Title
Functional Assessment of Variants Associated with Wolfram Syndrome

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Abstract

Wolfram Syndrome (WS) is a heterogeneous multisystem neurodegenerative disorder with two allelic variations in addition to a separate subtype known as WS type 2. The wide phenotypic spectrum of WS includes diabetes mellitus and optic atrophy which is often accompanied by diabetes insipidus, deafness, urological and neurological complications in combination or in isolation. To date, the understanding of the genotype phenotype relationship in this complex syndrome remains poorly understood. In this study we identified and explored the functionality of rare and novel variants in the two causative WS genes \( WFS1 \) and \( CISD2 \) by assessing the effects of the mutations on the encoded proteins Wolframin and ERIS, in a cohort of 12 patients with autosomal recessive WS, dominant WS and WS type 2. The identified pathogenic variants included missense changes, frameshift deletions and insertions in \( WFS1 \) and an exonic deletion in \( CISD2 \) which all altered the respective encoded protein in a manner that did not correlate to the phenome previously described. These observations suggest the lack of genotype phenotype correlation in this complex syndrome and the need to explore other molecular genetic mechanisms. Additionally, our findings highlight the importance of functionally assessing variants for their pathogenicity to tackle the problem of increasing variants of unknown significance (VUS) in the public genetic databases.
Introduction

Wolfram syndrome 1 (WS) (OMIM 222300), also known as DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness), is a widely heterogeneous autosomal recessive multisystem neurodegenerative disorder that was first described by Wolfram and Wagener in 1938 (1). The first and earliest diagnosing feature of WS is non-autoimmune and non-HLA linked diabetes mellitus (DM) which appears at the average age of six years and in most cases is insulin dependent (2). The second identifying manifestation of WS is optic atrophy which manifests at the average age of eleven years and frequently results in near total blindness by the second decade of life (3). Additional common but not essential features of WS include diabetes insipidus, sensorineural deafness (D), renal abnormalities (R), neurological dysfunction (N), gastrointestinal dysregulation, sexual development abnormalities and psychiatric manifestations (2,4).

Three types of WS are recognized. The two WS type 1 forms are caused by either recessive or dominant mutations in WFS1 (5,6) which encodes for a tetrameric protein, Wolframin, that plays an integral role in the regulation of endoplasmic reticulum stress (7). The majority of reported WFS1 mutations are missense/nonsense variants followed by frameshifts, deletions and insertions (8). Most of the mutations are in exon 8 which encodes for Wolframin’s transmembrane and C terminal domains (2). There have also been various reports of heterozygous dominant mutations in WFS1 in patients with non-syndromic low frequency sensorineural hearing loss (LFSNHL) and autosomal dominant optic atrophy (ADOA) with hearing impairment (9) which widens the phenotypic spectrum of WS. The third subtype of WS is known as WS type 2 (OMIM 604928) that is distinguishable from WS type 1 by the increased risk of bleeding, development of gastrointestinal ulcers and the absence of diabetes.
insipidus even though the latter symptom has been described once in a patient with WS type 2 (10). This type of WS is caused by mutations in \textit{CISD2} (11) which encodes for the endoplasmic reticulum IFN stimulator (ERIS) protein which plays a role in maintaining the structural and functional integrity of the endoplasmic reticulum and the mitochondria (12).

There is no clear genotype phenotype relationship in WS as there appears to be wide heterogeneity even amongst the same family (9).

In this manuscript we explore the allelic variations of WS type 1 in addition to WS type 2 in a cohort of 12 patients in an effort to expand our current understanding of the genotype-phenotype correlation in WS, and encourage the functional assessment of identified variants that are often reported as variants with unknown significance (VUS). Additionally, we aim to critically interpret our genetic and functional results while taking into account the growing evidence for the role of gene modifiers, enhancers and the untranslated genome regions in driving certain complex diseases (20, 21).

\textbf{Results}

\textit{Detection of pathogenic mutations in patients A-J}

Homozygosity mapping analysis showed overlapping loss of heterozygosity (LOH) loci on chromosome 4p of patients A-D harboring \textit{WFS1} and subsequently their genomic DNA was Sanger sequenced for \textit{WFS1}. Homozygosity mapping beta allele plots can be found in supplementary figure 3.1, 3.2, 3.3. A homozygous missense mutation in exon 8 of \textit{WFS1}, c.1885C>T; p.(Arg629Trp), was identified in patients A and B (Figure 3A). A homozygous three base pair deletion in \textit{WFS1} was identified in patient C, c.1243_1245del; p.(Val415del) (Figure 3B). Similarly, a homozygous three base pair deletion in \textit{WFS1} was identified in patient D, c.1716_1718delTTC; p.(Ile572_Leu573delMet) (Figure 3C). As for patient E, beta allele frequency plots showed LOH segregation on chromosome 4p, that was
subsequently Sanger sequenced. This revealed a novel frameshift insertion c.2826_2827insA p.(Phe886fs*54), in patient E that prolongs Wolframin by 54 amino acids (Figure 3D). In patients F and G, whole exome sequencing analysis showed a \textit{WFS1} homozygous deletion c.1230_1233delCTCT; p.(Val412Serfs*29), which was then confirmed by Sanger sequencing (Figure 3E). Similarly, in patients H and I, whole exome sequencing identified a homozygous missense change in \textit{WFS1}, c.376G>A; p.(Ala126Thr), which was then confirmed by Sanger sequencing (Figure 3F). Whole exome sequencing filtering strategies used for analyzing the variants for patients F, G, H and I are available in supplementary table 2. As for patient J, homozygosity mapping analysis showed homozygosity enrichment in chromosome 4q which harbors \textit{CISD2} (11). Several attempts of amplifying exon 3 of \textit{CISD2} by PCR failed (supplementary Figure 1) and furthermore, whole exome sequencing analysis failed to provide sequence of exon 3, since it is a region of segmental duplication that shares homology with at least four different genes, making its mapping and alignment almost impossible (supplementary Figure 2). It was concluded that there is a novel deletion of \textit{CISD2} exon 3 in patient J (chr4:83007475-113025264), which was supported by the protein expression results discussed below. Table 2 shows the details of the mutations identified in patients A-J, including information on their cytogenetic location, transcript and protein variants, conservation, translation impact, existing population frequencies and pathogenicity software predictions.

\textit{Pathogenic mutations in patients K and L}

In the first report that linked autosomal dominant optic atrophy and hearing loss to \textit{WFS1}, Eiberg et al., (2006) (13) described a novel heterozygous mutation in exon 8 of \textit{WFS1}, c.2590G>A; p.(Glu864Lys) (Figure 2B) in patient K and three other affected individuals from the same family (Figure 2A). Similarly, Rendtorff et al., (2011) (14) described a
heterozygous WFS1 mutation in patient L, c.2051C>T; p.(Ala684Val) (Figure 2D) who is a member of the first family described with isolated autosomal dominant optic atrophy and sensorineural hearing loss (Figure 2C).

Expression analysis of Wolframin and ERIS

Protein expression analysis of both Wolframin and ERIS was performed by lysing fibroblasts cultured from the patients’ skin biopsies which were then probed by their respective antibodies using the western blotting technique. Wolframin expression levels were found to be altered in all the patients with mutations in WFS1 (patients A-I, K & L). Wolframin expression levels were very clearly reduced in patients A, B, H, I, K & L and completely absent in patients C, D, E, G & H as compared to control fibroblasts that were cultured and lysed under identical conditions (Figure 4). As for the expression of ERIS protein in patient J, Figure 4E shows an almost complete absence of ERIS in the patient fibroblasts compared to control fibroblasts.

Discussion

Allelic variations and genetic heterogeneity in a cohort of 12 WS patients

The genetic and phenotypic profile of our WS patient cohort clearly shows the wide genetic heterogeneity of this complex syndrome and the multifaceted mechanisms involved in regulating variant penetrance as well as disease expressivity. For example, the missense WFS1 mutation in patients A and B, (c.1885C>T; p.(Arg629Trp)) has been previously reported in patients with additional syndromic features such as urological and neurological manifestations (15). Similarly, the WFS1 deletion (c.1242_1244delCGT) identified in patient C has been reported before in three patients with only DM and OA contrasting to patient C who presented with DM, OA, D, DI and renal reflux (16). Additionally, the WFS1 deletion (c.1716_1718delTTC) in patient D has been identified previously in a female that presented
with DM, OA and D (17), contrasting to patient D who does not have deafness but has DI and renal reflux.

Another puzzling paradigm in understanding the genetics of WS is the previously described reduced penetrance in the dominant form, so that not all carriers of mutations in \textit{WFS1} show manifestations of the disease including hearing impairment and optic atrophy (\textbf{Figure 3F}) (13,14). Additionally, various reports have linked \textit{WFS1} carriers to the development of psychiatric manifestations as for instance reported in the grandfather of a patient with the missense homozygous \textit{WFS1} mutation c.1885C>T; p.(Arg629Trp) (15). This same homozygous mutation has been identified in patients A and B, and given that they come from a consanguineous family, some family members, including their parents are expected to be carriers for this change. However, there are no reports of psychiatric complications in this family. Moreover, it should be noted that all of the identified \textit{WFS1} mutations affect the transmembrane and C terminal domains of Wolframin and yet there is no phenotypic concordance.

Our findings raise the question of the involvement of other potential mechanisms that could modulate penetrance such as non-sense-mediated mRNA decay pathways, methylation signatures, post transcriptional regulation or any disruption to the transcriptome or the genome as a whole that remains unexplored in general and particularly in monogenic syndromes of DM. Nucleotide changes in promoter or enhancers have been linked to influencing gene expression and phenotype severity as a whole. By looking for an explanation for genetic heterogeneity only in the coding regions of \textit{WFS1} and \textit{CISD2} specifically and other protein coding regions of known genes we would be missing the vast majority of our genome where on average there are 3.5 million genomic variants per
individual of which only 0.65% are in protein coding regions. The possibility of this difference in phenotypic severity across WS patients being due to variants in certain key enhancers for WFS1 or other closely associated genes should not be disregarded when interpreting the pathogenicity of protein changing mutations in WFS1 that have a poor genotype-phenotype correlation as shown in our cohort.

Protein expression is decreased in all the patients despite differences in genotypes and phenotypic severity

Nonsense and frameshift mutations resulted in the complete absence of Wolframin in patients C, D, E, F and G which is consistent with previous findings (18) and suggests that the disease phenotype is likely to be caused by a dosage effect rather than a functional defect of Wolframin. The combined results of our and the previous study show that the frameshift and stop mutations in WFS1 lead to the complete absence of protein rather than the production of a truncated product which explains the loss of function of Wolframin in those patients. Similarly, to the frameshift and stop mutations, missense mutations in WFS1 lead to the cellular depletion of Wolframin, albeit partially. Decreased levels of Wolframin are seen in patients A, B, H and I which is consistent with findings reported by previous studies (18,19). Unexpectedly, Wolframin levels were found to be decreased in patients K and L who harbor missense heterozygous mutations in WFS1. It was speculated (14) that the p.(Ala684Val) heterozygous mutation leads to misfolded Wolframin which is partly degraded prompting the remainder of this protein to act as a dominant negative mutant leading to the autosomal dominant disease. In an attempt to understand the impact of this mutation, HEK cells were transfected with plasmids expressing wild-type and mutant Wolframin which were then analyzed for protein expression that showed decreased expression levels of the mutants (14). The reduced Wolframin expression results of patient K & L are consistent with the analyses
of the transfected HEK cells and provide the first report of protein expression analyses of dominant WS performed on patient cell lines.

These results provide compelling evidence for a deleterious effect of the mutations in patients K and L, but do not provide a clear association between Wolframin expression and disease severity. Patients K and L have fewer symptoms than patients A and B who showed similar low protein expression levels. This data indeed shows that the c.2590G>A and c.2051C>T mutations in families 8 and 9, respectively, are disease causing but it does not provide proof that these mutations can cause a dominant phenotype.

When interpreting our protein expression results in light of our patients’ phenotypes, a clear association could not be seen. Table 3 shows details of how the genotype, phenotype and protein expression in the two allelic variations of WS type 1 and WS type 2 do not correlate. For instance, patients with absent Wolframin (C, D, E, F) have different phenotypes where patient D does not have Deafness, patient E does not have DI nor neurological symptoms and patient F does not have any urological/renal manifestations.

As for our patient with the novel deletion of exon 3 in CISD2, this provides the first report of ERIS expression in a patient cell line and we report that there is no clear phenotypic signature of WS type 2, since patient J is not the only patient in our cohort to lack DI, that in any way has been disregarded as a differentiating feature of WS type 2 (10). Also, patient J does not have a history of increased bleeding tendencies and abnormal platelet aggregation which similarly to WS type 1 also questions the genotype-phenotype correlation in this subset of WS.

**Conclusion**
This study provides the first functional mutational report in a cohort of patients with the two types of WS and the two allelic variations of WS type 1 which highlights the poor correlation not only at the genotype phenotype level but also at the molecular phenotypic level by examining the effect the mutations have on the respective encoded proteins. Additionally, our functional studies have contributed to confirming pathogenicity of identified variants which in turn reduces to some extent the frequency of VUS in WS. This strengthens the notion that mutations in WS are of variable penetrance with differing expressivity and severity and highlights the possibility of having other genetic, epigenetic or environmental mechanisms driving this variable expressivity process such as pre and post transcriptional changes upstream and downstream of the genome that could drive or restrict mutations in the protein coding parts of the genes in question. The underexplored untranslated regions of the genome have been recently reported to play significant roles in driving or protecting against certain genetic diseases (20,21). These findings shed a light on the importance of examining and comparing different variations of one complex disease that has been historically known to be purely monogenic and interpreting the data in light of other potential driving factors such as enhancers across the entire genome which is in line with the growing field of population genetics and high throughput sequencing that could shed a light on the ‘still mysterious’ world between the transcriptome and the proteome.

Materials and Methods

Research inclusion consent was obtained from all the patients (or caregivers when applicable) and ethical consent for this study was obtained from the Research & Development office at UCL GOS Institute of Child Health. Handling of all patient samples was in accordance with the declaration of Helsinki.

Blood and 4 mm skin punch biopsies from ten patients (A-J) with typical and atypical phenotypic features of WS type 1 and type 2 from seven unrelated Turkish consanguineous
families were obtained from various regions in Istanbul. **Figure 1** shows the patients’ family pedigrees and any relevant family history for patients A-J, **Figures 2A/2C** respectively show the same details for patients K and L, and **Table 1** contains a summary of the patients’ clinical details which shows the phenotypic heterogeneity of our patients. Established fibroblast cell lines of the two remaining patients (K and L) were obtained from Rendtorff et al., (2011).

Blood genomic DNA for patients A-J was extracted at GOSH North East Thames Regional Genetics Service Laboratories using the Maxwell 16 Blood DNA Purification Kit (Promega, USA). **WFS1** primers, **CISD2** primers and PCR/sequencing cycling conditions are available in supplementary **Table 1**. Primary fibroblast cell lines were established for each patient (A-J) in a biosafety level-2 cell culture laboratory and were cultured using Dulbecco’s Modified Eagle Medium GlutaMAX supplement (Thermo Fisher Scientific, USA) and Fetal Bovine Serum from South American origins (Thermo Fisher Scientific, USA). Protein expression of the encoded **WFS1** protein Wolframin was analyzed by western blotting using a **WFS1** anti-rabbit antibody (Thermo Fisher Scientific, USA, #PAI-16923) and a **GAPDH** housekeeping gene anti-rabbit antibody (Cell Signalling Technology, USA, #2118S). Protein expression of the encoded **CISD2** protein ERIS was analyzed by western blotting using a **CISD2** anti-rabbit antibody (Thermo Fisher Scientific, USA, #PA5-34545). Whole exome sequencing was performed at UCL Institute of Neurology using the Illumina HiSeq 2000 platform (Illumina, San Diego, USA). The sample enrichment and library preparation were based on the SureSelect Human All Exon v4 and SureSelect v4 protocols (Agilent, Santa Clara, USA). Samples were sequenced at a final coverage of 30x. Data filtering and interpretation was done using the Ingenuity Variant Analysis software (Qiagen, USA). Homozygosity mapping was performed at UCL Genomics core facility at UCL GOS ICH. All the steps were carried...
out according to the Infinium HD Ultra Assay protocol (Rev B, 2010, Illumina Inc, San Diego, USA) using the cytoSNP-12 v2.1 bead-chip array.

Acknowledgments

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Conflict of Interest

We have no conflict of interest.

References


Figure 1: Family pedigrees of patients A-J. (A) Patients A and B are siblings born to Turkish consanguineous parents with a maternal family history of insulin dependent DM (maternal grandmother). (B) Patient C is born to Turkish consanguineous parents with a maternal family history of insulin dependent DM (maternal uncle). (C) Patient D is born to Turkish consanguineous parents with no known family history relating to DM. (D) Patient E is born to Turkish consanguineous parents with a family history of undiagnosed WS. The patient’s paternal cousins have DM, OA, DI and D. (E) Patients F and G are siblings born to Turkish consanguineous parents. Their paternal cousin has manifestations of undiagnosed WS and those include DM, OA, DI and congenital hypothyroidism, in addition to insulin dependent DM diagnosed in their great paternal uncle. (F) Patients H and I are siblings born to Turkish consanguineous parents with a family history of insulin dependent DM (great grandfather). (G) Patient J is born to Turkish consanguineous parents with a family history of insulin dependent DM (mother, maternal aunt and paternal uncle).
Figure 2: Family pedigrees and Sanger sequencing chromatograms of patients K and L. (A) Family pedigree of patient K showing a strong family history of hearing impairment and optic atrophy. (B) gDNA Sanger sequencing chromatogram obtained from Eiberg et al., (2006) showing a novel heterozygous mutation in exon 8 of WFS1, c.2590G>A; p.(Glu864Lys) in patient K. (C) Family pedigree of patient L displaying a family history of hearing loss and optic atrophy. (D) gDNA Sanger sequencing chromatogram showing a heterozygous WFS1 mutation in patient L, c.2051C>T; p.(Ala684Val) obtained from Rendtorff et al., (2011).
Figure 3: Genomic DNA Sanger sequencing chromatograms for WFS1. (A) Homozygous missense mutation c.1885C>T; p.(Arg629Trp) in patients A and B, wild type allele C seen in control. (B) Homozygous three base pair deletion in patient C, c.1242_1244delCGT; p. (del415Val). (C) Homozygous three base pair deletion in patient D, c.1716_1718delTTC; p. (del516Phe). (D) Novel frameshift insertion c.2826_2827insA; p. (Phe886fs*54) in patient E. (E) Homozygous frameshift deletion c.1230_1233delCTCT; p.(Val412Serfs*29), in patients F and G. (F) Homozygous missense mutation c.376G>A; p.(Ala126Thr) in patients H and I.
**Figure 4:** Wolframin and ERIS protein expression analysis in patients normalised to GAPDH and Calnexin respectively. (A) Wolframin levels are reduced in patients A and B compared to control 1 and control 2. (B) Wolframin is absent in patients C and D compared to control 1. (C) Wolframin is absent in patient E compared to control 2 and control 3. (D) Wolframin is absent in patients G and F and reduced in patients H and I compared to controls 1 and control 2. (E) ERIS is almost completely absent in patient J compared to control 1. (F) Wolframin is reduced in patients K and L compared to controls 1, 2, 3 and 4.
Table 1. Summary of the patients’ clinical features (DM: diabetes mellitus, DI: diabetes insipidus, OA: optic atrophy, D: deafness). The majority of the patients (83%) have DM, 100% of the patients have OA, 66% have additional clinical features, 67% have DI, 41% have D and 100% of the patients with autosomal recessive WS are from consanguineous families. All the patients have been screened for additional features.

Table 2: Summary of the mutations identified in 10 patients and their respective population prevalence (%) and pathogenicity prediction results which are consistently disease causing in the probands. Conservation of the affected amino acids was checked using Clustal Omega in humans, orangutan, chimpanzee, frog and chicken.

Table 3: Summary of the patients’ phenotypes combined with their respective genetic and functional characterizations which show a clear absence of any genotype-phenotype correlation.

Abbreviations

Wolfram syndrome (WS), diabetes insipidus (DI), diabetes mellitus (DM), optic atrophy (OA), deafness (D), renal complications (R), neurological complications (N), low frequency sensorineural hearing loss (LFSNHL), autosomal dominant optic atrophy (ADOA), endoplasmic reticulum IFN stimulator (ERIS), loss of heterozygosity (LOH), Variant of Unknown Significance (VUS)
# Table 1

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