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CLINICAL & BASIC RESEARCH

A Novel Splice-site Allelic Variant is Responsible for Wilson Disease in an Omani Family

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طفرة وراثية جديدة في موقع الاستقطاع هي المسئولة عن مرض ويلسون في أسرة عُمانية

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الملخص: **الهدف:** توصيف مرض ويلسون وراثيا واختبار المرادفات في المورث الخاص بنقل النحاس المسئولة عن هذا المرض في الأسرة العمانية. **الطريقة:** تم تشخيص ثلاث حالات دالة مصابة بمرض عن مرض من عائلة عُمانية يعانون من أعراض وعلامات عصبية. قمنا بفحص ستة وأربعين من الأقرباء في العائلة لتقصي مرض عن مرض، وكانت نتائج الفحص ايجابية لأحد عشر فردا، ولكن الأعراض غير ظاهرة. **النتائج:** تم تحديد ثلاثة عشر من المرادفات الجينية غير المسببة للمرض (والتي وصفت في وقت سابق) في الجين ATP7B عند 46 فردا من الأسرة. تم الكشف عن مرادف فريد و جديد في منطقة مفترض أن تكون المسببة للمرض في موقع القطع (c.2866–2A>G) ، والتي لم يُبَلَغ عنها سابقا. يقع هذا التغير في منبع exon 13 الذي يشفر جزء من قناة النحاس عبر الغشاء الخلوي (Ch/Tm6). وقد استخدم تفاعل البلمرة النسخ لتضخيم منطقة الحمض النووي التكميلي (cDNA) التي تحتوي على (exons 12، 13 and 14). من خلال نتائج التفاعل، لوحظ أنه تم تخطي exon 13 من الشفرة الوراثية والذي ربما يؤدي إلى بروتين ATP7B معيب وغير فعال. **الخلاصة:** وُجِدَ مرادف جديد في المورث ATP7B في موقع القطع، بين المرضى الاربعة عشر المصابين بمرض ويلسون وقد انتقل مع المرضى بطريقة متنحية. وهذا يشير الي انه المرادف المسبب للمرض.

مفتاح الكلمات: مرض ويلسون؛ ATP7B؛ بديل النحاس الأليلي؛ موقع قطع؛ الطفرة؛ عمان

ABSTRACT: ***Objectives:*** The objective of this study was to characterise Wilson's Disease (WD) [OMIM 277900] genetically and test for allelic variants in the copper transport gene (ATPase, Cu^{++} transporting, beta polypeptide, ATP7B) responsible for the disease in an Omani family. ***Methods:*** Three index patients from an Omani family had been previously diagnosed with WD. All three patients suffered neurological symptoms and signs. Forty-six relatives in the family were screened for WD. Eleven more individuals were positive, but asymptomatic. ***Results:*** Thirteen non-disease-causing allelic gene variants, described previously, were identified in the ATP7B gene from 46 family members. A putative novel disease-causing splice-site variant (c.2866-2A>G), which has not been reported previously, was detected in this family. It is located upstream of exon 13 which encodes part of transmembrane copper channel (Ch/Tm6). Reverse transcription polymerase chain reaction was used to amplify a complementary DNA (cDNA) fragment containing exons 12, 13 and 14. Exon 13 was entirely skipped from the transcript which probably would result in a defective ATP7B protein. ***Conclusion:*** A new ATP7B splice-site allelic variant, found among the 14 WD patients segregated with the disease in a recessive manner, suggests it is a disease-causing variant.

Keywords: Wilson Disease; ATP7B; Copper; Allelic Variant; Splice site; Mutation; Oman

Advances in Knowledge

1. *This is the first time that the molecular genetic cause of Wilson Disease is revealed in an Omani family. It adds to the body of knowledge of pathophysiology and genetic heterogeneity of the disease.*

application to patient care

1. *A new Omani-specific molecular diagnostic test could be developed to confirm diagnosis of Wilson Disease and screen asymptomatic family members for prevention and early treatment.*

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Wilson Disease (WD, OMIM 277900)[1] is an autosomal recessive disorder of copper metabolism resulting from allelic variants of the ATPase, Cu^{++} transporting, beta polypeptide (ATP7B) gene on chromosome 13q14.3. The gene is 78,826 bp long, with 21 exons coding for a copper transporting ATPase.[2,3] In WD, there is failure of copper excretion from the liver and defective incorporation of copper into ceruloplasmin by the ATP7B (EC 3.6.3.4). The disease typically manifests at any age from 5 to 50 years with hepatic or neurologic symptoms, alone or in combination, and occasionally with psychiatric, haematological or rheumatological problems.[4] The disorder usually manifests early as chronic liver disease and/or later as neurologic impairment due to the toxic accumulation of copper in several tissues, principally the liver and the brain.[5] Kayser-Fleischer (KF) rings result from copper deposition in Descemet's membrane of the cornea and reflect a high degree of copper storage in the body. WD has been described in every region of the world and its incidence is estimated at 30 per million worldwide.[4] It is more common (1 in 3,000–10,000) in communities where consanguineous marriages are prevalent such as the Druze, Yemenite and Iranian Jews and Palestinians.[6] WD is lethal if left untreated.

Analysis of disease-causing allelic variants reveals enormous molecular heterogeneity consisting of a small number of frequent variants that are population specific, as well as a much greater number of rare individual alleles. To date, more than 300 different variants have been characterised in WD patients from varying ethnic origins.[3,7-19] The majority are missense variants, with splice-site variants accounting for 10% only.[14,15] Here we describe WD in an extended highly consanguineous Omani family, with a possible novel splice-site disease-causing allelic variant.

Methods

One Omani family of Asian origin, referred to previously,[20] was included in the study. Three patients from the family were previously diagnosed at Sultan Qaboos University Hospital using standard tests for WD and are under continuous treatment now. A total of 46 siblings, parents or relatives from the family were examined. Several standard tests were used to screen for WD: serum ceruloplasmin, penicillamine challenge test and Kayser-Fleischer ring (KF ring). Eleven more family members tested positive for WD, but were all asymptomatic. Fifty DNA samples were obtained from medical students exhibiting no clinical disease or aliment, to be used as controls. Ethical clearance for the study was obtained from the Ethics Committee of the College of Medicine & Health Sciences, Sultan Qaboos University. All individuals gave their consent for inclusion in the study.

DNA was extracted from whole blood using the standard procedure of phenol-chloroform method. All 21 exons of the ATP7B gene with a flanking region from the introns, the 5'UTR, 3'UTR and the promoter region were amplified using primers specified previously,[5] with minor modifications. Gene variant detection was performed by direct sequencing using the ABI3100 DNA sequencer (Applied Biosystems, USA). The sequencing result for each sample was aligned with the reference sequence retrieved from the National Center for Biotechnology Information (NCBI) Genome website (NC_000013.10).[2] Alignments were processed using three software programmes: BioEdit, (2007, IbisBiosciences, Carlsbad, CA, USA), ChromasPro (Version 15, Technelysium Pty Ltd., Brisbane, Australia), and LaserGene (2010, DNASTAR Inc., Madison, USA).

Fresh blood samples were collected from two WD patients and one normal control. Ribonucleic acid (RNA) was extracted using ABI PRISM™ 6100 Nucleic Acid Prep Station (Applied Biosystems, USA). Reverse transcription of DNA was performed using the high capacity complementary DNA (cDNA) Archive kit (Applied Biosystems, USA). A cDNA fragment containing exons 12, 13 and 14 was amplified using forward primer: 5'AGCTGGCTGACCGGTTTA3' and reverse primer: 5'CAAGGGGTGTTCACTGCTG3' giving a product size of 470bp in normal controls. The size of the fragment (470bp) is smaller than the total size of exons 12, 13 and 14 (514bp) because of the inward positioning of the primers. Agarose gel electrophoresis was used to separate cDNA fragments obtained from WD patients and normal control.

Table 1: All identified allelic variants of the ATPase, Cu^{++} transporting, beta polypeptide, (ATP7B) gene found in a large extended consanguineous family with Wilson disease (WD) in Oman

Location	Polymorphism#	SNP ID@	Type
Promoter	-521T>C	rs9563084	Substitution
5'UTR	-119 ins CGCCG	rs28362531	Insertion
5'UTR	-75C>A	rs2277448	Substitution
Exon 2	1216T>G	rs1801243	Non-synonymous
Exon 3	1366G>C	rs1801244	Non-synonymous
Intron 3	1544-53A>C	rs2147363	Substitution
Intron 9	2448-25G>A	rs10870860 rs9526811	Substitution
Exon 10	2485A>G	rs1061472	Non-synonymous
Exon 12	2855G>A	rs732774	Non-synonymous
Intron 12	2866-90G>T	rs2296246	Substitution
Intron 12	c.2866-2A>G*	-	Splicing
Exon 13	3045G>A	rs1801248	Synonymous
Exon 16	3419T>C	rs1801249	Non-synonymous
Intron 18	3903+6C>T	rs2282057	Substitution

Note:
 # Numbering is in reference to first translation site
@ Numbers in the SNP database for Wilson Disease (NCBI / dbSNP)
** Suggested disease causing mutation.*

Results

All 21 exons were sequenced in each of the 46 individuals in the family. Overall, 14 variants were detected in the ATP7B gene [Table 1]; 13 of them were identified as disease non-causing polymorphisms. There was one in the promoter region, two in the untranslated region (5'UTR), six in the coding region, one in a splice site and four in intronic regions. The 13 known variants were previously recorded in the WD SNP database[16] and the NCBI/dbSNP.[19] Most are substitution polymorphisms while there is only one insertion. Only one of the detected variants (c.2866-2A>G),[21] at genomic location 52,520,615 (NCBI, NC_000013.10),[19] was suspected as a cause of WD. It is located at -2 of the splice acceptor site of intron 12. The splice acceptor dinucleotide AG has been replaced by GG upstream of exon 13. It was genotyped in all WD patients, all relatives of patients and in fifty normal controls. It was found in 3 patients with WD and 11 asymptomatic individuals within the family who tested positive for the disease, but not in the normal controls. The c.2866-2A>G variant segregated in a recessive manner with the disease in all 14 individuals who were all homozygous for the allelic variant [Figure 1]. This variant has not been documented in the WD-Human Gene Mutation (HGMD)-NCBI databases,[16-19] nor cited in any publication on WD; therefore, it was necessary to confirm as a disease-causing variant, using reverse transcription polymerase chain reaction (RT-PCR). RNA was extracted from two patients and one normal control. A cDNA fragment containing Exons 12, 13 and 14 was amplified. Agarose gel electrophoresis showed that the cDNA PCR product of WD patients is 275 bp compared to 470 bp in normal control [Figure 2]. Upon sequencing this amplified region, it was found that the entire Exon-13 (195 bp) was skipped in WD patients [Figure 3]. The ATP7B protein will probably, therefore, be non-functional.

Discussion

Allelic variants of the ATP7B gene causing WD can be common or specific for certain ethnic groups. One of the aims of this study was to examine whether Omani WD patients harbour common

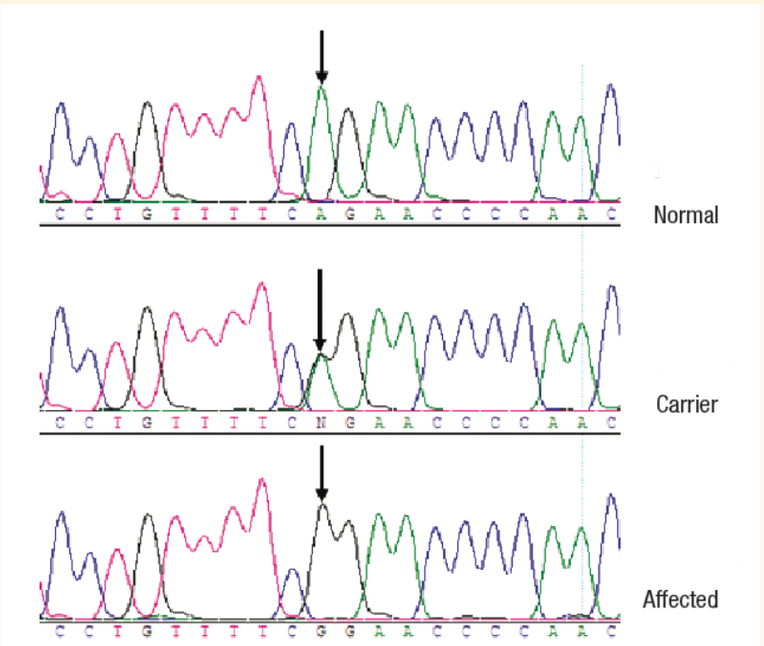

Figure 1: Exon-13 sequence showing the novel c.2866-2A>G variant in a Wilson Disease affected individual, one parent and a normal control.

or specific variants. A novel splice-site disease-causing allelic variant (c.2866-2A>G) was found in 14 individuals who belonged to an Omani large consanguineous family of Asian origin.

In the c.2866-2A>G variant, the normal allele A has been substituted by G at -2 position of the consensus sequence of the acceptor splice-site in intron 12, upstream of exon 13 (TTC**A**GAAC>TTC**G**GAAC). It appears that the highly conserved splice acceptor AG dinucleotide has been replaced by GG, resulting in complete skipping of the downstream exon 13. To confirm

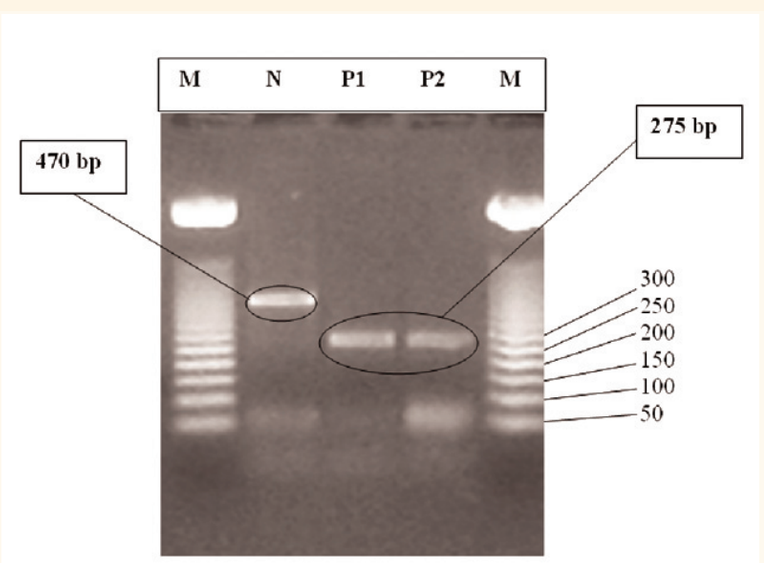

Figure 2: Polymerase chain reaction (PCR) of exons 12, 13 and 14 where M = Marker (50 bp); N = Control sample; P1 and P2 are Wilson disease (WD) patients. The amplified cDNA fragment of WD patient is smaller (275 bp) than that of normal control (470 bp).

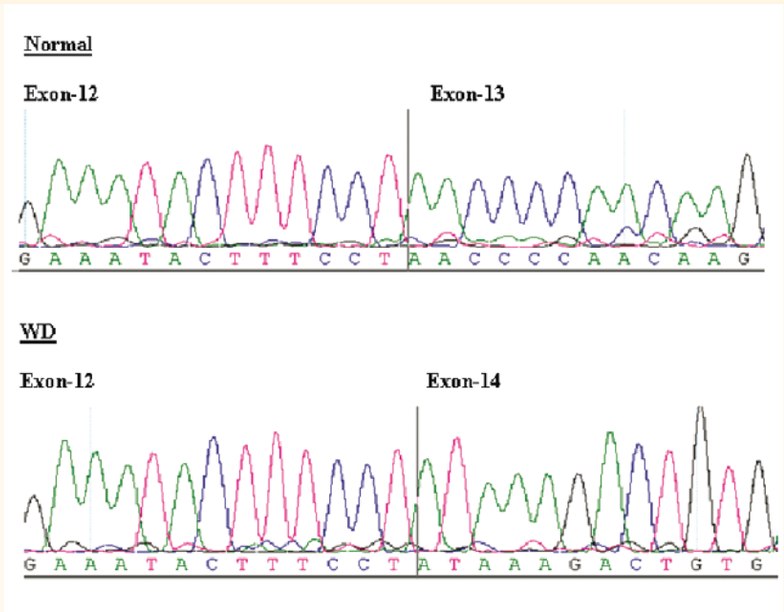

Figure 3: The complementary DNA (cDNA) sequence of the junction of exon 12-13 in normal individuals and junction 12-14 in a Wilson Disease patient, where exon 13 has been entirely skipped.

the variation, transcript analysis using RT-PCR was performed. Transcripts from WD patients showed complete deletion of exon 13. A similar novel splice-site disease-causing variant in -6 position at the intron/exon junction (IVS12-6G>A) has been reported previously.[14] Exon 13 encodes part of the transmembrane copper channel (Ch/Tm6) which is essential for copper transport. It is probable, therefore, that the c.2866-2A>G mutation would result in a defective protein.

Further analysis of the 3 patients and 11 positive, but asymptomatic, relatives showed that all 14 individuals were homozygous for the c.2866-2A>G variant. The disease appears to segregate with the allelic variant in a recessive manner. This new allelic variant is not documented in the NCBI/HGMD database;[17] therefore, it seems to be the most probable novel variation causing WD, specific to this Omani family.

However, the study has been limited by the paucity of reports on WD in Oman. Very few patients have been recorded previously. It is also essential that further molecular functional studies be done, to firmly establish the pathogenicity of the described mutation, which at present is not feasible in Oman.

Conclusion

A novel splice-site variant of the ATP7B gene that segregated with Wilson Disease in a recessive manner in an Omani family, strongly suggests it is the disease causing mutation.

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CONFLICT OF INTEREST

The authors reported no conflict of interest.

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