Silencing of Wilson Disease gene (*ATP7B*) induces transcriptional changes in copper-related genes

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Abstract

Wilson Disease is an autosomal recessive disorder of copper metabolism due to mutation in cellular copper transporter - ATP7B. The mutant protein is unable to export copper, leading to its accumulation that results in various harmful molecular changes in cells. In the present study using human hepatoma (HepG2) and neuroblastoma (SH-SY5Y) cells, we report the transcriptional variations in major copper-related genes on *ATP7B* knockdown. The cells were transfected with specific siRNA to silence *ATP7B* and the expression of copper-related genes was studied at mRNA level by quantitative real-time PCR. In both cell lines there was significant upregulation of Antioxidant1 (*Atox1*), human Copper homeostasis protein (*hCutC*) and Ceruloplasmin (*Cp*), while the mRNA expression of copper transporter gene *hCTR1* (*Slc31a1*) was not significantly affected. Effect of copper overload was studied by supplying $CuSO_4$ in medium (100 μM/ 24hour) that resulted in further upregulation of these genes. The cell viability was also found to be reduced significantly upon *ATP7B* silencing. Total cellular copper was estimated by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES). Findings of this study show the alterations in expression of major genes involved in cellular copper network and consequent imbalance in the copper homeostasis during intracellular copper overload.

Index Terms

Wilson Disease, ATP7B, hCutC, Copper homeostasis, Ceruloplasmin, Knockdown, siRNA, Gene expression, ICP-AES, Cell viability.

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1 INTRODUCTION

Copper is an essential micro element required in diet that plays significant role in cellular physiology. Both eukaryotic as well as prokaryotic cells contain copper proteins with specific roles in metabolism. This trace metal mediates various redox reactions which is vital to cells. An imbalance in intracellular copper initiate cellular dysfunction and eventual cell death [19]. In human liver and brain, copper export is mediated by a copper specific P-type ATPase called ATP7B. Mutations in the *ATP7B* leads to impairment in copper export resulting in deposition of copper in concerned tissues. The accumulated copper causes hepato-lenticular degeneration or Wilson Disease (WD) which is clinically presented as hepatic or neurological disorder or both [2], [6], [21]. The deposition of copper in cytoplasm and cellular organelles leads to various harmful effects due to initiation of active free radical toxicity [11]. There are more than 400 mutations of *ATP7B* reported worldwide till date [2].

Human CTR1 (hCTR1) is a high affinity copper transporter located in plasma membrane and encoded by the gene *Slc31a1*. This protein helps in the copper uptake and overexpression of hCTR1 is found to be harmful to cells as it leads to increased cellular copper sensitivity [3]. The human antioxidant protein Atox1 is a copper chaperone that delivers Cu^+ to ATP7B which is localized at the trans-Golgi network [8], [13]. This protein functions as a copper-dependent transcription factor and also has a protective role during free radical stress and consequent cellular injuries [7], [10]. Human homolog of copper homeostasis protein (hCutC) is an intracellular copper binding protein, virtually expressed in all tissues and distributed in cytoplasm and nucleus [14]. Human CutC exists as tetramer in solution and its monomers bind Cu^+ in 1:1 ratio. This protein has a TIM barrel structure and its exact function in higher organisms is unclear. [15].

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Ceruloplasmin is the major copper binding protein in cells which exhibits ferroxidase activity also. The blood level of total copper and ceruloplasmin is generally found to be decreased in WD patients due to impaired export of ceruloplasmin bound copper [6], [18].

In the present study, we used Neuronal and Hepatic cell lines (SH-SY5Y and HepG2 respectively) and gene expression of ATP7B, Atox1, hCutC, hCTR1, and Ceruloplasmin were looked into. The study reveals the transcriptional changes of major genes involved in cellular copper protein network on *ATP7B* silencing and provides evidence of the harmful effects of copper overload.

2 PROCEDURE AND METHODS

2.1 Cell Culture

The cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune, India. Human hepatoma (HepG2) cells were cultured in MEM (Sigma) with 10% FBS (Sigma). Human neuroblastoma (SH-SY5Y) cells were grown in 1:1 cocktail of DMEM (Sigma) and Ham's F12 (HiMedia) with 10% of FBS. The cell lines were provided with antibiotic cocktail of penicillin (Sigma, 100 units/mL) and streptomycin (Sigma, 100 µg/mL). The cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

2.2 Gene Knockdown

In a 6 well culture plate (Eppendorf) about 1×10^5 cells were seeded and grown to about 70% confluency. Washed the attached cells with Opti-MEM (Gibco) and added fresh serum-free basal media. These cells were then transfected with specific siRNA (OriGene, USA) of sequence AGAGAAUUCAUGUGACUAGCGCCTG using the transfection reagent Oligofectamin (Invitrogen). Antibiotics were avoided to minimize the cytotoxicity. After 4 hours of incubation (for transfection) at 37°C, the cells were supplied with 20% FBS in media. A universal scrambled siRNA sequence was used as the negative control. About 80% (or more) knockdown was achieved at 10nM siRNA concentration, which was verified by quantitative real-time PCR at 52 to 56 hours. To check the effect of copper overload, 100 µM $CuSO_4$ (Sigma) was supplied in media along with 20% FBS and 5mM L-Glutamine after 24 hours of siRNA transfection.

2.3 Gene expression by Real-time PCR

Total cellular RNA was isolated using PureLink RNA kit (Ambion). Manufacturer's instructions were followed to extract the total RNA. The RNA integrity was checked by 1.5% agarose gel and quantified using microplate reader (Synergy HT). Total cDNA library was made from this RNA extract using RevertAid first strand cDNA synthesis kit (Thermo) according to manufacturer's instructions. The mRNA expression was quantified using gene-specific primers and Fast SYBR green master mix (Applied Biosystems) in quantitative real-time PCR machine (Bio-Rad IQ5). Human GAPDH was used as house-keeping gene. Technical and biological triplicates were run and relative mRNA expression was calculated from CT values using Livak's $2^{-\Delta\Delta CT}$ method [17]. MIQE guidelines were followed to obtain true and error-free data [4].

2.4 Total cellular copper by ICP-AES

In a 6 well culture plate about 2×10^5 cells were seeded and the *ATP7B* knockdown was carried out as described above. After 24 hours of incubation 100 µM $CuSO_4$ was supplied in media and incubated for another 24 hours. The cells were then trypsinized, washed twice with PBS, centrifuged, freeze dried (MTG-1314) and weighed (Sartorius). Then the cells were re-suspended in reagent-grade water and infused into ICP-AES analyzer (Perkin-Elmer). The results were expressed in micrograms of copper per milligram of dry weight. Student *t*-test (GraphPad Prism) was used for the statistical analysis of data.

2.5 Cell viability assay

The *ATP7B* knockdown was carried out in 96-well (Corning) format followed by $CuSO_4$ treatment (as previously described). After the final incubation, cell viability was checked by MTT assay (HiMedia). The yellow colored water soluble Tetrazolium dye 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) was reduced to insoluble purple formazan crystals by metabolically active cells. The crystals were solubilized and absorbance was measured at 570nm in spectrophotometer (Tecan). Four biological and technical replicates were run and the data was statistically analyzed using Student *t*-test.

3 RESULTS

Knockdown of ATP7B

In both cell lines *ATP7B* silencing was effective at 10nM siRNA concentration. In HepG2 cells, about 84% of knockdown was achieved while SH-SY5Y cells showed 81% *ATP7B* downregulation (Fig. 1).

Total intracellular copper

The intracellular copper level was found to be significantly elevated in ATP7B siRNA treated cells when compared to wild type (untreated) cells (Fig. 2). When *ATP7B* silenced cells were treated with $CuSO_4$, the intracellular copper level increased further. Both cell lines showed similar results.

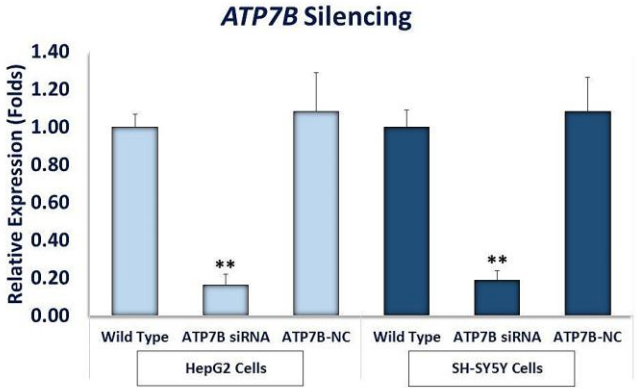


Fig. 1. Knockdown of *ATP7B* using siRNA. A universal scrambled siRNA sequence was used as the negative control (ATP7B-NC). Student *t*-test **p <0.01.

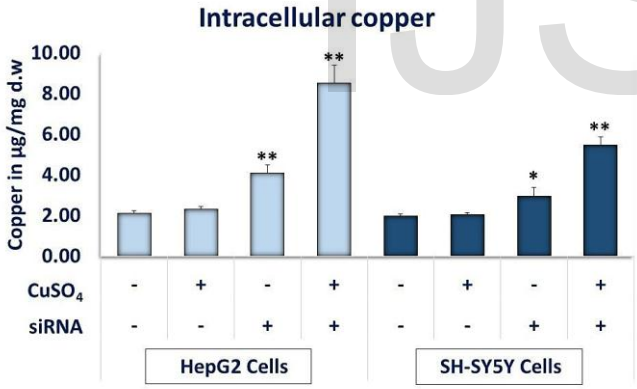
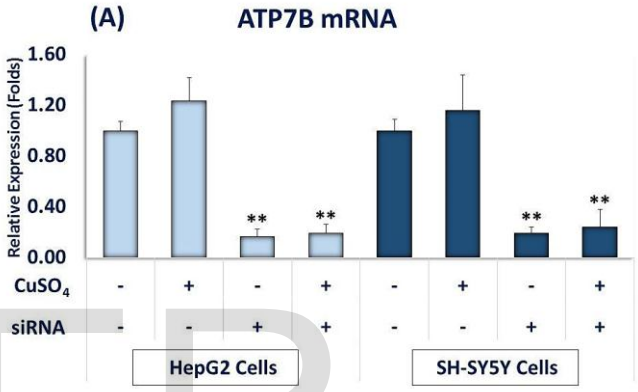
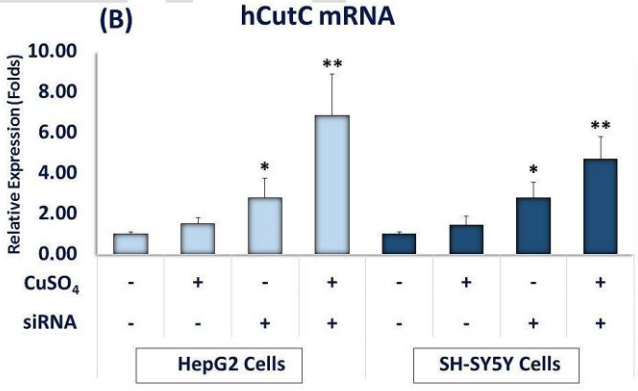


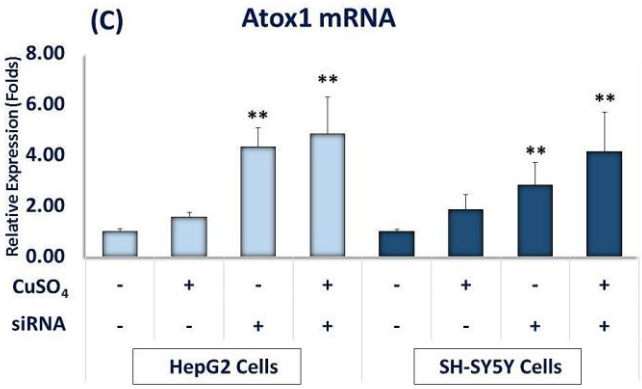
Fig. 2. Effect of *ATP7B* knockdown on intracellular copper levels. Total intracellular copper as estimated by ICP-AES. The ATP7B siRNA treatment resulted in significant accumulation of copper in cells. *p < 0.05, **p < 0.01.

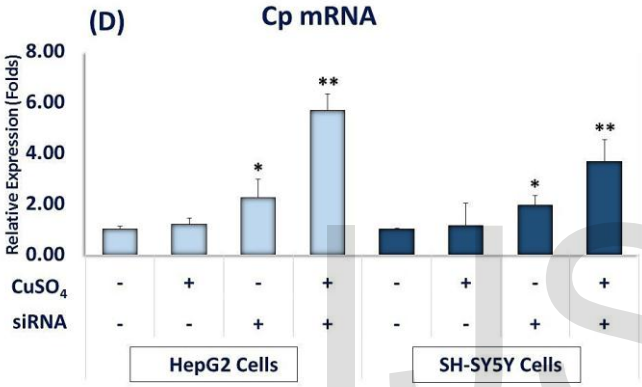
Effect of ATP7B silencing on copper-related gene expression

Silencing of *ATP7B* (Fig. 3 (A)) was confirmed by the downregulated ATP7B mRNA in both cell lines (P<0.01). The mRNA expression of human CutC (Fig. 3 (B)), Antioxidant1 (Fig. 3 (C)) and Ceruloplasmin (Fig. 3 (D)) was increased on *ATP7B* silencing with further upregulation on copper treatment. Both HepG2 and SH-SY5Y cell lines showed similar expression levels for the genes under study. The copper overload was created by supplying $CuSO_4$ at high levels in medium (100 µM/24 hour), which is higher than the plasma levels of copper observed in normal subjects. When cells were treated only with copper, there was no significant change in the expression of these genes, but addition of copper after silencing of *ATP7B* produced significant variation in expression, confirming the deleterious effect of *ATP7B* knockdown. The expression of hCTR1 mRNA was not significantly affected by *ATP7B* silencing (Fig. 3. (E)).









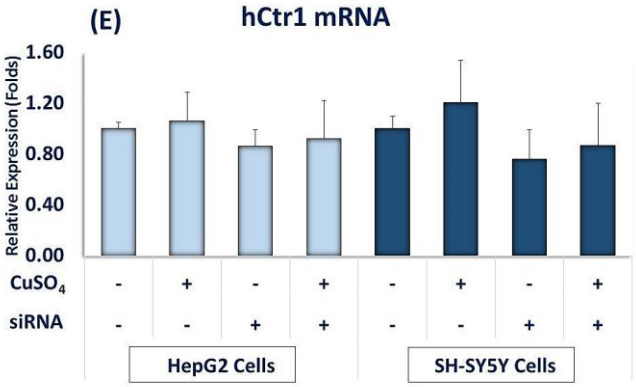


Fig. 3. Effect of *ATP7B* knockdown on expression of copper-related genes. (A) The ATP7B mRNA was downregulated on ATP7B siRNA treatment. The mRNA expression of hCutC (B), Atox1 (C) and Ceruloplasmin (D) was upregulated significantly on *ATP7B* knockdown. Addition of $CuSO_4$ resulted in further increase in mRNA expression. No significant change was observed in hCTR1 mRNA expression. Student *t*-test, *p <0.05, **p <0.01.

Effect of ATP7B silencing on cell viability

Silencing of *ATP7B* resulted in the reduction of mean cell viability to 73.93% in HepG2 cells (P<0.01) and 62.53% in SH-SY5Y cells (P<0.01). Addition of $CuSO_4$ in media further decreased the viability of HepG2 and SH-SY5Y cells to 48.58% and 37.77% respectively. However, there was no significant change observed in the HepG2 cell viability on treatment with copper alone (Fig. 4).

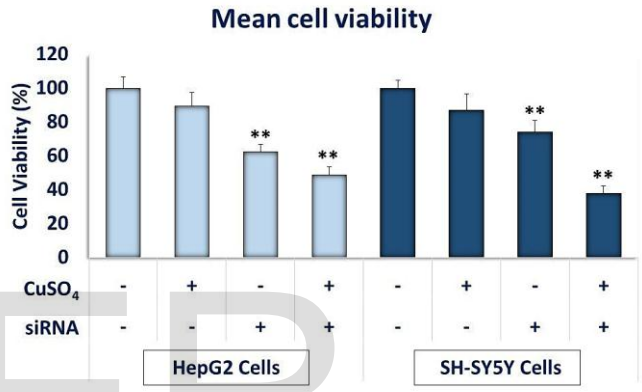


Fig. 4. Effect of *ATP7B* knockdown on mean cell viability. The cell viability was significantly decreased on *ATP7B* knockdown. Addition of copper caused further decrease in cell viability. Student *t*-test, **p <0.01.

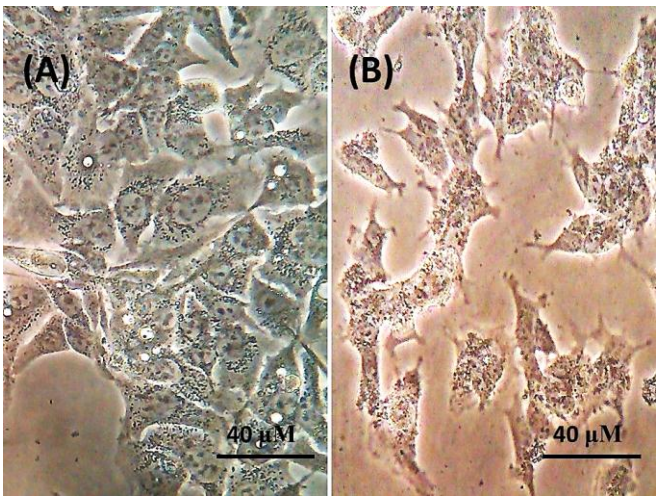


Fig. 5. Phase contrast images of HepG2 (A) and SH-SY5Y (B) cells (Nikon Ecliplse Ti-S).

Table showing Gene-specific primers used in the study.

No.	Gene	Primer Sequence	Product size
1	Human *CutC*	FP 5'- GGTGCTGATGGTTTGGTTTT RP'- GCGTTCAAATCCCAAGGTTA	177 bp
2	Human *ATP7B*	FP 5'- GCCAGCATTGCAGAAGGAAAG RP 5'- TGATAAGTGATGACGGCCTCT	200 bp
3	Human *Atox1*	FP 5'- GTGCTGAAGCTGTCTCTCGG RP 5'- GGCCAAGGTAGGAAACAGTCTTT	156 bp
4	Human *Cp*	FP 5'- GGGCCATCTACCCTGATAACA RP 5'- TTAAAGGTCCGATGAGTCCTGA	198 bp
5	Human *CTR1*	FP 5'- GGGGATGAGCTATATGGACTCC RP 5'- TCACCAAACCGGAAAACAGTAG	211 bp
6	Human *GAPDH*	FP5'- AGGGCTGCTTTTAACTCTGGT RP 5'- CCCCACTTGATTTTGGAGGGA	197 bp

4 DISCUSSION

Cellular degeneration in Wilson Disease is a consequence of site-specific accumulation of copper and its redox activity [19]. Knockdown of *ATP7B* mimics cellular pathological condition as seen in WD and is used here to study the transcriptional changes in copper-protein network. Accumulation of copper initiates cascade of hazardous changes including release of Acid Sphingomyelinase and ceramide in cells, ultimately leading to apoptosis [12].

Copper levels as estimated by ICP-AES showed an increase in intracellular copper under conditions of *ATP7B* knockdown, indicating copper accumulation as in WD pathology. A proportional increase was observed during copper overload when supplied in the medium. There are reports showing uncontrolled oxidative stress generated in neurons and other tissues leading to ageing and neurodegenerative diseases [22]. In our study, cell viability was reduced significantly on *ATP7B* silencing. Arnal *et al*., reported significant increase in cell death in HepG2 cells on copper overload using LDH assay [1]. The decrease in the viability was clear on addition of copper (Fig. 4).

Expression of copper importer hCTR1 was found to be slightly downregulated (not statistically significant) on *ATP7B* silencing. This suggests a compensatory response to minimize or not to enhance the influx of copper under conditions of copper overload. There are reports by Petris *et al*., showing decreased expression of surface hCTR1 on copper overload in HEK293 cells [20]. All other genes studied among the copper homeostasis proteins showed upregulation on *ATP7B* silencing. This may be an effort to clear off or nullify the effect of excess copper accumulated in the cells.

Accumulation of copper due to inability of cells to excrete it maybe a trigger for overexpression of genes of copper network proteins. These genes play significant role to buffer the oxidative stress induced. Atox1 has not only the antioxidant role but also acts as a transcription factor. Atox1 moves into the nucleus, inducing expression of cell proliferation proteins and rescues cells with defective copper-induced cell proliferation [10]. Studies conducted in neurons and yeasts show that Atox1 has a protective role during free radical stress and consequent injury [9], [16].

Copper is incorporated to apoceruloplasmin by ATP7B to form holo-ceruloplasmin [18]. The upregulation of ceruloplasmin on *ATP7B* knockdown is natural to allow effective export of excess copper being accumulated in the cells. In WD, in the absence of efficient copper excretion, apoceruloplasmin cannot be converted to functional ceruloplasmin and is excreted as such. This apo-ceruloplasmin rapidly degrades in plasma [9]. This is the reason why in spite of increased gene expression, serum ceruloplasmin activity is low in WD patients.

In the present study hCutC mRNA was also seen to be upregulated 2.78 folds on *ATP7B* silencing and 6.84 folds on further copper treatment. Till date, there are no studies on hCutC describing its physiological function in detail. In lower organisms CutC plays significant role in copper transport but its function is taken up by more sophisticated proteins in higher order animals like mammals. Study by Sara *et al* in *C. elegans* showed increased copper sensitivity and severe copper toxicity on *cutc*-1 silencing [5]. We assume that upregulation of hCutC would help to bind off excess intracellular free copper and minimize cytotoxicity. Keeping all these observations in mind, it can be stated that the tetrameric structure of hCutC binds copper (Cu^{+}) at 1:4 ratio [15] and points to an adaptive response of cells against copper toxicity.

5 CONCLUSION

There are various intracellular changes brought about by copper overload and consequent cellular copper deposition. The final outcome of copper accumulation is impairment in cellular physiology and apoptosis. The present study shows significant changes in cell viability and gene expression of copper protein network, especially hCutC due to *ATP7B* silencing. This may be the part of adaptive response by the cells to copper overload. These variations may be comparable with WD pathology.

6 ACKNOWLEDGEMENT

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