Research

Identification of Three Novel and One Known Mutation in the *WFS1* Gene in Four Unrelated Turkish Families; the Role of Homozygosity Mapping in the Early Diagnosis

Sherif M et al. Mutation Analysis of WFS1 Patients

*Maha Sherif[1], *Huseyin Demirbilek[1,2,3], Atilla Cayir[4], Sophia Tahir[1], Busra Cavdarli[5], Meliha Demiral[6], Ayşe Nurcan Cebeci[7], Dogus Vuralli[3], Sofia Asim Rahman[1], Edip Unal[6], Gonul Buyukyilmaz[9], Riza Taner Baran[2], Mehmet Nuri Ozbek[2,6], Khalid Hussain[1,9]

[1]Developmental Endocrinology Research Group, Clinical and Molecular Genetics Unit, Institute of Child Health, University College London, WC1N 1EH, London, United Kingdom
[2]Department of Paediatric Endocrinology, Diyarbakir Children's Hospital, 21100 Diyarbakir, Turkey
[3]Hacettepe University Faculty of Medicine, Department of Pediatric Endocrinology, Ankara, Turkey
[4]Regional Training and Research Hospital, Clinics of Paediatric Endocrinology, Erzurum, Turkey
[5]Ankara City Hospital, Department of Medical Genetics, Ankara Turkey
[6]Gazi Yaşargil Research and Training Hospital, Pediatric Endocrinology, Diyarbakır, Turkey
[7]Derince Training and Research Hospital, Clinics of Paediatric Endocrinology, Kocaeli, Turkey
[8]Ankara City Hospital, Department of Pediatric Endocrinology, Ankara Turkey
[9]Department of Pediatrics, Division of Endocrinology, Sidra Medicine, Doha, Qatar
*These two authors have contributed equally as first authors

What is already known on this topic?
Wolfram syndrome 1 (WS1) is a clinically heterogeneous disease with variable manifestations and progression pattern depending on the underlying molecular genetics aetiology. Patients may present with incomplete phenotype, but the disease has a progressive nature with a negative impact of poor glycaemic control. Identification of molecular genetics aetiology provides early diagnostic confirmation, thereby, opportunity to detect and manage the subtle symptoms more appropriately.

What does this study add?
Our study expands the mutation database of WFS1 with three novel variants and provides further insights into the genotype and phenotype association.
We use the homozygosity mapping as an adjunctive tool which contributed to early detection of molecular genetics etiology in cases who presented with incomplete WS1 phenotype.

Abstract
Objective: Bi-allelic mutations in *wolframin gene (WFS1)* cause Wolfram syndrome 1 (WS1 or DIDMOAD) characterized with non-autoimmune diabetes mellitus (DM), optic atrophy (OA), diabetes insipidus (DI), sensorineural deafness (SND), urinary tract abnormalities, and neuropsychiatric disorders. We evaluated patients presented with an incomplete phenotype of WS1 using homozygosity mapping and subsequent whole-exome sequencing.
Patients and methods: Four unrelated consanguineous Turkish families (7 affected children), and their unaffected parents and siblings were evaluated. Homozygosity mapping (HZM) was performed, followed by whole-exome sequencing of *WFS1*. Mutations were classified according to results of *"in silico"* analyses, protein prediction, and functional consequences.
Results: Homozygosity mapping confirmed shared homozygous regions on chromosome 4 (chr4p16.1) between the affected individuals, and absent from their unaffected siblings. Exome sequencing identified three novel (c.1215T>A, c.554G>A, c.1525_1540dup) and one known (c. 1522_1523delTA) mutations in *WFS1* gene. All mutations were predicted

1

to cause stop codon leading to early termination of protein synthesis and complete loss-of-function. All patients were found to be homozygous for the change, with parents and other unaffected siblings being carriers.

Conclusion: Our study expands the mutation spectrum of *WSF1* mutations with three novel mutations. Homozygosity mapping may provide enrichment for molecular genetic analysis and early diagnosis of WS1 patients with incomplete phenotype, particularly in consanguineous pedigrees.

Keywords: Wolfram syndrome, WFS1, diabetes mellitus, diabetes insipidus, optic atrophy, sensorineural deafness

Professor Khalid Hussain, Division Chief – Endocrinology, Vice Chair for Research Department of Paediatric Medicine, Sidra Medical & Research Center, OPC, C6-337
PO Box 26999 | Doha, Qatar
+974-4003-7608 | MOB +974-30322007
khussain@sidra.org| www.sidra.org
05.07.2020
06.09.2020
https://orcid.org/0000-0002-5480-7112

Introduction

Wolfram Syndrome (WS), first described in 1938 by Wolfram and Wagener in four siblings, is an autosomal recessive disorder characterized with early-onset diabetes mellitus (DM) progressive neurodegeneration, endocrine dysfunction, and psychiatric disorders (1,2). WS1 is also known by the acronym DIDMOAD syndrome, which describes the frequent clinical features of the disease; diabetes insipidus (DI) and DM with optic atrophy (OA) and deafness. As WS1 is a progressive degenerative disease, additional clinical features including ataxia, urinary tract and renal disorders, and psychiatric disorders may present later in life (2).

WS1 is a rare cause of early-onset non-autoimmune DM, which is the most common clinical feature, usually occurs within the first decade of life (median age: 6 years). This is followed by progressive OA (median age of presentation at around 11 years), which first begins with colour and peripheral vision loss and can eventually lead to blindness over the next decade of life, as neurodegeneration progresses (3). All four clinical features described by DIDMOAD were observed in around 66% of patients in a review of 392 WS patients (4).

Biallelic loss-of-function mutations in wolframin ER transmembrane glycoprotein (*WFS1*), located at chromosomal position 4p16.1, account for the molecular genetic aetiology of WS1(5). The *WFS1* encodes for the protein wolframin, which is expressed ubiquitously, while the steady-state levels vary significantly among organs (6)**.** It is highly expressed in brain neurons, pancreas, heart and muscle, and lower expression is observed within the liver and skeletal muscle, and lowest expression in kidney and spleen (6). Within the pancreas, wolframin has a higher expression in the islet cells than the pancreas exocrine cells (5). Wolframin, a transmembrane glycoprotein composed of a cytoplasmic N-terminal domain, a central nine-transmembrane domain, and a luminal C-terminus, is predominantly localized in the ER (7). It involves in the regulation of ER-stress, which is critically important in establishing intracellular homeostasis, integrity and survival of the cell (8,9). Wolframin primarily involves in the unfolded protein response (UPR), which transduces the stimulus for increased unfolded proteins and maintain the balance between anti-apoptotic and pro-apoptotic processes (9,10). Unfolded protein response regulates ER-stress by eliminating misfolded proteins or attenuating the protein translation (11,12). Loss-of-function in wolframin causes decreased UPR activity, and thereby a chronic ER-stress mediated apoptosis and cell death (8,9). This eventually causes neurodegeneration and loss of beta-cell mass (13). Wolframin also plays an essential role in stimulus-response coupling mechanism, which regulates beta-cell insulin synthesis and secretion (13,14).

WS1 is a clinically heterogeneous disease with variable presentation as well as progression pattern depending on the underlying molecular genetics aetiology (4,15,16). Such diversity can make diagnosing WS1 difficult, especially in the context of an outbred population, multiple genes may need to be explored and sequenced. However, in consanguineous families, homozygosity mapping can prove to be an efficient tool to localize causative genes for recessive traits. It allows us to narrow down to specific chromosomal regions of DNA which are shared only by affected individuals, thereby facilitating the process of finding candidate genes and detecting mutations, when used alongside exome sequencing.

In the present study, we evaluate 7 affected individuals and their apparently healthy relatives from four unrelated consanguineous Turkish families with a variable clinical phenotype due to 3 novel and one previously described *WFS1* mutation.

Patients and Methods

In this study, we evaluated 7 affected patients (6 males and one female) presented with rare forms of DM and sensorineural deafness (SND) and their apparently healthy parents and siblings with a total number of 21 individuals from 4 unrelated families. All patients are from first-degree consanguineous parents. We performed homozygosity mapping on all patients and their unaffected siblings, from all families. We identified specific chromosomal regions which were shared by the patients alone, and absent in their unaffected siblings. In these regions we identified candidate genes, and used exome sequencing data alongside to detect mutations in the region of interest.

2

Family 1

Family 1 consists of three male children, two affected with DM and SND, and one unaffected belong to first-cousin parents (Figure 1A).

Patient 1 was born at term with a birth weight of 2.6 kg. At the age of 7 years, he was presented with polyuria and polydipsia. Blood glucose level was **340 mg/dl (18.9mmol/l)** at the time of diagnosis with no diabetic ketoacidosis (DKA). Diabetes autoantibodies (ICA, IAA, and GAD65) were negative. He was started on insulin therapy. He also developed SND when he was about 13 years old. A DNA sample was collected due to non-autoimmune early-onset DM and SND. Although he had a history of decreased visual acuity observed at the age of 8 years, the diagnosis of OA was considered after reassessment due to genetically proven WS1 diagnosis (Table 1). During the follow-up, he developed all clinical features of WS1 as displayed in Table 1.

Patient 2 was born at term with a birth weight of 2.8 kg. At the age of 11 years, he was presented with polyuria and polydipsia. Blood glucose level was **301 mg/dl (16.7mmol/l)** with no DKA at the time of diagnosis. Diabetes autoantibodies (ICA, IAA, and GAD65) were negative. He also had a history of decreased visual acuity, which was first observed at the age of 8 years. Blood sample for DNA was collected due to his early onset non-autoimmune diabetes and history of his elder brother. A diagnosis of OA was also considered when a full ophthalmological evaluation performed after the results of DNA analysis. These two patients from family 1, unfortunately, did not come to their regular follow-up visits. At the latest follow-up visit when he was 16 years old, his audiological evaluation also revealed the diagnosis of SND, although the patient was not suffering from a hearing problem (Figure 1A). The clinical features of WS1 and the age of onset for symptoms in this patient are displayed in Table 1.

Family 2

Family 2 is a large consanguineous family with two affected male siblings (Figure 1B). *Patient 3 (P3)* was born at term with a birth weight of 2.9 kg. At the age of 2 years, he was diagnosed with DM. At the time of the presentation, his blood glucose level was **270 mg/dl (15mmol/l)**, and the diabetes autoantibodies (ICA, IAA, and GAD65) were negative. He was started on insulin therapy. He also had a history of SND, which was noticed at the age of 2 years. He had no other features of WS1 at the time of the DNA sampling while developed central DI at the age of 20 years. He also had mild-moderate mental retardation and emotional instability (Table 1).

Patient 4 (P4) was born at term with a birth weight of 2.7 kg. At the age of 5 years, he was diagnosed with DM. The blood glucose level at admission was **306 mg/dl (17mmol/l),** and the diabetes autoantibodies (ICA, IAA, and GAD65) were negative. Insulin therapy was commenced. He also had a history of SND detected at the age of 2 years. At his latest follow-up visit at the age of 20 years had no other feature of WS1, while a mild developmental delay was observed (Table 1).

Family 3

Patient 5 (P5) is a female patient belong to first-cousin parents (Figure 1C). She had two unaffected male siblings and history of one sister and one first-cousin with DM and SND who both died with unknown aetiology. She was born at term with a birth weight of 2.5 kg. She had SND, which was first noticed at the age of 2 years and required hearing aid at the age of 5 years-old. She was presented with polyuria and polydipsia at the age of 6 years. At the time of the presentation, she had a blood glucose level of **360 mg/dl (20mmol/l)** with no DKA. The diabetes autoantibodies (ICA, IAA, and GAD65), were negative. A diagnosis of DM was considered, and insulin therapy commenced. She developed decreased visual acuity, which was first noticed at the age of 10 years, and a diagnosis of OA was considered at the age of 13 years. She developed central DI at the age of 16 and had all cardinal features of WS1 at her latest follow-up visit of 18 years old with no renal and psychiatric disorders (Table 1).

Family 4

Family 4 is a first-degree consanguineous family with two affected male (Patient 5 and 6) and one unaffected female (Figure 1D).

Patient 6 (P6) was born at term with a birth weight of 2.7 kg. At the age of 5 years, he had presented with polyuria and polydipsia. His fasting blood glucose level **was 340 mg/dl (18.9mmol/l),** and diabetes autoantibodies (ICA, IAA, and GAD65) were negative. A diagnosis of DM considered, and insulin therapy commenced. He had subsequently developed visual and hearing deficits around the age of 7 years. His developmental history achieved appropriately for age. The younger brother *(P7)*, was born at term with a birth weight of 2.9 kg. At the age of 6 years, he was presented with polyuria and polydipsia, and a fasting blood glucose level of **234 mg/dl (13mmol/l).** Diabetic auto-antibodies (ICA, IAA, and GAD65) were also negative. He had no visual or hearing loss at the time of this study (Table 1).

Ethics

The ethical approval was granted by the University College of London (UCL), Institute of Child Health, Great Ormond Street Hospital for Children. Informed consent was obtained from all patients or their legal guardians and unaffected family members.

Molecular genetics analysis

Families were originally recruited alongside a cohort of other families in a study to identify rare causes of DM and SND. Not all patients had evidence of OA at the time of the presentation, and therefore WS1 was not considered initially. Genomic DNA was isolated through standard techniques at the UCL Genomics centre. DNA samples from these patients were sent for homozygosity mapping at the UCL Genomics centre, as all patients belonged to consanguineous parents. The Illumina microarray platform was used for the genotyping, following the Infinium HD Ultra Assay protocol (Rev B, 2010, Illumina Inc, San Diego, USA). Results were generated using the Illumina Genomestudio software, and copy number variation and loss of heterozygosity data was generated (cnvPartition v3.1.6, Illumina). The minimum homozygous region size was 1Mb, with a minimum of 50 consecutive SNPs. Further to identify rare variants possibly explaining early-onset DM and SND, exome sequencing was performed out of UCL. Primer 3 software was used to

design the primers for the *WFS1* gene. The sequencing reaction was conducted using the BigDye Terminator V1.1 Cycle Sequencing kit (Applied BioSystems, Foster City, CA, USA). The sequences were compared to a reference sequence using the Sequencher® 5.3 software. The variants was classified based on the 2015 American College of Medical Genetics and Genomics and Association for Molecular Pathology guidelines (ACMG-AMP) using InterVar (17). We also classified the variants effects on protein synthesis, genotypic classification and functional consequences using the classifications described by Heredia et al.(4) and Rohayem et al.(16) (Table 2)

Results

Homozygosity mapping results has shown shared homozygous regions on chromosome 4 (chr4p16.1) between the affected individuals, and absent from their unaffected siblings. We narrowed our search to this region. The 4p16.1 locus contains 81 genes which 51 out of them are protein-coding. Although 23 of these genes are defined in Online Mendelian Inheritance in Man database (OMIM), only 4 genes (*WFS1, HMX1, SLC2A9, DRD5*) are associated with a phenotype in the OMIM morbid list (Figure 2). We performed whole-exome sequencing that identified *WFS1* gene mutations which was confirmed by Sanger sequencing (Table 2). In total, we analyzed samples from 21 individuals, including affected subjects and their apparently unaffected family members.

A novel nonsense c.1215T>A (p.Tyr405Ter) variant was detected in exon 8 of the *WFS1* gene in family 1 (P1 and P2) (Table 2). This variant has not been listed in mutation databases (HGMD, Clinvar), sequence variant databases (Exome Variant Server, dbSNP, EXAC and 1000genome) or not published elsewhere in the literature search including google and pubmed databases. The pathogenicity and classification of the variant according to various classifications are displayed in Table 2.

In family 2 (P3 and P4), we identified another novel nonsense variant in exon 5 of *WFS1*, where the base pair change c.554G>A, leads to early termination of the protein chain (p.Trp185Ter), leading to synthesis of a truncated protein (Table 2). The pathogenicity and classification of the variant according to various classifications are displayed in Table 2. We first presented this mutation in ESPE 2014 meeting(18), but shortly after our report, another group published the same variant in a Jordanian family(19).

In family 3 (P5), a known frameshift/nonsense mutation (c. 1522-1523delTA, p.Y508X) was identified in *WFS1* gene. This mutation has been reported only once before, in 2006, in two Turkish male siblings with WS1 features and suicidal behaviour (20). The pathogenicity and classification of the variant according to various classifications are displayed in Table 2.

In family 4 (P6 and P7), a novel C.1525_1540dup15 duplication mutation was identified. This variant has not been listed in mutation databases (HGMD, Clinvar), sequence variant databases (Exome Variant Server, dbSNP, EXAC and 1000genome) and not published elsewhere in the literature search including google and pubmed databases. The pathogenicity and classification of the variant according to various classifications are displayed in Table 2.

Discussion

In the present study, we evaluated the clinical characteristics, underlying molecular genetics and follow-up of 7 patients with WS and their unaffected 14 relatives from 4 unrelated consanguineous Turkish families. As the patients' phenotypes were incomplete for WS1 diagnosis, we used the homozygosity mapping to enrich the molecular genetic analysis and identified three novel variants, and one previously reported mutation in another Turkish family from same geographical location (20).

WFS1 gene is intolerant to the loss-of-function mutations (The score of probability for being loss-of-function intolerant is 1.0) (21). Functional studies and protein analysis of fibroblast cell lines of WS1 patients have shown that nonsense, splicing site and frameshift mutations of *WFS1* cause nonsense transcripts that are unstable in vivo and rapidly degraded by nonsense-mediated mRNA decay(6,22,23). Missense variants have shown to cause WS1 phenotype by affecting post-transcriptional modifications, protein stability and regulation of the degradation of wolframin transcripts (12). Besides, missense mutations are predicted to cause reduced half-time and low steady-state level of the wolframin, thus, suggested to have a dosage-sensitive effect (6,22,23).

More than 330 variants have been described in the *WFS1* gene (HMGD database website: http://www.hgmd.cf.ac.uk/ac/index.php, latest access 22nd May 2020) so far. Of which around 230 have been reported associated with WS1 phenotype. The common type of mutations include missense, nonsense, frameshift, splice-site mutations, in-frame deletions/insertions or duplications. The majority of *WFS1* mutations have been detected in exon-8, which accounts for about 86% of variants detected (24). Mutations detected in our case series were nonsense (n=2), deletion (n=1) and dupplication (n=1) mutations leading to a stop codon, thereby, early termination of the protein wolframin.

The novel nonsense c.1215T>A (p.Tyr405Ter) variant is located on the third transmembrane domain of the *WFS1* gene (Figure 2), and results in a premature stop codon and early termination of the protein chain (PVS1) (Table 2). This nonsense mutation is predicted leading to nonsense-mediated mRNA decay and complete depletion of wolframin protein, therefore, complete loss-of-function. Homozygous affected members and heterozygous/wild variants in the unaffected family members showed a phenotype and genotype co-segregation.

The novel nonsense c.554G>A (p.Trp185Ter) variant , leads to early termination of the protein chain, thereby synthesis of a truncated protein (Table 2). This is an "N terminal" stop-gain variant that causes complete loss-of-function due to premature termination of protein synthesis and rapid degradation of the truncated transcripts (Table 2 and Figure 2). We first presented this mutation in ESPE 2014 meeting(18), but shortly after our report, another group published the same variant in a Jordanian family(19). Although this mutation is predicted to severely affect wolframin expression leading to complete loss-of-function, the clinical phenotype in our cases was relatively milder compared to patients from the Jordanian family. The age of onset for DM was similar and between 2-5 years-old in both families. However, in our patients SND was observed earlier (2 years vs 5 years). The most striking discrepancy was the development of OA and DI, which were detected around the ages of 4 years (DI) and 5 years (OA) in Jordanian patients. However,

4

none of our cases developed OA until their latest follow-up visit at the age of 20 and 22 years. None of our cases developed urinary tract abnormalities which were observed in one of the Jordanian patients (hydronephrosis and gall bladder stones). Patients from both families had a moderate intellectual disability.

The previously published frameshift/nonsense, c.1522-1523delTA (p.Y508X) mutation causes deletion of 2-base pairs (TA) in exon-8 of *WFS1*. It causes disturbance in the normal reading frame which result in early termination of the amino acid sequence and synthesis of a truncated protein. This mutation has been reported only once before, in 2006, in two Turkish male siblings with WS1 features and suicidal behaviour (20). The previously reported two siblings were from the same city with our case, while both families were not related. The age of DM in the first report and our case were similar, while, our case developed hearing loss at an earlier age of 2 years, suggesting congenital SND. Besides, our case developed other clinical features at a later age compared to the first cases and still has not yet developed psychiatric complications, and urinary tract problems.

The novel C.1525_1540dup15 mutation duplicates 15-base pairs in the 5 amino acids from V509_Y513. This mutation causes stop codon and changes reading frame, thereby, termination of protein synthesis at the 34th amino acid sequence. Sanger sequencing confirmed that two affected siblings had homozygous mutation, whereas, their unaffected family members were heterozygote carriers.

WS1 is a clinically heterogeneous disorder with variable age of onset for the clinical features. DM and OA are the most common presenting features with a frequency of 98.2% and 82.1%, respectively(4). The disease has a progressive nature, and other components of DIDMOAD develop within a variable timeframe depending on mutation characteristics. Therefore, mutation analysis of the *WFS1* gene in patients with any two DIDMOAD symptoms is warranted for early detection of WS1 patients(4,15,16). In our case series, the age of onset for DM was similar to previous reports and consistent with the mutations characteristics, while, OA was detected in a lower rate and variable timing. Besides, at the time of the genetic analysis, OA was not confirmed in the majority of cases (only 2 out of 7). SND was the second most prominent feature which was detected in 5 out of 7 patients at the time of the genetic analysis. We, therefore, performed homozygosity mapping which provides enrichment for detection of mutations in *WFS1*. Homozygosity mapping has previously been reported as contributing to the detection of novel mutations as well as a new coding region responsible from the WS phenotype (25). Mutations in this second locus cause a distinct WS phenotype with upper gastrointestinal tract bleeding and the absence of DI (WS2) (25).

Age of presentation for deafness can be quite variable ranging from severe congenital deafness to late-onset mild and progressive hearing loss (4,26). The age of presentation in 3 out of 6 patients who developed SND was 2 years. Heterozygous mutations in *WFS1* are also associated with a dominant form of hearing loss; low-frequency sensorineural hearing loss (LFSNHL) (27). Although we were not able to test the audiological evaluation for our family members who were carrying a heterozygous mutation, none of those subjects had declared symptoms of hearing loss. Nevertheless, diagnostic evaluation based on parents or patients' declaration or examination without using standardized method may result in underdiagnosis of hearing loss which suggest a need for using standardized audiological methods for the evaluation of patients presenting with WS1 symptoms (26).

Urinary tract abnormalities including upper urinary tract dilatation (hydroureteronephrosis), recurrent urinary tract infections, urinary incontinence due atonic bladder, and end-stage renal failure are common features of WS1 (2,28). The rate of urinary system problems has been reported up to 90% with a median age of 20 years and three peaks observed at the age of 13, 21 and 33 years (2). Indeed, two patients with the c.554G>A and c. 1522-1523delTA mutations have been previously reported to have urinary tract problems (19,20). Nevertheless, until their latest follow-up visits at the age of 22 years, none of our patients with identical mutations developed urinary tract problem.

Mutations that cause earlier presentation of DM are suggested to cause accelerated neurodegeneration as well (4,16). Besides, glucotoxicity is also associated with and increased risk of neurodegenerative disorders in WS1 patients(16). Good glycaemic control (HbA1c<7.5%) has been shown correlated with lower rate of diabetes insipidus, deafness, neurological and psychiatric symptoms (16). In our case series, early-onset DM and SND were seen in the majority of cases. However, other neurological features of WS1 developed later within a wide range of ages. Notably, two patients with the earliest presentation for DM and SND had not developed OA at their age of 20 and 22 years-old. One of these patients also has not yet developed other features of WS1 except for DM and SND, while the other developed DM, DI and SND in addition to psychiatric symptoms (Table 1).

WS1 patients present with a wide variety of clinical symptoms and signs due to the clinically heterogenous nature of the disease. Furthermore, the varying ages of presentation of symptoms, and the different rates of progression, makes WS1 a very difficult syndrome to diagnose in the early stages, especially when only one symptom may be apparent, consequently leading to delay in the treatment and management. With the use of homozygosity mapping, we were able to narrow down on specific regions of the human genome, which were shared only by affected individuals of different families, allowing us to fast track the search for the causative gene.

In conclusion, our study expands the mutation spectrum of *WSF1* with three novel nonsense variants in three unrelated consanguineous families confirming variable phenotypical expression and heterogeneity in presenting features as well progressive nature of the disease. The prominent WS1 features in our cases were early onset SND, lower rate and delay in development of OA, and lack of urinary tract problems. Homozygosity mapping proved to be a useful tool for enrichment of molecular genetic analysis in the early diagnosis of WS1 patients with an incomplete phenotype, particularly those with no OA, or belonging to consanguineous pedigrees.

References

1. Wolfram DJ. Diabetes mellitus and simple optic atrophy among siblings. Mayo Clin Proc 1938;13:715-718.
2. Pallotta MT, Tascini G, Crispoldi R, Orabona C, Mondanelli G, Grohmann U, Esposito S. Wolfram syndrome, a rare neurodegenerative disease: from pathogenesis to future treatment perspectives. J Transl Med 2019;17:238-238.

3.   Barrett TG, Bundey SE, Macleod AF. Neurodegeneration and diabetes: UK nationwide study of Wolfram (DIDMOAD) syndrome. Lancet (London, England) 1995;346:1458-1463. Epub 1995/12/02

4.   de Heredia ML, Cleries R, Nunes V. Genotypic classification of patients with Wolfram syndrome: insights into the natural history of the disease and correlation with phenotype. Genetics in medicine : official journal of the American College of Medical Genetics 2013;15:497-506. Epub 2013/02/23

5.   Inoue H, Tanizawa Y, Wasson J, Behn P, Kalidas K, Bernal-Mizrachi E, Mueckler M, Marshall H, Donis-Keller H, Crock P, Rogers D, Mikuni M, Kumashiro H, Higashi K, Sobue G, Oka Y, Permutt MA. A gene encoding a transmembrane protein is mutated in patients with diabetes mellitus and optic atrophy (Wolfram syndrome). Nature genetics 1998;20:143-148. Epub 1998/10/15

6.   Hofmann S, Philbrook C, Gerbitz K-D, Bauer MF. Wolfram syndrome: structural and functional analyses of mutant and wild-type wolframin, the WFS1 gene product. Human molecular genetics 2003;12:2003-2012.

7.   Takeda K, Inoue H, Tanizawa Y, Matsuzaki Y, Oba J, Watanabe Y, Shinoda K, Oka Y. WFS1 (Wolfram syndrome 1) gene product: predominant subcellular localization to endoplasmic reticulum in cultured cells and neuronal expression in rat brain. Human molecular genetics 2001;10:477-484. Epub 2001/02/22

8.   Fonseca SG, Burcin M, Gromada J, Urano F. Endoplasmic reticulum stress in beta-cells and development of diabetes. Current opinion in pharmacology 2009;9:763-770. Epub 2009/08/12

9.   Fonseca SG, Ishigaki S, Oslowski CM, Lu S, Lipson KL, Ghosh R, Hayashi E, Ishihara H, Oka Y, Permutt MA, Urano F. Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells. The Journal of clinical investigation 2010;120:744-755. Epub 2010/02/18

10.   Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nature reviews Molecular cell biology 2007;8:519-529. Epub 2007/06/15

11.   Vembar SS, Brodsky JL. One step at a time: endoplasmic reticulum-associated degradation. Nature reviews Molecular cell biology 2008;9:944-957. Epub 2008/11/13

12.   Guo X, Shen S, Song S, He S, Cui Y, Xing G, Wang J, Yin Y, Fan L, He F, Zhang L. The E3 ligase Smurf1 regulates Wolfram syndrome protein stability at the endoplasmic reticulum. The Journal of biological chemistry 2011;286:18037-18047. Epub 2011/04/02

13.   Ishihara H, Takeda S, Tamura A, Takahashi R, Yamaguchi S, Takei D, Yamada T, Inoue H, Soga H, Katagiri H, Tanizawa Y, Oka Y. Disruption of the WFS1 gene in mice causes progressive beta-cell loss and impaired stimulus-secretion coupling in insulin secretion. Human molecular genetics 2004;13:1159-1170. Epub 2004/04/02

14.   Lipson KL, Fonseca SG, Ishigaki S, Nguyen LX, Foss E, Bortell R, Rossini AA, Urano F. Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. Cell metabolism 2006;4:245-254. Epub 2006/09/05

15.   Çelmeli G, Türkkahraman D, Çürek Y, Houghton J, Akçurin S, Bircan I. Clinical and Molecular Genetic Analysis in Three Children with Wolfram Syndrome: A Novel WFS1 Mutation (c.2534T>A). Journal of clinical research in pediatric endocrinology 2017;9:80-84. Epub 2016/07/29

16.   Rohayem J, Ehlers C, Wiedemann B, Holl R, Oexle K, Kordonouri O, Salzano G, Meissner T, Burger W, Schober E, Huebner A, Lee-Kirsch MA. Diabetes and neurodegeneration in Wolfram syndrome: a multicenter study of phenotype and genotype. Diabetes care 2011;34:1503-1510. Epub 2011/05/24

17.   Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in medicine : official journal of the American College of Medical Genetics 2015;17:405-424. Epub 2015/03/06

18.   Maha Sherif HD, Atilla Cayir, Mehmet Nuri Ozbek, Riza Taner Baran, Ayşe Nurcan Cebeci, Sophia Tahir, Sofia Asim Rahman, Khalid Hussain. Two families with diabetes mellitus and sensorineural deafness. Horm Res Paediatr 2014; 82:1-507.

19.   Bodoor K, Batiha O, Abu-Awad A, Al-Sarihin K, Ziad H, Jarun Y, Abu-Sheikha A, Abu Jalboush S, Alibrahim KS. Identification of a novel WFS1 homozygous nonsense mutation in Jordanian children with Wolfram syndrome. Meta gene 2016;9:219-224. Epub 2016/09/13

20.   Aluclu MU, Bahceci M, Tuzcu A, Arikan S, Gokalp D. A new mutation in WFS1 gene (C.1522-1523delTA, Y508fsX421) may be responsible for early appearance of clinical features of Wolfram syndrome and suicidal behaviour. Neuro endocrinology letters 2006;27:691-694. Epub 2006/12/26

21.   Lek M, Karczewski KJ, Minikel EV, Samocha KE, Banks E, Fennell T, O'Donnell-Luria AH, Ware JS, Hill AJ, Cummings BB, Tukiainen T, Birnbaum DP, Kosmicki JA, Duncan LE, Estrada K, Zhao F, Zou J, Pierce-Hoffman E, Berghout J, Cooper DN, Deflaux N, DePristo M, Do R, Flannick J, Fromer M, Gauthier L, Goldstein J, Gupta N, Howrigan D, Kiezun A, Kurki MI, Moonshine AL, Natarajan P, Orozco L, Peloso GM, Poplin R, Rivas MA, Ruano-Rubio V, Rose SA, Ruderfer DM, Shakir K, Stenson PD, Stevens C, Thomas BP, Tiao G, Tusie-Luna MT, Weisburd B, Won HH, Yu D, Altshuler DM, Ardissino D, Boehnke M, Danesh J, Donnelly S, Elosua R, Florez JC, Gabriel SB, Getz G, Glatt SJ, Hultman CM, Kathiresan S, Laakso M, McCarroll S, McCarthy MI, McGovern D, McPherson R, Neale BM, Palotie A, Purcell SM, Saleheen D, Scharf JM, Sklar P, Sullivan PF, Tuomilehto J, Tsuang MT, Watkins HC, Wilson JG, Daly MJ, MacArthur DG. Analysis of protein-coding genetic variation in 60,706 humans. Nature 2016;536:285-291. Epub 2016/08/19

22.   Frischmeyer PA, Dietz HC. Nonsense-mediated mRNA decay in health and disease. Human molecular genetics 1999;8:1893-1900. Epub 1999/09/02

23. Daiho T, Yamasaki K, Suzuki H, Saino T, Kanazawa T. Deletions or specific substitutions of a few residues in the NH(2)-terminal region (Ala(3) to Thr(9)) of sarcoplasmic reticulum Ca(2+)-ATPase cause inactivation and rapid degradation of the enzyme expressed in COS-1 cells. The Journal of biological chemistry 1999;274:23910-23915. Epub 1999/08/14

24. Astuti D, Sabir A, Fulton P, Zatyka M, Williams D, Hardy C, Milan G, Favaretto F, Yu-Wai-Man P, Rohayem J, López de Heredia M, Hershey T, Tranebjaerg L, Chen J-H, Chaussenot A, Nunes V, Marshall B, McAfferty S, Tillmann V, Maffei P, Paquis-Flucklinger V, Geberhiwot T, Mlynarski W, Parkinson K, Picard V, Bueno GE, Dias R, Arnold A, Richens C, Paisey R, Urano F, Semple R, Sinnott R, Barrett TG. Monogenic diabetes syndromes: Locus-specific databases for Alström, Wolfram, and Thiamine-responsive megaloblastic anemia. Human mutation 2017;38:764-777.

25. El-Shanti H, Lidral AC, Jarrah N, Druhan L, Ajlouni K. Homozygosity mapping identifies an additional locus for Wolfram syndrome on chromosome 4q. American journal of human genetics 2000;66:1229-1236. Epub 2000/03/31

26. Karzon R, Narayanan A, Chen L, Lieu JEC, Hershey T. Longitudinal hearing loss in Wolfram syndrome. Orphanet journal of rare diseases 2018;13:102. Epub 2018/06/28

27. Lesperance MM, Hall JW, 3rd, San Agustin TB, Leal SM. Mutations in the Wolfram syndrome type 1 gene (WFS1) define a clinical entity of dominant low-frequency sensorineural hearing loss. Archives of otolaryngology--head & neck surgery 2003;129:411-420. Epub 2003/04/23

28. Yuca SA, Rendtorff ND, Boulahbel H, Lodahl M, Tranebjærg L, Cesur Y, Dogan M, Yilmaz C, Akgun C, Acikgoz M. Rapidly progressive renal disease as part of Wolfram syndrome in a large inbred Turkish family due to a novel WFS1 mutation (p.Leu511Pro). European journal of medical genetics 2012;55:37-42. Epub 2011/10/05

Table 1. Age of onset for clinical characteristics for the WS1 features in 7 cases with homozygous *WFS1* mutations

Patient (Sex)/Family number	Age of the mutation analysis	Age at the latest F/up visit	DM	SND	OA	DI	Neuro-psychiatric disorders and other symptoms
P1 (M) (Family 1)	15 years	19 years	7 years-old	13 years-old (HA) (Presumably earlier as the symptoms were present earlier)	15 years-old (Age of onset for symptoms was reported at 8-9 years)	13 years	Mild mental retardation, emotional disability Renal USG normal Developed dysphagia at the age of 20 and declared died with an aspiration problem during feeding presumably developed due to problem in swallowing
P2 (M) (Family 1)	12 years	18 years	11 years-old	16 years-old (HA) (Presumably earlier as the symptoms were present earlier)	13-years (Age of onset for symptoms was reported at 8-9 years)	Absent at the latest fw/up visit	Has mild anxiety and depressive mood which observed at the age of 16 years-old. Renal USG and urine protein excretion normal.
P3 (M) (Family 2)	17 years	22 years-old	2 years-old	2 years-old (HA)	Absent	20 years-old	Mild-moderate mental retardation, Has severe emotional disability since 15 years-old
P4 (M) (Family 2)	15 years	20 years-old	5 years-old	2 years-old (HA)	Absent	Absent	Mild mental retardation No psychiatric symptoms observed Renal USG and ECO: normal
P5 (F) (Family 3)	13 years	18 years old	7 years-old	2 years old (HA)	10 years-old	16 years-old*	Absent
P6 (M) (Family 3)	10 years	10 years	5 years-old	7 years (HA)	7 years	Absent	Absent
P7 (M) (Family 4)	7 years	7 years	5 years-old	Absent	Absent	Absent	Absent

M: male, F: Female, SND: Sensorineural deafness, OA: Optic atrophy, DI: Diabetes insipidus, HA: Hearing aid, USG: ultrasonography, ECO: Echocardiography

Table 2. Mutations characteristics, results of *"in silico"* analyses, protein prediction, functional consequences and individuals with particular mutations

NM_006005.3	Family 1	Family 2	Family 3	Family 4
Mutation	c.1215T>A	*c.554G>A	c. 1522_1523delTA	c.1525_1540dup
Exon	8	5	8	8
Protein change	p.Tyr405Ter	p.Trp185Ter	p.Tyr508CysfsTer34	p.Leu514ArgfsTer34
Mutation type	NS	NS	NS/FS	Dup/FS
Location	Transmembrane domain (3rd segment)	N- terminal domain	Transmembrane domain (5th segment)	Transmembrane domain (5th segment)
Zygosity	Homozygous	Homozygous	Homozygous	Homozygous
In silico **analyses**				
Mutation Taster	Disease-causing	Disease-causing	Disease-causing	Disease-causing
DANN score	0.9825	0.9971	NA	NA
Eigen score	Benign	Pathogenic		
FATHMM-MKL(coding prediction)	Neutral	Damaging		
GERP (conservation prediction)	Not-conserved	Conserved		
ACMG classification	Pathogenic PVS1 PM1 PM2 BP4	Pathogenic PVS1 PM1 PM2 PP3	Pathogenic PVS1 PM1 PM2	Pathogenic PVS1 PM1 PM2
Mutation effect (Rohayem et al.) (16)	Group 1 (Complete loss of function)	Group 1 (Complete loss of function)	Group 1 (Complete loss of function)	Group 1 (Complete loss of function)
Mutation Type (Heredia et al.) (4)	Type 3	Type 1	Type 3	Type 3
Genotypic Class (Heredia et al.) (4)	C	A1	C	C
Phenotype-genotype co-segregation	Both parents and unaffected sibling are heterozygous carriers	Parents are heterozygous carriers for the mutation, an unaffected sibling is homozygous for the normal allele.	Parents and one unaffected sibling are heterozygous carriers, an unaffected sibling is homozygous for the normal allele.	Both parents and unaffected sibling are heterozygous carriers of the mutation

DANN: Deleterious Annotation of Genetic Variants; GERP: Genomic Evolutionary Rate Profiling; ACMG: American College of Medical Genetics and Genomics; NS: Nonsense, FS: Frameshift, Dup: Duplication, HM: Homozygous, *We first described this mutation (presented at ESPE annual meeting 2014)(18), but was later published by another group in 2016 (19).
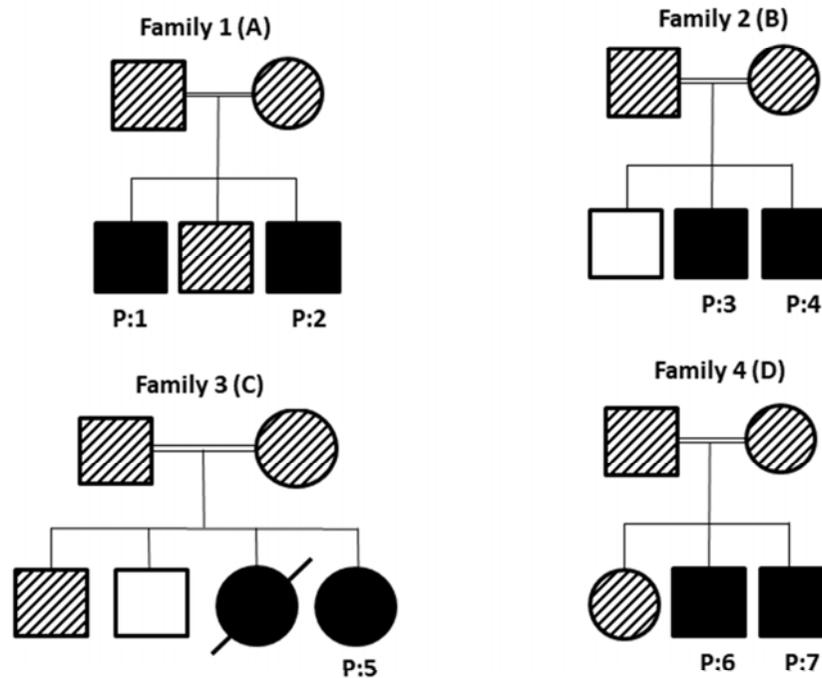
9

Figure 1 (A-D). Family pedigrees of Wolfram syndrome patients. Black-filled boxes refer to homozygous and clinically affected members, while black shaded boxes refer heterozygous and completely empty boxes refer to mutation-negative unaffected family members. (A female sibling of P5 was not undertaken for mutation analysis, but she had clinical features of WS1 similar to P5. It was therefore thought that she presumably had an identical mutation and displayed with black-filled box).
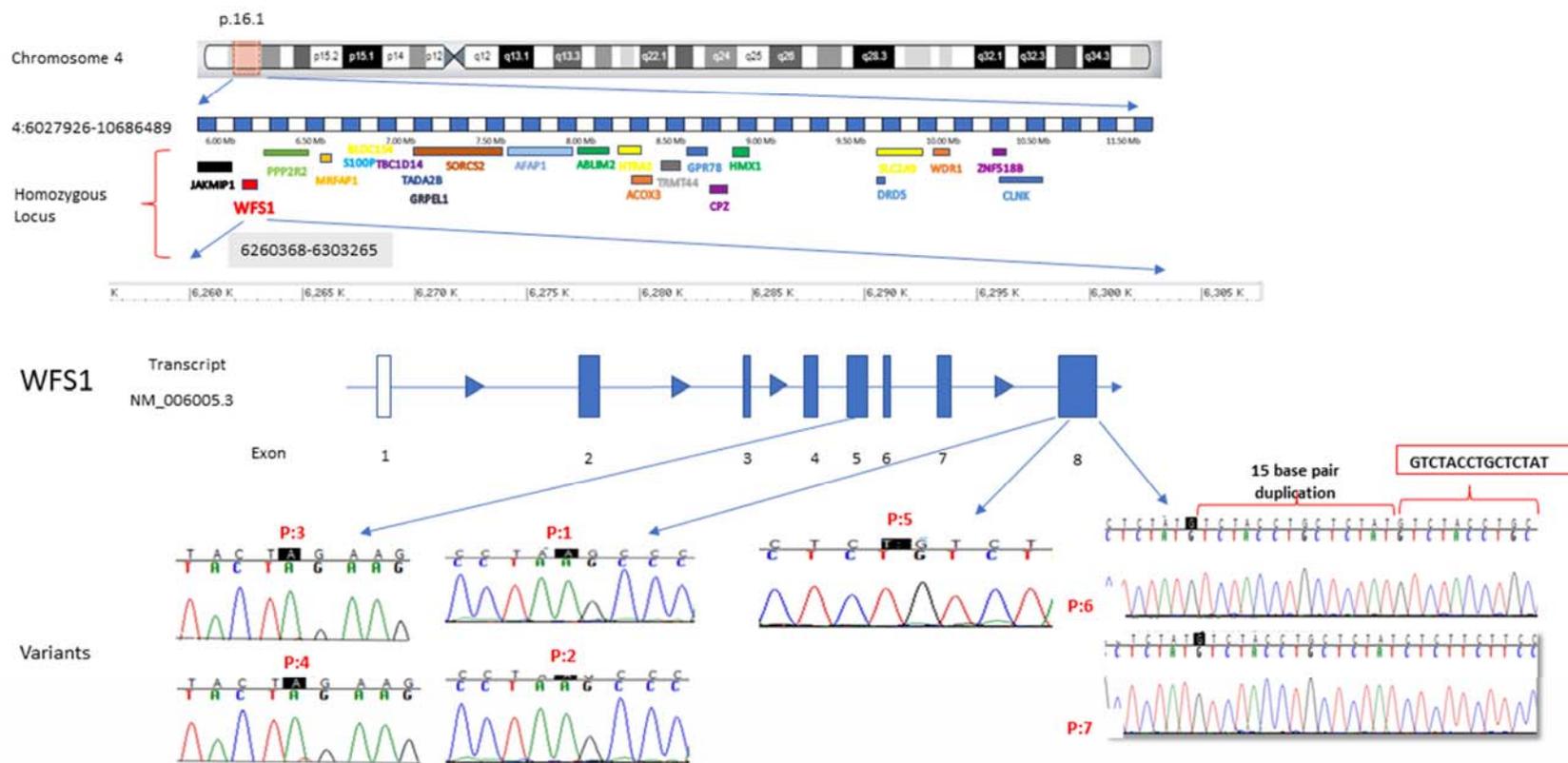
10

Figure 2. Homozygosity mapping showed a shared region between affected members where the WFS1 gene was located in the same region. Electropherogram of the WFS1 analysis for patients 1-7.
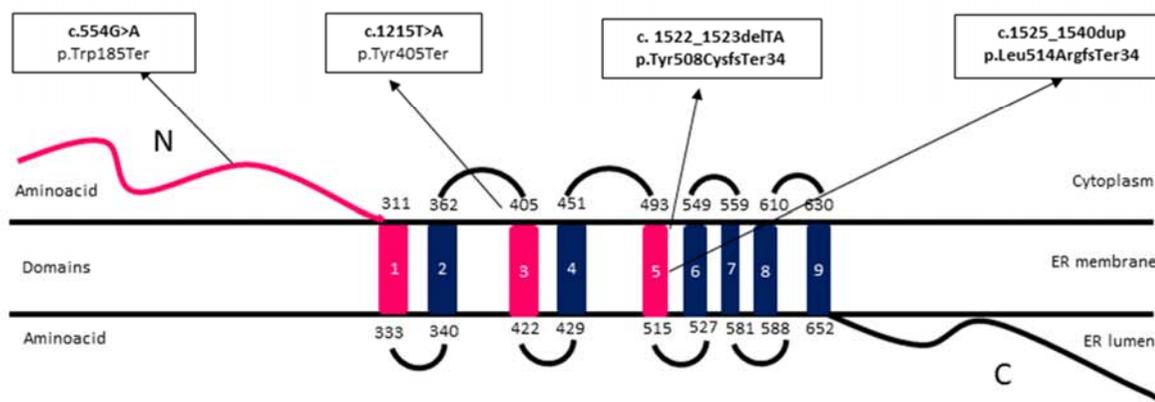
Figure 3. A motif of *WFS1* showing NH2-terminal, 9 transmembrane and -COOH terminal domains and location of mutations detected in the present report.