

Analysis of the human Atox 1 homologue in Wilson patients

Isabel Simon, Mark Schaefer, Jürgen Reichert, Wolfgang Stremmel

Isabel Simon, Mark Schaefer, Jürgen Reichert, Wolfgang Stremmel, Department of Gastroenterology, University of Heidelberg, Germany

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Correspondence to: Mark Schaefer, MD, Department of Gastroenterology, University of Heidelberg, INF 410, D-69120 Heidelberg, Germany. kontakt@dr-schaefer-praxis.de

Telephone: +49-6221-28029 Fax: +49-6221-162321

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Abstract

AIM: To analyze the metallochaperone antioxidant-1 (Atox1) gene sequence in Wilson disease patients.

METHODS: Mutation analysis of the four exons of the Atox1 gene including the intron- exon boundaries was performed in 63 Wilson disease patients by direct sequencing.

RESULTS: From 63 selected patients no mutations were identified after the entire coding region including the intron- exon boundaries of Atox1 were sequenced. One known polymorphism within the Atox1 gene (5'UTR -99 T>C) in 31 (49%) of the Wilson patients as well as one previously undescribed variation (5'UTR -68 C>T) in 2 of the Wilson patients could be detected. Statistical analyses revealed that the existence of a variation within the Atox1- gene showed a tendency towards an earlier onset of the disease.

CONCLUSION: Based on the data of this study, no major role can be attributed to Atox1 in the pathophysiology or clinical variation of Wilson disease.

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Key words: Antioxidant-1; Wilson disease; Wilson's disease protein; Mutation analysis

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INTRODUCTION

Copper is an essential trace element for prokaryotes and eukaryotes. It acts as a cofactor for a number of proteins such as cytochrome c oxidase, dopamine β -hydroxylase (production of catecholamines), superoxide dismutase (free radical detoxification), lysyl oxidase (cross-linking collagen and elastin) and ceruloplasmin^[1]. At the same time, excess or free copper is toxic to the cell.

The inherited disorders of copper metabolism Menkes and Wilson disease (WD) result from a disturbance of copper balance, resulting in either a deficiency (Menkes) or an accumulation of copper (WD) in the body^[2]. WD occurs in about one of 30 000 people^[3-5] and is characterized by the accumulation of copper primarily in the liver but also in the brain, kidney, cornea (Kayser-Fleischer-Rings), and spleen. WD is caused by a genetic defect in the ATP7B gene, located on chromosome 13q14.3^[6]. This gene encodes a polytopic membrane protein containing several motifs characteristic of P-type ATPases, highly expressed in the liver^[7]. Under steady-state conditions the gene product of ATP7B WNDP (Wilson's disease protein) resides in the trans-Golgi network (TGN)^[8] where it delivers copper to such secreted copper-dependent enzymes as ceruloplasmin. As the copper content of the hepatocytes increases, this ATPase moves from the TGN to a cytoplasmic vesicular compartment near the canalicular membrane. As copper is transported into this compartment, the intracellular copper concentration falls and the WNDP is recycled back to the TGN while copper is exported from the cell^[9].

There are more than 230 mutations in ATP7B^[10] accounting for Wilson Disease, and no mutation is predominant^[11]. Patients present, typically between the ages of 5 and 40 years, with quite various hepatic (40%), neurological (40%), psychiatric (14%-18%) or other symptoms^[12,13]. Although the specific type of mutation might have in part influence on disease severity, even patients with identical mutations show high clinical variability regarding the age of onset, signs and syndromes, ceruloplasmin levels, hepatic copper levels and presence of Kayser-Fleischer-rings^[14,15]. The following factors are

known to influence the disease: There are certain sex-specific differences: female patients for example show a higher prevalence of acute liver failure than male patients. Schiefermeier *et al* reported that an APO E ε3/3 genotype delays the onset of signs and symptoms^[16]. Merle *et al* described influence on onset of symptoms of Wilson disease depending on prion protein status^[17]. It has been proposed that genes influencing human copper metabolism might modify the clinical picture caused by a mutated Wilson disease gene.

As noted above copper is not free within the cell^[18]. The trafficking of copper from donor and acceptor proteins is mediated by a unique class of proteins termed copper chaperones^[19,20] that were first identified in the yeast *Saccharomyces cerevisiae*. The yeast ATX1p encodes a small cytosolic copper-binding protein that binds copper via the copper-binding MxCxxC motif and delivers this metal to CCC2^[21] - the yeast homologue of WNDP - for subsequent transport into the secretory pathway and incorporation into the ceruloplasmin homologue FET3^[22]. The human homologue Antioxidant-1 (Atox1) is an 8 kDa cytosolic protein that contains a single copy of the highly conserved MxCxxC motif^[23] in the amino terminus that is repeated 6-fold in WNDP. This metallochaperone interacts directly with the Wilson ATPase^[2] and can regulate its copper occupancy^[24]. By modulating the amount of copper bound to the protein Atox1 can regulate the intracellular localization^[25], the posttranslational modification^[26,27] and the enzymatic activity of WNDP^[24].

MATERIALS AND METHODS

Patients

The Genomic DNA of 63 WD patients was sequenced, which include 42 female and 21 male patients. The average age was 34 years (19-56). Twenty eight (44%) patients presented primarily with hepatic symptoms (including elevated liver enzyme test, ascites, liver cirrhosis, and acute liver failure), 20 (32%) patients primarily with neurological symptoms, 7 (11%) patient with both, 8 (13%) patients were diagnosed preclinically by family screening.

In all patients the definitive diagnosis of WD was established either by DNA analysis or by typical clinical and laboratory constellations [reduced copper levels (< 10 μmol/L) and ceruloplasmin levels (< 0.2 g/L) in the serum, raised free plasma copper levels, increased urinary copper excretion (≥ 2 μmol/24 h), detection of Kayser-Fleischer-rings, elevated hepatic copper concentrations (> 250 μg/g dry tissue)] or both. The ATP7B gene has been sequenced in most patients in part so far in cooperation with Professor Ferenci, Department of Gastroenterology and Hepatology, Vienna, including the analysis of the H1096Q mutation status in all patients and sequencing of exons 8, 13, 14, 15 and 18 in most patients not homozygous for H1096Q. In 36 patients two mutations could be detected within the Wilson disease gene. In 16 patients one mutation and in 11 patients no mutations could be detected within the Wilson gene so far.

Methods

Genomic DNA was isolated from peripheral EDTA-

Table 1 Oligonucleotide primers used to amplify Atox1

Exon	Sequence	Fragment size
Exon 1-Forward primer	5'-GGAGTGGGAGGGGC CTCCGGGACC-3'	273 bp
Exon 1-Reverse primer	5'-GTAAGCTAGGGGAC AACAGCGCTC-3'	
Exon 2 - Forward primer	5'-GCACITGTGGGGG TCACTCTACAG-3'	320 bp
Exon 2-Reverse primer	5'-GTGAGGATTAATG ATGTGATTCAC-3'	
Exon 3-Forward primer	5'-GAACTCTTCTTGCT GTAACGGGAG-3'	344 bp
Exon 3-Reverse primer	5'-GAGGGCTCTCCCGC TCCAACAAG-3'	
Exon 4-Forward primer	5'-TGCAATGTCGCTAT GTCCACACCA-3'	326 bp
Exon 4-Reverse primer	5'-GATCACACAGCAA AGAATCAGAATC-3'	

blood using the QIAamp® DNA Blood mini Kit (Quiagen, Hilden/Germany) according to the manufacturer's instructions.

The four exons of the gene including the intron-exon boundaries were amplified by PCR, using the Oligonucleotide primers shown in Table 1, designed from the published cDNA sequence of the human homologue of Atox1 gene^[23].

The 50 μL volume of each PCR reaction contained 100 ng of template DNA, 500 μmol/L of each oligonucleotide primer, 200 μmol/L each dNTP, 2.5 units of Stratagene Pfu-DNA Polymerase (Qiagen, Hilden, Germany) in 10 × QIAGEN PCR Buffer (Qiagen, Hilden).

PCR reactions were performed in a Progene FPROG050 cycler (Techne, Cambridge/ UK) starting with the initial denaturation of the DNA at 95°C for 10 min; followed by 45 cycles (exon 1, 3, 4)/40 cycles (exon 2) of: 45 s denaturation at 95°C, 45 s annealing at 60°C (Exon 1)/56.5°C (Exon 2)/50°C (Exon 3 + Exon 4) and 1 min extension at 72°C, then with a final extension of 72°C for 10 min.

PCR products were purified with the MinElute™ Purification Kit (Quiagen, Hilden/Germany). The sequencing reaction was performed by SEQLAB (Sequence Laboratories Göttingen, Göttingen/Germany).

Sequences obtained by sample sequencing were compared with <http://www.ncbi.nlm.nih.gov/BLAST>.

Statistical analyses

Statistical analyses were performed with the SPSS, version 13.0 (Statistical Package for the Social Science, SPSS Inc., Chicago, IL, USA.). *P* < 0.05 was taken as significant.

RESULTS

We analysed Atox1 in 63 WD patients, diagnosed either by DNA analysis or by typical clinical constellations.

There could be no alterations in the Atox1 coding exon sequence or splice junction sequence be detected in any of these individuals.

Direct sequencing of the Atox1 gene within the 5'-UTR region located before exon 1 of the 63 Wilson disease

Table 2 Atox1 gene analysis in Wilson disease patients

Mutations in the ATP7B-gene	No. of patients	Distribution of the variations detected in the <i>Atox1</i> -gene among the <i>ATP7B</i> -genotypes				No variation within the sequence
		5'UTR -99 T>C		5'UTR -68 C>T		
		Heterozygous	Homozygous	Heterozygous	Homozygous	
H1096Q/H1096Q	22	6	4	-	-	12
R1041W/R1041W	2				-	2
G12810N/G12810N	1				-	1
G1266R/G1266R	1	1			-	
G710A/G710A	1					1
K844k-fs/K844k-fs	1					1
W779X/W779X	1		1			
D765N/Y741X	2		1			1
3400 Del C/2299InsC	1					1
3400 Del C/H1096Q	1	1				
3400 Del C/G982V	1	1				
3400 Del C/W779X	1					1
H1096Q/M769V	1	1				
m.n.d./m.n.d.	11	7		2		4
H1096Q/m.n.d.	8	3	1			4
W 778 X/m.n.d.	1	1				
W779X/m.n.d.	2		1			1
D765N/m.n.d.	2					2
G710S/m.n.d.	1		1			
M769V/m.n.d.	1					1
2299 InsC/m.n.d.	1	1				
Total	63	22	9	2	0	32

m.n.d.: Mutation not detected.

patients examined revealed one known polymorphism within the Atox1 gene in 31 (49%) of the Wilson patients (Table 2). Thirty one of 63 (49%) Wilson patients had detectable Atox1 gene changes, with the heterozygous T/C at 5'UTR -99 being the most common with 22 (35%) patients.

In 2 Wilson patients the previously undescribed 5'UTR -68 C>T (heterozygote) genetic variation could be detected.

Seven of the 11 patients where no Wilson disease gene mutation could be found so far and 8 of the 16 patients with a single identified Wilson disease gene mutation showed one of the variations in Atox1.

5'UTR -99 T>C

This polymorphism 5'UTR -99 T>C (Figure 1A) is already described in the database (NCBI Sequence Viewer: NM_004045: "dbSNP: 1549921"). 35% (22 patients) showed this substitution of the base T by C heterozygous, the other nine patients (14%) were homozygous for this variation.

Among these 22 patients with the heterozygous variation 7 persons presented with primarily hepatic symptoms, 7 patients with primarily neurological symptoms, and 3 patients with hepatic and neurological symptoms. Five were presymptomatic at diagnosis.

The nine patients with homozygous variation are composed of 3 patients with primarily hepatic symptoms, 3 persons with primarily neurological symptoms, 1 patient with hepatic and neurological symptoms, and 2 patients diagnosed preclinically by family screening (Figure 1B).

The heterozygote variation could also be found in one healthy control person, homozygote variation could be found in two persons without WD. The healthy control person, [ALT 24 U/L, AST 31 U/L (-35), copper

21 µmol/L (12-35), ceruloplasmin 0.27 g/L (0.2-0.6)] and two persons had no signs of Wilson disease (based on clinical and basic laboratory testing). They had normal levels of serum ceruloplasmin, copper and liver function tests.

5'UTR -68 C>T (heterozygot)

This additional variation of the 5'UTR could be found in two patients. One of them presented with the hepatic presentation, the other one had a mixed presentation (Figure 2).

The detected nucleotide changes in the 5'UTR region of the Atox1 gene did not have significant association with or influence on the average age of initial manifestation of Wilson disease (Table 3). Statistical analyses revealed that the existence of a variation within the Atox1-gene showed no significance towards an earlier onset of the disease (Table 4).

DISCUSSION

When sequencing the exons of the Wilson disease gene in clinically proven Wilson disease patients, only a single or even no mutation can be detected in 5%-10% of all patients depending on the population examined and the analyzing laboratory. In our study, a single Wilson disease gene mutation could only be identified in 16 patients and no Wilson disease gene mutation could be found in 11 patients so far. However complete sequencing is still under way in most of these patients.

Sixty three patients were analyzed as potential candidates of an Atox1 caused Wilson disease like disease. 31 out of these 63 patients had variations within the Atox1 gene. In terms of clinical presentation (age of onset, hepatic/

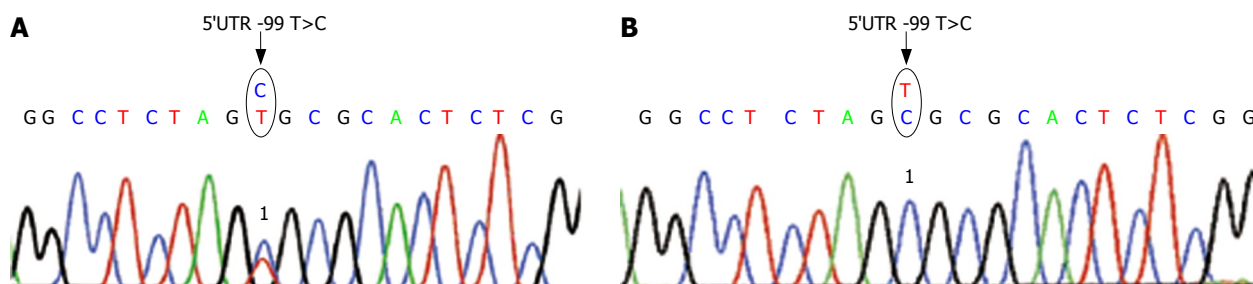


Figure 1 Sequence analysis of genomic DNA of the *Atox1*- gene 5'UTR -99T>C site. **A:** Heterozygote in this site. ¹Heterozygote substitution of the base T by C on position -99 in front of the beginning of the start codon located in exon 1; **B:** Homozygote in this site. ¹Homozygote substitution of the base T by C on position -99 in front of the beginning of the start codon located in exon 1.

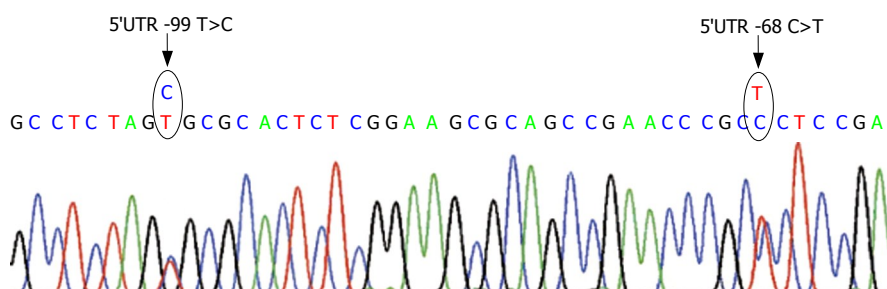


Figure 2 Localization of the 5'UTR -68 C>T gene variation in relation to the 5'UTR -99 T>C variation.

Table 3 Analysis of average ages of initial manifestation with regard to the variations detected in the *Atox1* gene

<i>Atox1</i> -variation	Average age of initial manifestation	n of patients	Standard deviation
No variation within the sequence	23.68	31	12.993
Variation within the sequence detected	18.88	25	9.909
Total	21.54	56	11.863

neurologic onset), laboratory tests (serum ceruloplasmin, serum copper, 24 h urinary copper excretion) and clinical course (improvements, drug reactions, side effects, initial neurological worsening) there were no significant differences between the 31 WD patients with *Atox1* changes compared to the remaining 32 patients without *Atox1* changes. As these sequence changes were in the 5'UTL region leaving the translated regions as well as the splicing sites and the classical translation initiation complex site of the gene intact, it seemed unlikely, that these detected changes could cause a Wilson disease like disease by themselves or be able to significantly influence the clinical course of Wilson disease. On the other hand, the highly conserved region of the translated *Atox1* gene in humans can be evidence for a vital role of *Atox1* protein in human metabolism or embryonic development. This also might explain the still unknown phenotype of *Atox1*-mutation associated diseases^[2], even though different roles and regulatory factors for *Atox1* in human metabolism are emerging out of recent studies^[28-31]. One might speculate about a Menkes disease like phenotype resulting from a complete disruption of both functional alleles of *Atox1* as suggested by *Atox1* knockout mice data^[32].

The association between the *Atox1* variations and the

Table 4 Statistical data for Analysis of age of initial manifestation

Model	Not standardized coefficients		Standardized coefficients		
	B	Standard error	Beta	T	Significance
ATP7B	4.042	1.970	0.272	2.052	0.045
<i>Atox1</i>	-5.312	3.079	-0.225	-1.725	0.091

changes in the age of onset was weak in this study similar to data published before^[2].

Taken together, *Atox1* associated modification of Wilson disease or Menkes disease are still not seen so far and the absence of mutations in the coding regions of the *Atox1* gene speak for an essential role of wild type *Atox1* in human metabolism.

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COMMENTS

Background

Cytoplasmic copper has to be transferred into the cellular excretory pathway by copper transport pumps. In patients with the copper storage disease "Wilson disease" the copper transporter ATP7B is defective. This transmembranous ATPase receives cytoplasmic copper from the copper chaperone antioxidant-1 (*Atox1*).

Research frontiers

Patients with Wilson disease show a wide variation in their clinical presentation.

Proteins interacting with the ATP7B copper transporter, such as Atox1, COMMD1 or chemical such as Platinum-complexes are one important area in explaining this phenomenon.

Related publications

Lutsenko S, LeShane ES, Shinde U. Biochemical basis of regulation of human copper transporting ATPases. *Arch Biochem Biophys* 2007; 463: 134-148 [PMID: 17562324]; Singleton C, Le Brun NE. Atx-1 like chaperones and their cognate P-type ATPases: copper-binding and transfer. *Biometals* 2007; 20: 275-289 [PMID: 17225061]; Merle U, Schaefer M, Ferenci P, Stremmel W. Clinical presentation, diagnosis and long-term outcome of Wilson's disease: a cohort study. *Gut* 2007; 56: 115-120, 314 [PMID: 16709660].

Innovations and breakthroughs

Proper ATP7B function is regulated by cytoplasmic factors (e.g. COMMD1, Atox1), cytoskeletal factors (e.g. dynactin subunit p62) and by hormones (e.g. prolactin, oestrogens). ATP7B and its sister protein ATP7A (Menkes protein) are regulated by tissue specific factors and by hormones and can be present simultaneously within one cell.

Applications

Research on modification factors of Wilson disease are aimed to identify protective factors within the clinical course of Wilson disease and providing them to affected patients.

Terminology

Atox1: antioxidant-1, small cytoplasmic protein with copper binding sites; ATP7B: copper transporting ATPase encoded by the Wilson disease gene; Wilson disease: autosomal recessive copper storage disease due to malfunction of ATP7B.

Peer review

This paper was well designed and analysed in large-scale patients. Although the results are in part negative, they are important to the scientific community.

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