

## Homozygosity Mapping Identifies an Additional Locus for Wolfram Syndrome on Chromosome 4q

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Wolfram syndrome, which is sometimes referred to as “DIDMOAD” (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness), is an autosomal recessive neurodegenerative disorder for which only insulin-dependent diabetes mellitus and optic atrophy are necessary to make the diagnosis. Researchers have mapped Wolfram syndrome to chromosome 4p16.1, and, recently, a gene encoding a putative transmembrane protein has been cloned and mutations have been identified in patients. To pursue the possibility of locus heterogeneity, 16 patients from four different families were recruited. These patients, who have the Wolfram syndrome phenotype, also have additional features that have not previously been reported. There is an absence of diabetes insipidus in all affected family members. In addition, several patients have profound upper gastrointestinal ulceration and bleeding. With the use of three microsatellite markers (D4S432, D4S3023, and D4S2366) reported to be linked to the chromosome 4p16.1 locus, we significantly excluded linkage in three of the four families. The two affected individuals in one family showed homozygosity for all three markers from the region of linkage on chromosome 4p16.1. For the other three families, genetic heterogeneity for Wolfram syndrome was verified by demonstration of linkage to chromosome 4q22–24. In conclusion, we report the unique clinical findings and linkage-analysis results of 16 patients with Wolfram syndrome and provide further evidence for the genetic heterogeneity of this disorder. We also provide data on a new locus that plays a role in the etiology of insulin-dependent diabetes mellitus.

### Introduction

Wolfram syndrome, which is also known as “DIDMOAD” (diabetes insipidus, diabetes mellitus, optic atrophy, and deafness [MIM 222300]) and which was first described by Wolfram and Wagner (1938), is associated with high morbidity and mortality. Only insulin-dependent diabetes mellitus (IDDM) and progressive optic-nerve atrophy (OA) are necessary to make the diagnosis, but associated findings include sensorineural hearing loss (SNHL), ataxia, peripheral neuropathy, urinary-tract atony, and psychiatric illnesses (Rose et al. 1966; Cremers et al. 1977; Swift et al. 1990; Salih and Tuvemo 1991). Typically, but not invariably, IDDM is detected before OA is detected, whereas diabetes insipidus (DI) and SNHL may or may not develop. Wolfram syndrome is an autosomal recessive disorder with probable locus heterogeneity, based on reported clinical heterogeneity (Rose et al. 1966; Page et al. 1976; Barrett and Bunday

1997). The literature contains a wealth of familial and individual reports as well as several comprehensive clinical reviews (Bretz et al. 1970; Blasi et al. 1986; Kinsley et al. 1995; Barrett and Bunday 1997).

Wolfram syndrome has previously been shown to be linked to markers at chromosome 4p16.1 (Polymeropoulos et al. 1994; Barrientos et al. 1996a, 1996b; Collier et al. 1996). Recently, mutations in a gene known as “wolframin” were identified in patients with Wolfram syndrome (Inoue et al. 1998; Strom et al. 1998). This gene, which encodes a novel protein containing predicted transmembrane domains, is expressed ubiquitously, with high expression in the pancreatic islets. However, evidence of locus heterogeneity exists, in that there was no linkage to this region in one small nuclear family in which OA presented one decade prior to diabetes mellitus (DM), which is an atypical Wolfram syndrome phenotype (Collier et al. 1996).

In this study, we present clinical data on 16 affected individuals with a clinical presentation that fits the diagnosis of Wolfram syndrome. These individuals are from four consanguineous Jordanian families. The genetic heterogeneity for Wolfram syndrome was verified by mapping of another locus to chromosome 4q22–24, by use of a DNA-pooling linkage strategy, and Neurex-1 receptor 3 (NKR3), a candidate gene that

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maps in the critical region, was screened for mutations in patients with Wolfram syndrome.

## Patients and Methods

### Patient Evaluation

Nineteen individuals affected with Wolfram syndrome were identified from seven families that were seen in the clinical service provided by the National Center for Diabetes, Endocrine, and Genetic Diseases in Amman, Jordan. Of these seven families, four families with several affected siblings ( $n = 16$ ) were recruited to participate in the study after informed consent was obtained from each individual or from a legal guardian. All recruited family members were examined thoroughly, and detailed family history and medical history were obtained. All available family members underwent a detailed ophthalmologic examination, and audiography was performed. Most of those who showed signs of the disease also underwent intravenous urography and magnetic-resonance imaging of the pituitary gland. A water-deprivation test was done for most family members. Any family member who showed at least two of the four cardinal signs of Wolfram syndrome was considered to be affected.

### Genotyping and Linkage Analysis

DNA was extracted from leukocytes in venous blood, by use of standard procedures. Four microsatellite DNA markers (D4S432, D4S3023, D4S2366, and D4S2639) known to be linked to the Wolfram syndrome locus on chromosome 4p16.1 were used to test for linkage in the four families (Polymeropoulos et al. 1994; Collier et al. 1996).

Homozygosity mapping was done with the use of a DNA-pooling strategy (Sheffield et al. 1995). Equimolar amounts of samples from families WS-3 and WS-4 were independently pooled into affected and unaffected pools from each family. Genotyping was performed by means of standard protocols (Lidral et al. 1997).

A 20-cM genomewide scan was performed, with an initial focus on those markers from the Weber mapping panel, version 8.0 (Yuan et al. 1997), that are on gene-rich chromosomes (Antonarakis 1994; Inglehearn 1997). Additional markers for fine mapping of the critical region were chosen from maps from the Center for Medical Genetics, Marshfield Medical Research Foundation (Broman et al. 1998), the Cooperative Human Linkage Center (CHLC) (Murray et al. 1994), and GeneMap'98 (Deloukas et al. 1998).

Two-point LOD score analysis was performed with the use of the LINKAGE (Lathrop et al. 1984) and GENEHUNTER (Kruglyak et al. 1996) programs, and multipoint analysis was performed with the use of GENEHUNTER, under the assumption of autosomal

recessive inheritance with 95% penetrance and a disease-gene frequency of .005. The reference genetic map and allele frequencies used for the analysis were generated from CHLC and CEPH data (Broman et al. 1998). The order and distances designated for the chromosome 4p16 markers were D4S432-.014-D4S3023-.0469-DS2366-.205-D4S2639, and those designated for the chromosome 4q markers were D4S3243-.051-D4S2361-.114-D4S1647-.020-D4S1591-.021-D4S3256-.036-D4S1531-.001-D4S1564-.014-D4S3240-.001-D4S2623-.024-D4S2945-.128-D4S2394. Recalculation of the LOD scores, by use of equal allele frequencies, resulted in minimal changes and in no change in significance (data not shown).

### Mutation Screen

The NKR3 gene for was screened for mutations with the use of primers designed to amplify the five exons, including splice sites and splice-branch sites, (Buell et al. 1992; primer sequences available on request). Sequences from one affected individual each from families WS-2–WS-4, along with sequences from a control individual, were sequenced with the use of the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) and were analyzed on an ABI 310 capillary DNA analyzer (PE Biosystems). Templates were prepared by means of PCR amplification and were purified with the use of Qiaquick PCR columns (Qiagen).

## Results

### Clinical Features

**Family WS-1.**—The two affected individuals in family WS-1 are a 17-year-old boy and a 10-year-old girl (table 1). Both presented with DM at the age of ~5 years; shortly thereafter, a visual deficit occurred in both, and a hearing deficit occurred in the boy. The visual deficit was attributed to progressive OA, whereas the hearing loss was caused by high-frequency SNHL. The boy has urinary-tract dilatation with impaired kidney function, and both the boy and the girl had a normal response (urine osmolarity doubled at the end of 3 h) to the water-deprivation test. They both have soft, dysmorphic features, probably as a result of decreased facial subcutaneous fat. Recently, after a severe chest infection, the boy died of respiratory failure of central origin. The parents in family WS-1 are first cousins (fig. 1).

**Family WS-2.**—Family WS-2 includes six affected individuals in one sibship (table 1; fig. 1). All affected individuals had DM develop in the early part of their second decade; this was soon followed by both visual and hearing deficits, except in the case of the 20-year-old man (individual V.9), who still has normal hearing. All affected individuals have documented OA and high-

**Table 1**  
**Diagnosis and Age at Diagnosis in Patients with Wolfram Syndrome**

CHARACTERISTICS	DIAGNOSIS AND AGE AT DIAGNOSIS IN AFFECTED INDIVIDUALS IN EACH FAMILY <sup>a</sup>															
	Family WS-1		Family WS-2				Family WS-3				Family WS-4					
	IV.2	IV.4	V.1	V.2	V.6	V.7	V.9	V.13	V.3	V.8	V.9	V.10	IV.1	IV.2	IV.3	IV.4
Age (years)	17	10	38	35	25	23	20	15	28	14	14	14	14	12	12	6
Sex	M	F	M	F	M	F	M	F	M	M	F	F	F	F	F	F
DM:	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No
Age at diagnosis (years)	5	5	15	15	12	14	15	12	10	10	10	10	13	NA	NA	NA
OA:	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Age at diagnosis (years)	7	7	20	22	18	18	19	14	20	12	12	12	13	11	11	5
SNHL:	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Age at diagnosis (years)	10	NA	25	25	18	18	NA	14	24	12	12	12	13	11	11	5
DI	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Urinary-tract dilatation:	Yes	No	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes	No	Yes	No	No	No
Age at diagnosis (years)	14	NA	35	34	24	NA	NA	24	12	12	NA	13	NA	NA	NA	
Peptic ulcer:	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	No	NA
Age at diagnosis (years)	NA	NA	20	20	17	20	18	14	26	13	13	13	NA	11	NA	NA
Diabetic retin	No	No	Yes	No	Yes	No	No	No	Yes	No	No	No	No	No	No	No
Status	Dead	Dead	Alive	Alive	Alive	Dead	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive	Alive

<sup>a</sup> M = male; F = female; NA = not available.

frequency SNHL, and all have severe complicated peptic-ulcer disease. They also all had a urine volume of <3 liters in 24 h. Individual V.6 had a normal response to the water-deprivation test. The parents in this family are distantly related (they are at least second cousins), and they come from a very inbred family.

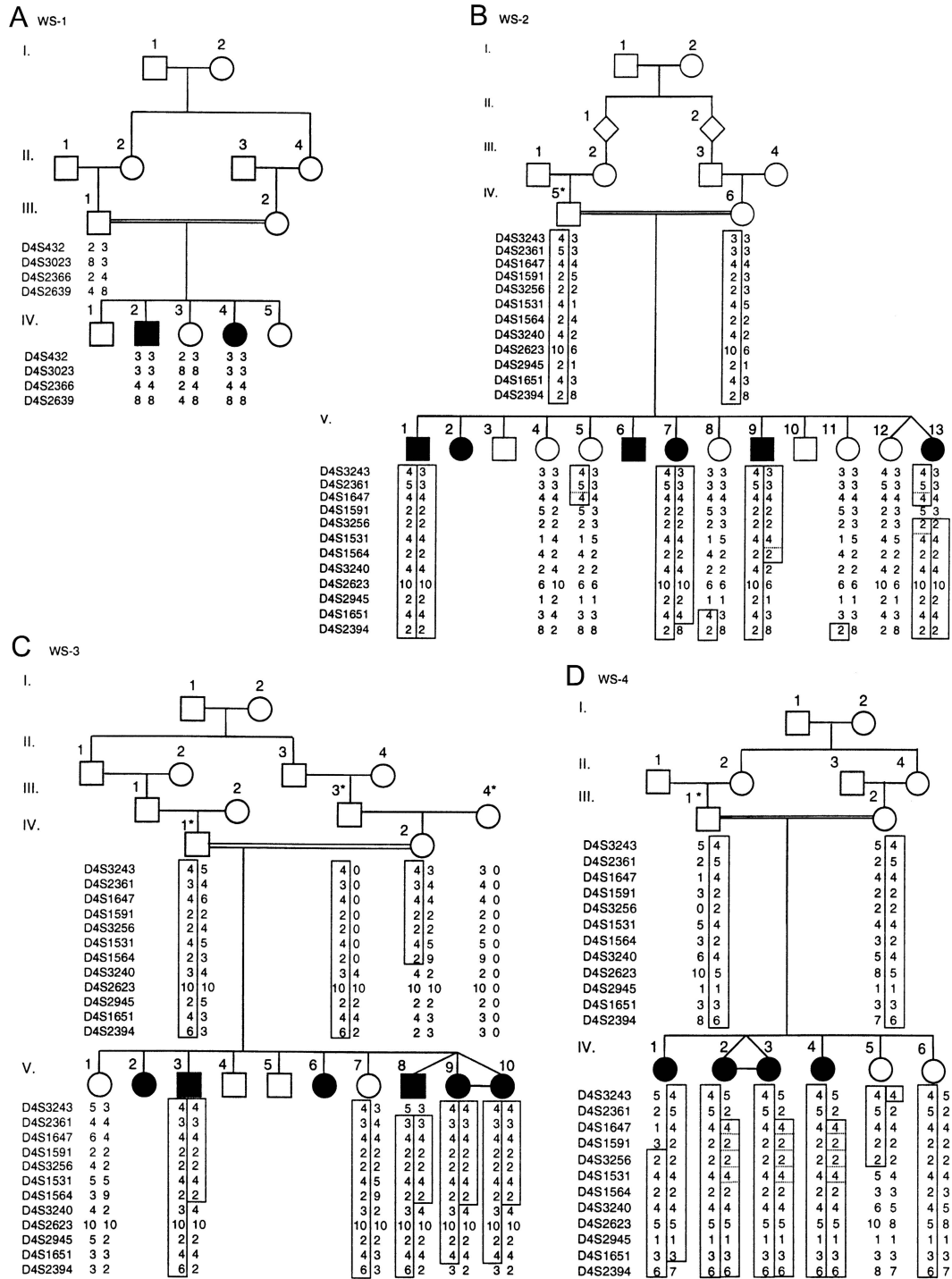
**Family WS-3.**—Family WS-3 includes naturally conceived triplets that are affected with Wolfram syndrome (table 1; fig. 1). Two additional members of this family were suspected of having Wolfram syndrome, but they were unavailable for study, since individual V.2 is living abroad and since individual V.6 had died of a cause unrelated to the disorder. The other four affected individuals all have DM that manifested when each individual was ~10 years old. They all have documented OA and high-frequency SNHL. Three of the individuals have urinary-tract dilatation, and one individual (V.3), who was 28 years old at the time of the present study, has end-stage renal failure. The triplets had a normal response to the water-deprivation test. The parents are second cousins.

**Family WS-4.**—Family WS-4 includes two affected siblings and an affected monozygotic twin pair born to parents who are first cousins (table 1; fig. 1). From a previous marriage to a woman to whom he was not related, the father has a daughter, who was age 16 years at the time of the present study (data not shown). The daughter presented with normal ophthalmologic findings, normal fasting blood glucose levels, and no other symptoms. The affected children include a girl (individual IV.1), who is currently 14 years old and who presented with DM at age 13 years. Although she had no

visual or hearing deficits, she has documented OA and high-frequency SNHL. In addition, she has urinary-tract dilatation with normal kidney function. The twins (individuals IV.2 and IV.3), who are currently 12 years old, have documented and asymptomatic OA and high-frequency SNHL. One of them had a high fasting blood glucose level that later normalized. A girl (individual IV.4), who is currently 6 years old, has documented and asymptomatic OA and high-frequency SNHL. All affected individuals in this family produced a urine volume of <3 liters per 24 h with high osmolarity. The sisters (individuals IV.5 and IV.6), who are 5 and 4 years old, have optic nerves that appeared to be normal on ophthalmologic examination.

*Excluding Linkage to the Chromosome 4p16 Locus*

Linkage to the previously described Wolfram syndrome locus on chromosome 4p16 was explored by genotyping of the markers D4S432, D4S3023, DS2366, and D4S2639 in members of all four families. Visual inspection revealed allelic heterozygosity for these markers in affected individuals from families WS-2–WS-4 (data not shown), whereas, affected individuals from family WS-1 were found to be homozygous for markers D4S432, D4S3023, D4S2366, and D4S2639 (fig. 1). For family WS-1, the results of two-point linkage analysis showed a maximum LOD score of 1.28 (table 1), and multipoint linkage analysis indicated a maximum LOD score of 1.87 near marker D4S3023 (data not shown). Linkage to this region was excluded for families WS-2–WS-4, each of which had a LOD score <2.0 (table 2).



**Figure 1** Pedigrees of families with Wolfram syndrome (DIDMOAD). A, Family WS-1 shows homozygosity for markers at the chromosome 4p16 WFS1 locus. B–D, Families WS-2–WS-4 show haplotypes for markers at chromosome 4q22–24. Recombinations in individuals V.9 and V.13 from family WS-2, individual IV.2 from family WS-3, and individual IV.1 from family WS-4 define the critical region. For individuals denoted by an asterisk, genotypes were inferred from the genotypes of existing family members, and maximum-likelihood haplotypes were deduced by use of GENEHUNTER. Blackened squares denote haplotypes and recombinants, and broken lines indicate alternative crossover events that could also explain the data.

**Table 2**  
Two-Point LOD Scores between Wolfram Syndrome and Chromosome 4p16 Markers

MARKER	LOD SCORE FOR			
	Family WS-1	Family WS-2	Family WS-3	Family WS-4
D4S432	1.28	-2.49	-2.57	-3.95
D4S3023	.88	-2.54	-2.11	-2.76
D4S2366	1.27	-2.67	-2.90	-2.50
D4S2639	1.27	-2.08	-.63	NA <sup>a</sup>

<sup>a</sup> NA = Not available.

*Linkage Mapping with the Use of Pooled DNA Samples*

Given the exclusion of linkage in three of the four families, a 20-cM genomewide scan for additional loci was performed with the use of a DNA-pooling strategy for families WS-3 and WS-4. Approximately 30% of the genome had been screened—initially, with the use of markers from gene-rich chromosomes (Antonarakis 1994; Inglehearn 1997)—before homozygosity was identified with the marker D4S1647 (data not shown). The results of linkage analysis of additional markers indicated a maximum two-point LOD score of 6.84 for marker D4S1564, at recombination fraction ( $\theta$ ) = 0 (table 3), and a multipoint maximum LOD score of 9.55 near markers D4S1531 and D4S1564 (fig. 2). Recombinations in individuals V.9 and V.13 in family WS-2, individual IV.2 in family WS-3, and individual IV.1 in family WS-4 defined the critical region as a 7.1-cM region between D4S1591 and D4S3240 (fig. 1). Haplotype analysis revealed a common haplotype for markers D4S3256, D4S1531, and D4S1564 in members of all three families, suggesting that they may have a common ancestor.

*Mutation Screen of the NKR3 Gene*

The NKR3 gene, which has been mapped near D4S411 and D4S3256 on GeneMap'98, was screened for mutations, since it is expressed in the brain and kidney and since it is involved in the regulation of neurotransmission, inflammation, and pain sensation (Buell et al. 1992). Sequencing of the five exons of the NKR3 gene did not indicate any coding or splicing mutations in affected individuals from families WS-2–WS-4.

**Discussion**

In the present study, the clinical symptoms of the affected members of four families demonstrate phenotypic heterogeneity, since DI was not diagnosed in any individual, although it was suspected in a few. The method (urine volume) used for diagnosis of DI is not the most accu-

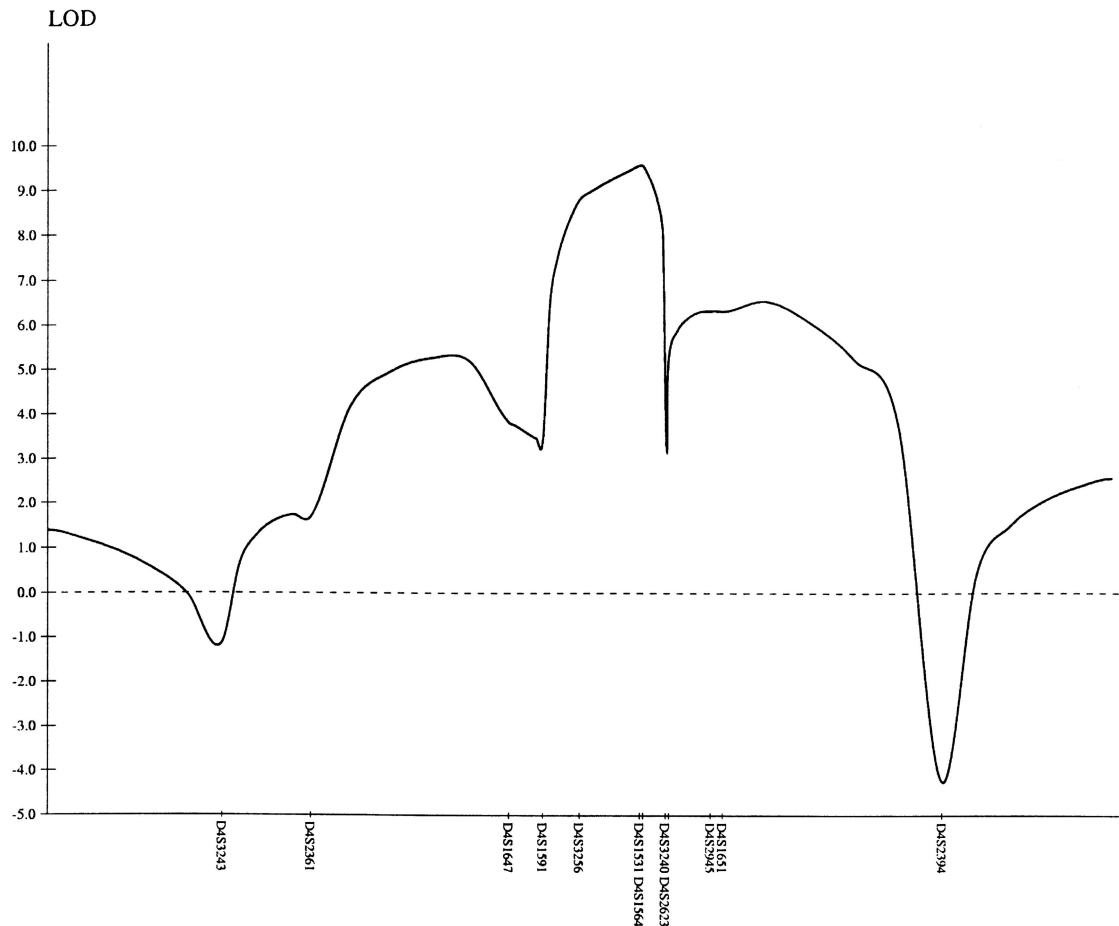
rate, but several patients underwent the water-deprivation test. Even if milder forms of DI exist, it is definitely less common than has been reported in previous series (Bretz et al. 1970). In family WS-1, onset of DM and OA occurred at an earlier age than it did in families WS-2–WS-4. Also, in family WS-1, the morbidity of the disease is more severe, resulting in early mortality. Patients from families WS-2 and WS-3 seem to have an unusual peptic-ulcer disease that has not previously been reported in patients with Wolfram syndrome. This finding is currently undergoing further study. DM was diagnosed in all affected individuals, with the exception of the three younger siblings from family WS-4, who presented with OA and SNHL (table 1). This is not the typical sequence of clinical symptoms; however, OA has been the initial presentation in other clinical series (Barrett and Bunday 1997).

Homozygosity mapping by means of a DNA-pooling strategy and subsequent linkage analysis successfully located the presence of another locus for Wolfram syndrome, WFS2, to a 7.1-cM region between D4S1591 and D4S3240. Previously, the NKR3 gene had been mapped to this interval. However, mutation analysis of the five coding exons did not reveal any mutations, thus excluding NKR3 as a gene for Wolfram syndrome. Within the WFS2 critical region, 22 genes have been mapped, in addition to multiple expressed sequence tags, which will be the basis for positional-cloning efforts.

The pathology of Wolfram syndrome has been speculated to be a result of nuclear mutations that cause deletions in the mitochondrial genome, and a semidominant model of inheritance, in which heterozygous carriers are at greater risk for these deletions and may manifest some of the clinical features of Wolfram syndrome, has been proposed (Barrientos et al. 1996a, 1996b). A high frequency of mitochondrial deletions

**Table 3**  
Two-Point LOD Scores between Wolfram Syndrome and Chromosome 4q Markers in Families WS-2, WS-3, and WS-4

LOCUS	LOD SCORE AT $\theta$ =						
	.0	.01	.05	.1	.2	.3	.4
D4S3243	-1.48	-1.22	-.43	.07	.32	.21	.04
D4S2361	1.10	1.31	1.61	1.61	1.25	.72	.20
D4S1647	-.67	.00	.42	.48	.37	.22	.08
D4S1591	-2.10	-1.41	-.56	-.09	.20	.18	.80
D4S3256	3.38	3.30	2.97	2.54	1.72	.96	.34
D4S1531	6.10	5.96	5.41	4.71	3.30	1.92	.68
D4S1564	6.84	6.70	6.11	5.35	3.82	2.31	.92
D4S3240	2.53	3.47	3.82	3.59	2.68	1.57	.53
D4S2623	2.21	3.10	3.32	3.08	2.29	1.40	.53
D4S2945	1.70	2.59	2.84	2.61	1.88	1.09	.39
D4S1651	2.85	3.74	3.90	3.56	2.58	1.51	.55
D4S2394	-4.77	-2.08	.19	.90	1.07	.77	.35



**Figure 2** Multipoint LOD plot for markers in the chromosome 4q22-24 region of families WS-2–WS-4

has been found in the CNS tissue of one patient (Barrientos et al. 1996b). However, studies of lymphocytes have been variable, ranging from no deletions (Barret et al. 1997) to a low frequency of deletions (Rotig et al. 1993; Barrientos et al. 1996a, 1996b). In addition, neither the tRNA<sup>Leu</sup>(3243) mutation associated with mitochondrial DM nor the mitochondrial deletions have been found in other studies of patients with Wolfram syndrome (Hofmann et al. 1997; Strom et al. 1998; Hardy et al. 1999), thereby leading researchers to question the role of mitochondrial genome anomalies in the etiology of Wolfram syndrome. It has been suggested that the wolframin gene may be a mitochondrial membrane protein and that mutations may affect mitochondrial function, although not through effects on the mitochondrial genome (Strom et al. 1998). Subcellular localization of Wolframin and identification of the WFS2 gene will help discern the role of the mitochondria in the pathophysiology of Wolfram syndrome.

Mapping of Wolfram syndrome loci may provide information regarding more common psychiatric and diabetic diseases. Given that the carrier frequency is es-

timated to be 1/350 (Barrett and Bunday 1997), carriers may contribute to a significant frequency of psychiatric illness and diabetes. The majority of patients with Wolfram syndrome have been shown to have had a history of psychiatric illness, including severe depression, psychosis, or organic brain syndrome (Swift et al. 1990). In addition, heterozygous carriers have a 26-fold increase in risk for psychiatric illness (Swift et al. 1998). Bipolar disorder, which is among a variety of psychiatric disorders that have been mapped to chromosome 4 (Kennedy et al. 1999), shows weak linkage to marker D4S1647, which maps to a location 5 cM proximal to the WFS2 region (Detara-Wadleigh et al. 1997). Also, weak linkage for schizophrenia exists at marker D4S2623 (Levinson et al. 1998), with a recent report showing suggestive linkage to D4S2917 (reviewed by Kennedy et al. 1999). D4S2917 is within the WFS2 critical region, and D4S2623 is very close to the distal critical region. Susceptibility loci for bipolar disorder have also been mapped to markers flanking the WFS1 locus on chromosome 4p16 (Blackwood et al. 1996; Ginns et al. 1998; reviewed by Kennedy et al. 1999),

further supporting a possible role for Wolfram syndrome genes in the etiology of mental illness.

Heterozygous carriers of WFS1 also have an increased risk for DM (Ohata et al. 1998). A locus contributing to insulin activity has been mapped near fatty acid-binding protein 2 (FABP2) in Pima Indians (Prochazka et al. 1993) and Mexican Americans (Mitchell et al. 1995). FABP2 maps to markers flanking the distal aspect of the WFS2 critical region. This mapping is in the same region that shows suggestive linkage for type 2 DM in Mexican Americans (Duggirala et al. 1999). Weak linkage of IDDM to D4S1604, which maps 30 cM distal to the WFS2 critical region, has previously been reported (Hashimoto et al. 1994). Thus, cloning of the WFS2 gene may provide an understanding of diabetes and psychiatric disorders, in addition to providing a further understanding of the pathophysiology of Wolfram syndrome.

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## Electronic-Database Information

The accession number and URLs for data in this article are as follows:

- Center for Medical Genetics, Marshfield Medical Research Foundation, [http://www.marshmed.org/genetics/Map\\_Markers/maps/indexmap.html](http://www.marshmed.org/genetics/Map_Markers/maps/indexmap.html) (for the reference genetic map and markers used to fine map the critical region)
- CEPH, <http://cephb.fr/> (for markers used to fine map the critical region and for allele frequencies)
- CHLC, <http://lpg.nci.nih.gov/CHLC/> (for allele frequencies, the reference genetic map, and markers used to fine map the critical region)
- GeneMap'98, <http://www.ncbi.nlm.nih.gov/genemap98/> (for the identification of candidate genes in the critical region)
- Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for DIDMOAD [MIM 222300])

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