

Non-syndromic progressive hearing loss *DFNA38* is caused by heterozygous missense mutation in the Wolfram syndrome gene *WFS1*

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Dominantly inherited progressive hearing loss *DFNA38* is caused by heterozygosity for a novel mutation in *WFS1*, the gene for recessively inherited Wolfram syndrome. Wolfram syndrome is defined by juvenile diabetes mellitus and optic atrophy and may include progressive hearing loss and other neurological symptoms. Heterozygotes for other Wolfram syndrome mutations generally have normal hearing. Dominant deafness defined by *DFNA38* is more severe than deafness of Wolfram syndrome patients and lacks any syndromic features. In a six-generation kindred from Newfoundland, Canada, *WFS1* Ala716Thr (2146 G→A) was shared by all deaf members of the family and was specific to deaf individuals. The causal relationship between this missense mutation and deafness was supported by two observations based on haplotype and mutation analysis of the kindred. First, a relative homozygous for the mutation was diagnosed at age 3 years with insulin-dependent diabetes mellitus, the central feature of Wolfram syndrome. Second, two relatives with normal hearing had an identical haplotype to that defining *DFNA38*, with the exception of the base pair at position 2146. Other rare variants of *WFS1* co-inherited with deafness in the family could be excluded as disease-causing mutations on the basis of this hearing-associated haplotype. The possibility that 'mild' mutations in *WFS1* might be a cause of non-syndromic deafness in the general population should be explored.

INTRODUCTION

Wolfram syndrome (OMIM 222300) is a recessive disorder defined by juvenile, insulin-dependent diabetes mellitus and

progressive optic atrophy, with variable manifestations including deafness, diabetes insipidus, cataracts, and neurologic symptoms (1, reviewed in 2). The gene responsible for Wolfram syndrome is *WFS1* (OMIM 606201), a member of a novel gene family encoding an 890 amino acid glycoprotein with 10 predicted transmembrane domains (3,4). The wolframin protein (GenBank accession no. O76024) localizes primarily in the endoplasmic reticulum and hence is likely to play a role in membrane trafficking, protein processing and/or regulation of calcium homeostasis (5). Wolfram syndrome is caused by homozygosity or compound heterozygosity for any of multiple protein terminating mutations, in-frame insertions or deletions, or missense mutations in *WFS1* (2).

In a six-generation kindred from Newfoundland, Canada, heterozygosity for a mutation in *WFS1* causes dominantly inherited deafness. Hearing loss in this family is progressive and non-syndromic, affecting the low frequencies first. The critical *WFS1* allele responsible for deafness in the family is a missense mutation. Although it can be difficult to demonstrate a causal relationship between a missense mutation and disease, in this case the structure of the kindred provided genetic clues that the suspect missense in *WFS1* was responsible for deafness.

RESULTS

Deafness phenotype in Family C

Hearing impairment in Family C is usually noted by affected individuals in the second decade although affected children can be identified before school age by an S-shaped pure tone audiogram pattern. Deafness is progressive: mild hearing loss restricted to the low frequencies in the young child progresses to moderate impairment affecting low and middle frequencies in the second decade, then to moderate to severe impairment across all frequencies after age 40 years (Fig. 1). Males appear more severely affected than females after age 40 years,

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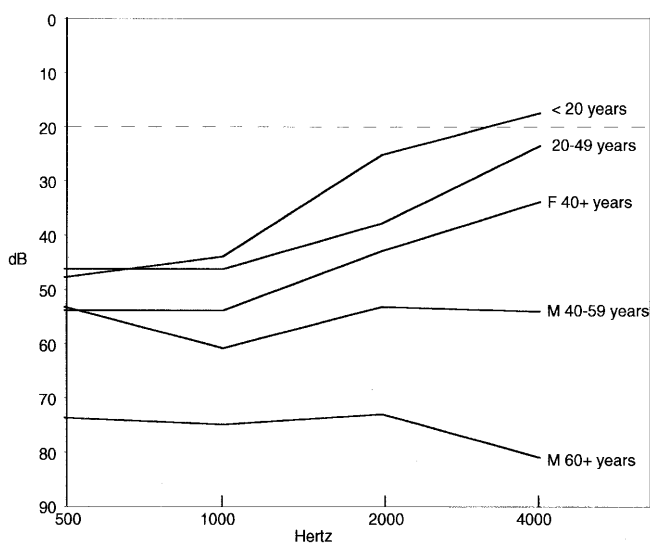


Figure 1. Audiograms defining *DFNA38* hearing loss among affected individuals in Newfoundland Family C. Normal hearing involves thresholds of 0–20 dB, as indicated by the dotted line. Hearing loss is progressive and bilateral, affecting low frequencies (500 and 1000 Hz) first. Males (M) and females (F) are affected similarly prior to age 40 years; hearing loss in males progresses more rapidly than in females after age 40 years.

possibly reflecting confounding by occupational exposure. There is no vestibular or visual involvement.

Positional cloning of *DFNA38*

Genome-wide linkage analysis of 47 informative relatives of Family C yielded a maximum LOD score of 11.58 for linkage of fully penetrant, dominant hearing loss to marker D4S431. Genotypes of multiple markers on chromosome 4p16 placed *DFNA38* between D4S2354 and D4S2983 (Fig. 2). The critical proximal and distal recombinants both appeared in unaffected individuals (IV:12 and V:15). Normal hearing was confirmed in these individuals at ages 33 and 29, respectively (data not shown). The region linked to *DFNA38* is ~1.5 Mb and overlaps the region linked to *DFNA14* and possibly that linked to *DFNA6* (6).

The *DFNA38* critical region of 4p16 includes *WFS1* (UCSC Human Genome Project Working Draft, WWW URL: <http://genome.ucsc.edu/>). Although Wolfram syndrome is recessive and includes many clinical features not present in deaf relatives of Family C, *WFS1* was a good candidate for *DFNA38* for two reasons: hearing loss can be a component of Wolfram syndrome (7) and mouse *WFS1* is expressed in cochlea (NIDCD Inner Ear Expression Database, WWW URL: <http://www.mgh.harvard.edu/depts/coreylab/genomics.html>). The *WFS1* sequence (GenBank accession no. AF084481) comprises eight exons spanning 33 kb of genomic sequence; translation begins in exon 2 (3,4). We sequenced genomic DNA from affected and unaffected individuals for the entire *WFS1* transcript and 50–100 bp flanking each exon. Eleven variant sites in *WFS1* were detected in all deaf individuals. Five variants were known polymorphisms; six were apparently novel (Table 1). Of the six previously unreported variants, three were amino acid substitutions: Gly107Arg, Ala716Thr

and Val871Met. Each of these appeared to cosegregate with deafness.

Amino acid substitutions, many of them individually rare, occur throughout *WFS1* (2–4,8–12). It is often not clear whether a specific amino acid substitution is associated with any disease phenotype, either Wolfram syndrome or any of the other conditions postulated to be associated with *WFS1*. The multiple rare variants in *WFS1* shared by deaf individuals in Family C presented two questions. Is *WFS1* associated with hearing loss in the family, and if so, which mutation is responsible?

Features of Wolfram syndrome in a homozygote

The likelihood that *WFS1* is responsible for disease in Family C was increased by the clinical phenotype of VI:3, who is homozygous for the haplotype linked to *DFNA38* (Fig. 2). Medical records of VI:3, now age 25 years, were thoroughly reviewed and he was evaluated for characteristics of Wolfram syndrome. According to his medical history, VI:3 was diagnosed with type I diabetes at age 3 years and has difficulty controlling his blood glucose levels. He had cataracts removed from his left eye at 13 years and right eye at 18 years of age.

On his recent clinical visit, dilated fundoscopic examination showed that VI:3 does not have optic atrophy but has mild non-proliferative diabetic retinopathy. Tonal audiometry revealed mild sensorineural hearing loss (20–30 dB) at 500 and 4000 Hz. The neurologic exam was completely normal. Renal ultrasound was normal. In summary, VI:3 has features of Wolfram syndrome, specifically juvenile diabetes mellitus and cataracts, but lacks the optic atrophy that is part of the definition of the complete syndrome. The attenuated disease phenotype of VI:3 may be due to the fact that the *WFS1* variants for which he is homozygous are missense mutations, whereas patients with complete, severe Wolfram syndrome are generally homozygous or compound heterozygous for protein-terminating mutations.

Ancestral haplotype of *WFS1* in unaffected individuals in Family C

In order to determine which, if any, of the *WFS1* variants shared by all deaf members of Family C were specific to disease, rather than rare neutral variants, we sequenced *WFS1* in informative relatives in Family C. *WFS1* sequences of two hearing individuals, V:8 and her daughter VI:1, were identical to the sequence shared by all deaf relatives at 10 of 11 variant sites (Table 1). The haplotype carried by V:8 and VI:1 is likely to be the ancestral chromosome on which the 2146 G→A mutation occurred. The haplotype apparently entered the kindred a second time by marriage into the family of a hearing individual distantly related to the founders of Family C (Fig. 2).

The only *WFS1* mutation specific to deaf individuals in Family C was 2146 G→A, which leads to substitution of threonine for alanine at codon 716 (Fig. 3). In wolframin, alanine at codon 716 is conserved in mouse (GenBank accession no. P56695) and rat (GenBank accession no. AAF61423) and is part of the putative intracellular C-terminus after the 10th transmembrane domain. None of the 150 unrelated individuals from Newfoundland with normal hearing carried the Ala716Thr mutation.

The nuclear family comprised of IV:13, V:8, VI:1 and VI:2 illustrates how the presence of an ancestral haplotype

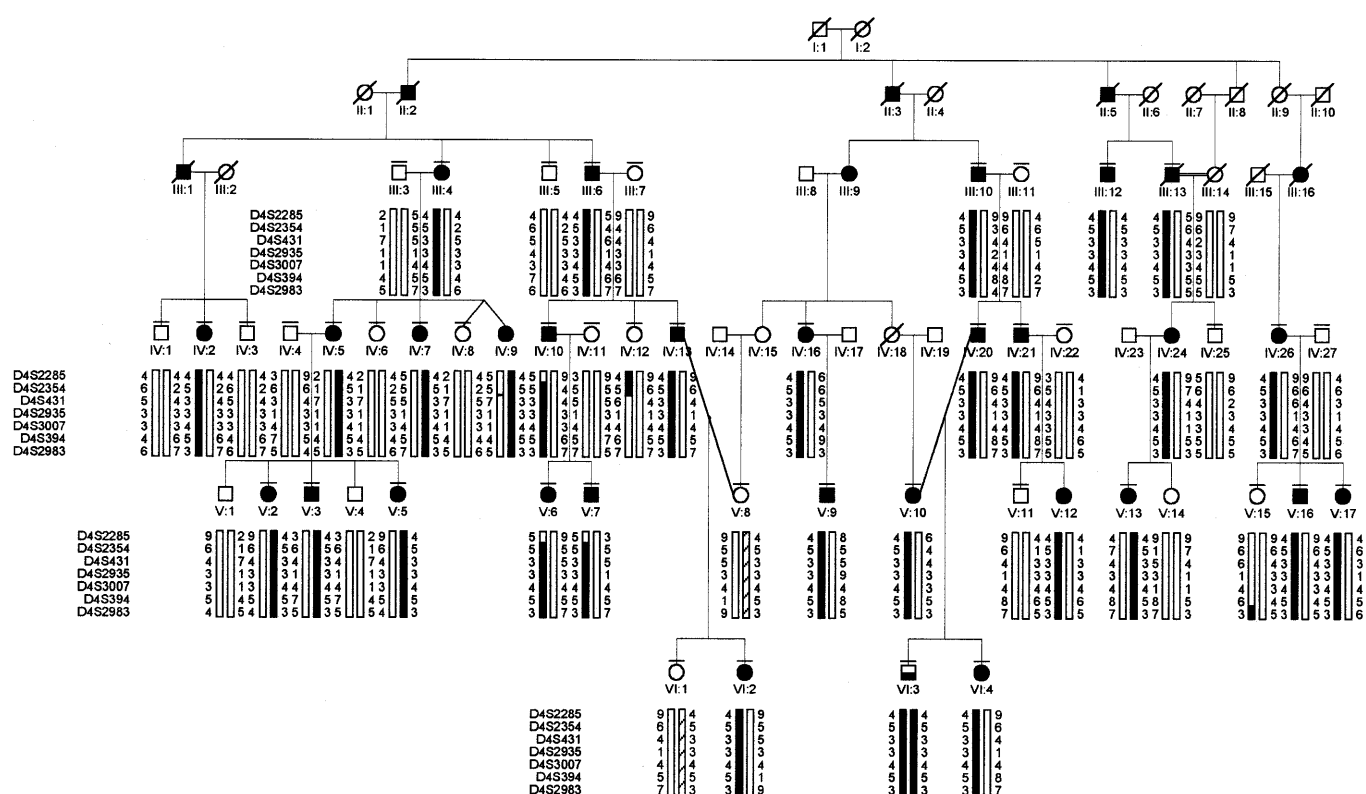


Figure 2. Pedigree of a portion of Newfoundland Family C demonstrating linkage of dominant, non-syndromic, progressive hearing loss to a 1.5 Mb region of chromosome 4p16. Dashes above symbols indicate hearing testing by pure tone audiometry. Hearing of untested individuals was evaluated by interview and/or medical history. Hearing status of I:1, I:2, II:8 and II:9 are not known. Black bars represent the haplotype co-segregating with deafness. Hatched bars represent the homologous ancestral haplotype that does not co-segregate with deafness, but has identical alleles at all linked marker sites. Critical recombinations were at D4S2354 (IV:12) and at D4S2983 (V:15). Individual VI:3 is homozygous for the deafness-linked haplotype and has clinical features of Wolfram syndrome.

homologous to a mutant haplotype can pinpoint which amino acid substitution among many could be associated with disease. Nuclear family members IV:13 and VI:2 carrying the *DFNA38* haplotype are deaf, whereas family members V:8 and VI:1, who carry the ancestral haplotype that differs from *DFNA38* in only one *WFS1* base pair, have normal hearing (Fig. 4).

The very severe deafness of VI:2 at a young age illustrates the variability of this phenotype among different members of Family C despite the same mutation in *WFS1*. In order to evaluate whether a mutation in mitochondrial DNA might influence expression of *DFNA38*, we screened deaf individuals in the family for mtDNA mutations postulated to be associated with hearing loss: A1555G, A3243G, A7445G, 7472insC, T7510C, T7511C, T7512C and 961_TC(n)ins (13). All family members were wild-type at all these sites.

WFS1 sequence of the tilted (*tl*) mouse

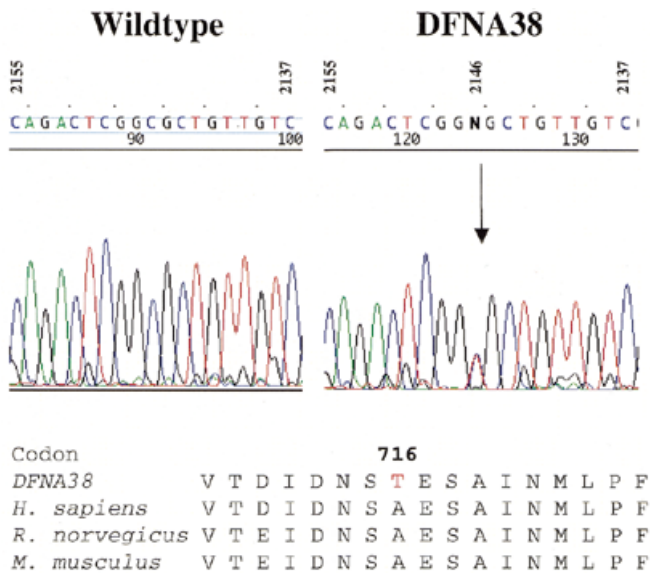
The mouse mutant *tilted* (*tl*) involves hearing loss and maps between D5Mit421 and D5Mit353, a region syntenic to the portion of human chromosome 4p16 that overlaps the *DFNA38* critical region (14). *Wfs1* coding regions, 5'- and 3'-UTRs, and flanking splice junctions were sequenced in DNA from *tl/tl* mice and from the parental C57BL/6J-*tl* strain and from wild-type *Mus musculus castaneus*. Sequences were wild-type in all three strains. *Wfs1* does not appear to be responsible for *tilted*.

DISCUSSION

In the past 5 years, the convergence of publicly available genome resources and the characterization of large informative kindreds has led to the discovery of 24 genes that control human hearing (Hereditary Hearing Loss Homepage, WWW URL: <http://dnalab-www.uia.ac.be/dnalab/hhh>). Historically isolated populations with traditions of large families, birth and marriage records, and well organized public health systems have been an especially rich source of kindreds for genetic studies. Family C from Newfoundland perfectly fits this profile. Newfoundland is an island province of Canada, 111 390 km² in size with a population of only 550 000. The population is highly endogamous and has been a collection of genetic isolates for ~200 years (15,16). Nearly all Newfoundland residents are descendants of persons who immigrated from England or Ireland in the early 1800s and settled in small coastal fishing communities ('outports') along the nearly 10 000 km coastline (17). Until very recently, virtually all transportation in Newfoundland was by boat, so most marriages were between members of the same or nearby outports. Families were large: Newfoundlanders now age 30 years or older have an average of eight siblings. Church records of marriages, births and deaths and public census data from 1621 to the present are accessible through public archives. Newfoundland geneticists have taken advantage of the historical demography of their province to identify genes

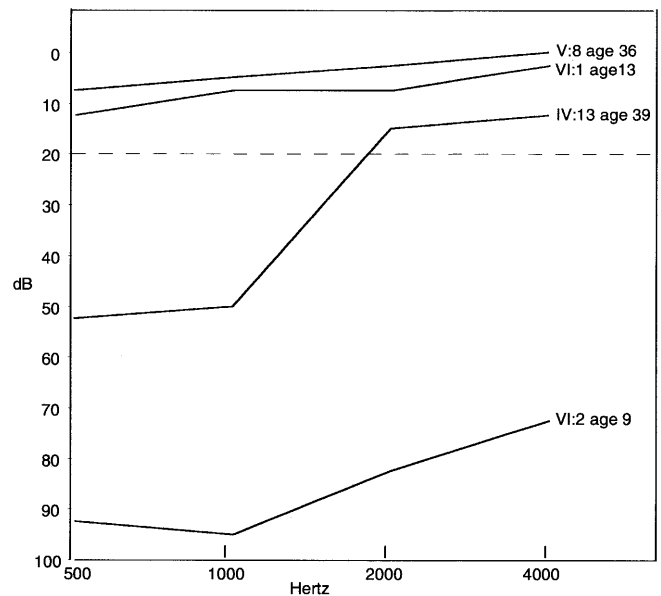
Table 1. Variant sites of *WFS1* shared by *DFNA38* and the homologous ancestral haplotype of Family C

<i>WFS1</i> nucleotide (1 = A of ATG)	Wild-type	<i>DFNA38</i> haplotype	Haplotype of hearing relatives V:8 and VI:1	Effect	Frequency of variant
-6 (+84)	T	C	C	Intronic	Private
319	G	A	A	G 107 R	Private
460 (-9)	A	G	G	Intronic	Private
997	G/A	A	A	I 333 V	$f(A) = 0.41$ in British (10)
1122	T	C	C	F 374 F	Private
1185	C/T	T	T	V 395 V	$f(T) = 0.50$ in British (10)
1674	G/A	A	A	R 611 H	$f(A) = 0.54$ in British (10)
2146	G	A	G	A 716 T	Variant associated with deafness
2433	A/G	G	G	K 811 K	$f(G) = 0.56$ in British (10)
2565	A/G	G	G	S 855 S	$f(G) = 0.35$ in British (10)
2611	G	A	A	V 871 M	Private

**Figure 3.** Mutant and wild-type sequences of *WFS1*. Portion of the electropherogram for *WFS1* exon 8 reveals a C→T transition at nucleotide position 2146 (arrow, reverse strand shown) changing an alanine to a threonine at codon 716. All hearing impaired individuals in the family are heterozygous for the mutation; III:6 is shown. All hearing individuals are wild-type at nucleotide position 2146: V:8 is shown. The *DFNA38* mutation lies in a highly conserved region of the *WFS1* gene.

critical to a wide variety of human diseases (18–20). In the same way, Family C offered the opportunity to identify a gene for inherited non-syndromic deafness.

Positional cloning of *DFNA38* in Family C led to a small genomic region including *WFS1*, and *WFS1* in Family C carried four novel amino acid substitutions co-segregating with deafness. Attribution of a pathogenic role to missense mutations is difficult in the absence of a functional assay. There is not yet an assay for wolframin function, nor a mouse model for loss of wolframin function. Fortunately, however, Family C included individuals whose genotypes and phenotypes provided clues to the significance of missense alleles. In

**Figure 4.** Audiograms associated with the *DFNA38* haplotype versus the homologous ancestral haplotype not linked to deafness. Affected individuals IV:13 and VI:2 are heterozygous for the Ala716Thr mutation in *WFS1*. In contrast, unaffected individuals V:8 and VI:1 inherit a haplotype that is wild-type at *WFS1* codon 716 but otherwise identical to the *DFNA38* haplotype at the *WFS1* locus.

Family C, a homozygote for a novel missense mutation in *WFS1* had clinical features of Wolfram syndrome, suggesting that *WFS1* is the relevant disease gene in this family. Furthermore, the only detectable sequence difference between a haplotype associated with deafness and a homolog not associated with deafness is the 2146 G→A mutation. Together, these features of Family C suggested that *WFS1* Ala716Thr is responsible for *DFNA38* hearing loss. It is formally possible that homozygosity for this mutation leads to juvenile diabetes mellitus, but that an unknown mutation in another gene in the *DFNA38* region leads to hearing loss among the Family C heterozygotes. However, were this the case, one would need to explain why

this hypothetical second allele does not lead to severe deafness in a homozygote. On the other hand, there is a precedent that homozygosity for disease mutations in *WFS1* may lead to mild to moderate hearing loss in the presence of Wolfram syndrome (7).

Wolfram syndrome can be caused by homozygosity or compound heterozygosity at multiple mutations of *WFS1*: 15 different nonsense mutations, 12 insertions or deletions leading to frameshifts, eight inframe deletions, splicing errors and deletion of the entire gene have been described in patients (2–4,8,10,12,21). With the exception of a single allele shared by most affected Spanish families (10), mutations are specific to one or a few families. At least 19 missense alleles have been identified in Wolfram patients, most of them in the same C-terminal domain of wolframin as Ala716Thr. Some of these other amino acid substitutions are likely to be disease alleles; others may be rare neutral variants, analogous to those on the ancestral hearing-associated haplotype in Family C.

Deafness is not generally a presenting feature of Wolfram syndrome patients, who are usually first seen for very early onset diabetes mellitus and optic atrophy. Among Wolfram syndrome patients who live to adulthood, hearing loss develops in most by age 40 years (21). Obligate heterozygotes generally hear normally (21). The one previously noted exception to normal hearing among carriers is a Japanese family in which Wolfram syndrome is due to homozygosity for a deletion of five amino acids, del508–513 (22). In this family, eight of 17 obligate heterozygotes older than age 20 years had hearing loss >20 dB (i.e. at least mild hearing loss). Hearing loss due to del508–513 was detected at low and high frequencies, but not at middle frequencies. In contrast, hearing loss due to Ala716Thr is fully penetrant, and more severe, with a different audiometric pattern.

Despite the fact that only insulin-dependent diabetes mellitus and bilateral progressive optic atrophy are necessary to make the diagnosis of Wolfram syndrome, many more symptoms may be associated with the disorder. Most of these variable symptoms are a consequence of neurological deficit due to degeneration of specific areas in the central nervous system (23,24). Expression of *Wfs1* in cochlea and the involvement of a specific amino acid substitution with maintenance of hearing are consistent with these observations. The progressive nature of *DFNA38* hearing loss suggests a role for wolframin in maintenance of sound transduction, rather than in the development and maturation of the cochlear cells.

Perhaps paradoxically, it may be reasonable to suggest that *WFS1* plays a role in age-related hearing loss or presbycusis. Mutations in *WFS1* have been identified in pedigrees with low frequency hearing loss. Presbycusis typically affects high frequencies. However, the latter stages of hearing loss among members of Family C, after low frequency hearing loss has occurred, closely resemble the pattern of high frequency loss associated with presbycusis. It may be revealing to evaluate whether 'mild' mutant alleles of *WFS1* are associated with age-related hearing loss.

MATERIALS AND METHODS

Family data

Family C was ascertained through the Newfoundland and Labrador Medical Genetics Program and Memorial University

of Newfoundland, St John's, Newfoundland, Canada. The founder of Family C migrated from England to Newfoundland in the early 1800s, settling in a tiny coastal community (population <1000) where many family members still live (25). After obtaining informed consent from each individual, family and medical history were collected from 314 informative relatives and pure tone audiometric evaluations performed on 81 relatives. Sixty-two family members were identified with low frequency hearing loss. Following consent, peripheral blood was collected by venipuncture from 83 individuals.

Linkage analysis

DNA was isolated using a salting out extraction method (26). Forty-seven informative family members were included in a genome-wide scan using 386 polymorphic markers from the ABI Prism Linkage Mapping Set-MD10. PCR products were size fractionated on an ABI 377 sequencer machine and alleles were scored with Genotyper software (v. 2.0 Applied Biosystems). Two-point linkage analyses were performed using the MLINK subroutine of FASTLINK (V4.0P) (27). Deafness was modeled as a fully penetrant autosomal dominant trait with a frequency of 0.001. Additional polymorphic markers were genotyped on family members in the vicinity of D4S431 to define the *DFNA38* critical region.

Sequence analysis

Coding sequence, regulatory regions and flanking intronic sequence of candidate genes were sequenced from genomic DNA via the dideoxy chain termination method using dye terminator and dye primer chemistries. Because the A716T mutation abolishes an *HaeII* restriction enzyme site, all family members who were not included in the genome scan were tested for the mutation by restriction enzyme analysis using *WFS1* specific primers. Restriction digest analysis was also used to test for mitochondrial mutations associated with hearing impairment: A1555G, A3243G, A7445G, 7472 ins C, T7510C, T7511C, T7512C, and 961_TC (n) ins (13).

Clinical analysis

Clinical evaluation of individual VI:3 for features of Wolfram syndrome included physical examination, ophthalmologic and audiologic assessments and renal ultrasonography (21,28).

DNA from *tlr* mice

Genomic DNA from the F₂ *tlr/tlr* progeny, parental C57BL/6J-*tlr* and wild-type *M.musculus castaneus* mice was obtained from the Jackson Laboratory (Bar Harbor, ME) and amplified with *WFS1*-specific primers. Sequencing was carried out as described above.

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