Knockdown of lncRNA HULC inhibits proliferation, migration, invasion, and promotes apoptosis by sponging miR-122 in osteosarcoma

Daliang Kong | Yang Wang

Department of Orthopaedics, China-Japan Union Hospital of Jilin University, Changchun, China

Correspondence
Yang Wang, Department of Orthopaedics, China-Japan Union Hospital of Jilin University, No.126, Xiantai Street, Changchun 130033, Jilin Province, China. Email: wangyang0917@sina.com

Abstract
Osteosarcoma is a rare malignant bone tumor with high degree of malignancy. HULC (highly upregulated in liver cancer), a long noncoding RNA (lncRNA) was involved in hepatocellular carcinoma development and progression, but its underlying mechanism in osteosarcoma is unknown. The aim of this study was to explore the functional role of HULC in osteosarcoma. The study was conducted in human osteosarcoma cell lines and the expression of HULC in the cell lines was detected by qRT-PCR. Furthermore, the effects of HULC on tumorigenicity of osteosarcoma cells were evaluated by in vitro assays. Results revealed that HULC was highly expressed in osteosarcoma MG63 and OS-732 cells compared to osteoblast hFOB1.19 cells. Suppression of HULC in osteosarcoma cells inhibited cell viability, migration, invasion, and promoted apoptosis. HULC functioned as an endogenous sponge for miR-122, and its silence functioned through upregulating miR-122. HNF4G was a target of miR-122, and the effect of HNF4G on OS-732 cells was the same as HULC. Furthermore, overexpression of miR-122 inactivated PI3K/AKT, JAK/STAT, and Notch pathways by downregulation of HNF4G. These findings suggest that knockdown of HULC inhibited proliferation, migration, and invasion by sponging miR-122 in osteosarcoma cells. HULC may act as a novel therapeutic target for management of osteosarcoma.

Keywords:
cell invasion, HULC, miR-122, osteosarcoma, tumor growth

1 | INTRODUCTION

Osteosarcoma is a rare malignant bone tumor, with high degree of malignancy, and high incidence of recurrence and metastasis.1,2 It is characterized by direct formation of immature bone or osteoid tissue by the tumor cells.1 The most common symptoms include pain and swelling in the affected bone and is more commonly observed in young adults and adolescents.1,3 Therapeutic strategies for treating osteosarcoma include surgical resection combined with radiotherapy and chemotherapy.3 However, the outcomes of patients with osteosarcoma are still unsatisfied, that the relative 5-year survival rate of young-onset osteosarcoma is 61.6% globally.4

Abbreviations: AKT, Protein kinase B; HNF4G, Hepatocyte nuclear factor 4 gamma; HULC, Highly upregulated in liver cancer; JAK, Janus kinase; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; STAT, Signal transducers and activators of transcription.
Genetically, osteosarcomas are extremely complex tumors since multifarious molecular changes are involved in the pathogenesis of osteosarcoma. Therefore, a better understanding of osteosarcoma will be helpful for discovering potential therapeutic targets, which may improve the overall survival rate in patients of osteosarcoma.

Long non-coding RNAs (lncRNAs) are non-coding transcripts longer than 200 nucleotides that do not contain significant open reading frames (ie, no open reading frames greater than 100 amino acids) and thus do not translate into proteins. LncRNAs comprise a heterogeneous group of RNA molecules that is involved in the regulation of gene expression, protein localization, and in the formation of essential protein complex substructures. Besides, lncRNAs are involved in many cellular processes, such as cell proliferation, cell cycle progression, cell growth, and cell apoptosis. Currently, lncRNAs are emerging as important molecular markers of metastatic disease in several human cancers, while the research on lncRNA is still at the beginning stage. Hepatocellular carcinoma upregulated long non-coding RNA (HULC), as one of the most upregulated lncRNAs, was first identified from a hepatocellular carcinoma-specific gene expression profiling. Many recent observations have indicated the importance of HULC in cellular invasion and metastasis of numerous human tumors such as gastric, pancreatic, and liver cancers.

MicroRNA-122 (miR-122) is the most abundant miRNA in the liver which accounts for about 70% of the total miRNA population. It has been reported to function as a tumor suppressor, and be downregulated in several cancers, such as hepatocellular carcinoma, non-small lung cancer, and bladder cancer. Besides, miR-122 has been revealed as a tumor suppressor in osteosarcoma.

Recently, many studies have shown that lncRNAs can serve as a competing endogenous RNAs (ceRNAs) to regulate miRNAs. In this regard, HULC has been reported to be a ceRNA for several miRNAs, such as miR-372/miR-373, miR-9, and miR-107. In this study, we aimed to explore the functional role of HULC in osteosarcoma, and to detect whether HULC affected osteosarcoma cells via interaction with miR-122.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

Human osteosarcoma cell line MG63 and human osteoblast cell line hFOB1.19 were purchased from the American Type Culture Collection (ATCC; Manassas, VA). OS-732 cell line was obtained from Orthopaedics Graduate School in Beijing Jishuitan Hospital. All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen). Cells were maintained in humidified 5% CO₂ incubator at 37°C and cultured for 2-4 days until confluence was reached. Subcultures were prepared using 0.05% trypsin solution (Sigma-Aldrich, St. Louis, MO) and seeded in 6- or 96-well culture plates.

2.2 | Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA) according to the manufacturer’s instructions. For detection of HULC and HNF4G levels, cDNA was synthesized by using mRNA Selective PCR kit (TaKaRa, Dalian, China), and the One Step SYBR®PrimeScript®PLUS RT-RNA PCR Kit (TaKaRa) were used for the qRT-PCR analysis. GAPDH level was tested and severed as an internal control for normalizing HULC and HNF4G levels. For miR-122 level detection, cDNA was performed by using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and qRT-PCR was performed by using the Taqman Universal Master Mix II (Applied Biosystems). miR-122 level was normalized to the level of U6. Fold changes were calculated by the classic 2^−ΔΔCt method.

2.3 | Transfection and generation of stably transfected cell lines

For suppressing the expression of HULC, two different sequences of short-hairpin RNA (shRNA) targeted HULC (GenePharma, Shanghai, China) was transfected into cells, and were respectively referred as to sh-HULC #1 and sh-HULC #2. For overexpressing or suppressing the expression of miR-122, miR-122 mimic, or miR-122 inhibitor (Life Technologies Corporation) were respectively transfected into cells. Mimic negative control (NC) and inhibitor NC were respectively transfected and used as controls. For the analysis of the HNF4G functions, the full-length HNF4G sequences and shRNA against HNF4G were constructed in pEX-2 and U6/GFP/Neo plasmids (GenePharma), respectively. And they were transfected into cells and referred as to pEX-HNF4G and sh-HNF4G. The plasmid carrying scrambled sequence or non-targeting sequence of short-hairpin RNA (shRNA) targeted HNF4G were respectively transfected and served as controls, referring p-EX and shNC. Transfections were performed by using the lipofectamine 3000 reagent (Life Technologies Corporation). At 48 h of transfection, the stably transfected cells were selected by the culture medium containing 0.5 mg/mL G418 (Sigma-Aldrich). After approximately 4 weeks, G418-resistant cell clones were established.

2.4 | Cell viability assay

Cell viability was detected by the classic trypan blue exclusion as previously described. In brief, cells were collected by 0.05% trypsin (Sigma-Aldrich), and then cell
suspension (1 × 10^5 cells) were stained with Trypan Blue solution (Sigma-Aldrich) which with final concentration of 0.04%. Living cells and dead cells were immediately counted under an inverted light microscope, and the viability calculation was made according to the following formula: viable cells (%) = (live cells/total cells) × 100.

2.5 | Apoptosis assay

Cell apoptosis analysis was performed using Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). Briefly, 1 × 10^5 cells were washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol. Fixed cells were then washed twice in PBS and stained in 200 µL Annexin V-FITC binding buffer containing 5 µL Annexin V-FITC, and 10 µL PI, in the presence of 50 µg/mL RNase A (Sigma-Aldrich). After incubation in the dark for 1 h at room temperature, the samples were analyzed using a FACS can (Beckman Coulter, Fullerton, CA). The data were analyzed by using FlowJo software (Treestar, Inc., San Carlos, CA).

2.6 | Migration and invasion assays

For migration assay, cells suspended in 200 mL of serum-free medium were seeded on 24-well Transwell culture chamber (8-mm pore-size filters; Corning, MA), and 600 mL of complete medium was added to the lower compartment. After incubation at 37°C for 48 h, traversed cells on the lower compartment were fixed with methanol, and counted under the microscope following by staining with 0.1% crystal violet (Sigma-Aldrich). The invasion behavior of was determined using 24-well Millicell Hanging Cell Culture inserts with 8 mm PET membranes (Millipore, Bedford, MA). Briefly, after the cells were treated for indicated condition, 5.0 × 10^5 cells in 200 µL serum-free DMEM medium were plated onto BD BioCoat™ Matrigel™ Invasion Chambers (8 µM pore size polycarbonate filters; BD Biosciences), while complete medium containing 10% FBS was added to the lower compartment. After processing the invasion chambers for 48 h (37°C, 5% CO2) in accordance with the manufacturer’s protocol, the non-invading cells were removed with a cotton swab; the invading cells were fixed in 100% methanol and then stained with crystal violet solution and counted microscopically. Five fields were randomly chosen from the upper and lower chamber. Relative migration/invasion (%) = (average cell number in lower chamber/average cell number in upper chamber) × 100.

2.7 | Reporter vectors constructs and luciferase reporter assay

The fragment from HULC containing the predicted miR-122 binding site was amplified by PCR and then cloned into a pmirGO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI) to form the reporter vector HULC-wild-type (HULC-wt). To mutate the putative binding site of miR-122 in the HULC, the sequence of putative binding site was replaced and was named as HULC-mutated-type (HULC-mt). Accordingly, the HNF4G 3′UTR that contains or excludes putative site of miR-122 was cloned into pmirGO Dual-luciferase miRNA Target Expression Vector to form HNF4G-wt and HNF4G-mt. Then the vectors and miR-122 mimic were co-transfected into cells, and the Dual-Luciferase Reporter Assay System (Promega) were used for testing the luciferase activity.

2.8 | Western blot

The protein used for western blotting was extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Basel, Switzerland). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer’s instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the Polyvinylidene Difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µL Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Hercules, CA).

2.9 | Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as mean ± SD. Statistical analyses were performed using Graphpad 6.0 statistical software (GraphPad Software Inc., San Diego, CA). P values were calculated using one-way analysis of variance (ANOVA). A P-value of <0.05 was considered to be statistically significant.

3 | RESULTS

3.1 | HULC was highly expressed in osteosarcoma cell lines

To explore the role of HULC in osteosarcoma cell lines, qRT-PCR was performed to examine the expression levels of HULC in osteoblast and osteosarcoma cells. The data of the
present study revealed that HULC expression was upregulated in osteosarcoma MG63 and OS732 cells compared to osteoblast hFOB1.19 cells ($P < 0.05$ or $P < 0.001$, Figure 1).

### 3.2 Suppression of HULC inhibited osteosarcoma cells viability, migration, invasion, and promoted apoptosis

OS-732 cells were separately transfected with two different sequence of shRNAs which were directly targeted HULC. qRT-PCR analysis was performed to detect the expression level of HULC after transfection. The expression level of HULC was significantly suppressed in OS-732 cells after treatment with sh-HULC#1 ($P < 0.01$) and sh-HULC#2 ($P < 0.001$), respectively, when compared to shNC (Figure 2A). Considering that sh-HULC#2 resulted in a much lower expression than sh-HULC#1, sh-HULC#2 was selected for use in the subsequent experiments. Also, MG63 cells were transfected with sh-HULC#2, and the transfected MG63 cells exhibited a significant decrease in HULC expression ($P < 0.01$, Figure 2B).

Cell viability of OS-732 and MG63 cells was determined following transfection with sh-HULC#2. Figures 2C and 2D demonstrated that suppression of HULC significantly inhibited the viability of OS-732 and MG63 cells when compared to shNC group ($P < 0.05$ or $P < 0.01$). Migratory and invasive capacities of OS-732 and MG63 cells were also determined. The results showed (Figures 2E-2I) that suppression of HULC significantly inhibited the migration and invasion of OS-732 and MG63 cells when compared to shNC ($P < 0.05$ or $P < 0.01$). Apoptosis assay was performed to determine the apoptotic rate of osteosarcoma cells. The results showed that suppression of HULC significantly promoted apoptosis ($P < 0.01$ or $P < 0.001$, Figures 2J-2K). Furthermore, results in western blotting demonstrated that Bcl-2 was downregulated, Bax was upregulated and two caspases (caspase-3 and caspase-9) were cleaved after sh-HULC#2 transfection (Figures 3A-3D).

### 3.3 HULC acted as an endogenous sponge for miR-122 in OS-732 cells

Given that the cross-regulation between miRNA and lncRNA has gained many interest in recent years, and miR-122 has been revealed as a tumor suppressor in osteosarcoma. Thus, in this study we detect the relationship between HULC and miR-122 in OS-732 cells. Figure 4A demonstrated that suppression of HULC significantly ($P < 0.001$) upregulated the expression of miR-122, indicating the expression of miR-122 was negatively regulated by HULC. Luciferase reporter assay revealed that the luciferase activity was significantly reduced in OS-732 cells when co-transfected with HULC-wt and miR-122 mimic compared with the control group ($P < 0.05$, Figure 4B). However, the luciferase activity revealed no significant difference in OS-732 cells co-transfected with HULC-mt and miR-122 mimic when compared with the control group. The data indicated that HULC was able to directly bind to miR-122, and HULC functioned as an endogenous sponge for miR-122 in OS-732 cells.

### 3.4 Suppression of HULC inhibited OS-732 cells viability, migration, invasion, but promoted apoptosis via upregulation of miR-122 in OS-732 cells

Next, the effects of miR-122 on HULC-modulated cell proliferation, migration, invasion, and apoptosis of OS-732 cells were investigated. Results identified that HULC silence-induced decreases in cell viability, migration and invasion, and HULC silence-induced increase in apoptotic cell rate, downregulation in anti-apoptotic protein (Bcl-2), and upregulations in pro-apoptotic proteins (Bax and cleaved caspase3/9), were all attenuated when miR-122 was knocked down ($P < 0.05$, $P < 0.01$ or $P < 0.001$, Figures 5A-5G). These data suggested that suppression of HULC inhibited OS-732 cells growth and metastasis via upregulation of miR-122.

### 3.5 HNF4G was a target of miR-122

To further study the mechanism via which miR-122 mediated osteosarcoma cells growth and metastasis, the regulation between miR-122 and HNF4G were detected. As shown in Figure 6A, both the mRNA and protein levels of HNF4G were low expressed in miR-122 overexpressing-cells ($P < 0.01$, **Figure 6A**). Further study revealed that HNF4G expression was downregulated in osteosarcoma MG63 and OS732 cells compared to osteoblast hFOB1.19 cells ($P < 0.05$ or $P < 0.001$, Figure 6A).}

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**FIGURE 1** HULC was highly expressed in osteosarcoma cell lines. The expression level of HULC in hFOB1.19, MG63 and OS-732 cells were detected by qRT-PCR. $n = 3$. Data represent the mean ± SD. *$P < 0.05$, ***$P < 0.001$ (ANOVA).
while were highly expressed in miR-122 suppressing-cells (P < 0.001), implying HNF4G was negatively regulated by miR-122. Further, we validated that miR-122 whether can bind HNF4G in OS-732 cells. Luciferase reporter assay revealed that the luciferase activity was significantly reduced in OS-732 cells when co-transfected with HNF4G-wt and miR-122 mimic compared with the control group (P < 0.001, Figure 6B). However, no significant difference in OS-732 cells were found when co-transfected with HNF4G-mt and miR-122 mimic when compared with the control group. The data indicated that HNF4G might be a target of miR-122 in OS-732 cells.

3.6 | miR-122 overexpression inhibited OS-732 cells viability, migration, invasion, but promoted apoptosis via downregulation of HNF4G in OS-732 cells

The effects of miR-122 and HNF4G on the proliferation, migration, invasion, and apoptosis of OS-732 cells were investigated. As results shown in Figures 7A, 7B, both the mRNA and protein levels of HNF4G were downregulated by transfection with miR-122 mimic (P < 0.01), while these downregulations were abolished by addition of pEX-HNF4G (P < 0.05 or P < 0.01). Besides, we found that miR-122
FIGURE 3  Suppression of HULC downregulated anti-apoptotic protein expression while upregulated pro-apoptotic protein expressions. (A-D) OS-732 and MG63 cells were transfected with sh-HULC#2, and then the protein expressions of apoptosis-related factors were determined. 

\( n = 3 \). Data represent the mean ± SD. **P < 0.01; ***P < 0.001 (ANOVA)

FIGURE 4  HULC acted as an endogenous sponge for miR-122. (A) OS-732 cells were transfected with sh-HULC#2, and then the expression level of miR-122 was detected by qRT-PCR. (B) The reporter vectors HULC-wild-type (HULC-wt) or HULC-mutated-type (HULC-mt) were co-transfected with miR-122 mimic into cells, and the Dual-Luciferase Reporter Assay System were used for testing the luciferase activity. 

\( n = 3 \). Data represent the mean ± SD. *P < 0.05; ***P < 0.001 (ANOVA)
overexpression significantly reduced cell viability, migration, and invasion (all $P < 0.01$), but increased apoptosis ($P < 0.001$, Figures 7C-7I). More importantly, HNF4G overexpression alleviated miR-122 overexpression-induced decreases in cell viability, migration, and invasion ($P < 0.05$ or $P < 0.01$), as well as alleviated miR-122 overexpression-induced increase in apoptosis ($P < 0.01$). The aforementioned data suggested that miR-122 inhibited OS-732 cells proliferation, migration, and invasion through downregulations of HNF4G.

3.7 PI3K/AKT, JAK/STAT, and Notch pathways were involved in miR-122-mediated OS-732 cells

Western blot analysis was performed to analyze the role of miR-122 in PI3K/AKT, JAK/STAT, and Notch pathways in OS-732 cells. The results demonstrated that overexpression of miR-122 inactivated the PI3K/AKT, JAK/STAT, and Notch pathways, as evidenced by the
downregulations of p-PI3K, p-AKT, p-JAK1, p-STAT3, Jagged-1, Notch1, and Hes-1 (P < 0.01 or P < 0.001, Figures 8A and 8B). miR-122 suppression impacted these proteins resulted in a completely opposite way, that all these proteins were upregulated after miR-122 inhibitor transfection (P < 0.01 or P < 0.001). Moreover, knocking down HNF4G by using its specific shRNA alleviated miR-122 inhibitor induced upregulations of these proteins (P < 0.01 or P < 0.001). These data indicated that PI3K/AKT, JAK/STAT, and Notch pathways might be importance in the miR-122-mediated OS-732 cells.

**DISCUSSION**

LncRNAs play important roles in controlling of cell growth and metastasis, and they are proposed as novel targets for the prevention of oncogenesis. Hence, identification of cancer associated LncRNAs and investigating their significance will be helpful for suppressing tumor growth and metastasis. Recently, several studies have focused the functional role of LncRNA HULC on cancers, and reported it as a predictor for various carcinomas as its expression level was associated with worse survival and high risk of cancer metastasis. 

Du et al in their study demonstrated that increased HULC levels in liver cancer cells lead to a higher proliferation rate and tumor growth. Zhao et al, indicated that HULC promoted proliferation and invasion while inhibited apoptosis in gastric cancer cells. Peng et al, revealed that HULC was upregulated in human pancreatic cancer tissues and knockdown of HULC inhibited tumor cells proliferation. These previous literatures suggested a carcinogenic role of HULC in cancers by modulation tumor cells growth and metastasis.

The functional involvement of HULC in the pathogenesis of osteosarcoma has also been reported. By qRT-PCR, HULC level in osteosarcoma tissues and cell lines were quantified, and higher expression of HULC were observed in osteosarcoma tissues and cell lines when compared to normal controls. In vivo data from 33 osteosarcoma patient tissues demonstrated that high levels of HULC were associated with low survival rates in osteosarcoma patients, both in terms of overall and event-free survival implying HULC could be a potential prognostic biomarker in this cancer. Furthermore, it has been reported that decreased expression of HULC markedly suppressed osteosarcoma U2OS cells proliferation, migration, and invasion. In this study, the anti-growth and anti-metastasis roles of HULC suppression in another two osteosarcoma cells (OS-732 and MG63) were also identified, as HULC silencing could significantly suppress OS-732 and MG63 cells viability, migration, and invasion, and could improve apoptosis.

HULC has been demonstrated to act as a miRNA sponge that binds to and reduces the expression of a number of miRNAs. Considering miR-122 has been revealed as a tumor suppressor in osteosarcoma, we detected the cross-regulation between HULC and miR-122 in order to reveal the underlying mechanisms via which HULC promoted osteosarcoma cells growth and metastasis. We for the first time discovered that HULC functioned as an endogenous sponge for miR-122. Moreover, miR-122 suppression could alleviate HULC silence-induced the inhibitions in osteosarcoma cells growth and metastasis. Therefore, we inferred HULC silence suppressed osteosarcoma at least in part via upregulation of miR-122.

To better understand the mechanism, we further detected the cross-regulation between miR-122 and HNF4G in OS-732 cells. In bladder cancer and esophageal squamous cell carcinoma, HNF4G has been reported as an oncogene as it...
could increase tumor cell growth and invasion. In the study of Coulouarn et al, miR-122 showed a strong positive correlation with the expression level of numerous liver-enriched transcription factors essential for hepatocytic differentiation, such as HNF1A, HNF3A, HNF3B, HNF4A, HNF4G, and HNF6. In contrast, our study demonstrated a negative correlation of miR-122 with the expression of HNF4G. Besides, the carcinogenic role of HNF4G was found in OS-732 cells, as HNF4G overexpression significantly abolished miR-122 overexpression-induced decrease in cell growth and metastasis. Together with our findings that HULC worked as a ceRNA for miR-122, these findings evidenced that HULC/miR-122/HNF4G axis played important roles in osteosarcoma.

In osteosarcoma cells, activation of PI3K/AKT pathway leads to promoted proliferation and the suppressed apoptosis. Besides, this pathway has been found to play a central role in the regulation of metastasis. In addition to PI3K/AKT, JAK/STAT, and Notch pathways were also discovered as osteosarcoma signaling pathways and their specific role in osteosarcoma has been summarized in a recent
review, in which the activation of JAK/STAT and Notch pathways has been demonstrated to promote the development of osteosarcoma. Interestingly, complex interactions between the components of the PI3K/AKT pathway, and with components of JAK/STAT and Notch pathways, appear to be essential for facilitating and fuelling meningioma progression.

Our study demonstrated that miR-122 suppression activated PI3K/AKT, JAK/STAT, and Notch pathways, and while HNF4G blocked this activation, which indicating miR-122-mediated osteosarcoma cells growth and metastasis possibly via regulation of these three signaling pathways.

In conclusion, our findings suggested a carcinogenic role of HULC in osteosarcoma, as OS-732 and MG63 cells growth and metastasis were suppressed by HULC silence. HULC functioned as a ceRNA for miR-122, to prevent HNF4G from degradation by miR-122. HULC/miR-122/HNF4G axis might play pivotal effects on the development of osteosarcoma, and PI3K/AKT, JAK/STAT, and Notch pathways might be involved in this procedure. This study provided evidence that HULC could serve as a potential biomarker and therapeutic target for osteosarcoma.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

ORCID

Yang Wang http://orcid.org/0000-0003-2800-0566

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