Long non-coding RNA NEAT1 promotes steatosis via enhancement of estrogen receptor alpha-mediated AQP7 expression in HepG2 cells

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ABSTRACT

Hepatic steatosis is one of the most important features of the pathogenesis for non-alcoholic fatty liver disease. Fat deposition in liver cells can influence hepatic lipogenesis along with other metabolic pathways and further lead to the irreversible liver cirrhosis and injury. However, the underlying mechanism of steatosis remains largely unexplored. Our previous study revealed that AQP7 played an important role in liver steatosis. In this study, we determined that the transcriptional level of AQP7 was up-regulated by estrogen receptor alpha (ERα) upon 17β-estradiol (E2) and oleic acids treated HepG2 cells. Furthermore, we identified long non-coding RNA nuclear enriched abundant transcript 1 (NEAT1) as a potential hallmark which was down-regulated in ERα silencing HepG2 cells by RNA-Seq. Finally, we validated that the 3’ terminal nucleotides of NEAT1 were contributed for the interaction with ERα to facilitate AQP7 transcription to suppress liver steatosis. Overall, our study gave evidence that NEAT1 played an important role in the activation of ERα to regulate AQP7-mediated hepatic steatosis.

Abbreviations: NEAT1: nuclear enriched abundant transcript 1; NAFLD: non-alcoholic fatty liver disease; TG: triglycerides; MTP: microsomal triglyceride transfer protein; TNF-α: tumour necrosis factor-α; RIP: RNA immunoprecipitation

Introduction

Non-alcoholic fatty liver disease (NAFLD), characterized as the excessive hepatic lipid accumulation, is found to closely bridge with a substantial increased risk of diabetes mellitus, dyslipidemia and cardiovascular disease [1]. The current theory of “two-hit” explained the pathogenesis and progression of NAFLD [2]. Initially, the overmuch fatty acids from peripheral adipose tissue or local synthesis and the accompanying insulin resistance-mediated triglycerides (TG) are stored in liver. Then, the microsomal triglyceride transfer protein (MTP) plays a crucial role in assembly and secretion of hepatic triglyceride as very low-density lipoprotein for steatosis and fibrosis. The second hit is derived from oxidative stress and excessive cytokine production. The enhancement of the mitochondrial β oxidation, reactive oxygen species and the oxidative stress cascade, as well as cytokines such as tumour necrosis factor-α (TNF-α), adiponectin [3] modulated by the free fatty acids, are all important in the pathogenesis of NAFLD.

The regarding mechanisms of NAFLD have yet to be fully elucidated. Previous epidemiology pointed to the presence that prevalence of NAFLD is lower in premenopausal women [4], which suggested that estrogen may protect from the development of NAFLD [5,6]. Consistently, we also reported that aquaporin 7, a water-glycerol transporter, enhanced by 17β-estradiol (E2) is benefit for suppression of the glyceral kinase activity and TG synthesis in HepG2 cells [7], which indicated that E2-induced pathway may regulate the gene transcription to affect NAFLD and steatosis. Although molecular effects of subtypes of estrogen receptors (ERs) namely ERα and ERβ on the effect of obesity have been both observed [8], the E2-response function and associated metabolic pathways of ERα and ERβ usually display different even opposite in many cases [9–11]. Moreover, one study also revealed that ERβ was failed to be detected in liver cells [12]. Overall, the further molecular mechanisms in relation to estrogen and NAFLD are still obscure. In the present study, based on our previous research, we first revealed that which ERs contributed to E2 response for steatosis. Furthermore, we focused on long non-coding RNA (lncRNA) regulating the corresponding estrogen receptor and attempted to illuminate the mechanism of lncRNA and transcriptional regulation for steatosis.

Materials and methods

Cell culture

Liver carcinoma cell line HepG2 was obtained from American Type Culture Collection (ATCC, USA) and cultured in DMEM
supplemented with 10% FBS (Thermo Fisher Scientific, USA) at 37°C with a humidified atmosphere with 5% CO2. 60 μg/ml sodium olate, 50 ng/ml E2, 5 μM MPP and 10 μM PHTPP (all purchased from Sigma) were treated with HepG2 cells accordingly. The siRNA oligos of ERα and NEAT1 were obtained, verified the effect and prepared shRNA of psiRNA-h7SK vector (GenePharma, China), and transfected into HepG2 cells using lipofectamine 3000 (Thermo Fisher Scientific, USA) and screened by 500 μg/ml G418. The full length of NEAT1.1 was amplified from the cDNA of HepG2 cells and conducted mutagenesis to generate truncations, and inserted in pcDNA3.1 for overexpression. All the primers used in this study are listed in Supplementary Table 2.

**Oil red O (ORO) staining**

In brief, HepG2 cells were cultured on the sterilized black well slides and cross-linked within 1% paraformaldehyde for 10 min. Cells were stained with ORO (Sigma-Aldrich, USA) and gently counterstained by hematoxylin and visualized using a Olympus BX-51 microscope.

**Triglyceride (TG) assay**

Triglyceride GPO-POD assay kit (Andybio, USA) was used to detect the TG level in HepG2 cells using manufacturer’s standard protocol. The TG levels were quantified by GPO Trinder reaction. Each group was repeated three individual experiments.

**Western blot**

After adding RIPA buffer to harvest cells, the total protein concentration of the different cells was determined using the bicinchoninic acid (BCA) assay. Aliquots of protein resolved by SDS-PAGE were immunoblotted with the appropriate primary antibodies (CST, USA) at 4°C overnight and then incubated with horseradish peroxidase-conjugated secondary antibody at 1:10,000 dilution at room temperature for 1 h. GAPDH was used as a protein loading control. Images of Western blots were acquired using Image Lab (Bio-Rad) and analyzed using Image J.

**Illumina RNA sequencing**

Total RNA of HepG2 cells was extracted using Trizol (Thermo Fisher Scientific, USA) according to the manufacturer’s protocol. Five micrograms of RNA in each group were used for library preparation using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer’s recommendations and were sequenced on an Illumina Hiseq platform. The raw data were trimmed adaptors and filtered out low quality reads using Trimmomatic [13] and checked the quality of clean reads using Fastqc [14]. Next, clean reads were aligned to the latest human genome assembly hg38 using Hisat2 [15]. The transcripts were assembled and estimated the expression levels by FPKM values using the StringTie algorithm with default parameters [16]. Differential mRNA and lncRNA expression among the groups were evaluated using a R package Ballgown [17] and computed the significance of differences by the Benjamini & Hochberg (BH) p values adjustment method. Gene annotation is described using Ensembl genome browser database (http://www.ensembl.org/index.html). The R package ClusterProfiler was used to annotate the differential genes with gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways [18].

**Chromatin immunoprecipitation (ChIP) assay**

Briefly, HepG2 cells were breaking by a Dounce Homogenizer within lysis buffer. Five percent whole cell lysis were harvested as input after genomic DNA was sonicated into 200–500 bp. One microgram ChIP grade antibody of ERα was incubated with the rest of lysis overnight, followed by further 2-h protein-A beads incubation at 4°C for targeted DNA pull down. Primers designed to detect the potential target of ERα at the regions of AQP7 promoter are listed in Supplementary Table 2.

**RNA pull down assay**

One microgram biotinylated NEAT1.1 with full length or truncations were activated to form the RNA secondary structure by the structure buffer (10 mM Tris pH 7.4, 100 mM KCl, 10 mM MgCl2) and denatured at 95°C for 1 min, then cooled in room temperature for 30 min to stabilize RNA, and resuspended by 50 μl T1 streptavidin magnetic beads (Thermo Fisher Scientific, USA) to incubated overnight at 4°C. The next day, RNA and beads mixture were centrifuged at 2500 rpm for 1 min and washed by wash buffer (25 mM Tris pH 7.4, 150 mM KCl, 5 mM EDTA, 0.5 mM DTT, 0.2% NP40, 100 U/ml RNAase inhibitor, 1× Protease inhibitors cocktail) three times, followed by the incubation with 1 ml cell lysate of 1×10^6 HepG2 at 37°C 2 h. RNA-beads-proteins mixture were centrifuged at 2500 rpm, washed by wash buffer, added loading buffer for denaturation at 95°C 10 min and run SDS-PAGE gel to detect ERα.

**RNA immunoprecipitation (RIP)**

1×10^7 HepG2 cells were washed and resuspended within the nuclear isolation buffer (40 mM Tris pH 7.5, 1.28 M sucrose, 20 mM MgCl2, 4% Triton X-100) and conducted cell lysis on ice for at least 30 min with occasional vortex. The pellet nuclei were centrifuged by 13,000 rpm for 15 min, resuspended by wash buffer (same with RNA pull down) and broken the chromatin through sonication by high power, 10 s on, 30 s off for 30 cycles. After that, 90% nuclei were incubated with 1 μg ERα primary antibody overnight and 40 μl Protein A/G beads 2 h by gentle rotation at 4°C while the rest of 10% were harvested as input. The pellet beads were centrifuged by 3000 rpm 3 min, washed three times. Both the input and pellet beads were purified using RNAiso plus and conducted reverse transcription which was described in RNA extraction.
**Realtime PCR**

Real-time quantitative PCR method was employed to detect the expression of target genes. Total RNA was extracted using the Trizol extraction kit (Invitrogen). First-Strand Synthesis System for reverse transcription (Thermo Fisher Scientific, USA) was used to synthesize cDNA from 1 μg total RNA according to the oligo (dT) version of the protocol. Realtime PCR was performed using Fast SYBR Green Master Mix (Roche, USA). The following cycle parameters were used in this study: 94°C 20 s, and 60°C 30 s, 72°C 30 s for a total of 40 cycles. All primer sequences used in this study are listed in Supplementary Table 2.

Ct value was analyzed to calculate enrichment using delta-delta methods. The relative mRNA levels of certain genes were normalized by Gapdh.

**Statistical analysis**

All statistical analyses were performed in SPSS 20. Student’s t-test was used to analyze the difference among the groups. p value less than .05 was considered as statistical significance.

**Accession number**

RNA sequencing data were deposited to ArrayExpress assigned with the accession number E-MTAB-7318.

**Results**

**ERα contribute to response upon E2 to regulate the transcription of AQP7 and affect steatosis in HepG2 cells**

Initially, the adipohepatic HepG2 cell model in vitro was prepared by 60 μg/ml sodium oleate treatment for 48 h. To investigate the regulatory manner of ERs in steatosis, HepG2 cells were further treated by the additional E2, ERα inhibitor (MPP) and ERβ inhibitor (PHTPP) respectively and detected the status of lipid accumulation by ORO staining and TG assay. We observed that the lipid highly enriched in oleic acid treated HepG2 cells but compromised upon E2 treatment while MPP rather than PHTPP treatment could obviously block the lipid suppressive effect of E2 (Figure 1(A)). Likewise, the changed trend of TG was consistent with ORO staining (Figure 1(B)). Meanwhile, the protein levels of AQP7, ERs, acetyl-CoA (ACC), fatty acid synthase (FAS) and glycerol-3-phosphate acyltransferase (GPAT) were also detected to confirm the effect of estrogen and ERs on steatosis (Figure 1(C)). Consistent with our previous study, the expression of AQP7, ACC, FAS and GPAT all compromised after E2 treatment. Interestingly, MPP but not PHTPP could robustly block the E2-mediated effect, which suggested that ERα might play a crucial role in responding to estrogen for anti-steatosis. Furthermore, ChIP assay was conducted to determine the substantial binding status of ERα on three sites of AQP7 promoter. We observed that the enrichment of ERα on −350 to −260 of AQP7 promoter sensitively responded to sodium oleate and E2 (Figure 1(D)). Taken together, the results above indicated that ERα played a crucial role in bridging between E2 and transcriptional regulation of downstream targets such as AQP7 for steatosis alleviation.

**NEAT1 enhance the effect of ERα on steatosis in HepG2**

Besides the transcriptional regulation of AQP7 by ERα, we further investigated the regulatory network of ERα on whole transcriptome in steatosis suppression. We generated the HepG2 cells with oleic acid, E2 and stable ERα silencing treatment and conducted RNA-seq. We observed that plenty of...
genes change their transcriptional level upon E2 treatment and ERα knockdown (Figure 2(A)). E2 treatment caused 654 mRNA (364 up-regulated and 291 down-regulated) and 355 lncRNA (183 up-regulated and 172 down-regulated) compared to non-E2 treated HepG2 cells while ERα knockdown led to 652 mRNA (347 up-regulated and 305 down-regulated) and 316 lncRNA (162 up-regulated and 154 down-regulated) compared to E2 treated HepG2 cells with normal ERα (Figure 2(B), Supplementary Table 1). And, the lipid-associated functions and pathways involved in differentially expressed genes including Arachidonic acid metabolism, steroid hormone biosynthesis and fatty acid biosynthesis (Figure 2(C,D)). Here, we noticed that the lncRNA NEAT1 was highly expressed upon E2 treatment (Fold change = 3.71, p = 3.72E-6) but significantly reduced upon ERα knockdown (FC = 0.27, p = 3.95 E-9). Due to two splicing isoforms of NEAT1 (NEAT1.1 3.7 kb and NEAT1.2 22.7 kb), which isoforms actually regulated by ERα was needed to be clarified. Thus, qPCR was conducted to verify that NEAT1.1, not NEAT1.2 was substantially downregulated upon ERα silencing or inactivation. Interestingly, mRNA level of AQP7 was observed a slighted reduction upon NEAT1.1 knockdown while an obvious promotion when NEAT1.1 was overexpressed in HepG2 cells, which suggested that NEAT1.1 exerting a dispensable manner could enhance the effect of ERα on AQP7 transcription (Figure 2(E)). Taken together, our results determined that NEAT1.1 facilitated ERα for the transcriptional regulation of targeted genes such as AQP7 through a dispensable manner in steatosis suppression.

The molecular interaction between NEAT1 and ERα in HepG2 cells

Now that we found the relationship between NEAT1.1 and ERα in HepG2 cells, the essential regulatory mode of the lncRNA and transcription factor were further explored. Here, we performed RIP assay to pull down the interacted RNAs with ERα and generated the biotin labelled NEAT1.1 to pull down the interacted proteins of NEAT1.1. Our results showed a substantial interaction between NEAT1.1 and ERα. We noticed that this interaction was apparently strong in E2 treated cells compared to the HepG2 cells with ERα inhibition (Figure 3(A,B)). And then we prepared six different region truncations of NEAT1.1 and conducted RIP assay again to figure out the key domain of NEAT1.1 contributing to the interaction with ERα in E2 treated HepG2 cells (Figure 3(C)). We determined that the 3' terminal (3278–3733) of NEAT1.1 played a crucial role in ERα interaction. Finally, overexpression this truncated NEAT1.1 failed to observe the significant promotion of AQP7 expression compared to NEAT1.1 wild type and other truncations (Figure 3(D)).

In conclusion, our data demonstrated that lncRNA NEAT1.1 could interact with ERα to facilitate it to regulate the genes transcription for preventing from steatosis in HepG2 cells.

Discussion

ERα and ERβ displayed a differential tissue-specific distribution and played the different ligand-effect roles in transcription regulation upon the estrogen response elements although they were highly homologous in protein structure. Our results in this study determined that ERα was the main cause to contribute to the E2-mediated lipid metabolism and steatosis inhibition in HepG2 cells. And ERβ, unlike the statement of undetectable in rat liver tissues in the previous study [12], was detectable but independent with E2 effect in this case of HepG2 cells. We also found that ERβ was substantially expressed but not enriched on AQP7 promoters in HepG2 cells.
cells by ChIP assay (Figure 1). Thus, we speculated that ERβ might be differentially expressed in normal liver and liver carcinoma cells.

LncRNA is a large and diverse group of RNA transcripts longer than 200 bases and without evident protein-coding capacity [19]. LncRNAs can be transcribed from antisense, intronic, intergenic, divergent of genes and numerously from enhancers and other regulatory elements in the genome [20]. Most lncRNAs are nuclear localization and are likely to exert their functions via chromatin modifiers recruitment or scaffolds within ribonucleoprotein complexes to repress or activate gene transcription [21]. Recently, the number of functional lncRNAs identified in a variety of diseases including cancer, neuron disease, cardiac disorder, obesity and endocrine physiology is increasing. NEAT1 was demonstrated to play an oncogenic role in breast and prostate cancers [22,23]. And, NEAT1 was one of the non-coding transcriptome signature driven by ERα and was required for the formation of FOXN3-SIN3A repressor complex to facilitate epithelial-to-mesenchymal transition process for tumour progression. Furthermore, NEAT1 was also determined to be involved in adipogenesis including lipolysis, lipid uptake and low-density lipoprotein oxidation [24–26]. In addition to this, LINC01116 [27], MIAT [28] and ElncRNA1 [29] displayed an obviously differential expression upon E2 treatment and ERα silencing of HepG2 cells. Here, we confirmed the connection between NEAT1 and ERα in regulatory network of steatosis.

We observed that NEAT1.1 transcription could be impacted by ERα silencing or inhibition, which was consistent with previous studies [22,23]. However, our pull down assay reinforced that NEAT1.1 could interact with ERα to enhance its effect, which implied that NEAT1 might be a manner of ERα self-regulation for modulating on lipid metabolism and steatosis. And, we further determined the crucial domain of NEAT1.1 for binding with ERα, which regulatory mode was similar with the other two well-studied lncRNAs that HOTAIR and MALAT1 could interact with ERs and recruit on the promoter to facilitate the transcription of estrogen-target genes [30]. Nevertheless, due to the obscure secondary structure of NEAT1.1, the underlying molecular mechanism of binding domain of NEAT1 on ERα is needed to be illustrated in further study.

Overall, NEAT1.1 is determined as an important lncRNA to co-regulated with ERα for the steatosis suppression upon E2 treatment and can be considered as a potential therapeutic target for obesity prevention.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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