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LncRNA NKILA inhibits invasion and migration of osteosarcoma cells *via* NF-κB/Snail signaling pathway

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Abstract. – OBJECTIVE: Our research explored the possible biological function of long non-coding RNA (lncRNA) NKILA in the pathogenesis of osteosarcoma and its underlying mechanism.

PATIENTS AND METHODS: NKILA expression in 60 cases of osteosarcoma and adjacent tissues was detected. The correlation between NKILA expression and clinical information was analyzed by Chi-square test. The overexpression plasmid or siRNA of NKILA were transfected into osteosarcoma cells by liposome. Cell proliferation was detected by cell counting kit-8 (CCK-8) assay. Transwell assay was used to check the migratory and invasive abilities. Western Blot was used to detect the expressions of nuclear factor-κB (NF-κB)-related proteins. In addition, we analyzed the cell invasion and migration after treatment of NF-κB inhibitor (JSH) to further verify whether NKILA can participate in the occurrence of osteosarcoma through the NF-κB / Snail signaling pathway.

RESULTS: The expression level of NKILA in osteosarcoma tissues was significantly lower than that in adjacent tissues, and was related to tumor size, Enneking stage, and metastasis. After KNKS/NP cells were transfected with NKILA-siRNA, cell proliferation, invasion and migration were enhanced. Transfection of the NKILA overexpression plasmid in Saos2 cells reduced cell proliferation, invasion and migration. NKILA knockdown downregulated the expressions of p65 and E-cadherin, but strikingly increased Snail expression. The RNA binding protein co-immunoprecipitation experiments illustrated that p65 could bind to NKILA. Additionally, JSH was found to reverse the inhibitory effect of NKILA on cell migration and proliferation.

CONCLUSIONS: NKILA was lowly expressed in osteosarcoma tissues. In addition, high expression of NKILA could suppress the migration and invasion of osteosarcoma cells by inhibiting the NF-κB/Snail signaling pathway.

Key Words:
NKILA, NF-κB/Snail, Proliferation, Osteosarcoma.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in adolescents. Its incidence is approximately (1-3) / 1000,000 per year[1]. The combined treatment of surgical operation and multiple chemotherapeutic agents has become the normative strategy for almost all patients with osteosarcoma. This combination has remarkably increased the survival rate of OS patients[2]. However, many OS patients exhibit resistance to available chemotherapeutic drugs and die from extensive metastases and neoplasm recurrence. The metastases and neoplasm recurrence are major obstacles to the treatment of OS[3]. At present, various long non-coding RNAs (lncRNAs) have been confirmed to play a crucial role in OS[4]. Therefore, lncRNAs are expected to become therapeutic targets, so as to strikingly improve the clinical treatment for OS.

LncRNAs with more than 200 nucleotides have been studied to be able to take part in the development of a variety of cancers[5,6]. Studies have revealed that over 90% of human genomic DNAs can be transcribed, but only 2% of them can encode proteins. It has been reported that small ncRNAs can participate in the oncogenesis, metastasis and chemoresistance of OS[7,8]. However, there is little research on relative lncRNAs in OS. Since lncRNAs account for more than 70% of ncRNAs[9], the unknown functions of lncRNAs involved in the pathogenesis of OS or mecha-

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nisms of drug resistance have been well explored nowadays. NKILA is an lncRNA that interacts with NF-κB and regulates the IκB kinase/nuclear factor-κB (IKK/NF-κB) signaling pathway. Although specific underlying mechanisms have not been fully elucidated, inflammation has been identified as a driver of cancer[10,11]. Among them, the IKK/NF-κB signaling pathway is a significant link between inflammation and tumor development[12]. The primary aim of this study was to investigate the role of NKILA in the pathogenesis of osteosarcoma to further understand the function of lncRNA in cancers.

Patients and Methods

Sample Collection

60 OS tissues were selected from patients of Yantaishan Hospital undergoing OS resection from July 2012 to July 2017. None of them received adjuvant therapy such as radiotherapy and chemotherapy preoperatively. All the specimens were proved to be OS by pathology. The samples in control group were from the peri-cancer tissue of the same patient (at least 3 cm away from the surgical margin). No cancer cells were found in peri-cancer tissues through pathological examination. All specimens were obtained and frozen in liquid nitrogen, and then stored in a refrigerator at -80°C. This experimental study was approved by the Ethics Committee of Yantaishan Hospital Medical.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

All cell and tissue samples were lysed with TRIzol (Invitrogen, Carlsbad, CA, USA), extracted with chloroform and precipitated with isopropanol to obtain total RNA. The extracted total RNA was stored at -80°C until use. After the concentration was measured with a micronucleus quantifier, complementary Deoxyribose Nucleic Acid (cDNA) was obtained by reverse transcription, and then SYBR Green was used for PCR detection. The primer sequences were: NKILA (F: 5'-TGGATTGTTGGGTATATTTTGGA-3'; R: 5'-TGTATGAAGAGGATGCTGAAGGC-3').

Cell Culture and Transfection

The normal bone cell line Hfob1.19 and the OS cell lines including KHOS/NP, U2OS MG63 and Saos2 were all purchased from obtained from the Institute of Biochemistry and Cell Biology (Beijing, China) and were cultured with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. OS cells with a good growth condition were selected for transfection experiment. The cells were counted first, and then evenly seeded in a six-well plate at a density of approximately 1×10^4 cells per well. The cells were transfected when grown to a density of 75-85%. After mixing the transfection reagent with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), the mixture was added to the cells with 1.5 ml of serum-free medium. The cells were then incubated at 37°C incubator. We changed the medium after 4-6 h of transfection, and cultured the cells for another 24 to 48 h. Then we proceeded to the next step according to the purpose of the experiment.

Cell Counting kit-8 (CCK-8) Assay

After transfection for 24 hours, the cells were collected and seeded in a 96-well plate at a density of 2×10^3 cells per well. Cell viability was detected at 24, 48, 72 and 96 h, respectively after cells were adherent. Subsequently, 10 μL of CCK-8 solution was added into each well and then the cells were cultured at 37°C for 2 h. The absorbance at a wavelength of 450 nm was detected with a microplate reader.

Cell Migration

24 h before the Transwell experiment, the cells with stable growth after transfection were selected and seeded into a 24-well transwell chamber at a density of 3×10^4 cells per well, and 5 replicate wells were set in each group. Next, 100 μL of serum-free medium were added to the lower chamber, and 500 μL of serum-containing medium was added to the upper chamber. The 24-well plate was placed in a 37°C incubator for 12 hours. After the plate was removed, 3 ml of 0.1% crystal violet staining solution was added to the chamber for 30 minutes at room temperature for sufficient staining. After the staining solution was discarded and rinsed with pre-warmed phosphate-buffered saline (PBS) for 2 times, unmigrated cells through the basement membrane of the upper chamber were wiped off with a cotton swab. Afterwards, a 10 × 20 microscope was used to observe the cells passing through the basement membrane of the chamber. Three fields were randomly selected from each well for photographing and cell counting.

Cell Invasion

Fibronectin (FN) was diluted to a concentration of 100 μg/mL. Matrigel was diluted with serum-free medium at a ratio of 1:9. 50 μL of FN was applied to the bottom of each cell and dried for 2 h in a clean bench. 100 μL of Matrigel was added to the inner side of the chamber, and then the cells were cultured overnight in the incubator. The cell density was adjusted to 1×10^6 cells/mL and 100 μL of cell suspension was pipetted to the upper layer of the Transwell chamber. 600 μL of medium containing 10% FBS was added to the outside of the chamber, which was then placed in the incubator for 24 h. After that, the chamber was fixed with methanol, stained with trypan blue and washed 3 times with PBS. The cells were photographed under a microscope.

Western Blot

The transfected cells were collected and the lysate was added to extract total protein. The cell lysate was placed on ice and centrifuged. The BCA (bicinchoninic acid) method was performed to quantitate the protein concentration. After the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein loading buffer was added, the protein was denatured by heating at 100°C. Sample was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Corresponding size of PVDF membrane was cut according to the molecular weight. Then, the antigen was sealed in blocking solution of 5% skim milk. Primary antibodies were used to incubate the protein bands overnight. In the next day, the membrane was incubated with secondary antibody, and then exposed.

RIP

After obtaining the cell lysate, magnetic beads were prepared and resuspended with Wash Buffer on ice. Subsequently, RNA-binding protein immunoprecipitation was performed. RNA was purified sequentially using phenol, chloroform, Salt Solution I, Salt Solution II, Precipitate Enhancer and absolute ethanol (no RNase). The final product was dissolved with 10-20 μL of diethyl pyrocarbonate (DEPC) water and stored at -80°C. Finally, the expression of NKILA in precipitates of p65 and IgG by qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (SPSS Inc., Chicago, IL, USA) was used for analysis. Measured data were expressed as mean ± standard deviation. The data was compared using the *t*-test. The χ^2-test was used to detect the correlation between clinical information and NKILA expression. p-value < 0.05 was considered statistically significant.

Results

NKILA was Lowly Expressed in Osteosarcoma Tissues

We detected the NKILA expression in 60 OS tissues and adjacent tissues. QRT-PCR results demonstrated that the expression of NKILA in OS tissues were strikingly lower than that in adjacent tissues ($p<0.001$) (Figure 1A and B). The median expression level of NKILA in OS tissues was calculated by statistical analysis, and then the case group was further divided into NKILA high expressing group and low expressing group. The correlation between clinical information and NKILA expression in OS patients was analyzed (Table I). We found that the expression of NKILA was not related to the age or sex of OS patients, but related to tumor size, Enneking stage and metastasis. OS patients were subdivided into Enneking stage I-II group and stage III group. The expression of NKILA in OS patients with stage III tumors was lower than those with stage I-II (Figure 1C). After that, OS patients were subdivided into lung metastasis group and non-pulmonary metastasis group. We found that the expression of NKILA in the tumor tissue of OS patients with lung metastasis was lower than those without metastasis (Figure 1D). These results indicated that NKILA might participate in the occurrence and development of OS.

Overexpression of NKILA Inhibited Migration and Invasion of Osteosarcoma Cells

NKILA expression was detected in the normal bone cell line Hfob1.19 and OS cell lines including KHOS/NP, U2OS, Saos2 and MG63. NKILA expression in OS cells was found to be remarkably lower than that of normal bone cells (Figure 2A). Since NKILA level was the highest in KHOS/NP cells and lowest in Saos2 cells, these cell lines were selected for the next study. We transfected NKILA-siRNA in KHOS/NP cells and found that NKILA expression was reduced (Figure 2B). Subsequent transfection of the overexpression plasmid of NKILA in Saos2 resulted in increased NKILA expression. CCK-8

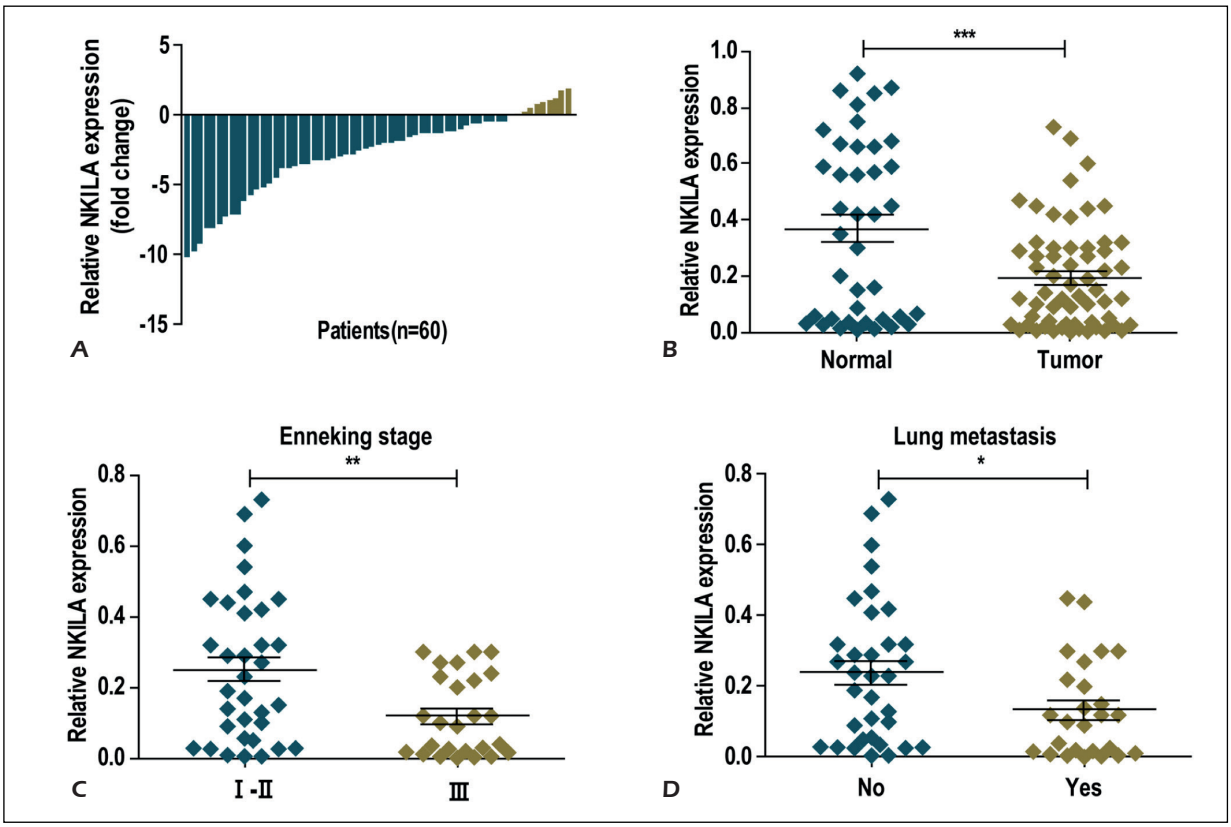

Figure 1. NKILA was lowly expressed in osteosarcoma patients. **A-B**, The expression of NKILA in tumor tissue was significantly lower than that in the normal group. **C**, NKILA expression in stage III tumor tissue was significantly lower than stage I-II. **D**, Expression of NKILA in tumor tissues of patients with lung metastasis was significantly lower than those without metastasis.

Table I. Relationship between *NKILA* expression and clinicopathologic characteristics of osteosarcoma.

Clinicopathological features	No. of cases	NKILA expression		*p*-value
		Low (n=30)	High (n=30)	
Age (years)				0.4383
<14	31	14	17	
≥14	29	16	13	
Gender				0.8056
Male	47	23	24	
Female	13	7	6	
Tumor size (cm)				0.0006*
<8 cm	23	18	5	
≥8 cm	37	12	25	
Enneking stage				0.0007*
I-II	27	20	7	
III	33	10	23	
Lymph metastasis				0.0097*
No	32	21	11	
Yes	28	9	19	

*$p<0.05$.

assay was used to detect cell proliferation at 24, 48, 72 and 96 hours, respectively. The results demonstrated that cell viability strikingly increased after NKILA-siRNA transfection (Figure 2D). However, after transfection of the NKILA overexpression plasmid, the cell viability of OS cells was reduced (Figure 2F). To further explore and analyze the function of NKILA in OS, we transfected NKILA-siRNA or overexpression plasmid in cells respectively, and then detected cell migration and invasion. We found that the invasion and migration of KHOS/NP cells significantly increased after knockdown of NKILA (Figure 2E), whereas and the invasion and migration of Saos2 cells were remarkably reduced after NKILA was overexpressed (Figure 2G). Those results indicated that NKILA could inhibit the invasion and migration of OS cells.

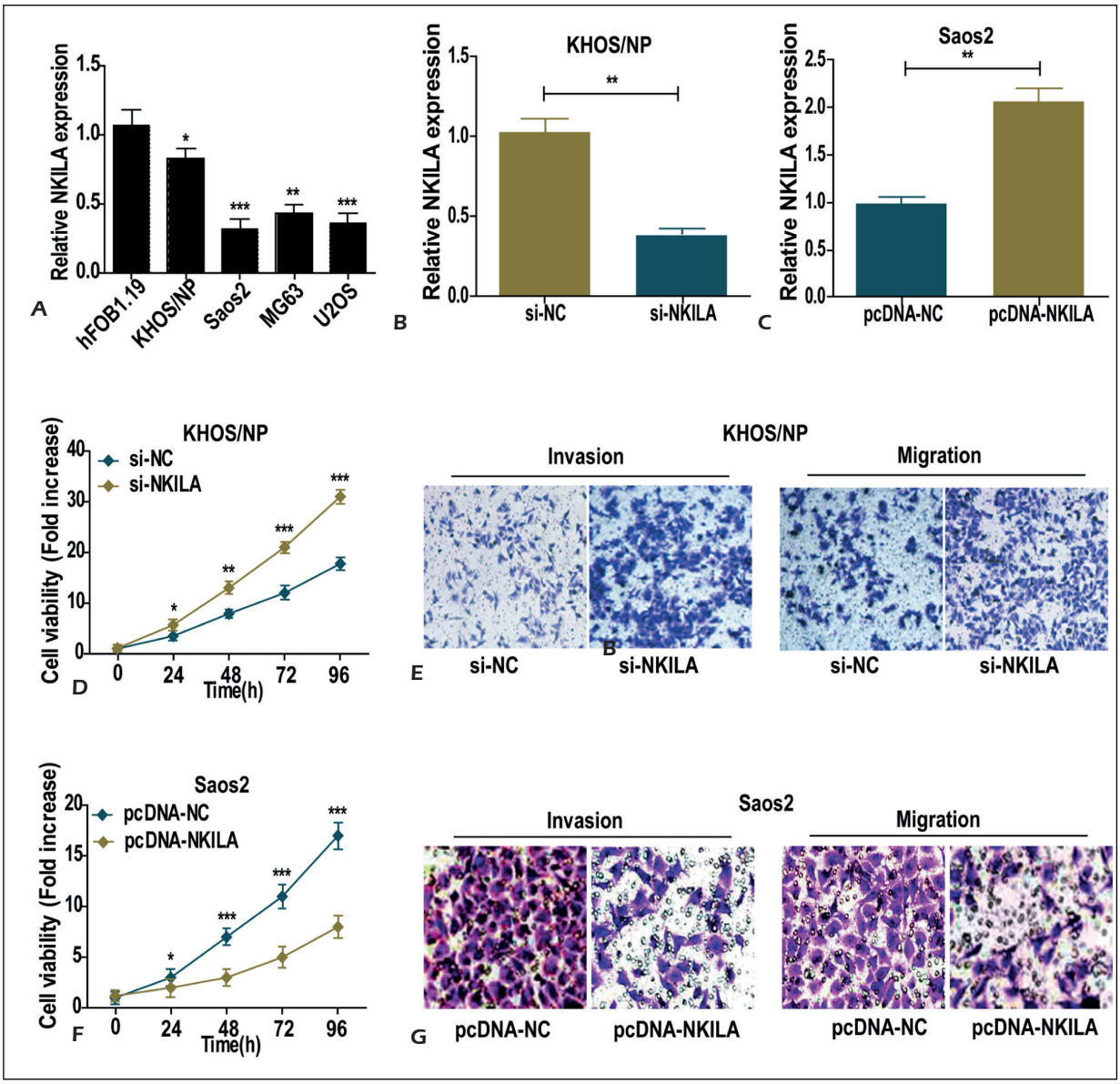

Figure 2. Overexpression of NKILA inhibited migration and invasion of osteosarcoma cells. **A**, The NKILA expression in the normal bone cell line Hfob 1.19 and osteosarcoma cell lines KHOS/NP, Saos2, MG63, U2OS. **B**, In the KHOS/NP cell line, the expression of NKILA significantly decreased after transfection of interfering sequences. **C**, In the Saos2 cell line, the expression of NKILA significantly increased after the overexpression plasmid was added. **D-E**, In the KHOS/NP cell line, NKILA knockdown increased the cell viability, migration and invasion. **F-G**, In the Saos2 cell line, NKILA overexpression decreased the cell viability, migration and invasion.

NKILA Affected Migration and Invasion of Osteosarcoma Cells by Regulating NF-κB/Snail Signaling Pathway

In the process of tumorigenesis, E-cadherin, NF-kb and Snail constitute a complex signaling network and influence with each other. After transfecting NKILA-siRNA in KHOS/NP cells, the expressions of p65 and E-cadherin were reduced but Snail expression strikingly increased (Figure 3A). Meanwhile, transfection of NKILA overexpression plasmid up-regulated the expressions of p65 and E-cadherin while, decreased Snail expression in Saos2 cells (Figure 3A). The RIP experiment performed in cells revealed that p65 could bind to NKILA (Figure 3B). Afterwards, the cells were divided into 3 groups, including control group, NKILA knockdown group and NKILA knockdown + NF-κB inhibitor (JSH) treatment group. We found that knockdown of NKILA increased the cell migration and invasion, whereas JSH treatment partially restored them (Figure 3C). These results indicated that NKILA could affect the invasion and migration of OS cells by regulating the NF-κB/Snail signaling pathway.

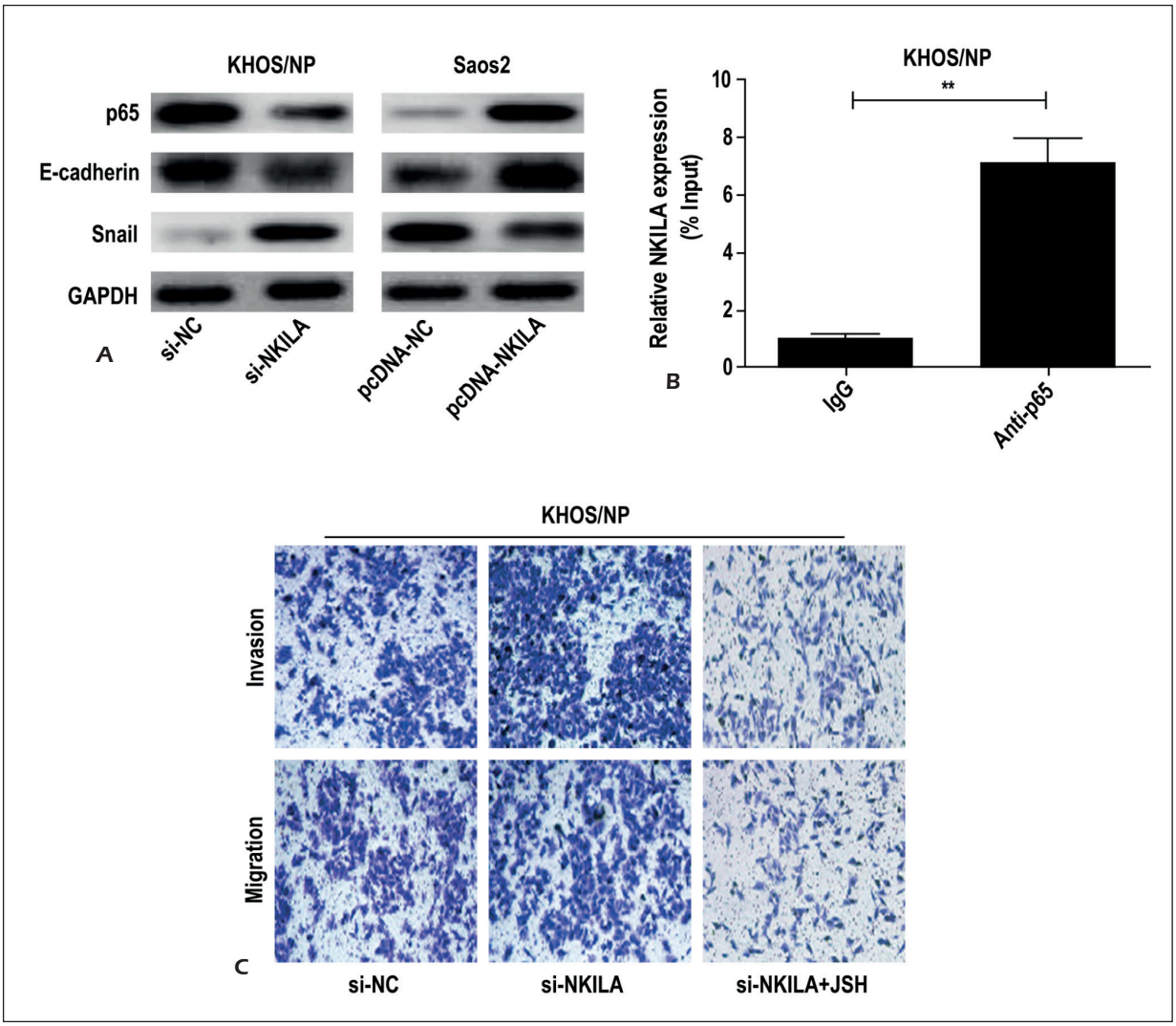

Figure 3. NKILA affected the migration and invasion of osteosarcoma cells by regulating the NF-κB/Snail signaling pathway. **A**, In the KHOS/NP cell line, NKILA knockdown decreased the expressions of p65 and E-cadherin decreased, but increased Snail expression. In the Saos2 cell line, NKILA overexpression increased the expressions of p65 and E-cadherin, but decreased Snail expression. **B**, QRT-PCR detection revealed a significant increase in the expression of NKILA in the RIP assay. **C**, The cells were divided into 3 groups: control group (si-NC), NKILA knockdown group (si-NKILA) and NKILA knockdown+NF-κ inhibitor JSH (si-NKILA+JSH) group. Knockdown of NKILA increased cell migration and invasion, which were reversed by JSH treatment.

Discussion

Osteosarcoma occurs mostly in the long bones, with 80-90% are femoral and luminal bones[13]. Less than 1% of this tumor occurs in the extremities of the hands and feet[14]. The major treatment of OS is surgical resection combined with radiotherapy or chemotherapy[15]. However, the prognosis of OS patients is poor, and the long-term survival rate of patients with metastases or relapses is still less than 20%[16]. Therefore, further understanding of the molecular mechanism related to the occurrence and development of OS could be beneficial to develop new prognostic molecular biomarkers and targeted therapeutic drugs. LncRNA was originally found in mouse transcripts, lacking of ability to encode proteins and significant open reading frame[17]. Therefore, lncRNAs are thought to be highly heterogeneous sequences of RNA molecules. Studies have demonstrated that lncRNA can act as a key regulator in different types of cancer. LncRNAs can be involved in tumor development, metastasis and regulation of chemotherapy resistance[18]. Studies have found that taurine upregulation gene 1 (TUG1) may be a therapeutic target for OS, which was closely related to the disease state[19]. Additionally, upregulation of UCA1 expression may have connections with tumor growth and distant metastasis, and may act as a biomarker for poor prognosis[20]. The oncogenic or tumor suppressive lncRNAs take part in the occurrence and development of OS, including migration, metastasis and apoptosis. LncRNA is expected to be an independent prognostic biomarker. Previous studies have shown that NKILA can participate in tumorigenesis. It can inhibit NF-κB activation induced by TGF-β and act as a target for preventing breast cancer metastasis by inhibiting EMT[21]. In laryngeal cancer, NKILA can inhibit cell proliferation and migration while promoting the apoptosis of cancer cells[22]. In liver cancer, NKILA can enhance the anticancer effect of safrole on hepatocellular carcinoma (HCC) *in vitro* and *in vivo* by modulating NF-κB signaling pathway[23]. In our study, NKILA expression in OS tissues was strikingly reduced, and its level was lower in stage III tumor tissues than that in stage I-II. In lung cancer tissue, the expression of NKILA in patients with metastasis was lower than that in non-metastasis patients. Meanwhile, the high expression of NKILA strikingly reduced the proliferation, invasion and migration of OS cells. All these results suggested that NKILA plays a crucial role in the occurrence and development of OS. NKILA expression was activated by NF-κB pathway. It can directly block the phosphorylation of IκB by interacting with the NF-κB/IκB complex, thereby inhibiting IKK phosphorylation and NF-κB activation. E-cadherin is involved in the cellular information transmission [24]. It is closely related to the differentiation of tumor cells, the loss of epithelial cell prototypes and the invasion process. E-cadherin plays a crucial role in the adhesion between cells as well as between cells and extracellular matrix. The loss of cell adhesion mediated by E-cadherin is an essential prerequisite for infiltration and metastasis of tumor cells[25]. In mesoderm and neural precursors, Snail triggers the transition from epithelium to mesenchymal, prompting their delamination and subsequent movement from the original stratum and neural tube. The interaction disruption between Snail-induced epithelial cells is not only limited to the destruction of the adhesive junction, but may also be related to the destruction of tight junctions[26]. In this study, we found that knockdown of NKILA strikingly reduced the expressions of p65 and E-cadherin, but increased the Snail level. At the same time, the invasive and migratory ability of OS cells was enhanced. Additionally, NF-κB inhibitor can reverse the effect of knockdown of NKILA on cell migration and proliferation.

Conclusions

We observed that NKILA can influence migration and invasion of osteosarcoma cells *via* NF-κB/Snail signaling pathway.

Conflict of Interests

No conflict of interest was declared.

References

1) Dai X, Ma W, He X, Jha RK. Review of therapeutic strategies for osteosarcoma, chondrosarcoma, and Ewing's sarcoma. Med Sci Monit 2011; 17: A177-A190.
2) Ottaviani G, Jaffe N. The etiology of osteosarcoma. Cancer Treat Res 2009; 152: 15-32.
3) Zhang XD, Wang YN, Feng XY, Yang JY, Ge YY, Kong WQ. Biological function of microRNA-30c/SOX9 in pediatric osteosarcoma cell growth and metastasis. Eur Rev Med Pharmacol Sci 2018; 22: 70-78.

4) Li CH, Chen Y. Targeting long non-coding RNAs in cancers: progress and prospects. Int J Biochem Cell Biol 2013; 45: 1895-1910.
5) Gutschner T, Diederichs S. The hallmarks of cancer: a long non-coding RNA point of view. RNA Biol 2012; 9: 703-719.
6) Da M, Ma J, Zhang Y, Yang J, Yao J, Huang B, Ma H, Ge L. High expression level of long non-coding RNA HOTAIR is associated with poor overall survival in gastric cancer patients: evidence from meta-analysis. J BUON 2017; 22: 911-918.
7) Wan X, Ding X, Chen S, Song H, Jiang H, Fang Y, Li P, Guo J. The functional sites of miRNAs and lncRNAs in gastric carcinogenesis. Tumour Biol 2015; 36: 521-532.
8) Yu X, Li Z. Long non-coding RNA growth arrest-specific transcript 5 in tumor biology. Oncol Lett 2015; 10: 1953-1958.
9) Yu X, Li Z. Long non-coding RNA HOTAIR: a novel oncogene (Review). Mol Med Rep 2015; 12: 5611-5618.
10) Dubbink HJ, Atmodimedjo PN, van Marion R, Krol N, Riegman P, Kros JM, van den Bent MJ, Dinjens W. Diagnostic detection of allelic losses and imbalances by next-generation sequencing: 1p/19q co-deletion analysis of gliomas. J Mol Diagn 2016; 18: 775-786.
11) Baumgarten SC, Frasor J. Minireview: Inflammation: an instigator of more aggressive estrogen re-ceptor (ER) positive breast cancers. Mol Endocrinol 2012; 26: 360-371.
12) Postler TS, Ghosh S. Bridging the gap: a regulator of NF-kappaB linking inflammation and cancer. J Oral Biosci 2015; 57: 143-147.
13) Baumhoer D. [Pathogenesis and genetics of osteosarcoma: current concepts and developments]. Pathologe 2018; 39: 139-145.
14) Poletajew S, Fus L, Wasiutynski A. Current concepts on pathogenesis and biology of metastatic oste-osarcoma tumors. Ortop Traumatol Rehabil 2011; 13: 537-545.
15) Carrle D, Bielack SS. Current strategies of chemotherapy in osteosarcoma. Int Orthop 2006; 30: 445-451.
16) Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. Nat Immunol 2008; 9: 839-845.
17) He S, Gu W, Li Y, Zhu H. ANRIL/CDKN2B-AS shows two-stage clade-specific evolution and becomes conserved after transposon insertions in simians. BMC Evol Biol 2013; 13: 247.
18) Rodriguez C, Borgel J, Court F, Cathala G, Forne T, Piette J. CTCF is a DNA methylation-sensitive positive regulator of the INK/ARF locus. Biochem Biophys Res Commun 2010; 392: 129-134.
19) Fan TM, de Lorimier LP, O'Dell-Anderson K, Lacoste HI, Charney SC. Single-agent pamidronate for palliative therapy of canine appendicular osteosarcoma bone pain. J Vet Intern Med 2007; 21: 431-439.
20) Kirpensteijn J, Kik M, Rutteman GR, Teske E. Prognostic significance of a new histologic grading system for canine osteosarcoma. Vet Pathol 2002; 39: 240-246.
21) Ke S, Li RC, Meng FK, Fang MH. NKILA inhibits NF-kappaB signaling and suppresses tumor me-tastasis. Aging (Albany NY) 2018; 10: 56-71.
22) Yang T, Li S, Liu J, Yin D, Yang X, Tang Q. lncRNA-NKILA/NF-kappaB feedback loop modulates laryngeal cancer cell proliferation, invasion, and radioresistance. Cancer Med 2018; 7: 2048-2063.
23) Yu X, Tang W, Yang Y, Tang L, Dai R, Pu B, Feng C, Xia J. Long noncoding RNA NKILA en-hances the anti-cancer effects of baicalein in hepatocellular carcinoma via the regulation of NF-kappaB signaling. Chem Biol Interact 2018; 285: 48-58.
24) Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. Science 1991; 251: 1451-1455.
25) Birchmeier W, Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta 1994; 1198: 11-26.
26) Sipos F, Galamb O. Epithelial-to-mesenchymal and mesenchymal-to-epithelial transitions in the colon. World J Gastroenterol 2012; 18: 601-608.