**WFS1** mutations are frequent monogenic causes of juvenile-onset diabetes mellitus in Lebanon

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Most cases of juvenile-onset diabetes (JOD) are diagnosed as type 1 diabetes (T1D), for which genetic studies conducted in outbred Caucasian populations support the concept of multifactorial inheritance. However, this view may be partly challenged in particular population settings. In view of the suggestive evidence for a high prevalence of Wolfram syndrome (WFS) in Lebanon, the phenotypic variability associated with **WFS1** mutations, and the high consanguinity rate in Lebanon, we aimed to evaluate the contribution of **WFS1** mutations as monogenic determinants to JOD in Lebanon. We performed a family-based genetic study, with linkage analysis followed by systematic mutation screening of **WFS1** exons in all JOD probands. The study population consisted of an unbiased recruitment of all juvenile-onset insulin-dependent diabetic patients from a specialized diabetes pediatric clinic in Beirut, Lebanon. Homozygous or compound heterozygous **WFS1** mutations were found in 22 of the 399 JOD probands (5.5%), resulting in WFS (17 probands) or in non-syndromic non-autoimmune diabetes mellitus (DM, five probands). These accounted for 12.1% (21/174) of probands in consanguineous families, compared with 0.4% (1/225) in non-consanguineous families. Of the 38 patients identified with homozygous or compound heterozygous **WFS1** mutations, 11 (29%) had non-syndromic DM, all of whom carried a particular **WFS1** mutation, **WFS1LIB**, encoding a protein with an extended C-terminal domain. This mutation resulted in a delayed onset or absence of extrapancreatic features. These results underscore the major impact of population-specific factors, such as population-specific mutations and founder effects, and family structure in the genetic determinism of JOD.

**INTRODUCTION**

Type 1 diabetes (T1D) is among the most common chronic childhood diseases in Caucasian populations. Its etiology is thought to be principally autoimmune, resulting in the destruction of insulin-producing pancreatic β cells, leading to complete dependence on insulin replacement therapy generally at a young age. Epidemiologic and genetic studies support the hypothesis of multifactorial contributions, with genetic and environmental risk factors (1). Genes located within the major histocompatibility complex locus are major risk factors of T1D, and genome-wide linkage and association scans in outbred Caucasian populations consistently support the model of multiple additional genes with minor contribution to T1D risk (2,3). Hence, monogenic factors are unlikely to play a major role in T1D in these populations. However, the extent of monogenic contributions may vary considerably between populations, depending on ethnic or
population-specific factors, including founder effects and the presence of consanguinity. They may also vary between families, depending on their structure (presence or not of multiple affected siblings, and inter-parental consanguinity).

Several rare recessive syndromes in which insulin-dependent juvenile diabetes is associated with other clinical manifestations have been described, including Wolfram syndrome (WFS), Wolcott–Rallison syndrome and thiamine-responsive megaloblastic anemia (4). Although these syndromes are very rare in outbred Caucasian populations, their incidence may be increased in some populations or contexts, due to founder effects and/or the high frequency of consanguineous marriages. WFS (DIDMOAB, OMIM 222300) is characterized by juvenile-onset diabetes (JOD) mellitus, optic atrophy (OA), diabetes insipidus (DI) and sensorineural deafness, and is caused by recessive mutations in the Wolfram gene (WFS1) (5,6). It is generally considered to be very rare, with an estimated prevalence of 1/770 000 in UK (7). However, based on previous reports, the prevalence of this syndrome may be much higher in some countries, including Lebanon (8). In addition to WFS, WFS1 mutations and genetic variants have been implicated in low-frequency non-syndromic hearing loss (dominant mutations) and psychiatric diseases (9,10), and common WFS1 variants have recently been shown to be associated with type 2 diabetes (T2D) (11,12). In view of the large phenotypic variability associated with WFS1 mutations with regard to diabetes and extrapancreatic manifestations, the possibility of some genetic overlap between typical WFS and non-syndromic diabetes could also be anticipated. In this study, we aimed to evaluate the contribution of WFS1 mutations and variants in syndromic and non-syndromic JOD in Lebanese families.

RESULTS

Strong contribution of WFS1 locus to JOD in Lebanon in WFS and non-syndromic DM detected by linkage analysis

We studied 408 nuclear families from Lebanon, which had been ascertained through a patient with insulin-dependent JOD, with a total of 455 JOD patients, including non-syndromic and syndromic cases. Twenty seven patients from 17 of these families had clinical features characteristic of WFS (Tables 1 and 2). The high frequency of WFS patients among Lebanese JOD probands (17/408, or 4.2%), based on our survey, confirms the high prevalence of this syndrome in Lebanon that had been suggested in a previous study (8).

Linkage analysis performed under a highly penetrant rare recessive model in all multiplex, consanguineous or extended families showed highly significant evidence of linkage near the WFS1 locus on chromosome 4p (Fig. 1), with LOD (logarithm of odds) scores with heterogeneity of 8.66 (estimated proportion of linked families: $\alpha = 13.8\%$). After excluding WFS families and the 11 neonatal or other syndromic diabetes families, there was evidence of residual linkage at WFS1 locus (LOD = 2.47, $\alpha = 7.1\%$) (Fig. 1), suggesting either that some WFS patients may have been underdiagnosed, or that some WFS1 mutations may result in an incomplete phenotype restricted to diabetes, or that frequent variants in the WFS1 gene or another gene near WFS1 may contribute to diabetes susceptibility in Lebanon.

WFS1 mutations in WFS and non-syndromic DM patients

By sequencing WFS1 exons in WFS and non-syndromic DM probands and their parents from multiplex, consanguineous or extended families, and in probands from simplex non-consanguineous families, we identified 173 WFS1 DNA variants, 46 of which were predicted to affect the primary sequence of the protein, 38 of which were novel (Supplementary Material, Table S2).

In the WFS patients, we identified 10 variants compatible with the status of WFS mutations, nine of which were novel (Table 1). All the WFS patients carried a homozygous mutation (or a combination of two mutations in complete linkage disequilibrium) that was predicted to have deleterious consequences on the protein function, as it resulted in a truncated protein, deleted one amino acid or was predicted to be damaging based on PolyPhen analysis. A double mutation, WFS1LIB, associating the 707VI non-synonymous change and the F884fs951X frameshift on the same haplotype, was homozygous in 7/17 (41.2%) of the WFS probands, and 10/27 of all WFS patients. F884fs951X frameshift affects the most C-terminal endoplasmic reticulum (ER) luminal domain, replacing the last seven amino acids by 67 other amino acids, while 707VI is predicted to have no major functional consequence on protein function.

In non-syndromic DM patients, two variants were compatible with the status of mutations, based on our criteria (Methods): WFS1LIB and F646fs708X (Supplementary Material, Table S2). WFS1LIB was homozygous in four non-syndromic DM probands (10 non-syndromic DM patients) and compound heterozygous with F646fs708X in one non-syndromic DM proband (Table 2). Re-analysis of linkage data after excluding the corresponding families cancelled the residual linkage peak near WFS1, indicating that WFS1 mutations in WFS and non-syndromic DM patients accounted for the totality of the evidence of linkage detected at WFS1 locus (Fig. 1).

WFS1LIB mutation is associated with delayed or absent extrapancreatic manifestations

Of the 38 JOD patients from our study population diagnosed with homozygous or compound heterozygous WFS1 mutations, 11 (27%) had non-syndromic DM; 11/21 (52.4%) of the patients homozygous or compound heterozygous for WFS1LIB mutation had only DM, without any evidence of OA, DI or deafness, compared with none (0/17) of the patients homozygous for any other mutation (Table 2). Using logistic regression analysis, we showed that the absence of extrapancreatic manifestations was dependant on the age of the patients at last examination ($P = 0.036$) and on the nature of the mutation ($P = 0.0001$) (Table 3A). In a Kaplan–Meier analysis, we showed that the appearance of OA was significantly delayed in patients homozygous for the WFS1LIB mutation compared with those homozygous for other WFS1 mutations (Fig. 2, $P = 0.0003$). Patients homozygous for the WFS1LIB mutation exhibited reduced expression of other extrapancreatic features, including hearing impairment, DI, cataract and...
<table>
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<th>Exon</th>
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<th>Protein domain</th>
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WFS, Wolfram syndrome; DM, diabetes mellitus; NA, not available. For references to known mutations, see Supplementary Material, Table S2.

\(^a\)Position on NM_006005.2.

\(^b\)Out: cytoplasmic domain, In: ER domain, TM: transmembrane domain.

\(^c\)PolyPhen prediction was used to assess the consequence of missense mutations (see Materials and Methods). We indicated as ‘damaging’ mutations scored as ‘possibly’ or ‘probably’ damaging by this program.

\(^d\)All the mutations identified in WFS patients were homozygous.

\(^e\)Mutations 461TS and 842LF were in complete LD and systematically associated with the same haplotype (461TS + 842LF).

\(^f\)Mutations 707VI and F884fs951X were in complete LD and systematically associated with the same haplotype (707VI + F884fs951X, referred to as WFS1\(^{LIB}\) in the text).

\(^g\)The double mutation 707VI + F884fs951X was also found to be homozygous in 10 non-syndromic DM patients and compound heterozygous (together with F646fs708X) in one non-syndromic patient (see text and Table 2).
neuropsychiatric complications, with the possible exception of epilepsy, showing the opposite trend (Table 3A); in contrast, ages at onset of diabetes were similar in both groups (WFS1LIB: 5.95, others: 5.24, P = 0.32) (Table 3B). With the exception of epilepsy reported in one patient, non-syndromic DM patients with WFS1 mutations had no extra-pancreatic features, at age up to 23 years. Glutamic acid dehydrogenase (GAD) autoantibody level was negative in all pancreatic features, at age up to 23 years. Glutamic acid dehydrogenase syndrome DM patients with the exception of epilepsy reported in one patient, non-disease process is non-autoimmune in all cases. 

Among WFS1LIB homozygous individuals, the risk of having WFS (compared with non-syndromic DM) was family dependent (P = 0.002, after correcting for the age at last examination). This observation could not be accounted for by other WFS1 variants, since the entire WFS1 sequence was identical in all the WFS1LIB haplotypes. This suggests the contribution of additional factors to the clinical outcome of WFS1LIB patients, which may be variants located in flanking regions of the WFS1 gene. Alternatively, this could be explained by other genetic or environmental factors that may be shared within families; the role of a differential diagnosis by family is unlikely, since all patients were examined by the same ophthalmologist.

### Impact of family structure on the risk of WFS1-monogenic contribution

Based on our sequence screening survey, 5.5% (22/399) of all Lebanese JOD probands (including all WFS and non-WFS syndromic cases) were homozygous or compound heterozygous for WFS1 mutations. We tested the impact of family
structure, namely parental consanguinity and multiplicity of diabetic siblings, on the frequency of monogenic determinism due to \( WFS1 \) mutations. As expected, the frequency of \( WFS1 \) mutations as a causal determinant of JOD (i.e. homozygous or compound heterozygous status for \( WFS1 \) mutations) was increased in probands with consanguineous parents, reaching 12.1% (21/174) overall, compared with 0.4% (1/225) in non-consanguineous simplex families. This risk reached 46.2% (12/26) in multiplex consanguineous families, compared with 6.1% (9/148) in simplex consanguineous families.

The \( WFS1_{\text{LIB}} \) mutation was found to be prevalent in Lebanese JOD. Although all other \( WFS1 \) mutations in our study were absent in non-transmitted parental alleles to WFS and non-syndromic DM probands (\( N = 395 \) alleles), \( WFS1_{\text{LIB}} \) mutation was present in one non-transmitted parental allele (allele frequency: 0.0025). This mutation was absent in 1000 Lebanese population Controls studied. Its presence in non-transmitted Control alleles is likely to be a consequence of familial endogamy.

There were no cases of T1D in parents of WFS or non-syndromic DM patients with \( WFS1 \) mutations (i.e. parents who are obligate \( WFS1 \) mutation carriers, all mutations combined). Comparable risk of T2D was observed among parents of patients with or without \( WFS1 \) mutations, after adjusting for age and sex (6.4 versus 6.3%, respectively, data not shown). Hence heterozygous status for these \( WFS1 \) mutations does not significantly affect the risk of T1D or T2D. Conversely, there was no distorted transmission of alleles at the T2D-associated common SNPs rs10010131 and rs734312 (611RH) to JOD patients in our study (Supplementary Material, Table S2).

**DISCUSSION**

\( WFS1 \) encodes wolframin, a transmembrane glycoprotein localized to the ER, with high expression in pancreatic \( \beta \) cells, neurons and some other tissues (13), where it regulates \( \text{Ca}^{2+} \) homeostasis (14). To date, \( WFS1 \) mutations have been implicated in WFS, low-frequency non-syndromic hearing loss and psychiatric diseases, and common variants have recently been shown to be associated with T2D. We now show evidence that some \( WFS1 \) mutations may result in non-syndromic JOD, and that \( WFS1 \) mutations are responsible for a large proportion of JOD in some population subgroups, reaching 12.1% of probands in Lebanese consanguineous families. This situation results from the combination of population-specific founder effects responsible for the high prevalence of \( WFS1_{\text{LIB}} \) mutation in Lebanese patients, the non-syndromic DM phenotype frequently associated with this mutation, and the high rate of consanguinity. Intra-population stratification further increases this effect, as all \( WFS1_{\text{LIB}} \) patients belonged to the same socio-cultural community, although they were geographically dispersed over Lebanon (data not shown). Similar clustering of homozygous mutations has been previously reported in Lebanon for \( \beta \)-thalassemia (\( \beta \)-globin gene) and familial Mediterranean fever (MEFV gene).
Table 3. Effect of the nature of the mutation on clinical manifestations of WFS

(A) Extrapancreatic clinical manifestations in patients homozygous for WFS1 mutations

| Trait                                | Number positive for the trait (%)         | Other (N = 17) | P-value
|--------------------------------------|------------------------------------------|----------------|----------
|                                      | WFS1LIB (N = 20)                         |                |          |
| Optic atrophy                        | 10 (50%)                                 | 17 (100%)      | 0.0001   |
| Hearing impairment                   | 1 (5%)                                   | 7 (41.2%)      | 0.008    |
| Diabetes insipidus                   | 3 (15%)                                  | 8 (47.1%)      | 0.06     |
| Cataract                             | 1 (5%)                                   | 5 (29.4%)      | 0.08     |
| Epilepsy                             | 5 (25%)                                  | 1 (5.9%)       | 0.03     |
| Neuropsychiatric complicationsb      | 3 (15%)                                  | 9 (52.9%)      | 0.01     |

(B) Age at onset of diabetes

| Phenotype (number)          | Homozygous for WFS1LIB mutation; numbers, mean (95% CI) | Homozygous for ‘other’ mutations; numbers, mean (95% CI) | P-value
|----------------------------|--------------------------------------------------------|--------------------------------------------------------|----------
| WFS (N = 27)               | N = 10, 6.50 (5.03–7.97)                               | N = 17, 5.24 (4.11–6.36)                               | 0.17     |
| DM (N = 10)                | N = 10, 5.40 (4.13–6.67)                               | N = 0                                                  | 0.32     |
| All (N = 37)               | N = 20, 5.95 (4.97–6.93)                               | N = 17, 5.24 (4.18–6.29)                               | 0.17     |

Notes:
- All individuals were homozygous for WFS1 mutations, either WFS1LIB, or all others grouped (other). We excluded one compound heterozygous WFS1LIB/other from this analysis.
- CI, confidence interval.
- Logistic regression test (likelihood ratio test), taking into account the age at last examination.
- Neuropsychiatric complications were: memory loss, disorientation to time, place or person, delusions or hallucinations, or confusion.
- ANOVA test comparing patients homozygous for WFS1LIB with those homozygous for other mutations.

(15,16), providing strong evidence for the combined impact of consanguinity and founder effect, resulting from the religious and socio-cultural ethnic endogamy in the Lebanese population. Hence, the impact of WFS1 mutations on JOD is strongly dependent on population-specific factors, ranging from almost none in outbred Caucasians patients (7), consistent with the absence of genome-wide significant linkage and SNP association observed at WFS1 locus in outbred Caucasian populations (16,17), to very high in Lebanese consanguineous families, and especially those with multiple affected siblings. WFS1 mutations may also have a strong impact in other populations or patients’ subgroups with high frequency of autoantibody negative T1D. Interestingly, association of common population-specific WFS1 variants with T1D has been reported in Japan (18), although the role of monogenic determinants has not been demonstrated in this population.

Although we were able to show that WFS1LIB mutation is associated with significant delay in the appearance of OA, compared with other WFS1 mutations, a longer follow-up of these patients, or a specific study of adult patient populations, would be needed in order to determine if a subset of the non-syndromic WFS1LIB patients may be exempted from extrapancreatic manifestations during their lifetime. Remarkably, some of these patients were still with non-syndromic DM, with no sign of OA and full visual acuity, up to 23 years of age.

A mild WFS phenotype has been previously described in two siblings homozygous for F884fs951X, included in the WFS1LIB mutation (707V1 mutation was newly identified in our study and was not described in these patients), who had DM and OA, but no deafness, DI or neurologic abnormalities (19). A compound heterozygous F884fs951X mutation has also been reported in another WFS patient who had no deafness or DI (20). WFS patients carrying a homozygous 4 bp deletion with similar protein consequences (F883fs950X) also had no DI or deafness (21,22). All these patients had OA and had been diagnosed as having WFS. Two hypotheses may explain the selective mild WFS/non-syndromic DM features of WFS1LIB mutation: (i) β cells may be more sensitive than other cells to the pathway affected in WFS; β cells are highly secreting cells, which makes them particularly sensitive to ER stress (23), a proposed mechanism by which WFS1 mutations may lead to WFS (24,25). This may result in an obligate diabetes phenotype, but no or delayed extrapancreatic features in case of ‘mild’ mutations; (ii) the C-terminal region of wolframin affected in the WFS1LIB mutation may be critical for β-cell-specific function. Recently, this region has been shown to interact with Na+/K+ ATPase β1 subunit, which is critical for β cell function and may involve mechanisms that are partly independent of ER stress pathways (26). Evidence for genotype/phenotype correlation on the clinical variability of WFS was shown in a recent meta-analysis, with a dose–effect association of the number of inactivating mutations on disease severity (20). However, the effect of specific mutations on this variability could not be detected in the previous study, owing to the large variety of mutations. In addition, the WFS1LIB mutation, which would have been considered as an inactivating mutation in this study (frameshift) is actually associated with a milder disease phenotype. Additional molecular studies in patients and Controls cell lines will be needed to determine the underlying mutation-dependant mechanisms resulting in variable clinical expression.

The full WFS or non-syndromic DM expression of WFS1LIB showed familial clustering, suggesting the contribution of additional modifier factors, most likely genetic; Wfs1-deficient mice appear to have a ‘milder’ disease than human WFS (27), as extrapancreatic features have not been described to date.
Further supporting this hypothesis. Some studies have suggested a role of mitochondrial mutations in WFS (28). In particular the possible role of Leber hereditary optic neuropathy mutations in WFS has been proposed (29). We did not identify significant differences between the entire mitochondrial sequence of full WFS and non-syndromic DM WFS1LIB patients that may explain this effect (data not shown); however, the small number of independent homozygous WFS1LIB patients provided limited power to this analysis.

Our findings extend the role of WFS1 mutations as a frequent monogenic determinant of JOD in some population subgroups and suggest the existence of significant genetic heterogeneity between patients with JOD, strongly dependent on populations and family structure. To our knowledge, our observation of non-syndromic DM caused by a specific recessive WFS1 mutation constitutes the first description of recessive monogenic inheritance in diabetes which is not syndromic or neonatal. Based on these results, we anticipate that in populations, or in particular subgroups of patients, monogenic causes of diabetes may largely exceed the estimation of 1–2%, with important consequences for diagnosis and patients management (30–32). The increasing genetic knowledge on these monogenic forms of diabetes suggests the need for some revision of the current diabetes classification, to integrate genetic characteristics, when applicable. Generally, our results highlight the need to consider population origin and family structure, in addition to ethnicity, in genetic studies of diabetes and other multifactorial diseases. We anticipate that monogenic determinants, which remain undetected in the general multifactorial background in outbred populations, may contribute significantly to multifactorial diseases in specific situations.

**MATERIALS AND METHODS**

**Patients and families**

We recruited 408 nuclear families through a proband with JOD, with a total of 455 patients and 1520 of their family members, who were followed at the Chronic Care Center (Beirut, Lebanon), a pediatric clinic. The Chronic Care Center is the country’s sole professional center for the treatment of JOD, where ~90% of patients from the various communities and social structures from Lebanon come for treatment. All patients and their families visiting the center between January 2001 and March 2003 were asked to participate in the current study, by answering a questionnaire and providing a blood sample for DNA studies (establishment of cell lines was not included in the study protocol). All patients and family members, or their guardians in case of children, who accepted to participate, gave their written informed consent after the study was explained to them. The study protocol was approved by the Research and Ethics Committee of the Chronic Care Center.

All patients included in the study were with ketoacidosis or ketosis with severe symptoms of acute onset at presentation and continuous dependence on insulin within 6 months of diagnosis, fulfilling the criteria for T1D according to the World Health Organization criteria (33). Patients from four families were suspected of Maturity Onset Diabetes of the Young on the basis of incomplete insulin-dependency and dominant transmission, and these families were excluded from this study. A secondary inclusion criterion for this study was that families had to have both parents, or one parent and at least one sibling, available for DNA study. All but 10 families had both parents available. In addition to the medical examination and routine biochemical analyses, a standardized medical history questionnaire and demographic information were obtained on all patients. Age at onset of diabetes was before 18 and 26 years for probands and all patients respectively (mean age at onset of all patients: 9.7 years). Epidemiologic characteristics of an earlier subset of this collection have been reported (34,35). Patients presenting with other clinical manifestations in addition to diabetes mellitus (DM) that may reveal known or unknown syndromic forms of diabetes, were not excluded, and these additional clinical manifestations were recorded. Hence this recruitment provides an unbiased representation of all patients diagnosed with JOD in Lebanon, and may include increased clinical heterogeneity compared with ‘typical’ T1D.

DNA was extracted from blood collected on EDTA, using standard procedures. The presence of anti-GAD autoantibodies was tested at disease onset or retrospectively in a subset of 97 consecutive diabetic patients, using the GAD-AB radioimmunoassay kit (RSR, Limited, Cardiff, UK) following recommended procedures. Positive values (>1.0 U/ml) were observed in 68% of patients tested within 5 years after disease onset. Patients were medically evaluated at 1- or 2-month-interval visits, with clinical examinations by the Center’s endocrinologist, dietician and clinical psychologist. Fundoscopic examination and other routine ophthalmic tests were performed by a specialized ophthalmologist twice a year, and every 2 months for WFS patients. Diagnosis of WFS was based on the presence of OA confirmed by the presence of white papilla with regular and well-demarcated borders, in addition to DM. All the diabetic patients who were discovered to have a mutation in the wolframin gene (WFS1) during the course of this study were re-examined and evaluated by the ophthalmologist and the endocrinologist.
for any clinical symptoms of WFS every 2 months following genetic diagnosis, and throughout the duration of the study.

Information on consanguinity between probands’ parents was obtained through a questionnaire, and statistically re-evaluated based on genome-wide microsatellite genotyping done on all multiplex, consanguineous or extended families, and on 40 additional probands from non-consanguineous families and their parents. Information on consanguinity obtained by questionnaire and inferred from microsatellite data were highly concordant. In particular, no consanguinity was detected in the 40 non-consanguineous probands in this analysis. Frequencies of first and all degree consanguineous marriages between probands’ parents were 25.7 and 43.4% respectively (general population estimates: 14 and 25%, respectively, 36).

Families were classified as consanguineous, multiplex (multiple affected siblings), extended (multiple related nuclear families), or simplex (single affected child) non-consanguineous. Of a total of 408 nuclear families, 220 belonged to consanguineous, multiplex or extended families, and 192 were consanguineous or multiplex. Several non-typical T1D patients were identified based on clinical diagnosis or/and subsequent genetic studies and re-examination of patients. These included 17 families with WFS, and 11 other families where patients had neonatal diabetes or were affected by additional extrapancreatic clinical manifestations, suggesting recessive monogenic inheritance. A role of WFS1 gene has been excluded in these 11 families, based on linkage analysis, WFS1 mutation screening and/or direct gene identification of the causative defect. These 11 families were included in our study in order to achieve an exhaustive prevalence survey of genetic causes of JOD in Lebanon.

One thousand unrelated consenting healthy adults (≥40 years) representative of the socio-cultural diversity of the Lebanese population were used as population Controls. These individuals are part of an ongoing larger population study in Lebanon (37).

Microsatellite genotyping in WFS1 gene region

We combined microsatellite genotyping data from a microsatellite genome scan performed in all consanguineous, multiplex or extended families, excluding those initially diagnosed as having WFS, and additional genotyping of three microsatellite markers located near WFS1 gene, D4S431, D4S2366 and D4S2933, was performed in all these families, including those with WFS. The microsatellite genome scan used >400 microsatellite markers (Linkage Mapping Set 2, Applied Biosystems, with modifications).

Mutation and polymorphisms screening in WFS1 gene

Screening of WFS1 mutations and polymorphisms was performed by DNA sequencing in all WFS probands (N = 17) and their parents (N = 32), in all non-syndromic diabetic probands except nine due to technical failure (N = 371) and all available parents from multiplex, consanguineous or extended families (N = 363). We amplified all WFS1 exons, including intron/exon boundaries, from genomic DNA by PCR using primers shown in Supplementary Material, Table S1A. Sequence interpretation was performed using the Genalys software (38). After identification of a prevalent WFS1 Lebanese mutation (WFS1LD), we genotyped it in duplicate in all study individuals, using a TaqMan assay (Applied Biosystems), with primers described in Supplementary Material, Table S1B. There was 100% concordance between sequencing and genotyping results. We also screened 1000 unrelated Lebanese Controls for this mutation using the same TaqMan assay.

Defining the mutation status of variants, in WFS and non-syndromic DM patients

The mutation status of variants was assessed based on WFS1 sequence data generated on patients (WFS: N = 17 and non-syndromic DM: N = 371) and their parents (N = 32 and 363 respectively). WFS1 variants were considered to be compatible with WFS mutations if they satisfied simultaneously the following four criteria: (i) being homozygous or compound heterozygous with other mutations in WFS patients; (ii) never being homozygous or compound heterozygous with other mutations in non-diabetic individuals (parents); (iii) being absent (or present at a very low allele frequency, in the case of more prevalent mutations) in parental alleles that were not transmitted to diabetic children (Control alleles); (iv) predicted to affect the coding sequence of the protein (coding variant or alteration of splice site). We used the same strategy to assess WFS1 mutation status for non-syndromic DM (test population: non-syndromic DM probands). In addition to the Control alleles from the study population (non-transmitted parental alleles), a large Control population (N = 1000) was used to assess the frequency of a more prevalent WFS1 mutation in the Lebanese population.

Prediction of the functional consequences of WFS1 missense variants

Missense variants that were compatible with WFS or non-syndromic DM mutations, based on the criteria mentioned earlier, were evaluated for their predicted impact on protein function using the PolyPhen computational program (39). We also assessed the species-conservation of amino acids by sequence alignment. Scores of ‘possibly damaging’ or ‘probably damaging’ given by this program for a conserved amino acid were estimated to be likely deleterious to protein function.

Linkage and association studies, and statistical analyses

We revised the family structures and corrected genotyping errors resulting in Mendelian inconsistencies, using the PEDCHECK program (40). Family structures and consanguinity estimates were determined using the IBSCHECK program (S. Heath, unpublished). Linkage analyses were performed under a highly penetrant recessive model (penetrance: 0.95, disease allele frequency: 0.001, no phenocopy), allowing for heterogeneity, using MERLIN (41). Complex or extended pedigrees were simplified when necessary to enable computation with this program, while maintaining the necessary consanguinity information. Hardy–Weinberg equilibrium testing, and family-based association and transmission Disequilibrium test studies were performed using the PLINK package (42). Additional statistical analyses, including logistic regression
analyses and Kaplan–Meier survival analysis were done using STATVIEW or JMP7 statistical packages.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

REFERENCES


