LncRNA KRAL suppresses cell growth and increases sensitivity to doxorubicin in osteosarcoma cells by sponging microRNAs-141

Jingwei Cai,1 Xiaohui Chen,2 Fei Ma,1 Jun Qian,3 Ming Niu,1 Yuzhen Gao,1 Junwei Li1 and Xiaoqiang Ren3

Abstract
Osteosarcoma (OS) is one of the most common types of malignant tumors characterized by uncontrolled proliferation ability and acquired drug resistance. The previous study indicated that lncRNA KRAL participated in the reversal of 5-FU resistance in liver cancer, but it remains unclear whether lncRNA KRAL involved in doxorubicin (DOX) resistance of osteosarcoma. The expression of lncRNA KRAL and MicroRNAs-141 (miR-141) were detected by RT-qPCR experiment. Also, we used the plasmids transfection to construct the lncRNA KRAL overexpressed OS cell lines. Subsequently, the cell proliferation ability and the sensitivity to DOX in OS cells upon upregulating lncRNA KRAL expression were analyzed by CCK-8 and EDU assay, while western blotting experiment was performed to detect the regulatory mechanism. We found that lncRNA KRAL was downregulated in OS tissues, and the OS patients with OS patients with lower expression of lncRNA KRAL were more likely to have advanced Enneking stage, larger tumor size and distant metastasis. Subsequently, we discovered that upregulation of lncRNA KRAL could inhibit cell proliferation and increase the sensitivity to DOX of OS cells. Interestingly, the western blot results showed that over-expression of lncRNA KRAL could lead to down-expression of P-gp protein and reversal of Epithelial–mesenchymal transition (EMT) pathway. Furthermore, we identified miR-141 as the downstream target gene of lncRNA KRAL, which was further confirmed by the luciferase reporter assay. More importantly, our data demonstrated that addition of miR-141 could reverse cell proliferation and drug sensitivity of lncRNA KRAL-overexpressed OS cells. LncRNA KRAL could suppress cell growth and increases sensitivity to DOX in OS cells by sponging miR-141.

Keywords
lncRNA KRAL, drug sensitivity, miR-141, osteosarcoma, proliferation

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Introduction
Osteosarcoma (OS), as a malignant tumor with highly incidence and mortality,1,2 which usually occurs in childhood and adolescents.3 The comprehensive treatment approaches for OS patients had made a big through in the past decades, but the 5-year survival rate was still at a low level due to the presence of primary and acquired resistance to chemo- or radiotherapy.4,5 Therefore, in order

1Department of Orthopedics, People’s Hospital of Ganzhou District, Zhangye, PR China
2Department of Anesthesiology, Gansu Provincial Hospital, Gansu Province, PR China
3Department of Orthopedics, Zhangye People’s Hospital Affiliated to Hexi University, Zhangye, PR China

Corresponding author:
Xiaoqiang Ren, Department of Orthopedics, Zhangye People’s Hospital Affiliated to Hexi University, Zhangye, Gansu 734000, PR China.
Email: renxiaoqianggs@163.com
to improve the prognosis of the patients with OS, it is of huge urgency and significance to identify potential bio-marker and overcome chemotherapy resistance.

Long noncoding RNAs (lncRNAs) are a type of noncoding RNA with more than 200 nucleotides, which play an important roles in diverse cellular processes, including cell proliferation, cell apoptosis and cell metastasis.\textsuperscript{6,7} For example, lncRNA HOST2 could enhance cell proliferation and metastasis of hepatocellular carcinoma cells through regulating JAK2-STAT3 signaling pathway.\textsuperscript{8} In addition, recent findings demonstrate that lncRNAs could serve as a potential target for drug sensitivity increasing of OS, including lncRNA NEAT1,\textsuperscript{9} Linc00161,\textsuperscript{10} lncRNA SNHG12,\textsuperscript{11} and et al. Importantly, lncRNA KRAL, also named Keap1 regulation-associated lncRNA, has been identified as a potential therapeutic target for overcoming 5-FU resistance in hepatocellular carcinoma cell.\textsuperscript{12} However, the role of lncRNA KRAL during the development of OS, worth further exploring.

Actually, accumulating studies identified that lncRNAs could serve as a tumor activator or suppressor via sponging miRNAs.\textsuperscript{13,14} LncRNA RP4, for example, had been reported to suppress cell proliferation in colorectal cancer via sponging miR-7-5p.\textsuperscript{15} Based on the bio-informatics results, we hypothesized that miR-141 might be the downstream target of lncRNA KRAL. MicroRNA-141 is an important member of the miR-200 family, which was closely associated with the development of ovarian cancer,\textsuperscript{16} prostate cancer,\textsuperscript{17} gastric cancer,\textsuperscript{18} and et al. However, its relationship between miR-141 and lncRNA KRAL, the potential role of miR-141 in OS remains unclear.

In this study, we aim to investigate whether lncRNA KRAL act as an anti-oncogene in the progression of OS. Herein, we firstly explore the relationship between the expression levels of lncRNA KRAL and the clinical pathologic features of patients with OS. Then, in vitro experiments were carried out to elucidate the impact of lncRNA on cell proliferation, drug resistance and EMT pathway in OS. Finally, we identified the potential downstream of lncRNA KRAL in OS, and validated the function of miR-141 in OS. Therefore, we predicted that lncRNA KRAL could suppress the progression of OS through binding with miR-141, while lncRNA KRAL might serve as an anti-oncogene and a promising therapeutic target for patients with OS.

\textbf{Materials and methods}

\textit{Patient tissue samples}

OS and adjacent non-tumor tissues were collected from 65 patients who had not been treated with radio- or chemical therapy in our center. The tissues were confirmed by veteran pathologists. All patients signed the informed consent before participating in this research. The medical ethics committee of our center approved this study.

\textit{Cell culture}

The osteosarcoma cell lines (143B, Saos-2, MG-63, HOS, os-732 and U2-OS) and human osteoblasts (hFOB1.19) were all purchased from American Type Culture Collection (ATCC, USA). In addition, these cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM, Hyclone, Logan, UT, USA) contained 10% fetal bovine serum (FBS; Gibco, Gran Island, NY), 100 U/mL penicillin (Invitrogen), and 100 μg/mL streptomycin (Invitrogen). In addition, the cells were incubated in a humid atmosphere with 5% CO\textsubscript{2} and 37°C.

\textit{Cell transfection}

In order to upregulate the expression levels of lncRNA KRAL in OS cells, lentiviruses (multiplicity of infection [MOI], 30) containing lncRNA KRAL plasmids were transfected into Saos-2 and MG-63 cells, while empty vectors were used as negative control (NC). Subsequently, the stable over-expression of lncRNA KRAL cell lines were screened by puromycin, and evaluated by the RT-qPCR.

\textit{RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)}

In brief, total RNAs were extracted from cell lines with Trizol reagent (Takara, Dalian, China), then reverse-transcribed into cDNAs with the PrimeScript RT Master Mix (Takara, Dalian, China) following the manufacturer’s procedure. RT-qPCR was performed with the standard SYBR-Green PCR kit (Roche, America). The relative
expression levels of target genes were calculated by the 2ΔΔCt method. Moreover, the primers sequences were shown as follow: lncRNA KRAL Forward-5′-CCA GTG GAC GGA CAT GCT TT-3′ and Reverse- 5′-CAC AGA GTT TGT GAG GGA GT-3′; GAPDH Forward-5′-ACG GAT TTG GTC GTA TTG G-3′ and Reverse-5′-TCC CGT TCT CAG CCT TG-3′; miR-141 Forward-5′-AGG GGT AAC ACT GTC TGG TAA-3′ and Reverse-5′-ACC CAG ACA CAA ACA TAG CC-3′; U6 Forward-5′-TCC GGG TGA TGC TTT TCC TAG-3′ and Reverse-5′-CGC TTC ACG AAT TTG CGT GTC AT-3′.

**CCK-8 assay**

The transfected cells (1×10^3 cells/well) were collected and planted in 96-well plates. After incubation for 0, 24, 48, 72 and 96 h, Cell Counting Kit-8 (CCK-8, Dojindo) solution was added to each well. 2 h later, the absorbance for each well was detected by the microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Moreover, to assay the drug sensitivity to DOX, OS cells were also pre-treated with different concentration of DOX, then detected its cell proliferation ability through CCK-8 assays.

**EDU assay**

The transfected cells (1×10^3 cells/well) were collected and planted in 96-well plates. Forty-eight hours later, the cells were stained with EdU cell proliferation kit (Ruibo, China), and finally captured with fluorescence microscope. Moreover, to assay the drug sensitivity to DOX, OS cells were also pre-treated with DOX (8 µg/mL), then detected its cell proliferation ability through EDU assay.

**Western blot analysis**

Total proteins were extracted through the RIPA lysis buffer (Beyotime Biotechnology, China) contained protease inhibitor (Roche, China). Then protein concentrations were quantified with the BCA™ Protein Assay Kit (Pierce, Appleton, USA). After that, proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, proteins were transferred into polyvinylidene difluoride (PVDF) membrane after electrophoresis. Subsequently, the PVDF membrane was incubated with 5% BSA for 1 h, then with primary antibodies at 4°C overnight. The following primary antibodies were used: anti-E-cadherin (cat. no. ab15148, 1:1000), anti-N-cadherin (cat. no. ab245117, 1:1000), anti-P-gp (cat. no. ab103477, 1:1000) and anti-GAPDH (cat. no. ab8245, 1:1000) (all from Abcam, Cambridge, MA, USA, 1:1000). The membranes were then incubated with an HRP-conjugated anti-rabbit (cat. no. RABHRP1, 1:1000) or anti-mouse (cat. no. RABHRP2, 1:1000) secondary antibody (both from Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Finally, GeneSnap in the SynGene system was used to evaluate the protein bands.

**Binding sites prediction**

In order to further elucidate the potential target microRNA of lncRNA KRAL, publicly available bioinformatic algorithms (lncRNABase) was utilized to predict the binding sites between target miRNA and lncRNA KRAL.

**Luciferase reporter assay**

The 293T cells were plated in 24-well plates at the concentration of 5×10^4 cells/well. Subsequently, cells were transfected with the wild-type lncRNA KRAL reporter (lncRNA KRAL-Wt) or the mutant-type lncRNA KRAL reporter (lncRNA KRAL-Mut). MiR-14 mimics or miR-NC were co-transfected into 293T cells. After 48h, Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was used to evaluate the relative luciferase activity.

**Statistical analysis**

Data were shown as the mean ± standard deviation (SD). All the experiments were repeated three times. All statistical analyses were conducted by using SPSS 20.0 software (Chicago, IL, USA). Differences among groups were measured by the student’s t-test and the one-way ANOVA. P < 0.05 was considered statistically significant.

**Results**

Down-regulation of lncRNA KRAL is closely associated with poor prognosis in OS

In order to determine the relationship between the expression of lncRNA KRAL and the clinicopathological characteristics of OS patients, RT-qPCR
was performed to analyze the lncRNA KRAL expression in OS tissues and adjacent normal tissues. The results displayed that OS patients with low expression of lncRNA KRAL accounted for 72.3% (47/65) of OS specimens (Figure 1(a)). In addition, compared with the adjacent non-tumor tissues, the expression levels of lncRNA KRAL were significantly down-regulated in OS tissues (Figure 1(b)). Besides, accumulative survival results exhibited that OS patients with lower expression of lncRNA KRAL might have shorter survival time (Figure 1(c)). Furthermore, OS patients with low expression of lncRNA KRAL were more likely to have advanced Enneking stage (Figure 1(d)), larger tumor size (Figure 1(e)) and distant metastasis (Figure 1(f)).

Figure 1. LncRNA KRAL is downregulated in OS tissues and closely related to OS patients' poor clinicopathological parameters. (a) Low expression of lncRNA KRAL accounts for 72.3% (47/65) in OS patients. (b) The expression levels of lncRNA KRAL are significantly lower in OS tissues than those in normal tissues. (c) The cumulative survival rates of OS patients with high or low expression of lncRNA KRAL. (d) The correlation between the expression of lncRNA KRAL and tumor Enneking stage. (e) The relationship between lncRNA KRAL expression and tumor size. (f) The relationship between lncRNA KRAL expression and metastasis. All experiments were repeated at least three times.

Overexpression of lncRNA KRAL suppresses cell growth and increases sensitivity to doxorubicin (DOX) in OS cells

Since lncRNA KRAL was proven to be downregulated in OS and capable to predict poor prognosis, we subsequently speculated that lncRNA KRAL might serve as an anti-oncogene in OS development. To investigate the potential biological function, we firstly detected the expression of lncRNA KRAL in OS cell lines. In our study, RT-qPCR results showed that, compared with the normal Hfob1.19 cell line, the expression levels of lncRNA KRAL in OS cell lines (143B, Saos-2, MG-63, HOS, os-732 and U2-OS cells) were down-regulated, with Saos-2 and MG-63 cell lines showing lowest expression (Figure 2(a)). Then, we constructed lncRNA KRAL-overexpressed (OE-lncRNA KRAL) Saos-2 and MG-63 cell lines via plasmids transfection. RT-qPCR results showed that the expression levels of lncRNA KRAL were not changed in the negative control group (OE-vector) compared with the blank group (OS cells without treatment), while the expression levels of lncRNA KRAL were up-regulated in the OE-lncRNA KRAL group (Figure 2(b) and (c)).
Subsequently, CCK-8 results indicated that lncRNA KRAL over-expression could significantly inhibit cell proliferation of Saos-2 and MG-63 (Figure 2(d) and (e)). Similarly, EDU assay also showed that there were less EDU-positive OS cells in OE-lncRNA KRAL group than that in OE-vector group (Supplementary files, Figure S1 A and B). Moreover, to detect the influence of lncRNA KRAL on drug sensitivity, we also detected the cell viabilities of OE-lncRNA KRAL OS cells in the presence of DOX, with the concentration ranged from 0 to 32 μg/ml. Compared to OE-vector group, OE-lncRNA KRAL OS cells showed increased sensitivity to DOX, and its concentration of IC50 was lower (Figure 2(f) and (g)). Consistent with the results of CCK-8 assay, at the present of DOX (8 μg/mL), less EDU-positive OS cells were found in OE-lncRNA KRAL group in comparison with the OE-vector group (Supplementary files, Figure S2 A&B). These findings generally elucidated the biological functions of lncRNA KRAL on cell proliferation and drug sensitivity abilities of OS cells.

LncRNA KRAL could down-regulate the expression of P-glyprotein (P-gp) via inhibiting epithelial-mesenchymal transition (EMT) signaling pathway

We subsequently detected the expression of drug resistance related P-gp protein, and EMT related proteins via western blotting. As shown in Figure 3 and Figure S3, the expression levels of P-gp proteins were dramatically down-regulated in OE-lncRNA KRAL OS cell lines. Meanwhile, we also discovered that the expression of E-ca protein was increased, while N-ca protein, beta-catenin and Vimentin were significantly down-regulated in OE-lncRNA KRAL group.

MicroRNAs-141 (miR-141) expression was negative related with lncRNA KRAL expression in OS patients

As lncRNAs have been acknowledged to regulate expression of certain miRNAs by sponging them at...
specific binding sites, and miR-141 was predicted to be the potential downstream target of lncRNA KRAL based on the public biological database. To address the issue, RT-qPCR was performed to evaluate the expression pattern of miR-141 in OS tissues. RT-qPCR results revealed that miR-141 was overexpressed in 73.8% (48/65) of OS specimens (Figure 4A). Consistently, the expression of miR-141 in OS tumors was significantly up-regulated compared with the adjacent normal tissues (Figure 4B). In addition, The correlation analysis showed that the expression levels of lncRNA KRAL were negatively correlated with the expression of miR-141 (Figure 4C, \( r = -0.5674, p < 0.0001 \)).

MicroRNAs-141 (miR-141) is the downstream target of lncRNA KRAL in OS

The binding sites between lncRNA KRAL and the miR-141 were showed in Figure 5(a). To further explore the relationship between lncRNA KRAL and miR-141, luciferase reporter assay was employed, which indicated that luciferase activity was significantly lower in wild-type (WT) cells treated with miR-141 mimics (Figure 5(b)). In addition, RT-qPCR results also indicated that the expression levels of miR-141 were downregulated in lncRNA KRAL-overexpressed MG-63 cells (Figure 5(c)). Moreover, from the results of CCK-8 assay, miR-141 could partly rescue the influence...
on cell proliferation and drug sensitivity induced by lncRNA KRAL’s overexpression (Figure 5(d) and (e)). Taken together, these findings suggest that miR-141 might be a downstream gene of lncRNA KRAL in OS.

Discussion

Osteosarcoma (OS) is one of the most common malignant tumor among teenagers, and the main treatment for patients with OS is radical surgical resection in conjunction with systemic chemotherapy.\textsuperscript{3,19} Doxorubicin (DOX) is the first-line chemotherapy drug for the treatment of various cancers.\textsuperscript{20} However, during the DOX chemotherapy, numerous OS patients exhibit primary or acquired drug resistance, which contributed to the high death rates of OS.\textsuperscript{21} In recent year, accumulating studies prove that lncRNAs play an important role in regulating tumorigenesis and drug resistance of cancer cells. The lncRNA GAS5, for example, overcomes adriamycin resistance in breast cancer via inhibiting the activation of Wnt/β-catenin signaling pathway.\textsuperscript{22} Another study identifies that the silencing of lncRNA CCAT1 could enhance paclitaxel-induced anti-tumor effect in prostate cancer via regulating miR-24-3p/FSCN1 axis.\textsuperscript{23} Therefore, it is of great important to identify the effective biological marker and clarify its regulatory mechanism.

Interestingly, a recent study discovers that lncRNA KRAL might function as a critical regulator in overcoming 5-FU resistance of hepatocellular carcinoma cells, indicating that lncRNA KRAL might play an important role in regulation of drug resistance.\textsuperscript{12} As DOX resistance is the pivot cause for high mortality of OS, therefore we performed in vitro experiments to identify whether lncRNA KRAL participate in regulating DOX resistance in OS. In current study, we evaluated the expression level of lncRNA KRAL in OS tissue samples, and discovered that of lncRNA KRAL was down-regulated in OS cells relative to adjacent normal tissues. After statistical analysis, our results demonstrated that the OS patients with lncRNA KRAL lowexpression was more likely to have advanced Enneking stage, larger tumor size, distant metastasis and shorter survival time. Therefore, lncRNA KRAL would be a significant bio-marker in OS progression. In order to further confirm the biological role of lncRNA KRAL in the development of OS and elucidate the potential regulatory mechanism, the lncRNA KRAL-overexpressed OS cells were constructed via plasmids transfection. Both of the results of CCK-8 and EDU assays demonstrated that the cell proliferation ability of OS cells could be suppressed upon lncRNA KRAL overexpressed. Furthermore, we discovered that overexpression of lncRNA KRAL could lead to lower IC50 values for DOX in both Saos-2 cells and MG-63 cells, indicating that lncRNA KRAL could sensitize OS cells to DOX. Taken together, these results consistently exhibited that lncRNA KRAL played a significant role in the reversal of DOX resistance in OS, but the potential regulatory mechanism was still not clear.

Epithelial-mesenchymal transition (EMT) is a biological process that epithelial cells transform into mesenchymal cells.\textsuperscript{24} Recently, increasing studies demonstrated that EMT signaling pathway way was closely involved in the development of cancer drug resistance.\textsuperscript{25} Importantly, previous studies identified that EMT inducers could enhance the function of P-gp protein, which efflux more chemotherapy drug out of cells contributed to drug resistance.\textsuperscript{26} Another study demonstrated that down-regulation of HIF-1α could inhibit EMT phenotype, subsequently recover the drug
sensitivity to cisplatin in hepatocellular carcinoma under hypoxia. In our study, western blot results showed that up-regulation of lncRNA KRAL led to the expression levels of drug resistance related P-gp proteins and reversal of the EMT pathway, with over-expression of E-cadherin and reduction of EMT marker (N-cadherin, beta-catenin and Vimentin). Taken together, lncRNA KRAL might sensitize the OS cells to DOX through suppressing EMT signal pathway.

More importantly, we screened in the publicly available bioinformatic algorithms (lncRNABase) and identified miR-141 as the downstream target of lncRNA KRAL. MiR-141 has been reported to be upregulated in various cancers and play a tumor activator role, including gastric cancer, colorectal cancer and liver cancer. In our study, RT-qPCR results showed that the expression levels of miR-141 were up-regulated in OS tissue samples relative to the normal tissues, and the expression levels were negatively associated with lncRNA KRAL. In addition, luciferase reporter assay confirmed that miR-141 was the target downstream gene of lncRNA KRAL. Furthermore, CCK-8 results revealed that the tumor suppressed function and enhanced drug sensitivity in lncRNA KRAL overexpressed MG-63 cells could be reversed with the supplement of miR-141 mimics. Consequently, our results consistently revealed that miR-141 was also involved OS cells’ proliferation and DOX resistance induced by lncRNA KRAL.

**Conclusion**

To sum up, this study confirmed that lncRNA KRAL sponged miR-141, then elicited its impact on cell

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**Figure 4.** MicroRNAs-141 (miR-141)’s expression showed negative relationship with lncRNA KRAL in OS patients. (a) MiR-114 was overexpressed in 73.8% (48/65) of OS specimens. (b) The expression of miR-141 in OS tumors was significantly up-regulated compared with the expression in the adjacent normal tissues. (c) The correlation analysis showed that the expression levels of lncRNA KRAL were negatively correlated with the expression of miR-141.
proliferation, drug sensitivity and EMT pathway in OS, which would be a promising treatment target for patients with OS. However, there is still some limitation in our study. Firstly, the downstream target of lncRNA KRAL/miR141 has not been explored. Secondly, the relationship between DOX sensitivity and EMT signal pathway was not fully discussed, and we would carry out more experiments to validate them. Thirdly, the sample size calculation was not done in current study.

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ORCID iD
Xiaoqiang Ren https://orcid.org/0000-0002-6026-2171

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Figure 5. MicroRNAs-141 (miR-141) is the downstream target of lncRNA KRAL in OS. The binding sites between lncRNA KRAL and the miR-141. (a) Relative luciferase activity in 293T cells after co-transfection with pmirGLO-lncRNA KRAL-WT or pmirGLO-lncRNA KRAL-MUT, along with miR-141 mimics or miR-141 NC. (b) The expression levels of miR-141 in lncRNA KRAL overexpressed MG-63 cells. (c) The effect of miR-141 on proliferation of lncRNA KRAL overexpressed MG-63 cells in vitro using CCK-8 assay. (d) The effect of miR-141 on DOX sensitivity of lncRNA KRAL overexpressed MG-63 cells using CCK-8 assay. All experiments were repeated at least three times. *** p < 0.001.
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