LINK-A lncRNA functions in the metastasis of osteosarcoma by upregulating HIF1α

BOMING ZHAO[1], KEBIN LIU[2] and LIN CAI[1]

[1]Department of Orthopaedic Surgery, Zhongnan Hospital Affiliated to Medical College of Wuhan University, Wuhan, Hubei 430071; [2]Department of Orthopaedic Surgery, First People's Hospital of Jingzhou, Jingzhou, Hubei 434000, P.R. China

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Abstract. Long intergenic non-coding RNA for kinase activation (LINK-A) long non-coding RNA (lncRNA) is an oncogenic lncRNA in triple-negative breast cancer. The involvement of LINK-A lncRNA in other diseases is unknown. The present study aimed to investigate the possible involvement of LINK-A lncRNA in osteosarcoma. The results demonstrated that plasma levels of LINK-A lncRNA were significantly higher in patients with metastatic osteosarcoma (MO) compared with healthy controls and patients with non-metastatic osteosarcoma (NMO). LINK-A lncRNA overexpression significantly promoted cancer cell migration and invasion in osteosarcoma cell lines MG-63 and U20S. Upregulated expression of hypoxia-inducible factor 1α (HIF1α) was observed in cancer cells following LINK-A lncRNA overexpression. Exogenous HIF1α treatment did not significantly affect the expression of LINK-A lncRNA in cancer cells, whereas treatment with an HIF1α inhibitor significantly attenuated the effects of LINK-A lncRNA overexpression on cancer cell migration and invasion. Based on the results it was concluded that LINK-A lncRNA participated in the metastasis of osteosarcoma by upregulating HIF1α; upregulation of LINK-A lncRNA may serve as a potential diagnostic biomarker for patients with MO but not in those with NMO.

Introduction

Tumor metastasis is a major challenge in the treatment of cancer (1). With the advances in cancer treatment techniques, including surgery and targeted therapies, treatment outcomes of patients with non-metastatic tumors have been improved significantly in the past decades (2,3). However, the survival of patients with tumor metastasis is still poor owing to the lack of a radical treatment strategy (1). Osteosarcoma is a type of bone cancer that mainly affects children, adolescents, and young adults (4). Although the incidence rate is low, osteosarcoma is still a major cause of cancer-related mortalities owing to its aggressive nature (4). It has been reported that ~20% of patients with osteosarcoma were diagnosed with tumor metastasis (5), resulting in a higher mortality rate.

Beside mRNAs that encode protein products, the human genome also transcribes functional non-coding RNAs that serve crucial roles in both physiological and pathological processes (6). A growing body of literature has demonstrated that long non-coding RNAs (lncRNAs), a subgroup of non-coding RNAs that are >200 nucleotides long, are key players in human diseases (7), including certain types of cancers (8). Long intergenic non-coding RNA for kinase activation (LINK-A) lncRNA is a known oncogenic lncRNA in triple-negative breast cancer (9). As one of the subunits of the heterodimeric transcription factor hypoxia-inducible factor 1 (HIF1), HIF1α responds to hypoxia during cancer development and participates in the regulation of tumor invasion of various types of human malignancies (10). HIF1α in some cases may participate in cancer biology through interactions with lncRNAs (11,12). In the progression of triple-negative breast cancer, LINK-A lncRNA activates normoxic HIF1α to promote cancer development (9). Therefore, it is reasonable to hypothesize that the interaction between LINK-A lncRNA and HIF1α may also participate in osteosarcoma. In the present study, LINK-A lncRNA was demonstrated to function in the metastasis of osteosarcoma possibly by upregulating HIF1α.

Materials and methods

Cell line and human materials. MG-63 and U2OS human osteosarcoma cell lines were bought from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultivated with ATCC-formulated Eagle's Minimum Essential Medium (cat. no. 30-2003; ATCC) supplemented with 10% fetal bovine serum (FBS; Sangon Biotech Co, Ltd., Shangai, China) in an incubator (37˚C; 5% CO_2). When required, cells were treated with HIF1α (cat. no. 776-826; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 10, 20 and 40 ng/ml for 24 h or with the HIF inhibitor LW6 (cat. no. S8441; Selleck

Correspondence to: Dr Lin Cai, Department of Orthopaedic Surgery, Zhongnan Hospital Affiliated to Medical College of Wuhan University, 169 East Lake Road, Wuchang, Wuhan, Hubei 430071, P.R. China
E-mail: cailin969@163.com

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Chemicals, Shanghai, China) at 10 ng/ml for 24 h prior to further experiments.

Plasma samples were obtained from 62 patients with osteosarcoma and 48 healthy volunteers who were admitted to Zhongnan Hospital Affiliated to Medical College of Wuhan University (Wuhan, China) between May 2015 and January 2018. Inclusion criteria were: i) Patients who were diagnosed with osteosarcoma through pathological examination; ii) patients who were diagnosed and treated for the first time; iii) patients who were willing to join the study. Exclusion criteria were: i) Patients with multiple diseases; ii) patients who received treatment within 90 days before admission. Among the 62 patients with osteosarcoma, distant tumor metastasis was observed in 28 cases, and these patients were classified into the metastatic osteosarcoma (MO) group based on imaging findings. The remaining 34 patients were classified into the non-metastatic osteosarcoma (NMO) group. The patient group included 34 males and 28 females, aged between 12 and 44 years (mean, 27.4±4.5 years). The healthy control group included 28 males and 20 females, aged between 15 and 45 years (mean, 28.8±4.3 years). This study was approved by the Ethics Committee of Zhongnan Hospital Affiliated to Medical College of Wuhan University; all participants signed informed consent. Clinicopathological characteristics of patients included in the study are available in Table I

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from plasma (1 ml) or MG-63 and U2OS cells ($3x10^4$ cells/ml) using RNAzol® RT RNA Isolation Reagent (GeneCopoeia, Guangzhou, China). RNA concentrations were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA extraction was repeated until all RNA samples had an A260/A280 ratio between 1.8 and 2.0. cDNA was synthesized using RevertAid RT Reverse Transcription kit (Thermo Fisher Scientific, Inc.) under the following conditions: 25˚C for 5 min, 55˚C for 30 min and 75˚C for 10 min. One-Step PrimeScript RT-PCR kit for Real Time RT-PCR (Clontech Laboratories, Inc., Mountainview, CA, USA) was used to prepare all PCR reaction conditions. Primer sequences were as follows: LINK-A, forward 5'-TTC CCCCATTTTTCCTTTTC-3', reverse 5'-CTCTGGTTG GGTGACTGGTT-3'; GAPDH, forward 5'-GAAGGTGAA GGTCGGAGT-3', reverse 5'-GAAGATGGTGATGGGATT TC-3'. qPCR reaction conditions were: Initial denaturation at 95˚C for 50 sec, followed by 40 cycles of 95˚C for 15 sec and 60.5˚C for 34 sec. Relative expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (13) and normalized to GADPH loading control.

LINK-A lncRNA expression vectors and cell transfection. LINK-A lncRNA overexpression vectors (pcDNA3.1) and empty vectors were designed and synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). MG-63 and U2OS cells were cultivated overnight to reach 80-90% confluence. Lipofectamine® 2000 reagent (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) was used to perform transfection with vectors at a dose of 15 mM. Vectors were incubated with $5x10^5$ cells at 37˚C for 6 h. Subsequent experiments were carried out 24 h post-transfection. Non-transfected cells were used as control cells; empty vector transfection was used as the negative transfection control (NC).

Table I. Clinicopathological characteristics of participants.

Characteristics	Osteosarcoma	Controls
Cases (n)	62	48
Sex		
Male	34	28
Female	28	20
Metastasis		
Yes	28	NA
No	34	NA
Tumor size (cm)		
<2	22	NA
2-4	20	NA
>4	20	NA
Age range (years)	12-44	15-45
Mean age (years)	27.4±4.5	28.8±4.3

NA, not applicable.

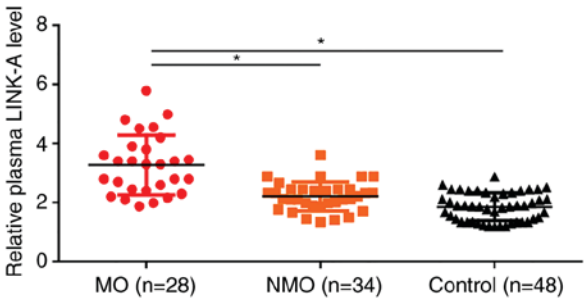

Figure 1. Plasma LINK-A lncRNA is upregulated in patients with osteosarcoma and related to tumor metastasis. Compared with the control group, significantly upregulated plasma LINK-A lncRNA expression was observed in MO groups. In addition, plasma levels of LINK-A lncRNA were also significantly higher in MO group compared with NMO group. *P<0.05. LINK-A, long intergenic non-coding RNA for kinase activation; lncRNA, long non-coding RNA; MO, metastatic osteosarcoma; NMO, non-metastatic osteosarcoma.

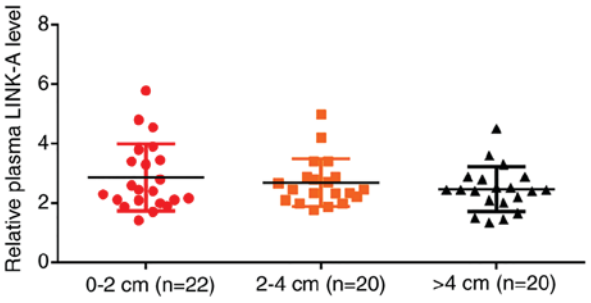

Figure 2. Plasma levels of LINK-A lncRNA in patients with osteosarcoma are not affected by tumor size. No significant differences were observed in plasma levels of LINK-A lncRNA among osteosarcoma patients with different tumor sizes. LINK-A, long intergenic non-coding RNA for kinase activation; lncRNA, long non-coding RNA.

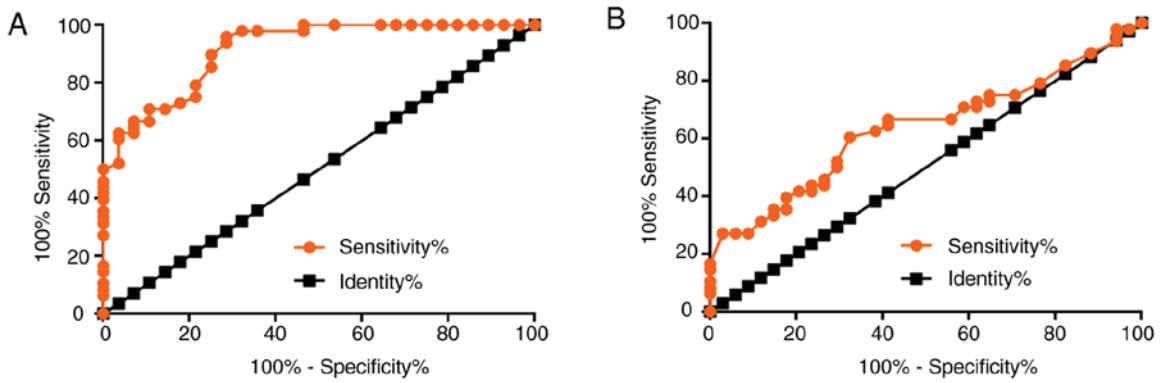

Figure 3. Upregulation of LINK-A lncRNA distinguishes patients with MO, but not NMO, from healthy controls. Receiver operating characteristic curve analysis showed that upregulation of LINK-A may serve as a potential diagnostic biomarker for (A) patients with MO but not (B) patients with NMO. LINK-A, long intergenic non-coding RNA for kinase activation; lncRNA, long non-coding RNA; MO, metastatic osteosarcoma; NMO, non-metastatic osteosarcoma.

Cell migration and invasion assay. Transfected MG-63 and U2OS cells were harvested and serum-free single cell suspensions with a cell density of $5x10^4$ cells/ml were prepared. Cell migration and invasion were examined by the following steps: The upper Transwell chamber (3 μm pore size) was filled with 100 μl serum-free cell suspension and the lower chamber was filled with ATCC-formulated Eagle's Minimum Essential Medium containing 20% FBS. Cells were cultivated in an incubator (37˚C; 5% CO_2) for 24 h and 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) staining was performed for 20 min at room temperature. Free cells were removed using a cotton swab. For the invasion assay, the upper chamber was pre-coated with Matrigel (cat. no. 356234; EMD Millipore, Billerica, MA, USA). Membranes were collected and invading or migrating cells were observed under a light microscope (magnification, x40; Olympus Corporation, Tokyo, Japan).

Western blotting. Proteins were extracted from cells ($3x10^4$ cells/ml) using ReadyPrep™ Protein Extraction kit (Total Protein; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein concentration was measured with the bicinchoninic acid assay. Following denaturing, proteins (35 μg) were separated by 10% SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membrane by semi dry method. Membranes were blocked in 5% milk for 2 h at room temperature. Membranes were then incubated with the rabbit anti-human primary antibodies against HIF1α (1:1,500; cat. no. ab2185, Abcam, Shanghai, China) and GAPDH (1:2,000; cat. no. ab9485; Abcam) at 4˚C overnignt, followed by secondary antibody horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:1,000; cat. no. MBS435036; MyBioSource, San Diego, CA, USA) at room temperature for 2 h. Signals were developed using ECL™ (Sigma-Aldrich; Merck KGaA) and scanned by MYECL™ Imager (Thermo Fisher Scientific, Inc.). Densitometric analysis was performed using ImageJ v1.46 software (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as endogenous control for data normalization.

Statistical analysis. All experiments were performed in triplicates and data were recorded as the mean ± standard deviation. SPSS 19.0 (IBM Corp., Armonk, NY, USA) was used to perform all statistical analyses. Comparisons among multiple groups were performed by one-way analysis of variance followed by Tukey test. Receiver operating characteristic (ROC) curve analysis was performed using patients with osteosarcoma as true positive cases and healthy controls as true negative cases. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Plasma LINK-A lncRNA is upregulated in patients with osteosarcoma and is related to tumor metastasis. RT-qPCR results revealed that, compared with the control and NMO groups, plasma LINK-A lncRNA expression was significantly upregulated in the MO group (Fig. 1). In addition, plasma levels of LINK-A lncRNA were also slightly higher in the NMO group than in control group (Fig. 1).

Plasma levels of LINK-A lncRNA in patients with osteosarcoma are not affected by tumor size. Based on the diameter of the primary tumor, patients with MO and NMO were divided into 0-2 cm group (n=22), 2-4 cm group (n=20) and >4 cm group (n=20) (Fig. 2). RT-qPCR results indicated no significant differences in plasma levels of LINK-A lncRNA among these groups. No significant differences in plasma levels of LINK-A lncRNA were found in MO patients with different tumor size (data not shown).

Upregulation of LINK-A lncRNA distinguishes patients with MO but not those with NMO from healthy controls. To investigate the diagnostic value of plasma LINK-A lncRNA for osteosarcoma, ROC curve analysis was performed patients with NMO or MO as true positive cases and healthy controls as true negative cases. For metastatic osteosarcoma, the area under the curve (AUC) was 0.9141, with standard error of 0.03214 and 95% confidence interval of 0.8511-0.9771 ($P<0.0001$; Fig. 3A). For non-metastatic osteosarcoma, the AUC was 0.6351, with standard error of 0.06082 and 95% confidence interval of 0.5159-0.7543 ($P=0.038$; Fig. 3B).

LINK-A lncRNA overexpression mediates the upregulation of HIF1α in MG-63 and U2OS cell lines. Compared with the Control and NC groups, LINK-A and HIF1α expression levels

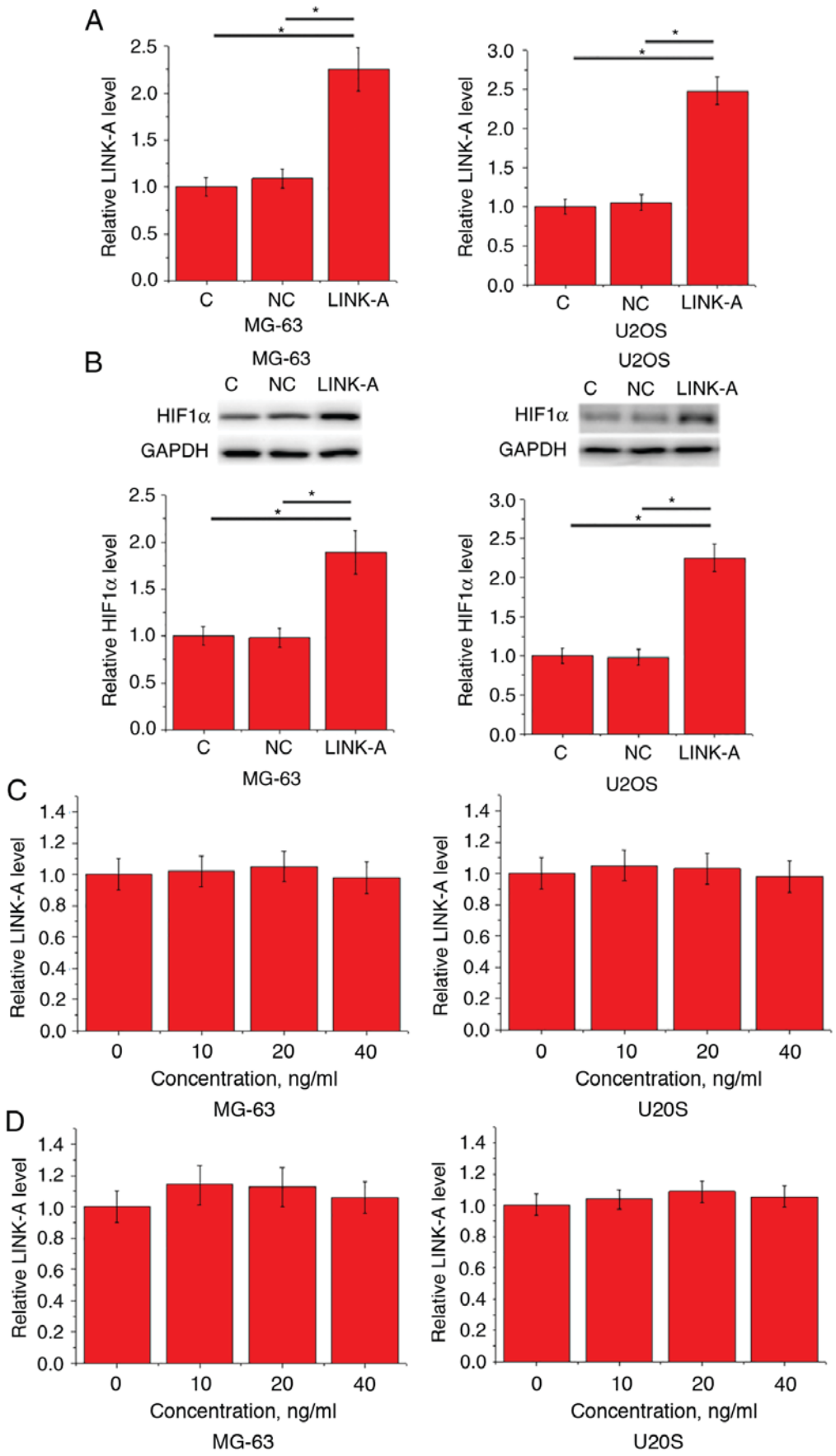

Figure 4. LINK-A lncRNA overexpression mediates the upregulation of HIF1α in MG-63 and U2OS osteosarcoma cell lines. (A) Successful transfection of LINK-A overexpression vectors in the two cell lines. (B) LINK-A overexpression led to significantly increased expression of HIF1α protein in MG-63 and U2OS cell lines; GADPH was used as loading control. (C and D) Treatment with HIF1α at doses of 10, 20 and 40 ng/ml failed to significantly affect LINK-A lncRNA expression in (C) untransfected cells and in (D) cells with LINK-A lncRNA overexpression. *P<0.05. C, control; HIF1α, hypoxia-inducible factor 1α; LINK-A, long intergenic non-coding RNA for kinase activation; lncRNA, long non-coding RNA; NC, negative control.

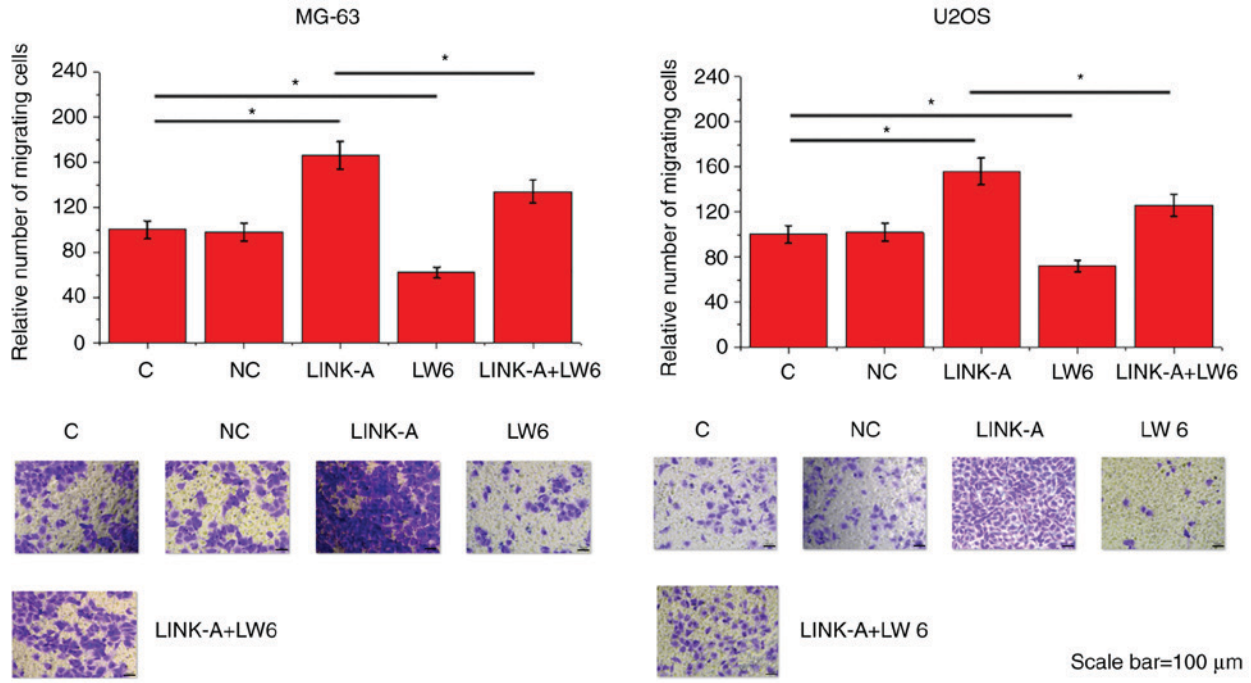

Figure 5. LINK-A lncRNA overexpression promotes migration of MG-63 and U2OS cells through HIF. LINK-A lncRNA overexpression led to significantly increased migration of MG-63 and U2OS cells. In addition, treatment with HIF inhibitor LW6 significantly attenuated the effects of LINK-A lncRNA overexpression on cancer cell migration and invasion. *P<0.05. C, control; HIF, hypoxia-inducible factor; LINK-A, long intergenic non-coding RNA for kinase activation; lncRNA, long non-coding RNA; NC, negative control.

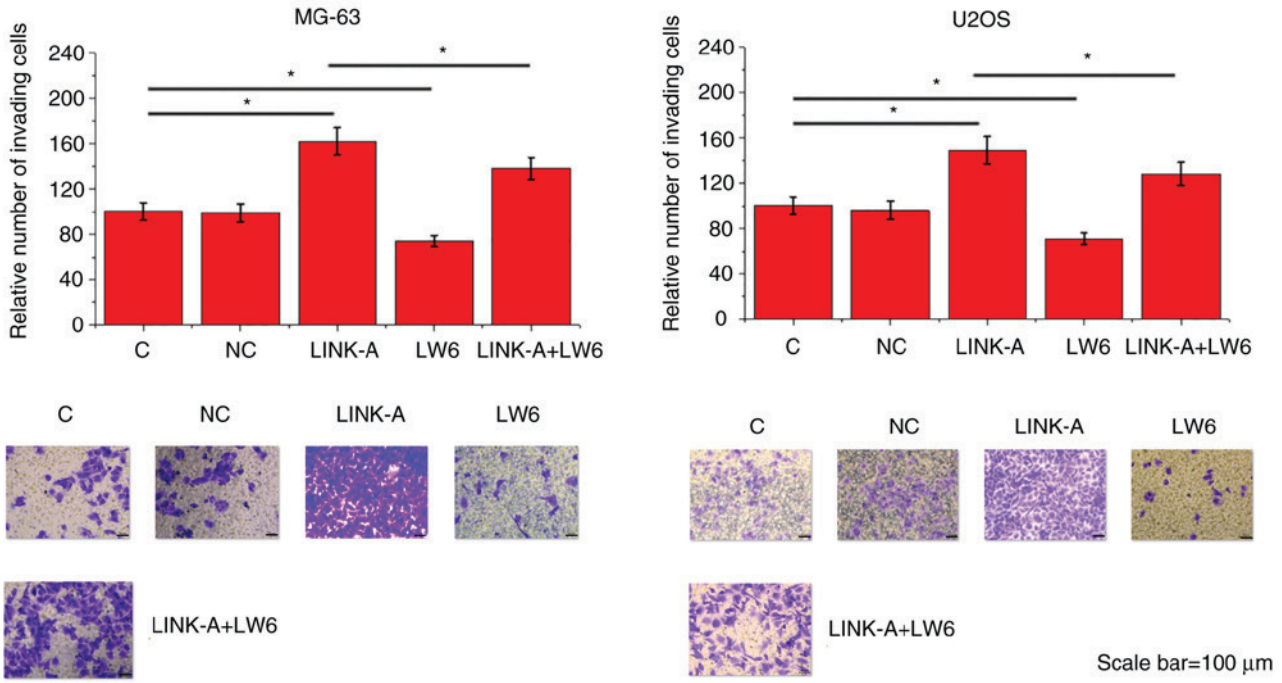

Figure 6. LINK-A lncRNA overexpression promotes invasion of MG-63 and U2OS cell lines through HIF. LINK-A overexpression led to significantly increased invasion of MG-63 and U2OS cells. In addition, treatment with HIF inhibitor LW6 significantly attenuated the enhancing effects of LINK-A overexpression on cancer cell migration and invasion. *P<0.05. C, control; HIF, hypoxia-inducible factor; LINK-A, long intergenic non-coding RNA for kinase activation; lncRNA, long non-coding RNA; NC, negative control.

were significantly upregulated following cell transfection (Fig. 4A). LINK-A lncRNA overexpression led to significantly increased expression of HIF1α protein in MG-63 and U2OS cells (Fig. 4C). By contrast, treatment with HIF1α at doses of 10, 20 and 40 ng/ml failed to significantly affect endogenous LINK-A lncRNA expression in untransfected cells (Fig. 4D) as well as in cells overexpressing LINK-A lncRNA (Fig. 4D).

LINK-A lncRNA overexpression promotes migration and invasion of MG-63 and U2OS cells through HIF1α. Compared with the Control group and the NC group, LINK-A lncRNA overexpression led to significantly promoted migration (Fig. 5) and invasion (Fig. 6) of MG-63 and U2OS cell lines. In addition, treatment with HIF inhibitor LW6 at a dose of 10 ng/ml significantly attenuated the enhancing effects of LINK-A overexpression on cancer cell migration (Fig. 5) and invasion (Fig. 6).

Discussion

A recent study reported that LINK-A lncRNA serves an oncogenic role in triple-negative breast cancer through the interaction with HIF1α (9). The present study confirmed the existence of the association between LINK-A lncRNA and HIF1α in osteosarcoma. Therefore, triple-negative breast cancer and osteosarcoma may share similar molecular pathological pathways.

Circulating biomarkers have been widely used in the diagnosis and prognosis of human diseases (14,15). Previous studies have revealed that the development of osteosarcoma induces altered expression of a large set of human genes, including lncRNAs (16,17). A number of these differentially expressed lncRNAs, such as urothelial cancer associated 1 (18) and hepatocellular carcinoma up-regulated lncRNA (19), have been demonstrated to have a diagnostic and prognostic potential for osteosarcoma. As an oncogenic lncRNA, expression of LINK-A lncRNA is altered in triple-negative breast cancer (9); however, the expression pattern of LINK-A lncRNA in other human diseases is unknown. The present study revealed that plasma circulating LINK-A lncRNA was only upregulated in patients with MO but not in patients with NMO; upregulation of LINK-A distinguished patients with MO, but not those with NMO, from healthy controls. These data suggested that LINK-A may be specifically involved in the distant metastasis of osteosarcoma.

In the present study, a comparison of patients with different diameters of primary tumors revealed no significant differences in plasma levels of LINK-A lncRNA, which suggested that LINK-A lncRNA was unlikely to be involved in the growth of osteosarcoma. The *in vitro* cell proliferation data also indicated that overexpression of LINK-A lncRNA had no significant effects on the proliferation of MG-63 and U2OS human osteosarcoma cells (data not shown).

It has been reported that LINK-A lncRNA in triple-negative breast cancer activates normoxic HIF1α signalling (9). The present study confirmed the existence of the association between LINK-A and HIF1α in osteosarcoma. LINK-A overexpression had inhibitory effects on HIF1α expression, whereas HIF1α had no effect on LINK-A. In addition, HIF inhibitor LW6 reduced the effects of LINK-A overexpression on cell migration and invasion. Therefore, LINK-A may be an upstream activator of HIF1α. However, the molecular mechanism of the regulation of HIF1α by LINK-A remains to be determined.

In conclusion, LINK-A lncRNA is upregulated in osteosarcoma and may participate in the metastasis of osteosarcoma by upregulating HIF1α.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LC and BZ did the experiments, analyzed the data and were major contributors in writing the manuscript. KL performed a part of the experimental work. All authors read and approved the final manuscript and confirmed its accuracy.

Ethics approval and consent to participate

Ethical approval was obtained from The Ethics Committee of Zhongnan Hospital Affiliated to Medical College of Wuhan University (Wuhan, China). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all patients and controls following an explanation the nature and possible consequences of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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