Original Article

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

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Abstract: Aim: 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 paeonol 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, adhesion 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 IncRNA 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
Paeonol inhibits the growth of gastric cancer cells via suppressing HULC expression

Table 1. List of primers and their applications

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences (5'-3')</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>HULC-assay-F</td>
<td>ATCTGAAGCCAGGAAAGGTTC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>HULC-assay-R</td>
<td>CTTGCTTGATGCTTGTTGCTGT</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>p18-assay-F</td>
<td>CGGAGGTTGTGTTGTTG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>p18-assay-R</td>
<td>TTTTGTTGCTTGTGTTG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>GAPDH-assay-F</td>
<td>CATCACCATTCCAGGACCC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>GAPDH-assay-R</td>
<td>TGACCTGCCCAGCCTT</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>MET-assay-F</td>
<td>TGCGAGAGGAGCAATGG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>MET-assay-R</td>
<td>ATTCTGGATGGTTCCGTG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>TGF-βRl-assay-F</td>
<td>GUCUUGCAUGAGGAACACG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>TGF-βRl-assay-R</td>
<td>UUCAGAAACUAGUUGAC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>Rac2-assay-F</td>
<td>CCGTGGCTTTCTTAGCAGA</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>Rac2-assay-R</td>
<td>GGATGACACACTGAAAT</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>cdc42-assay-F</td>
<td>CTCAGAAACTCAACACC</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>cdc42-assay-R</td>
<td>GACGCAAGGCTTCAACG</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>si-HULC</td>
<td>AACCTCCAGAACTGTGATCCA</td>
<td>Knockdown</td>
</tr>
</tbody>
</table>

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-ΔΔCt 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 examined 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) supplement- ed 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 adher- ent cells were collected, washed and resus-
Paeonol inhibits the growth of gastric cancer cells via suppressing HULC expression.

- **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 examined 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).

- **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 were loaded and separated on SDS-PAGE, transferred to polyvinylidene 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, 4×10⁴ 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 examined 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
Paeonol inhibits the growth of gastric cancer cells via suppressing HULC expression.

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Figure 2. Effects of down-regulated HULC on cell proliferation and cell cycle. A. The interference effect of HULC in SGC-7901 cell lines. 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.

Figure 2. Effects of down-regulated HULC on cell proliferation and cell cycle. A. The interference effect of HULC in SGC-7901 cell lines. 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 S phase 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.
Paeonol inhibits the growth of gastric cancer cells via suppressing HULC expression

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

(*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 examined 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 inhibitory 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 inhibi
t 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 IncRNAs 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 IncRNAs 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 anti-cancer 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.

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**References**

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


