

Overexpression of Long Non-Coding RNA HOTAIR Promotes Tumor Growth and Metastasis in Human Osteosarcoma

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Human osteosarcoma usually presented a high tendency to metastatic spread and caused poor outcomes, however, the underlying mechanism was still largely unknown. In the present study, using a series of *in vitro* experiments and an animal model, we investigated the roles of HOX antisense intergenic RNA (HOTAIR) during the proliferation and invasion of osteosarcoma. According with our results, HOTAIR was commonly overexpressed in osteosarcoma, which significantly correlated with advanced tumor stage, highly histological grade and poor prognosis. *In vitro* and *in vivo* experiments demonstrated that knockdown of HOTAIR could notably suppress cellular proliferation, inhibit invasion and decrease the secretion of MMP2 and MMP9 in osteosarcoma. Collectively, our results suggested that HOTAIR might be a potent therapeutic target for osteosarcoma.

INTRODUCTION

Osteosarcoma is the most common primary malignance of bone (Bielack et al., 2009). Globally, the incidence of osteosarcoma is around 0.2-3/100000 per year, which is even higher in the age group of 15-19 years-old (0.8-11/100000 per year) (Mirabello et al., 2009). Most of osteosarcoma arise at the metaphysis of a long extremity bone and the involvement of axial skeleton or craniofacial bones is also observed in the adults population (Ritter et al., 2010). Osteosarcoma usually presents a high tendency to metastatic spread. Previous studies proved that more than 20% osteosarcoma patients already occurred lung metastases at the initial diagnosis (Bacci et al., 2008;

Messerschmitt et al., 2009). The 5-year survival rate is about 65% for patients with localized osteosarcoma, however, it sharply declined to about 20% for these cases with metastasis (Bielack et al., 2002; Eccles et al., 2007; Hughes, 2009). Thus, further elucidating the underlying mechanisms of metastatic osteosarcoma is urgently required.

Long non-coding RNAs (lncRNAs) are a group of transcribed RNA consisting of more than 200 nucleotides while not encoding any proteins (Mercer et al., 2009; Ponting et al., 2009). lncRNAs are demonstrated to regulate the gene expression at various levels and widely involved in the physiological and pathological process (Kapranov et al., 2007; Ponting et al., 2009). In recent years, it has been found that lncRNAs are closely correlated with human malignancies, acting as oncogenes or tumor suppressors (Gupta et al., 2010; Ji et al., 2003; Li et al., 2014; Matouk et al., 2007). HOTAIR is one of the well-studied lncRNAs, which localized at human chromosome 12q13 within the anti-sense strand of the HOXC gene cluster (Rinn et al., 2007). Several researches showed that HOTAIR could directly bind with the polycomb repressive complex 2 (PRC2) and subsequently silence these HOXD genes (Rinn, 2007). Moreover, HOTAIR also could interact with the LSD1/CoREST/REST complex and remodified histone H3K4 demethylation (Tsai et al., 2010). In the recent years, a large body of evidences supported that HOTAIR was involved in the regulation of tumor invasion and metastasis (Hajjari et al., 2013; Ishibashi et al., 2013; Kim et al., 2013; Liu et al., 2013; Lu et al., 2012; Sorensen et al., 2013). In breast cancer, overexpression of HOTAIR significantly increased the invasiveness and metastasis through altering the methylation at histone H3 lysine 27; on the contrary, knockdown of HOTAIR could block the tumor invasion and metastasis (Gupta et al., 2010). In gastric cancer, HOTAIR was proved to be able to promote cellular invasion/migration through enhancing the epithelial-mesenchymal transition (Xu et al., 2013). Since osteosarcoma was also characterized by highly invasion and widespread metastasis, we herein aimed to investigate the biological functions of HOTAIR in osteosarcoma and its underlying mechanisms.

MATERIALS AND METHODS

Samples collection

The tumor tissues and their adjacent non-malignant tissues were obtained from a panel of 40 pathological diagnosed os-

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Table 1. The clinical and pathological characteristics of osteosarcoma patients

	No.	%
Total patients	40	
Gender		
Male	23	57.5
Female	17	42.5
Age		
Median	13.9	
Range	3-35	
Anatomical site		
Femure	17	42.5
Tibia	15	37.5
Humerus	5	12.5
Pelvis	2	5
Other	1	2.5
Histologic subtype		
Osteoblastic	22	55
Chondroblastic	11	27.5
Fibroblastic	7	17.5
Histologic grade		
III	15	37.5
IV	25	62.5
Stage		
IIA	16	40
IIB/III	24	60

osteosarcoma patients at the First Affiliated Hospital of Dalian Medical University (China) from June 2005 to June 2009. This study was approved by the Ethical Committee of our hospital and written consents were obtained from all the participants. Patients' parameters were provided in Table 1.

Osteosarcoma cell lines and reagents

The immortalized human osteosarcoma cell lines U2OS, MNNG/HOS, 143B and MG63 were purchased from ATCC (American Type Culture Collection, USA) and used for this study. The cells were routinely cultured in DMEM with 10% fetal bovine serum (FBS, Gibco, USA) at 37°C, supplemented with 5% CO₂. All the reagents were purchased from Sigma-Aldrich (USA) if not specifically mentioned.

RNA Extraction and real time PCR

The total RNA was extracted using the Trizol reagent (Invitrogen, USA). The expression of HOTAIR was determined by real time PCR using SYBR Premix ExTaq II kit (Takara, Dalian, China). The primers were as follows: HOTAIR forward, 5'-GGTAGAAAAAGCAACCACGAAGC-3'; HOTAIR reverse, 5'-ACATAAACCTCTGTCTGTGAGTGCC-3'; GAPDH forward, 5'-GTCAACGGATTTGGTCTGTATT-3'; GAPDH reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'. GAPDH was set as the endogenous control and the relative expression of HOTAIR was determined by the 2^{-ΔΔCt} method.

MTT assay

Cellular growth ability was determined by the MTT assay. In brief, 3 × 10³ cells/well were plated into 96-well plates and rou-

tinely cultured. At the indicated time point, 5 μl MTT solution (5 mg/ml) was added into each well and incubated two hours at room temperature. Then the reaction was terminated by 100 μl DMSO and the absorbance at 570 nm was measured on a microplate-reader.

Construction of the shRNA-HOTAIR vector

The pENTR-shHOTAIR vector was constructed as previously described (Liu et al., 2014). In brief, the specific oligonucleotides targeting HOTAIR were synthesized at Genepharma Technology (China): sense, 5'-GATCCGCCACATGAACGCC-CAGAGATTTTCAAGAGAAATCTCTGGGCGTTCATGTGGTT TTTTG-3'; anti-sense, 5'-AATTCAAAAACCACATGAACGCC-CAGAGATTTCTCTTGAAATCTCTGGGCGTTCATGTGGC G-3'. Then the pENTR-shHOTAIR plasmids and empty vectors were transfected into U2OS cells and the subclones with HOTAIR overexpression were selected with G418 (400 μg/ml). Real time PCR was performed to confirm the upregulation of HOTAIR.

Transwell assay

Transwell chamber system (Applied Biosystems, USA) was used to investigate the effects of HOTAIR on cellular invasion. The 1 × 10⁵ cells/well were plated onto the upper chamber and 400 μl DMEM medium without FBS was added in the bottom chamber. Twenty-four hours later, the cells at the bottom membrane were fixed, stained and counted under a microscope.

Wound healing assay

The 3 × 10⁵ osteosarcoma cells (per well) with knockdown of HOTAIR were plated in the 6-well plates and the cell monolayer was scarred with the tip of a 10 μl pipet tip at start (0 h). Then the cells were cultured in the serum-free medium for 24 h. Migration rate (%) = final distance/initial distance × 100%.

ELISA for the determination of MMP-2 and MMP-9

The 5 × 10⁵ cells/well were seeded into 12-well plates and routinely cultured for 48 h. Then the supernatants were collected and centrifuged at 200 × g and the concentrations of human MMP-2 and MMP-9 were determined by a commercial ELISA kit (R&D systems, USA) according to the manufacturer's instructions.

Establishment of the nude mice model

To investigate the *in vivo* roles of HOTAIR, we established a nude mice model bearing the U2OS xenografts. Our mice handling procedures were approved by the Institutional Animal Care and Use Committee of Dalian Medical University. In brief, six to eight weeks old balb/c nude mice were randomly divided into four groups (n = 5 in each group): blank, empty vector and sh-HOTAIR. On day 1, 2 × 10⁶ cells U2OS cells with or without HOTAIR knockdown were subcutaneously injected into the left posterior flank of the nude mice. Then tumor diameters were checked once a week and tumor volume was calculated using the formula: tumor volume = (length × width²)/2. On day 28, all the mice were sacrificed and the tumor tissues were collected for further analysis.

Immunohistochemical staining (IHC)

The antigen retrieval procedure was performed in EDTA buffer (pH 8.0, Maixin Biotechnology, China) using a microwave for 20 min. Then the slides were incubated with primary antibodies overnight at 4°C. On the next day, the slides were incubated with secondary antibodies for one hour at room temperature.

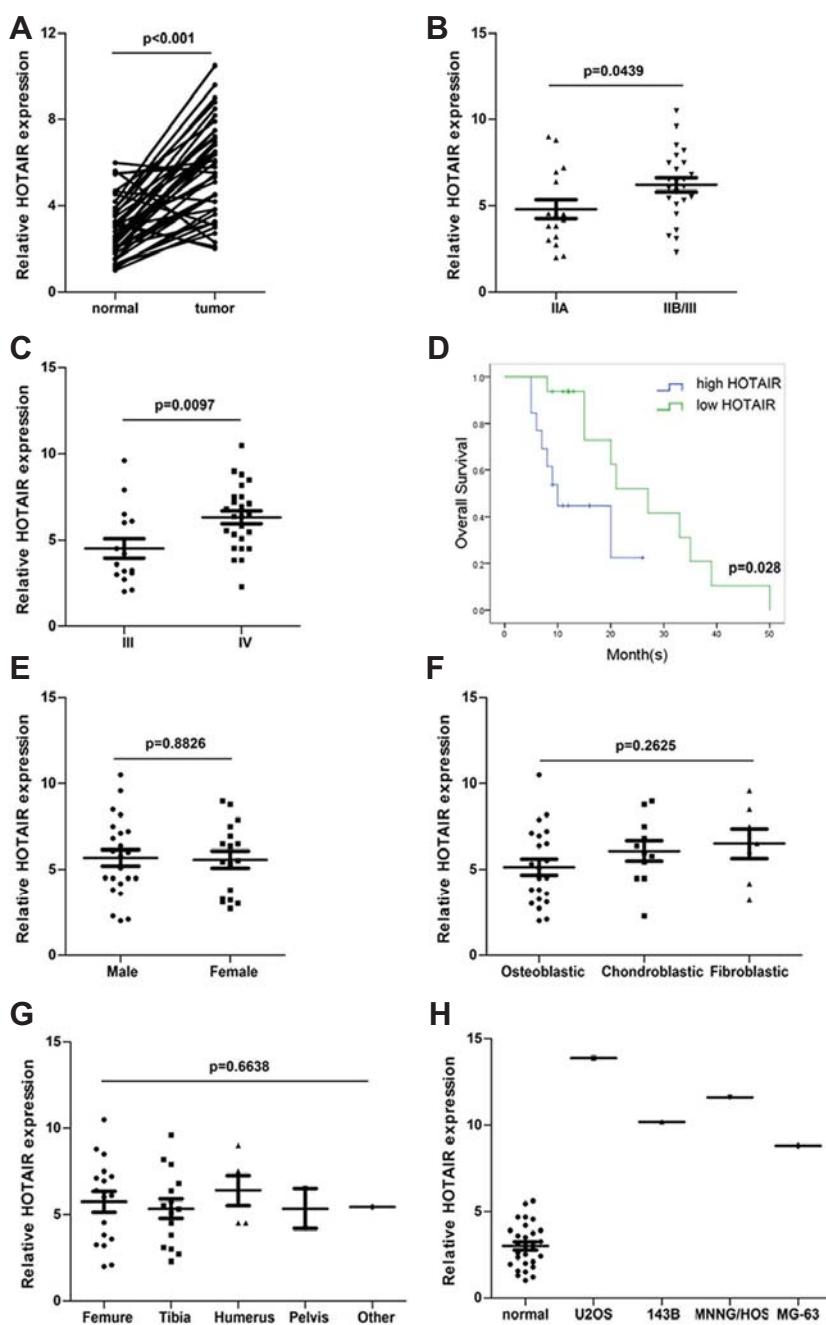


Fig. 1. HOTAIR was frequently upregulated in osteosarcoma. (A) compared to the adjacent non-malignant tissues, the expression of HOTAIR was significantly upregulated in osteosarcoma samples ($p < 0.001$); (B) the upregulation of HOTAIR was significantly correlated with advanced tumor stage (stage IIA vs. stage IIB/III, $p = 0.0439$); (C) the expression of HOTAIR was positively related with histological grade ($p = 0.0097$); (D) high level of HOTAIR predicted a shorter overall survival in osteosarcoma patients ($p = 0.028$); (E) there was no significant difference of HOTAIR expression between the male and female osteosarcoma patients ($p = 0.8826$); (F) the level of HOTAIR was not associated with osteosarcoma histological subtypes ($p = 0.2625$); (G) there was no difference among the anatomical sites of osteosarcoma ($p = 0.6638$); (H) HOTAIR in the four human immortalized osteosarcoma cell lines were significantly upregulated than those in the non-malignant tissues.

The staining was performed using the Histostain Plus IHC kit (Mingrui Biotechnology, China) and evaluated by two pathologists separately. The primary antibodies used IHC analysis were anti-Ki-67 (1:100, Cell Signaling Technology), anti-MMP2 (1:150, Cell Signaling Technology) and anti-MMP9 (1:100, Cell Signaling Technology).

Statistical analysis

All the *in vitro* experiments were repeated in triplicate and at least three times. Students' *t*-test was used to determine different HOTAIR level between normal and osteosarcoma tissues. Correlations between HOTAIR level and clinico-pathological

characteristics were analyzed using one-way analysis of variance (ANOVA). $p < 0.05$ was defined to be of statistical difference.

RESULTS

HOTAIR was commonly upregulated in osteosarcoma

Compared to that in normal tissues, the expression of HOTAIR was notably upregulated in the osteosarcoma samples ($p < 0.001$, Fig. 1A). In addition, HOTAIR overexpression was closely correlated with advanced tumor stage (stage IIA vs. stage IIB/III, $p = 0.0439$, Fig. 1B) and highly histological grade (grade

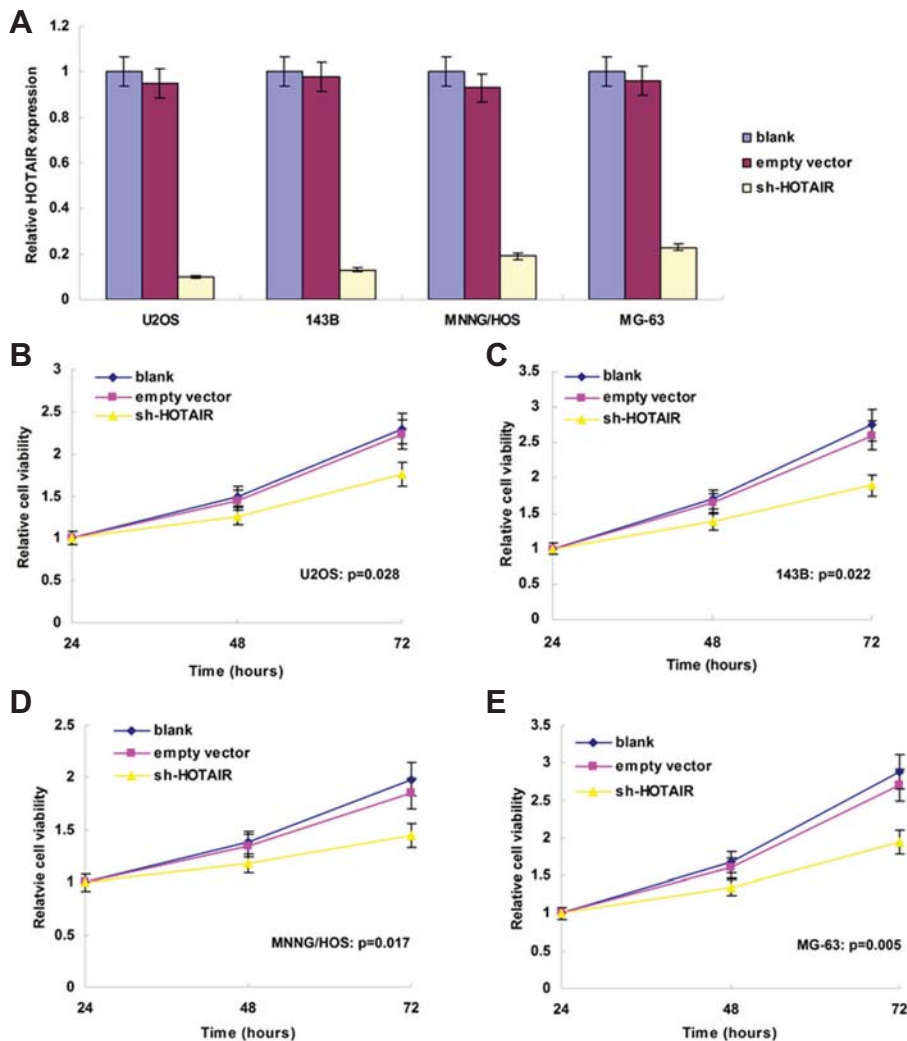


Fig. 2. Downregulation of HOTAIR suppressed the growth of osteosarcoma cells. (A) compared to the blank and empty vector groups, transfection with specific shRNA-HOTAIR (sh-HOTAIR) notably downregulated the expression of HOTAIR in all the four osteosarcoma cell lines; (B-E) downregulation of HOTAIR significantly suppressed cellular growth in the four cell lines ($p = 0.028, 0.022, 0.017, 0.005$ for U2OS, 143B, MNNG/HOS and MG-63 respectively).

III vs. grade IV, $p = 0.0097$, Fig. 1C). At the end of the follow-up (5-50 months), 18 deaths were reported. The high HOTAIR expression was defined to be the tumor tissues which showed more than two-fold upregulation of HOTAIR than their adjacent non-malignant tissues. As the Kaplan-Meier curve shown, high level of HOTAIR was significantly associated with a shorter overall survival ($p = 0.028$, Fig. 1D). However, there was no relationships between HOTAIR expression and patients' gender ($p = 0.8826$, Fig. 1E), histological subtype ($p = 0.2625$, Fig. 1F) or the anatomical sites ($p = 0.6638$, Fig. 1G).

Consistently, the expression of HOTAIR in the four immortalized human osteosarcoma cell lines were also much higher than that in normal tissues (Fig. 1H).

Downregulation of HOTAIR suppressed the growth of osteosarcoma cells

Then we further investigated the roles of HOTAIR on the growth of osteosarcoma cells. As shown in Fig. 2A, we successfully knockdown the expression of HOTAIR in the four cell lines. In MTT assay, we detected that downregulation of HOTAIR significantly suppressed cellular growth of the four osteosarcoma cell lines ($p = 0.028, 0.022, 0.017, 0.005$ for U2OS, 143B,

MNNG/HOS and MG-63 respectively, Figs. 2B-2E). These results implied an important role of HOTAIR in manipulating the growth of osteosarcoma.

Downregulation of HOTAIR inhibited the invasion and migration of osteosarcoma cells

Then we further used the Transwell system to study the alterations of cellular invasion pre- and pro-HOTAIR knockdown. As Fig. 3A shown, the cells which invaded through the Matrigel-coated membrane were notably decreased when HOTAIR was downregulated in the U2OS cells. Similar results were also investigated in the other three cell lines ($p = 0.039, 0.03, 0.009, 0.016$ for U2OS, 143B, MNNG/HOS and MG-63 respectively, Fig. 3B).

As the wound healing assay shown, the migration of U2OS with knockdown of HOTAIR was significantly inhibited than that in the blank and empty vector groups (Fig. 3C). Consistent results were also found in the other three cell lines ($p = 0.01, 0.044, 0.021, 0.035$ for U2OS, 143B, MNNG/HOS and MG-63 respectively, Fig. 3D). Collectively, our data suggested that HOTAIR positively regulated cellular invasion and migration of osteosarcoma.

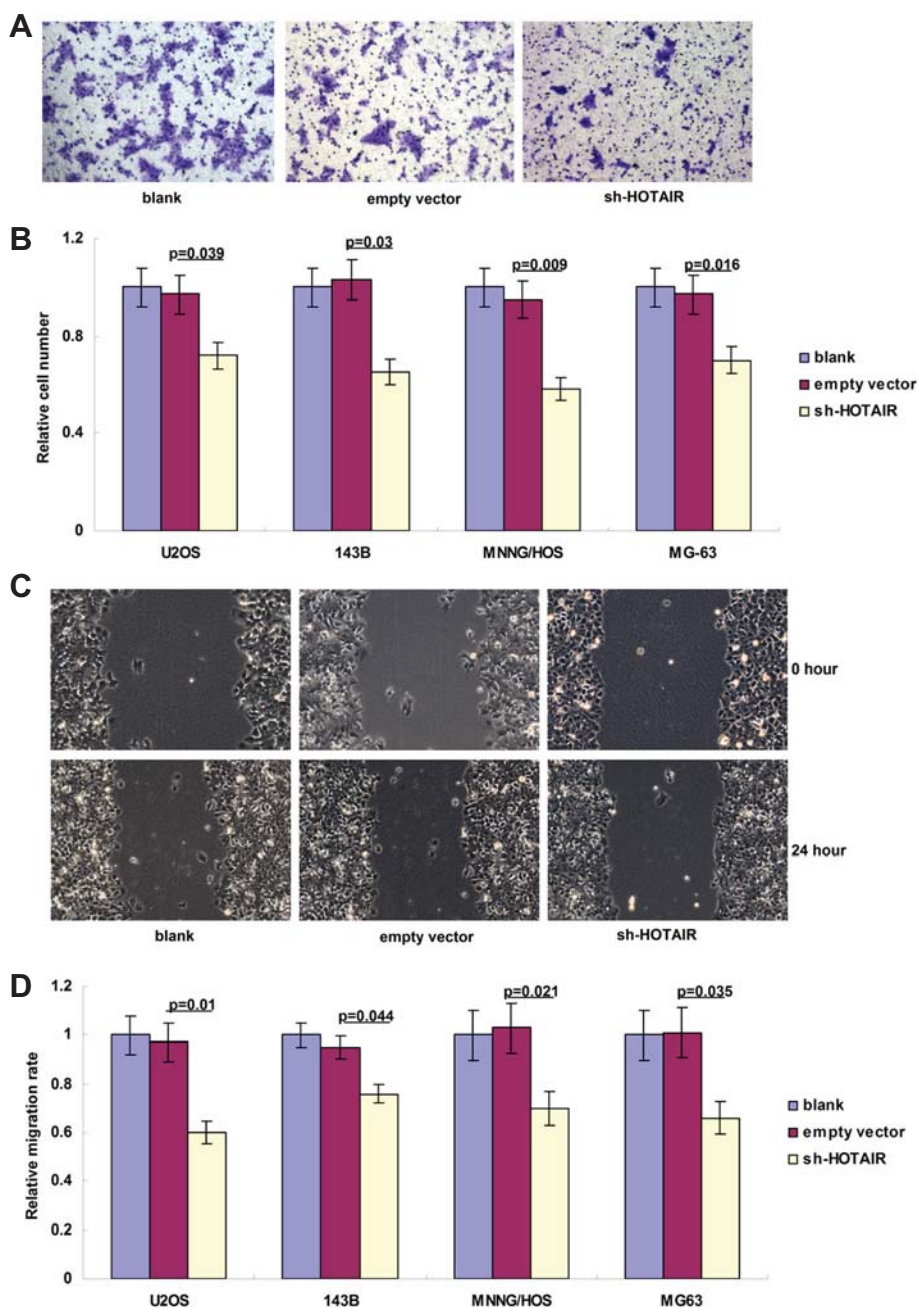


Fig. 3. Downregulation of HOTAIR inhibited the invasion and migration of osteosarcoma cells. (A) the representative images of the affects of HOTAIR-knockdown on cellular invasion in U2OS cells; (B) the quantification of the results in cellular invasion assay for osteosarcoma cell lines ($p = 0.039, 0.03, 0.009, 0.016$ for U2OS, 143B, MNNG/HOS and MG-63 respectively). (C) the representative images of the affects of HOTAIR-knockdown on the migration of U2OS cells; (D) the quantification of the results in wound healing assay for the four osteosarcoma cell lines ($p = 0.01, 0.044, 0.021, 0.035$ for U2OS, 143B, MNNG/HOS and MG-63 respectively).

Downregulation of HOTAIR suppressed the secretion of MMP2 and MMP9

Thus, we further detected the alterations of MMP2 and MMP9 secreted by osteosarcoma. As our data shown, the levels of MMP2 and MMP9 in the medium were both significantly decreased by the knockdown of HOTAIR (MMP2: $p = 0.035, 0.023, 0.018, 0.006$ for U2OS, 143B, MNNG/HOS and MG-63 respectively, Fig. 4A; MMP9: $p = 0.015, 0.011, 0.029, 0.031$ for U2OS, 143B, MNNG/HOS and MG-63 respectively, Fig. 4B). Our results further demonstrated that HOTAIR could promote the invasion of osteosarcoma via upregulating the secretion of MMP2 and MMP9.

Downregulation of HOTAIR suppressed the *in vivo* growth of osteosarcoma

In order to study the *in vivo* functions of HOTAIR, we then established the mouse model bearing U2OS xenografts. During the four weeks growth-period, the U2OS sh-HOTAIR xenografts grew much slower compared to the blank and empty vector groups ($p < 0.001$, Fig. 5A). Consistently, tumor weights of the sh-HOTAIR group were also suppressed by the downregulation of HOTAIR ($p < 0.001$, Fig. 5B). According to the IHC analysis, downregulation of HOTAIR significantly inhibited the expression of Ki-67 (a proliferative marker), MMP2 and MMP9 ($p = 0.032, 0.021$ and 0.01 respectively, Figs. 5C and 5D).

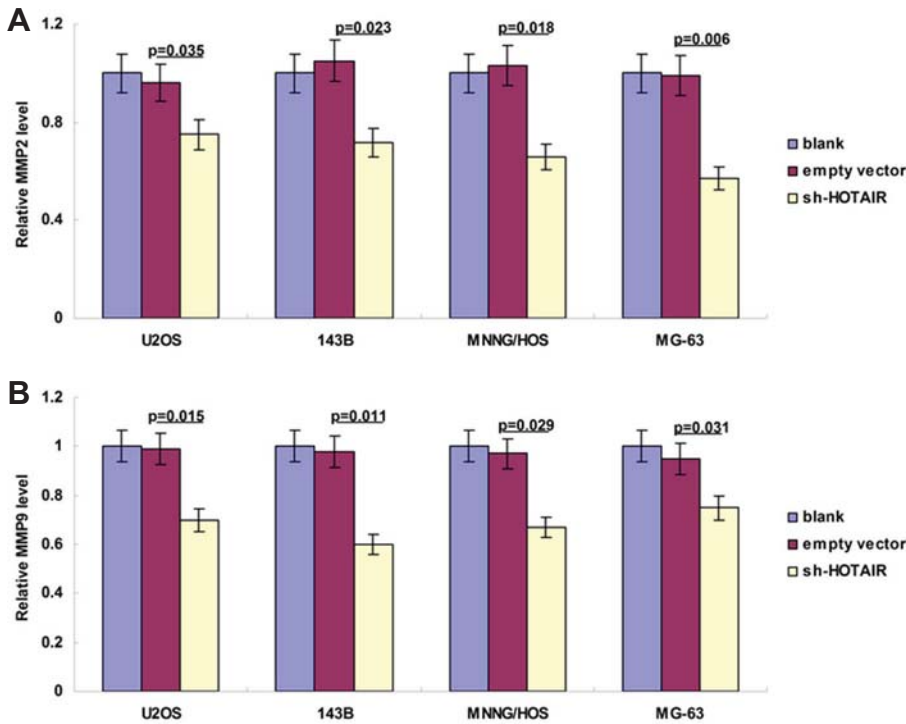


Fig. 4. Downregulation of HOTAIR suppressed the secretion of MMP2 and MMP9 in osteosarcoma cells. (A) compared to the blank and empty vector groups, the level of MMP2 secreted by osteosarcoma cells was significantly suppressed ($p = 0.035, 0.023, 0.018, 0.006$ for U2OS, 143B, MNNG/HOS and MG-63 respectively); (B) consistently, downregulation of HOTAIR also notably suppressed the secretion of MMP9 in osteosarcoma cells ($p = 0.015, 0.011, 0.029, 0.031$ for U2OS, 143B, MNNG/HOS and MG-63 respectively).

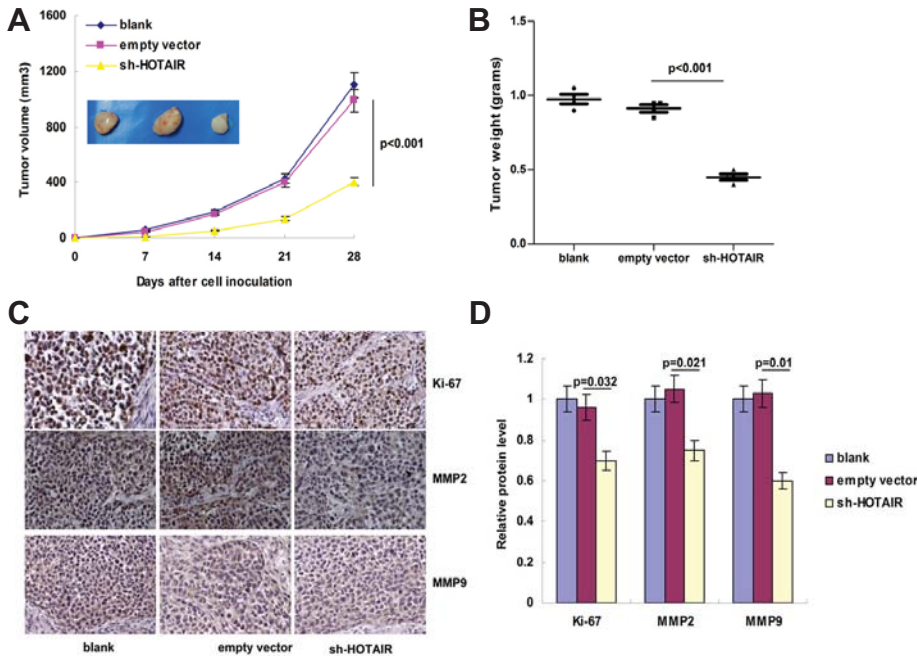


Fig. 5. Downregulation of HOTAIR suppressed the growth of U2OS xenografts *in vivo*. (A) compared to the blank and empty vector groups, the growth of U2OS xenografts was significantly suppressed by the downregulation of HOTAIR ($p < 0.001$); (B) consistently, tumor weights of the sh-HOTAIR group were also notably suppressed with HOTAIR-knockdown ($p < 0.001$); (C, D) in the IHC analysis, downregulation of HOTAIR significantly inhibited the expression of Ki-67 (a proliferative marker), MMP2 and MMP9 ($p = 0.032, 0.021$ and 0.01 , respectively).

These findings supported that targeting HOTAIR could inhibit the growth of osteosarcoma *in vivo*.

DISCUSSION

As the most common bone cancer in children and young adults, osteosarcoma remains the second leading cause of cancer-

related death in this age group (Siclari et al., 2010). However, the molecular mechanisms underlying the initiation and progression of osteosarcoma are still largely unknown yet. According to previous studies, lncRNAs could regulate the gene expression via genomic, transcriptional and post-transcriptional modulating (Ponting et al., 2009). It has been well demonstrated that lncRNAs were involved in almost every aspects of phy-

biological and pathological processes in the human body (Ernst et al., 2013; Gibb et al., 2011; Khalil et al., 2009; Lipovich et al., 2010).

In the past years, several lncRNAs are notably correlated with various tumors and served as oncogenes or tumor suppressors (Endo et al., 2013; Huarte et al., 2010; Matouk, 2007; Nie et al., 2014; Pandey et al., 2014; Xue et al., 2014). However, there were few reports about the roles of lncRNAs in osteosarcoma (Pasic et al., 2010; Zhang et al., 2013). In this study, we systematically investigated the roles of HOTAIR during the proliferation and invasion of osteosarcoma. According to our data, HOTAIR was frequently upregulated in osteosarcoma tissues, especially in these cases with advanced tumor stage and highly histological grade. In addition, high level of HOTAIR was significantly related with a short overall survival. These data suggested an important role of HOTAIR in the development of osteosarcoma.

Osteosarcoma is characterized by frequent local and distant metastasis (Mialou et al., 2005; PosthumaDeBoer et al., 2011). Up to date, treating metastatic osteosarcoma remains a huge challenge. During the past decades, in spite of the significantly improved surgical techniques, the chemotherapy was almost as same as that applied in the early 1980s and the prognosis of osteosarcoma patients was almost unchanged since then (Hughes, 2009; Ritter, 2010). Therefore, to further elucidate the mechanisms of invasion and metastasis in osteosarcoma is urgently required.

Since HOTAIR was reported to be involved in manipulating the key steps of metastasis like invasion, migration and epithelial-mesenchymal transition (Gupta et al., 2010; Jing et al., 2014; Li, 2014; Padua Alves et al., 2013), we herein explored its effects on the metastasis of osteosarcoma. Results from the Transwell assay demonstrated that knockdown of HOTAIR could inhibit the invasion in all the four kinds of human osteosarcoma cells, indicating that HOTAIR served as an enhancer in the invasion of osteosarcoma. Consistently, our results were supported by a panel of previous studies. In pancreatic cancer, both cellular proliferation and invasion were significantly enhanced by the overexpression of HOTAIR, while inhibited when HOTAIR was knockdown (Kim, 2013). In esophageal squamous cell carcinoma cells, knockdown of HOTAIR could reduce cellular ability to proliferate, migrate and invade the extracellular matrix (Li et al., 2013).

As previous studies reported, the final formation of distant tumor metastasis consisted of the following steps: invade through the extracellular matrix, survive in the circulation, arrest at a target organ site, adhere to and survive in new microenvironment (Eccles, 2007; Hanahan et al., 2011; Kashima et al., 2003; Steeg, 2006; Worth et al., 2002). MMPs were found to play a critical role during tumor cells invading through the extracellular matrix (Davies, 2014; Gupta et al., 2014). Aberrant expression of MMPs including MMP2 and MMP9 was common in osteosarcoma, which was associated with tumor metastasis and poor prognosis (Han et al., 2012; Himelstein et al., 1998; Peng et al., 2002). Many *in vitro* and *in vivo* researches proved that inhibition of the expression or activity of these MMPs significantly suppressed cellular growth and invasion of osteosarcoma (Cho et al., 2007; Ma et al., 2013; Zhang et al., 2015). Intriguingly, MMP2 and MMP9 were also two known targets of HOTAIR (Padua Alves, 2013; Wu et al., 2014). Thus, we further investigated the alterations of MMP2 and MMP9 in osteosarcoma cells before and after the HOTAIR knockdown. Our data showed that both MMP2 and MMP9 were significantly inhibited by the knockdown of HOTAIR *in vitro* and *in vivo*. Consistent

with our findings, similar effects of HOTAIR on other MMPs like MMP1, MMP3 and MMP13 were also reported in other human malignancies (Qiu et al., 2014; Xu, 2013). Collectively, these results evidenced that HOTAIR could promote the formation of metastasis via activating MMPs and enable tumor cells to invade through the extracellular matrix and enter into the circulation.

In conclusion, we firstly demonstrated that HOTAIR was commonly upregulated in human osteosarcoma, which could predict a worse prognosis. *In vitro* and *in vivo*, we proved that knockdown of HOTAIR suppressed tumor growth and reduced cellular invasion, proposing HOTAIR as a novel target for the treatment of osteosarcoma.

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