MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2018; 24: 5754-5760
DOI: 10.12659/MSM.910220

Received: 2018.03.26
Accepted: 2018.04.09
Published: 2018.08.18

Long Non-Coding RNA HULC Promotes Progression of Bone Neoplasms

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: Departmental sources

Background: Bone neoplasms are common in humans and have high lethality. Recently, great progress has been made in understanding the pathophysiological mechanisms, but little is known about the molecular and genetic networks involved.

Material/Methods: qRT-PCR assays were conducted to detect the expression levels of lncRNA HULC in various cell lines. MTT assay, Transwell assay, and wound-healing assay were performed to investigate the proliferation speed, invasion ability, and migration ability of each cell line, respectively. Western blot analysis was also done to assess the expression level of EMT-related factors. Statistical analysis was performed using the *t* test, Kaplan-Meier method, and log-rank test.

Results: Compared to the human normal bone cell line, we found lncRNA HULC was over-expressed in all 6 bone neoplasm cell lines, and we finally chose HT1080 and Saos-2 cell lines, which possessed the highest lncRNA HULC expression level, for the subsequent studies. We then observed that the expression level of lncRNA HULC was negatively correlated with overall survival rate of bone neoplasm patients, which means that lncRNA HULC has prognostic value in patients with bone neoplasms. Thus, we assessed the influence of lncRNA HULC down-regulation on proliferation, invasion, and migration abilities of bone neoplasm cells, and found a significant decrease in these abilities. Finally, we found that down-regulating lncRNA HULC led to decreased expression of EMT-related factors in bone neoplasm cells.

Conclusions: LncRNA HULC can promote the tumorigenesis of bone neoplasms through increasing the proliferation, invasion, and migration abilities and the expression level of EMT-related factors.

MeSH Keywords: Bone Neoplasms • Cell Proliferation • Neoplasm Invasiveness

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/910220

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Background

Bone neoplasms, which are tumors that occur in the bones or other affiliated tissues, are common in humans. In 2013, the WHO [1] divided bone neoplasms into 11 types: chondrogenic tumor, osteogenic tumor, fibrogenic tumor, fibrohistiocytic neoplasm, osteoclastic giant cell-rich tumor, notochordal tumor, vascular tumor, myogenic tumor, lipogenic tumor, tumor of undefined neoplastic nature, and miscellaneous tumor. Alternatively, bone neoplasms can also be divided into 2 types – benign and malignant – according to whether they possess the abilities of infiltration and metastasis [2]. Benign tumors are generally not harmful to human health and can be cured through surgery. Because of their invasive growth and metastatic ability, malignant tumors have potential recurrence after surgery and thus cannot be completely cured, which leads to a high mortality rate. Although great progress has recently been made in understanding the pathophysiological mechanisms involved in bone neoplasms [3–5], the overall survival time of bone neoplasm patients has not changed significantly. Therefore, a deeper understanding of the molecular and genetic networks controlling the initiation and progression of bone neoplasms is imperative.

Long non-coding RNAs (lncRNAs) are recently discovered transcripts of the genome and are longer than 200 nucleotides [6]. The discovery of lncRNAs is an important research development in molecular biology, epigenetics, and genomics. LncRNA cannot be translated into proteins and were initially thought to be the spurious transcriptional noise, but there was a large amount of research evidence indicating that the disorder adjustment and expression of lncRNAs contribute to the occurrence of diseases such as cancer [7–9], immunity [10], and neurological dysfunction [11]. In recent years, researchers have found multiple lncRNAs whose expression levels in bone neoplasms were changed [12–15], but few studies have elucidated the function and mechanism of these lncRNAs. HULC was such a long non-coding RNA, which was first discovered in liver cancer [16], and had a high expression level, so it was named HULC. Studies indicated that lncRNA HULC was related to colorectal cancer [17], pancreatic cancer [18], and even osteosarcoma [19,20].

In the present study, we found lncRNA HULC was commonly over-expressed in bone neoplasms and its expression level was negatively correlated with overall survival rate of bone neoplasm patients. We further detected the influence of lncRNA HULC down-regulation on proliferation, invasion, and migration abilities, and expression of EMT-related factors of bone neoplasm cells, and found a significant decrease in these parameters. We concluded that lncRNA HULC can promote the tumorigenesis of bone neoplasms through elevating the proliferation, invasion, and migration abilities and the expression level of EMT-related factors.

Material and Methods

Cell lines and culture

The cell lines (OUMS-27, HT1080, U20S, Saos-2, MG-63, XPTS-, and hFOB) used in this study were purchased from ATCC (American Type Culture Collection, USA). The cell lines were regularly cultured in DMEM at 37°C and with 10% fetal bovine serum (FBS, Gibco, USA) added and 5% CO_2 supplemented.

RNA Extraction and qRT-PCR

The total RNA extraction reagent was RNAiso Reagent (TaKaRa, USA), following the manufacturer's instructions. The qRT-PCR was performed to detect the expression level of lncRNA HULC using the SYBR® Premix ExTaq™ II kit (TaKaRa, USA) and the corresponding primers were: The forward primer of lncRNA HULC was 5'-ACTCTGAAGTAAAGGCCGGA-3'; The reverse primer of lncRNA HULC was 5'-TGCCAGGAAACTTCTTGCTTG-3'; The forward primer of GAPDH was 5'-GTCAACGGATTTGGTCTGTATT-3'; The reverse primer of GAPDH was 5'-AGTCTTCTGGGTGGCAGTGAT-3'. GAPDH was the endogenous control of qRT-PCR assays, and the calculation method of qRT-PCR was $2^{-\Delta\Delta Ct}$.

Construction of the shRNA-HULC vector

ShRNA-HULC vector was constructed to down-regulate the expression level of lncRNA HULC, and the steps were: Synthesizing the specific oligonucleotides 5'-GCCTTTACAAGGGAATGAAGA-3' for targeting lncRNA HULC (Genewiz, China), ligating the specific target sequence with AAV plasmid, and the shRNA-HULC vector was successfully constructed.

MTT assay

MTT assay was performed to reflect the proliferation speed based on colorimetric method, and the steps were: Inoculating 1000–10 000 cells into 96-well plates with 200 µl culture; 3 to 5 days later, adding 10 µl MTT solution (5 mg/ml, pH=7.4) into each well for 4 h, and then terminating the reaction; After sweeping away the supernatant, adding 100 µL DMSO into each well and shaking for 10 min, and then measuring the absorbance using an enzyme-linked immune detector at 570 nm. Time was the abscissa and absorbance was the ordinate used to plot the cell proliferation curve.

Transwell assay

The invasion ability of cells was detected by Transwell invasion assay. The detail steps were: Collecting cells after transfection for 24 h; Suspending 5×10^4 cells into 100 µL of serum-free medium; Seeding the medium above to the upper chamber precoated with Matrigel Matrix (BD Biosciences); Adding 600 µL

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medium which contained 10% FBS into the lower chamber; Incubating for 24 h; Removing the cells that did not invade through the membrane using a cotton swab; Fixing the cells on the bottom surface of the membrane by 4% paraformaldehyde for 10 min; Staining the cells with 0.4% crystal violet solution; and Imaging the invaded cells by digital microscopy. The invasion ability was determined by the number of invaded cells.

Wound-healing assay

The migration ability of cells was detected by wound-scratch assay. The detail steps were: Seeding cells with concentration of 3×10^5 cells/well in a 6-well plate; Exposing to various treatments accordingly; Using a sterilized pipette tip to make a straight scratch in each well after cells reached 100% confluence; and Capturing images at every indicated time point by digital microscopy. The migration ability was determined by the wound closure area.

Western blot analysis

Western blot assay was conducted with the following steps: Lysing the stimulated cells by RIPA buffer purchased from Sinogene Biotechnology, Beijing, China; Separating 20–100 μg lysates above on SDS PAGE gels with concentration of 8–12%; Transferring to a PVDF membrane; Incubating the membrane with primary antibodies specific for Snail, E-cadherin, Vimentin, and ZEB1(1: 5000; Cell Signaling Technology) at 4°C overnight; Incubating with HRP-conjugated secondary antibodies; Detecting the bound antibodies by ECL substrate; and normalizing protein levels to GAPDH (1: 10 000; Cell Signaling Technology).

Statistical analysis

SPSS version 20.0 software was used for statistical analysis. All data are presented as the mean ±SD, and the differences between samples were assessed by *t* test. Kaplan-Meier analysis was performed to plot the overall survival curves, and the differences of survival rates between samples were indicated by log-rank test. The difference was considered statistically significant only if the P value was lower than 0.05.

Results

lncRNA HULC is over-expressed in multiple bone neoplasm cell lines

We performed qRT-PCR to detect the expression level of lncRNA HULC in 6 bone neoplasm cell lines (chondrosarcoma OUMS-27, fibrosarcoma of bone HT1080, osteosarcoma U20S, Saos-2, osteoid osteoma MG-63, and hemangioma XPTS-1) and 1 human normal bone cell line (hFOB) to assess whether it was commonly elevated in bone neoplasms. As shown in Figure 1, the hFOB cell line possessed the lowest lncRNA HULC expression level. Compared to the hFOB cell line, the lncRNA HULC expression level in all 6 bone neoplasm cell lines was significantly increased, which suggested that lncRNA HULC was commonly over-expressed in bone neoplasms. In addition, we selected HT1080 and Saos-2 cell lines for use in subsequent experiments because they had the highest lncRNA HULC expression levels.

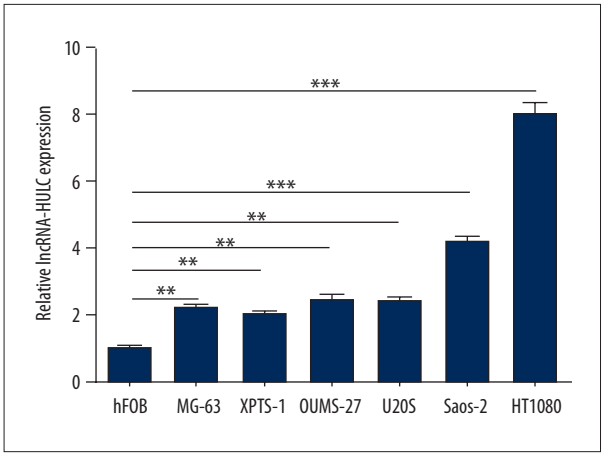

Figure 1. Relative lncRNA HULC expression level in multiple bone neoplasm cell lines. The lncRNA HULC expression level of 6 bone neoplasm cell lines and 1 human normal bone cell line was detected by qRT-PCR and normalized to GAPDH expression. ** P<0.01, *** P<0.001.

lncRNA HULC expression level is negatively correlated with overall survival rate of bone neoplasm patients

We used Kaplan-Meier method and log-rank test to find the correlation between lncRNA HULC expression and overall survival rate of bone neoplasm patients. Figure 2 shows a significantly negative correlation between lncRNA HULC expression level and overall survival rate of bone neoplasm patients, which indicates that lncRNA HULC had prognostic value for bone neoplasm patients, and further study was indeed necessary.

lncRNA HULC down-regulation inhibits the proliferation of bone neoplasm cells

We then further researched the influence of lncRNA HULC on the proliferation of bone neoplasm cells by MTT assays. The result shown in Figure 3A and 3B show that we successfully down-regulated the expression of lncRNA HULC in both HT1080 and Saos-2 cell lines. Figure 3C and 3D are growth curves of HT1080 and Saos-2 cell lines, respectively, which both indicated that down-regulating the expression level of lncRNA HULC inhibited the proliferation of bone neoplasm cells.

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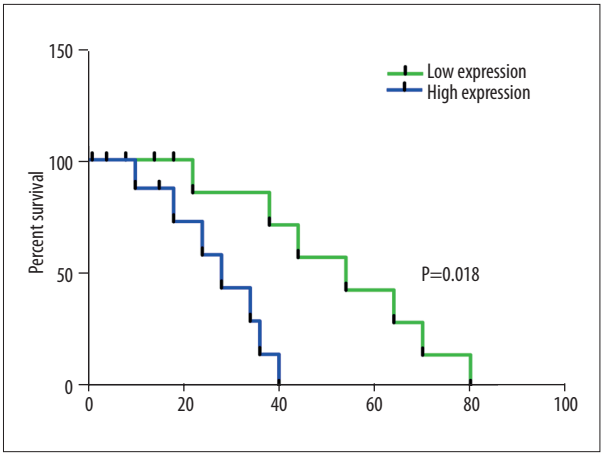

Figure 2. Kaplan-Meier survival curves of bone neoplasm patients. Patients with high lncRNA HULC expression level had significantly lower overall survival than those with low expression level (P<0.05).

lncRNA HULC down-regulation weakens the invasion and migration abilities of bone neoplasm cells

Invasion and metastasis of cancer cells are the most common causes of cancer-related deaths. Therefore, we further performed Transwell assay and wound-healing assay to investigate the effect of lncRNA HULC on the invasion and migration abilities of bone neoplasm cells. The grey value in Figure 4A shows that, compared to the blank (cell lines transfected with nothing) and empty vector groups, the number of cells invading through the membrane was sharply decreased when HULC was down-regulated in HT1080 and Saos-2 cell lines, which means that down-regulating the expression level of lncRNA HULC weakens the invasion ability of bone neoplasm cells. Figure 4B and 4C show that the wound-healing area was clearly smaller when HULC was down-regulated in HT1080 and Saos-2 cell lines, which means that down-regulating the

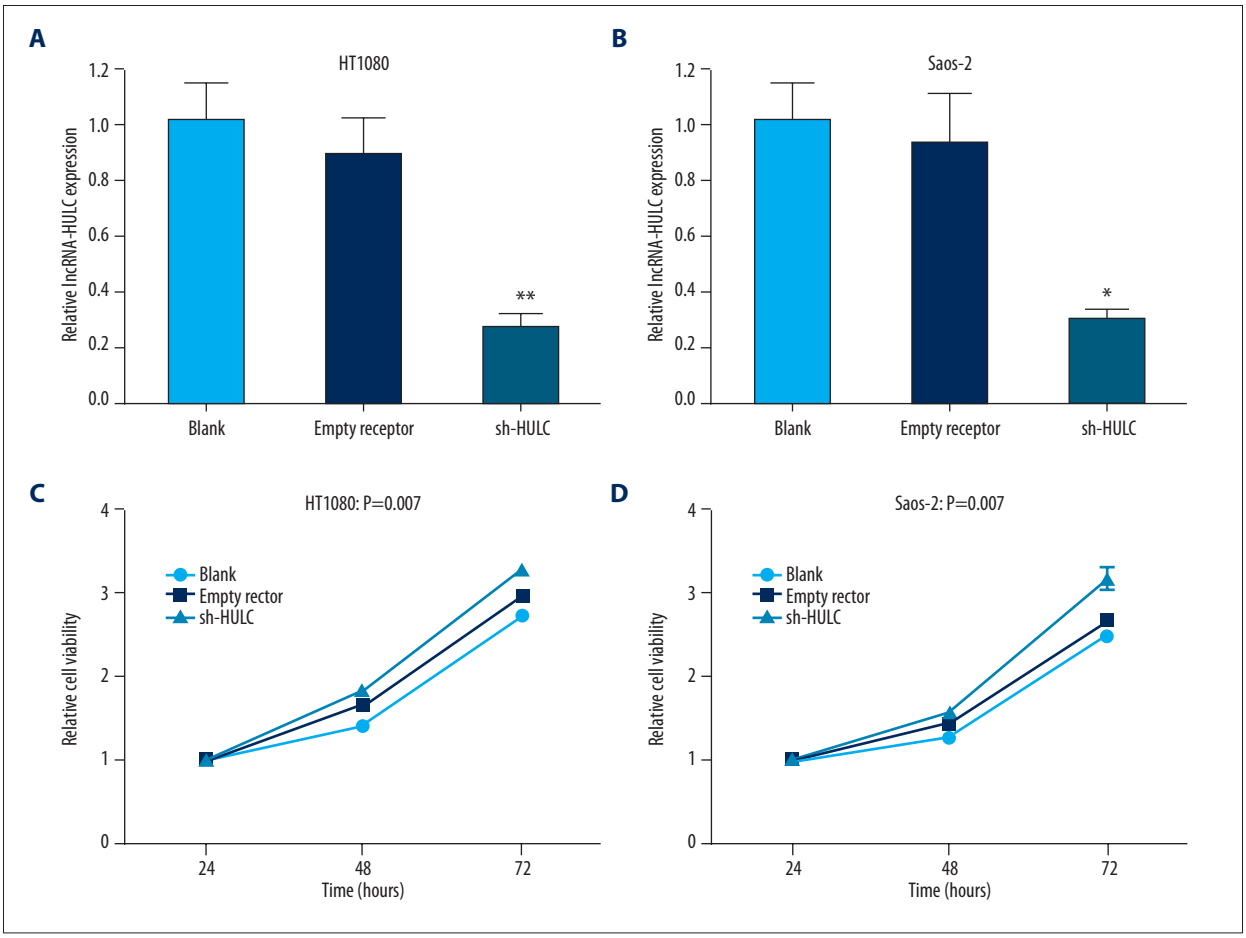

Figure 3. LncRNA HULC down-regulation inhibits the proliferation of bone neoplasm cells. (**A**) lncRNA HULC expression level was clearly down-regulated by transfecting with specific sh-HULC compared to the blank and empty vector groups in the HT1080 cell line (P<0.01); (**B**) lncRNA HULC expression level was clearly down-regulated by transfecting with specific sh-HULC comparing to the blank and empty vector groups in Saos-2 cell line (P<0.05); (**C**) Growth curve of HT1080 cell line by performing MTT assay (P=0.007); (**D**) Growth curve of Saos-2 cell line by performing MTT assay (p=0.007).

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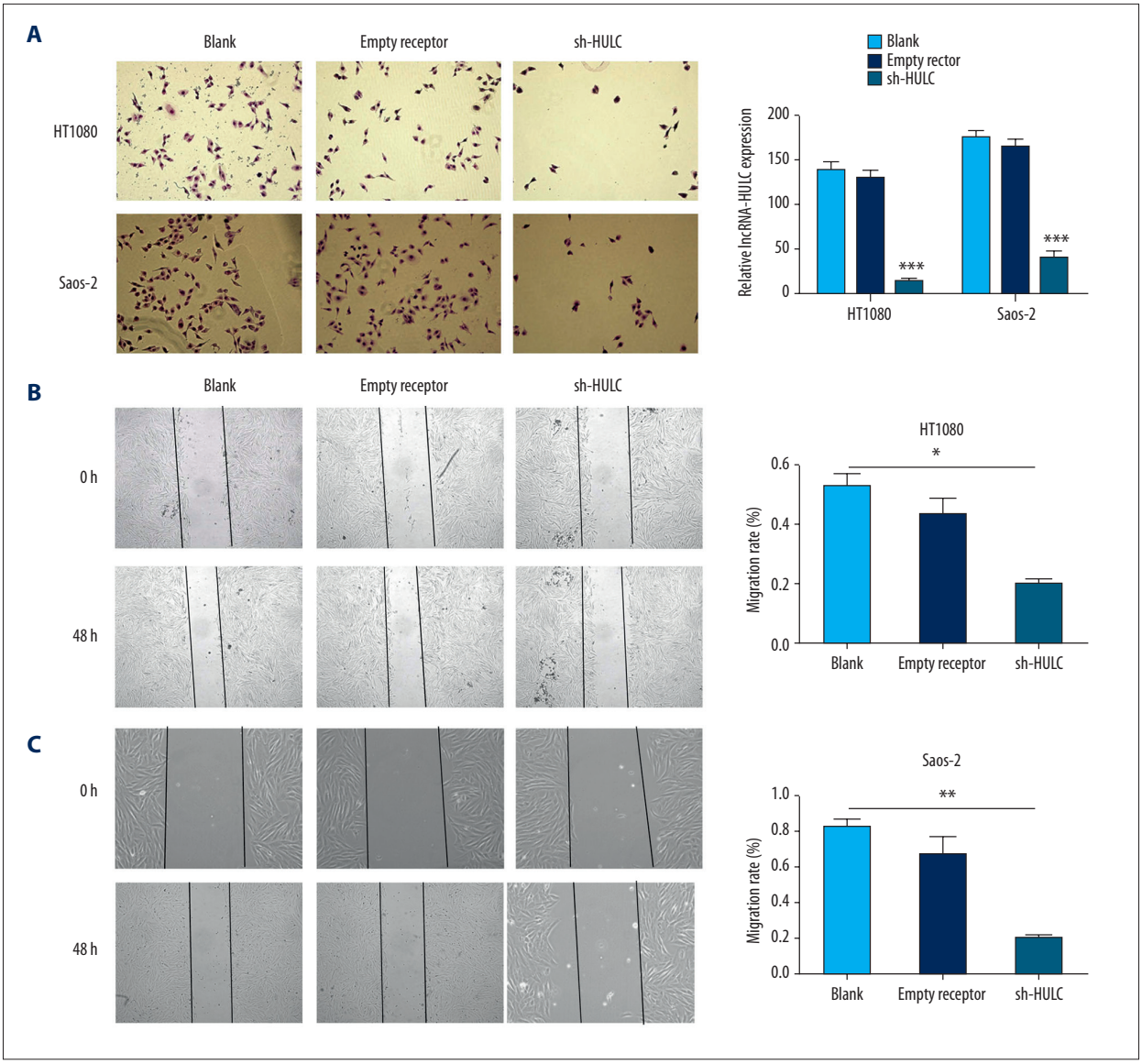

Figure 4. lncRNA HULC down-regulation weakens the invasion and migration abilities of bone neoplasm cells. (**A**) Transwell assays of HT1080 and Saos-2 cell lines, in which the grey value indicates that lncRNA HULC down-regulation sharply decreased the number of cells invading through the membrane (P<0.001); (**B**) Wound-healing assays of HT1080 cell line, in which the grey value indicates that lncRNA HULC down-regulation sharply decreased the wound-healing area (P<0.05); (**C**) Wound-healing assays of Saos-2 cell line, in which the grey value indicates that lncRNA HULC down-regulation sharply decreased the wound-healing area (P<0.01).

expression level of lncRNA HULC weakens the migration ability of bone neoplasm cells.

lncRNA HULC down-regulation leads to decreased expression of EMT-related factors in bone neoplasm cells

We further detected the expression levels of EMT (epithelial-mesenchymal transition)-related factors using Western blot analysis to explore the mechanism by which lncRNA HULC affects tumorigenesis of bone neoplasms. As shown in Figure 5A and 5B, the expression level of EMT-related factors, including Snail, E-cadherin, Vimentin, and ZEB1, was notably decreased in HT1080 and Saos-2 cell lines when down-regulating the expression level of lncRNA HULC, which indicates that lncRNA HULC promotes the tumorigenesis of bone neoplasms through elevating the expression level of EMT-related factors.

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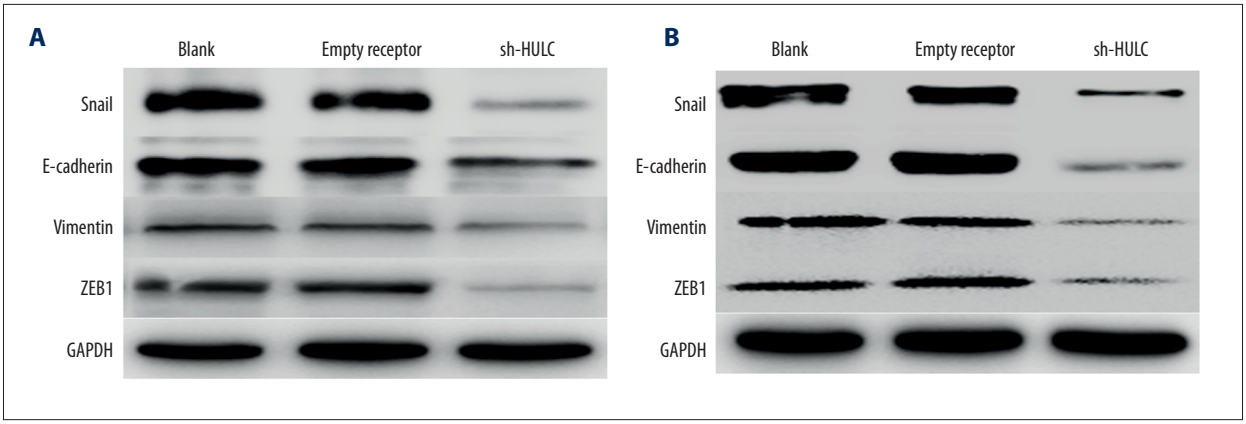

Figure 5. lncRNA HULC down-regulation leads to decreased expression of EMT-related factors in bone neoplasm cells. (**A**) The expression levels of EMT-related factors in HT1080 cell line as assessed by Western blot; (**B**) The expression levels of EMT-related factors in Saos-2 cell line as assessed by Western blot.

Discussion

Long non-coding RNA (lncRNA) is a novel transcript whose length is longer than 200 nucleotides of the genome. Because it lacks the ability of translating to protein, lncRNA was initially thought to be spurious transcriptional noise. Recently, there were more and more studies showing the important role of lncRNA in the occurrence of cancer, immunity, and neurological dysfunction, as well as in bone neoplasms, but only a few studies had elucidated the function and mechanism of lncRNA involved in bone neoplasms.

Bone neoplasms have various types depending on the origin of disease, and osteosarcoma is the most-studied type. Multiple lncRNAs were reported to be involved in osteosarcoma, such as UCA1 [21,22], HOTAIR [23], FENDRR [24], and MALAT1 [25–28]. Taking MALAT1 as an example, Dong et al. [29] found it was over-expressed in human osteosarcoma tissues and the expression level was positively correlated with lung metastasis of osteosarcoma. Taniguchi et al. [30] further validated that MALAT1 promotes proliferation, invasion, and metastasis through the PI3K/AKT signaling pathway, and concluded that MALAT1 was a potential prognostic marker for osteosarcoma patients. There have been few studies on the relationship between lncRNA and other bone neoplasm types, such as chondrosarcoma [31], fibrosarcoma of bone, osteoid osteoma, and hemangioma.

lncRNA HULC was first discovered in liver cancer [16], and had a high expression level, so it was named HULC. Studies indicated that lncRNA HULC was related to colorectal cancer [17], pancreatic cancer [18], and even osteosarcoma [19,20,32,33]. Sun et al. [34] reported that lncRNA HULC was over-expressed in osteosarcoma tissues and cell lines, and the expression level had a significant correlation with clinical stage, distant metastasis, and overall survival. Down-regulating the expression level of lncRNA HULC inhibited the proliferation, invasion, and metastasis of osteosarcoma cells, which suggested that lncRNA HULC is a prognostic marker of osteosarcoma patients.

Therefore, we assessed the expression level of lncRNA HULC in 6 bone neoplasm cell lines of different types, and found they were commonly increased compared to the normal expression level. We then observed that the expression level of lncRNA HULC was negatively correlated with overall survival rate of bone neoplasm patients, which means that lncRNA HULC has prognostic value. Thus, we further detected the influence of lncRNA HULC on proliferation, invasion, and migration abilities of bone neoplasm cells, and found significant decreases when down-regulating the expression level of lncRNA HULC.

Conclusions

We found that down-regulating lncRNA HULC decreased the expression of EMT-related factors in bone neoplasm cells. The results presented above suggest that lncRNA HULC can promote the tumorigenesis of bone neoplasms through increasing the proliferation, invasion, and migration abilities and the expression level of EMT-related factors.

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