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***WFS1* mutations in Spanish patients with diabetes mellitus and deafness**

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Wolfram syndrome (WS) is an autosomal recessive neurodegenerative disorder characterised by early onset diabetes mellitus and progressive optic atrophy, as well as other clinical features such as deafness, diabetes insipida, renal tract abnormalities and diverse psychiatric illnesses. A gene responsible for WS was identified in 4p16.1 (*WFS1*). It encodes a putative 890 amino acid transmembrane protein expressed in a wide spectrum of tissues. Recently, a new locus for WS has been located on 4q22-24, providing additional evidence for the genetic heterogeneity of this syndrome. We have studied the presence of *WFS1* variants in three groups of individuals: patients with diabetes mellitus, patients with deafness and patients with both conditions. A fourth group of healthy subjects was used as control. We have identified a total of 18 nucleotide changes in the *WFS1* gene: three mutations and 15 polymorphisms. Six of these changes were previously undescribed. Four of the 15 polymorphisms studied among the patients group present statistical differences in the allelic and genotypic distribution when comparing affected vs control groups.

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Introduction

Wolfram syndrome (WS, MIM 222300, also named DIDMOAD, the acronym of diabetes insipida, diabetes mellitus, optic atrophy and deafness) is a rare autosomal recessive neurodegenerative disease. WS patients typically develop diabetes mellitus in the first decade of life, followed by deafness and bilateral optic atrophy in the second decade of life.¹ Other clinical features such as psychiatric disorders, renal tract abnormalities, diabetes insipida or peripheral neuropathy have been described among WS patients.^{2–4}

Linkage between WS phenotype and a marker located in the short arm of chromosome 4 was demonstrated by Polymeropoulos *et al*⁵ and later, in 1998, a gene responsi-

ble for WS named *WFS1*, was identified on 4p16.1.^{6,7} *WFS1* encodes a putative 890 amino acid protein named by some groups wolframin, with no functional homology with any known family of proteins. Biochemical studies and immunofluorescent cell staining showed that wolframin is an endoglycosidase H-sensitive membrane glycoprotein that localizes primarily in the endoplasmic reticulum.⁸

Recently, linkage analysis has identified an additional locus for WS in the region 4q22-24.⁹ Phenotypically, the WS patients in that study do not show diabetes insipida and some of them suffer gastrointestinal ulceration, proving once more the genetic and phenotypic complexity of this syndrome.^{9,10}

We wanted to ascertain the presence of *WFS1* gene mutations in patients with diabetes mellitus and/or neurosensorial deafness, since these two conditions are characteristic of WS patients. Mutations in one of the two copies of the *WFS1* gene in an individual could be contri-

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buting to the development of diabetes mellitus, deafness or other symptoms described in WS patients, but not the full syndrome.

A large number of variants in the *WFS1* gene has been reported previously.⁴ Considering this, the main objective of this study is to identify mutations in the *WFS1* gene in a Spanish population affected with diabetes mellitus and/or neurosensory deafness. We will also analyse to what extent these changes could contribute to the phenotype studied.

Materials and methods

Patients and controls

Individuals studied in this work belonged to four categories:

- (1) Twenty-three unrelated subjects affected of type 2 diabetes mellitus and neurosensory deafness (DM+F) (10 men and 13 women, range: 29–74 years).
- (2) Forty-eight unrelated subjects with autosomal recessive deafness (F) (24 men and 24 women, range: 4–83 years).
- (3) Thirty-eight type 2 diabetes mellitus patients (DM) (20 men and 18 women, range: 42–81 years).
- (4) Forty-nine healthy unrelated controls also participated in this work (24 males and 25 females, range: 26–61 years). None of them had previous history of diabetes mellitus or deafness.

Analysis of *WFS1* gene

DNA was extracted from peripheral blood samples obtained after informed consent following standard procedures.

The *WFS1* gene has eight exons. In this study we screened exons 2 to 8, the latter being subdivided into seven regions 8-1 to 8-7, following previous works.⁷ The thirteen pairs of primers and conditions for the PCRs are reported elsewhere.⁷ After PCR, SSCP analysis was performed, we ran premade 12.5% acrylamide SSCP gels (ExcelGel DNA analysis kit, Pharmacia Biotech, Sweden) at 5°C. A 20-min prerun at 100 V, 23 mA and 5 W was performed, followed by a 3-h 20-min run at 600 V, 30 mA and 18 W. Silver staining of these gels was completed with the DNA Silver Staining kit on an automatic gel stainer (Pharmacia Biotech).

Sequencing reactions were carried out with the same primers used in the previous PCRs. The sequencing reaction mix contained primers, forward and reverse for each sample at a final concentration of 0.3 µM, 2 µl of Big Dye Terminator Cycle Sequencing ready Reaction Kit (Applied Biosystems, USA), 2 µl of purified PCR product (QIAquick Gel Extraction Kit, Qiagen, Germany) and 3 µl of distilled water, in a final volume of 10 µl.

Sequencing PCR consisted of an initial denaturation step at 94°C for 5 min, 30 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 15 s and a long extension at 60°C for

4 min, then a final extension at 60°C for 10 min. PCR products were purified through G-50 columns (Pharmacia Biotech) and loaded in an ABI Prism 377 sequencer (Applied Biosystems). Sequences were then analysed using the programme Sequencer v 3.0 (Gene Codes Corporation, USA).

The sequence variants are considered mutations when they imply an amino acid change and are not found in controls. Other variants not included in this category are considered polymorphisms.

Statistical analysis

Hardy-Weinberg equilibrium of the different genotypes was evaluated for each polymorphism by Chi-squared with Jurg Ott's HWE-program version 1.5. Control genotypes follow H-W equilibrium (data not shown), so we can assume no stratification for our population and panmixy. Statistical significance of differences between distributions for alleles and genotypes was evaluated using Fisher's exact test with a 2 × 2 or 3 × 2 contingency table, respectively, comparing the three patient groups vs control group. Significance was considered when $P < 0.05$. Yule's coefficient was evaluated for each polymorphism with statistical differences in its allelic distribution, in order to ponder the intensity of association.

Results

WFS1 mutations

Two new mutations, as well as a previously described one, were found in the *WFS1* gene among DM+F and F patients. Figure 1 shows the heterozygotic pattern of these mutations. Changes 2185G>A and 2269C>A, both on exon 8-6, were found in two unrelated DM+F patients, each in a different subject. These mutations cause the amino acid changes D729N and L757I, respectively. Mutation D729N represents the change from a charged amino acid to a polar one, whereas L757I mutation keeps the aliphatic characteristic of the amino acid replaced. The third mutation 2611G>A,¹¹ in exon 8-7, encodes for the V871M amino acid change. It represents the change of a small aliphatic amino acid to a hydrophobic. This variation has been found in a DM+F subject and in a deaf patient (a 74 year old man and a 15 year old woman, respectively). The segregation of the mutation V871M among the family of the deaf patient was then studied. The mutation was found in heterozygosis in her 14 year old deaf sister and her father, a non affected 37 year old man.

Allelic frequencies of the polymorphisms

We have analysed the frequencies of alleles of the polymorphisms found in the four groups studied. Fifteen polymorphisms have been identified, four of them previously unreported: T346T, L432V, Y455Y and V813A (see Table 1). Significant statistical differences in the allelic distribution of polymorphisms V395V ($P = 0.0002$), H611R ($P < 0.0001$), K811K ($P < 0.0001$) and S855S ($P < 0.0001$) were

Figure 1 Electropherograms of the three mutations found in the *WFS1* gene. (A) and (B) Correspond to exon 8-6, whereas C to exon 8-7. Electropherogram A is shown as the reverse sequence. * Indicates the mutated nucleotide. II: SSCP pattern of these three mutations. Arrows indicates the heterozygous SSCP pattern.

found in the DM+F group compared to control group (Table 1). In addition, there were significant statistical differences in the allelic distribution of polymorphisms K811K ($P=0.035$) and S855S ($P<0.0001$), among the DM and control groups (Table 1). No significant statistical differences in the allelic distribution of any polymorphism in the F group has been found (Table 1).

Yule's coefficient was evaluated for those alleles with significant statistical distribution, revealing low intensity of association (data not shown).

Genotypic frequencies of the polymorphisms

Significant statistical differences in the distribution of genotypes of polymorphisms V395V ($P=0.0004$), H611R ($P<0.0001$), K811K ($P=0.0002$) and S855S ($P=0.0006$) were found when comparing DM+F vs control groups (Table 1). The DM group showed significant statistical differences for H611R ($P=0.016$) and S855S ($P<0.0001$) polymorphisms in their genotypic frequencies, when comparing with the control group. No significant differences were found for the F group (Table 1).

Discussion

We have identified three mutations in heterozygosis (Figure 1) in three patients affected with type 2 diabetes mellitus and deafness. These are amino acid substitutions that conserve the main biochemical properties. According to the predicted structure of the wolframin,⁴ the mutations found are located in the intra-cytoplasmic domain, near to the C-Terminal end. The lack of knowledge about the function of *WFS1* makes it difficult to explain the possible contribution of these mutations to the above mentioned diseases.

Mutations D729N and L751I were found in the DM+F group. At this moment, we can not postulate with which disease, diabetes mellitus or deafness, these mutations could be related. A study of the familial transmission for these mutations in the families of affected subjects may help to clarify this point.

Mutation V871M, detected in a DM+F subject, was also found in heterozygosis in two deaf sisters and in her unaffected father. By studying the frequencies of alleles and genotypes of the *WFS1* polymorphisms in the four groups

Table 1 Allelic and genotypic frequencies of the polymorphisms found in WFS1 gene

Region	Polymorphism	Frequencies of alleles					Frequencies of genotypes				
8-1	997A>G; I333V	A		G		Odds	A/A	A/G	G/G		
	C, n=48‡	72	0.75	24	0.25		25	0.52	22	0.46	1 0.02
	DM+F, n=23	40	0.87	6	0.13	0.45	17	0.74	6	0.26	0 0
	DM, n=31	50	0.81	12	0.19	0.72	19	0.61	12	0.39	0 0
	F, n=46	72	0.78	20	0.22	0.83	26	0.57	20	0.43	0 0
8-1	1023C>T; F341F	C		T			C/C	C/T	T/T		
	C, n=48‡	89	0.93	7	0.07		41	0.85	7	0.15	0 0
	DM+F, n=23	41	0.89	5	0.11	0.31	19	0.83	3	0.13	1 0.04
	DM, n=31	53	0.85	9	0.15	2.15	22	0.71	9	0.29	0 0
	F, n=46	81	0.88	11	0.12	1.72	36	0.78	9	0.20	1 0.02
8-2	185T>C; V395V	T		C			T/T	T/C	C/C		
	C, n=49	71	0.72	27	0.28		23	0.47	25	0.51	1 0.02
	DM+F, n=23*	45	0.98	1	0.02	0.06	22	0.96	1	0.04	0 0
	DM, n=32	39	0.61	25	0.39	1.68	10	0.31	19	0.59	3 0.1
	F, n=47	68	0.72	26	0.28	1.00	22	0.47	24	0.51	1 0.02
8-2	1294C>G; L432V	C		G			C/C	C/G	G/G		
	C, n=49	97	0.99	1	0.01		48	0.98	1	0.02	0 0
	DM+F, n=23	46	1	0	0	0.69	23	1	0	0	0 0
	DM, n=32	63	0.98	1	0.02	1.54	31	0.97	1	0.03	0 0
	F, n=47	94	1	0	0	0.34	47	1	0	0	0 0
8-2	1308C>T; T436T	C		T			C/C	C/T	T/T		
	C, n=49	96	0.98	2	0.02		47	0.96	2	0.04	0 0
	DM+F, n=23	46	1	0	0	0.42	23	1	0	0	0 0
	DM, n=32	63	0.98	1	0.02	0.76	31	0.97	1	0.03	0 0
	F, n=47	91	0.97	3	0.03	1.58	45	0.96	1	0.02	1 0.02
8-2	1364C>T; Y455Y	C		T			C/C	C/T	T/T		
	C, n=49	98	1	0	0		49	1	0	0	0 0
	DM+F, n=23	46	1	0	0		23	1	0	0	0 0
	DM, n=32	64	1	0	0		32	1	0	0	0 0
	F, n=47	93	0.99	1	0.01	3.16	46	0.98	1	0.02	1 0.02
8-2	1367G>A; R456H	G		A			G/G	G/A	A/A		
	C, n=49	91	0.93	7	0.07		42	0.86	7	0.14	0 0
	DM+F, n=23	41	0.89	5	0.11	1.58	18	0.78	5	0.22	0 0
	DM, n=32	53	0.83	11	0.17	2.70	21	0.66	11	0.34	0 0
	F, n=47	87	0.93	7	0.07	1.05	41	0.87	5	0.11	1 0.02
8-3	1500T>C; N500N	T		C			T/T	T/C	C/C		
	C, n=29‡	39	0.67	19	0.33		12	0.41	15	0.52	2 0.07
	DM+F, n=23	36	0.78	10	0.22	0.57	14	0.61	8	0.35	1 0.04
	DM, n=8	10	0.63	6	0.37	1.23	2	0.25	6	0.75	0 0
	F, n=32	39	0.61	25	0.39	1.36	10	0.31	19	0.60	3 0.09
8-4	1725C>T; A575A	C		T			C/C	T/T			
	C, n=48‡	87	0.91	9	0.09		39	0.81	9	0.19	0 0
	DM+F, n=23	43	0.93	3	0.07	0.75	20	0.87	3	0.13	0 0
	DM, n=33	55	0.83	11	0.17	1.93	22	0.67	11	0.33	0 0
	F, n=44	81	0.92	7	0.08	0.83	37	0.84	7	0.16	0 0
8-4	1832A>G; H611R	A		G			A/A	G/A	G/G		
	C, n=48‡	57	0.59	39	0.41		14	0.30	29	0.60	5 0.10
	DM+F, n=23**	43	0.93	3	0.07	0.10	20	0.87	3	0.13	0 0
	DM, n=33	33	0.50	33	0.50	1.93	11	0.33	11	0.33	11 0.33
	F, n=44	59	0.67	29	0.33	0.83	21	0.48	17	0.38	6 0.14
8-6	2322G>A; K774K	G		A			G/G	G/A	A/A		
	C, n=48‡	87	0.91	9	0.09		39	0.81	9	0.19	0 0
	DM+F, n=23	41	0.89	5	0.11	0.10	19	0.83	3	0.13	1 0.04
	DM, n=26	48	0.92	4	0.08	1.46	22	0.85	4	0.15	0 0
	F, n=48	85	0.89	11	0.11	0.71	38	0.79	9	0.19	1 0.02
8-6	2433G>A; K811K	G		A			G/G	G/A	A/A		
	C, n=48‡	32	0.33	64	0.67		4	0.08	24	0.50	20 0.42
	DM+F, n=23**	32	0.70	14	0.30	0.22	11	0.48	10	0.43	2 0.09
	DM, n=26*	27	0.52	25	0.48	0.46	7	0.27	13	0.50	6 0.23
	F, n=48	34	0.35	62	0.65	0.88	6	0.12	22	0.46	20 0.42
8-6	2438T>C; V813A	T		C			T/T	C/T	C/C		
	C, n=48‡	95	0.99	1	0.01		47	0.98	1	0.02	0 0
	DM+F, n=23	46	1	0	0	0.68	23	1	0	0	0 0
	DM, n=26	52	1	0	0	0.61	26	1	0	0	0 0
	F, n=48	95	0.99	1	0.01	1.00	47	0.98	1	0.02	0 0

Continued

Table 1 (Continued)

Region	Polymorphism	Frequencies of alleles				Frequencies of genotypes					
		C	T			C/C	C/T	T/T			
8-7	2469C>T; 18231										
	C, n=49	98	1	0	0	49	1	0	0	0	0
	DM+F, n=23	46	1	0	0	23	1	0	0	0	0
	DM, n=37	74	1	0	0	37	1	0	0	0	0
	F, n=41	80	0.98	2	0.02	6.12	40	0.98	0	0	0.02
8-7	2565A>G; 5855S										
	A			G		A/A		A/G		G/G	
	C, n=49	29	0.30	69	0.70		4	0.08	21	0.43	0.49
	DM+F, n=23**	1	0.02	45	0.98	18.91	0	0	1	0.04	0.96
	DM, n=37**	0	0	74	1	63.25	0	0	0	37	1
	F, n=41	33	0.40	49	0.60	0.62	8	0.20	17	0.41	0.39

Previously undescribed polymorphisms are shown in italic. n=number of individuals analysed. *= $P<0.05$, **= $P<0.0001$. ‡ indicates when PCR amplification was not possible in all samples.

of individuals analysed, we have found significant statistical differences in the distribution of substitutions 1185T>C, 1832A>G, previously related with type 1 diabetes mellitus,¹² and in the distribution of the changes 2433G>A and 2565A>G, in the diabetic groups (DM and/or DM+F) (Table 1). Previous works described the polymorphisms R456H, H611R and I720V in *WFS1* gene, related with non-autoimmune type 1 diabetes mellitus.¹² We have observed significant statistical differences in the allelic and genotypic distribution in H611R change in patients with type 2 diabetes mellitus, when compared with the control group. This suggest a possible contribution of this variant with diabetes mellitus.

Three of the four new polymorphisms (T436T, Y455Y, and V831A), as well as mutation V871M, were found in the F group. This fact suggests that other possible variants could exist in the *WFS1* gene, which would contribute to the pathogenic phenotype. Association between deafness and some markers in heterozygosis in the *WFS1* gene was previously described.^{11,13,14} In this work, we have not found such a correlation but, perhaps, the analysis of a larger number of deaf patients could reveal association. Unfortunately, we have not been able to perform an association study due to the reduced number of patients available.

We cannot assure whether there is a direct contribution of those alleles with significant statistical differences between affected and control groups in any phenotype.

In spite of all the as yet unclear genetic aspects on WS, we can postulate that missense polymorphisms described in the literature with significant statistical differences in their allelic and genotypic distribution may modulate, directly or indirectly, the normal function of the protein encoded by the *WFS1* gene.

In this work, we report a total of five new nucleotide changes and significant statistical differences in distribution of some polymorphisms in a relatively small cohort of patients for each disease. In view of our results and in agreement with previous works, we think that the analysis of the *WFS1* gene should be considered in the cases of

diabetes mellitus and deafness in which the genetic origin is unknown.

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