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Additional Information

Short Report

Genetics of Wilson disease and Wilson-like phenotype in a clinical series from eastern Spain

Short running: **Genetics of Wilson phenotypes**

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ABSTRACT

Wilson's disease (WD) is an autosomal recessive disorder caused by *ATP7B* mutations. Subjects with only one mutation may show clinical signs and individuals with biallelic changes may remain asymptomatic. We aimed to achieve a conclusive genetic diagnosis for 34 patients clinically diagnosed of WD. Genetic analysis comprised from analysis of exons to WES (whole exome sequencing), including promoter, introns, UTRs (untranslated regions), besides of study of large deletions/duplications by MLPA (Multiplex Ligation-dependent Probe Amplification). Biallelic *ATP7B* mutations were identified in 30 patients, so that four patients were analyzed using WES. Two affected siblings resulted to be compound heterozygous for mutations in *CCDC115*, which is involved in a form of congenital disorder of glycosylation. In sum, the majority of patients with a WD phenotype carry *ATP7B* mutations. However, if genetic diagnosis is not achieved, additional genes should be considered because other disorders may mimic WD.

KEYWORDS

Wilson's disease, Wilson-like phenotype, genetic diagnosis, *ATP7B* gene, *CCDC115* gene, targeted next-generation sequencing, whole exome sequencing

INTRODUCTION

Wilson's disease (WD; MIM 277900) is an autosomal recessive disorder resulting in abnormal copper accumulation mainly in liver, brain, kidney and cornea, caused by mutations in *ATP7B*. Currently, 995 different mutations are known (HGMD® Professional 2019.2; accessed November 14, 2019). The genetic heterogeneity makes difficult the molecular diagnosis, but also the clinical presentation that varies enormously among patients even with the same genotype. The main goal was to obtain a conclusive genetic diagnosis for the 34 WD patients belonging to our series. We developed a comprehensive workflow from exon analysis of *ATP7B* to WES (whole exome sequencing) (Figure S1) that allowed us to diagnose 32 patients.

SUBJECTS AND METHODS

This clinical series comprised 34 patients belonging to 25 families (Tables S1 and S2) supervised at Hospital Universitari i Politècnic La Fe (València) and Hospital General Universitari d'Elx (Alacant). Informed consent was obtained from all participants and the study was approved by the ethics committees of both hospitals. Details of the clinical assessment are provided in the Supporting Information.

The genetic analysis comprised several steps: (1) Sequencing of the 21 exons and their intronic flanking sequences of *ATP7B*; (2) Analysis of the promoter (1,438 bp of 5' upstream sequence) and searched for large deletions and duplications by MLPA (Multiplex Ligation-dependent Probe Amplification); (3) Sequencing of the whole sequence of *ATP7B* using a custom target enrichment library; (4) WES. An in-detail description of the genetic analysis and of the subsequent bioinformatics analysis is provided in the Supporting information.

Methods for the luciferase reporter assay for analysis of the promoter variant, and transcript analysis and minigene assays for study of the splicing mutations are included in the Supporting Information.

RESULTS

Clinical findings

The 34 patients were clinically diagnosed of WD because they had a Leipzig score of at least 3, except EW111 (Leipzig score = 2; Tables S1 and S2). The EW111's brother (EW110) presented with a severe clinical picture (Leipzig score = 5), and hence, both siblings were clinically diagnosed of WD.

Mutational screening of *ATP7B*

We analysed the exons and the intronic flanking regions of *ATP7B.* In 20 probands (80%) genetic analysis was definitive. In one case, only one mutation was detected, and when the study was extended to MLPA, a large deletion of exon 1 was identified (Figure S2A). In another patient, the analysis of promoter revealed the novel c.-447C>T variant in homozygosis. Therefore, 22 cases were solved (88%; Table S1 and S3). Importantly, other two novel mutations were detected: c.3254dupC (p.E1086Rfs*32) and c.4125-1G>C. Altogether, we detected 25 distinct mutations (Table S3, Figure S3).

Families fEW-36 and fEW-66 deserve a special mention (Figure S4). The fEW-36's proband had three *ATP7B* mutations in heterozygosis, c.3932T>C (p.I1311T), c.3254dupC (p.E1086Rfs*32) and c.3359T>A (p.L1120*), two of them *in cis* inherited from his mother. In family fEW-66, the proband's father was compound heterozygous for c.-447C>T / c.1995G>A (p.M665I). He was evaluated clinically and presented no

WD symptoms, which allowed us to discard c.1995G>A as a pathological variant, also supported by previous studies that classified it as a tolerated change or as a VUS (variant of uncertain significance).[1,2]

The promoter activity was investigated for the novel c.-447C>T mutation and resulted to be decreased by 25% compared to reference allele (Figure S5), which may support its pathogenicity.

Whole sequencing of *ATP7B*

In EW13 and in EW27, we investigated the whole *ATP7B* using a custom target enrichment library with the aim of identifying causative variants located on deep intronic regions, and on UTRs; we used EW24 as a control who harbored a deletion of exon 1 detected by MLPA (Figure S2A). We did not analyze the fEW-60's proband, since the two affected siblings did not share the same haplotype for the *ATP7B* locus (data not shown), and hence, *ATP7B* could not be the responsible gene in fEW-60.

This new diagnostic tool was robust in terms of coverage (>350x) and read depth (99% of bases covered with >10 reads) (Table S4), and revealed one novel deep intronic variant in heterozygosis in EW13, c.2865+467A>G. Moreover, this genetic tool allowed us to detect that that EW24 harbored a larger deletion that spanned 8.7 Kb of the target region, c.(?_-1627)_(51+1_52-1)del (Figure S2B).

Analysis of splicing mutations

The transcript analysis was performed for six mutations (Table S5), since c.51+4A>T and c.3061-12T>A were previously demonstrated to alter the splicing.[3,4] Three mutations led to the skipping of a whole exon (c.1708-1G>A skipping of exon 5, and both, c.4022G>T and c.4124+5G>A, skipping of exon 20), and the variant c.4125-

1G>C caused the deletion of the last 11 bp in exon 20 and the first 175 bp in exon 21 (Figure 1).

No aberrant transcripts by analysis of the RNA's patient was observed for c.2447+1G>T (Figure 2A). Strikingly, we observed that only one allele was sequenced because the polymorphism c.2495A>G (rs1061472) in exon 10 was previously detected in heterozygosis by Sanger sequencing, and in the transcript analysis, we identified it in homozygosis in patient EW45 (data not shown). The *in silico* tools predicted the disruption of the natural donor splice site and the activation of a cryptic one, which would originate a new exon 9 lacking 22 bp (Figure 2B). The minigene assay confirmed this computational analysis and an additional minor transcript lacking exon 9 (Figure 2C).

The novel deep intronic c.2865+467A>G variant was detected in compound heterozygosis with c.482T>C in the proband EW13 and in his unaffected sister EW15. *In silico* tools predicted possible alterations of the proper splicing, although no aberrant transcripts were detected by analysis of the RNA's patients or by minigene assay, which may rule out that c.2865+467A>G could alter the splicing process (Figure S6).

Exome Sequencing

ATP7B was discarded as the gene responsible for disease in three cases that were ultimately investigated by WES (Table S2, Figure S7). For families fEW-12 and fEW-18, we did not have variants of interest (Tables S6 and S7). In family fEW-60, both patients were compound heterozygous for the novel c.379G>T (p.G127*) and c.383T>A (p.I128N) changes in *CCDC115* (Figure 3). In the Supporting Information, an in-depth clinical description is provided.

DISCUSSION

Genetics provides an accurate diagnosis and an early diagnosis is vital because patients suffer from disease complications if there is a long delay before receiving a therapy. In 30 patients (88%), we achieved a conclusive genetic diagnosis. Using a similar strategy, the overall mutation detection frequency ranged from 88-98%.[1,5] Therefore, applying standard genetic approaches, the majority of WD patients can be diagnosed.

To establish the impact of genetic results on the phenotype is difficult. We focused on investigating the consequences of six splicing mutations. Mostly, variants residing at splice site consensus sequences lead to single exon skipping,[6,7] as it occurs with c.1708-1G>A and c.4124+5G>A. Exonic changes, such as c.4022G>T, can also cause exon skipping,[8] although they are usually presumed as changes on the protein. In occasions, a mutation located at the splice site can produce the inclusion of an intron fragment or the removal of an exon fragment because a nearby cryptic site is activated,[6,7] as it happens with c.4125-1G>C and c.2447+1G>T. The c.2447+1G>T change only presented an altered splicing with the minigene approach, which could be caused by a non-sense mediated decay (NMD) mechanism, since in the transcript analysis, only one allele was sequenced. Moreover, different transcripts were obtained for c.2447+1G>T in the minigene assay. This phenomenon depends on factors such as the strength of the splice site or the length of the resulting exon/intron.[9,10] Finally, c.2865+467A>G was discarded as a clinical mutation because its study showed a WT splicing pattern.

WD is a well-established monogenic disorder. However, other liver diseases can display a clinical presentation resembling WD. We concluded that fEW-60's patients, first diagnosed as WD patients, suffered from a congenital disorder of glycosylation

type IIo (CDG2O; MIM 616828) caused by biallelic mutations in *CCDC115*. WES allowed us to obtain a diagnosis for this family, which emphasizes the capacity of genetics to resolve complex clinical profiles.

In conclusion, the majority of patients with WD can be diagnosed with the analysis of the codified regions and promotor of *ATP7B*. However, if this is not achieved, WES, and also WGS (whole genome sequencing), should be considered. WD is caused by *ATP7B* mutations, but other hereditary disorders can mimic a WD phenotype.

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FIGURE LEGENDS

FIGURE 1 Transcript analysis of *ATP7B* intronic variants. Schematic representation of the nested PCR design. A, c.1708-1G>A caused the skipping of exon 5 (310 bp; band and electropherogram 1), whereas the control yielded a band of 472 bp (band and electropherogram 2). B, c.4022G>T led to the skipping of exon 20 (589 bp; band and electropherogram 3). The control showed a band of 692 bp; electropherograms corresponded to the sequences of the splice sites related to exons 19, 20 and 21 of a WT allele (band and electropherogram 4). C, c.4125-1G>C and c.4124+5G>A produced the loss of 186 bp affecting exons 20 and 21 (506 bp; band and electropherogram 5), and the skipping of exon 20 (589 bp; band and electropherogram 6), respectively. For these two mutations, the nested PCR design and the used control was the same than for c.4022G>T.

FIGURE 2 Functional analysis of *ATP7B* c.2447+1G>T. A, Agarose gel of PCR products of patient EW45 and a control that showed one band of 500 bp (band and electropherogram no. 1). Schematic representation of the nested PCR design; B, *In silico* predictions. The strength for main (WT exon 9; 92 bp) and alternative (70 bp) donor splice sites; C, Agarose gel shows the band pattern of transcripts obtained after overexpression of pSPL3-derived constructs. Schematic representation of mRNA transcripts and its electropherograms for WT allele (354 bp; band and electropherogram 2), for the mutated allele resulting of the major transcript (332 bp; band and electropherogram 3), and of the minor one (263 bp; band and electropherogram 4), with the predicted protein. SD6 and SA2 are the codified exons of the pSPL3 reporting vector.

FIGURE 3 Analysis of the *CCDC115* c.379G>T and c.383T>A mutations. A, Pedigree of family fEW-60. Only DNA from the affected siblings was available. B, Detection of each change by exome sequencing in the proband's sample (EW110), and its corresponding validation by Sanger sequencing. The proximity of both mutations makes that the two variants are detected in the same amplicon. C, *In silico* predictions. The c.379G>T would generate a premature stop codon and c.383T>A is predicted to be deleterious. D, G127 as well as I128 are evolutionary conserved amino acids.