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Refining the Position of Wilson Disease by Linkage Disequilibrium with Polymorphic Microsatellites

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Summary

Wilson disease (WND) is an autosomal recessive disorder that is due to an inability of the liver to eliminate copper. Copper buildup in the liver, brain, kidney, and other tissues can result in liver cirrhosis, neurologic and psychiatric defects, and other problems. We have localized the disease-containing region to between D13S31 and D13S59, with >70 multiply affected families, and have constructed a YAC contig of >4.5 Mb that spans these loci and orders nine highly polymorphic microsatellites. Here we present an analysis of disequilibrium with markers in this interval and provide evidence for strong allelic associations between AFM084xc5 alleles and WND alleles in European, Middle Eastern, and East Asian populations. Significant but weaker allelic associations were also observed between WND alleles and alleles at D13S137 and D13S169. The strength of the association between AFM084xc5 and WND in all non-Sardinian populations combined (linkage-disequilibrium coefficient [Φ] = .61) suggests that the number of mutations accounting for WND is less than expected on the basis of the variety of clinical symptoms that are observed.

Introduction

Wilson disease (WND) is an autosomal recessive disorder of copper metabolism; it results in an accumulation of copper in a variety of tissues, but primarily the liver, basal ganglia, and kidneys (Scheinberg and Sternlieb 1984, pp. 25–105). The disorder is rare, with a gene frequency of .0056 worldwide (Danks 1989), although the incidence is probably underestimated because many cases are undetected because of inadequate diagnosis. WND was recognized at the beginning of this century (Wilson 1912), but its biochemical defect is not known. Although affected individuals have severely reduced levels of ceruloplasmin, the disease is not caused by a molecular defect in this gene, which has been localized to chromosome 3, unlike WND, which has been localized to chromosome 13. This is supported by studies by our group (Frydman et al. 1985; Bonne-Tamir et al. 1986; Bowcock et al. 1987, 1988) and others (Yuzbasiyan-Gurkan et al. 1988; Figus et al. 1989; Chuang et al. 1991; Scheffer et al. 1992).

Our search for the WND gene was directed to the 13q14 region, on the basis of the initial demonstration of linkage between WND and the esterase-D locus in three kindreds from the Middle East (Frydman et al. 1985; Bonne-Tamir et al. 1986). Acquisition of additional families and saturation of the chromosome with polymorphic DNA markers has localized the gene to a 0.3-cM interval between the loci D13S31/D13S227/D13S228 and D13S59 (Farrer et al. 1991; Stewart et al. 1993). We currently have 74 families from Europe, North America, the Middle East, Russia, and East Asia.

Once a disease gene has been confidently mapped to within 1 cM, recombinational mapping is unlikely to provide additional information on the location of the gene, without the analysis of significantly more fami-

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lies. Linkage disequilibrium is a type of allelic association where alleles at two loci occur together more frequently in a population than would be expected on the basis of the allele frequencies at the two loci. In the case of disease genes, one would expect that, if a mutation accounting for the majority of disease states were to arise on a particular chromosome, tightly linked markers unlikely to undergo recombination between the disease locus would be maintained with the disease allele. If linkage disequilibrium between a marker and disease locus can be detected, it is a powerful approach for narrowing the critical region to which physical mapping efforts should be directed.

We have previously been unable to detect linkage disequilibrium between WND and close flanking markers D13S31 and D13S59 (Farrer et al. 1991; Stewart et al. 1993), and it was unclear whether this was due to the existence of several ancestral mutations or to excessive distance between the disease and marker loci. We recently constructed a 4.5-Mb YAC contig that spanned these loci, and we physically mapped nine polymorphic microsatellites to this interval (White et al., in press). In the study described here we present genotypes for polymorphic microsatellites that we have localized to between D13S31 and D13S59 in the three recombinant families described elsewhere (Stewart et al. 1993) and in one other recombinant family. These analyses refine the location of WND to between D13S155/D13S169 and D13S133. We also present evidence for linkage disequilibrium, in several populations, between WND alleles and alleles at three highly polymorphic microsatellite loci (D13S169, D13S137, and AFM084xc5) in this interval.

Subjects, Material, and Methods

DNAs and Marker Typing

DNA was prepared from fresh blood or Epstein-Barr virus–transformed lymphoblastoid cell lines from affected members of WND pedigrees and from their unaffected relatives. Ascertainment of cases and diagnostic methods have been described elsewhere (Frydman et al. 1985; Bowcock et al. 1987; Farrer et al. 1991; Stewart et al. 1993). Multiallelic polymorphisms at D13S133, D13S137, D13S155, D13S164, D13S169, and AFM084xc5 were typed as described elsewhere (Stewart et al. 1993). PCR primers and amplification conditions for D13S133 and D13S137 (Petrukhin et al. 1993), D13S155 and D13S169 (Weissenbach et al. 1992), and AFM084xc5 (White et al., in press) have also been described elsewhere.

Test of Linkage Disequilibrium

Differences in the overall distribution of alleles on disease-bearing and normal chromosomes were tested by Fisher's exact test with a $2 \times k$ contingency table, where k is the number of marker alleles present in the sample. This test is more sensitive than the χ^2 test, which is traditionally used for testing linkage disequilibrium (Weir 1990), when the data set includes individual cells having fewer than five observations (Kendall and Stuart 1979). The problem of small or empty cells was particularly acute for stratified analyses. Computations were carried out using the SAS (1990) computer program. Analyses were stratified by ethnic and geographical background of the families, as follows: (1) Caucasian, including 28 families originating from all regions of Europe, except Scandinavia, Sardinia, and Russia; (2) Arab and Druze (3 families); (3) Sephardic Jewish (3 families); (4) Sardinian (8 families); and (5) East Asian, including 13 families from Korea and the People's Republic of China. The degree of association was measured as $\sqrt{\chi^2/n}$, where n = number of chromosomes (Vogel and Motulsky 1986, pp. 162–164).

Results

DNA Markers in WND Gene Region

The physical map surrounding loci D13S25, D13S31, and D13S59, with the location of polymorphic and nonpolymorphic sequence-tagged sites (STSs), is shown in figure 1 (White et al., in press). Five polymorphic microsatellites have been localized to the interval between D13S31/D13S227/D13S228 and D13S59 that has been demonstrated by linkage analysis to contain the WND gene (Farrer et al. 1991; Stewart et al. 1993). These are the polymorphic "CA" repeat–containing loci D13S133 and D13S137 (Petrukhin et al. 1993), D13S155 and D13S169 (Weissenbach et al. 1992), and AFM084xc5 (White et al., in press).

Recombination Mapping

To establish the smallest cosegregating region for WND, we genotyped the polymorphic microsatellites shown in figure 1 in (*a*) the three families in which crossovers were observed for either D13S31/S227/S228 or D13S59 (Stewart et al. 1993) and (*b*) families that were uninformative for D13S59 (Farrer et al. 1991; Stewart et al. 1993). Haplotypes were constructed assuming the most parsimonious linkage phase, and families exhibiting crossing-over in this region between WND and DNA markers are shown in figure 2. Crossovers have previously been reported between D13S31/

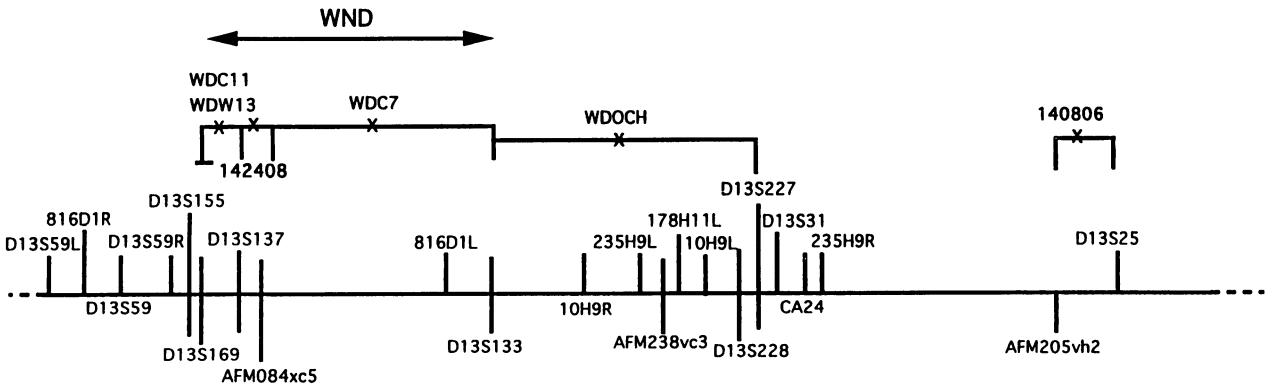

Figure 1 STS-content map around the WND gene, as described elsewhere (White et al., in press). The locations of STSs used to construct the map are indicated by vertical lines. Those that extend below the map are polymorphic microsatellites. The position of recombination breakpoints in WND (WDW13, WDC7, and WDOCH) and CEPH families (142408 and 140806), as well as the location of WND, are indicated above the map.

D13S227/D13S228 in two families of European origin: OCH and C7. When these families were genotyped for D13S133, the crossover was retained in the OCH family but was absent in family C7. Crossovers were removed from both families, with all markers distal to D13S133. A crossover has also been described with D13S59 in the Middle Eastern family W13. This crossover was still evident with D13S169 and D13S155 but not with any marker proximal to these loci. A fourth family (C11) was also informative for these markers, and one individual inherited an affected chromosome with a recombination event between D13S137 and D13S169 while still retaining the WND allele. These recombinants localize WND to between D13S155/D13S169 and D13S133.

Linkage Disequilibrium for Closely Linked Markers

Failure to detect any recombinants between WND and either AFM084xc5 or D13S137 in the WND families suggested that a search for linkage disequilibrium with these and other tightly linked markers is a feasible approach. We typed AFM084xc5 and D13S137 in 52 additional WND families with at least one affected child. In some cases parental haplotypes were reconstructed on the basis of analyses of marker alleles in unaffected offspring and other members of the extended family.

AFM084xc5.—In the total sample (table 1), linkage disequilibrium was evident because of both a significant excess of allele 5 and a deficiency of alleles 4 and 9 on WND chromosomes relative to normal chromosomes ($P = .03$). Stratification of the sample by ethnic and geographical background showed even more striking linkage disequilibrium in several populations. In non-Sardinian Europeans, 60% of both the normal and mutant WND genes are linked to allele 4 or allele 5, but they are distributed very differently in the two groups: 44% of the normal WND gene and 19% of the mutant WND gene are linked to allele 4, whereas 15% of the normal WND gene and 41% of the WND gene are linked to allele 5. When these data are combined with the frequencies of the other alleles (table 1), there is significant linkage disequilibrium ($P = .004$). A small sample of 11 chromosomes in Arabs and Druze (table 1) did not reveal linkage disequilibrium ($P = .6$). Analysis of the same number of chromosomes in Sephardic Jews gave a remarkably significant result ($P = .004$), which is due to the presence of (*a*) alleles 5 and 6 on WND chromosomes only and (*b*) alleles 4 and 9 on normal chromosomes only (table 1). The Sardinian families gave the most conclusive evidence for linkage disequilibrium ($P = .0003$). Fourteen (82%) of 17 mutant WND genes were linked to allele 4; this allele was linked to only 23% of the normal alleles (table 1). Linkage disequilibrium was also present among East Asian patients ($P = .04$) mainly because of an excess of allele 5 and a deficiency of alleles 4 and 9 linked to the mutant WND gene (table 1). Linkage disequilibrium in the total sample excluding the Sardinians, who show an association pattern different from that of non-Sardinians, was surprisingly very high ($\Phi = .61$; $P = .004$).

D13S137.—Table 2 shows the marker alleles for D13S137 linked to WND and normal chromosomes. Among the various strata of families, linkage disequilibrium was significant in Europeans only. In non-Sardinian Europeans there was (*a*) a disproportionately high

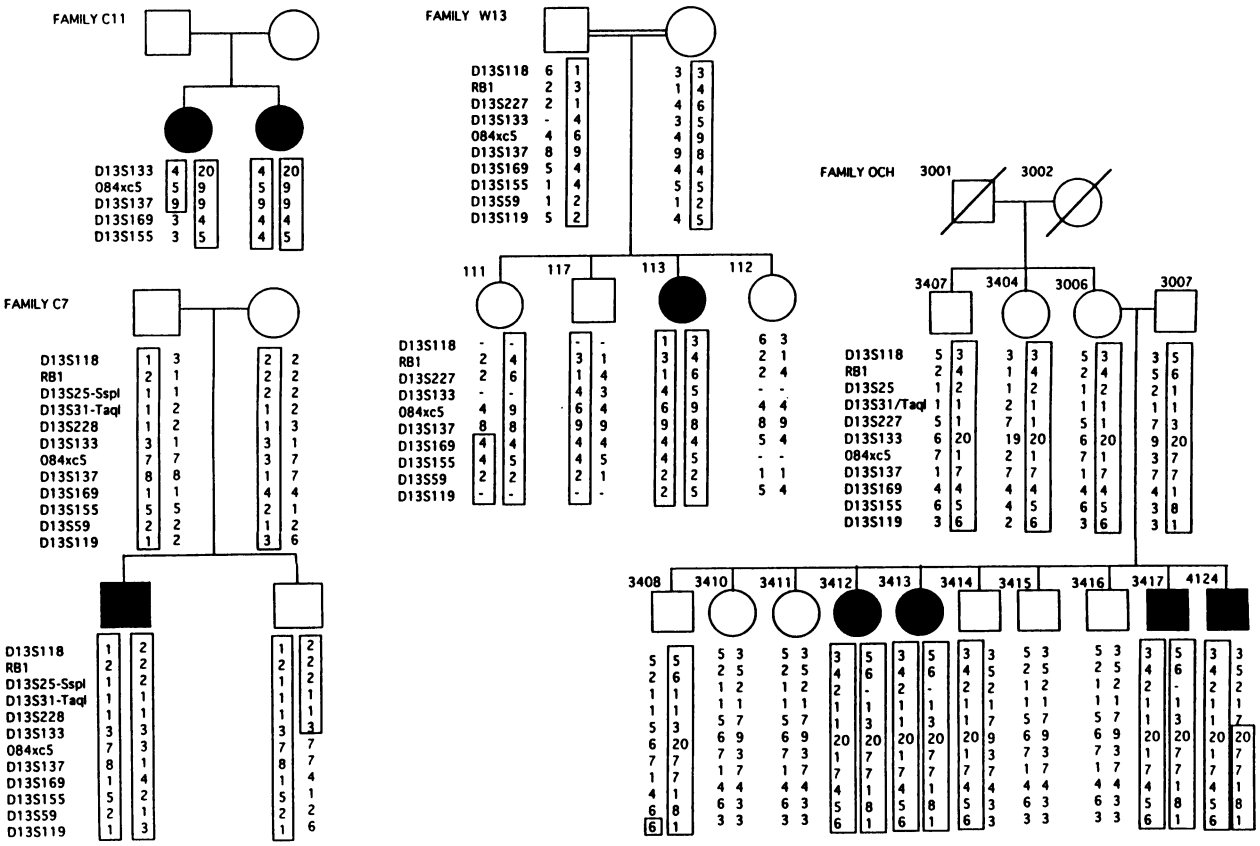

Figure 2 Genotypes at polymorphic microsatellite loci in families demonstrating crossovers near the WND gene. Genotypes of previously described loci are also provided. Loci are shown to the left of each pedigree, and the genotype for each individual at each locus is shown underneath that individual's pedigree symbol. In some cases, when they were unambiguous, genotypes were inferred. The haplotype carrying the WND allele is boxed. Haplotypes for the parents in family C11 were unavailable but do not affect the conclusions that were drawn. For family C7 an alternate phase is possible, but the conclusions are the same. In family OCH, haplotypes of 3006 and 3007 were reconstructed on the basis of information on the genotypes of children and sibs of 3006.

number of alleles 2 linked to the mutant WND gene and (*b*) an underrepresentation of alleles 8 and 9, compared with normal chromosomes (P = .005). In contrast, in Sardinian patients, the mutant WND gene was almost exclusively linked to allele 9, whereas linkage of this allele to normal chromosomes was only half as frequent (P = .026).

Linkage Disequilibrium in Sardinia

In an attempt to establish the position of the WND gene relative to AFM084xc5 and D13S137, we evaluated linkage disequilibrium with several polymorphic microsatellites in the region between D13S31 and D13S59. Theoretically, linkage disequilibrium should decay as one moves farther from the WND gene, because of the increased likelihood of recombination in the interval. We restricted these analyses to Sardinian families in which we had seen the greatest Φ with AFM084xc5 (see above). Sardinia is a Mediterranean island with a surface area of about 10,000 square miles and a population of <2 million inhabitants. The population has remained relatively stable throughout the past century. All of the families are unrelated and geographically dispersed but are of Sardinian origin (Giagheddu et al. 1985).

Table 3 shows that linkage disequilibrium was absent for all loci proximal to AFM084xc5 (D13S133, P = .5; D13S227, P = .12; and D13S228, P = .07). For the markers distal to D13S137, linkage disequilibrium was present for D13S169 (P = .04) but was absent for D13S155 (P = .4). Comparison of the Φ values (table 4) shows that the strength of allelic association at AFM084xc5 is moderately high and approximately 1.5 times greater than that at D13S137 and D13S169. Al-

Table 1

Distribution of AFM084xc5 Alleles on Normal and WND Chromosomes, by Ethnic and Geographic Origin

	No. of Normal Chromosomes; No. of WND Chromosomes					
Allele (size)	Caucasian	Arab and Druze	Sephardic Jewish	Sardinian	East Asian	Total
11 (99 bp)	1; 0	0; 0	0; 0	0; 0	0; 0	1; 0
1 (95 bp)	0; 1	0; 0	0; 0	0; 0	0; 0	0; 1
2 (93 bp)	2; 0	0; 0	0; 0	0; 0	0; 0	2; 0
3 (91 bp)	1; 1	0; 0	0; 0	0; 1	0; 1	1; 3
4 (89 bp)	22; 11	2; 3	2; 0	4; 14	4; 0	34; 28
5 (87 bp)	9; 26	2; 0	0; 4	3; 0	10; 17	24; 47
6 (85 bp)	2; 0	0; 1	0; 2	1; 0	0; 0	3; 3
7 (83 bp)	3; 3	0; 0	0; 0	0; 0	0; 0	3; 3
8 (81 bp)	0; 0	0; 0	0; 0	0; 1	0; 1	0; 2
9 (79 bp)	13; 18	1; 2	4; 0	9; 1	13; 10	40; 31
Total	53; 60	5; 6	6; 6	17; 17	27; 29	108; 118

though the order of D13S155 and D13S169 could not be determined on the basis of recombination breakpoint or physical mapping (White et al., in press), disequilibrium mapping suggests that D13S169 is proximal to D13S155, if it is assumed that decay in linkage disequilibrium and distance are related.

In order to avoid a false interpretation of the linkage-disequilibrium results because of differences in the population frequencies of the WND-associated alleles of the marker loci, haplotypes of the four loci (D13S133, AFM084xc5, D13S137, and D13S169) in the region showing the strongest linkage disequilibrium were constructed for the 34 Sardinian chromosomes. Analysis of these haplotypes suggested that the 20-4-9-1 haplotype was most likely to carry the Sardinian WND ancestral mutation. Although this haplotype accounts for only 3 of the 17 WND chromosomes, 3 other haplotypes can be explained by stepwise mutations (with a gain or loss of one repeat unit) in the dinucleotide repeats of D13S133 and D13S137 (see table 5). Another seven haplotypes can be explained on the basis of recombination between D13S133 and AFM084xc5 and between D13S137 and D13S169. Three of the WND chromosomes can be accounted for only by recombination between AFM084xc5 and D13S137 involving descendants of the ancestral WND chromosomes. The combination of the allele 4 (or the presumed derivative allele 3) for AFM084xc5 and of the allele 9 (or the

Table 2

Distribution of D13S137 Alleles on Normal and WND Chromosomes, by Ethnic and Geographic Origin

	No. of Normal Chromosomes; No. of WND Chromosomes					
Allele (size)	Caucasian	Arab and Druze	Sephardic Jewish	Sardinian	East Asian	Total
11 (127 bp)	0; 0	0; 0	0; 0	0; 0	0; 0	2; 5
1 (125 bp)	1; 0	0; 2	0; 0	0; 1	2; 4	3; 7
2 (123 bp)	2; 17	1; 0	0; 0	2; 0	8; 10	13; 27
3 (121 bp)	7; 6	0; 2	3; 3	2; 0	3; 2	15; 13
4 (119 bp)	0; 1	0; 0	1; 0	0; 0	3; 0	4; 1
5 (117 bp)	1; 1	0; 0	0; 0	0; 0	1; 1	2; 2
6 (115 bp)	1; 0	0; 0	0; 0	0; 0	0; 0	1; 0
7 (113 bp)	1; 2	0; 0	0; 0	0; 0	1; 2	2; 4
8 (111 bp)	11; 7	4; 3	1; 3	6; 2	3; 0	25; 15
9 (109 bp)	23; 17	4; 2	3; 2	7; 14	4; 4	41; 39
10 (107 bp)	0; 0	0; 0	0; 0	0; 0	0; 1	0; 1
Total	108; 114	47; 51	9; 9	8; 8	17; 17	27; 29

Table 3

Distribution of Alleles for Several Microsatellite Marker Loci on Normal and WND Chromosomes in Sardinian Families

Marker and Allele (size)	No. of Chromosomes	
	Normal	WND
D13S227:		
2 (156 bp)	1	1
3 (154 bp)	2	3
4 (152 bp)	1	5
5 (150 bp)	2	2
6 (148 bp)	7	2
7 (146 bp)	3	0
8 (144 bp)	1	2
9 (142 bp)	0	2
Total	17	17
D13S228:		
1 (134 bp)	7	6
2 (132 bp)	1	6
3 (130 bp)	3	0
4 (128 bp)	6	5
Total	17	17
D13S133:		
22	0	1
1	1	0
2	2	2
3	0	2
4	1	1
5	0	1
6	3	0
19	1	0
20	7	8
21	2	2
Total	17	17
D13S169:		
1 (189 bp)	3	9
3 (185 bp)	3	0
4 (183 bp)	11	8
Total	17	17
D13S155:		
2 (218 bp)	2	0
3 (216 bp)	0	2
4 (214 bp)	3	4
5 (212 bp)	2	2
7 (208 bp)	1	5
8 (206 bp)	2	3
9 (204 bp)	1	0
Total	11	16

presumed derivative allele 8) at D13S137 occurred on 14 (82%) of 17 WND chromosomes and on only 4 (24%) of 17 normal chromosomes. The next most frequent combination (alleles 20/21/22 for D13S133 and allele 4 for AFM084xc5) is distributed on 11 (65%) of 17 WND chromosomes but did not occur on any of the 17 normal chromosomes. The results in tables 4 and 5 indicate that the candidate region for the WND gene is likely to be limited to the region between D13S133 and D13S137.

Table 4

Relative Φ Values for Marker Loci in Sardinian Families

Locus	Φ	P^a
D13S227	.57	NS
D13S228	.44	NS
D13S133	.52	NS
AFM084xc5	.73	.0008
D13S137	.52	.026
D13S169	.44	.038
D13S155	.52	NS

[a] NS = not significant.

Table 5

Haplotypes at Loci Tightly Linked to WND, Associated with WND and Normal Chromosomes, in Sardinian Families

Haplotype	No. of Chromosomes	
	WND	Normal
20-4-9-1	3	0
21-4-9-1	2	0
20-4-8-1	1	0
22-4-8-1	1	0
20-4-9-1	3	2
3-4-9-1	1	0
3-4-9-4	1	0
4-4-9-4	1	0
2-3-9-4	1	0
20-4-1-4	1	0
5-9-9-1	1	0
2-8-9-4	1	0
2-4-9-3	0	1
2-4-9-4	0	1
20-9-8-1	0	1
20-9-8-4	0	2
20-9-9-4	0	1
6-9-8-4	0	2
6-9-9-1	0	1
20-9-2-4	0	1
1-6-9-3	0	1
19-5-9-3	0	1
21-5-3-4	0	1
21-5-2-4	0	1
4-9-3-1	0	1

NOTE.—Order of loci in each haplotype is D13S133–AFM084xc5–D13S137–D13S169.

Discussion

Recombinational mapping with markers localized to a 4.5-Mb contig spanning the WND region localized the disease gene to between D13S133 and D13S155/D13S169, within a region that includes AFM084xc5 and D13S137. The close proximity of AFM084xc5 to WND is strongly supported by our ability to detect linkage disequilibrium between these two loci in a large group of families from different areas of the world. Linkage disequilibrium between WND and AFM084xc5 was highly significant in the Sardinian families but was also evident in Sephardic Jews, East Asians, and non-Sardinian Europeans. The detection of very strong linkage disequilibrium in the total sample of families excluding the Sardinians was unexpected because this is a heterogeneous group that includes racially and ethnically distinct families separated as far as the British Isles to the Orient.

Linkage disequilibrium has been described for a number of polymorphic DNA markers and disease loci. Markers XV2c and CS.7 were shown to be in linkage disequilibrium with the cystic fibrosis gene (Farrall et al. 1987) and subsequently were shown to lie within 100 kb of CFTR (cystic fibrosis transconductance regulator gene) (Kerem et al. 1989). The loci D4S95 and D4S127 were shown to be in linkage disequilibrium with the Huntington disease (HD) gene candidate region (MacDonald et al. 1992) and subsequently was shown to lie within 200 kb of the 5′ end of the HD gene (Huntington's Disease Collaborative Research Group 1993). Recently, linkage disequilibrium has been reported between DNA markers on chromosome 5 and diastrophic dysplasia in Finland (Hastbacka et al. 1992), and it has been predicted that these markers lie within 60 kb of the gene. In the case of diastrophic dysplasia, allelic associations were higher for RFLP-based markers than for microsatellite-based markers, which appeared to reduce the level of association. One explanation that was provided was that mutation rates at microsatellite loci may affect allele frequencies so significantly that the detection of linkage disequilibrium would be obscured. However, mutation rates at the microsatellite-based markers linked to the diastrophic dysplasia locus were higher than normal (current estimates are 10^{-4}; Hastbacka et al. 1992 describes markers with mutation rates as high as 1/250). We have been able to detect highly significant linkage disequilibrium with polymorphic microsatellites and WND, although it is not yet clear (*a*) how the high mutation rate at polymorphic microsatellites has affected its value and (*b*) how its value can be related to physical distance. In general, linkage-disequilibrium values between two loci decrease with physical distance. This has been demonstrated in the HLA region in humans (Migone et al. 1985). Within isolated populations, where admixture is unlikely to confound inverse correlations of linkage disequilibrium values with physical distance, linkage disequilibrium mapping may be an effective way to predict the distance between a particular marker and a gene of interest. Nevertheless, phenomena such as gene conversion and recombination cold spots would confound this type of analysis and cannot be ignored, and in fact the region studied here is likely to be a recombination cold spot (White et al., in press) where the ratio of recombination to physical distance is 6,000 kb/cM. Although one would expect, on the basis of the linkage-disequilibrium value, that WND is within 200 kb of 084xc5, the fact that this is a recombination cold spot may have facilitated the detection of linkage disequilibrium over a region larger than this.

One explanation for the variety of clinical symptoms of WND is that there is allelic heterogeneity. This may abolish the ability to detect linkage disequilibrium with DNA markers and the disease. The ability to detect linkage disequilibrium in a variety of populations suggests that the number of mutations is less than expected and that the variability in WND phenotypes is due to environmental effects or modifier genes. This also suggests that the number of ancestral WND mutations may be fewer than expected on the basis of its frequency of 1/180 (Danks 1989).

Failure to detect linkage disequilibrium with AFM084xc5 in the Arab and Druze families may be a consequence of our small sample size, a high mutation rate at this locus in these populations, or the presence of multiple mutations. It is surprising that the AFM084xc5 alleles linked to the mutant WND gene are the same in the Chinese, Korean, and Russian populations, suggesting a common WND mutation.

Allele association studies in the Sardinian families with multiple markers in the WND region suggest that, among the markers that we evaluated, AFM084xc5 is the closest to the WND gene. Although it may be argued on the basis of Φ values (table 4) that WND could lie on either side of AFM084xc5, inference from their magnitude suggests that the candidate region is either the <40-kb interval between AFM084xc5 and D13S137 (which have both been localized to the same cosmid; authors' unpublished results) or the interval between AFM084xc5 and D13S133, which spans approximately 500 kb. Both the identification of addi-

tional polymorphic markers between AFM084xc5 and D13S133 and an analysis of linkage disequilibrium with these new markers would clarify which region is likely to contain WND.

Haplotype analysis of WND and normal chromosomes in Sardinians identifies a common-core haplotype that is likely to contain a major WND mutation. In most instances, a mutation event at one of the polymorphic microsatellite-containing loci, AFM084xv5 and D13S137, resulting in gain or loss of one repeat unit will explain an alteration in the "AFM084xc5/4 allele-D13S137/allele 9" core WND-carrying haplotype. Although the cloning of the WND gene and an elucidation of its defects will determine the number of mutations accounting for this disease and possibly will explain the ethnic and familial variation in age at onset and clinical presentation of the illness, linkage-disequilibrium results provided here suggest that a limited number of mutations account for the disease in a variety of populations and that variability in clinical presentation is due to other genes or environmental effects.

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