Effect of Homoharringtonine on Proliferation and Apoptosis of Osteosarcoma Cells via LINC00857/miR-340

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ABSTRACT

The objective of this study was to investigate the effect of homoharringtonine on proliferation and apoptosis of osteosarcoma cells through long non-coding RNA (lncRNA) LINC00857/miRNA (miR) -340. U2OS of osteosarcoma cells was treated with harringtonine at a concentration of 0.25, 0.5, 1, 2, 4 μg/ml. P21 and cysteine-containing aspartic protease 3 (Caspase-3) protein expression in U2OS cells were detected by western blot, thiazole blue (MTT) and plate cloning experiments to determine cell survival and colony formation, flow cytometry to measure apoptosis, and quantitative real-time polymerase chain reaction (qRT-PCR) to assay LINC00857 and miR-340 expression. Bioinformatics and dual luciferase reports analyzed the targeting relationship between LINC00857 and miR-340. Cells were transfected with pcDNA3.1-LINC00857 or anti-miR-340, and treated with homoharringtonine to observe their effects on U2OS protein expression, survival, clone formation and apoptosis. We found that different concentrations of homoharringtonine significantly increased the expression of P21, Caspase-3 protein, apoptotic rate, and miR-340 expression in U2OS cells, and obviously decreased the cell survival rate, the number of colony formation, and the expression of LINC00857 (P<0.05). All were dose-dependent. LINC00857 targets and regulates miR-340. Overexpression of LINC00857 or inhibition of miR-340 evidently reduced the expression levels of P21, Caspase-3 and apoptosis in U2OS cells after homoharringtonine treatment, and remarkably improved cell survival rate and colony formation (P<0.05). It is concluded that homoharringtonine can inhibit the proliferation of osteosarcoma cells and induce apoptosis by regulating LINC00857/miR-340.

INTRODUCTION

Osteosarcoma, the most common primary malignant bone tumor, is widespread in children and young people (Li et al., 2020). Standard therapies for osteosarcoma include neoadjuvant chemotherapy, surgical resection and further chemotherapy after resection (Lu et al., 2018). Despite advances in surgical techniques, relapse and chemotherapy resistance remain major challenges for osteosarcoma. The past 30 years have witnessed almost no change in survival rate of patients with recurrent or metastatic osteosarcoma, and the overall 5-year survival rate is about 20% (Zuo et al., 2017). Therefore, it is essential to develop more effective therapies for osteosarcoma. Homoharringtonine, as a known inhibitor for protein synthesis, has been clinically used to treat chronic myeloid leukemia (Yakhni et al., 2019). Homoharringtonine with extensive pharmacological effects is viewed as an effective anti-tumor component, which is cytotoxic to cervical cancer Hela, breast cancer MCF-7 and osteosarcoma U2OS cells (Wang et al., 2016). However, homoharringtonine-mediated anti-osteosarcoma activity and underlying mechanisms have not been fully elucidated. Long non-coding RNA (lncRNA) (longer than 200 nucleotides) and micro RNA (miRNA/miR) (22-25 nucleotides in length) belong to non-coding RNA. lncRNA and miRNA have been proven to act as oncogenes or cancer suppressor genes in the occurrence and development of various human cancers including osteosarcoma by regulating target expression (Li et al., 2019; Zhu et al., 2020; Yu et al., 2019). Studies have shown that lncRNA LINC00857 is highly expressed in hepatocellular carcinoma, the silencing of LINC00857 significantly inhibits the proliferation of hepatocellular carcinoma Hep-3B and SNU449 cells and inhibits cell
and apoptosis in vitro as well as the underlying mechanism. LINC00857 and miR-340 expression, thereby further determining its effect on osteosarcoma cell proliferation. Nevertheless, it is unknown regarding biological functions and significance of LINC00857 and miR-340 in homoharringtonine-induced osteosarcoma cells. This study aims to study the effect of homoharringtonine on LINC00857 and miR-340 expression, thereby further determining its effect on osteosarcoma cell proliferation and apoptosis in vitro as well as the underlying mechanism.

MATERIALS AND METHODS

Cells and reagents

Osteosarcoma cells U2OS were purchased from the Cell Bank of China Center for Type Culture Collection, high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM medium), fetal calf serum (FBS), bicinchoninic acid (BCA) assay kit were purchased from Thermo Scientific, USA, homoharringtonine (purity ≥98%) was purchased from Shanghai Yuanye Biotechnology Co., Ltd., methyl thiazolyl tetrazolium (MTT) was purchased from Beijing Solarbio, Lipofectamine 2000 reagent was purchased from Invitrogen, USA, P21, Cysteinyl aspartate specific proteinase 3 (Caspase-3), glyceraldehyde 3 phosphate dehydrogenase (GAPDH) primary and secondary antibodies were purchased from Abcam, UK.

Cell culture, transfection and grouping

U2OS cells were cultured in high-glucose DMEM medium supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, and incubated in a humidified, 37°C, 5% carbon dioxide incubator for passage at a ratio of 1:3. The cells in the logarithmic growth phase were selected and treated with homoharringtonine at concentrations of 0.25, 0.5, 1, 2 and 4 µg/ml for 48 h, which were recorded as Experiment 1, Experiment 2, Experiment 3, Experiment 4 and Experiment 5 groups, respectively. The cells without drug treatment were recorded as the control group.

After transfection, the U2OS cells were divided into: Experimental 5 group + pcDNA3.1 group, Experimental 5 group + pcDNA3.1-LINC00857 group, Experimental 5 group + anti-miR-NC group, Experimental 5 group + anti-miR-340 Group, pcDNA3.1-LINC00857 group, pcDNA3.1 group, si-LINC00857 group and si-NC group. At the time of transfection, U2OS cells (5×10⁴ cells) were inoculated into 6-well plates 1d in advance, and pcDNA3.1-LINC00857, anti-miR-340, si-LINC00857 and si-LINC00857 and respective negative controls were transfected with U2OS cells. The experiment 5 group was treated with 4 µg/ml homoharringtonine. Cells were collected 48 h after transfection, and quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the expression of LINC00857 or miR-340, followed by other detections.

Western blot to detect the expression of P21 and Caspase-3 in U2OS cells

U2OS total protein was prepared in radio immunoprecipitation (RIPA) buffer supplemented with protease inhibitor. Protein was quantified by BCA assay kit after centrifugation at 12,000 g for 20 min at 4°C. Protein sample (40 µg) was then separated by SDS-PAGE and transferred to nitrocellulose membrane and blocked in non-fat milk. The membrane was incubated with anti-P21, Caspase-3, GAPDH primary antibodies, and then stayed overnight at 4°C. The membrane was then incubated with the secondary antibody coupled to HRP for 1 h at room temperature. The protein in the membrane was detected using an enhanced chemiluminescence kit, and the optical density of the bands was determined by Image J 1.47 software.

MTT detection of U2OS cell survival

U2OS cells (1×10⁴) were inoculated in 96-well plates, and after incubation overnight were incubated with homoharringtonine serial diluents for 48 h. Then 20 µl of MTT solution was added and incubated for 4 h. After adding dimethyl sulphoxide (DMSO), the absorbance was read at 490 nm using a microplate reader.

Plate clone detection of U2OS cell clone formation

U2OS cells (500) were inoculated in a 6-well plate and evenly dispersed by gentle shaking. After cells adherence, grouping was performed. After 10-14 d culture, when the cells showed clones visible to the naked eye, the medium was discarded, and the cells were washed twice with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 20 min, washed with PBS, stained for 15 min with 0.1% crystal violet, and washed with PBS. Clones larger than 50 cells were counted under a normal optical microscope.

Detection of U2OS apoptosis by flow cytometry

FITC Annexin V/PI apoptosis kit was used to assess the apoptosis of U2OS cells by double staining. After corresponding treatment, U2OS cells were collected,
washed with PBS, and resuspended in binding buffer. Then, 5 μL FITC-Annexin V and 5 μL PI were added to the binding buffer and incubated for 15 min at room temperature in the dark. The percentage of apoptotic cells was analyzed using flow cytometry.

**qRT-PCR detection of LINC00857 and miR-340 expressions in U2OS cells**

Total RNA in U2OS cells was isolated using TRIzol reagent, and RNA was quantified using NanoDrop 1000 spectrophotometer according to the manufacturer’s operating instruction. Reverse transcription was performed by cDNA synthesis kit, followed by qRT-PCR. LINC00857 and miR-340 expressions were calculated by 2-ΔΔCt method, and the results were normalized using GAPDH and U6, respectively. Primer sequences:

- LINC00857 F 5’-CCCCTGCTTCATGTTCCTCCC-3’
  R 5’-AGCTTGTCCTTCTTGGGTACT-3’
- miR-340 F 5’-TTATAAAGCAATGAGA-3’
  R 5’-GTGCAGGGTGTCGAGTG-3’
- GAPDH F 5’-GGTGGTCTCCTCTCTTGGT-3’
  R 5’-TCTCTTCCTCTTGCTTGCT-3’
- U6 F 5’-CTCGCTTCGGCAGCACATATACT-3’
  R 5’-ACGCTTCACGAATTTGCGTGTC-3’

**Dual-luciferase report validates the targeting of LINC00857 to miR-340**

StarBase website (http://starbase.sysu.edu.cn/) was used to identify potential binding sites between LINC00857 and miR-340. The wild type (WT) -LINC00857 and mutant type (MUT) -LINC00857 miR-340 binding sites were established and cloned into the luciferase reporter gene vector. For dual-luciferase activity assay, U2OS cells with a density of 2 × 10⁵ cells/well were inoculated into 24-well plates, and WT-LINC00857 or MUT-LINC00857 was co-transfected with miR-NC or miR-340 using Lipofectamine 2000 reagent. Luciferase activity was measured after 48 h, and the ratio of Renilla luciferase to firefly luciferase was calculated for each well.

**Statistical analysis**

The experimental data were counted and analyzed by SPSS 22.0 software, and the results were expressed as mean ± standard deviation (x̅±s). Differences between the two groups were compared using t test, comparisons between multiple groups were performed using one-way analysis of variance, and multiple comparisons between groups were performed using SNK-q test. P < 0.05 suggested significant difference.

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**RESULT**

**U2OS cell proliferation**

Western blot, MTT and plate cloning test results show that compared with the control group, homoharringtonine at different concentrations significantly increases P21 protein expression in U2OS cells, while significantly reducing the cell survival rate and clone number (P < 0.05) in a dose-dependent manner (Table I, Fig. 1).

**Apoptosis of U2OS cells**

Western blot and flow cytometry detection results show that compared with the control group, homoharringtonine at different concentrations significantly increases Caspase-3 protein expression level and apoptosis rate of U2OS cells (P < 0.05) in a dose-dependent manner (Table II, Fig. 2).
Fig. 2 Effect of homoharringtonine on U2OS apoptosis and Caspase-3 protein expression.

Table II. Effect of homoharringtonine on apoptosis of U2OS cells (x̅±s, n=9).

<table>
<thead>
<tr>
<th>Control / Experiment</th>
<th>Caspase-3 (x̅±s, n=9)</th>
<th>Apoptosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.23±0.02</td>
<td>8.19±0.86</td>
</tr>
<tr>
<td>1</td>
<td>0.26±0.02*</td>
<td>10.93±1.00*</td>
</tr>
<tr>
<td>2</td>
<td>0.37±0.03*</td>
<td>13.68±1.15*</td>
</tr>
<tr>
<td>3</td>
<td>0.51±0.03*</td>
<td>16.24±1.29*</td>
</tr>
<tr>
<td>4</td>
<td>0.63±0.04*</td>
<td>19.37±1.46*</td>
</tr>
<tr>
<td>5</td>
<td>0.77±0.04*</td>
<td>22.57±1.51*</td>
</tr>
<tr>
<td>F</td>
<td>425.452</td>
<td>168.375</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: Compared with the control group, *P<0.05. For details of groups, see Table I.

Overexpression of LINC00857 can alleviate the effects of homoharringtonine on proliferation and apoptosis of U2OS cells

The results of qRT-PCR, western blot, MTT, plate cloning and flow cytometry show that compared to homoharringtonine 4 μg/ml and pcDNA3.1 transfection groups, homoharringtonine 4 μg/ml and pcDNA3.1-LINC00857 transfection group have significantly increased expression of LINC00857 in U2OS, significantly reduced P21 and Caspase-3 protein levels, significantly increased cell survival rate and clone number, and significantly reduced apoptosis rate (P <0.05, Table IV, Fig. 3).

LINC00857 targets and regulates miR-340

The starBase website prediction indicates that LINC00857 and miR-340 have targeted binding sites (Fig. 4). The dule-luciferase reporting experiment found that compared to co-transfection of miR-NC and WT-LINC00857, co-transfection of miR-340 and WT-LINC00857 significantly reduces the luciferase activity of U2OS cells (P <0.05), but co-transfection of miR-NC or miR-340 with MUT-LINC00857 shows no significant change in luciferase activity of U2OS cell (Table V). qRT-PCR data shows that transfection of pcDNA3.1-LINC00857 significantly reduces miR-340 expression compared to transfection of pcDNA3.1, and transfection of si-LINC00857 significantly increases miR-340 expression compared to transfection of si-NC (Table VI).
Effect of Homoharringtonine on Proliferation and Apoptosis of Osteosarcoma Cells

Fig. 3 Overexpression of LINC00857 can alleviate the effects of homoharringtonine on U2OS apoptosis as well as P21 and Caspase-3 protein expressions

Table IV. Overexpression of LINC00857 can alleviate the effects of homoharringtonine on proliferation and apoptosis of U2OS cells (x̅±s, n=9).

<table>
<thead>
<tr>
<th>Groups</th>
<th>LINC00857</th>
<th>P21</th>
<th>Caspase-3</th>
<th>Survival rate (%)</th>
<th>Clone number</th>
<th>Apoptosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+pcDNA3.1</td>
<td>0.99±0.05</td>
<td>0.60±0.04</td>
<td>0.76±0.04</td>
<td>48.8±4.05</td>
<td>62±4.27</td>
<td>22.56±1.46</td>
</tr>
<tr>
<td>+pcDNA3.1-LINC00857</td>
<td>2.76±0.07*</td>
<td>0.22±0.03*</td>
<td>0.29±0.03*</td>
<td>94.1±5.49*</td>
<td>131±6.03*</td>
<td>10.26±1.03*</td>
</tr>
<tr>
<td>t</td>
<td>61.728</td>
<td>22.800</td>
<td>28.200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: Compared with experiment 5 group+pcDNA3.1 group, *P<0.05. For details of group 5, see Table I.

Fig. 4. LINC00857 targets miR-340

Inhibition of mir-340 can alleviate the effects of homoharringtonine on proliferation and apoptosis of U2OS cells

The results of qRT-PCR, western blot, MTT, plate cloning and flow cytometry show that compared with homoharringtonine 4 μg/ml and anti-miR-NC transfection groups, homoharringtonine 4 μg/ml and anti-miR-340 transfection groups have significantly reduced miR-340 expression in U2OS cells, significantly reduced P21 and Caspase-3 protein levels, significantly increased cell survival rate and clone number, and significantly reduced apoptosis rate (P <0.05, Table VII, Fig. 5).

Table V. Dule-luciferase reporting experiment (x̅±s, n=9).

<table>
<thead>
<tr>
<th>Group</th>
<th>WT-LINC00857</th>
<th>MUT-LINC00857</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-NC</td>
<td>0.98±0.05</td>
<td>0.97±0.05</td>
</tr>
<tr>
<td>miR-340</td>
<td>0.31±0.03*</td>
<td>1.01±0.05</td>
</tr>
<tr>
<td>t</td>
<td>34.471</td>
<td>1.697</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>0.109</td>
</tr>
</tbody>
</table>

Note: Compared with miR-NC group, *P<0.05.

Table VI. LINC00857 regulates miR-340 expression (x̅±s, n=9).

<table>
<thead>
<tr>
<th>Group</th>
<th>miR-340</th>
</tr>
</thead>
<tbody>
<tr>
<td>pcDNA3.1</td>
<td>0.97±0.05</td>
</tr>
<tr>
<td>pcDNA3.1-LINC00857</td>
<td>0.33±0.03*</td>
</tr>
<tr>
<td>si-NC</td>
<td>0.98±0.05</td>
</tr>
<tr>
<td>si-LINC00857</td>
<td>1.91±0.07#</td>
</tr>
<tr>
<td>F</td>
<td>1410.306</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Note: Compared with pcDNA3.1 group, *P<0.05; Compared with si-NC group, #P<0.05.
DISCUSSION

As the most common invasive cancer in the human skeletal system, osteosarcoma is becoming the second major cause of cancer-related deaths in children and adolescents (Dong et al., 2018). Although osteosarcoma has significantly improved prognosis in the past few decades, it is still one of the solid tumors with the highest mortality (Chang et al., 2017), and no significant change has occurred in clinical outcome and treatment of patients so far (Gianferante et al., 2017). Therefore, there is an urgent need to identify less toxic and more effective treatments for osteosarcoma. Natural products with the advantages of high biological activity and low toxicity have received more and more attention (Jin et al., 2018; Yang et al., 2018; Liu et al., 2017). Inhibition of proliferation and induction of apoptosis are considered as two important treatments for tumor including osteosarcoma (Chu et al., 2018). This study evaluated the effects of homoharringtonine treatment on osteosarcoma in vitro. The results indicated that homoharringtonine effectively inhibited the proliferation of osteosarcoma cells and induced apoptosis of osteosarcoma cells in a dose-dependent manner, demonstrating effective antitumor activity. Moreover, its role is related to the regulation of LINC00857/miR-340 expression in osteosarcoma cells, thus providing a novel treatment option for osteosarcoma patients.

Homoharringtonine, a plant alkaloid with antitumor properties, is derived from cephalotaxus plants. Since the 1970s, it has been widely used in China to treat hematological malignancies (Wu et al., 2019; Bohlander, 2020). According to reports, homoharringtonine has anti-proliferative and pro-apoptotic effects on colorectal cancer cells, and the mechanism is closely related to blocking mammalian target of rapamycin (mTOR) signaling pathway (Wang et al., 2018). Yin Junqiang screened the anti-osteosarcoma effect of 16 traditional Chinese medicine monomers, finding that homoharringtonine, bufalin, etc. had significant inhibitory effects on osteosarcoma cell growth and proliferation. Homoharringtonine significantly inhibits the growth of lung tumors in vitro and in vivo, demonstrating effective anticancer activity (Weng et al., 2018).
However, its mechanism of action in osteosarcoma remains unclear. This study found that homoharringtonine at different concentrations significantly increased P21 and Caspase-3 protein expression as well as apoptosis rate in U2OS cells, while significantly reducing cell survival rate and clone number in a dose-dependent manner. It indicates that homoharringtonine can inhibit the proliferation of osteosarcoma cells and induce apoptosis, thus having certain anti-tumor ability, which is consistent with the previous studies (Wang et al., 2018; Weng et al., 2018).

Increasing evidence shows that IncRNA can affect various cellular processes, such as cell proliferation, cell cycle regulation, tumor growth and apoptosis (Wo et al., 2019). LINC00857 has been reported to have carcinogenic effects. For example, LINC00857 is highly expressed in esophageal adenocarcinoma. Knocking down LINC00857 can reduce the proliferation, invasion and migration of esophageal adenocarcinoma cells, and increase apoptosis (Su et al., 2019). LINC00857 is up-regulated in lung adenocarcinoma tissues and cell lines. Knockdown of LINC00857 results in inhibition of lung adenocarcinoma cell proliferation and glycolysis, while apoptosis is increased (Wang et al., 2019). This study observed that homoharringtonine significantly reduced the expression of LINC00857 in osteosarcoma cell U2OS in a dose-dependent manner. The results of functional experiments indicated that overexpression of LINC00857 significantly reduced the apoptosis rate of homoharringtonine-induced U2OS cells and the expression levels of P21 and Caspase-3 proteins, while significantly increasing cell survival rate and clone number. It suggests that homoharringtonine can inhibit the proliferation and promote apoptosis of osteosarcoma cells by down-regulating the expression of LINC00857.

miRNA is a small non-protein-coding RNA that binds to a specific region of 3’ untranslated region (3’UTR) of the target mRNA to inhibit or promote the translation or degradation of mRNA, which becomes a key mediator of cancer (Huang et al., 2018). Data show that miR-340 is down-regulated in cervical cancer tissues, and overexpression of miR-340 can inhibit the migration and invasion of cervical cancer cells. MiR-340 can target EphA3 as an anti-tumor factor in cervical cancer metastasis (Xiao et al., 2018). MiR-340 expression is down-regulated in ovarian cancer tissues, and ectopic expression of miR-340 inhibits the growth and metastasis of ovarian cancer cells in vitro and in vivo (Huang et al., 2019). MiR-340 is also down-regulated in osteosarcoma (Yan et al., 2018), suggesting possibility of consistency with previous reports (Xiao et al., 2018; Huang et al., 2019) that miR-340 acts as an anticancer factor in osteosarcoma. This experiment found that homoharringtonine at different concentrations significantly increased miR-340 expression in U2OS cells in a dose-dependent manner. Bioinformatics prediction found that miR-340 may be a functional target gene of LINC00857, which is validated by dual-luciferase. That is, LINC00857 targets and regulates miR-340. In addition, inhibition of miR-340 significantly reduced the apoptosis rate of homoharringtonine-induced U2OS, the expression level of P21, Caspase-3 protein, and significantly increased the cell survival rate and clone number, suggesting that homoharringtonine activity against osteosarcoma may be achieved by inhibiting LINC00857 and targeting miR-340.

CONCLUSION

To conclude, this study found that homoharringtonine exerts an anti-osteosarcoma effect by inhibiting osteosarcoma cell proliferation and inducing apoptosis. In addition, regulating the expression of LINC00857/miR-340 is one of the mechanisms by which homoharringtonine inhibits the growth and progression of osteosarcoma, which demonstrates the potential and value of homoharringtonine in treatment of osteosarcoma.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES


