Int J Clin Exp Med 2016;9(7):13900-13908
www.ijcem.com /ISSN:1940-5901/IJCEM0021452

Original Article

Paeonol inhibits the growth of gastric cancer cells via suppressing HULC expression

Shimin Yang[1], Xu Wang[2], Gang Zhong[1]

[1]*Department of Gastrointestinal Surgery, Tianjin Nankai Hospital, Tianjin 300100, China;* [2]*Medical Department of Oncology, Tianjin Medical University Cancer Institute Hospital, Tianjin 300060, China*

Received December 8, 2015; Accepted March 19, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: Amis: The long noncoding RNAs (lncRNAs) are closely associated with tumor proliferation, differentiation, apoptosis and metastasis. The traditional Chinese medicine, paeonol, has been reported to have an obvious anti-cancer effect in many types of tumors. However, it was unknown whether paenonl could regulate lncRNA expression to inhibit tumorigenesis. Methods: SGC-7901 cells were treated with paeonol, and the lncRNA HULC expression of which was measured by qRT-PCR assays. The effect of decreased HULC expression on proliferation, cell cycle, apoptosis, adhension and invasion of SGC-7901 transfected with si-HULC was detected via MTT, flow cytometric analysis, transwell, qRT-PCR, western-blot and so on. Results: Paeonol could significantly inhibit the expression of HULC in SGC-7901 cells. Down-regulated HULC obviously suppressed the cell growth and invasion, and promoted cell apoptosis and adhesion. Conclusions: These results strongly suggested that the anti-cancer mechanism of paeonol might be down-regulating the expression level of HULC in cancer cells.

Keywords: lncRNA, paeonol, HULC, gastric cancer, tumorigenesis

Introduction

The long noncoding RNAs (lncRNAs) are non-protein-coding transcripts that are ≥ 200 nucleotides in length. LncRNAs participate in many physiological processes by modulating gene expression at the epigenetic, transcriptional and posttranscriptional levels; dysregulated lncRNA expression plays a crucial role in human carcinogenesis [1]. Several lncRNAs have been identified as being linked to human disease especially cancer and exerting specific functions [2, 3]. Increasing evidence indicated that abnormal expression of lncRNAs is closely related to tumor proliferation, differentiation, apoptosis and metastasis. Highly upregulated in stomach cancer (HULC), first identified as a novel mRNA-like lncRNA up-regulated dramatically in HCC by Panzitt et al., is ~1.6 k nucleotides long containing two exons but not translated [4, 5]. Many studies have reported that HULC was closely related to tumorigenesis.

Paeonol is a traditional Chinese medicine characterized by its broad pharmacological activity, high efficiency and low toxicity. It has been demonstrated that paeonol can inhibit the proliferation of a variety of tumor cell lines such as hepatoma and lung cancer; its anti-tumor mechanisms have become a hot research topic [6-8]. Currently, the relationship between the mechanisms of Paeonol action and lncRNAs has not been reported. The preliminary microarray results of this project team showed that the HULC expression in human gastric cancer SGC-7901 cells was up-regulated after treatment with paeonol. But the mechanism is not clear. Accordingly, in this study, we are interested in the roles of lncRNA HULC in the development of HCC. Our results show that HULC contributes to the proliferation, apoptosis, cell cycle, adhesion, and invasion of human hepatoma SGC-7901 cells. Our finding gives a new insight into the anti-tumor mechanisms of paeonol and will provide the theoretical basis for clinical treatment of hepatoma.

Materials and methods

Cell lines and cell culture

Human hepatoma SGC-7901 cell line was purchased from Cell Bank of Shanghai Institutes

Table 1. List of primers and their applications

Primer	Sequences (5'-3')	Application
HULC-assay-F	ATCTGAAGCCAGGAAGAGTC	qRT-PCR
HULC-assay-R	CTTGCTTGATGCTTTGGTCTGT	qRT-PCR
p18-assay-F	CGGGAGGTTCTTGTTCTG	qRT-PCR
P18-assay-R	TTTGTTGGCTTGCTTGAC	qRT-PCR
GAPDH-assay-F	CATCACCATCTTCCAGGAGCG	qRT-PCR
GAPDH-assay-R	TGACCTTGCCCACAGCCTT	qRT-PCR
MET-assay-F	TGGTGCAGAGGAGCAATGG	qRT-PCR
MET-assay-R	ATTCTGGATGGGTGTTTCCG	qRT-PCR
TGF-βRII-assay-F	GUCUUGCAUGAGCAACUGC	qRT-PCR
TGF-βR-assay-R	UUCAGAACGUACUCGUUGACG	qRT-PCR
Rac2-assay-F	CCTGTGGCGTTTCTTAGCAGA	qRT-PCR
Rac2-assay-R	GGATGCAGCACCTGCAAAT	qRT-PCR
cdc42-assay-F	CTCCGGAAACTCAACCCAAA	qRT-PCR
cdc42-assay-R	GACGCAGAGGCTTTCAAACAG	qRT-PCR
si-HULC	AACCTCCAGAACTGTGATCCA	Knockdown

for Biological Sciences of Chinese Academy of Sciences, and maintained at 37°C in high-glucose DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco) in the presence of 5% CO_2. The medium was routinely changed every 2 days. The cells in logarithmic growth phase were used for experiments.

Drug treatment

SGC-7901 cells in logarithmic growth phase were plated in 6-well plates until cell attachment, and then treated with paeonol at final concentrations of 62.5 mg/L for 24 hours. The cells treated with phosphate-buffered saline (PBS) were used as negative control.

Total RNA extraction and quantitative real-time (RT)-PCR

Total RNA was extracted from the SGC-7901 cells using TRIzol (Invitrogen) reagent according to the manufacturer's recommended instructions. RNA purity and integrity were analyzed using an Agilent Bioanalyzer 2100 (Agilent Technologies).

The complementary DNA (cDNA) was synthesized using an iScript cDNA Synthesis Kit (BIO-RAD) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Premix ExTaqTM II kit (TaKaRa). The conditions of qRT-PCR were as follows: 94°C for 10 s, 94°C for 5 s, 52°C for 30 s to anneal, 72°C for 15 s followed by 40 cycles. The detection was set at 62°C. PCR amplifications were performed in three duplicates for each sample. The relative RNA expression was calculated using the 2-ΔCt method. And the specific primers sequences were listed in **Table 1**.

Small interfering RNAs and transfection

Small interfering RNAs (siRNAs) targeting human HULC mRNA (si-HULC) and negative control siRNA (si-Ctrl) were synthesized by GenePharma (Shanghai, China) (see **Table 1** for details). For transfection, cells were plated in 6- or 96-well plates, incubated for 24 h and then transfected with 50 nmol/L of si-HULC or si-Ctrl using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended instructions. And the transfection efficiency was examed after harvested for 24 h and 48 h, respectively. The transfection efficiency was monitored using qRT-PCR to quantify the post-transfection changes in HULC expression level; the data were analyzed using the same 2-ΔΔCt method.

Methyl thiazolyl tetrazolium (MTT) assay

SGC-7901 cells were seeded in 96-well plates, cultured for 24 h and then transfected with siRNA or treated with paeonol as described above. Cell viability was analyzed using MTT (Sigma) assay at the indicated time points as described previously [9]. In brief, 1 μl/well of MTT was added and incubated at 37°C for an additional 4 h. Then, the medium was discarded and cells were lysed in DMSO (150 μl/well). The absorbance at 490 nm was measured on a plate reader. Each experiment was performed in triplicate and repeated three times.

Cell cycle assay

Appropriate cells as earlier described were collected, washed and then resuspended in PBS and fixed in 75% ethanol. The fixed cells were stained with propidium iodide (PI) supplemented with RNaseA (Sigma) and analyzed with a FACScan flow cytometer (BD Biosciences). Data were collected and analyzed with the ModFit software (BD Biosciences).

Flow cytometric analysis of apoptosis

For detection of apoptosis, appropriate adherent cells were collected, washed and resus-

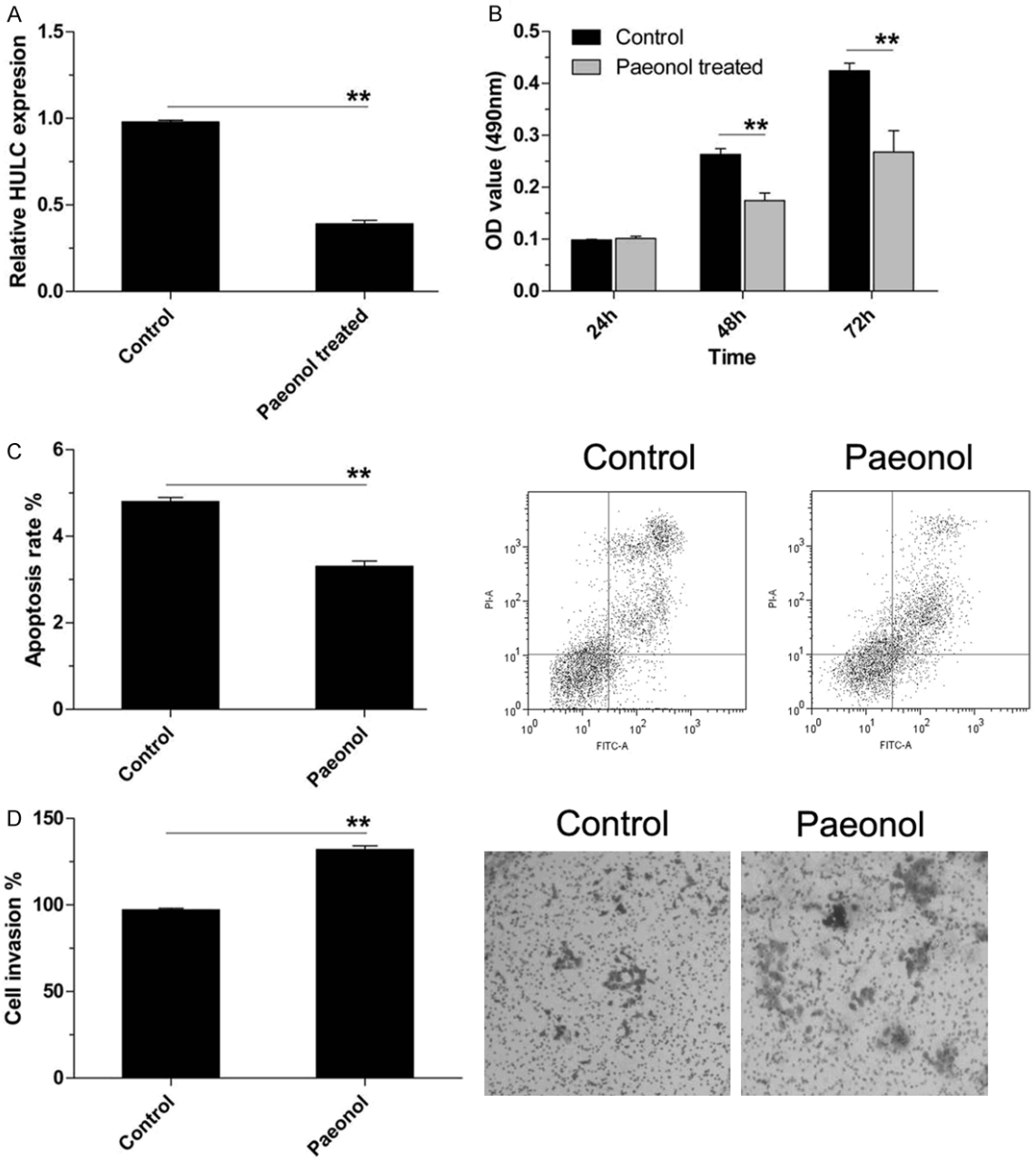

Figure 1. Effects of paeonol on HULC expression and cell growth of SGC-7901 cell lines. A. The expression level of HULC in SGC-7901 cells treated with paeonol was detected using qRT-PCR. B. The effect of paeonol on cell proliferation. The cell viability was measured using the MTT assay at 24, 48, and 72 h after paeonol treated. C. The effect of paeonol on cell apoptosis. The cells treated with paeonol for 24 h and the apoptosis rate was examed using flow cytometric analysis. D. The effect of paeonol on cell invasion. The invasiveness of SGC-7901 cells was measured by transwell assays. The cells treated with PBS were as negative control in all these experiments above. Data are at least three independent experiments and are shown as mean ± SD (**P<0.01 as compared with control group).

pended in cold PBS for analysis. Apoptosis was detected using the Alexa Fluor® 647/7-AAD apoptosis kit (BioLegend) according to the manufacturer's recommended instructions. Data were assessed by flow cytometry (BD Biosciences).

Western-blot

Western blotting was carried out as previously described (S. Li et al., 2010). Briefly, Cells were collected and lysed with complete cell lysis (Beyotime) with protease inhibitors cocktail

(Rocha). Identical quantities of proteins was loaded and separated on SDS-PAGE, transferred to polyvinyli dene fluoride membranes (Millipore), and then incubated with appropriate antibodies, which are against Bcl-2 (Cell Signaling Technology), Bax (Cell Signaling Technology), cleaved caspase 3 (Cell Signaling Technology), caspase 3 (Cell Signaling Technology), cleaved caspase 9 (Cell Signaling Technology), caspase 9 (Cell Signaling Technology), JNK (Cell Signaling Technology), p-JNK (Cell Signaling Technology), and GAPDH (Abcam). The immuno-reactive bands were visualized using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL) and obtained using a CCD camera system (Tanon). The density of which was measured by Image J.

Transwell invasion assay

Transwell invasion assay was performed using the Biocoat Matrigel Invasion Chamber (BD Biosciences) according to the manufacturer's recommended instructions. In brief, 4×10^4 cells were plated in the upper chamber consisting of 8-mm membrane filter inserts coated with Matrigel (BD Biosciences). The bottom chamber contained DMEM medium with 10% FBS as an inducer of invasion. After 24 h, cells on the upper surface were removed, and those attached on the lower side of the membrane were fixed and stained with crystal violet before counting under a microscope in five randomly selected fields.

Statistical analysis

All experiments were performed independently for at least three times. All data are presented as the mean values ± standard deviations (SD) of each group. Statistically significant differences were calculated by two-tailed Student's *t*-test using SPSS software (version 19.0). The graphs were generated with GraphPad Prism 5.0.

Results

HULC expression is down-regulated after paeonol treatment in SGC-7901 cell lines

Our preliminary tests using microarray have shown that lncRNA HULC is down-regulated in SGC-7901 cells after paeonol treatment. To confirm this finding, we assessed the HULC expression levels in SGC-7901 cell lines treated with paeonol using qRT-PCR. As presented in **Figure 1A**, the expression levels of HULC were remarkably decreased by 59% in the paeonol treatment group relative to the expression in the untreated SGC-7901 cells ($P = 0.0023$). Furthermore, we also monitored the growth of the cells, and the result showed that the proliferation and invasiveness of SGC-7901 cells treated with paeonol was obviously inhibited, while the apoptosis was remarkably promoted (**Figure 1B-D**).

The biological effect of down-regulated HULC

In order to verify whether these anti-cancer effects of paeonol are relevant to the change of HULC expression level, we need to investigate the biological effects of decreased HULC. For this purpose, we inhibited HULC expression by transfecting HULC-siRNA (si-HULC) into SGC-7901 cells, using original cells and control siRNA (si-Ctrl) as control and the negative control, respectively. After 24 h and 48 h of RNA interference, we used qRT-PCR to analyze the interference effect, and it showed that HILC expression level of 24 h and 48 transfection decreased to 26% and 17%, respectively. It means that HILC expression could be efficiently inhibited in SGC-7901 cells transfected with HULC-siRNA relative to the controls (**Figure 2A**). According to this result above, the cells transfected with HULC-siRNA for 48 h were used in the subsequent functional experiments.

Down-regulated HULC suppresses cell proliferation via elevating p18 expression

We first studied the effect of down-regulated HULC in cell proliferation. As expected, the MTT results revealed that silencing HULC resulted in a significant decrease of cell viability at 24 and 48, and 72 h, respectively (**Figure 2B**). As we all known, as a regulator, HULC is a regulator so it could not have a direct effect on cell proliferation. And it has been reported that HULC could promote cell proliferation via down-regulating tumor suppressor gene p18 in hepatoma cells [10-12]. So we further examed whether the p18 expression changed. Our result was also in accordance with previous research that the knockdown of HULC led to an obvious increase of p18 promoter activity in SGC-7901 cells (**Figure 2C**).

Moreover, the results of cell cycle assays indicated that repressing HULC expression mainly

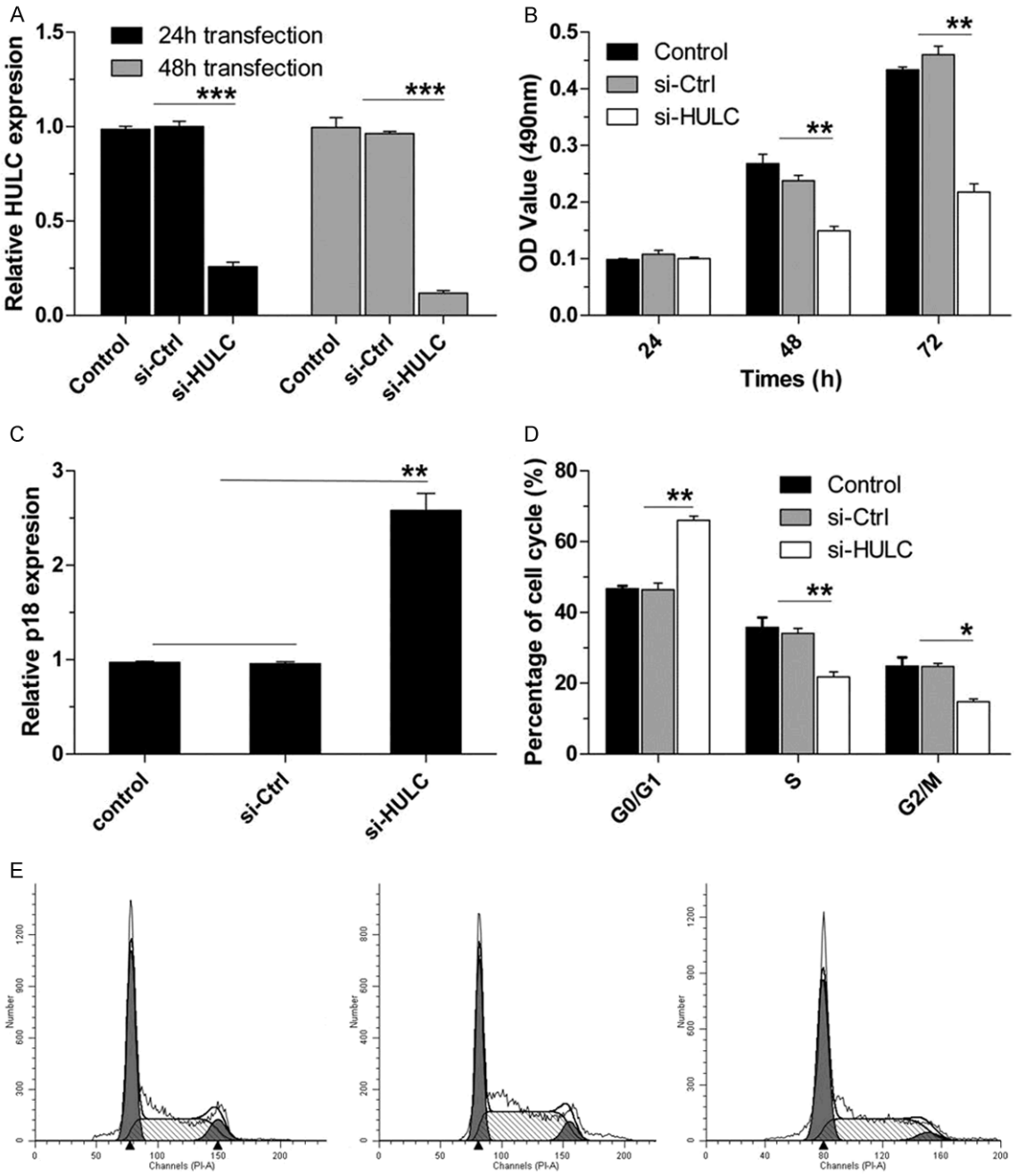

Figure 2. Effects of down-regulated HULC on cell proliferation and cell cycle. A. The interference effect of HULC in SGC-7901 cell lines. in The cells were transfected with si-HULC and the decreased expression of HULC was measured using qRT-PCR 24 h and 48 h post-transfection. B. SGC-7901 cells were transfected with si-HULC, and cell viability was measured using the MTT assay at 24, 48, and 72 h after transfection. C. The expression level of p18 in the cell transfected with si-HULC was detected using qPR-PCR. D, E. SGC-7901 cells with si-HULC transfection were collected and stained with PI and the cell cycle distribution was analyzed with flow cytometer.

led to a G0/G1 accumulation at 66.00±2.13% compared with 46.67±1.46%, 46.42%±3.28% of the controls, but a decrease of Sphase at 21.75±2.57%, compared with 35.75±4.91%, 34.09±2.47% of the controls (**Figure 2D**, **2E**).

In these experiments above, the original cells and the cells transfected with si-Ctrl were adopted as control and negative control, respectively. Data are at least three independent experiments and are shown as mean ± SD

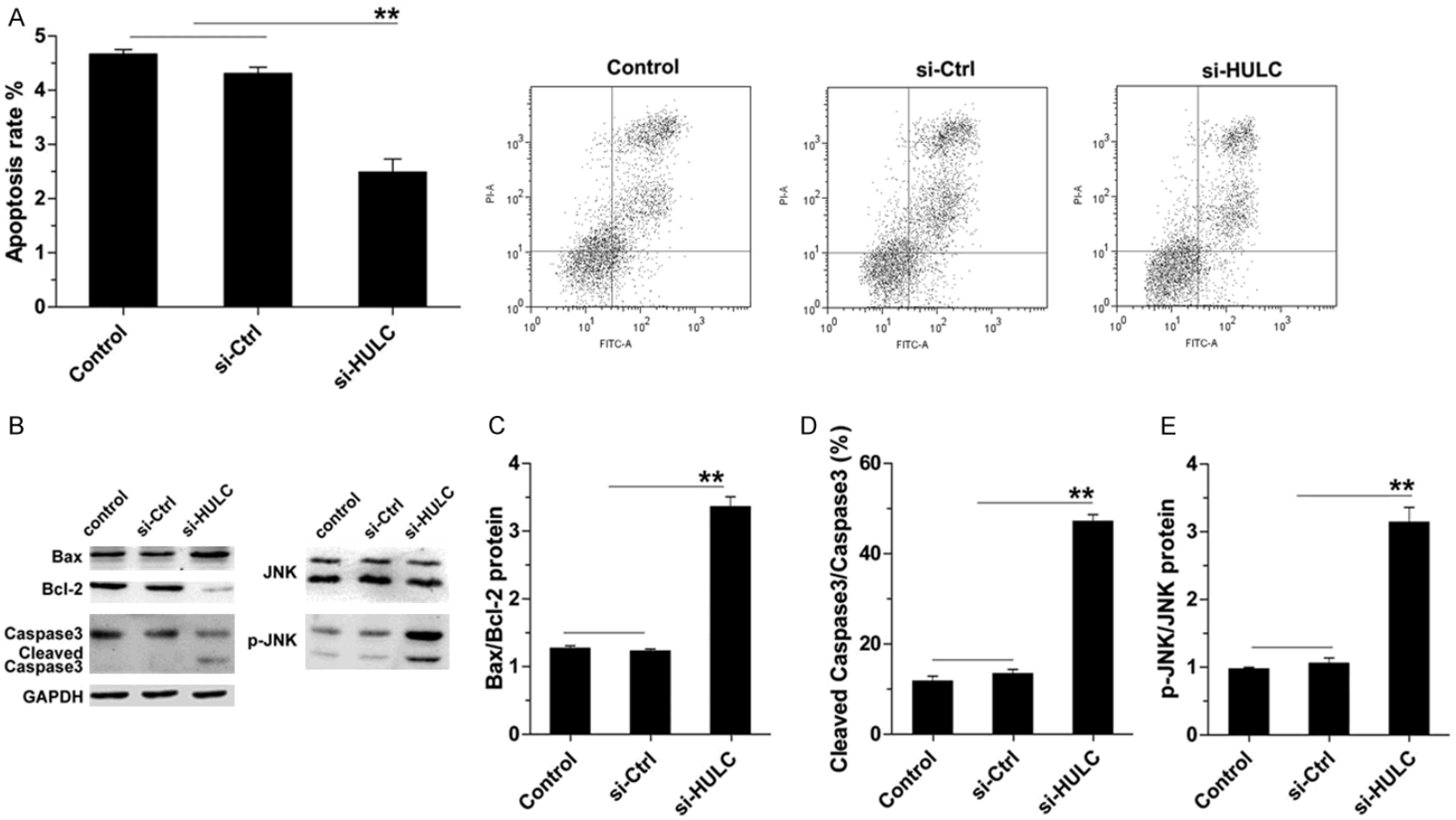

Figure 3. Effects of down-regulated HULC on cell apoptosis. (A) The cells transfected with si-HULC for 48 h and the apoptosis rate was examed using flow cytometric analysis. (B) The expression of apoptosis-related protein, Bax, Bcl-2, caspase 3 and cleaved caspase 3, p-JNK/JNK were examined using western blot analyses and the ratios of Bax/Bcl-2 (C), cleaved caspase 3/caspase 3 (D), and (E) pJNK/JNK were determined. GAPDH was used as a reference. The results show the means ± SD from at least 3 separate experiments. **, P<0.01.

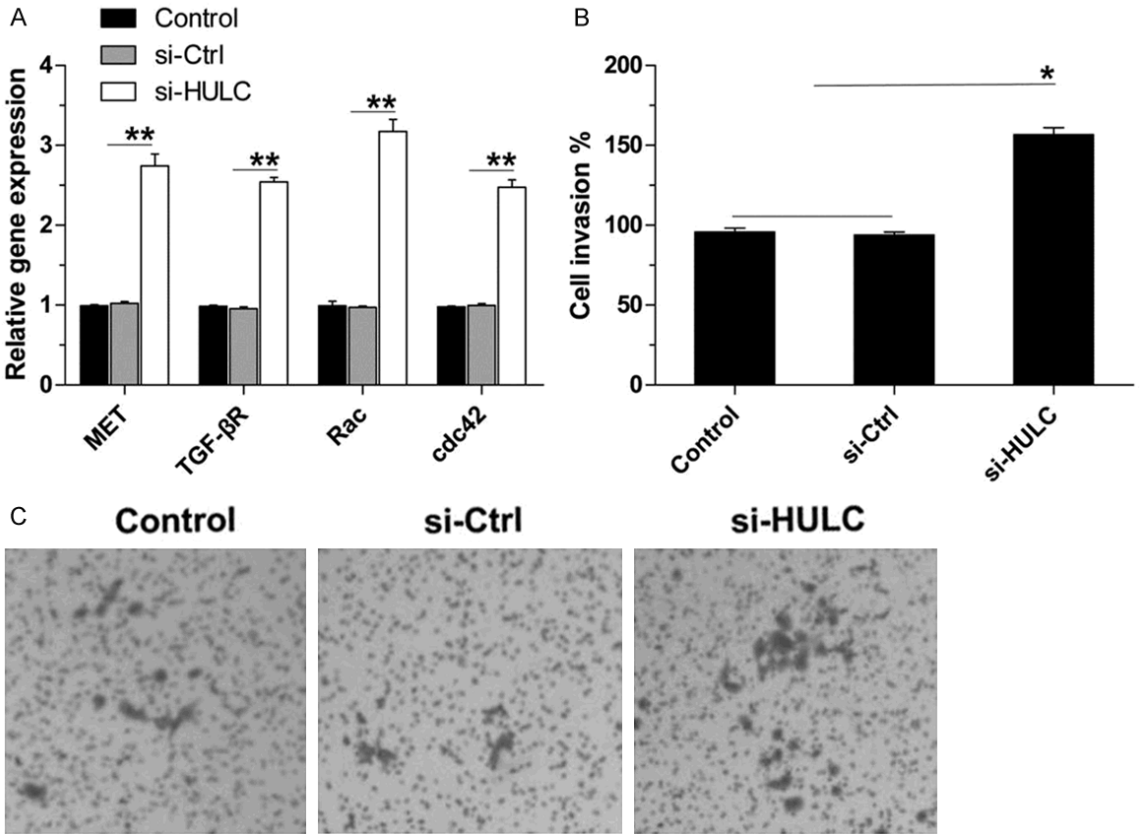

Figure 4. Effects of down-regulated HULC on cell adhesion and invasion. A. The expression level change of adhesion-related gene in SGC-7901 cells was analyzed by qRT-PCR assays. B, C. The invasiveness of SGC-7901 cells transfected with si-HULC was measured by transwell assays. In these experiments above, the original cells and the cells transfected with si-Ctrl were adopted as control and negative control, respectively. Data are at least three independent experiments and are shown as mean ± SD (*P<0.05, **P<0.01 as compared with control group).

(*P<0.05, **P<0.01 and ***P<0.001 as compared with control group).

Down-regulated HULC suppresses cell apoptosis via activating JNK MAPK signaling pathway

In addition, we also investigated its effect on cell apoptosis using Flow cytometric analysis. As depicted in **Figure 3A**, decreased expression of HULC promote cell apoptosis. In the meanwhile, we examed the expression levels of some apoptosis-related proteins using western blot analysis. It is shown that Bax/Bcl-2 protein levels of the HULC knockdown group obviously increased compared with the control cells (**Figure 3B**, **3C**), and cleaved caspase 3 was also up-regulated significantly in cells that had been transfected with HULC-siRNA (**Figure 3B**, **3D**).

Mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, are mediators of cellular responses to extracellular signals, and these related signaling pathway were involved in cell survival, apoptosis, proliferation, differentiation, migration, inflammatory response and so on [13]. JNK/MAPK was believed to play a pivotal role in cell apoptosis [14, 15]. So we next analyzed the activation of the JNK/MAPK pathway after HULC was depressed. The western blot assays showed that p-JNK was only significantly up-regulated in cells transfected with si-HULC (**Figure 3B**, **3E**). It is suggested that HULC probably suppressed the cell apoptosis via regulating the activation of JNK/MAPK signaling pathway.

Down-regulated HULC promotes cell adhesion

The finding of growth inhibitiory effect of decreased HULC promoted us to investigated its effect on cell adhesion and invasion. We

analyzed the expression level of some adhesion-related genes using qRT-PCR. As shown in **Figure 4A**, these genes are obviously up-regulated in the t knockdown cells, such as MET (1.7 fold change), TGF-βR (1.5 fold change), and the two members of the Rho-family GTPase, Rac (2.2 fold change fold change), and cdc42 (1.5 fold change). Moreover, recent research has been reported that JNK/MAPK signaling pathway was also closely correlated with cell adhesion [16, 17], and our western blot assays showed that the JNK/MAPK signaling pathway was remarkably activated, which is in accordance with previous studies. So all these results are suggested that HULC could inhibit cell adhesion and down-regulated HULC could significantly promote cell adhesion.

Down-regulated HULC suppresses cell invasion

The effects of HULC on the invasiveness of SGC-7901 cells were checked by Transwell assays. An in vitro Matrigel invasion assay revealed that invasiveness of SGC-7901 cells transfected with HULC were suppressed compared with original SGC-7901 and negative control cells (**Figure 4B**). It means that HULC plays an important role in cell invasion, and down-regulated HULC could remarkably suppress the invasiveness of hepatoma cells.

Discussion

In recent years, accumulating evidence indicates that lncRNAs are not the "dark matter" of the genome, but that they play significant roles in various biological processes including cell differentiation, cell apoptosis, stem cell pluripotency, and so on [18]. And it is noteworthy that an increasing number of studies have demonstrated that lncRNAs might act as oncogenes or tumor suppressors, which were involved in human cancer pathogenesis, including HCC [19-21].

Paeonol, as a traditional Chinese medicine, has been reported to have several biological effects, such as anti-inflammatory, anti-oxidant, anti-allergic, anti-oxidation and antitumor effects [22]. However, the anti-cancer molecular mechanism of paeonol was still not researched clearly.

In this study, we found that the growth and invasiveness of SGC-7901 cells were suppressed after paeonol treatment, while the HULC expression was also remarkably down-regulated. Additionally, our data showed that decreased HULC could inhibit cell proliferation via elevating p18 expression, and promote cell apoptosis by activating JNK/MAPK signaling pathway. Moreover, down-regulated HULC also has effects on promoting cell adhesion and suppressing cell invasion. Collectively, all these results suggested that paeonol could suppress cancer cell growth and invasion via down-regulating the expression level of HULC. This is the first report to demonstrate the potential anticancer mechanism of paeonol in Hepatocellular carcinoma, and the results may provide a theoretical support for the application of paeonol in clinical treatment of hepatoma.

Disclosure of conflict of interest

None.

Address correspondence to: Xu Wang, Medical Department of Oncology, Tianjin Medical University Cancer Institute Hospital, Tianjin 300060, China. E-mail: xuwang_tjch@126.com

References

[1] Mercer TR, Dinger ME, Mattick JS. Long noncoding RNAs: insights into functions. Nat Rev Genet 2009; 3: 155-159.

[2] Meseure D, Drak Alsibai K, Nicolas A, Bieche I, Morillon A. Long noncoding RNAs as new architects in cancer epigenetics, prognostic biomarkers, and potential therapeutic targets. Biomed Res In 2015; 2015: 320214.

[3] Wapinski O, Chang HY. Long noncoding RNAs and human disease. Trends Cell Biol 2011; 6: 354-361.

[4] Xu D, Yang F, Yuan JH, Zhang L, Bi HS, Zhou CC, Sun SH. Long noncoding RNAs associated with liver regeneration 1 accelerates hepatocyte proliferation during liver regeneration by activating Wnt/beta-catenin signaling. Hepatology 2013; 2: 739-751.

[5] Panzitt K, Tschernatsch MM, Guelly C, Moustafa T, Stradner M, Strohmaier HM, Zatloukal K. Characterization of HULC, a novel gene with striking up-regulation in hepatocellular carcinoma, as noncoding RNA. Gastroenterology 2007; 1: 330-342.

[6] Cai J, Chen S, Zhang W, Hu S, Lu J, Xing J, Dong Y. Paeonol reverses paclitaxel resistance in human breast cancer cells by regulating the expression of transgelin 2. Phytomedicine 2014; 7: 984-991.

[7] Yin J, Wu N, Zeng F, Cheng C, Kang K, Yang H. Paeonol induces apoptosis in human ovarian cancer cells. Acta Histochem 2013; 8: 835-839.
[8] Li M, Tan SY, Wang XF. Paeonol exerts an anticancer effect on human colorectal cancer cells through inhibition of PGE (2) synthesis and COX-2 expression. Oncol Rep 2014; 6: 2845-2853.
[9] Shan C, Xu F, Zhang S, You J, You X, Qiu L, Zhang X. Hepatitis B virus X protein promotes liver cell proliferation via a positive cascade loop involving arachidonic acid metabolism and p-ERK1/2. Cell Res 2010; 5: 563-575.
[10] Park BJ, Kang JW, Lee SW, Choi SJ, Shin YK, Ahn YH, Kim S. The haploinsufficient tumor suppressor p18 upregulates p53 via interactions with ATM/ATR. Cell 2005; 120: 209-221.
[11] Zhang YQ, Xiao CX, Lin BY, Shi Y, Liu YP, Liu JJ, Guleng B, Ren JL. Silencing of Pokemon enhances caspase-dependent apoptosis via fas- and mitochondria-mediated pathways in hepatocellular carcinoma cells. PLoS One 2013; 7: e68981.
[12] Ko YG, Park H, Kim S. Novel regulatory interactions and activities of mammalian tRNA synthetases. Proteomics 2002; 9: 1304-1310.
[13] Arthur JS, Ley SC. Mitogen-activated protein kinases in innate immunity. Nat Rev Immunol 2013; 9: 679-692.
[14] Bubici C, Papa S. JNK signalling in cancer: in need of new, smarter therapeutic targets. Br J Pharmacol 2014; 1: 24-37.
[15] Sui X, Kong N, Ye L, Han W, Zhou J, Zhang Q, Pan, H. p38 and JNK MAPK pathways control the balance of apoptosis and autophagy in response to chemotherapeutic agents. Cancer Lett 2014; 2: 174-179.
[16] Li S, Dong P, Wang J, Zhang J, Gu J, Wu X, Liu Y. Icariin, a natural flavonol glycoside, induces apoptosis in human hepatoma SMMC-7721 cells via a ROS/JNK-dependent mitochondrial pathway. Cancer Lett 2010; 2: 222-230.
[17] You H, Lei P, Andreadis ST. JNK is a novel regulator of intercellular adhesion. Tissue Barriers 2013; 5: e26845.
[18] Sun J, Bie B, Zhang S, Yang J, Li Z. Long noncoding RNAs: critical players in hepatocellular carcinoma. Int J Mol Sci 2014; 11: 20434-20448.
[19] Maruyama R, Shipitsin M, Choudhury S, Wu Z, Protopopov A, Yao J, Polyak K. Altered antisense-to-sense transcript ratios in breast cancer. Proc Natl Acad Sci U S A 2012; 8: 2820-2824.
[20] Lin R, Maeda S, Liu C, Karin M, Edgington TS. A large noncoding RNA is a marker for murine hepatocellular carcinomas and a spectrum of human carcinomas. Oncogene 2007; 6: 851-858.
[21] Gibb EA, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. Mol Cancer 2011; 10: 38.
[22] Lee MH, Koria P, Qu J, Andreadis ST. JNK phosphorylates beta-catenin and regulates adherens junctions. FASEB J 2009; 11: 3874-3883.