Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

lncRNA HULC promotes the growth of hepatocellular carcinoma cells via stabilizing COX-2 protein

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ARTICLE INFO

Article history:
Received 24 May 2017
Accepted 17 June 2017
Available online xxx

Keywords:
HULC
HCC
COX-2
USP22
Tumor growth

ABSTRACT

Highly upregulated in liver cancer (HULC), a lncRNA overexpressed in hepatocellular carcinoma (HCC), has been demonstrated to be involved in the carcinogenesis and progression of HCC. However, the mechanisms of HULC promoting the abnormal growth of HCC cells are still not well elucidated. In the present study, we for the first time demonstrated that HULC promoted the growth of HCC cells through elevating COX-2 protein. Moreover, the study of the corresponding mechanism by which HULC upregulated COX-2 showed that HULC enhanced the level of ubiquitin-specific peptidase 22 (USP22), which decreased ubiquitin-mediated degradation of COX-2 protein by removing the conjugated polyubiquitin chains from COX-2 and finally stabilized COX2 protein. In addition, knockdown of USP22 or COX-2 attenuated HULC-mediated abnormal growth of HCC cells. In conclusion, our results demonstrated that "USP22/COX-2" axis played an important role in HULC promoting growth of HCC cells. The identification of this novel pathway may pave a road for developing new potential *anti*-HCC strategies.

1. Introduction

HCC is the second leading cause of cancer-related death in worldwide [1]. Although numerous advances in the understanding of the molecular basis of HCC, the prognosis of HCC is poor because of limited effective treatment options [2]. During the development of HCC, it is also accompanied with multiple molecular changes [3]. Therefore, it is so urgent to identify the key molecular changes and clarify the corresponding signaling pathway.

Recently, mounting studies have proved that long non-coding RNAs (lnc RNAs) play an important role in the carcinogenesis and development of HCC [4,5]. Highly upregulated in liver cancer (HULC) is the first identified lncRNA that is strongly overexpressed in human HCC samples [6]. Recently, HULC has been reported to be correlated with other cancers such as nasopharyngeal carcinoma [7], gastric cancer [8] and colorectal carcinoma [9]. Moreover, high expression levels of HULC has been demonstrated to be an independent prognostic biomarker for poor overall survival (OS) and metastasis in general human tumors [10]. As an oncogenic lncRNA, HULC could be upregulated by cAMP responsive element binding protein (CREB) in HCC [11]. Hepatitis B virus X (HBX) protein has been proved to elevate the level of HULC, leading to the suppression of p18 protein, which contributes to the proliferation of HCC cells [12]. Collectively, previous studies have demonstrated that HULC is involved in the proliferation of HCC cells. However, the related mechanism by which HULC promotes the proliferation of HCC cells is still not that clear.

Cyclooxygenase (COX)-2, also called prostaglandin-endoperoxide synthase 2 (PTGS2), could be induced by a large number of factors such as cytokines, growth factors, hormones and oncogenes [13,14]. As a key enzyme of arachidonic acid metabolism, COX-2 can catalyze arachidonic acid into prostaglandins and mediate many physiological and pathological processes. Previous studies have demonstrated that COX-2 is up-regulated in many different kinds of human caners such as colorectal [15], prostate [16], breast [17], gastric [18], lung [19] and liver [20]. It has been reported that the level of COX-2 is associated with decreased overall and disease-free survival and a worse prognosis [21]. Inhibition of COX-2 shows significant anti-tumor effects while overexpression of COX-2 lead to spontaneous HCC formation [22]. Previous studies have demonstrated that COX-2 could be regulated by transcriptional factors [23], miRNAs [24] and several lncRNAs [25,26]. However, it is still unknown whether COX-2 could be

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http://dx.doi.org/10.1016/j.bbrc.2017.06.103
0006-291X/

regulated by HULC.

In the present study, we for the first time demonstrated that HULC promoted the growth of HCC cells through elevating COX-2. Moreover, mechanistic study showed that HULC increased the level of ubiquitin-specific peptidase 22 (USP22), which decreased the ubiquitin-mediated degradation of COX-2 protein by removing the conjugated polyubiquitin chains from COX-2, and finally led to the upregulation of COX-2 protein. Collectively, our results demonstrated that "USP22/COX-2" axis plays an important role in HULC-triggered growth of HCC cells.

2. Materials and methods

2.1. Reagents

Cycloheximide (CHX) was purchased from Xiya Reagent (Chengdu, china). Lipofectamin 2000 and Optim-medium were purchased from Invitrogen (Carlsbad, CA, USA). RNA OUT reagent were purchased from TIANDZ (Beijing, China). SYBR Green Mix and reverse transcript kit were purchased from TAKARA corporation (Kyoto, Japan). RIPA lysis buffer and BCA protein assay kit were purchased from Beyotime Biotechnology (Shanghai, China). Cocktail were purchase from Roche (Basel, Switzerland). Cell Counting Kit-8 (CCK-8) was from Dojindo (Shanghai, China). SiRNAs for USP22, COX-2 and control siRNA were synthesized by GenePharma (Shanghai, China).

2.2. Cell culture

Human HCC cell lines including HepG2 and Hep3B were originally tested and authenticated by American Type Culture Collection (Manassas, VA, USA) and passaged less than 6 months in the lab. All the cells were cultured in DMEM medium containing 10% fetal bovine serum at 37 °C in 5% CO_2 incubator.

2.3. The construction of plasmid

The vector pcDNA-3.1 (Invitrogen, Carlsbad, CA, USA) was digested by *Eco*R I and *Xho* I and then the large fragment was pure and collected. Thereafter the DNA fragment encoding HULC chemically synthesized by Sangon Biotech Corporation (Shanghai, China) was inserted into the large fragment, and the resulting plasmid was named pcDNA-HULC.

2.4. Quantitative RT-PCR

Total RNA was extracted using RNA OUT reagent according to the manufacturer's instructions. After that, the first-strand cDNA was synthesized using reverse transcriptase M-MLV. Quantitative RT-PCR (qRT-PCR) was performed by One-Step RT-PCR kit (ComWin, Beijing) and SYBR Green Mix respectively. Primers of qRT-PCR are listed as follows:

Gene	Forward primer	Reversed primer
HULC	5′-ATCTGCAAGCCAGGAAGAGTC-3′	5′-CTTGCTTGATGCTTTGGTCTGT-3′
COX-2	5′-GTTCCACCCGCAGTACAGAA-3′	5′-AGGGCTTCAGCATAAAGCGT-3′
USP22	5′-GGCGGAAGATCACCACGTAT-3′	5′-TTGTTGAGACTGTCCGTGGG-3′
β-actin	5′-GTGAAGGTGACAGCAGTCGGTT-3′	5′-GAAGTGGGGTGGTTTTAGGA-3′

2.5. Transient transfection

HepG2 and Hep3B cells were respectively cultivated in 6-well plates overnight. siRNAs or plasmids were transfected into HepG2 and Hep3B cells by lipofectamin 2000 according to the manufacturer's recommendations. Six hours later, the cells were treated with 10% FBS DMEM medium. The sequence of siRNAs for targeted genes are listed as follows.

Target	sequence
HULC	5′-AACCUCCAGAACUGUGAUCCA-3′
COX-2	5′-AACUGCUCAACACCGGAAU-3′
USP22	5′-CCACUGCAACUGCAUCAUA-3′
NC	5′-UUCUCCGAACGUGUCACGU-3′

2.6. Western blot (IB: Immunoblot)

Cells were lysed and then the protein concentrations were detected by BCA kit following the protocol. Proteins were transferred into polyvinylidene difluoride (PVDF) membrane. Primary antibody used to detect COX-2 (#12282) was purchased form Cell Signaling Technology Corporation (Boston, MA, USA). USP22 antibody (AP2148B) was from Abgent company (San Diego, CA, USA), the antibody against ubiquitin (sc-271289) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Primary antibodies against GAPDH (AG019) and Tubulin (AT819) were purchased from Beyotime corporation (Shanghai, China). HRP labeled goat anti-rabbit and anti-mouth immunoglobulin G are secondary antibody.

2.7. Immunoprecipitation (IP)

After grown to an 80% confluence in 10 cm-dishes, the cells were given different treatments. Then the protein of the HCC cells was extracted following the manufacturer's instructions (Beyotime, Shanghai, China). Thereafter, the primary antibodies were added to the lysate in the ratio of 2 μg per 1 mg total protein. After incubation at 4 °C overnight, 40 μl protein A/G agarose beads (Santa Cruz, CA, USA) was separately added to each sample, and incubated at room temperature for 2 h. Then the mixtures were washed using ice-cold PBS for 5 times and denatured with 1 × SDS-PAGE loading buffer followed by Western blot.

2.8. Cell proliferation assay

HepG2 cells were cultivated in 96-well plates overnight, then transfected with indicated plasmids or siRNA for 48 h. After that, OD value of 450 nm was measured by microplate reader using CCK-8 reagent according to the instructions. Each experiment was done in triplicate.

Please cite this article in press as: H. Xiong, et al., lncRNA HULC promotes the growth of hepatocellular carcinoma cells via stabilizing COX-2 protein, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.06.103