Journal of Molecular and Genetic Medicine

Gupta et al., J Mol Genet Med 2018, 12:4
DOI: 10.4172/1747-0862.1000380

Research Article Open Access

Spectrum of Mutations in *WFS1* Gene in Six Families with Wolfram Syndrome: Identification of Five Novel Mutations

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Abstract

Background: Wolfram syndrome is a neurodegenerative disorder characterized by the acronym DIDMOAD (Diabetes Insipidus (DI), Diabetes Mellitus (DM), Optic Atrophy (OA) and Deafness). Homozygous/compound heterozygous mutations in *WFS1* gene causes autosomal recessive form of Wolfram syndrome (AR-WS) whereas heterozygous mutations are associated with autosomal dominant-low-frequency non-syndromic hearing loss (AD-LFNSHL). Clinical symptoms and degree of severity is reported to be heterogeneous in WS patients.

Aim: To characterize clinical features and molecular gene mutations in patients with WS in India and compare them with data from other countries.

Patients and Methodology: Eleven patients from 6 families were enrolled. In nine patients from 4 families with phenotypic features of diabetes mellitus, optical atrophy and hearing loss *WFS1* gene was sequenced. Two patients of the other 2 families presented with hearing loss only and were analysed for targeted deafness genes panel by next generation sequencing.

Results: Nine patients from 4 families had biallelic mutations in *WFS1* gene. Two patients harboured heterozygous mutation in *WFS1* gene. Seven different mutations *WFS1* were identified, of which 5 mutations were novel. All the identified mutations were present in exon 8 of *WFS1* gene.

Conclusion: Pathogenic variations in *WFS1* gene can cause both AR-WS and AD-LFNSHL. We recommend a protocol in which patients with WS should be first sequenced for the hotspot exon 8. If no mutation is identified, then the full gene should be sequenced. Further, for patients with hearing loss with/without diabetes and/or optical atrophy, WS should be considered as one of the differential diagnosis.

Keywords: Wolfram Syndrome (WS); Non-syndromic hearing loss (AD-LFNSHL); *WFS1* gene; Mutation; Hotspot

Introduction

Wolfram syndrome (WS) is a neurodegenerative disorder characterized by features denoted by the acronym DIDMOAD (Diabetes Insipidus (DI), Diabetes Mellitus (DM), Optic Atrophy (OA) and Deafness). Although diabetes and optic atrophy comprise the minimal diagnostic criteria, patients may present with neurological, urological and psychiatric manifestations later in their life [1,2]. The prevalence is approximately 1 in 1,00,000 for WS, with a carrier frequency of 1 in 354 in the UK and 1 in 100 in the North American population [3-5].

Genetic mutations in *WFS1* gene cause both recessive and dominant forms of WS. Homozygous or compound heterozygous mutations in *WFS1* gene cause autosomal recessive form of Wolfram syndrome (WS), and heterozygous mutation in one of the alleles is associated with autosomal dominant WS like syndrome [6,7]. Several other, *WFS1*-related disorders have been described such as low-frequency non-syndromic hearing loss (LFNSHL) with autosomal dominant transmission [8]. Mutations in the *WFS1* gene are also implicated in autosomal dominant DM, with OA (11), with DM and OA together, with HL and OA and psychiatric problems [6-16].

WFS1 gene is mapped on chromosome 4p16 and spans 33.4-kb of genomic DNA. It has eight exons, of which the first exon is non-coding. This gene encodes a transmembrane glycoprotein called wolframin (890 amino acid long), located primarily in the endoplasmic reticulum (Figure 1). This protein is ubiquitously expressed in brain, pancreas, heart, and insulinoma beta-cell lines [5]. Its function has not been fully characterised, but it is reported to have a crucial role in the regulation of Na+/K+ ATPase β-1 subunit, regulation of Ca^{2+} homeostasis, negative regulation of ER stress and the regulation of insulin biosynthesis and secretion in pancreatic β-cells [17-21].

Different types of mutations, including missense, out of frame or in frame deletions, nonsense and splicing are reported in patients with WS. Majority (80-90%) of the mutations are inactivating and reside in exon 8 [22]. There is a paucity of data on genetic studies in WS in India [23]. We report series of eleven cases from six families, diagnosed to have WS by identification of underline genetic cause in *WFS1* gene.

Materials and Methods

Patients

Eleven probands from 6 families were enrolled in the study. Blood samples were collected from patients and parents (if available), after

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Received October 22, 2018; **Accepted** November 10, 2018; **Published** November 14, 2018

Citation: Gupta D, Bhai P, Saxena R, Bijarnia-Mahay S, Puri RD, et al. (2018) Spectrum of Mutations in *WFS1* Gene in Six Families with Wolfram Syndrome: Identification of Five Novel Mutations. J Mol Genet Med 12: 380 doi:10.4172/1747-0862.1000380

obtaining an informed consent from the parents/guardian. Ethical clearance was obtained from the Ethics Committee of Sir Ganga Ram Hospital, New Delhi. Detailed information about age of onset of symptoms, clinical presentation, and family history was collected.

Molecular analysis of *WFS1* gene

DNA was isolated from leukocytes using standard methods [24]. Coding regions as well as exon– intron boundaries of the hotspot exon 8 of *WFS1* genes was amplified by polymerase chain reaction (PCR) for families F1, F2, F3 and F4. Exon 8 (~2.6 Kb), was amplified in four PCR fragments using four sets of primers designed using web primer software (http://www.yeastgenome.org/cgi-bin/web-primer) (sequence available on request). Thermal profile was as follows: initial denaturation at 95°C for 5min, cycle denaturation 95°C for 1min, annealing at 60°C for 1 min, elongation at 72°C for 1min 30 sec (for 35 cycles), final elongation at 72°C for 7min. Amplified PCR products were purified and sequenced using BigDye Terminator sequencing kit and 3500 genetic analysers (Thermo Fisher Applied Biosystems, Foster city, CA, USA) according to the manufacturer's protocol. Chromatograms obtained after sequencing were analysed using the Chromas pro software (Technelysium.com.au) and matched to the wild type *WFS1* (NM_006005) gene sequence. Mutations were classified as novel, if not earlier reported in dbsnp, HGMD, ExAc, 1000 genome, Clinvar and LOVD databases.

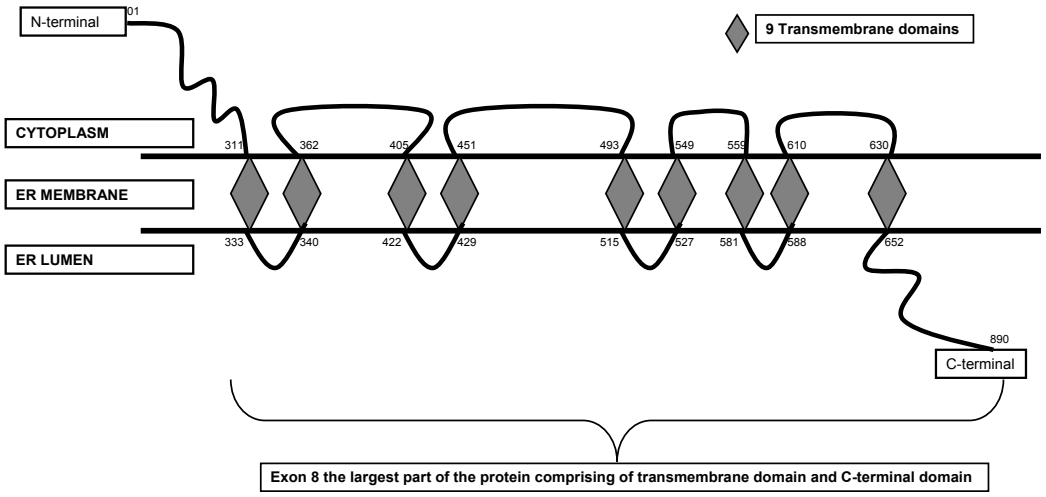


Figure 1: Hypothetical structure of the wolframin describing the transmembrane domains in the ER (Endoplasmic reticulum) membrane and amino terminal (in cytoplasm) and carboxy terminal domain of the protein in ER lumen.

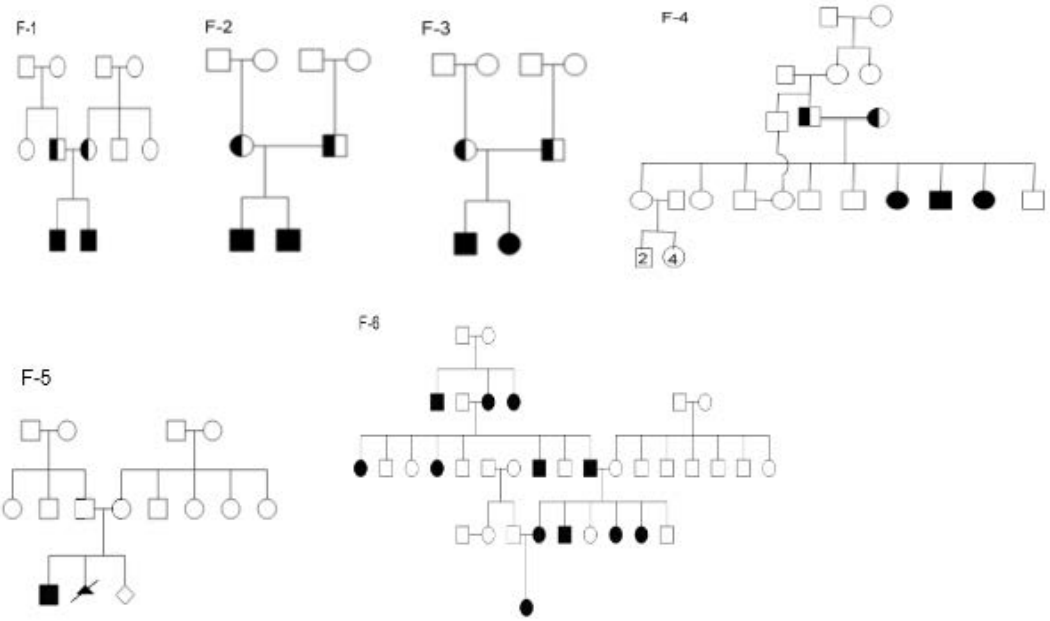


Figure 2: Pedigree charts of wolfram families enrolled labeled as F-1 to F-6. Families (F-1 to F-4) were autosomal recessive WS while families (F-5 and F-6) were autosomal dominant - low-frequency non-syndromic hearing loss (AD-LFNSHL).

Affected children of family F5 and F6 had bilateral sensorineural hearing loss alone, with no other significant phenotypic feature (Table 1). In view of this, targeted deafness gene panel (involving more than 300 genes) was performed using next generation sequencing platform for both the samples. The raw data output files were analyzed for the putative genetic variants using standard Bioinformatics pipeline including base calling, alignment of reads to GRCh37/hg19 genome assembly, primary filtering out of low-quality reads and probable artefacts, and subsequent annotation of variants. All variants related to the phenotype of the proband, except benign or likely benign variants were further evaluated. Heterozygous variant in *WFS1* gene was identified in both the families, also confirmed by Sanger sequencing.

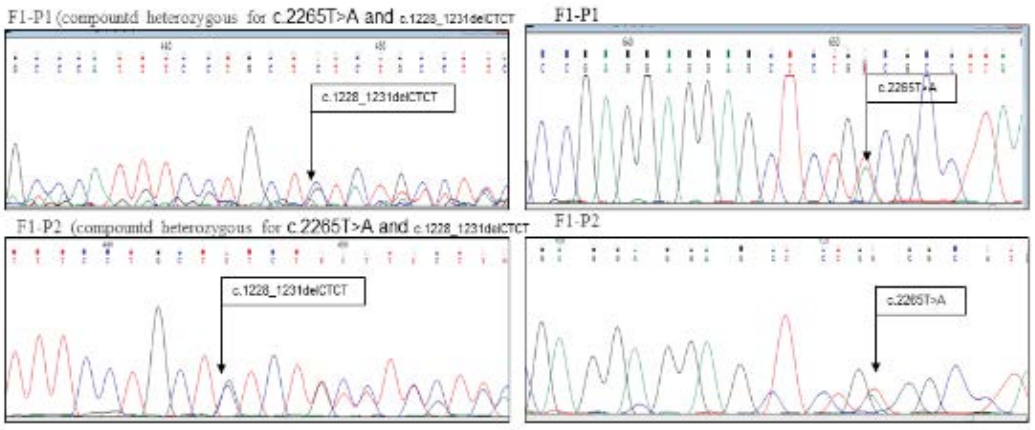
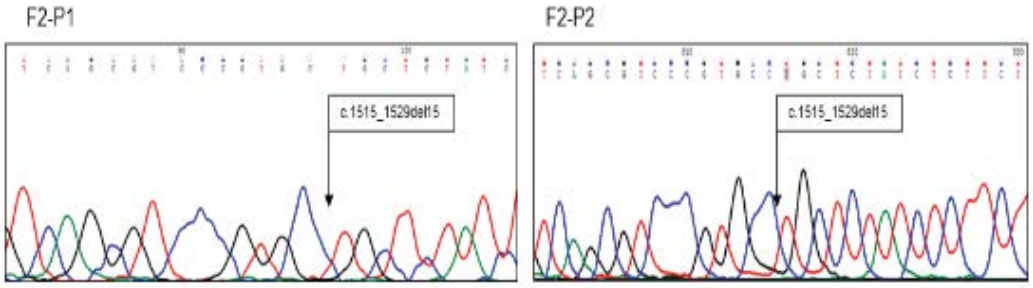
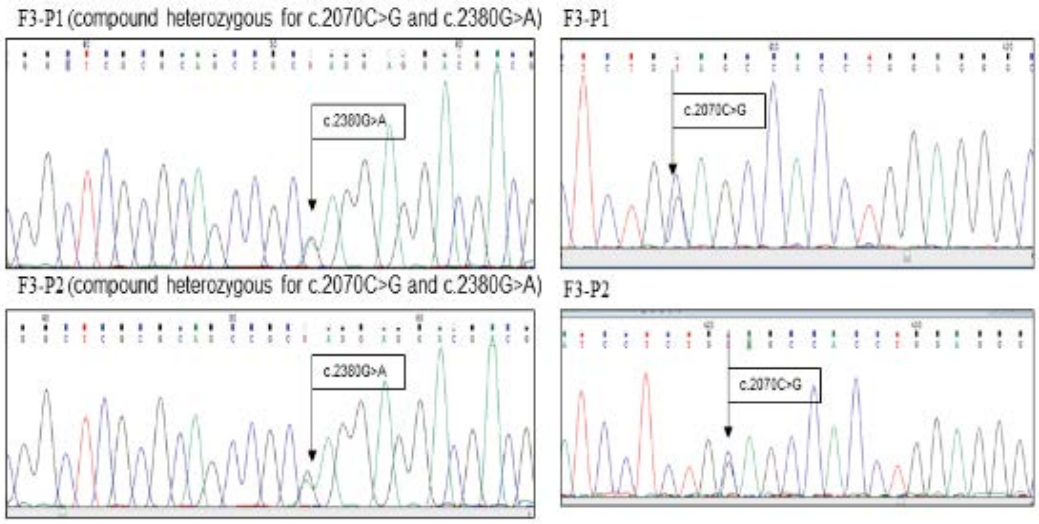


Figure 3: Chromatograms pictures of the families (F1-F-5) of wolfram syndrome. *Chromatograms could not be made available for family F-6.
3A: Chromatograms of both the probands of family F-1.



3B: Chromatograms of both the probands of family F-2.



3C: Chromatograms of both the probands of family F-3.

Annotation of novel variations

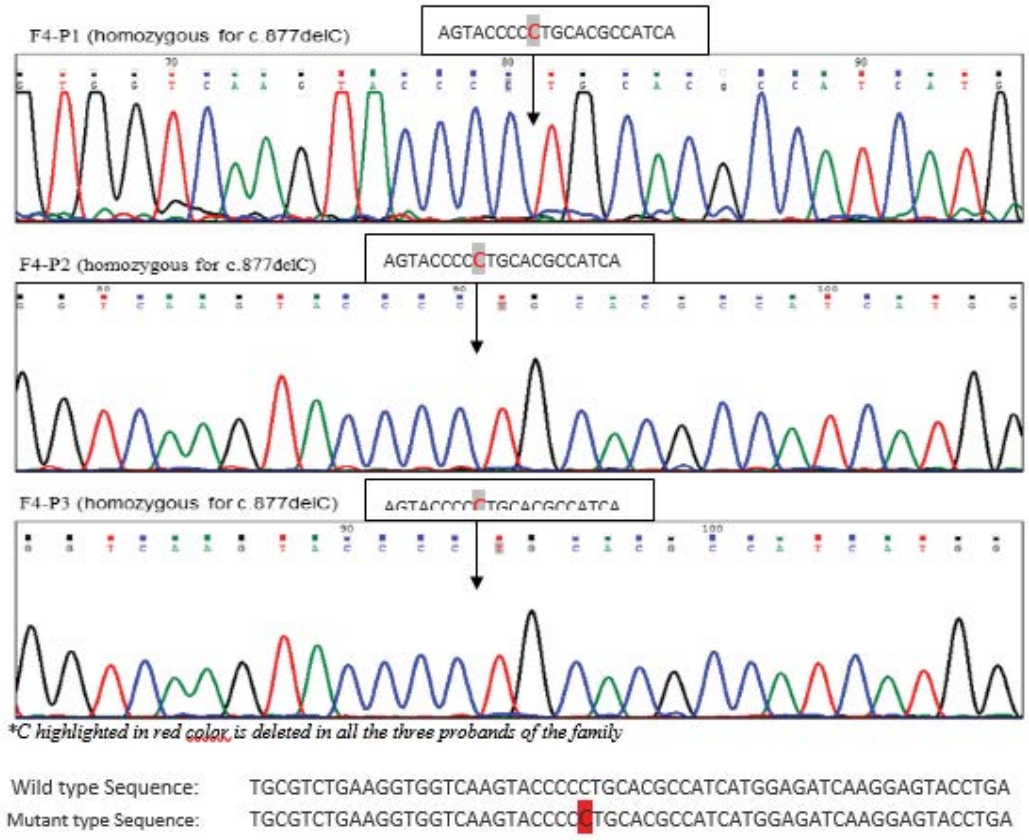
In silico tools like PolyPhen2, SIFT, Mutation Taster, LRT, Mutation Assessor and FATHMM were used to predict the effect of novel variations (Table 2) [25-30]. Conservation of the amino acids in case of missense change was checked in the homologous protein of different species by multiple sequence alignment using Clustal-Omega web server [31]. Segregation analysis was done where ever possible.

Results

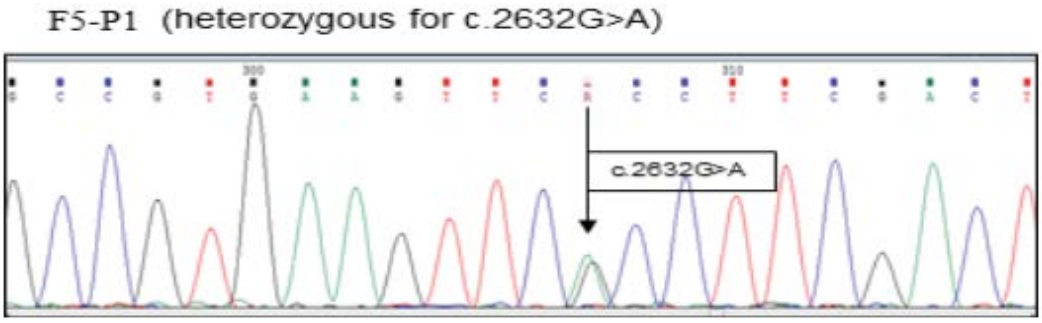
Nine patients from 4 families (F1-F4) met the minimal diagnostic criteria of hearing loss, optic atrophy and diabetes mellitus. Diabetes insipidus was present in 7 patients from 3 families (F2, F3 and F4). Bi-allelic mutations in *WFS1* gene were identified in these patients, confirming a diagnosis of autosomal recessive WS.

Affected probands of family F5 and F6 had no phenotypic features of WS other than deafness. Heterozygous mutation was identified in *WFS1* gene in these patients, confirming diagnosis of autosomal dominant low frequency non-syndromic hearing loss (LFNSHL). Clinical details are set out in Table 1. Pathogenic variants were identified in all 11 probands of 6 families. Eight different mutations were detected of which 6 were novel. All the identified mutations were present in exon 8 of *WFS1* gene (Figure 2).

In family F1, both the probands were compound heterozygous for two novel pathogenic variants in *WFS1* gene (Figure 3A). One was a



3D: Chromatograms of all three probands of family F-4.



3E: Chromatograms of the probands of family F-5.

nonsense change (c.2265T>A; p.Cys755Ter) and the other variation (c.1228_1231delCTCT, p.Val412SerfsTer29) was a deletion of 4 base pairs (CTCT) at 1228 nucleotide position. Both the variations were predicted to be unstable or non-functional due to early premature termination codon (PTC) that would undergo mechanism of nonsense mediated decay (NMD) [32-34].

In family F2, both affected probands were homozygous for deletion of 15bp (c.1525_1539del15) resulting in an in-frame deletion of five amino acids (p.Val509_Tyr513del) (Figure 3B). This mutation is previously reported with a different nomenclature (c.1522_1536del15 (p.Tyr508_Leu512del)) and was predicted to produce a protein of shorter length.

In family F3, both the probands were compound heterozygous for c.2070C>G (p.Cys690Trp), and c.2380G>A (p.Glu794Lys) pathogenic variants (Figure 3C). These missense pathogenic variations are unique to the family and are predicted to be deleterious by in silico tools (Table 2).

In family F4, All the 3 probands of this family were homozygous for a novel single base pair deletion (c.877delC, p.Leu293CysfsTer11) (Figure 3D). The deletion of one base pair result in frameshift with the occurrence of an early stop codon (11 amino acids downstream). This further leads to formation of truncated protein which is expected to undergo NMD.

In family F5, a novel heterozygous missense change, (c.2632G>A, p.Ala878Thr) was identified in the affected proband (Figure 3E). This

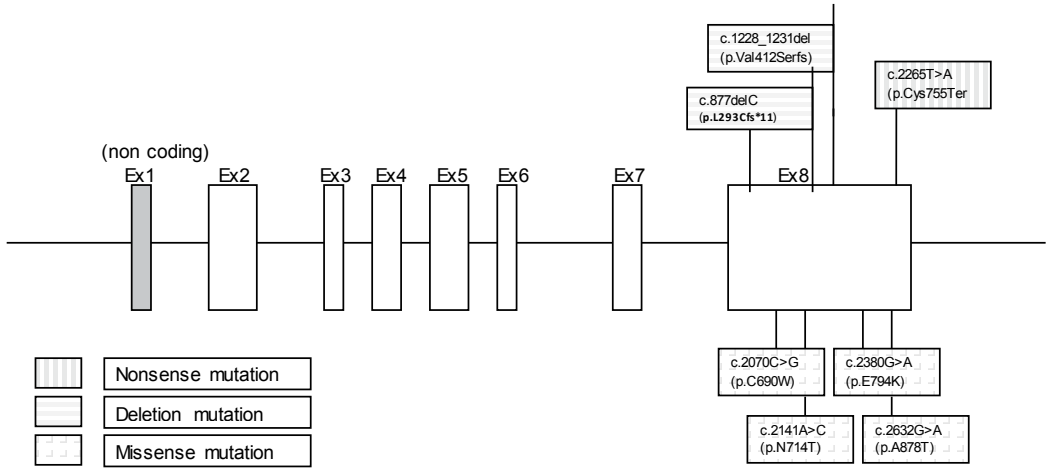


Figure 4: Representation of the structure of *WFS1* gene comprising of 8 exons (exon 1 is non-coding), showing the mutations identified in this study. Different types of mutations are shown with dissimilar shaded boxes.

Family	Age/ Sex	Age of onset	Clinical details	Mutation identified	Protein change	Exon	Zygosity	Final Diagnosis
F1:P1	25-9-09/M	7.5y	DM, OA, HL, DI absent	c.2265T>A#, c.1228_1231delCTCT	p.Cys755Ter#, p.Val412SerfsTer29	8	Compound Heterozygous	AR-WS
F1:P2	03-3-12/M	5y	DM, OA, HL, DI absent	c.2265T>A, c.1228_1231delCTCT	p.Cys755Ter, p.Val412SerfsTer30	8	Compound Heterozygous	AR-WS
F2:P1	22Y/M	20y	DI, DM, HL, OA	c.1525_1539del15	p.Val509_Tyr513del	8	Homozygous	AR-WS
F2:P2	12Y/M	12y	DI, DM, HL, OA	c.1525_1539del15	p.Val509_Tyr513del	8	Homozygous	AR-WS
F3:P1	20Y/F	20y	DI, DM, HL, OA	c.2070C>G#, c.2380G>A#	p.Cys690Trp#; p.Glu794Lys#	8	Compound Heterozygous	AR-WS
F3:P2	22Y/M	22y	DI, DM, HL, OA	c.2070C>G, c.2380G>A	p.Cys690Trp; p.Glu794Lys	8	Compound Heterozygous	AR-WS
F4:P1	19Y/F	5y	DI, DM, HL, OA	c.877_877delC#	p.Leu293CysfsTer11#	8	Homozygous	AR-WS
F4:P2	18Y/M	5y	DI, DM, HL, OA	c.877_877delC	p.Leu293CysfsTer11	8	Homozygous	AR-WS
F4:P3	15Y/F	5y	DI, DM, HL, OA	c.877_877delC	p.Leu293CysfsTer11	8	Homozygous	AR-WS
F5	28-12-11/M	5y	HL, OA absent, DM not present	c.2632G>A#	p.Ala878Thr#	8	Heterozygous	AD-LFNSHL
F6	7y/F	7y	HL, OA absent, DM not present	c.2141 A>C	p.Asn714Thr	8	Heterozygous	AD-LFNSHL

Novel mutations identified in this study, HL: Hearing Loss, OA: Optical Atrophy, DM: Diabetes Mellitus, AR-WS: Autosomal Recessive Wolfram Syndrome, AD-LFNSHL: Autosomal Dominant Low Frequency Non-Syndromic Hearing Loss

Table 1: Clinical and molecular data of the families with WS.

S.No.	Mutation identified	Protein change	Novel/ Reported	Mutation taster	Polyphen 2	SIFT	LRT	Mutation Assessor	FATHMM	dbSNP
1	c.2265T>A	p.Cys755Ter	Novel	Disease Causing	NA	NA	Deleterious	NA	Damaging	Not present
2	c.1228_1231delCTCT	p.Val412SerfsTer29	Reported (48)	Disease Causing	NA	NA	NA	NA	NA	1.132E+09
3	c.1525_1539del15	p.Val509_Tyr513del	Reported (33)	Disease Causing	NA	NA	NA	NA	NA	781262017
4	c.2070C>G	p.Cys690Trp	Novel	Disease causing	Damaging	Damaging	Deleterious	Medium	Damaging	Not present
5	c.2380G>A	p.Glu794Lys	Novel	Disease causing	Benign	Tolerated	Deleterious	Medium	Damaging	201078003
6	c.877_877delC	p.Leu293CysfsTer11	Novel	Disease Causing	NA	NA	NA	NA	NA	Not present
7	c.2632G>A	p. Ala878Thr	Novel	Disease causing	Damaging	Damaging	Deleterious	Medium	Damaging	570527044
8	c.2141 A>C	p. Asn714Thr	Reported#	Disease causing	Damaging	Damaging	Deleterious	Medium	Damaging	397517196

*NA=Not available #Laboratory for Molecular Medicine, Partners HealthCare Personalized Medicine (unpublished data).

Table 2: Prediction of mutations using different bioinformatics software.

mutation is predicted to be deleterious by bioinformatic softwares (Table 2).

In family F6, a previously reported missense mutation, c.2141A>C (p.Asn714Thr) was noted ((rs397517196), unpublished data from Laboratory for Molecular Medicine, Partners HealthCare Personalized Medicine, reported in ClinVar). Based on computational analysis, the variant has been classified as pathogenic.

Discussion

Genetic studies in six Asian Indian families with WS identified biallelic mutation in *WFS1* gene in 9 patients from 4 families, and heterozygous mutations in 2 patients with AD-LFNSHL. Only a few clinical reports describe phenotype of patients with WFS from India [35-39]. To the best of our knowledge, only one case of WS has been published mentioning homozygous mutation in exon 8 of the *WFS1* gene [23].

In autosomal recessive Wolfram syndrome (AR-WS), 268 different mutations in *WFS1* gene have been reported in HGMD public database and more than 90% of WS patients carry mutations that result in a loss of function of the wolframin protein [34,40]. Majority of these pathogenic variants reside in exon 8 coding for transmembrane domain and C-terminal domain of wolframin protein [1,34,41-43]. In this study, all the pathogenic variants were identified in exon 8, suggesting that this is a mutational hotspot (Figure 4). This exon covers almost half portion of the gene coding for the functionally relevant region of protein (transmembrane domain and C-terminal domain) and thus inactivating mutations in this region result in disease phenotype.

In autosomal dominant low frequency sensorineural hearing loss (AD-LFNSHL) majority of variations observed are missense in exon 8 of *WFS1* gene [1]. The present study showed comparable findings, with 2 missense variations in exon 8 identified in patients with hearing loss. Exon 8 of *WFS1* gene contains the conserved C-terminal domain which has a crucial function in the cochlea. A mutation in this region of the gene is likely to cause hearing loss by affecting the regulation of inner ear homeostasis [42]. WS like syndrome is associated with autosomal dominant inherited hearing loss with/without optical atrophy and/or diabetes mellitus [6,14,15,44]. In addition, some *WFS1* variations are known to cause type 1 and 2 DM and psychiatric problems [7,9,16]. Our patients had only hearing loss but no eye involvement or diabetes mellitus.

Eight mutations were identified in 11 probands of 6 different families with WS. Five of the 8 mutations were novel, observed for the first time in patients with WS. All missense mutations c.2070C>G (p.Cys690Trp), c.2380G>A (p.Glu794Lys), c.2632G>A (p.Ala878Thr) except c.2141A>C (p.Asn714Thr), 1 deletion mutation, c.877delC (p.Leu293CysfsTer11), and 1 nonsense mutation, c.2265T>A (p.Cys755Ter) were noted to be novel. Nonsense and missense pathogenic variants were present in the C-terminal domain of wolframin protein. These novel variants were checked for the pathogenicity using bioinformatics prediction tools and were found to be deleterious. Nonsense mutation, c.2265T>A (p.Cys755Ter), results in formation of truncated protein with a deletion of half of the C-terminal segment. This region is highly conserved across species and seems to have crucial function in the wolframin protein. It is speculated that this domain is interacting with some other unknown proteins [34]. Deletion pathogenic variants, c.877delC (p.Leu293CysfsTer11) and c.1228_1231del (p.Val412SerfsTer29), causes shift in reading frame, leading to a stop codon at 11 and 29 positions downstream respectively, which results in formation of a truncated protein. This truncated protein is predicted to undergo the known phenomenon of nonsense mediated decay (NMD) or form unfunctional protein with half of the transmembrane and C-terminal region being deleted. Thus, it is predicted to be a severe mutation as no protein/or truncated protein will be formed [32,33]. Two of the five novel pathogenic variants (c.2380G>A (p.Glu794Lys) and c.2632G>A (p. Ala878Thr)) were present in dbSNP database but have not been correlated with the disease phenotype of the WS patient (Table 2). Since we are associating these mutations with the disease for the first time, we label these variations as novel.

Two pathogenic variants identified in the present study were previously reported. The variant c.1525_1539del was reported earlier with a different nomenclature of c.1522_1536del15 by Ouwland et al. (one patient was homozygous, and another patient was heterozygous) [33]. Another reported mutation, c.2141A>C is mentioned in Clinvar as pathogenic, identified in an Indian patient with hearing loss (unpublished data from Laboratory for Molecular Medicine, Partners Health Care Personalized Medicine).

Few mutations are known to be frequent in certain regions; e.g., c.424_425ins16 is common in the Spanish population and variation c.1362_1377del16 is prevalent in Italian patients [45,46]. However, no frequent mutation has been observed in the present case series. All the patients in this study harboured different mutations.

Wolframin protein is an ER membrane protein with nine transmembrane domains. It has a C-terminal domain located in ER lumen and N-terminal domain present in cytoplasm. The 9 transmembrane segment, forms loops facing both side of ER membrane (Figure 1) [17]. Wolframin protein's C- terminal and transmembrane domain interact with Na/K ATPase b1 subunit in the inner ear. Therefore, mutations in any of these segments result in alteration of K^+ circulation resulting in hearing loss [1,17]. In the present study 5 mutations were in the C-terminal domain and 3 mutations were in the transmembrane region of the protein, leading to deafness as a common symptom in this cohort (both AR-WS and AD-LFSNHL).

Wolframin is highly expressed in β cells of pancreas and may help in maturation of proinsulin to insulin that controls blood glucose levels. Thus, inactivating mutations lead to deficient level of this protein, which explains the occurrence of diabetes mellitus in these patients. Apart from the inactivating mutations, missense mutations in the C-terminal domain of the protein could also cause diabetes mellitus in these probands as it is an important segment for the proper functioning of the protein. It is also reported that a mutation in any of the last seven amino acids leads to a severe disease phenotype underlying the main role of this region in the protein [1]. Seven probands of 3 families (F1, F2 and F4) had inactivating mutations and two probands of 1 family (F3) having missense mutations had impairment of glucose regulation.

Two patients with AD-LFNSHL also had mutations in the C-terminal domain but these patients did not have diabetes mellitus. The reason of this could be that the mutations responsible for LFNSHL do not inactivate the *WFS1* protein. These mutations have an altered gene product that opposes the wild-type allele, and thus, these mutations are presumed to have a dominant negative effect on the normal protein [47].

Wolframin is abundant in retinal cells and deficiency of this protein ultimately leads to optical atrophy [1,48]. We predict that the probands with AR-WS having deleterious mutations result in deficient protein leading to optic atrophy. Patients with AD-LFNSHL do not have optic atrophy, as these are not loss of function mutations and thus have an antimorphic effect on normal protein.

Genotype could not be correlated with phenotype as the patient cohort is small. However, the findings in the present study are similar to those reported the literature that loss-of-function mutations such as terminations and deletions result in severe phenotype [22,32]. It is reported that compound heterozygosity for 2 missense mutations may cause milder phenotype [22,33]. In contrast, 2 probands of family (F3) in the present cohort had 2 different missense mutations, but they have all the severe features of Wolfram syndrome (DM, DI, HL and OA).

Conclusion

In conclusion, mutations in *WFS1* gene can lead to autosomal recessive WS as well as autosomal dominant LFNSHL. Most of the mutations were present in transmembrane and C-terminal domain of wolframin making it an important segment for the functioning of the protein. All the mutations identified in the present cohort are located in exon 8 of *WFS1* gene making it a hotspot region. We recommend a protocol in which WS patients should be first screened for the hotspot exon 8, and if no mutation is identified then the full gene should be sequenced. This study adds to the molecular data on WS from India. Further, patients with hearing loss with/without diabetes and or optical atrophy should be considered as one of the differentials for diagnosis of WS.

Declaration of Interest

All the authors declare that there is no conflict of interest with regards to preparation and submission of the manuscript.

Funding

There was no source of funding for the study.

Author contribution statement (optional):

DG, PB: Study design; DG, PB, SBM, RDP, LD, AB, ICV: Patient contribution and clinical details analysis; DG, PB, RS: Molecular studies and data analysis; DG, PB: Manuscript preparation; RS, ICV: Critical analysis for important intellectual content.

Acknowledgements

We thank our colleagues at Institute of Medical Genetics and Genomics and to patients whom contributed in this study.

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