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Investigating the Effect of RNAi Mediated Silencing of Gene NPC1L1 on Cholesterol Absorption in an in Vitro Cell Based Model System

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Abstract: **The present study here is an attempt to define the role of Niemann-Pick C1-like 1 (NPC1L1), a cholesterol transporter and a key regulator in cholesterol uptake as well as intracellular cholesterol trafficking. Using an RNA interference apparoach, four different siRNAs were designed against the gene (targeting different regions), synthesized by in vitro transcription and validated in HepG2 cell based assay system. The inhibitory effects of siRNA molecules individually and in different combinations were compared in this model system to analyse the impact of siRNAs on the function of the cholesterol transporter gene studied presently. The pooling of siRNAs lead to approximately 78% decrease in the percentage of cholesterol absorbed. This cell based assay validated the silencing effects of siRNAs against NPC1L1 gene, thus establishing its role in cholesterol absorption. Conclusively, knocking down the expression of NPC1L1 can significantly reduce the uptake of cholesterol in the cell based experiment performed presently. Further experiments could be designed for better understanding of the mechanism of cholesterol absorption by NPC1L1 along with other transport proteins vis-a-vis cell lines and in vivo systems. Moreover this could also provide a lead to new line of treatment based on gene silencing against cardiovascular diseases.**

Keywords: **RNA interference, cholesterol homeostasis, NPC1L1, Insilco analysis**

1. INTRODUCTION

Whole body cholesterol homeostasis is a complex interplay of de novo biosynthesis, intestinal absorption and biliary excretion. In humans, there is a significant and positive correlation between the level of serum LDL cholesterol and the efficiency of intestinal cholesterol absorption. The proximal small intestine represents a major position for entry of cholesterol into the body pool. This cholesterol is derived primarily from the bile and diet, having a potential impact on the plasma low density lipoprotein cholesterol (LDLc) concentrations. There are three main phases involved in cholesterol absorption via intestine. The first phase occurs intraluminally and culminates in micellar solubilization of unesterified cholesterol, which facilitates its movement up to the enterocyte. The second phase involves transport of cholesterol across the membrane by Niemann-Pick C1 Like-1 (NPC1L1), whereas the third phase entails a series of steps within the enterocyte involving esterification of cholesterol and its incorporation, along with other lipids and apolipoprotein B-48, into nascent chylomicrons. Therefore, control of intestinal cholesterol absorption serves as a pharmacologically effective way of lowering serum LDL-cholesterol concentrations in the general population. Based on this hypothesis a cell based assay system targeting inhibition of NPC1L1 gene via RNAi was developed to establish its role in cholesterol absorption.

2. MATERIALS AND METHODS

RNA secondary structure prediction and siRNA selection

The *in silico* designing of siRNAs is the most crucial step for commencing RNAi experiment. Three servers were used here for the analysis: siSearch (http://sisearch.cgb.ki.se/); MWG (http://www.mwg-biotech.com/html/) and siDirect (http://design.RNAi.jp/).The targeted region of all the predicted siRNAs was checked and only those which fulfilled all the parameters as given by Uei-tei et al.2004, [**1**] high score value, GC content from 30 -50 %, minimal blast hits and accessibility to the targeted mRNA region were selected (Table 1).

Table 1: List of siRNAs selected

	mRNA Sequence/siRNA	GC (%)	Position	Score	Blast hits	HBI
1.	GGGGC)CCAAGAACU UCAGCGGAAU(CCUG G) AUU CCG CUG AAG UUC UUG Gtt CCA AGA ACU UCA GCG GAA Utt	47.3	1342-1360	6	12	**14**

2.	(CCCCU)CGACCGUCA ACUCUCUGAA(CUGCC) UUCAGAGAGUUGACG GUCGtt CGACCGUCAACUCUC UGAAtt	52.3	3001-3019	6	9	**5**
3.	(CCC)**CUCGACCGUCA ACUCUCU**G(AACUGC C CAGAGAGUUGACGGU CGtt CGACCGUCAACUCUC UGAAtt	50	2999-3017	6	12	**4**
4.	**AA|CUCACAGGAUUA CACAGAA|GC CUCACAGGAUUACA CAGAA**	**42.1**	**3308**	**6**	**14**	**11**

HBI: Hydrogen bond Index

The target accessibility was checked according to the structure of the mRNA target as predicted in the minimum free energy (MFE) structure obtained using default settings on the M fold web server version 3.2, (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html) .This also helped us to calculate hydrogen bond index (HBI).

SiRNA synthesis: In Vitro Transcription Method

The siRNAs were synthesized by in vitro transcription method. Modified methodologies for in vitro transcription as given by Donze et al, 2002 [2] & Zhang et al., 2004 [3] were used. The concentrations of siRNAs, thus prepared were calculated spectrophotometrically with absorbance measured at λ260 & λ280. The ratio λ260/λ280 for RNA samples was >= 1.8.

Cell Culturing

Human hepatocellular liver carcinoma cell line (HepG2), a liver cell line derived from a human hepatoblastoma was selected for the study as it has been reported to have a higher endogenous expression of NPC1L1. The cell line was procured from NCCS, Pune and revived.

Transfection Reaction

Reverse transfection method was used here as it produces equivalent or improved transfection efficiency over the standard pre-plated method for many of the cell types tested.

NPC1L1 gene expression quantification

The total mRNA was isolated by using RNA Isolation Kit followed by a two-step NPC1L1 gene specific RT PCR to quantify gene expression. The first step involved cDNA synthesis followed by PCR reaction with NPC1L1 specific primers Primers FP: 5'tatggtcgcccgaagcacag3' RP: 5'gtccaaacacgcaccggtag3'.The RT-PCR product was visualized on 8% PAGE (figure 1).

The Band intensities were then measured by scanning the gel with Gel Doc 1000 (Bio-Rad, Hercules, CA).Quantity One® software was used for Lane-based quantitation involving calculating the average intensity of pixels across the band width and integrating over the band height. Experiment was repeated at least three times.The inhibition percentage (%) was calculated according to the following formula: Inhibition percentage = (1-*A*sample/*A*control) ×100.

Where ***Asample*** = the intensity of NPC1L1 RT PCR product in cells transfected with siRNA and HiPerfect Transfection Reagent; ***Acontrol*** = the intensity of NPC1L1 product in cells transfected with Hiperfect Transfection Reagent alone [**3**].

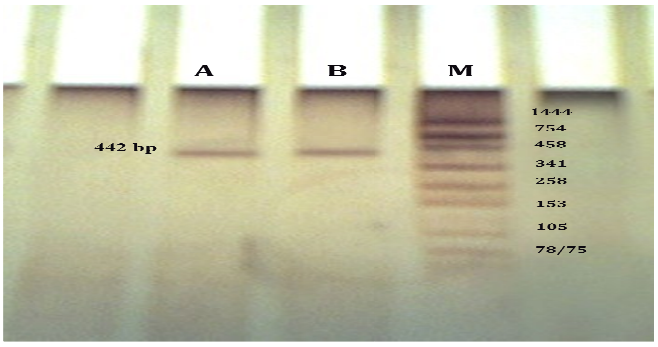

Figure 1: The 8% PAGE depicting NPC1L1 gene specific RT-PCR product

3. CHOLESTEROL ESTIMATION

HepG2 cells were cultured to 70%-80% confluency. The cells were transfected with pooled siRNA with concentrations of 5 nM, 10 nM and 20 nM. The cells were harvested after 72 hrs and washed with PBS and then incubated in 2ml media containing 50mg/ml of cholesterol for 8 hours. After the recommended time, media was removed and cells were washed twice with PBS. The cholesterol was estimated by Folch Method [**4**].

4. RESULTS AND DISCUSSION

Determination of gene knockdown of NPC1L1 using siRNAs targeting different sequence regions

Four siRNAs designed were compared individually and in different combinations to estimate their silencing potential. The cells were transfected with each siRNA at different concentrations ranging from 2.5 nM, 5 nM, 7.5 nM, 10 nM and 20nM respectively.The gel picture (figure 2) depicts that

the siRNA molecule 3 shows maximum inhibition as compared to other three siRNAs. Thus, comparing the inhibitory effect, the sequences follow the pattern from siRNA3>siRNA 2>siRNA 4> siRNA1 (figure 3).

The higher silencing efficiency of siRNA3 can well be explained on the basis of its local target structure. As is depicted in table 1 siRNA3 has lowest hydrogen bond index. Hydrogen Bond Index represents the average number of hydrogen bonds formed between nucleotides in the target region and the rest of mRNA. Moreover it is correlated inversely with the gene silencing efficiency [**5, 6**]. Among the four siRNAs selected the first and fourth have higher hydrogen bond index with 14 and 11 bonds whereas 2nd and 3rd have 5 and 4 respectively. Moreover siRNA 3 produces desired silencing effect at minimal (nanomolar) concentration of 2.5 nM & 5.0 nM as compared to other molecules to minimize unintended effects like off target effect (concentration-dependent parameter) [**7**].Thus, explaining the selection of siRNA3 as an efficient gene silencer molecule.

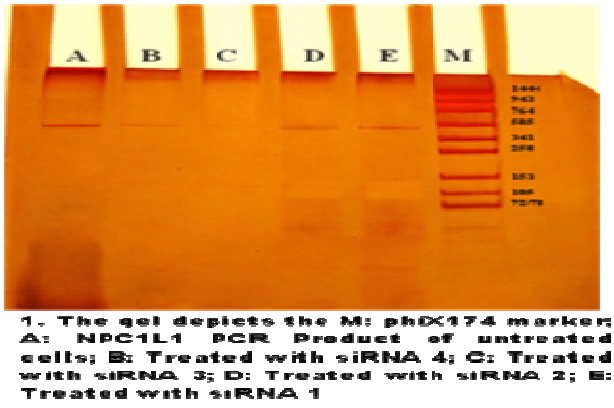

Figure 2. 12% PAGE depicting the silencing effect of all the four siRNAs

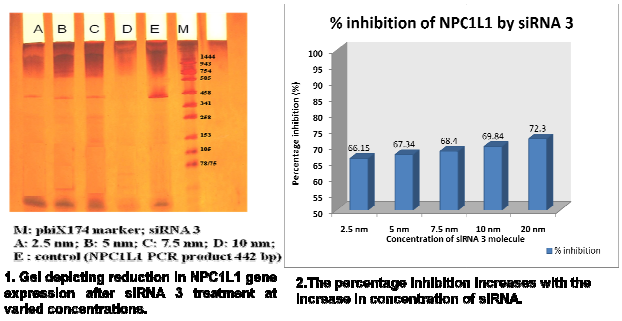

Figure 3: The silencing effect of the siRNA3 molecule

NPC1L1 gene silencing effect on cholesterol absorption in the cellular assay system

The effect of silencing of gene NPC1L1 also reduced cholesterol absorption (Figure 4). The results depicted that siRNA combination at a higher concentration of 20nM causes reduction in percentage absorption by almost 78% followed by a lower concentration of 10nM (≈ 69%).

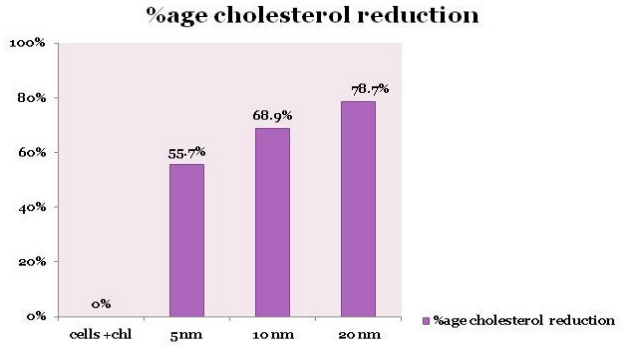

Figure 4: The graph depicts the decrease in the rate of absorption of cholesterol on transfecting the cells with siRNA (1+2+3) molecules at varied concentrations

These results indicate that NPC1L1 contributes to absorption of cholesterol and its inhibition would definitely lead to less of cholesterol absorption. This can serve as basis for determination of the complete pathway of cholesterol absorption. Moreover as these results were based on predicted MFE structures, thus they demonstrate the utility of mRNA secondary structure predictions in designing effective siRNAs.

REFERENCES

[1] Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., Ueda, R., and Saigo, K. (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res. 32:936–948.

[2] Donze, O., and Picard, D. (2002) RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. Nucleic Acids Res, 30: e46.

[3] Zhang,S-Q., Du, Q-Y.,Ying,Y., Ji, Z-Z., Wang, S-Q.(2004) Polymerase synthesis and potential interference of a small-interfering RNA targeting hPim-2. World J Gastroenterol, 10(18):2657-2660.

[4] Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, J. Biol. Chem. 226: 497–509.

[5] Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res, 31: 3406-3415.

[6] Luo, K.Q. and Chang, D.C. (2004), The gene silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. Biochem. Biophysc. Res. Commun., 318:303-310.

[7] Persengiev, S.P., Zhu, X., and Green, M.R. (2004) Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA 10(1):12-8.