RESEARCH STUDIES

Classification of mutations in *ATP7B* in Wilson's disease patients

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Abstract

Background: The impact of individual *ATP7B* mutations on the diversity of the clinical spectrum of Wilson's disease (WD) is not understood yet.

Material and methods: The functional activity of *ATP7B* has been assessed and compared to the reports of the homozygous WD patients. Ten rare and two frequent mutations H1069Q and R969Q have been selected after the analysis of the literature in question. Chinese hamster ovary cell lines lacking ATP7B expression and carrying the selected mutations have been generated. The cells have been characterized by transgenic *ATP7B* activity by the determination of copper accumulation and copper toxicity.

Results: The highly concordant results have been observed in the diverse functional activities of *ATP7B* within the groups of mutations that were established with regard to the disease onset reported in the patients. Whereas a low (< 29 ± 3% of wild type of ATP7B) or no *ATP7B* activity has been found in the group of the mutations (n = 5) observed in the patients with early (or sudden) onset of the disease, its high activity (77.6 ± 7% to 118.6 ± 7%) has been observed in the group of the patients with the late onset of the disease (n = 3). Notably, the mutations H1069Q and R969Q of the third group (n = 4) showing predominately the intermediate time of the disease onset have had a moderate level of *ATP7B* activity.

Conclusions: The data suggest that in the functional assessment of *ATP7B* mutations in homozygous patients we can single out the groups that have distinct grades of biologic activity which improves our understanding of the high degree of phenotypic variation observed in WD patients as well as the data on the onset of the disease.

Key words: Wilson's disease, Western Blot, MTT-test, ATP7B.

Introduction

Wilson's disease (WD) is an autosomal recessive disorder resulting from the mutation of ATP7B gene [2]. The WD gene consists of 21 exons that span a genomic region of about 80 kb and is located on the long arm of chromosome 13 (13q14.1) [5, 35]. *ATP7B* encodes a large membrane protein of 1.465 amino acids that has been characterized to be a copper-transporting P-type adenosine triphosphatase (ATPase) which has a high homology of the amino acid sequence of the genes responsible for Menkes' disease. Worldwide, the frequency of WD is approximately 1:30 000. ATP7B is mainly expressed in the liver and to a lesser extent in the brain and other organs. ATP7B has two functions in the liver which are central for the copper homeostasis [21]. ATP7B transports copper into the Trans Golgi Network (TGN) where the metal is transferred into apoceruloplasmin that is finally released into the blood as ceruloplasmin. The excess of copper is sequestered by ATP7B in vesicles that are subsequently released from the body via bile canaliculi.

WD is a fatal disease when it is not appropriately treated, e.g. by anti-coppering drugs like penicillamine, and often leads to death [26, 31]. Hallmarks of WD are a copper accumulation in liver, a low ceruloplasmin activity and the presence of Kayser-Fleischer corneal rings; though the diagnosis is difficult to be made since the individual abnormalities can be absent or on the borderline. A wide spectrum of clinical presentations is observed, including a liver damage and/or neurologic/psychologic symptoms ranging from the patients with asymptomatic phenotypes, which show only mild abnormalities of copper homeostasis, to the patients with liver cirrhosis, acute liver failure, mild psychologic symptoms or severe neurologic disability. The onset of the disease is also highly variable and is often observed in childhood as well as in adolescence and even in elderly adults [9]. At present, the molecular mechanisms that underlie the complex clinical manifestation of WD have not been understood. The carrier proteins such as metallothionine, gluthatione, superoxide dismutase and heat-shock proteins that mediate the uptake, delivery and efflux of copper have been implicated to modify the disease clinics.

It has been suggested that the type and location of a mutation within ATP7B is a single determinant of the disease clinics indicating that the individual mutations of *ATP7B* may be linked to a phenotype. More than 600 mutations of *ATP7B* are known and have been summarized in the public data base (www.hgmd.org). The majority of mutations present missense mutations that are mostly located throughout the open reading frame of *ATP7B,* deletions and insertions; and other mutations leading to a premature stop codon are observed. Since most WD patients have the compound mutations of *ATP7B* that may modulate the phenotypic expression of the disease by the two alleles which have distinct biologic activity, the analysis of homozygous mutations has helped our understanding in the investigation of the possible links of *ATP7B* genotype and phenotype, e.g. in the studies of the large families in specific regions [3]. While the frame shift and nonsense mutations of *ATP7B* have been implicated to result in the early onset and more severe manifestations of WD in various studies of

patients from Poland, Greece, and Germany [12, 28], such findings have been challenged by others [25, 27]. For some of the most frequently found homozygous missense mutations like H1069Q an association with late and predominantly neurological clinical presentation has been observed in WD patients [22, 9, 34], although such a link could not be detected in other cohorts [6, 10, 33]. The presence of uncharacterized disease-modifying factors has been postulated by the studies describing siblings and twins that have an identical mutation but different onsets and severity of the disease. Therefore, it has been suggested that the conflicting reports include the observations of several *ATP7B* genotypes and are possibly caused by the disease modifying, e.g. due to ethnic or specific genetic factors of the region, alternate splicing, single nucleotide polymorphisms (SNP) and also by such factors as diet or lifestyle, that cannot be well controlled by a sole analysis of clinical data of WD patient cohorts. Thus, in addition to the clinical studies of the patient cohorts a functional characterization of *ATP7B* mutations has been helpful for understanding the impact of a given mutation [29, 14, 15 16].

We have addressed the question whether a functional characterization of *ATP7B* mutations may be suitable to classify the impact of an individual mutation on the clinical presentation when compared to the respective phenotype of homozygous WD patients. A set of 12 mutations of *ATP7B* has been chosen from the data base. The mutations have been grouped with regard to the early and late onset of the disease as reported for the rare homozygous WD patients. A third group has contained the mutations that have been found in WD patients showing intermediate (neither early nor late) time of the disease onset which is predominantly observed in the two frequently found mutations – H1069Q and R969Q. The mutations have been stably expressed in mammalian cell line of Chinese hamster ovary (CHO) that lacks intrinsic *ATP7B* expression. The analysis of *ATP7B* protein expression and cell growth has been made for 12 cell lines. The data on the functional characterization of *ATP7B* mutant cell lines have been compared to the clinical presentation reported for the homozygous WD patients.

Material and methods

Mutations phenotype

The data on most patients have been derived from the literature search of Pubmed, Medline, HGMD. The criteria for the selection of mutations have been the presence of a homozygous *ATP7B* allelic variant and the availability of clinical information about the time of the disease onset and the predominant clinical presentation in patients having it. Ten mutations have been arbitrarily chosen. Two mutations of WD patients, who have been studied during the last 10 years, have been chosen from Klinische und Experimentelle Transplantationshepatologie, Universitätsklinikum Münster (UKM) data base. The clinical parameters, laboratory data, and *ATP7B* mutation analysis have been evaluated by UKM clinicians according to the common guidelines (Ferenci 2003). The data are summarized in table 1. The genomic DNA has been extracted from the peripheral blood samples. The blood has been obtained after getting the informed consent, and all procedures have been approved by the local Ethics Review Board. Twenty one exons of the WD gene have been amplified, and the sequencing of the polymerase chain reaction (PCR) products has been performed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) with an ABI-Prism 3100 genetic analyzer (Applied Biosystems).

Generation of mutant ATP7B cell lines

The studies have been carried out using CHO cells lacking ATP7B, which were obtained from the German tissue culture collection (DSMZ) and cultivated in 5% CO_2 at 37°C in humid atmosphere. The culture medium used has been DMEM F12 (Lonza), 10% FCS (PAA), 1% penicilline-streptomycine (PAA), 0.075% sodium bicarbonate (Lonza). Plasmid pGCsamENATP7B has been cloned into retroviral vector (kind gift of O. Wildner, Ruhr Universität Bochum) using cDNA that contained human wild type cDNA of *ATP7B* [32]. Plasmid pGCsamENATP7B has been mutagenized using the primers by means of site-directed mutagenesis (QuikChange II XL site-directed mutagenesis kit, Stratagene). The mutagenized cDNA has been sequenced using an Applied Biosciences 3100 sequencer to confirm the presence of the selected variant and the absence of secondary mutations. The sequence of *ATP7B* in the cell lines has been also confirmed at the end of the experiment. The retroviral vectors have been generated as reported above [32]. The transductions of CHO cells have been carried out in the presence of polybrene (Sigma-Aldrich). The stable cell lines have been selected by gentamycin (Gibco; 400 μg/ml) that has been included into the tissue culture medium during the cultivation of the cells. The CHO cells that carried empty vector, untransduced CHO cells and CHO cells harboring wild type pGCsamENATP7B plasmid have been used as control ones.

Western Blot analysis

The cells have been grown in a tissue culture dish, rinsed twice with ice cold Phosphate Buffered Saline (PBS; produced by PAA company) and incubated for 10 min on ice in the lysis buffer (1% Triton X-100, 30 mM Tris, pH 8.0, 75 mM NaCl, 7.5 mM EDTA, 1% Na-deoxycholate, 2.5% SDS) using protease inhibitor cocktail (Complete; Roche Applied Science). The cell lysate has been collected by scraping and incubated for 15 min on ice followed by a centrifugation at 14,000 rpm at 4°C for 30 min. 20 μg of protein has been divided into fractions on a 9% SDS polyacrylamide gel and blotted onto nitrocellulose membrane (Amersham Hybond ECL; GE Healthcare Life Sciences). The membrane has been blocked in PBS containing 0.1% Tween 20 (produced by USB) and 5% fat free dry milk (AppliChem) at 4°C overnight. After washing five times with 0.05% Tween/PBS the membrane has been incubated with polyclonal rabbit anti-*ATP7B* (kind gift of Dr. I. Sandoval, Madrid, Spain) diluted 1:2 000 in 0.1% Tween/PBS, 3% BSA/PBS for 1.5 hours. To control the protein loading goat polyclonal antiserum – anti-HSC70 – (produced by Santa Cruz, # C2906) has been used. A peroxidase-labelled secondary antibody produced by Sigma has been used for the detection by Enhanced Chemi-Luminescence (ECL) (Western

Blotting Detection Reagent; GE Healthcare Life Sciences). A densitometric analysis has been performed using ImageQuant TL Plus 7.0 (GE Healthcare Life Sciences) software. A relative expression has been compared to CHO cells expressing the wild type of ATP7B.

Cell growth

2 x 10^4 cells have been seeded into three wells in a 96-well plate (Becton Dickinson) and cultivated for 24 hours in 100 µl of medium (DMEM high glucose without phenol red; produced by PAA company). On the next day, at a cell confluency of 90-100% the medium has changed to 100µl of the same medium containing $CuCl_2$. The cells have been cultivated for 48 hours. After that the cells have been washed with PBS once and 100 µl of MTT solution (2 mg/ml in phenol red free medium, Sigma) has been added. After a 2-hour incubation 100 µl of sodium dodecyl sulphate (15%; Sigma)/dimethyl sulfoxide (6 M; Roth; pH 4.5) has been added. The optical density has been determined at 560 nm in a multiwell plate reader (Multiskan EK, Thermo Labsystems) after a 24-hour incubation of the plates at room temperature. For each cell line three 96-well plates have been analysed in the experiments. Three independent experiments have been performed. The results of the mutant cells have been calculated as a percentage of the respective cells that received no copper (100%).

Table 1

ATP7B Variants Evaluated for Copper Transport Activity in CHO cells

	Protein position	Age of onset	Clinic		Exon	Location Type Postulated effect			KF ring
			Gastro intestinal	Neurological					
Group$_E$	p.E583R fs	5		Dystonia tremors	5	Cu 6	Frameshift		+
	p.T766R	17		Severe dysarthria	8	TM 4	Missense		+
	p.G691R	7-9	Liver cirrhosis		7	TM 2	Missense		+/-
	p.G1341D	5			20	TM7	Missense		
	p.L1071W	8		Dysarthria	14	ATP N-binding			+
Group$_L$	p.R616Q	38, 55		Ataxia, Dysarthria	5	Cu 6		Conservative amino acid change	+
	p.A874V	27, 34			11	TD			
	p.T1288R	30	Liver cirrhosis		18	ATP hinge			
Group$_I$	p.H1069Q			+	14	ATP loop SEPHL	Missense	Disruptive ATP binding	
	p.R969Q	8-9 20±11	Hepatic cirrhosis		13	Tm5 Tm6	Missense		-
	p.C1079Y	8-11		Dysarthria, dystonia	14	ATP N-binding			
	p.I1102T	11	Chronic liver disease	+	15	ATP N-binding			

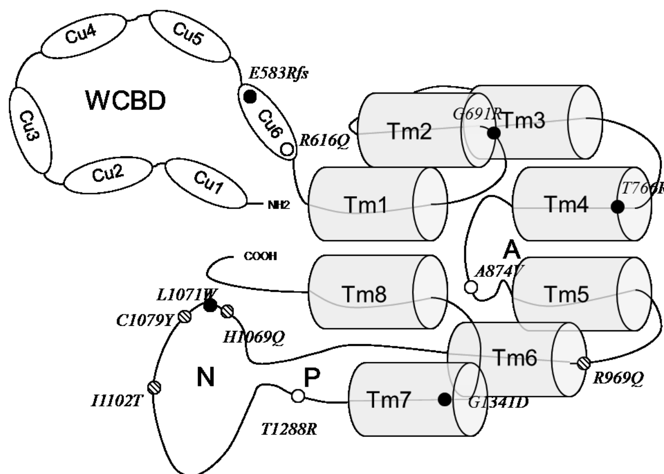

Fig. 1. Scheme of ATP7B protein, featuring the N-terminal domain (Wilson's disease copper-binding domain) and the 6 N-terminal metal binding sites marked Cu1-Cu6, phosphatase (A-domain), phosphorylation (P-domain) and ATP-binding (N-domain). The cylindrical regions labeled Tm1-Tm8 represent the trans-membrane domains. Selected mutation sites are marked.

Results

Establishment of stable cell lines expressing mutants of *ATP7B*

Twelve mutations observed in *ATP7B* homozygous patients showing different clinical manifestation of WD have been selected from the literature as well as from UKM data base (tab. 1).

Eleven missense and one frame shift mutation (E583R-fs) have been located throughout the open reading frame of *ATP7B* (fig. 1).

For our study *ATP7B* mutations have been grouped according to the reported onset of the disease observed in the respective homozygous patients (tab. 1). The first two groups have contained mutations observed in the reports of early or sudden (E583R-fs, G691R, T766R, L1071W and G1341D), or late (R616Q, A874V and T1288R) onset of the disease (group$_E$

and $group_L$, respectively). The third group of the mutations has contained the mutations R969Q, H1069Q, C1079Y and I1102T that have been reported for WD patients predominantly showing an intermediate onset of the disease ($group_I$). Most mutations have been reported in a few patients (n < 5) and families while mutations R969Q and H1069Q have been more frequently observed. To generate stable cell lines that express mutant *ATP7B* CHO cells that lack *ATP7B* expression have been chosen. Untransduced CHO cells and CHO cells expressing *ATP7B* wild type have been used as a reference value for the determination of the biologic *ATP7B* function during the study.

***ATP7B* mutant cell lines display highly characteristic growth curves at elevated copper concentrations**

To assure that the biologic function of the *ATP7B* mutant cell lines is a valid methodology for the classification of the functional *ATP7B* activity the growth of cell lines has been followed using various copper concentrations (fig. 2).

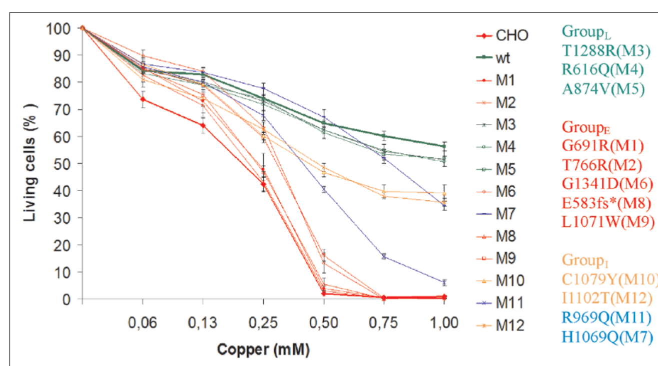

Fig. 2. Graphical representation of the results obtained after the calculation of the mean value and SE after 3 independent experiments (9 plates have been used).

The growth of the cells has been determined by MTT assay after 48 hours period. All five *ATP7B* mutant cell lines of $group_E$ (E583R-fs, G691R, T766R, L1071W and G1341D) have showed a rapid decline of cell growth at increasing copper concentrations that has leveled to a value of 0% at the copper concentrations above 0.5 mM. The cell growth rates of the five cell lines have been almost identical to that of the CHO control cell line which lacks *ATP7B* expression. On the contrary, all three *ATP7B* mutant cell lines of $group_L$ (R616Q, A874V and T1288R) have displayed a uniform growth characteristic with high cell growth rates (> 50.7 ± 2%) at copper concentrations above 0.5 mM. The growth rates of the three *ATP7B* mutant cell lines have very much resembled the values obtained with CHO cells expressing wild type of *ATP7B*. *ATP7B* mutant cell lines of $group_I$ (R969Q, H1069Q, C1079Y and I1102T) have displayed the growth curves that have been highly elevated as compared to CHO control cells but have had intermediate values as compared to CHO cells expressing wild type of *ATP7B*. It's notable that the growth rates of mutant cell line R969Q have been significantly lower at the copper concentrations above 0.5 mM (> 6.1 ± 1%) as compared to the other mutant cell lines of this group (> 34.5 ± 1%). The statistical analysis of the growth rates among the three groups of *ATP7B* mutant cell lines has showed the highly significant differences suggesting that the biological activity of *ATP7B* mutants may fall into three distinct classes that mostly correlate with the onset of the disease observed in homozygous WD patients (tab. 1).

Highly diverse *ATP7B* protein expression in mutant cell lines

Next, the *ATP7B*-specific protein expression has been determined in *ATP7B* transduced CHO cell lines (fig. 3).

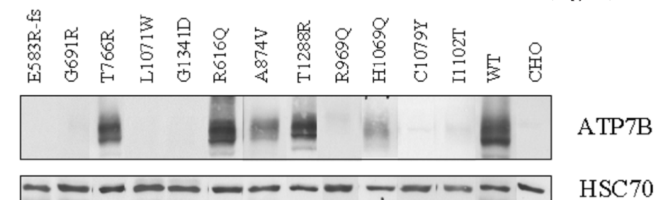

Fig. 3. CHO cells transfected with wild type or mutant ATP7B have been lysed and the protein expression has been determined by Western Blot analysis using anti-ATP7B and anti-actin.

Densitometric analyses of Western Blots (tab. 2) has revealed that *ATP7B*-specific protein has been poorly expressed (< 5% of wild type) in $group_E$ of mutants (E583R-fs, G691R, L1071W, and G1341D) with the exception of T766R (37.2 ± 7%). In $group_L$ of mutants (R616Q, A874V, and T1288R) the high levels of *ATP7B* protein (up to 90%) have been detected as compared to CHO cells expressing *ATP7B* wild type (100%). Within $group_I$ of mutants (R969Q, H1069Q, C1079Y, and I1102T) only mutant cell line H1069Q has showed moderate levels (18.5 ± 5%) of *ATP7B* protein expression while the other three mutants have been poorly detected (< 5% of wild type).

Table 2

Characteristics of *ATP7B* mutant cell lines as compared to the wild type

Mutant cell line	Protein (%)	Growth (1 mM Cu) (A_{560} nm) (%)
E583R-fs	0.3 ± 0	0.9 ± 1
G691R	3.4 ± 0	0.0 ± 0
T766R	37.2 ± 7	0.0 ± 0
L1071W	3.3 ± 1	0.3 ± 0
G1341D	2.3 ± 1	0.0 ± 0
R616Q	77.3 ± 8	89.5 ± 4
A874V	35.3 ± 8	82.7 ± 3
T1288R	84.7 ± 6	90.8 ± 3
R969Q	2.3 ± 1	9.4 ± 4
H1069Q	18.0 ± 5	56.7 ± 9
C1079Y	0.3 ± 0	63.3 ± 11
I1102T	3.3 ± 1	62.4 ± 2

The percentage has been calculated relative to CHO expressing *ATP7B* wild type. Mean value ± SE of 3-6 experimets are shown. The value of CHO background has not been considered.

Discussion

Many mutations have been reported in some patients without the knowledge of their impact on the course of WD. This is the first report on the functional characterization of *ATP7B* mutations in a cohort of WD patients having rare homozygous mutations. A high correlation has been observed between *ATP7B* protein expression, cell growth and the time of the disease onset. The early onset and most severe disease have correlated with an almost complete lack of the respective *ATP7B* activities in transgenic tissue culture cells. A late onset of the disease has correlated with high *ATP7B* activities which almost reached levels of the wild type *ATP7B*. An intermediate time of the disease onset with mostly milder disease has been found in the group of mutations showing intermediate activities of A*TP7B*. The *in vitro* characterization of the *ATP7B* activities, as it has been established in this study, has allowed us to single out the distinct groups of mutations that have similar biologic activity correlating to the onset of the disease. The functional classification of *ATP7B* mutations may have importance for the disease prognosis of patients, at least in the subset of homozygous WD patients and may also further extend our knowledge about the molecular principles of the wide phenotypic variations observed in individual mutations including H1069Q and R969Q.

Various exogenous expression systems have been used to analyze the function of *ATP7B* mutations including the cell lines of yeast, insect cells and mammalian tissue culture derived from e.g. human kidney or liver [29, 14, 15]. CHO cells used for our study proved to be well suited for the analysis of *ATP7B* function [11, 18]. Generally the trafficking of *ATP7B* through different cellular compartments with regard to copper concentration has been studied while various mutations have also been characterized by biochemical and biological analyses including the assessment of copper toxicity, copper retention and growth. According to our knowledge this is the first report combining two functional characterizations of *ATP7B* in a set of mammalian mutant cell lines. The molecular mechanisms leading to the rescue from toxic copper in hepatocytes and in extrahepatic cells is not completely understood, the current knowledge suggests that the copper is transferred by complex mechanisms including trafficking of the copper mediated by *ATP7B* to a location of reduced oxidative stress, e.g. by incorporation of the copper into vesicles and/or by the copper secretion (Singleton 2010). The highly concordant results of the two functional assays that have determined a cellular growth at elevated copper concentrations have been mostly observed in a given cell line. Interestingly, two *ATP7B* mutant cell lines, L1071W and G1341D, have showed the low but significantly elevated values of copper retention while the growth has been severely impaired suggesting that the capability to accumulate the copper within the cells is a different functional entity of transgenic *ATP7B* as compared to the escape of the cell growth at high copper concentration.

A lot has been learned about the association of the *ATP7B* genotype and phenotype by means of studying the patient cohorts. The most studied mutation in such cohorts, H1069Q mutation, has however got rather conflicting results of the study. Yet a predominately later onset of the disease and neurological presentation have been observed in many H1069Q homozygous patients' different clinical manifestations, e.g. early and severe hepatic disease has been noticed in other patients [22, 9, 34, 6, 10, 33]. It is, therefore, interesting that our functional characterization of H1069Q has showed rather high activity for a mutant protein of *ATP7B* suggesting that a moderate activity of *ATP7B* may represent a prerequisite for the observed wide spectrum of clinical presentations which are likely caused by the secondary disease modifying factors. Thus, the classification of *ATP7B* activity as shown here may predict the overall possibility of a given mutation to be modulated by the secondary disease modifying factors that are obviously operational in the patients. In this line, the diverse clinical presentations observed in R969Q mutation of this group might also be associated to the intermediate *ATP7B* activity observed in this mutant. Mutations E583R-fs, G961R, T766R, L1071W and G1341D that have been proved to have significantly reduced *ATP7B* activity may, thus, have a more limited spectrum of phenotypic presentations and mutations of this group might, therefore, be predominately associated with an early onset of the disease. The mutations belonging to the group that have retained a high *ATP7B* activity close to the level observed in the wild type (R616Q, A874V and T1288R) may, thus, be associated to a later onset and a milder disease. It should be noted, however, that the extrapolation of our findings is limited to WD patients having the respective homozygous mutation. In addition, our conclusions are based on a limited number of mutations and some of the chosen mutations have been obtained from the patients that may show a region-specific phenotype. However, the cell culture approach that has been used here to classify the mutations possibly excludes the influence of many disease-modifying factors that may reside in the 80 kb *ATP7B* gene and elsewhere, e.g. in the variations of the promoter region, splicing events, polymorphisms (SNPs), differences in poly-adenylation sequences and the influence caused by environmental factors. Such analysis of *ATP7B* activity, therefore, seems to be valuable to predict the impact of a given mutation in the absence of disease-modifying factors.

Two of the missense mutations (E583R-fs and R616Q) studied here are located in copper binding domain 6 of *ATP7B*. The deletion studies have shown that the last two copper binding domains (Cu5 and Cu6) are required for copper transport across membranes while the other copper binding domains may have a regulatory role [13, 1, 15]. The mutation E583R-fs leads to a premature stop of the protein translation. Such mutations have been implicated to result in early and severe disease by analysis of WD cohorts; however, these findings have been challenged by others [12, 22, 28, 24, 25, 27]. As it is shown here, the expression of *ATP7B* cDNA encoding a frame shift mutation leads to a complete loss of *ATP7B* activities suggesting that in the absence of disease-modifying factors such mutations are likely to cause a severe disease as opposed to the missense mutations that retain a residual biologic activity of *ATP7B*. The second mutation of the Cu6 domain (R616Q) is a missense mutation that has been described in two separate homozygous patients with a late onset and a mild phenotype of WD. The change of the bulky amino acids arginine to

glutamine at amino acid position 616, therefore, has had only a low impact on *ATP7B* activity.

Four mutations studied here are located within (G691R, T766R and G1341D) or between (R969Q) the trans-membrane regions of *ATP7B*. The three of these mutations (G691R, T766R and G1341D) are found to have a highly reduced activity of *ATP7B*. The homozygous mutation G691R is found in a large family in Lebanon with a severe hepatic disease and the early time of the disease onset [4]. The homozygous mutation T766R is found in a patient that showed a sudden severe onset of the disease with a neurologic manifestation [30]. The homozygous mutation G1341D has been observed in the patients from Eastern Europe having an early onset of the disease and a severe hepatic/neurologic manifestation. The mutations located within the trans-membrane region of *ATP7B* may, thus, significantly reduce the biologic activity of *ATP7B*. In contrast to the three mutations which have showed almost no biologic activity of *ATP7B*, the mutation R969Q has showed an intermediate activity of *ATP7B*. The homozygous mutation R969Q is frequently found and has been also observed in the families from Pakistan and Greece having a variety of mostly hepatic disease manifestations that have included the asymptomatic course of the disease as well as a rapid progression to a fulminant disease [28].

The mutation A874V that is located in the transduction domain (A domain) of *ATP7B* has had almost no detrimental effect on *ATP7B* activity. The mutation A874V is commonly found in Asian cohorts and a homozygous mutation A874V has been observed in the families having hepatic and hepatic-neurological diseases with a late onset [17]. The mutation T1288R is located in P domain (hinge region) and has not showed a significant impact on *ATP7B* activity. The homozygous mutation T1288R has been observed in a family from Sicilia that has showed a late onset of a hepatic disease with no signs of a neurologic syndrome [19].

Four mutations are located in the adenosine triphosphate (N-domain) of *ATP7B* important for copper binding. The mutation L1071W has almost completely lost *ATP7B* activities while the other mutations (H1069Q, C1079Y and I1102T) have showed an intermediate *ATP7B* activity. The homozygous mutation L1071W has been observed in a family that showed an early onset of a severe neurological disease, and it has been suggested that the bulky amino acid tryptophan in position 1071 can significantly affect the activity of *ATP7B*. Notably, the homozygous mutation H1069Q, C1079Y and I1102T have been found in the patients having a broad range of mostly intermediate time of the disease onset. Our finding of a moderately reduced *ATP7B* activity of the mutation H1069Q located in N domain is supported by other biochemical characterizations. The configuration of the ATP binding site is not affected by this mutation although the affinity of N-domain to ATP is markedly decreased [8, 23].

Conclusions

The results indicate that the characterization of *ATP7B* activity in mammalian cell cultures has resulted in the classification of mutants having distinct degrees of biologic activity which correlate with the onset of the disease in rare homozygous WD patients.

For the two frequently found mutations, H1069Q and R969Q, an intermediate *ATP7B* activity has been observed, what contributes to our understanding of the role of the disease-modifying factors for the clinical presentation in the group of mutations having significant residual activity. An early onset and most severe disease has been observed in the group of mutations having none or marginal *ATP7B* activity including one frame shift mutation.

The prediction of the onset of the disease worked out by the functional classification may not be applicable on an individual basis; the characterization of *ATP7B* mutations as it is demonstrated here may help to further understand the general impact of a mutation, e.g. of novel mutations observed in young *ATP7B* homozygous patients where the clinical phenotype of WD may not be fully developed.

In order to achieve a higher accuracy while predicting the course of the disease and to gain more knowledge concerning the protein function more tests are to be made, for example, regarding ATP7B protein stability, copper retention, copper induced toxicity.

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Clinical and age peculiarities of non-Hodgkin's lymphomas with primary involvement of lymph nodes

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Abstract

Background: Non-Hodgkin's lymphomas (NHLs) are a heterogenous group of malignant tumors developing from the lymphoid tissue and having a wide range of clinical manifestations and varied evolution and prognosis.

Material and methods: We have studied the clinical peculiarities of 228 patients of different age groups with NHLs and a primary involvement of lymph nodes.

Results: The frequency of the lymph nodes primary involvement has constituted 37.6%. It has been established that NHLs most frequently had their primary onset in the peripheral lymph nodes (61.8%), less frequently – in the abdominal (23.3%) and mediastinal (14.9%) ones. NHLs most frequently begin their development in the peripheral lymph nodes, first in patients over 60 years old (84.6%), in the abdominal lymph nodes – in children (57.2%), in the mediastinum – in children and people aged between 19 and 39 (48.2%).

Conclusions: Children develop only aggressive NHL forms, these forms also predominate in adults. Aggressive NHLs in adults have been most often diagnosed in the patients having the primary tumor focus location in the mediastinal and abdominal lymph nodes. The frequency of indolent NHLs is higher in the cases with the primary involvement of the peripheral lymph nodes, the patients' age being over 60. Metastases in the bone marrow have most frequently been recorded in NHL patients with the primary involvement of peripheral lymph nodes (53.5%). The involvement of CNS has taken place most frequently in the patients with NHLs, having the onset in the abdominal (34.4%) and mediastinal (30.0%) lymph nodes.

Key words: non-Hodgkin's lymphomas, lymph nodes, age.