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Association of a c.1084A>G (p.Thr362Ala)Variant in the *DCTN4* Gene with Wilson Disease

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Purpose: Wilson disease is an autosomal recessive disorder which causes excessive copper accumulation in the hepatic region. So far, *ATP7B* gene is the only disease-causing gene of Wilson disease known to date. However, *ATP7B* mutations have not been found in ~15% of the patients. This study was performed to identify any causative gene in Wilson disease patients without an *ATP7B* mutation in either allele.
Materials and Methods: The sequence of the coding regions and exon-intron boundaries of the five *ATP7B*-interacting genes, *ATOX1*, *COMMD1*, *GLRX*, *DCTN4*, and *ZBTB16*, were analyzed in the 12 patients with Wilson disease.
Results: Three nonsynonymous variants including c.1084A>G (p.Thr362Ala) in the exon 12 of the *DCTN4* gene were identified in the patients examined. Among these, only p.Thr362Ala was predicted as possibly damaging protein function by *in silico* analysis. Examination of allele frequency of c.1084A>G (p.Thr362Ala) variant in the 176 patients with Wilson disease and in the 414 normal subjects revealed that the variant was more prevalent in the Wilson disease patients (odds ratio [OR]=3.14, 95% confidence interval=1.36-7.22, P=0.0094).
Conclusion: Our result suggests that c.1084A>G (p.Thr362Ala) in the *ATP7B*-interacting *DCTN4* gene may be associated with the pathogenesis of Wilson disease.

Key Words: Wilson disease, *DCTN4*, *ATP7B*-interacting genes, Polymorphism, Association

Introduction

Wilson disease is an autosomal recessive disorder caused by the defect in copper transportation, resulting in hepatic copper accumulation. Generally, copper is metabolized in the enterocyte and transported into the hepatocyte. The defect in hepatic exportation of copper causes Wilson disease. *ATP7B* mutation is the single cause of Wilson disease known to date[1)]. *ATP7B* plays a major role in transporting copper to apoceruloplasmin so that it binds copper and changes its form to a holo-ceruloplasmin. This ceruloplasmin is secreted into the bloodstream with copper. Furthermore, *ATP7B* transfers copper to bile canaliculi via *trans*-Golgi network, eventually excreting into bile. *ATP7B* has been suggested to interact with five proteins such as *ATOX1*, *COMMD1*,

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GLRX1, *ZBTB16*, and *DCTN4*[1]. *ATOX1* is a cystolic protein that serves as an intracellular donor of copper[2]. This protein also forms a complex with *ATP7B*[3]. *COMMD1*, also previously known as *Murr1*, is involved in the pathway of hepatic biliary copper excretion[4]. *GLRX1* is known to interact with the N-terminal of *ATP7B*. *GLRX1* also catalyzes the reduction of intramolecular disulphide bonds and deglutathionylation of the cystine residues of the CxxC motifs of *ATP7B*, which results in facilitation of copper binding on *ATP7B*[5]. *ZBTB16* is known to colocalize with *ATP7B* on the *trans*-Golgi network. *ZBTB16* plays a significant role in the ERK signaling pathway of the hepatocyte[6]. *DCTN4* is involved in vesicle transportation of copper. The interaction of *DCTN4* with *ATP7B* suggests that *DCTN4* facilitates copper-induced trafficking of *ATP7B*[7]. The existence of these five *ATP7B*-interacting proteins suggests that *ATP7B* interacts with various types of proteins that are critical for supporting the copper transportation by *ATP7B*.

Approximately, 72-90% of patients with Wilson disease have a mutation in the *ATP7B* gene[8]. Our main focus is on the remaining 10-28% of Wilson disease patients who do not have an *ATP7B* mutation. Although there is a possibility that mutation might reside in promoter or deep intron region of the *ATP7B*, we hypothesized that a mutation in the *ATP7B*-interacting proteins will cause defects in the transportation of copper in the liver and ultimately manifesting a same phenotype as Wilson disease. Thus, in this study, we screened five *ATP7B*-interacting genes to identify new disease-causing genes for Wilson disease.

Materials and Methods

1. Subjects

This study consisted of a total of 176 unrelated patients with Wilson disease. Patients were diagnosed based on decreased serum ceruloplasmin level (<15 mg/dL) and increased urinary excretion of copper (>100 ug/day) as well as clinical symptoms, majority of them showing hepatic or neurological manifestations. A total of 414 healthy individuals were also recruited for this study as control group. The study was approved by the Institutional Review Board of Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea. All patients or their parents provided a written informed consent.

2. Mutation Screening

To identify any causal mutations in the *ATP7B*-interacting genes, genomic DNA was isolated from peripheral blood, all the exons and their respective flanking regions of the 5 *ATP7B*-interacting genes were analyzed in 12 patients with Wilson disease without an *ATP7B* mutation in either allele. Genetic variation was identified by comparing the individual's sequence to the reference sequence. The PCR products were sequenced and analyzed with an ABI Prism 3730 sequencer (Applied Biosystems, Foster City, CA, USA) and Polyphred program (http://www.droog.gs.washington.edu/PolyPhred.html). *In silico* prediction of functional alterations by the novel genetic variants were performed using Polyphen (http://genetics.bwh.harvard.edu/pph/). Multiple alignments of amino acid sequences from different species were done by clustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

3. TaqMan genotyping

To validate the association of c.1084A>G (p.Thr362Ala) variant of *DCTN4* with Wilson disease, Wilson disease samples (N=176) and normal control samples (N=414) were investigated using TaqMan genotyping method with a HT7900 real time PCR machine (Applied Biosystems, Foster City, CA, USA).

4. Statistical Analysis

Statistical analyses were performed using the Hap-Analyzer program (http://hap.ngri.go.kr/) and SPSS programs (version 18) (SPSS Inc., Chicago, IL, USA).

To test the association with Wilson disease, χ^2 test was used to compare allele frequencies between cases and controls, and Fisher's exact test was used when an expected cell count was less than five.

Results

1. Mutation screening of ATP7B-interacting genes in patients with Wilson disease

Among the five *ATP7B*-interacting genes (*ATOX1*, *COMMD1*, *DCTN4*, *GLRX1* and *ZBTB16*), a total of 11 variants were found in the 12 patients with Wilson disease without an *ATP7B* mutation in either allele (Table 1). Among these, three variants were nonsynonymous, which were exclusively found in the *DCTN4* gene. All of them are known single nucleotide polymorphisms (SNP), rs11954652, rs3733923, and rs117873033. *In silico* prediction of functional effects of three nonsynonymous variants of *DCTN4*, using PolyPhen, showed only c.1084A>G (p.Thr362Ala) variant to be "possibly damaging". Two patients are heterozygotes for this variant. (Table 1). In addition, this site, p.Thr362 of the *DCTN4* gene, is highly conserved among various species. These results predict that c.1084A>G (p.Thr 362Ala) might alter the biological function of *DCTN4* significantly.

2. Association of c.1084A>G (p.Thr362Ala) in the *DCTN4* gene with Wilson disease

To determine whether c.1084A>G (p.Thr362Ala) in the *DCTN4* gene is associated with Wilson disease, we performed TaqMan genotyping in large control samples (N=414) and patients samples (N=176), including the samples of the 12 patients with Wilson disease without an *ATP7B* mutation in either allele. The c.1084A>G (p.Thr362Ala) was found in 9 patients of Wilson disease patient group (5 AG heterozygotes and 4 GG homozygotes), whereas 7 individuals in control group harbor c.1084A>G (p.Thr362Ala) (4 AG heterozygotes and 3 GG homozygotes) (Table 2). The c.1084A>G (p.Thr 362Ala) in the *DCTN4* gene was significantly more prevalent in patients with Wilson disease than in normal population (odds ratio [OR]=3.14, 95% confidence interval=1.36-7.22, *P*-value=0.0094).

Discussion

Our purpose of the mutation screening of five *ATP7B*-interacting genes in patients with Wilson disease was to discover any novel causative variants that develop a Wilson disease-mimicking phenotype. No causal mutation in the *ATP7B*-interacting genes has been

Table 1. Genetic Variants Identified in the Five ATP7B-interacting Genes by Direct Sequencing in 12 Patients with Wilson Disease

Gene	SNP (rs# or new)	Nucleotide change	Amino acid change	Allele (1:2)	Flanking sequences
*ATOX1**	n/a	n/a	n/a	n/a	n/a
COMMD1	new	c.180+70C>G		C:G	actctccccc[C/G]cttgccttcc
DCTN4	rs7706089	c.-79T>C	5'-UTR	T:C	AAGTCGAAAG[T/C]AGGGAAGGCA
	new	c.-27A>C	5'-UTR	A:C	ATGCGCCGGG[A/C]GCGTCATCGC
	new	c.969G>A	synonymous	G:A	GCTGGTCGCT[G/A]Tgtaagtatt
	rs11954652	c.1047C>G	p.Phe349Leu	C:G	TGACTCTCTT[C/G]GAGTGTGAGG
	rs117873033	c.1084A>G	p.Thr362Ala	A:G	TATCAACAGC[A/G]CTGCTAAGgt
	new	c.1299A>G	synonymous	A:G	ATGATTTTAA[A/G]AACCTGGCAG
	rs3733923	c.1334G>A	p.Ser445Asn	G:A	ATTGAAGAAA[G/A]TGACCAGGGA
GLRX1	rs4561	c.225T>C	synonymous	T:C	GAGTCTTTAT[T/C]GGTAAAGATT
ZBTB16	new	c.2016C>T	synonymous	C:T	GCCCAGCCTT[C/T]GAGGAGCCAA
	new	c.1624+46C>T		C:T	tgggagccag[C/T]gtctatattt

Abbreviations: n/a, not available (no detection of polymorphism in *ATOX1* gene); SNP, single nucleotide polymorphism; UTR, untranslated region
*No genetic variation was identified in the ATOX1 gene

Table 2. Genetic Association of *DCTN4* Polymorphism (c.1084A>G) in Wilson Disease

Group	Genotype			MAF	Allelic OR (95% CI)*	*P*-value
	AA	AG	GG			
Wilson disease	167	5	4	0.037	3.14 (1.36-7.22)	0.0094
Control	407	4	3	0.012		

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval
*Allelic test was performed using HapAnalyzer program for calculating OR (95% CI) and corresponding *P*-values

reported in humans to date. Among *ATP7B*-interacting genes, the *COMMD1* gene can cause early onset of Wilson-like disease in dogs[9]. However, no mutation of the *COMMD1* gene was found in Wilson disease patients who were negative for *ATP7B* mutations[10]. Only intronic SNPs and synonymous SNPs were detected in the *COMMD1* gene[11]. Most variants in the five *ATP7B*-interacting genes, identified in our patients, were also previously known synonymous SNPs and nonsynonymous SNPs with prediction of none functional alternation. Only one variant in *DCTN4* gene was predicted to alter the function of *DCTN4* protein significantly. The importance of *DCTN4* as an *ATP7B*-interacting gene is not completely clarified. *DCTN4* is expected to play an important role in the vesicle exportation of copper from the liver[7]. In addition, *DCTN4* interacts with the N-terminal of *ATP7B*, which might contribute to the development of Wilson disease. Particularly, it has been reported that the amino acid residues 200- 460 region of *DCTN4* protein interact with the N- terminal of *ATP7B* protein[7]. As the c.1084A>G (p.Thr 362Ala) variant of *DCTN4* is located in the center of *DCTN4*-*ATP7B* interaction, c.1084A>G (p.Thr362Ala) might affect the interaction of *DCTN4* with *ATP7B* and subsequently may have a critical or additionally detrimental effect on copper transportation. In addition, we demonstrated that the c.1084A>G (p.Thr362Ala) variant of *DCTN4* is significantly associated with Wilson disease. These data indicate that c.1084A>G (p.Thr362Ala) variant of *DCTN4* might modify the clinical outcome of Wilson disease, although the variant is not a direct cause of Wilson disease. Further study is necessary to determine whether the c.1084A>G (p.Thr362Ala) variant of *DCTN4* can directly affect the interaction of copper with *ATP7B* protein and subsequently the copper transport.

In the pedigree analysis of the *DCTN4* variant in the two patients, the variant was inherited from respective parent (data not shown), indicating that the c.1084A>G (p.Thr362Ala) is not a *de novo* variant. We also analyzed some clinical characteristics of the 9 patients, who carry c.1084A>G (p.Thr362Ala) variant of *DCTN4*, either heterozygotes of Thr/Ala or homozygotes of Ala allele, to find specific combination with *ATP7B* mutation types and any correlations with clinical features of Wilson disease. However, we could not observe any specific patterns or combinations with *ATP7B* mutation types, indicating that the c.1084A>G (p.Thr362Ala) variant of *DCTN4* does not directly influence the phenotype of Wilson disease as a modifier of *ATP7B* mutation. Although modifier gene mutation of Wilson disease such as *Murr1* in dog was identified, no cases were reported in the human patients with Wilson disease. Therefore, it assumed to be less likely that multiple minor loci may play a role for the occurrence of Wilson disease by influencing the *ATP7B*-mediated copper transport. However, c.1084A>G (p.Thr362Ala) variant of *DCTN4* still has a genetic epidemiological significance.

In summary, we identified a novel variant, c.1084A>G (p.Thr362Ala) in the *ATP7B*-interacting *DCTN4* gene which is highly conserved among species. This variant was significantly associated with Wilson disease. These results indicate that this variant might affect the *ATP7B*-mediated copper transport in liver. These finding will provide a new insight into the understanding of Wilson disease pathogenesis.

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국문초록

목 적: 윌슨병은 간조직에 구리의 과도한 침착으로 발병하는 상염색체 열성 유전질환이다. 지금까지 *ATP7B* 유전자가 유일한 원인유전자로 알려져 왔다. 그러나, 약 15%의 환자에서는 *ATP7B* 유전자 돌연변이가 발견되지 않는다. 본 연구는 *ATP7B* 유전자의 돌연변이가 발견되지 않은 윌슨병 환자를 대상으로 새로운 원인 유전자를 발견하기 위하여 시행되었다.

대상 및 방법: *ATP7B* 돌연변이가 발견되지 않은 12명의 윌슨병 환자를 대상으로 *ATP7B*와 상호작용을 하는 것으로 알려진 *ATOX1, COMMD1, GLRX, DCTN4*와 *ZBTB16* 유전자의 전사부위와 엑손-인트론 경계부위의 염기서열을 분석하였다.

결 과: *DCTN4* 유전자의 12번 엑손에 존재하는 c.1084A>G (p.Thr362Ala)를 포함하는 3가지의 변이가 환자에서 발견되었다. *in silico* 분석을 통해 3가지 변이 중 c.1084A>G가 유일하게 단백질 기능 변화를 일으킬 것으로 예측되었다. 176명의 윌슨병 환자와 414명의 정상인을 대상으로 이 변이의 빈도를 조사한 결과, 정상인보다 윌슨병 환자에서 더 높은 빈도를 나타내었다(상대비, odds ratio [OR]=3.14, 95% 신뢰도=1.36-7.22, P=0.0094).

결 론: 본 연구의 결과는 *ATP7B*와 상호작용하는 *DCTN4* 유전자의 c.1084A>G (p.Thr362Ala) 다형성이 윌슨병의 발병과 연관이 있음을 시사한다.

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