Phenotype-Genotype Correlations in a Series of Wolfram Syndrome Families

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OBJECTIVE — Wolfram syndrome is an extremely rare autosomal-recessive disorder that predisposes the development of type 1 diabetes in association with progressive optic atrophy. The genetic basis of this disease has been shown to be due to mutations in the WFS1 gene. The WFS1 gene encodes a novel transmembrane protein called wolframin, which recent evidence suggests may serve as a novel endoplasmic reticulum calcium channel in pancreatic β-cells and neurons. Genotype-phenotype correlations in this syndrome are becoming apparent and may help in explaining some of the variable characteristics observed in this disease.

RESEARCH DESIGN AND METHODS — In this report, we have studied 13 patients with Wolfram syndrome from nine families to further define the relationship between mutation site and type with specific disease characteristics.

RESULTS — A severe phenotype was seen in patients with mutations in exon 4 and with a large deletion encompassing most of exon 8. In total, nine novel mutations were identified as well as three new silent polymorphisms.

CONCLUSIONS — Similar to all other mutation reports, most causative changes identified in the WFS1 gene occurred in exon 8, and only one was identified outside this region in exon 4.

In 1998, a nuclear gene for Wolfram syndrome, WFS1, was discovered and mapped to chromosome 4p16.1 by positional cloning (12,13). The WFS1 gene is composed of eight exons spanning 33.4 kb of genomic DNA. The 3.6-kb mRNA encodes an 890–amino acid protein named wolframin (12) with nine predicted transmembrane domains belonging to a novel gene family. Biochemical studies in cultured cells indicate WFS1 to be an integral, endoglycosidase H-sensitive membrane glycoprotein that primarily localizes in the endoplasmic reticulum (14). Recent evidence suggests that WFS1 is either a novel endoplasmic reticulum calcium channel or a regulator of channel activity (15).

In the present study, we performed mutational analysis of the WFS1 gene in a series of 13 patients from nine families. The aim was to determine the spectrum of WFS1 mutations in this cohort and to establish any correlation between genotype and observed phenotypes.

RESEARCH DESIGN AND METHODS — DNA samples were collected from 13 affected individuals (7 women and 6 men) and 10 available relatives. The minimum criteria for diagnosis of Wolfram syndrome were type 1 diabetes and optic atrophy. The clinical features of the affected individuals are summarized in Table 1. The study was performed with the approval of the Hunter Area Research Ethics Committee and with the informed consent of each patient and/or their parents.

Genomic DNA was isolated from EDTA whole blood by the salt precipitation method (16). PCR amplification was performed using primers previously described (12), except for the following: 1F 5′-tcg tgc aga agg ccc ggc tag-3′, 1R 5′-aag agg aca gtc cct cag gg-3′, 8-6F 5′-ggg cag agt cgg cgg c-3′, 8-6R 5′-agg gcc acc tgg agg gc-3′, 8-6R 5′-agg gcc act tgc tcc cca ggc-3′, and 8-7F 5′-agg cct gct gct cag ctt gcg-3′. The PCRs were performed in a 25-μl volume containing 100 ng DNA; 0.8 μmol/l of each forward and reverse primer; 200 μmol/l each of dATP, dCTP,
<table>
<thead>
<tr>
<th>Family and patient number</th>
<th>Age (years)</th>
<th>Sex (M/F)</th>
<th>Diabetes mellitus</th>
<th>Optic atrophy acuity</th>
<th>Diabetes insipidus</th>
<th>Hearing loss</th>
<th>Renal tract abnormalities</th>
<th>Neurologic abnormalities</th>
<th>Other complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>F</td>
<td>8 years</td>
<td>7 years</td>
<td>—</td>
<td>6 years</td>
<td>—</td>
<td>Swallowing defect</td>
<td>Legally blind at 16 years</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>F</td>
<td>12 years, 9 months</td>
<td>14 years, 6/36</td>
<td>14 years</td>
<td>—</td>
<td>—</td>
<td>Central sleep apnea at 15 years</td>
<td>Depression requiring therapy</td>
</tr>
<tr>
<td>3I</td>
<td>17</td>
<td>F</td>
<td>10 years</td>
<td>13 years, 20/40</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>3II</td>
<td>18</td>
<td>M</td>
<td>6 years</td>
<td>14 years, 20/40</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>M</td>
<td>4 years, blind 16 years</td>
<td>14 years, 20/40</td>
<td>14 years</td>
<td>16 years</td>
<td>Bladder dysfunction at 21 years, suprapubic catheter at 27 years</td>
<td>One choking episode requiring hospitalization</td>
<td>Depression and aggression, collapsed and died of intracerebral hemorrhage, Manic episodes and depression, In psychiatric care with depression and psychosis, Aggressive behavior, Poor school performance</td>
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<tr>
<td>5</td>
<td>27</td>
<td>F</td>
<td>12 years</td>
<td>13 years</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>44</td>
<td>F</td>
<td>28 years</td>
<td>42 years</td>
<td>—</td>
<td>—</td>
<td>Urinary incontinence</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>M</td>
<td>7 years</td>
<td>6 years</td>
<td>—</td>
<td>8 years</td>
<td>—</td>
<td>—</td>
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<tr>
<td>8I</td>
<td>18</td>
<td>F</td>
<td>11 years</td>
<td>11 years</td>
<td>—</td>
<td>18 years</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8II</td>
<td>13</td>
<td>M</td>
<td>9 years</td>
<td>9 years</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9I</td>
<td>21*</td>
<td>M</td>
<td>5 years</td>
<td>3.5 years</td>
<td>18 years</td>
<td>—</td>
<td>Enuresis</td>
<td>Epilepsy from 6 years, Central sleep apnea at 10 years, Brain stem atrophy at 10 years, Dementia at 19 years</td>
<td>—</td>
</tr>
<tr>
<td>9II</td>
<td>26*</td>
<td>M</td>
<td>11 years, 7 months</td>
<td>11 years, 7 months</td>
<td>—</td>
<td>—</td>
<td>Bladder dysfunction</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9III</td>
<td>16*</td>
<td>F</td>
<td>6 years, 8 months</td>
<td>6 years, 8 months</td>
<td>Partial</td>
<td>—</td>
<td>Enuresis, resolved with DDAVP</td>
<td>Central sleep apnea at 8 years, Brain stem atrophy, Epilepsy at 13 years</td>
<td>Arhythmia; Cause of death, Psychiatric problems, suicidal depression, Eating disorder, Psychiatric problems, Eating disorder</td>
</tr>
</tbody>
</table>

*Death of patient.
dGTP, and dTTP; 1.0 or 1.5 mmol/l MgCl₂; 1× PCR buffer (20 mmol/l Tris-HCl [pH 8.4] and 50 mmol/l KCl); and 0.5 units, platinum Taq (Invitrogen, Rockville, MD). PCR amplification was achieved by an initial denaturation step of 94°C for 5 min, followed by 14 cycles of 94°C for 30 s, 7°C touchdown range decreasing at 0.5°C/cycle for 45 s, and 72°C for 1 min, then 20 cycles using an annealing temperature 0.5°C lower than the bottom of the touchdown range, with a final extension step of 72°C for 10 min. This was followed by a final denaturation step of 94°C for 2 min and a slow annealing step from 94°C to 60°C over 30 min to promote heteroduplex formation. All PCRs were performed on an OmniGene Thermal Cycler (Hybaid, Middlesex, U.K.).

Denaturing high-performance liquid chromatography was performed on a Varian Helix system (Varian, Walnut Creek, CA) equipped with a Helix column (Varian). The PCR products were examined for heteroduplexes by injecting 3 μl of each PCR product directly onto a preheated column. The oven temperature for optimal heteroduplex separation under partial DNA denaturation was determined using the DHPLC melt program available at http://www insertion.stanford.edu/ melt.html. PCR fragments were eluted from the column by an increasing acetonitrile gradient. Sequence variations were detected by comparing the patient elution profiles to a wild-type control subject.

For each abnormal elution profile, the PCR products were directly sequenced in both directions. Sequencing was performed using a BigDye Terminator Cycle Sequencing Reaction kit and analyzed on an ABI 310 automated sequencer (Applied Biosystems, Foster City, CA).

**RESULTS** — Ten affected individuals from six families and 3 individual patients were studied. Four families (numbers 1, 2, 7, and 9) and two unrelated patients (family 4 and family 5) were from Australia; however, family 7 was of Spanish descent. One Canadian family (number 3), one patient from Punjab, India (family 6), and one Kurdish family from Iraq (family 8) were also studied. The clinical characteristics of the affected patients are detailed in Table 1. In family 9, only one mutation had been previously identified (13).

The entire WFS1 gene was screened for mutations, including the noncoding exon 1, by DHPLC and sequencing. Mutation analysis revealed a total of 19 different mutations, 9 of which are novel (Table 2). These include 3 deletions, 2 insertions, 4 nonsense mutations, and 10 missense changes. Only two of the mutations were found outside of exon 8. Apart from one consanguineous family (family 8), all other patients were compound heterozygotes.

Deletions were found in three of the families studied. A very large novel deletion was discovered in family 9. A heterozygous 2,514-bp deletion was revealed, starting 1,357 bases before the start of exon 8 and eliminating 1,157 nucleotides (64%) of the coding region of exon 8. This resulted in the predicted loss of exon 8 and subsequently the entire predicted transmembrane domains and the hydrophilic carboxy tail of the WFS1 protein. The clinical characteristics of this family will be described in its entirety in a separate report. In family 1, an in-frame deletion of three nucleotides at position 1243 was found, removing a valine residue at codon 415. A heterozygous TC deletion at nucleotide 2642 was detected in family 5, resulting in the removal of the normal stop codon at 891 and an elongated protein of 937 amino acids.

Two insertions were detected in this series. A novel homozygous in-frame insertion of nine nucleotides (GC CTT CTTC) at position 1032 was detected in family 8. This resulted in the insertion of three amino acids (AFF) at codon 344. The parents of the affected individuals in this family are known to be cousins. Family 7 harbored a 16-bp insertion at position 425, resulting in a frameshift at residue 141 and premature termination of the protein at codon 251. This mutation has been previously described in patients of Spanish origin (17).

A total of four nonsense mutations were detected in the families analyzed. A novel nonsense mutation in exon 4, W129X, was found in family 7. This mutation predicted a severely truncated protein of 129 amino acids, lacking both the transmembrane domains and the hydrophilic carboxy terminus of the WFS1 protein. A G1838A substitution in family 6 has been previously described in patients with NCLA (18).

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**Table 2—Mutations in the WFS1 gene**

<table>
<thead>
<tr>
<th>Family</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Type of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>1243-1245delGTC</td>
<td>V415del</td>
<td>Deletion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1517T&gt;G</td>
<td>L506R</td>
<td>Missense</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>2327A&gt;T</td>
<td>E776V</td>
<td>Missense</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>2578C&gt;G</td>
<td>H860D</td>
<td>Missense</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>1673G&gt;A</td>
<td>R558H</td>
<td>Missense</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>2590G&gt;T</td>
<td>E864X</td>
<td>Nonsense</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>2100G&gt;T</td>
<td>W700C</td>
<td>Missense</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2254G&gt;T</td>
<td>E752X</td>
<td>Nonsense</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>1838G&gt;A</td>
<td>W613X</td>
<td>Nonsense</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>425-426ins16bp</td>
<td>A141-V142ins-fsX251</td>
<td>Frameshift/truncation</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>1511C&gt;T</td>
<td>P504L</td>
<td>Deletion</td>
</tr>
</tbody>
</table>

Novel mutations are in boldface. *On the same allele as 387G>A; †mutation only present in father’s DNA; ‡homozygous mutation.
A novel heterozygous missense mutation, H860D, was found in the affected proband from family 2. This mutation was not detected in either parent and is likely to have arisen sporadically. Several other missense mutations were also found, all within exon 8 of the WFS1 gene. These include L506R in family 1, E776V in family 2, R558H in family 3, W700C in family 4, A559T in family 5, A671V in family 6, and P504L in family 9. Another missense change, T449I, was detected in family 7 and was present on the same chromosome as the W129X mutation. An R818C change was found in both family 7 and family 8. This change was identified in the father of the proband from family 7 but was not on the transmitting allele in the proband.

Sequencing analysis also showed 17 polymorphisms in the individuals analyzed (Table 3). These were composed of 2 intronic variants, 12 silent variants, and 3 missense variants that resulted in a conservative amino acid change. Nine of the 17 polymorphisms (53%) occurred at a frequency >5%, suggesting that they are common variants, and 4 of the changes (23.5%) were deemed to be rare, occurring at a frequency <5%. The remaining four polymorphisms were not assigned a population frequency but were considered rare because they have not previously been identified (Table 3).

The data collected were analyzed for any relationship between the genotype and observed phenotypes of affected individuals. Several interesting observations were noted. Two families in our study (family 7 and family 9) have children who have developed neurodegenerative features before adolescence, whereas these are not usually seen until the third or fourth decade. The single affected child from family 7 harbored a nonsense mutation and a frameshift insertion, both in exon 4. These mutations resulted in severely truncated proteins of 129 and 251 amino acids, respectively, which lack both the nine predicted transmembrane domains and the hydrophilic COOH-terminus of the WFS1 protein. The patient suffers from a severe form of Wolfram syndrome including central and obstructive sleep apnea, which were diagnosed at a very young age. Interestingly, his father has diabetes.

The three affected children from family 9 carried the missense change, P504L, in a predicted transmembrane domain and a 2,514-bp deletion, removing the last 1,357-bp of intron 7 and 1,157-bp (64%) of the coding region of exon 8. This family also has a very severe phenotype.

CONCLUSIONS — Similar to all other mutation reports, most of the causative changes identified in the WFS1 gene occurred in exon 8, and only one was identified outside of this region in exon 4. There were a number of unique changes identified in exon 8, including 4 nonsense changes, 10 missense changes, and 3 deletions, 1 of which encompassed a 2,514-bp region starting in intron 7 and extending into codon 673 of exon 8. This large deletion was predicted to result in a null allele, because the stop codon at the end of exon 8 would not be recognized. The other deletion has previously been reported and results in an in-frame deletion that is presumed to alter one of the transmembrane domains in the wol-
framin protein (see Fig. 1). Of the four nonsense mutations, three were identified for the first time and one (E752X) has been reported previously in a British patient (20). The three novel mutations were distributed across the entire gene. Intriguingly, the E864X mutation removed the last 26 amino acids of the protein, implying that this section of the protein is important for its function. The remaining missense changes all occurred in exon 8 and are presumed to affect the function of the protein.

A series of silent polymorphisms, most of which have been identified previously, were also present in the patient series reported herein. Because 53% were identified multiple times, they were considered to be common polymorphisms that did not affect protein function, even if they were associated with amino acid changes. The remaining rare polymorphisms and those that have not been identified previously are problematic because they cannot be unequivocally determined as causative. Until a functional assay is established to assess these polymorphisms, they should be considered suspicious.

One of the striking features of our clinical series is the severity of the phenotype in two families. We have defined “severe” as the development of neurodegenerative changes in the first decade. Brain stem atrophy causing central sleep apnea that requires ventilation does not usually develop until the third or fourth decade (21). In our index family reported in 1998 (13), the extent of the maternal deletion has now been defined and shown to encompass the whole of exon 8. Two of the three affected siblings died from brain stem atrophy before 21 years of age. In the third affected sibling, diabetes developed at 12 years of age, 6 years later than in the other two siblings, and in his mid-20s, no brain stem symptoms had yet developed. On haplotype analysis, this subject had a genetic recombinant event distal to the WFS1 gene. The recombinant event seems to have protected him from the severe phenotype or hints that there is some other modifying effect. Nevertheless, he died at 26 years of age after a fulminating illness with pneumonia and secondary adrenal insufficient. The second Australian family with a severe phenotype has a child with central sleep apnea that began at 8 years of age. He has two mutations in exon 4 (one of these being the common Spanish mutation, a 16-bp insertion) resulting in a severely truncated protein. It has recently been reported that 7 patients who were homozygous for the Spanish mutation had a mean age at onset of diabetes of 5.3 years, compared with 13.5 years in the 10 patients with other mutations (17). The presence of severely truncating mutations seems to correlate with a particularly severe phenotype, which is likely to be related to the complete loss of function of the WFS1 gene product. A high index of clinical suspicion is needed to diagnose central sleep apnea, and assessment by sleep study is recommended.

Sequencing studies of Wolfram kinds have shown that some mutations are more common in certain ethnic groups. One of our patients was believed to have Italian heritage, but after we identified a known Spanish mutation (family 7), the family insisted that the “Italian” soldier in a family photograph only to discover he was in a Spanish uniform. This suggests there is a founder effect in some populations.

We identified two mutations in all clinically affected patients in our series, but some series have failed to identify a second mutation in every case. Initially, the primer 8-7F from Strom et al. (12) was used for amplifying the last section of exon 8. However, this primer potentially results in the amplification of only one allele by PCR. To circumvent this problem, a new primer was designed 15 bases downstream from the original primer, which was capable of amplifying both strands by PCR. Two mutations (H860D and 2642delTC) were initially missed in this study, and one mutation (E864X) seemed to be homozygous when the original 8-7F primer was used. Several studies have used the original primers from Strom et al. (12) for not only mutational analysis of patients with Wolfram syndrome but also studies attempting to determine the significance of psychiatric illness and deafness in heterozygous carriers. A significant proportion of mutations could have been missed, thereby underestimating the importance of the WFS1 gene in the development of psychiatric illness and deafness.

Diabetes mellitus is usually the first symptom to present at a median age of 6 years, followed by onset of optic atrophy at a median age of 11 years (21). In our series, the average age at onset of diabetes was 8.4 years (excluding the patient from family 6 because we were uncertain of the exact age of onset of diabetes). The de-
WFS1 mutations in DIDMOAD patients

opment of polyuria and/or enuresis can indicate diabetes insipidus, which does not usually appear until the second or third decade and may initially be partial. Therapy with desamino d-arginine vasopressin (DDAVP) can be very successful, even if the urine osmolality is >700 mOsmol/kg.

In our many years of managing some of these families, it is apparent that some patients develop other features of hypothalamic dysfunction in addition to the most obvious one of diabetes insipidus. Menstrual disturbances are common, but some of the older women have had successful pregnancies. Central hypothyroidism was seen, as well as documented secondary adrenal insufficiency in one patient. This latter problem may be easily overlooked when there are so many other pressing medical problems, and measurement of serum cortisol is important. We have also seen cardiac arrhythmias (paroxysmal atrial tachycardia). It is unclear whether this is an intrinsic cardiac defect, because wolframin is expressed in the heart (13), or whether it is related to central autonomic instability.

Optic atrophy was often detected at the same time as diabetes or was symptomatic before the onset of diabetes (average age of diagnosis 9.75 years). Occasionally, optic atrophy was diagnosed by an ophthalmologist during routine screening for diabetic retinopathy (family 3). Although visual acuity deteriorates mainly on the basis of optic atrophy, some children may have an element of myopia, which is correctable with eyeglasses (family 9).

Deafness due to high-tone hearing loss was reported in five patients, but in three patients it was a major clinical problem. One child (family 2) has profound deafness from infancy requiring a cochlear implant. Such early onset of severe deafness has not been reported in Wolfram syndrome. This child has also gone on to develop relatively early central sleep apnea at 15 years of age. Both of her mutations are in the COOH-terminus. Heterozygous mutations in this region have been found in patients with low-frequency sensorineural hearing loss. Intriguingly, only one of the two families with the severe phenotype had deafness (family 7).

Neuropsychiatric manifestations are reported in the third decade, but in some of our patients, behavioral problems or depression were evident at much younger ages. Two patients with significant psychiatric illness were found to harbor mutations detected previously in screening studies of psychiatric patients (18,19). One patient with mania had the mutation that has been associated with affective disorder (A559T). The second patient (A671V) has a mutation found in a study of suicide. In many cases, it can be difficult to tease out the effect of multiple disabilities and reactive depression from a true depressive illness. It is important to involve a pediatric psychiatrist as part of the multidisciplinary team, particularly if there is a seminal event such as a first seizure. Such events can have devastating psychological consequences for an adolescent.

The clinical course of Wolfram syndrome varies, and the reasons for this are only slowly emerging. Our results suggest that there is a correlation between genotype and phenotype. We found the strongest links so far to be in those patients with the most severe phenotype and severely truncated proteins and those changes in the COOH-terminus associated with deafness. Most mutations are found in exon 8. Although there is a wide variety of mutations, the literature suggests that there are certain “hot spots” related to psychiatric manifestations and deafness. The relative rarity of this condition also means that the number of kindreds able to be studied is limited. The ultimate correlation will be to determine the exact functional role of each region of the wolframin protein, so that specific targeted therapies can be developed to prevent or attenuate the long-term consequences of the disease.

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References


