Wolfram syndrome: a clinical and molecular genetic analysis

Philipp Eller, Bernhard Föger, Roland Gander, Teresa Sauper, Monika Lechleitner, Gerd Finkenstedt, Josef R Patsch

Wolfram syndrome (OMIM 222300) is a progressive neurodegenerative disorder characterised by the association of juvenile, non-autoimmune, insulin dependent diabetes mellitus and optic atrophy. The physician D J Wolfram, who reported four cases in 1938, is credited with the first description.1 With the identification of other clinical features, Wolfram syndrome was also referred to as DIDMOAD syndrome (diabetes insipidus, diabetes mellitus, optic atrophy and deafness). The initial manifestation of Wolfram syndrome is typically, but not invariably, insulin deficient diabetes mellitus at a median age of 6 years, followed by optic atrophy at 11 years.2 In the second decade, many patients develop central diabetes insipidus and sensorineural deafness. Additional, but less frequent neurological and endocrinological abnormalities are congenital blindness, ataxia, myoclonus, peripheral neuropathy, hypogonadism, and a relatively high incidence of depression and psychotic behaviour.3–5 Death occurs between 25 and 49 years (median 30 years).

The prevalence of Wolfram syndrome was estimated at 1/100 000 in a North American population and 1/770 000 in the United Kingdom.6 8 Family studies indicate autosomal recessive inheritance.

Genetic linkage analysis has localised Wolfram syndrome to the short arm of chromosome 4 (4p16),7 8 and the primary genetic defect of the syndrome was attributed to the WFS1 gene, which consists of eight exons encoding a protein of molecular weight of 100 kDa.9 10 Hydrophobicity analysis suggested a transmembrane protein comprising three structural domains: a hydrophilic amino terminus separated from a hydrophobic carboxy-terminal tail by a hydrophilic region containing nine predicted transmembrane segments. Homologous genes in Mus musculus, Rattus norvegicus, and Drosophila melanogaster have been described (GenBank AF084482/AJ011971, AF136378; FlyBase Y18064), consisting of exons 2–8, and amplified in the index patients with intronic primers, as described previously.10 11 Polymerase chain reaction (PCR) analysis was in a total volume of 20 μl containing 0.5 μmol/l of each forward and reverse primer, 0.5 U HotStarTaq polymerase (Qiagen), 1.5 mmol/l MgCl₂, and 200 μmol/l dNTP mix. After an initial activation step at 95°C for 15 minutes, cycling was performed at 94°C for one minute, 57°C for one minute, 72°C for one minute (30 cycles), followed by 10 minutes at 72°C in a T3 Thermocycler (Biometra). Dye modified primers were used in conjunction with a commercial dideoxy sequencing kit (Amersham Pharmacia) to obtain sample reaction products, which were electrophoresed in 4.3% acrylamide/7 mol/l urea Long Ranger gels (FMC Bioproducts) on a Licor 4000L fluorescent sequencer (MWG, Germany), and analysed by means of BaseImagIR version 4 software (Licor Biotechnologies).

The 1038ins(C) mutation was detected with a labelled PCR fragment on a denaturing polyacrylamide gel. As we could not directly sequence the PCR products in patient 2.1, a fragment of exon 8 containing the suspected mutation was subcloned into pCR2.1 vector (Invitrogen) as described by the manufacturer and at least four colonies sequenced for each allele. The 2315ins(T) mutation was also detected with PCR (primer 8-6, as previously described),12 digestion with BsaOI (Promega), and 2% agarose gel electrophoresis, with three bands in the wild type allele (165 bp, 128 bp, 64 bp), and two bands (229 bp, 128 bp) in the

Moreover, a recent publication suggested a further nuclear gene locus (chromosome 4q22-24) associated with Wolfram syndrome.13

In the present study, we report three cases of Wolfram syndrome carrying novel mutations of the WFS1 gene and describe mRNA heterogeneity in the 5′ non-coding region of the WFS1 gene, which is caused by alternative splicing.

Methods

Blood samples were collected from available family members after informed consent was obtained. For genotyping, DNA was extracted from peripheral blood lymphocytes using QIAamp DNA Blood kit (Qiagen). The entire WFS1 coding sequence (GenBank AF084481/Y18064), consisting of exons 2–8, was amplified in the index patients with intronic primers, as described previously.10 11 Polymerase chain reaction (PCR) analysis was in a total volume of 20 μl containing 0.5 μmol/l of each forward and reverse primer, 0.5 U HotStarTaq polymerase (Qiagen), 1.5 mmol/l MgCl₂, and 200 μmol/l dNTP mix. After an initial activation step at 95°C for 15 minutes, cycling was performed at 94°C for one minute, 57°C for one minute, 72°C for one minute (30 cycles), followed by 10 minutes at 72°C in a T3 Thermocycler (Biometra). Dye modified primers were used in conjunction with a commercial dideoxy sequencing kit (Amersham Pharmacia) to obtain sample reaction products, which were electrophoresed in 4.3% acrylamide/7 mol/l urea Long Ranger gels (FMC Bioproducts) on a Licor 4000L fluorescent sequencer (MWG, Germany), and analysed by means of BaseImagIR version 4 software (Licor Biotechnologies).

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Electronic letter

The 2638-2643del(GACTTC) mutation destroys a TaqI (Roche) cleavage site with an additional band of 212 bp in the mutant allele compared to the wild type (48 bp, 49 bp, 164 bp) site in a fragment of exon 8 (forward primer CCT CAA CTG CAT GGC CCA GC, reverse primer AGG TCT CTG CAG CCA CAG TC). The 1675G→A mutation eliminates a CfoI cleavage site (data not shown).20

Total RNA was extracted from peripheral lymphocytes using Rneasy kit (Qiagen). Poly A+ RNA of human pancreas, brain, heart, and lung was purchased from Clontech. First strand cDNA was synthesised using SuperScript II (Gibco BRL) for reverse transcriptase-PCR (RT-PCR). cDNA was subcloned into pCR2.1 vector (Invitrogen) and at least three colonies sequenced for each splice variant. In order to estimate the relative relationship of the two isoforms to each other, we performed a competitive PCR with the fluorescently labelled forward primer (IRD800-CCC GAA CAA CTT TTC TGC CG) and reverse primer (AGG CTG TGG CAT TGA GTC CAA CTT TTC TGC CG) and reverse primer (AGG CTG TGG CAT TGA GTC CAG CCA CAG TC). The PCR product was diluted to a final concentration of 5 pg/µl to guarantee an unsaturated signal-noise ratio. The splice variants of 171 and 167 bp were separated by polyacrylamide gel electrophoresis, and the signal intensity of the respective bands was measured by means of the Quantity One version 4.1.1 software (Biorad).

Statistical analyses were performed with SPSS version 8.0 software. Data are shown as means (SD) for the indicated number of experiments. The ratio of the splice variants in different tissues was compared by the one-way ANOVA analysis.

Patients

Patients were recruited at the local referral centre. Two unrelated families of Austrian descent were studied (table 1).

PATIENT 1.2

The 20 year old man presented with IDDM at the age of 6. Ophthalmological examination showed a progressive loss of visual acuity owing to bilateral optic atrophy (0.6 and 0.8 at the age of 7, 0.1, and 0.3 at the age of 13 for the right and left eye, respectively). Alcohol abuse precipitated several (hypoglycaemic) generalised seizures. Electroencephalographic recordings performed after the seizures were normal. GAD antibodies were normal (0.23 U/ml, reference values <6 U/ml), as determined at the age of 20.

PATIENT 2.1

The patient was a 22 year old woman, who developed IDDM at the age of 8 years. There was no family history of IDDM or consanguinity. Islet cell and glutamic acid decarboxylase (GAD-65) antibodies, as determined at the age of 21, were not detected. There was no macroalbuminuria and neurothesiometry and electromyography were normal. Interestingly, the patient underwent a cataract extraction of the right eye at the age of 2 even before the onset of diabetes mellitus, two operations for strabismus at the ages of 3 and 4, and a Nd:YAG posterior capsulotomy of the optic lens at the age of 18. She began to complain of progressive loss of visual acuity at the age of 16 and four years later bilateral atrophy of the optic nerves was detected. Neurological examination showed only low visual acuity, but cranial magnetic resonance imaging at 21 years showed atrophy of the median brainstem and the optic nerves. Additionally, there was a diffuse area of high signal on PD and T2 weighted images in the ventral pons, as recently described.23 The visual field test showed peripheral constriction, and ophthalmoscopic examination uncovered diabetic non-proliferative retinopathy with pale optic discs.

Table 1  Clinical manifestations in patients with Wolfram syndrome

<table>
<thead>
<tr>
<th>Family and patient</th>
<th>Age*</th>
<th>Gender</th>
<th>Diabetes mellitus</th>
<th>Progressive optic atrophy</th>
<th>Abnormal audiogram</th>
<th>Diabetes insipidus</th>
<th>Renal tract abnormalities</th>
<th>Other complications (age at onset)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>29 y</td>
<td>F</td>
<td>7 y</td>
<td>11 y</td>
<td>11 y</td>
<td>20 y</td>
<td>20 y</td>
<td>Hypogonadism</td>
</tr>
<tr>
<td>1.2</td>
<td>20 y</td>
<td>M</td>
<td>6 y</td>
<td>7 y</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>Seizures (19 y)</td>
</tr>
<tr>
<td>2.1</td>
<td>22 y</td>
<td>F</td>
<td>8 y</td>
<td>16 y</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Cataract (2 y)</td>
</tr>
</tbody>
</table>

*Age at time of the study.

ND, not determined.

mutant. The 2638-2643del(GACTTC) mutation destroys a TaqI (Roche) cleavage site with an additional band of 212 bp in the mutant allele compared to the wild type (48 bp, 49 bp, 164 bp) site in a fragment of exon 8 (forward primer CCT CAA CTG CAT GGC CCA GC, reverse primer AGG TCT CTG CAG CCA CAG TC). The 1675G→A mutation eliminates a CfoI cleavage site (data not shown).20

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Patients

Patients were recruited at the local referral centre. Two unrelated families of Austrian descent were studied (table 1).

PATIENT 1.1

The patient was a 29 year old woman with unaffected parents, three healthy sibs, and one affected brother (patient 1.2). There was no reliable information in regard to consanguinity in the family. One grandmother had diabetes mellitus type 2 diagnosed at the age of 64.

At the age of 7, the patient developed insulin dependent diabetes mellitus (IDDM) after a measles infection. At the age of 11, bilateral optic atrophy developed with reduced colour vision and reduced visual acuity. There was no evidence of diabetic retinopathy. A visual field test showed central scotomas. At the same age, bilateral high tone sensorineural hearing loss greater than 50 decibels above 4000 Hz emerged. At the age of 20, desmopressin was started for central diabetes insipidus. The bone mineral density of the lumbar spine and proximal femur as measured by dual energy x ray absorptiometry was normal. The neurogenic bladder (with overactive, hypococontractile, dis-inhibited detrusor function and low capacity of 180 ml) was initially treated with several intravesical electrical stimulations. The patient was managed conservatively with clean intermittent self-catheterisation and anticholinergic medication. As vesicoureteral reflux developed at the age of 28 years, an ileoceleal bladder augmentation using the Mainz Pouch I technique was performed to protect the upper urinary tract.21 22 The patient was prescribed a biphasic hormone combination (oestradiol valerate plus norgestrel) to regularise the menstrual cycle.

PATIENT 2.1

The patient was a 22 year old woman, who developed IDDM at the age of 8 years. There was no family history of IDDM or consanguinity. Islet cell and glutamic acid decarboxylase (GAD-65) antibodies, as determined at the age of 21, were not detected. There was no macroalbuminuria and neurothesiometry and electromyography were normal. Interestingly, the patient underwent a cataract extraction of the right eye at the age of 2 even before the onset of diabetes mellitus, two operations for strabismus at the ages of 3 and 4, and a Nd:YAG posterior capsulotomy of the optic lens at the age of 18. She began to complain of progressive loss of visual acuity at the age of 16 and four years later bilateral atrophy of the optic nerves was detected. Neurological examination showed only low visual acuity, but cranial magnetic resonance imaging at 21 years showed atrophy of the median brainstem and the optic nerves. Additionally, there was a diffuse area of high signal on PD and T2 weighted images in the ventral pons, as recently described.23 The visual field test showed peripheral constriction, and ophthalmoscopic examination uncovered diabetic non-proliferative retinopathy with pale optic discs.
Results

Sequences obtained from the family members were compared to the WFS1 sequence and to normal control chromosomes. Analysis of the complete coding sequence of the WFS1 gene in the index patients showed three novel mutations (table 2).

![Image of restriction fragment length polymorphism analysis](http://img.bmj.com)

**Table 2 Mutations and polymorphisms 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>1038ins(C)†</td>
<td>Ins346fs/ter542</td>
<td>Frameshift/truncation</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>2315ins(T)</td>
<td>Ile776fs/ter776</td>
<td>Frameshift/truncation</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2638-2643del(GACTTC)</td>
<td>De880-881DF</td>
<td>In frame deletion of 6 bp</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>1675G→A</td>
<td>A899T</td>
<td>Missense</td>
</tr>
<tr>
<td>1,2</td>
<td>8</td>
<td>1832A→G</td>
<td>H611R</td>
<td>Conservative</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>997A→G</td>
<td>I333V</td>
<td>Conservative</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>684G→C</td>
<td>R228R</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>1023C→T</td>
<td>F341F</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>1,2</td>
<td>8</td>
<td>1185T→C</td>
<td>V395V</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>1500T→C</td>
<td>N500N</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>1724C→T</td>
<td>A575A</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>2322G→A</td>
<td>K774K</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>1,2</td>
<td>8</td>
<td>2433A→G</td>
<td>K811K</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>2565G→A</td>
<td>S855S</td>
<td>Polymorphism</td>
</tr>
</tbody>
</table>

* | Nucleotide positions are given counting from the first base of the start codon.
† | Homozygous mutation.
‡ | On the same allele as 2315ins(T).

A discrete nuclear cataract of the left myopic eye was present. There was no evidence of diabetes insipidus and the audiogram was normal.

Fig 1 illustrates in index patient 1.1 homozygosity for a 1038ins(C) insertion of 1 bp at position 1038 in exon 8 predicting a frameshift starting at amino acid 1675G. The next out of frame stop codon at nucleotide positions 1185-1187 was predicted to be read through because of the homozygous polymorphism 1185T→C down to the stop codon of nucleotides 1622-1624. The putative protein would lack the transmembrane domains and the hydrophilic carboxy tail. Patient 1.2 was also homozygous for the 1038ins(C) mutation.

As shown in fig 2, patient 2.1 was found to be a compound heterozygote for (a paternal) 2315ins(T) mutation resulting in an Ins772fs/ter776 frameshift and premature truncation of the predicted protein, and (a maternal) 2638-2643del(GACTTC) mutation resulting in an in frame deletion of aspartate and phenylalanine of codons 880-881. Both amino acids were reported to be conserved in the respective WFS1 sequences of *Mus musculus* and *Rattus norvegicus* (GenBank AF084482/AJ011971, AF136378).

Sequence analysis also showed a number of polymorphisms in both families (table 2). Patient 1.1, for instance, was not only homozygous for the 1038ins(C) mutation, but also for the following polymorphisms: 684G→C, 997A→G, 1185T→C, 1500T→C, 1832A→G, 2322G→A, 2333A→G, and 2565G→A. The rare coding variant 1675G→A was regarded recently as a risk allele for affective disorders. We found no evidence for psychiatric illness (as judged by history, examination, and hospital records) in patient 2.1 or in her unaffected father, both heterozygous for the 1675G→A variant. However, the functional relevance of this coding variant was difficult to clarify, as it was localised to the same allele as the 2315ins(C) mutation.

By screening cDNA of the WFS1 gene, we found mRNA heterogeneity in the 5' untranslated region of the WFS1 gene. The two mRNA isoforms were isolated from human lymphocytes and designated type 1 and type 2, respectively. They differed from each other only in their Kozak sequence: type 2 was identical to type 1, except for being 4 bp shorter, corresponding to the four bases immediately upstream from the ATG translation initiation codon (fig 3A). Thus, the strong context of the putative ATG start codon of isoform type 1 was changed to a weak one of isoform type 2 with a cytidine instead of the original adenosine in nucleotide position −3 (that is, three nucleotides upstream from the ATG codon). We directly sequenced both exons 1 and 2 with the adjacent splice sites to exclude any genomic deletions. However, instead of reflecting a genomic mutation, the deletion of 4 bp in isoform type 2 was generated by the use of alternative 3' splice sites (fig 3B). mRNA isoform type 1 corresponded to the previously published sequence (GenBank AF084481/Y18064), whereas isoform type 2 was a novel splice variant of the WFS1 gene. Each splice variant was found in patients with Wolfram syndrome as well as in all negative controls (n=9). To test the tissue distribution of the
Figure 3. Splice variants of the WFS1 gene. (A) cDNA was subcloned into the pCR2.1 vector and several colonies sequenced for each mRNA isoform. Two representative samples are written in the single letter code (A = adenosine, G = guanosine, R = A or G (purine), N = any Nucleotide). Nucleotide positions are given counting from the first base of the start codon. Exonic and intronic sequences are indicated in upper case and lower case letters, respectively. 3' splice site consensus sequence, where a pyrimidine was substituted for the highly conserved purine at position –3. Neither the upstream leader sequence nor the open reading frame were affected. Thus, the final WFS1 protein of the two splice variants appeared to be ubiquitous.

Discussion

In the work presented here, three novel mutations were identified in the WFS1 gene in patients with Wolfram syndrome. All three patients suffered from the typical association of insulin dependent, non-autoimmune diabetes mellitus and optic atrophy. Additional typical findings were hypoaacusis, central diabetes insipidus, hypogonadism, and cataract.

The sibs from family 1 both carried a homozygous 1038ins(C) mutation, predicting a severely truncated protein, whereas patient 2.1 appeared to be compound heterozygote for a 2315ins(T) and a 2638-2643del(GACTTC) mutation. All three mutations were localised to exon 8. The two insertions of 1 bp are typical loss of function mutations and confirm the notion that lack of the putative gene product causes the disease. The in frame deletion of 6 bp in patient 2.1 (del880-881DF), which is very close to the carboxy-terminus of WFS1 pointed, together with other mutations (del882fs/ter937, del883fs/ter949, P885L), to the functional relevance of the cytoplasmic carboxy-terminus of the WFS1 gene product, which is hypothesised to interact with other, as yet unidentified, proteins. The location and nature of WFS1 mutations might thus help in understanding the molecular basis of Wolfram syndrome and the role of the WFS1 protein in protecting neurons and islet β cells from premature cell death.

Additionally, we present evidence for mRNA heterogeneity of the 5’ non-coding region of the WFS1 gene. mRNA heterogeneity is the result of alternative splicing of intron 1, which contains a redundant 3’ splice site. Competition of the tandemly repeated tetranucleotide CAGG (12-20 bp downstream from the putative branchpoint, fig 3) for the small nuclear ribonucleoprotein U2AF is in line with previous reports. Tandemly repeated AGs competed actively when placed 13-22 nucleotides downstream of the branchpoint in a yeast intron.

When the branch to 3’ splice site distance was significantly longer (at least 35 nucleotides), a linear search mechanism in the 5’→3’ direction showed strong preference for the 5’ most AG.

The two splice variants of the WFS1 gene differed from each other only in their Kozak consensus sequence, where a pyrimidine was substituted for the highly conserved purine at position –3. Neither the upstream leader sequence nor the open reading frame were affected. Thus, the final WFS1 protein of the two splice variants would be expected to be identical. Moreover, no major differences in the relative density of the two splice variants was evident in the tissues examined. To address the functional relevance, if any, of alternative splicing of WFS1, further molecular genetic and functional analysis will be required. Taken together, our data extend the mutational spectrum and provide, to our knowledge, the first evidence for natural variation in the translation regulatory region of the WFS1 gene.

We thank our patients for their participation in this study. This work was supported by a grant from the “Jubiläumsfond der Österreichischen Nationalbank” (grant 11693-MED to JP).


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J Med Genet 2001 38: e37
doi: 10.1136/jmg.38.11.e37

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