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Long noncoding RNA lncARSR promotes hepatic cholesterol biosynthesis *via* modulating Akt/SREBP-2/HMGCR pathway

Jiabin Huang*,[1], Shangjun Chen[1], Dongliang Cai, Deqiang Bian, Fengling Wang

Department of Geratology, First Affiliated Hospital of Jiamusi University, 348 Dexiang Street, Jiamusi, Heilongjiang Province 154002, China

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ABSTRACT

Aims: Disruption of cholesterol homeostasis has been identified as a major factor in the pathogenesis of atherosclerosis, myocardial infarction, and strokes. Long noncoding RNAs (lncRNAs) have emerged as critical players in cellular cholesterol metabolism, but their functions are still largely unknown.
Materials and methods: C57BL6/j mice were fed with high cholesterol diet (containing 4% cholesterol) or chow diet. Adenoviruses-lncARSR and lncARSR shRNA were used to overexpress or knockdown lncARSR expression.
Key findings: The expression of lncARSR were increased both in patients with hypercholesterolemia and mice with high cholesterol diet feeding. Overexpression of lncARSR in mice resulted in elevated lipid levels in both serum and liver fragments. However, knockdown of lncARSR in mice fed with high cholesterol diet showed decreased lipid levels in serum and liver fragments compared with control mice. Furthermore, we found that the expression of HMG-CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol synthesis was increased with lncARSR overexpression, which was accompanied with the increase of hepatic *de novo* cholesterol synthesis rate. Mechanistically, we found that lncARSR increased the expression of mature SREBP-2, which is a primary transcription factor of HMGCR. And lncARSR activated the PI3K/Akt pathway. When PI3K/Akt pathway was blocked by LY294002, the inhibitor of PI3K, the effect of lncARSR on SREBP-2 and HMGCR disappeared.
Significance: Our data indicated upregulated lncARSR promotes hepatic cholesterol biosynthesis *via* modulating Akt/SREBP-2/HMGCR pathway, and implied that lncARSR may serve as a therapeutic target for cholesterol disorder.

1. Introduction

Cholesterol plays important roles in animal and cellular membranous structures and serves as a precursor for steroid hormones and bile acids [1,2]. Disruption of cholesterol homeostasis has been identified as a major factor in the pathogenesis of atherosclerosis, myocardial infarction, and strokes [3–5]. Thus, maintaining cholesterol homeostasis is essential to human health.

Liver is the most important organ for cholesterol metabolism. Cholesterol homeostasis is maintained by a complex network involving cholesterol uptake, synthesis, transport, and excretion [6,7]. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR) is an endoplasmic reticulum-bound, cytoplasmic protein [8]. HMGCR catalyzes the conversion of HMG-CoA to mevalonate (MVA), which is the rate-limiting step in cholesterol biosynthesis [9]. The proper function of HMGCR is essential under both normal physiologic conditions, and its dysregulation has been reported to be associated with many diseases such as hypercholesterolemia, coronary artery disease, and stroke. Some researchers have even argued that HMGCR is a potential candidate 'metabolic oncogene' [10,11].

Long non-coding RNAs (lncRNAs) are a kind of non-coding RNAs (ncRNAs) with > 200 nucleotides in length [12]. Although lncRNAs have limited protein coding potential, it has been found that lncRNAs have critical functions in various pathophysiological processes and their dysregulation contributes to the pathogenesis of many diseases [13]. Several lncRNAs have been found to regulate cholesterol metabolism. Liver-specific triglyceride regulator (LncLSTR) was highly expressed in the liver and regulated the expression of sterol 12-alpha-hydroxylase (CYP8B1), a critical enzyme involved in bile acid synthesis [14]. LncRNA-RNCR3 was expressed in atherosclerotic plaques, where its expression is enhanced by ox-LDL *in vitro* [15]. Lnc-HC interacted with hnRNPA2B1 forming a RNA-protein complex, which could bind to target mRNAs, including Cyp7a1 and Abca1 [6].

LncARSR is a recently identified lncRNA with 591 nucleotides in length [16]. It has been found to play important role in cancer. LncARSR promoted renal tumor initiating cells expansion *via* physically

* Corresponding author.
E-mail address: dr_hang123@126.com (J. Huang).
[1] These authors contributed equally to this work.

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interacting with Yes-associated protein (YAP) and facilitating YAP nuclear translocation [17]. And it also could promote doxorubicin resistance in hepatocellular carcinoma *via* modulating Phosphatase and tensin homolog (PTEN)-PI3k/Akt pathway [18]. However, if lncASAR participates in the regulation of cholesterol metabolism is still unknown.

In this study, we demonstrated a novel role of lncARSR in the regulation of hepatic cholesterol biosynthesis. We measured the expression of lncARSR in hypercholesterolemia patients and mice with high cholesterol diet feeding. We also established lncARSR overexpression and knockdown mice model to observe the effect of lncARSR on cholesterol metabolism. Furthermore, we explored the underlying molecular mechanisms responsible for the effects of lncARSR on hepatic cholesterol biosynthesis.

2. Materials and methods

2.1. Human subjects

The protocol of this study was approved by the ethics committee of First Affiliated Hospital of Jiamusi University. Thirty patients with hypercholesterolemia and twenty healthy controls were enrolled in this study. Each participant completed a standardized questionnaire that included demographic characteristics, life factors, family and personal medical history, general health status and current medications. Written informed consent was obtained from each participant after explaining the purposes and procedures of the study prior to the questionnaire and blood draw. All experiments were performed in accordance with relevant guidelines and regulations. We excluded participants based on the following criteria: 1) missing vital data, such as age, sex, or serum lipid profiles; 2) presence of complications or conditions such as pregnancy, lactation, acute cardiovascular or cerebrovascular disease, malignant tumors, or severe renal dysfunctions; 3) use of medications that affect lipid metabolism over the preceding three months, including glucocorticoids, statins, fibrates, thyroid hormones, anti-thyroid drugs, nonsteroidal anti-inflammatory drugs, or thiazide diuretics. Blood samples were collected from all subjects following overnight fasting. Serum was isolated by centrifugation at 2000*g* for 15 min and stored at −80 °C.

2.2. Mice model

The protocol was approved by Research Ethics Committee of First Affiliated Hospital of Jiamusi University. C57Bl/6 male mice (6–8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., China. The mice were housed at 23 °C in a 12-hour light-dark cycle (7 A.M. on, 7 P.M. off) and humidity-controlled (60%) environment. For high-cholesterol diet feeding experiments, mice were fed a diet containing 4% cholesterol (cholesterol 4%, protein 20%, fat 6%, and carbohydrates 58%) or chow diet for 20 weeks (n = 10/groups). The mice were sacrificed after fasting for approximately 8 h. Blood samples from the animals were obtained and serum was isolated by centrifugation at 2000*g* for 15 min. In addition, liver fragments from all animals were collected. A partial fragment sample was fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline for histological analysis. The remaining fragment was immediately stored in liquid nitrogen until further analysis.

For virus transfection. C57BL/6J mice (6–8 weeks, male) were adapted to their environment for 1 week before injection with viruses. Adenoviruses (5.0×10^8 pfu) lncARSR, lncARSR shRNA, or control GFP were dissolved in sterile PBS and injected through the caudal vein to mice (n = 10/groups). The mice were fed with chow diet or high cholesterol diet for 20 weeks post adenovirus transduction for 4 weeks. The mice were anesthetized with sodium pentobarbital after fasting for approximately 8 h.

2.3. Cell culture

The human HepG2 hepatocellular carcinoma cell line was obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. These cells were grown in DMEM medium (Gibco/Invitrogen Life Technologies) supplemented with 10% FBS at 37 °C with 5% CO_2 humidity.

2.4. Intracellular and hepatic TC determination

Cholesterol was extracted from cells and fragments using a Cholesterol Assay Kit (Solarbio Inc. Shanghai, China) according to the manufacturer's instruction, and the cholesterol content was normalized by the corresponding protein content. In brief, cells or liver fragments were extracted with 200 μl of chloroform:isopropanol:NP-40 (7:11:0.1) in a microhomogenizer. After centrifugation at 15,000*g* for 10 min at room temperature, the organic phase was transferred and evaporated to dryness at 50 °C. The dried lipid was re-suspended in 200 μl of Cholesterol Assay Buffer. and then the cholesterol content was measured.

2.5. RNA isolation and real-time PCR

Total RNA from cells and mice liver fragments were isolated using Trizol reagent (Takara, Tokyo, Japan), following manufacturer's instructions. RT reaction (20 μl) was carried out using reverse transcriptase kits (RT) (Takara). SYBR green (Takara) was used to detect the amplification of cDNA in a total volume of 20 μl with the absolute quantitative, ΔCt method [19]. The PCR primers were shown in Table 1. β-Actin was used as an internal control to normalize the data.

2.6. Total, nuclear and cytoplasmic protein extraction

Liver fragment samples were ground into powder in liquid nitrogen. The fragment powder and cells were lysed in RIPA buffer to obtain total protein according to the manufacturer's protocol. The nuclear and cytoplasmic proteins were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). The protein concentration was measured using the BCA method.

2.7. Western blotting

Protein from nuclear extracts (40–60 μg), cytoplasmic extracts (80–100 μg) or total protein (100 μg) were mixed with 2XSDS loading buffer and subjected to SDS/PAGE on a 10% gel. Proteins were electrotransferred on to polyvinylidine fluoride membrane (Millipore, Billerica, MA, USA) and the blots were probed with the following antibodies at the dilution as indicated: SREBP-2 (1:500; Cayman), p-Akt (1:1000; CST), Akt (1:1000, CST), p-CREB (1:1000, CST), CREB (1:1000, CST), HMGCR (1:1000; Sigma), LAMB1 (1:3500, Proteintech) and β-actin (1:3500, Proteintech). The appropriate secondary

Table 1
The primers used for qPCR in this study.

Name	Forward	Reverse
lncARSR	TTTGAAATGCTCTTTGAGGGAT	TGCAGGTTGTCTGAAGTTGGA
Srebp-2	CCAAAGAAGGAGAGAGGCGG	CGCCAGACTTGTGCATCTTG
Hmgcr	CTTGTGGAATGCCTTGTGATTG	AGCCGAAGCAGCACATGAT
Hmgcs	TTTGATGCAGCTGTTTGAGG	CCACCTGTAGGTCTGGCATT
Mvd	ATGGCCTCAGAAAAGCCTCAG	TGGTCGTTTTTAGCTGGTCCT
Insig1	TGCAGATCCAGCGGAATGT	CCAGGCGGAGGAGAAGATG
Sqs	CCAACTCAATGGGTCTGTTCCT	TGGCTTAGCAAAGTCTTCCAACT
Cyp7a1	GAAGCAATGAAAGCAGCCTC	GTAAATGGCATTCCCTCCAG
Ldlr	GGTACTGGCACAACAACTTGGG	GCCAATCGACTCACGGGTTCAG
Abca1	GGACTTGCCTTGTTCCGAGAG	GCTGCCACATAACTGATAGCGA
actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT