FUNCTIONAL ANALYSIS AND GENOTYPE-PHENOTYPE CORRELATIONS IN WILSON DISEASE

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Keywords: Wilson disease, ATP7B, H1069Q, genotype, phenotype

Abstract: Knowledge of how mutations other than p.H1069Q translate into the basic defect in Wilson disease (WD) is scarce due to the low incidence of homozygous index cases. A total of 12 homozygous mutations of *ATP7B*, were examined for their functional activity. Transfected Chinese hamster ovary cells (CHO-K1) exposed to elevated copper levels was used as a model for predicting the severity of different WD mutations. The results of this research have direct implications for WD diagnosis. Our data strongly confirms that phenotypic presentation of the patients is related to the *ATP7B* mutation, providing evidence for genotype - phenotype correlations and can explain in part the variable clinical features observed in patients with WD. The results we have provided help to highlight the information still needed for understanding the function and malfunction of ATP7B and its role in the disease.

INTRODUCTION

Wilson disease (WD) is an autosomal recessive disorder resulting from mutation of the *ATP7B* gene [1] with a worldwide frequency of approximately 1:30 000. The gene encodes for a copper transporting P-type ATPase, which plays a key role in copper distribution inside the cells [5;4]. Recent experimental data in cell culture showed that ATP7B plays a dual functional role in the hepatocyte. Localized to the Golgi apparatus, it has a biosynthetic role, delivering copper atoms to apoceruloplasmin [7]. The other role of ATP7B (under conditions of copper overload) is to translocate to the biliary pole of hepatocytes and transport excess copper out of the cell into the bile canaliculus for subsequent excretion from the body via bile [9]. Mutations are a useful guide to examine the function of ATP7B. However, most WD patients are compound heterozygous making it difficult to establish a clear link between phenotype and genotype. The major mutations p.H1069Q and p.R778L are found in about 20-50% population of WD alleles in Europe/USA and Asia [8;6] respectively. In addition, all other (more then 700) known disease-causing mutations are rare with a frequency ranging from 2% to less than 0.01%, many of which are population specific. In order to examine the consequences of different WD mutations, we aimed to investigate the functional effect of ATP7B homozygous mutations (using stable transfection of CHO-K1 cells) and to clarify their ‘‘disease-causing’’ status.

MATERIALS AND METHODS

Clinical features, laboratory data, and *ATP7B* mutation analysis were evaluated in patients who were investigated in a multinational study. The inclusion criteria in our study were the presence of a homozygous pathogenic *ATP7B* gene mutation and the availability of clinical information. Genomic DNA was extracted from peripheral blood samples. Blood was obtained after informed consent, and all procedures were approved by local Ethics Review Board.

Selection of Mutations in WD Patients: Our homozygote cases were identified during the last 10 years by WD mutation analysis of large patient populations in Europe, Asia and USA. WD shows significant phenotypic diversity even in patients carrying identical mutations. Phenotypic diversity described in some patients carrying the same mutation in different ethnic groups and among siblings of the same family has made it more difficult to define the link between the genotype and phenotype. The clinical data for the patient are shown in Table 1.

Cell culture: Studies were carried out using Chinese hamster ovary (CHO-K1) cells obtained from the German tissue culture collection (DSMZ) and cultivated under 5% CO2 at 37° C in humid atmosphere. The cells were cultured in a mixture of MEM (EBSS), 10% FBS, 2mM L-Gglutamine, 1% non-essential amino acids; confluent cultures were split 1:3-1:6 using trypsin/EDTA; seeded at 2-3 x 10^4 cells/cm2. Cells were routinely checked for mycoplasma contamination.

Cell Viability Assays

MTT: Function of mutant variants relative to WT and parental CHO human (*ATP7B*) was determined by Copper Resistance Assay. $2x10^4$ cells from copper-resistant CHO-K1 variants were seeded in 96-well plates (Becton Dickinson) and cultivated for 24 h in 100µl of medium (DMEM high glucose without phenol red; PAA). Next day, at a cell confluency of 90-100%, 100µl of copper chloride (Sigma-Aldrich) in six different concentrations resuspended in phenol red-free DMEM was added to the wells and cultivated for 48 hours. Cells were washed with PBS, resuspended in MTT

solution (5 mg/ml; Sigma) and incubated for 2 h. The incubation was stopped by addition of 100µl sodium dodecyl sulphate (15% SDS Sigma)/dimethyl sulfoxide (6M DMSO; pH 4.5). Optical density was determined at 560 nm on a multiwell ELISA reader (Multiskan EK, Thermo Labsystems). All samples were assayed in triplicates. Results were expressed as a percentage of the respective control cells that received no copper (100%).

FACS: Cells were fixed in ice-cold ethanol (600 µl) and stored at -20 °C. For flow cytometry, cells were kept on ice, spun down, washed with PBS, and then resuspended in 300 µl of PBS containing RNase A (50µg/ml) and propidium iodide (62.5µg/ml) and analyzed by FACS (FACS excalibur, BD Biosciences).

Copper Accumulation Assay: Parental and transfected CHO-K1 cell lines were seeded into separate 75 cm^2 flasks and cultured for 24 hours. The medium was then replaced with either fresh basal or 1 mM/L CuCl2-supplemented medium, and the cells were further incubated for 24 hours. Total amounts of copper in wild-type and 12 mutant variants of ATP7B were analyzed by atomic absorption spectrophotometry (Atomic Absorbance Spectrophotometer Shimadzu AA6300, Japan). Prior to AAS analysis, samples were digested in 1 ml 65% nitric acid (Suprapur, Merck, Germany) at 90°C. After 24 hr of acid digestion, samples were diluted into a 2% nitric acid solution. For each cell line and treatment condition, three independent experiments were performed and the average copper concentration was used for comparisons.

RESULTS AND DISCUSSION

Five homozygous mutations found in our patients (p.R616Q, p.G691R, p.A874V, p.H1069Q and p.G1341D) and seven distinct WD-associated mutations (p.T766R, p.T1288R, p.E583fs, p.L1071W, p.C1079Y, p.R969Q and p.I1102T) (Table 1) were introduced in the ATP7B complementary DNA (cDNA). To study the effects of these mutations, the widely used CHO-K1 cell line was used as a model for *ATP7B* expression and function. All cell lines were transiently transfected with wild-type (WT) and mutant (*ATP7B*) cDNA. Sequencing confirmed the absence of secondary mutations in all variants, and wild-type ATP7B control.

TABLE 1. ATP7B Missense Variants Evaluated for Copper Transport Activity in CHO cells

№	Mutation	Exon	Domain	Patients	Ethnic origin	Summary
1	p.G691R, c.2071G>A SNP p.S406A, p.R832K p.K952R, p.A1140V	7	TM2	Big Family (50members), 3 Generations, 5 homozygote 3 Male and 2 Female	Lebanon (Turkey)	Hepato (Severe) Patients were diagnosed between 3 and13y, two patients with liver cirrhosis and KF and three with biochemical evidence of subclinical hepatitis. 3 patients have died at 3, 7, 12y of age.
2	p.T766R, c.2297C>G	8	TM4	One Homozygote	UK	Neuro (Severe) 17y, homozygosity for p.T766R present with a sudden onset with severe neurologic syndrome
3	p.T1288R, c.3863C>G	18	ATP Hinge	One family-4 Generations, 3 homozygote patients	Sicilia	Hepato (Late age of onset) 30y man with liver cirrhosis, his two brothers 24y and 32y with liver damages witout Neurologic symptoms.
4	p.R616Q, c.1847G>A	5	Cu6	Two unrelated homozygote patients from two different	Bulgaria (England, Poland, Czech,	Neuro (Mild clinic and Late age of onset) 38y (Male), 55y

№	Mutation	Exon	Domain	Patients	Ethnic origin	Summary
				families	Serbia)	(Female) p.R616Q mutation is associated with pseudo sclerotic type of WD.
5	p.A874V, c.2621C>T	11	Td	Three unrelated families - 5 patients (25, 27, 27, 35 and 34yrs.	Bulgaria, Japan (widespread in Asia,)	Mix (variable clinic with late age of onset).
6	p.G1341D, c.4022G>A	20	TM7	Six Homozygote patients from 5 unrelated families in East Europe (5-7yrs)	Bulgaria, Moldova, Romania, Poland (Egypt)	Mix hepato-neurologic type of WD with early (5-7yrs) and severe clinic.
7	p.H1069Q, c.3207C>A	14	ATP N-binding, SEHPL	Many homozygote patients with European origin	Europe, North America	Variable manifestation and severity. Relatively late age of onset (21yrs) and predominant Neurologic presentation in results of a meta-analysis
8	p.E583fs, c.1745_1746insT	5	Cu6	Two sisters (5yrs)	Pakistan	Neuro (severe) homozygosity for p.E583fs present with early (5yrs) and severe Neurologic presentation (Dystonia tremors)
9	p.L1071W, c.3212T>G	14	ATP N-binding, SEHPL	One Homozygote	Pakistan	Neuro (severe) homozygosity for p.C1071W present with early (8yrs) and severe onset (Dysarth&Writing difficulty)
10	p.C1079Y, c.3236G>A	14	ATP N-binding	One Homozygote	Pakistan	Neuro (severe) homozygosity for p.C1079Y present with relatively early (11yrs) and severe onset
11	p.R969Q, c.2906G>A	13	Tm5/Tm6	Four families. 8 patients - 2Female (32 and 41yrs) and 6Male (8, 8,12, 16, 17, 17 and 18yrs)	Pakistan Greece	Hepatic only (variable severity and age of onset)
12	p.I1102T, c.3305T>C	15	ATP N-binding	Two Homozygote (11yrs and 12yrs)	Pakistan	Chronic hepatitis in age (11-12y) severe. Second case with Subtle Neurologic signs

To investigate how ATP7B variants excess intracellular copper, copper accumulation was measured in CHO-K1 cell lines that stably expressed both WT-ATP7B and 12 mutated ATP7B proteins. Cells were cultured in either basal or copper supplemented medium (1 mM/L CuCl2) for 24 hours, and their resultant intracellular copper levels were measured by atomic absorption spectrophotometry (AAS). When cultured in medium supplemented with copper (Fig 1), the exogenous expression of WT-ATP7B and mutant ATP7B proteins had a marked effect on copper levels in CHO-K1 cells. Exogenous WT-ATP7B temporarily increased cellular copper levels [2].

CHO-K1 cells transfected with with p.G691R, p.T766R, p.E583fs and partly p.G1341D accumulated very low amount of copper similar to non-transfected CHO cells (Fig 1).

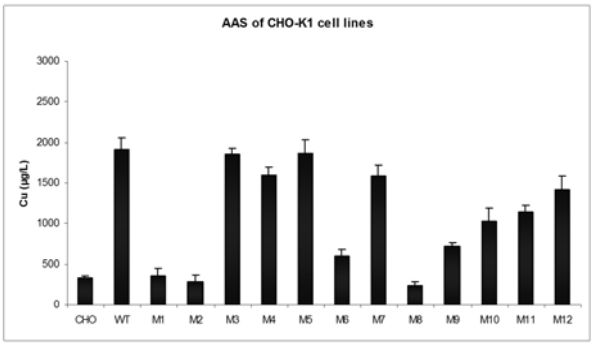

Figure 1. Accumulated intracellular copper was measured as total Cu (AAS). Cell cultures were exposed to extracellular copper concentration - 1 mM $CuCl_2$.

In contrast, exogenous expression of WT-ATP7B, p.R616Q, p.A874V, p.R969Q, p.H1069Q, p.I1102T and p.T1288R mutants resulted in a 3- 6-fold increase in copper accumulation compared with the parental cell line. Net Cu accumulation in CHO-K1 cells during 24 hr. These results were confirmed by FACS assay, an additional technique to count cell viability (Fig 2).

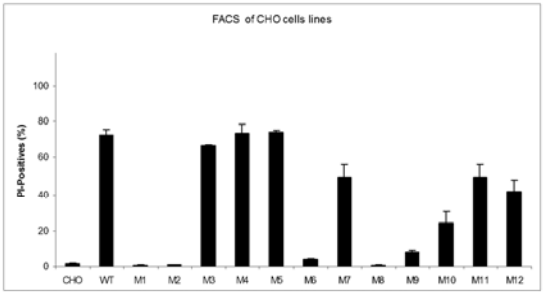

Figure 2. Growth rates in copper supplied media (0,5mM) of CHO-K1 mutant cells transformed with 12 *ATP7B* variants. Growth rates of ATP7B variants were measured in 48h and expressed as percentage. Mean +/- SEM of 3 independent experiments is indicated.

Effects of extracellular copper concentrations on CHO-K1 cell growth and proliferation were tested by incubating the cells in growth medium supplemented with up to 1 mM/L CuCl2 (Fig 3).

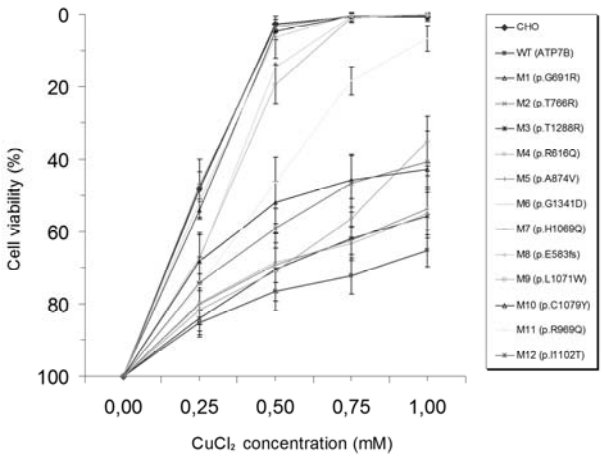

Figure 3. Effects of copper on cell growth and viability in CHO cells.

CHO clones expressing wild-type and mutant ATP7B were grown in media containing the indicated copper concentrations for 2 days, and growth inhibition was then measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The results are expressed as a percentage of control cells. Mean +/- SEM of three independent experiments is indicated.

The normal Cu concentration in human serum is reported to range from 15-35 μM [3]. Cellular viability parameters (cell growth and proliferation) were examined for 48hr incubation periods. ATP7B mutant CHO cells treated with copper supplied medium markedly differed in level on confluence state (Figure 1). After 48 hr cell growth number in group of mutations (p.H1069Q, p.C1079Y and p.I1102T) was reduced by 40%, compared with the WT-ATP7B control. Number of viable cells in group with severe mutations (p.G961R, p.T766R, p.G1341D, p.E583fs, p.L1071W and p.R969Q) was decreased to 5-18% even in 0,5mM CuCl2 concentration in our MTT test.

So we classified the mutants into three groups:

CHO cells transfected with mutations p.T1288R, p.R616Q and p.A874V had cell viability curves similar to WT-ATP7B construct, indicating light impaired copper transport. CHO transfected with mutations p.G691R, p.T766R, p.G1341D, p.L1071W, p.R969Q and p.E583fs had defective WD mutations completely unable to transport copper in our assay. Intermediate to severe deficits were observed for p.H1069Q, p.I1102T, p.C1079Y and p. R969Q.

First group of mutants (p.T1288R, p.R616Q and p.A874V) were described in patients with late onset and chronic progression. In our test these three mutations had light impaired copper transport activity (Fig 2, Fig 3). Based on clinical data mutations p.G691R, p.T766R, p.G1341D, p.L1071W and p.E583fs represented severely defective mutations and were completely unable to transport copper in our assay.

AAS and MTT results taken together suggested that p.T1288R, p.R616Q, p.A874V and p.H1069Q mutation does not affect the copper-dependent trafficking of ATP7B, but may cause structural instability, indicating that these mutations do not affect *ATP7B* expression or the copper-translocation activity of ATP7B and only partially reduced copper transport activity. The other five patient mutations – p.G1341D, p.R969Q (Transmembrane domain), p.I1102T,

p.C1079Y (ATP Nucleotide - binding domain) and p.A874V (Transduction domain) could also obstruct the correct folding of the molecule, which is consistent with the difficulty in isolating transformants expressing equivalent levels of protein to that obtained with WT-ATP7B. Probably some of ATP7B variant proteins retaining all, or most copper activity are mislocalized in the cells or ATP7B variant proteins retaining at least some copper transport activity but are unable to undergo copper-depending trafficking from *trans*-Golgi to the vesicular compartment. They could transport still sufficient copper for incorporation into ceruloplasmin. In p.G691R, p.T766R, p.G1341D and p.E583fs mutation, copper-translocation activity of ATP7B is severely impaired, and these mutants are completely unable to transport copper in transfected cells. These results suggest that the mutants would mislocalize *in vivo* and consequently would be unable to participate in hepatic copper homeostasis, accounting for the Wilson disease phenotype observed in patients with this mutation.

The goal of the present study was to determine any potential changes in copper dependent trafficking of ATP7B mutant proteins in order to gain further insight into WD pathology. By a combination of copper accumulation assay, cell culture tests determining the rate of viability of transfected cells and efficacy studies in WD patients it appears to be possible today to make a prediction on the clinical effect. *ATP7B* exogenously expressed in CHO-K1 cells can mediate vesicular sequestration of excess copper. To characterize the functional activity of selected WD patient mutants involved in copper-regulated ATP7B trafficking, CHO-K1 expressing transfected variants were exposed to elevated copper levels. The copper viability assay measures the capacity of ATP7B variants to transport copper, a requirement for normal function. These mutant proteins functionally impaired in our CHO-K1 cell model are indicative of an improperly functioning protein and likely to be the cause of the WD phenotype in the patient.

Changes in ATP7B function due to mutations in the gene can explain in part the diagnostic clinical variations in patients with WD. Mutations such as p.R616Q, p.A874V and p.T1288R that result in ATP7B proteins with altered transport activity (not effectively mediate copper efflux) are of importance for the diagnosis of pre-symptomatic individual affected with WD. Early detection and treatment of pre-symptomatic patients is critical to prevent irreversible liver damage. These mutations could be important for future studies to correct the mutant phenotype by improving protein stability.

Copper Resistance assay was used as a method for assessing the functional effect of *ATP7B* mutation. It was developed to distinguish between mutations that are disease-associated and those that appear to be less harmful in patients with WD. These results in our functional tests match completely to WD phenotypes found in patients with studied mutants. Our data have direct implications for WD diagnosis, indicating that, we can distinguish variants in which copper transport is disrupted from those in which intracellular traffic is impaired. Copper Resistance assay can be used to answer the question and to predict whether a mutation is likely to have a deleterious effect on the phenotype or not. It could be speculated that there is an association in determining the progression of the disease – mutations with fast or slow progressive cell death in the Copper Resistance assay may represent frequently early/ acute or late/chronic form of the disease respectively.

For in vitro cultured cells, Cu homeostasis should be considered more difficult to maintain, since it can be regulated by cellular adaptation mechanisms only. The CHO cells used in our study do not normally express ATP7B and therefore this endogenous protein is not detectable in these cells. ATP7B is expressed primarily in the liver, kidney and brain, and finding a biologically relevant cell line that does not express endogenous ATP7B is difficult. The rarity of

most WD mutations is such that the likelihood to obtaining the hepatocytes homozygous for a given mutation is small and the procedure to obtain such hepatocytes is invasive. Therefore CHO cells provide a relatively good experimental model for investigation of ATP7B function.

Cell Viability Assays can be used as a model for predicting the functional effect of different WD Mutations. Naturally in vitro and in vivo WD mutant phenotypes could not completely match. Other mechanisms are probably operating in the patients which may be absent in the heterologous host cells in vitro. Patients homozygous for the WD mutation in combination with non disease causing variants or polymorphisms as a background may have a more severe disease phenotype or an earlier onset. This makes the point that in vitro findings on recombinant mutants should be interpreted with caution when utilised for counselling of patients and their relatives.

In other words, the molecular pathology of mutants is hard to predict, because ATP7B topology, function and processing are complex. If sequence analysis uncovers a new or yet uncharacterised DNA change, diagnostic assays and in-depth clinical examination of the index cases need to be pursued in order to classify the clinical impact as neutral, benign or severe. Our highly selected study cohorts of individuals with homozygous (ATP7B) genotypes were associated with mild manifestation if ATP7B function was in the intermediate or normal range in both MTT and AAS. The homozygotes who developed symptoms of WD during adolescence, and were diagnosed at the oldest age, showed in our tests light progression and greater cell viability. This positive correlation between the amount of residual ATP7B function and severity of the disease was observed in all 12 mutations.

CONCLUSIONS

Functional data from our studies strongly suggest that patients phenotype is related to the *ATP7B* mutation type, providing evidence for genotype - phenotype correlations and can explain in part the variable clinical features (time of onset, severity and progression) observed in patients with WD.

On the other hand, there is considerable phenotypic variation in WD. The type or location of a mutation alone cannot explain these variations. Large sets of both clinical and molecular data are needed to study the association between *ATP7B* mutation type and severity of the disease and the specificity of organ involvement in relation to a mutation type.

Copper Resistance assay can help to establish whether a new sequence variation in *ATP7B* is responsible for the observed phenotype in WD patients.

Our data and analysis of variants has expanded our understanding of 12 sequence changes in *ATP7B* for their abilities to transport copper and their putative characterizations as disease-causing variants.

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Acknowledgements: The study was supported by a grant from the Deutsche Forschungsgemeinschaft to BT (SFB 621, project C7). I would like to express my very great appreciation to Teodor Todorov. My special thanks are extended to the staff of Klinische und Experimentelle Transplantationshepatologie, Universitätsklinikum Münster.