, and 15 children. Diagnosis of WD in the American families was based on the criteria described by Scheinberg and Sternlieb (Scheinberg and Sternlieb 1984). Clinical indices such as ceruloplasmin activity and hepatic copper levels were determined by measurement of the rate of paraphenylenediamine oxidation in a Perkin-Elmer UV/VIS spectrophotometer, model 559A (Morell et al. 1968), and by polarized atomic absorption spectrophotometry using a Hitachi Z-8270 spectrophotometer with a graphite furnace, respectively.

Haplotype Analysis

Individuals with confirmed diagnosis of WD, together with the available parents and unaffected siblings, were genotyped with six microsatellite markers that surround and flank the WD locus. The oligonucleotide primers and PCR amplification conditions for markers *D13S295, D13S296, D13S297, D13S298, D13S301,* and *D13S133* have been described elsewhere (Petrukhin et al. 1993). Haplotypes were determined for affected individuals after the derivation of phase for the parental genotypes. All patient samples showing a unique homozygous haplotype were analyzed further by direct DNA sequencing.

SSCP Analysis

For a subset of compound heterozygotes, all 21 exons of the WD gene were PCR amplified and were subjected to mutation analysis by SSCP analysis (Orita et al. 1989). For the majority of exons, amplification was performed with the set of intronic primers described elsewhere (Petrukhin et al. 1994). In addition, several new intronic and exonic primer sets were designed, to improve exon amplification or the efficiency of mutation detection, and included the following: 5′-CGCAACTTTGAATCATCCGT-3′ and 5′-AACGCGGGGAGGAAAATCCT-3′, for exon 1; 5′-AGAAGCTGGGATGTTGTAGAAAATATTAGG-3′ and 5′-AATGGAGCTGACACAGGACTG-3′, 5′-ATGGGCTTCGAGGCCAGGAT-3′ and 5′-TGGTTAGCAGAAGATAAAGGTCTCTT-3′, 5′-CCCAAAGAGACCTTTATCTTCTGC-3′ and 5′-AATTCCCAGGTGGAAGTGCC-3′, and 5′-TGAAGGCATGATCTCCCAAC-3′ and 5′-CCTATACCACCATCCAGGAG-3′, for exon 2; 5′-AAATGTCCTTATGTGATTAGAGTTCTGG-3′ and 5′-GGCTTTTCTCTCAATGTGAAATAGTAAA-3′, for exon 13; and 5′-GAAATAACCACAGCCTCTTTTG-3′ and 5′-AAGGAAGGCAGAAGCAGAAG-3′, for exon 16. Individual PCR reactions contained 0.2–1.0 μg genomic DNA; 50 pmol each of forward primer and backward primer; 50 mM Tris (pH 8.3); 1.5 mM $MgCl_2$; 50 mM KCl; 1 mM each of dATP, dTTP, and dGTP; 12.5 μM dCTP; 2.5 μCi [α32P]-dCTP; and 0.75 units *Taq* polymerase (Perkin Elmer Cetus), for a total volume of 25 μl. Amplification was performed in an MJ Research PTC-100 Programmable Thermal Controller, by use of a modified protocol of touchdown PCR (Don et al. 1991), for which primer annealing temperatures were decreased stepwise from 68°C to 55°C. In order to enhance the detection of sequence alterations, amplified products exceeding a length of 240 bp were digested

with the restriction enzymes listed as follows: *Mae*II for exon 2; *Hpa*II for exons 3, 5, and 11; *Mbo*I for exon 4; *Fok*I for exons 8, 14, 15, 16, and 19; *Mse*I for exon 9; *Ava*II for exon 10; *Alu*I for exon 15; *Msc*I for exon 13; *Pst*I for exon 17; and *Sac*I for exon 18. The samples then were diluted with 4 vol of a solution of 10 mM EDTA and 0.1% SDS. Two-fifths of this dilution mixture then was diluted with 1 vol of loading buffer (99% formamide, 0.5 mM EDTA, and 0.1% each of xylene cyanol and bromophenol blue), was denatured at 95°C for 5–10 min, and was electrophoresed through 6% mutation-detection-enhancement gels (AT Biochem), at 5 W either at room temperature for 7–10 h or at 4°C for 7–15 h. The gels subsequently were dried and exposed to film, at room temperature for 1–5 d. When analyzed, patient samples exhibiting mobility shifts relative to normal samples were allocated for direct sequencing.

Exon Amplification and Sequencing

When haplotype analysis indicated that samples were homozygous for a WD mutation, exonic DNA was amplified and directly sequenced. Select heterozygous samples also were sequenced. PCR amplification was performed in reaction volumes of 200 μl, containing 1.5 μg genomic DNA, 40 pmol of the forward and backward primers flanking the exon of interest, 2.5 mM dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), and 10 units *Taq* polymerase (Perkin Elmer Cetus), according to the conditions described above. Products were purified by electrophoresis through a 2% low-melting-point agarose gel (Boehringer Mannheim). The amplification bands were excised and purified over Qiagen tip-20 columns (Qiagen) or by a phenol/chloroform extraction method (Maniatis et al. 1989). Sequence analysis of the polyadenylation site involved the use of primers 5′-GATGGTCAAAGTGTAAGGAGTTTTTT-3′ and 5′-GTGCCTCACAGAAGCCCTC-3′, in the amplification reactions described above. DNA sequencing was performed by use of the *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (ABI), in accordance with the supplier's instructions. The reactions were analyzed on an ABI model 373A automated sequencer. Nucleotide changes in heterozygous individuals were detected reliably by manual inspection of characteristic double peaks. Detection of exon 4 deletion/insertion mutations required the synthesis of internal primers 5′-TATGACCCAGAGGTCATCCA-3′ and 5′-ATGAACTGAGCTATCTCGAG-3′.

RDBH

With a reliability of >90% (data not shown), we used RDBH to determine the population frequencies of individual mutations. Amino-labeled oligonucleotides containing seven WD mutations (table 1) were synthesized by use of an Applied Biosystems model 392 DNA synthesizer and then were bound to nylon membranes by use of a modification of the protocol used by Zhang et al. (1991). The terminal amino groups were introduced during the final coupling step, with the Aminolink 2 reagent (ABI). A CE-phosphoramidate (Clontech) that incorporates an 18-atom arm was used to introduce two spacer groups immediately prior to addition of the 5′ amino group. Oligonucleotides containing aminolink (Al), spacer groups (Sp), and disease-specific mutations (underlined in the following list) were designed for the His1069Gln mutation at exon 14 (table 1), as shown in the following examples: 5′-Al-Sp-Sp-GTGAACAC-CCTTGGG-3′ (wild-type oligonucleotide, sense strand); 5′-Al-Sp-Sp-GTGAACAACCCTTGGG-3′ (mutant oligonucleotide, sense strand); 5′-Al-Sp-Sp-CCCAAGG-GGTGTTCAC-3′ (wild-type oligonucleotide, antisense strand); and 5′-Al-Sp-Sp-CCCAAGGGTTGTTCAC-3′ (mutant oligonucleotide, antisense strand).

Restriction-Enzyme Analysis

The A→G transition at the exon 12 splice acceptor site creates an *Nla*III restriction-endonuclease recognition site. Since the original primers for exon 12 amplification produced a fragment with additional *Nla*III sites, new primers were designed (5′-TGGTCTTGGTGT-TTTATTTTCAT-3′ and 5′ATGATGATGATAAAT-GGGACAAAA-3′) to amplify a product of 87 bp that would be digested once with *Nla*III only if the substitution was present. *Nla*III reactions were performed at 37°C for 2 h by use of 25–50 ng DNA, 50 mM KOAc, 20 mM Tris-acetate, 10 mM MgOAc, 1 mM DTT, 100 μg BSA/ml, and 1 unit of enzyme. All restriction products were analyzed on a Nusieve 4% agarose gel.

Reverse-Transcriptase (RT) PCR

To assay for the A→G transition at the exon 12 splice acceptor site, poly(A^+) RNA (mRNA Purification Kit, Pharmacia Biotech) was prepared from lymphoblast cultures from control individuals and from WD-affected individuals who were either heterozygous or homozygous for the putative mutation. First-strand cDNA was synthesized by use of random hexamers as primer (Advantage RT for PCR kit, Clontech). By use of the same amplification programs mentioned previously for RDBH, PCR was performed in reaction volumes of 50 μl containing ~0.1 μg lymphoblast poly(A^+) RNA or 1 μg placenta or liver poly(A^+) RNA (Clontech), 10 pmol of the forward primer flanking exon 11 and of the backward primer flanking exon 14 (Petrukhin et al. 1994), 2.5 mM dNTPs, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), and 2.5 units *Taq* polymerase (Perkin Elmer Cetus). Two microliters of template from this reaction were used for another round of amplification using nested primers flanking exons 11 and 13 (Petrukhin et al. 1994). Products were analyzed on a 2% low-melt-

Table 1

Frequencies of WD Mutations in Various Ethnic Populations

Exon	Mutation[a]	Affected Region or Codon Change	Mutation Frequency (%), by Population[b]			
			North American	Russian	Sicilian	Swedish
14	His1069Gln	SEHPL motif	83/218 (38)	14/36 (39)	0/16 (0)	16/42 (38)
15	Frameshift	CCC_{3402}→CC	7/215 (3.3)	9/48 (19)	0/16 (0)	1/42 (2.4)
16	Frameshift	T_{3552}→TT	1/216 (.5)	0/48 (0)	0/16 (0)	0/42 (0)
17	Asp1222Tyr	ATP binding site	1/214 (.5)	2/48 (4.1)	0/18 (0)	0/42 (0)
18	Asn1270Ser	Hinge domain	2/198 (1)	0/40 (0)	2/16 (12.5)	0/42 (0)
8	Frameshift	C_{2304}→CC	6/198 (3)	2/48 (4.1)	0/16 (0)	0/42 (0)
7	Frameshift	7-bp deletion	0/198 (0)	0/48 (0)	0/16 (0)	0/42 (0)

[a] Mutations reported elsewhere (Tanzi et al. 1993; Bull et al. 1993) are underlined.
[b] Expressed as the number of chromosomes containing a particular mutation divided by the total number of chromosomes.

ing-point agarose gel and were recovered (Qiaex II, Qiagen) for sequencing.

Functional Assay for Mutant WD Protein

Cu^{2+}-dependent ATPase activity was measured from crude membranes prepared from lymphoblastoid cell lines derived from four patients with different WD mutations as well as from a control individual. Crude membranes were obtained by hypotonic lysis of the frozen cells in a buffer (10 mM Tris-HCl [pH 8.0], 0.2 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride), removal of nuclei pellet (8,000 g for 10 min), and collection of membrane pellets by high-speed centrifugation (100,000 g for 60 min). Membrane pellets were suspended in Tris/EDTA/$MgCl_2$ buffer containing 1 mM sodium azide and 100 μM ouabain (Lopez de Castro 1984). Protein concentrations were adjusted by dilution, and samples containing a total of 5 μg of protein were preincubated with 150 μM $ZnSO_4$. γ-[^{32}P]-ATP hydrolysis was measured in the presence or the absence of 10.5 μM Cu^{2+}. Sodium azide, ouabain, and zinc sulfate were used to inhibit endogenous non–copper stimulated ATPase activity. Copper-stimulated ATPase activity was calculated as a difference between two reactions (plus copper and minus copper) after 30 min incubations at 37°C. Four independent experiments were performed for each membrane preparation.

Results

Mutation Detection

Haplotype analysis was performed on a total of 141 WD-patient lymphocyte DNA samples, by use of six microsatellite markers that flank the WD locus (see Subjects, Material, and Methods). Patient DNA samples characterized by two identical copies of a novel haplotype (homozygotes) were analyzed by direct DNA sequencing, as described in Subjects, Material, and Methods. Those samples containing at least one novel haplotype were analyzed by either SSCP analysis or by direct DNA sequencing. This process allowed us to identify 25 disease-specific mutations (table 2) and 21 polymorphisms (table 3), within the WD gene.

Of the 25 putative mutations detected in our sample, 5 are small insertions/deletions (leading to frameshift mutations), 16 are missense mutations, 2 are nonsense mutations, and 2 affect splice-site sequences. For all mutations, the parent samples (when available) were shown to be heterozygous for the disease mutation. Among the frameshift mutations, one results from a single thymidine nucleotide deletion at nucleotide position 845 within the third copper-binding motif. A Middle Eastern WD patient was found to be homozygous for this mutation, and both of the patient's parents were heterozygous for the mutation, as was the patient's first cousin. Interestingly, a single cytosine nucleotide deletion at position 846 had been reported in another Middle Eastern WD individual in a previous study (Thomas et al. 1995). Another frameshift mutation results from a 2-bp deletion at amino acid position 4092 within the penultimate exon, which encodes a predicted Tm loop. We suspect that such a mutation would affect the ion-transporting capabilities of the WD protein. This region is highly conserved in eukaryotes and in prokaryotes; studies show that the analogous Tm segment of the non–heavy metal–transporting P-type ATPases contains a number of residues that are critical for cation binding and translocation (Andersen and Vilsen 1995).

Three of the putative missense mutations produce relatively conservative amino acid substitutions at positions that are highly conserved among eukaryotic and prokaryotic copper-transporting ATPases. In one case, a G→T transversion at amino acid position 1035 alters a highly conserved glycine residue to a hydrophobic valine residue, in the phosphorylation domain (table 2).

A highly conserved leucine residue at amino acid position 795 within the fourth Tm loop was replaced by phenylalanine, in an Italian WD patient heterozygous

Table 2

Mutations in the WD Gene

Mutation	Patient Ethnicity[a]	Frequency[b] (%)	Exon	Gene Region	Effect(s) on Protein Function or Structure
Deletion:					
845delT	Middle Eastern	2	2	Third copper-binding domain	Frameshift
3472del11 (GGTTTAACCAT)	Polish	<1	16	Second cytoplasmic loop	Frameshift
1340del4 (AAAC)[c]	Czech	<1	3	Between the fourth and fifth copper-binding domains	Frameshift
4092del2 (GT)	British	<1	20	Tm 7	Frameshift
2304insC	Russian/American	2.6	8	Tm 4	Frameshift
Missense:					
Gly1186Cys	Jewish	<1	17	Second cytoplasmic loop	Change in tertiary structure
His1069Gln	Polish/Swedish[d]	37	14	SEHPL motif	Disrupts ATP binding
Arg778Trp	American	<1	8	Tm 4	Affects copper transport
Trp1353Arg	Irish	<1	20	Tm 7	Affects copper transport
Gly869Arg	Anglo Saxon	<1	11	Phosphatase domain	Disrupts phosphatase activity
Met645Arg	Jewish	<1	6	Sixth copper-binding domain to Tm 1	Affects copper transport
Leu708Pro	American	<1	8	Tm 2	Affects copper transport
Gly1266Val	American	<1	18	Hinge domain	Affects hinge domain
Gly626Ala	Jewish[e]	<1	6	Sixth copper-binding domain to Tm 1	Affects copper transport
Asn1270Ser	Costa Rican[f]	7	18	Hinge domain	Affects hinge region
Asp1222Tyr	Russian	<1	17	ATP binding site	Disrupts ATP binding
Leu795Phe	Italian	<1	9	Tm 4 to the transduction domain	Affects copper transport
Gly710Ser	Italian/Middle Eastern[g]	<1	8	Tm 2	Affects copper transport
Arg1322Pro	Anglo Saxon	2	19	Tm 7	Affects copper transport
Glu1064Ala	Jewish	1	14	Proximal to SEHPL motif	Disrupts ATP binding
Gly1035Val	Japanese	<1	14	Phosphorylation domain	Affects phosphorylation
Nonsense:					
Trp779Stop	Anglo Saxon[h]	<1	8	Tm 4	Truncates protein
Arg1319Stop	British	<1	19	Tm 7	Truncates protein
Splice site:					
AG→GG	Puerto Rican/British	3	12	3′ acceptor	Cryptic-site usage; exon skipping
AG→GG	Sicilian[j]	<1	19	3′ acceptor	Cryptic-site usage; exon skipping[k]

[a] Refers to the individual in whom the initial mutation was detected.

[b] Refers to the entire clinical sample in this study (see Material and Methods) and was determined by use of a combination of mutation detection and identification of common haplotypes.

[c] Also could be designated as 1339del4 (CAAA). Ambiguity results from the nature of the sequence surrounding the deletion.

[d] Mutation was identified previously in the following populations: American and Russian (Petrukhin et al. 1993; Tanzi et al. 1993); German, French, British, and Eastern European (Thomas et al. 1995); continental Italian, Turkish, and Albanian (Figus et al. 1995); Dutch (Houwen et al. 1995); and northern European (Waldenström et al. 1996).

[e] Mutation was identified previously in Sardinian, continental Italian, and Turkish populations (Figus et al. 1995).

[f] Mutation was identified previously in a Sicilian population (Tanzi et al. 1993) and in continental Italian and Turkish populations (Figus et al. 1995).

[g] Mutation was identified previously in a Kurdish population (Waldenström et al. 1996).

[h] Mutation was identified previously in a northern European population (Waldenström et al. 1996).

[i] Mutation was identified previously in a continental Italian population (Figus et al. 1995) and in a British population (Thomas et al. 1995).

[j] Mutation was identified previously in a continental Italian population (Figus et al. 1995).

[k] Effects are speculative, since this mutation has not been analyzed by RT-PCR.

Table 3

Polymorphisms in the WD Gene

Polymorphism[a]	Codon Change	Amino Acid(s)	Exon
C1366G	CTG→GTG	Leu→Val	3
A2495G	AAG→AGG	Lys→Arg	10
C(−5)T	...	...	17
T3419C	GTC→GCC	Val→Ala	16
C3498T	AGC→AGT	Ser	16
T(−7)A	...	...	16
T3399C	CTT→CTC	Leu	15
A2855G	AAA→AGA	Lys→Arg	12
A287C	GAC→GGC	Asp→Gly	2
T1216G	TCT→GCT	Ser→Ala	2
G1336C	GTG→CTG	Val→Leu	3
C(−53)A	CCC→ACC	...	4
C1878T	GGC→GGT	Ser	6
T2379N	GCT→GCN	Ala	9
G2973A	ACG→ACA	Thr	13
C(9)G	CTC→GTC	...	19
C(35)T	TCC→TTC	...	11
C(40)T	CTT→TTT	...	11
T(49)C	...	...	9
T(13)C	CTT→CTC	...	19
A(−74)C	ACG→CCG	...	5′ UTR
C3548G	GCT→GGT	Ala→Gly	16
A2305G	ATG→GTG	Met→Val	8
G3381A	CTG→CTA	Leu→Leu	15

[a] Nucleotide positions are reported with nucleotide-position 1 corresponding to nucleotide A of the initiating codon ATG. Numbers in parentheses indicate positions within an intron, relative to the intron/exon boundary, with negative values corresponding to the distance, in bp, from the splice acceptor site of an exon and with positive values corresponding to the distance, in bp, from the splice donor site of an exon.

for this mutation. The haplotype associated with this mutation has been detected in only one other individual, from Sicily. Yet another missense mutation leads to an asparagine-to-serine substitution at amino acid position 1270 within the hinge domain. The mutation, previously described for our Sicilian sample (Tanzi et al. 1993), accounts for 61% of all WD mutations in a sample of 15 families from Costa Rica.

Two mutations consist of A→G transitions at the invariant −2 positions of splice acceptor sites. One mutation, present in the homozygous or the heterozygous state in four individuals who were of either Puerto Rican or British descent, results in the creation of an additional *Nla*III site at exon 12. These putative mutations appear to be rare in the general population, since restriction-enzyme analysis of 100 control individuals did not detect the *Nla*III site created by the nucleotide change. Furthermore, individuals with the splice-site substitution at exon 12 share a very similar modification of a unique haplotype.

Two nonsense mutations, present in two affected individuals from the American sample, result from a G→A transition at amino acid position 2337 and a C→T transition at amino acid position 3957, respectively. The population frequencies of the newly identified mutations were estimated by restriction-enzyme analysis, by haplotype-frequency analysis, or by RDBH (tables 1 and 2).

Transcriptional Assay for a WD Splice-Site Mutation

To determine whether the A→G transition in the exon 12 splice acceptor site alters the pattern of WD gene expression, lymphoblast poly(A^+) RNA from WD patients who were either heterozygous or homozygous for the substitution was isolated. The transcriptional assay is illustrated in figure 1*a*. As shown in figure 1*b*, heterozygous and normal individuals produced the full-length 326-bp band, whereas the individual homozygous for this splice-site mutation produced a 365-bp fragment along with the 192-bp alternative transcript (see the legend of fig. 1). By using DNA sequence analysis, we showed that the 365-bp RT-PCR product contains an in-frame 39-bp insertion derived from the recognition of a cryptic splice site at the 3′ end of intron 11. The first 7 bp of the cryptic splice site is identical to a wild-type acceptor site and is followed by a pyrimidine tract (fig. 1*c*). This mutation produces 13 additional amino acids (VVISHGLGVLFSW) in the region between the transduction motif and the fifth Tm region. We found no evidence for low levels of full-length transcript in the homozygous individual.

We presume that the 13–amino acid insert is indeed the disease mutation, since no other alterations were detected in the remaining 20 exons and the intron/exon junctions nor was this splice-site mutation detected in 100 control individuals. DNA sequencing of the PCR-amplification products also showed that the 192-bp band from the homozygous individual and the 192-bp and the 326-bp bands from the heterozygous and normal individuals are identical to the full-length and alternatively spliced products characteristic of controls. The larger bands (>370 bp) seen in the normal individual N1 were observed in other control lymphoblasts, in liver and placental poly(A^+) RNA, and in a construct containing the full-length WD cDNA (data not shown). DNA sequencing showed that the larger band consisted of wild-type sequences identical to the 326-bp transcript, suggesting that these bands arise from concatemerizaton of PCR products and that the cryptic splice site normally does not compete with the normal splice site. Surprisingly, the individual heterozygous for this mutation does not produce detectable levels of the 365-bp abnormal band and possesses the same homozygous haplotype as the individual homozygous for this mutation.

Population Frequencies of Select WD Mutations

We used an RDBH procedure to determine population frequencies for seven disease mutations (table 1).

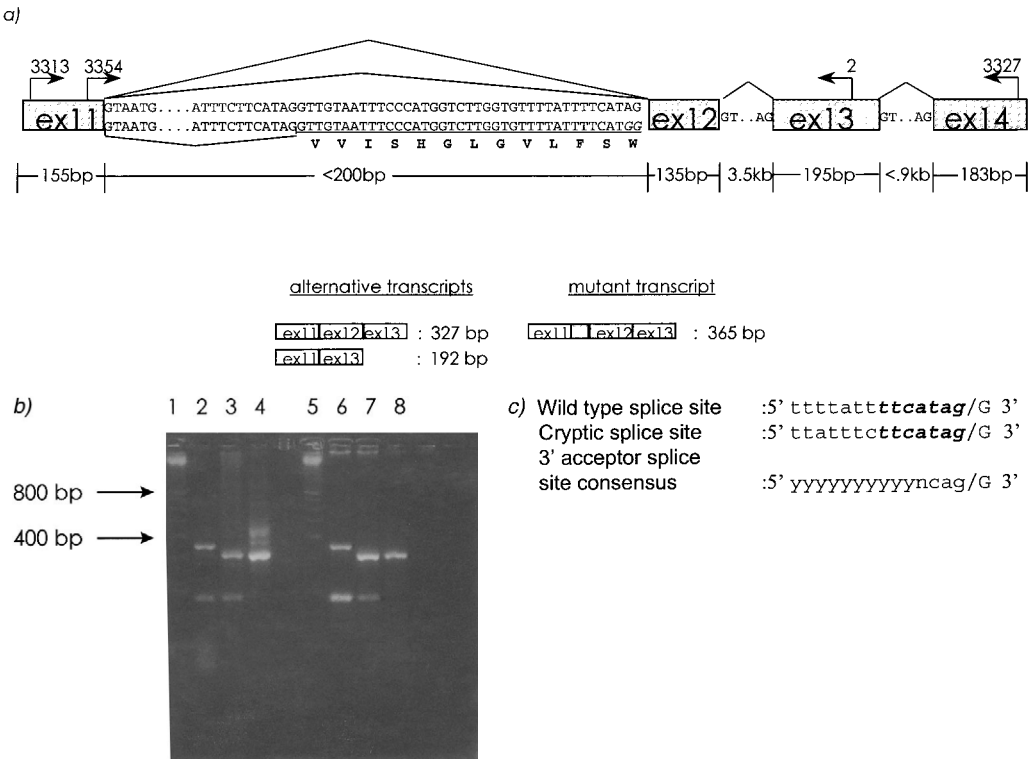

Figure 1 RT-PCR analysis for splice-site mutations. *a,* Genomic structure of exons 11–14 of the WD gene. PCR amplification was performed in two successive rounds. The primers used for first-round PCR (primers 3327 and 3313) and the nested primers used for a second round of PCR (primers 3354 and 2) are shown above. Controls were included to distinguish transcripts with exon 12 (placental and liver poly[A^+] RNA) and without exon 12 (brain WD cDNA), which frequently is omitted in the brain and the kidney (Petrukhin et al. 1994). The 326-bp product includes exon 12, whereas the 192-bp alternative product lacks exon 12. However, the presence of the A→G splice-acceptor-site mutation at the invariant −2 position (italicized) produced either a 192-bp product, by skipping exon 12 entirely, or a 365-bp product with an additional 39 bp (underlined), resulting in the presence of 13 additional amino acids (boldface letters). *b,* Gel electrophoresis of fragments produced by RT-PCR in samples from individuals homozygous (lanes 2 and 6) or heterozygous (lanes 3 and 7) for this mutation and in samples from normal individuals (lanes 4 and 8), from two different mRNA preparations. The sizes of the fragments were determined by comparison with a 100-bp ladder standard (lanes 1 and 5). *c,* Comparison of 3′ wild-type and cryptic splice-acceptor-site sequences of exon 12 of the WD gene with the consensus splice-acceptor-site sequence (Krawczak et al. 1992). Identical base-pair sequences between the wild-type and the cryptic sequences are italicized and boldface.

The most common WD mutation, His1069Glu, appeared in very similar frequencies in the North American, Russian, and Swedish samples but was not detected among the eight unrelated patients from Sicily. The exon 15 frameshift mutation (CCC_{2337}→CC) was relatively common in the North American and Swedish samples (Waldenström et al. 1996) and quite common in the Russian sample (found in 19% of WD chromosomes) but also was undetected in the small Sicilian sample. A missense mutation (Asn1270Ser) in the hinge domain accounted for 2 of 16 disease alleles in the Sicilian sample.

Genotype/Phenotype Correlation

In order to evaluate the functional significance of WD mutations, we searched for correlations between genotypes and several phenotypic manifestations of the illness, including age of onset, neurological versus hepatic onset of the illness, the level of ceruloplasmin activity, hepatic copper levels, and the presence of Kayser-Fleischer (KF) rings. In table 4 we evaluate our North American population for these five clinical indices of WD. This sample included 118 WD patients and included 30 individuals homozygous for the His1069Gln mutation and 23 heterozygous for the mutation. The His1069Gln mutation is the only WD mutation that occurs with sufficient frequency to allow the evaluation of multiple individuals who are homozygous for the mutation. As shown in table 4, individuals homozygous for the most common mutation (n = 20) had an average age of onset of 20 years, compared with 15.4 years for heterozygotes (n = 16) and 17.2 years for the entire sample. Homozygous individuals showed a nearly equal incidence of hepatic and neurological presentation. These findings are in agreement with those reported elsewhere (Thomas et al. 1995). Ceruloplasmin activity levels for our entire sample (n = 79) were within the range of 0–32 mg/dl, with a mean value of 9.4 mg/dl, and

Table 4

Evaluation of Genotype/Phenotype Correlation among North American WD Patients

Patient Sample[a]	*n*	Average Age at Onset (*n*) [years]	No. of Patients with Types of Clinical Presentation Evaluated (*n*)	Ceruloplasmin-Level Range/Mean (*n*)[b] [mg/dl]	Hepatic Copper–Level Range/Mean (*n*) [μg/gdw]	No. of Patients with KF Rings (*n*)
North American	118	17.6 (65)	37 hepatic, 24 neurological, 4 hepatic/neurological,[d] 2 hepatic/renal[d] (67)	0–32/9.4 (79)	156–1530/819.9 (30)	58 (79)
North American His1069Gln homozygotes	30	20 (20)	10 hepatic, 8 neurological, 2 hepatic/neurolocical[d] (20)	<1–25.7/10.12 (25)	40–1467/609.3 (11)	19 (24)
North American His1069Gln heterozygotes	23	15.4 (16)	6 hepatic, 10 neurological (16)	0–20.4/9.77 (17)	281–743/541.5 (4)	14 (17)

[a] Described in Subjects, Material, and Methods.
[b] Measured by assaying of oxidase activity, by use of the substrate paraphenylenediamine.
[c] Determined by use of atomic absorption spectrophotometry (see Subjects, Material and Methods).
[d] More than one type of clinical presentation was seen in these affected individuals.

hepatic copper levels (n = 30) were within the range of 156–1,530 μg/gdw, with a mean value of 819.9 μg/gdw. The normal range for ceruloplasmin and for hepatic copper is 20–35 mg/dl and <250 μg/gdw, respectively. For these two clinical indices, the mean values for His1069Gln homozygotes and heterozygotes do not differ significantly from the entire sample. Although for most other mutations there were too few individuals for the derivation of significant genotype/phenotype correlations, the Asn1270Ser mutation represented 61% of all mutations in the Costa Rican sample. This cohort has a very high incidence of fulminant WD cases; however, the correlation between phenotype and genotype remains unclear, since the single Sicilian individual with this genotype does not show signs of fulminant WD. Of the 79 individuals from our North American cohort tested for the presence of KF rings, 58 had corneal copper deposits, regardless of which WD mutation they possessed. A majority of the 21 individuals who did not manifest KF rings received chelation therapy at a young age, owing to early diagnosis.

Preliminary Functional Study of WD Mutations

We performed a preliminary assay to evaluate the effects of four independent WD mutations on copper-stimulated ATPase activity in lymphoblasts. Crude membrane preparations were isolated from lymphoblastoid cell lines derived from four WD patients and from one normal individual. In figure 2, we see that the copper-stimulated ATPase activity from normal lymphoblast membrane fractions is low, ~0.08 nmole Pi/mg protein/hour, or slightly >20% of total ATPase activity under conditions designed to inhibit endogenous non–copper stimulated ATPase activity. This result is consistent with our western blot analysis of lymphoblast membrane preparations and also with a more sensitive assay using RT-PCR, which confirmed the presence of the WD mRNA transcripts in lymphoblast RNA preparations (data not shown). Each of the 10 ATPase activities (5 with copper and 5 without copper) represents the average of three replicate assays. Estimates of copper-stimulated ATPase activities in each lymphoblast fraction are shown (fig. 2). As predicted, the cell lines from the four WD patients each demonstrated reduced activity, compared with the normal cell line.

Discussion

In our analysis of 109 WD patients from North America, we show that 38% of WD chromosomes harbor the His1069Gln mutation, while an additional five mutations account for ~8% of all disease mutations. We present evidence that a rare mutation, initially detected in a Sicilian population, accounts for 61% of all WD mutations in Costa Rica. The present-day population in Costa Rica is derived largely from a small number

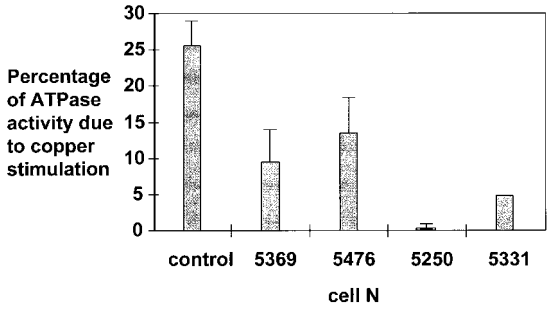

Figure 2 Loss of copper-stimulated ATPase activity in lymphoblasts from WD patients. Copper-stimulated ATPase activity was measured relative to the lymphoblast cell number derived from normal and from affected individuals. Patient 5369 is homozygous for the Asn1270Ser mutation. Patient 5476 is homozygous for a C insertion at position 2304 in exon 8. Patient 5250 is homozygous for the most common mutation, His1069Glu, in exon 14. Patient 5331 is heterozygous for a T insertion at position 2487 in exon 16. Error bars are indicated.

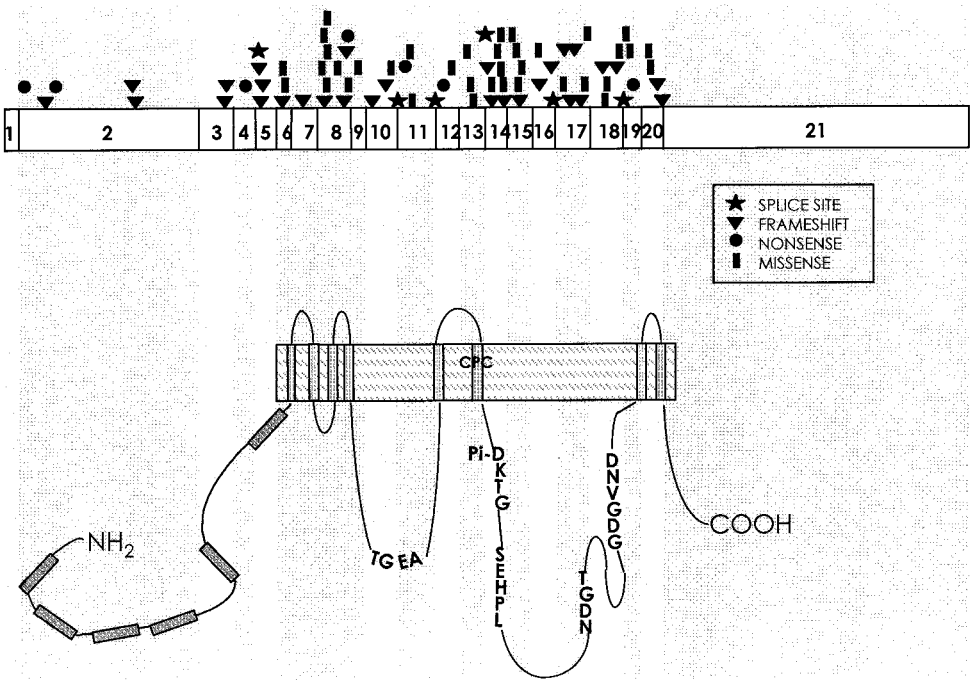

Figure 3 Summary of all published WD mutations in exonic and splice-site-junction sequences (Bull et al. 1993; Tanzi et al. 1993; Figus et al. 1995; Thomas et al. 1995; Loudianos et al. 1996; Waldenström et al. 1996; Kemppainen et al. 1997).

of founding families dating back to the eighteenth century (Freimer et al. 1996). Because the Sicilian and Costa Rican mutations appear on identical, presumptive ancestral haplotypes, we speculate that the mutation originated in southern Europe and was transported to Costa Rica with the founding families.

We report a total of 27 mutations in our WD sample—7 frameshift, 16 missense, 2 nonsense, and 2 splice-site mutations. The population-frequency values for these mutations confirm that the spectrum of WD mutations consists of a small number of relatively frequent mutations, with a large number of rarely occurring mutations. Among the ~66 mutations published to date (fig. 3) (Bull et al. 1993; Tanzi et al. 1993; Figus et al. 1995; Shimizu et al. 1995; Thomas et al. 1995; Loudianos et al. 1996; Waldenström et al. 1996; Kemppainen et al. 1997), several amino acid residues have been the target of multiple mutations. In particular, the arginine residue at position 778 has been substituted with leucine (Thomas et al. 1995), glycine (Figus et al. 1995), or tryptophan (this study), in different affected individuals. Similarly, a glycine residue at position 1266 in the highly conserved hinge region has been replaced with lysine (Thomas et al. 1995) or valine (this study). In addition, the glutamic acid residue at position 1064 has been exchanged for an alanine (this study) or a lysine (Figus et al. 1995) residue. Also, adjacent base pairs were deleted from the third copper-binding domain in unrelated individuals of Middle Eastern decent. Likewise, a 2-bp deletion in Tm loop 7, reported in a British individual in a previous study (Thomas et al. 1995), differed in location from the deletion that we also detected in a British individual, by 2 bp. The Tm regions encoded by exons 6–9 and 19–20 are also sites for frequent mutation. Twelve of our 27 mutations map to these regions of the gene and most likely affect both copper transport across the membrane and copper-dependent phosphorylation of the enzyme, by ATP, as shown elsewhere for the sarcoplasmic reticulum Ca^{2+}-ATPase (Clarke et al. 1990).

To discriminate between polymorphism and mutation, we analyzed a few mutations in the context of their conservation among various copper ATPases from humans, mouse, rat, yeast, and bacteria. A methionine-to-valine amino acid substitution in Tm loop 4, identified in a homozygous American individual, also was reported by another group (Thomas et al. 1995). Although we failed to detect this alteration in 100 control individuals, we suspect that this change actually may be a polymorphism, since valine residues have been found at this position in prokaryotic copper-transporting ATPases from *Proteus mirabilius* and *Synechococcus* sp. (Genbank U42410 and U04356, respectively). We and others (Figus et al. 1995) have reported a seemingly conservative glycine-to-alanine substitution at position 626. Analysis of this residue, among eight copper-transporting P-type ATPases (three prokaryotic and five eukaryotic), has shown this glycine residue to be conserved in all eight copper ATPases (data not shown). Therefore, we have classified this substitution as a mutation.

The WD and MNK genes are distinguished among metal-transporting ATPases by the presence of six highly homologous, putative copper-binding motifs. DNA-sequence comparison of the WD and MNK motifs indicates that the 600 amino acid N-terminal segment of these genes arose via gene duplication events that preceded the splicing of this segment with an ATPase core, forming the common ancestral molecule for both genes. Recently, the rat homologue to the human WD gene was shown to lack the fourth copper-binding motif (Wu et al. 1994), suggesting that the duplicated segments are functionally redundant. Consistent with this interpretation, no missense mutations have been reported in any of the six copper-binding motifs of the WD or the MNK genes. As shown in figure 3, all mutations in this segment of the gene are due to frameshifts that predictably would lead to production of a truncated protein. A number of MNK patients were reported to harbor splice-junction or missense mutations in the 77-bp exon located between the sixth copper-binding site and the first Tm region (Das et al. 1994). We report two WD mutations in this region. At least two studies indicate the presence of intronic or regulatory mutations among their WD patient sample. In a study of Swedish WD patients, 5 of 44 WD chromosomes did not harbor detectable mutations in any of the 21 known exons examined by DNA sequencing (Waldenström et al. 1996). In studies of WD patients of Sardinian or Mediterranean descent, 55% of all WD chromosomes harbored no detectable mutations in the 21 exons (Figus et al. 1995; Loudianos et al. 1996).

The most common WD mutation, His1069Gln, appears frequently (10%–40%) in diverse populations, including those of North America, Great Britain, Holland, Sweden, continental Italy (but not Sardinia or Sicily), and several Mediterranean countries. The mutation occurs on the same haplotype in each of the population samples, indicating that the mutation is relatively old. We found a statistically significant skewing of His1069Gln mutations toward the homozygous condition, in our sample. In the North American population, 83 of 218 disease alleles result from the His1069Gln mutation. Of the 83 mutations, 60 disease alleles were found in homozygous individuals (i.e., 30 individuals), whereas only 23 individuals were heterozygous for the mutation. From Hardy-Weinberg (HW) equilibrium predictions, the ratio of heterozygotes to homozygotes, within the population, would be $2pq/q^2 = 2(.62)(.38)/(.38)^2 = 3.26$. Thus, approximately three times as many heterozygous individuals as homozygous individuals would be predicted for this mutation. From the North American population, we calculated a ratio of 0.77 heterozygotes to homozygotes. Other studies appear to support this observation, by the inclusion of ratios of 0.6 for a study of Dutch WD families (Houwen et al. 1995), 1.8 for families of primarily European origin (Thomas et al. 1995), and 2.0 for 22 unrelated Swedish families (Waldenström et al. 1996). One explanation for these findings may be that admixture accounts for the deviation from HW equilibrium. Alternately, the skew toward homozygotes may be an artifact of detection or may be owing to the fact that other "mild" WD mutations in combination with the relatively mild His1069Gln mutation do not produce typical WD.

A number of splice-site mutations have been reported for both the WD and MNK genes (Das et al. 1994; Kaler et al. 1994; Shimizu et al. 1995; Thomas et al. 1995; Loudianos et al. 1996). In the case of the MNK gene, "mild" splice-site mutations have been described, which apparently allow for the production of some functional protein and which lead to the presentation of OHS, or cutis laxa, a mild phenotype characterized by hyperelastic and bruisable skin, hernias, bladder diverticulae, hyperextensible joints, varicosities, and multiple skeletal abnormalities (Das et al. 1995). These mild mutations may arise predominantly from aberrations at the 5′ donor splice junctions, whereas 3′ acceptor-junction mutations are more likely to lead to MNK (Das et al. 1994; Kaler et al. 1994). Two groups have reported splice-site mutations from the 3′ end of exon 5 of the WD gene, which produce very different WD phenotypes. In one case, it appeared that the recognition of a cryptic splice site led to production of a truncated protein, in a severely affected Indian patient (Thomas et al. 1995), whereas, in another case, exon skipping apparently produced a partially functional protein and a mild disease presentation (Shimizu et al. 1995).

We have described a splice-site mutation that forces the recognition of a cryptic splice site, which then produces a protein with 13 additional amino acid residues. This extra peptide segment presumably would alter the protein tertiary structure or would interfere with the flanking cation channel and/or the transduction domain, thereby hindering protein function. The individual with this mutation was diagnosed at age 14 years with evidence of both neurological symptoms (swallowing difficulties) and liver disease (abnormal transaminases). Levels of functional ceruloplasmin and hepatic copper were 1 mg/dl and 1,531 μg/gdw, respectively, and KF rings were not present. In comparison, the individual heterozygous for the splice-site mutation, diagnosed at age 17 years, presented with severe neurological manifestations, KF rings, and functional ceruloplasmin levels <1 mg/dl. Furthermore, detectable levels of the aberrant transcript were not produced, even though the splice-site mutation occurs on the same haplotype in both the homozygous and the heterozygous individuals. One possibility is that an in *trans* competition for transcription factors occurs such that the wild-type sequence is greatly preferred to the mutant sequence. Alternatively, in the heterozygous individual, the homologue with the splice-site mutation may harbor additional polymorphisms

that reduce the efficiency of the cryptic splice-site sequences so that exon skipping is favored.

Investigation of potential correlations between genotype and phenotype is hampered in the study of WD by the rarity of most mutations. Nonetheless, several groups have now reported on the phenotypic manifestations of patients who are homozygous for the His1069Gln mutation (Figus et al. 1995; Houwen et al. 1995; Thomas et al. 1995; Waldenström et al. 1996). In a study of 38 Dutch patients, it was shown that 9/10 homozygous patients presented with neurological or psychiatric conditions (Houwen et al. 1995). In contrast, our North American sample and our predominantly European samples showed a roughly equal presentation of liver disease and neurological presentations (Thomas et al. 1995; Waldenström et al. 1996). Our study further shows that His1069Gln homozygotes vary greatly in their levels of ceruloplasmin and hepatic copper and present with and without KF rings. These findings are consistent with many recent reports, which illustrate that a variety of genetic, epigenic, and environmental factors can influence the phenotypic outcome of a disease mutation. Previous reports noted that phenotypic variability among WD patients could be influenced by dietary copper intake, by metallothionein inducibility (Thomas et al. 1995), and by the individual's capacity for dealing with copper stress, which would likely include factors such as glutathione, superoxide dismutase, and heat-shock proteins (Luza and Speisky 1996).

Acknowledgments

We thank Geera Butala for her assistance with exonic DNA amplification and purification. This work was supported by NIH grant DK47383 (to T.C.G).

References

Andersen JP, Vilsen B (1995) Structure-function of cation translocation by Ca(2+)- and Na+, K+- ATPases studied by site directed mutagenesis. FEBS Lett 359:105–106

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 5:327–335

Chelley 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

Clarke DM, Loo TW, MacLennan DH (1990) Functional consequences of alterations to amino acids located in the nucleotide binding domain of the Ca^{2+}-ATPase of sarcoplasmic reticulum. J Biol Chem 265:22223–22227

Das S, Levinson B, Vulpe C, Whitney S, Gitschier J, Packman S (1995) Similar splicing mutations of the Menkes/mottled copper-transporting ATPase gene in occipital horn syndrome and the blotchy mouse. Am J Hum Genet 56:570–576

Das S, Levinson B, Whitney S, Vulpe C, Packman S, Gitschier J (1994) Diverse mutations in patients with Menkes disease often lead to exon skipping. Am J Hum Genet 55:883–889

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991) "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res 19:4008

Freimer NB, Reus VI, Escamilla MA, McInnes LA, Spesny M, Leon P, Service SK, et al (1996) Genetic mapping using haplotype, association, and linkage methods suggests a locus for severe bipolar disorder (BPI) at 18q22-q23. Nat Genet 12:436–441

Figus A, Anguis A, Loudianos G, Bertini C, Dessi V, Loi A, Deiana M, et al (1995) Molecular pathology and haplotype analysis of Wilson disease in Mediterranean populations. Am J Hum Genet 57:1318–1324

Houwen RH, Juyn J, Hoogenraad TU, Ploos van Amstel JK, Berger R (1995) H754Q mutation in Wilson disease is associated with late, neurological presentation. J Med Genet 32:480–482

Jungmann J, Reins HA, Lee J, Romeo A, Hassett R, Kosman D, Jentsch S (1993) MAC5, a nuclear regulatory protein related to Cu-dependent transcription factors is involved in Cu/Fe utilizations and stress resistance in yeast. EMBO J 12:5051–5056

Kaler SG, Gallo LK, Proud VK, Percy AK, Mark Y, Segal NA, Goldstein DS, et al (1994) Occipital horn syndrome and a mild Menkes phenotype associated with splice site mutations at the MNK locus. Nat Genet 8:195–202

Kemppainen R, Palatsi R, Kallioinen M, Oikarinen A (1997) A homozygous nonsense mutation and a combination of two mutations of the Wilson disease gene in patients with different lysyl oxidase activities in cultured fibroblasts. J Invest Dermatol 108:35–39

Krawczak M, Reiss J, Cooper DN (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet 90:41–54

Lopez de Castro JA (1984) Purification of human HLA-A and HLA-B class I histocompatibility antigens. Methods Enzymol 108:582–586

Loudianos G, Dessi V, Angius A, Lovicu M, Loi A, Deiana M, Akar N, et al (1996) Wilson disease mutations associated with uncommon haplotypes in Mediterranean patients. Hum Genet 98:640–642

Luza SC, Speisky HC (1996) Liver copper storage and transport during development: implications for cytotoxicity. Am J Clin Nutr 63:812S–820S

Maniatis T, Fritsch EF, Sambrook J (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Mercer JF, Livingston J, Hall B, Paynter JA, Begy C, Chandrasekharappa S, Lockhart P, et al (1993) Isolation of a partial candidate gene for Menkes disease by positional cloning. Nat Genet 3:20–25

Morell AG, Windsor J, Sternlieb I, Scheinberg IH (1968) Measurement of concentration of ceruloplasmin in serum by determination of its oxidase activity. In: Sunderman FW, Sunderman FW Jr (eds) Laboratory diagnosis of liver diseases. WH Greene, St Louis, pp 193–195

Orita M, Suzuki Y, Sekiya T, Hayashi K (1989) Rapid and sensitive detection of point mutation and DNA polymor-

phisms using the polymerase chain reaction. Genomics 5: 874–879

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, Gilliam TC (1994) Genetic disorders of copper metabolism. Curr Opin Pediatr 6:698–701

Petrukhin K, Lutsenko S, Chernov I, Ross BM, 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 9:1647–1656

Scheinberg IH, Sternlieb I (1984) Wilson's disease. Vol 23. WB Saunders, Philadelphia

Shimizu N, Kawase C, Nakazono H, Hemmi H, Shimatake H, Aoki T (1995) A novel RNA splicing mutation in Japanese patients with Wilson disease. Biochem Biophys Res Commun 217:16–20

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, Forbes JR, Roberts EA, Walshe JM, Cox DW (1995) The Wilson disease gene: spectrum of mutations and their consequences. Nat Genet 9:250–257

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

Waldenström E, Lagerkvist A, Dahlman T, Westermark K, Landegren U (1996) Efficient detection of mutations in Wilson disease by manifold sequencing. Genomics 37: 303–309

Wu J, Forbes JR, Chen HS, Cox DW (1994) The LEC rat has a deletion in the copper transporting ATPase gene homologous to the Wilson disease gene. Nat Genet 7:541–545

Yuan DS, Stearman R, Dancis A, Dunn T, Beeler T, Klausner RD (1995) The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. Proc Natl Acad Sci USA 92:2632–2636

Zhang Y, Coyne MY, Will SG, Levenson CH, Kawasaki ES (1991) Single-base mutational analysis of cancer and genetic diseases using membrane bound modified oligonucleotide. Nucleic Acids Res 19:3929–3933