lncRNA NORAD promotes the progression of osteosarcoma via targeting of miR-155-5p

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Abstract. Osteosarcoma (OS) is the most common malignant bone tumor in teens. Non-coding RNA activated by DNA damage (NORAD), a long non-coding RNA (lncRNA), has been reported to be involved in cancer biology, although its role in OS remains largely unknown. In the present study reverse transcription-quantitative PCR (RT-qPCR) was used to determine the expression levels of NORAD and miR-155-5p in samples from patients with OS. OS cell lines (Saos-2 and U2OS) were used as cell models. The biological influence of NORAD on OS cells was studied *in vitro* using Cell Counting Kit-8 and Transwell assays. The interaction between NORAD and miR-155-5p was clarified by bioinformatics analysis, RT-qPCR, luciferase reporter assay and RNA immunoprecipitation. NORAD was significantly increased in OS samples in comparison with controls, while miR-155-5p was reduced. Knockdown of NORAD and transfection of miR-155-5p mimics markedly inhibited the viability, migration and invasion of OS cells. There was a negative correlation between NORAD and miR-155-5p expression levels in OS samples. Taken together, the results of the present study indicated that the NORAD/miR-155-5p axis played a crucial role in regulating the proliferation, migration and invasion of OS cells. It is hypothesized that NORAD and miR-155-5p may serve as potential novel therapeutic targets for OS management.

Introduction

Osteosarcoma (OS) is the most common malignant bone tumor in teenagers and often occurs during rapid-growth of the metaphysis of long bones. Its clinical incidence is rather high, accounting for 60% of malignant bone tumors in adolescents (1,2). Previous trauma history, genetic factors, virus infection and radioactive stimulation are all closely related to the etiology of OS (3). Due to the high malignancy, rapid growth and invasiveness and early metastasis of OS, most OS patients are diagnosed at an advanced stage (4). It is therefore necessary to investigate the molecular mechanism underlying OS pathogenesis and explore potentially valuable biomarkers and therapeutic targets for OS treatment.

Long non-coding RNA (lncRNA) is a form of non-coding RNA with a length of ~200 nucleotides in eukaryotic cells. lncRNA plays an important role in genomic imprinting, chromatin modification, transcriptional activation and intranuclear transport, as well as in the proliferation, apoptosis, migration and invasion of cancer cells (5,6). Accumulating evidence suggests that many lncRNAs participate in the tumorigenesis and progression of multiple cancers, including OS. lncRNA HOXA11-AS promotes the proliferation and invasion of gastric cancer by scaffolding the chromatin modification factors polycomb repressive complex 2, lysine-specific histone demethylase 1A and DNA (cytosine-5)-methyltransferase 1 (7). lncRNA SNHG1 acts on microRNA (miR)-326, regulating the expression of RNA binding protein NOB1 and promoting the tumorigenesis of OS (8). Highly conserved and enriched in many types of cells, non-coding RNA activated by DNA damage (NORAD) is an lncRNA which plays an important role in cancer biology (9). As a novel competitive endogenous RNA (ceRNA), NORAD can enhance hypoxia-induced epithelial-mesenchymal transition by targeting miR-125a-3p, thereby promoting the metastasis of pancreatic cancer (10). However, the role and mechanism of NORAD in OS have not been fully elucidated.

miRs are a class of small non-coding RNAs consisting of ~18-22 nucleotides. miRs can inhibit the translation or regulate the degradation of their target mRNAs by binding to their 3'-untraslation regions (3'-UTRs) (11). miRs also participate in many cellular processes, including the differentiation, proliferation, metastasis and apoptosis of cancer cells (11-14). miR-486 inhibits the progression of OS by regulating the protein kinase C-δ pathway (15). The down-regulation of miR-664a disrupts the migration of OS cells via the regulation of maternally expressed 3 (16) and miR-491 inhibits lung metastasis and chemotherapy resistance of OS by targeting αB-crystallin (17).

miR-155 plays an important role in the progression of multiple types of cancers and other human diseases. It has been suggested that macrophage-derived exosomes

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containing miR-155 inhibit the proliferation of fibroblasts and promote fibroblast inflammation in myocardial injury (18). Down-regulation of miR-155 can inhibit the anus kinase-signal transducer and activator of transcription 3 pathway by up-regulating the expression of suppressor of cytokine signaling 3, thus suppressing the proliferation and promoting the apoptosis of lymphoma cells (19). miR-155 can also regulate the proliferation, invasion and apoptosis of renal cancer cells via regulation of the GSK-3β/β-catenin pathway (20). The function of miR-155 in OS has also been reported. Specifically, through the regulation of the phosphatase and tensin homolog deleted on chromosome ten/phosphoinositide 3-kinase/AKT/mechanistic target of rapamycin pathway, miR-155 can modulate doxorubicin-induced autophagy in OS cells (21). However, to the best of our knowledge, no previous studies have explored the mechanism of miR-155 dysregulation in OS.

Through the Starbase database, a potential binding site between NORAD and miR-155-5p was identified. As NORAD has been reported to be an oncogenic lncRNA in several cancers (9,10), it was hypothesized that NORAD could promote OS progression by regulating miR-155-5p. In the present study, the expression and biological functions of NORAD and miR-155-5p were investigated in OS. Additionally, NORAD was validated to contain a conserved target site of miR-155-5p. Based on this discovery, it was concluded that NORAD could regulate the proliferation, migration and invasion of OS cells by targeting miR-155-5p.

Materials and methods

Sample collection. OS tissues and paired adjacent tissues were surgically removed from patients (aged 7-35 years old; mean age, 25.2 years old) in Zhongnan Hospital of Wuhan University between July 2016 and November 2018. The collection and use of samples was approved by the Ethics Committee of Zhongnan Hospital and the Ethics Review Board of Ezhou Central Hospital. Patients or the parents of patients that were minors provided their written informed consent prior to study commencement. The tissue samples were obtained during surgery and the surgery was a part of comprehensive treatment program that aimed to prolong the prognosis of the patients. No patients had received radiotherapy or chemotherapy before surgery. The detailed parameters of all patients are shown in Tables I and II. All samples were confirmed as OS through clinical, imaging and histological methods (22). After collection, the samples were immediately stored at -80˚C until use. To facilitate subsequent analyses, the above 30 patients were divided into a low expression group (n=15) and a high expression group (n=15) according to the cutoff value of 2.72 for NORAD expression. in their tumor tissues.

Cell culture and transfection. The human OS cell lines 143B, HOS, MG63, Saos-2 and U2OS, the normal osteoblast cell line hFOB and HEK-293T were purchased from the American Type Culture Collection. Both OS cell lines (Saos-2 and U20S) and HEK-293T cells were cultured in a Dulbecco's Modified Eagle's medium (DMEM; HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The culture conditions were 5% CO_2 and 37˚C. hFOB cells were cultured under the same conditions in a DMEM/Ham's F-12 (1:1) medium supplemented with 10% FBS and 0.3 mg/ml G418 (Gibco; Thermo Fisher Scientific, Inc.). NORAD plasmids, NORAD siRNA and its control (si-NC), miR-155-5p mimics and its control (miR-NC), miR-155-5p inhibitors (miR-155-5p-in) and its control (inh-NC) were designed and constructed by GenePharma Co., Ltd. (Shanghai, China). Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) was used to conduct transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA in tissues and Saos-2/U2OS cells was extracted with a TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) miR was extracted using a mirPremier® microRNA Isolation Kit (Sigma-Aldrich; Merck KGaA). Total RNA was reverse transcribed using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). PCR was performed using a LightCycler FastStart DNA Master plus a SYBR Green I kit (Roche Diagnostics) to analyze the expression of NORAD and GAPDH according to the manufacturer's protocol. A TaqMan microRNA assay and TaqMan Universal Master Mix II (Applied Biosystems) were used to analyze the contents of miR-155-5p and U6. The PCR cycles were as follows: Initial denaturation at 95˚C for 5 min, 35 cycles of denaturation (45 sec at 95˚C), annealing (45 sec at 60˚C) and extension (8 min at 68˚C), and a final extension at 68˚C for 10 min. The relative expressions were calculated using the $2^{-\Delta\Delta Cq}$ method (23). The sequences of primers were as follows: NORAD forward 5'-GCCATTGGG CGAGACCTACCT-3' and reverse 5'-GTTCGGGACTTCGCT CACCTT-3'; miR-155-5p forward 5'-ACACTCCAGCTGTAA ACATCCTACACTCT-3' and reverse 5'-CTCAACTGGTGT CGTGGA-3'; U6 forward 5'-GCTTCGGCAGCACATATA CTA-3' and reverse 5'-CGAATTTGCGTGTCATCCTTG-3'; GAPDH forward 5'-TGTTCGTCATGGGTGTGAAC-3' and reverse 5'-ATGGCATGGACTGTGGTCAT-3'.

Luciferase reporter assay. The NORAD sequence was amplified and inserted to a pmirGLO plasmid (Promega Corporation) downstream of the luciferase gene to produce pMIR-NORAD-wild type (NORAD-wt). To obtain the mutant (mut) plasmid (NORAD-mut), a fragment containing a target region of the mutation was designed. Then, NORAD-wt, NORAD-mut, miR-155-5p mimics and miR-NC were transfected into HEK-293T cells at a final concentration of 50 nM using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, luciferase activity was analyzed using a Dual-Luciferase Reporter Assay System (Promega Corporation). The ratio of firefly luciferase activity to *Renilla* luciferase activity was calculated and used as relative luciferase activity.

RNA immunoprecipitation (RIP). An EZ-Magna RIP RNA-binding protein immunoprecipitation kit (EMD Millipore) was used according to the manufacturer's instruction. Cells were lysed in a complete RIPA buffer containing protease and RNase inhibitors. Cell ($1x10^7$ cells) extractions were incubated with a RIP buffer containing magnetic beads tagged with human anti-AGO 2 antibody (EMD Millipore) or IgG antibody. Finally, the combined RNA was purified with TRIzol, and the miR-155-5p and NORAD expression were evaluated using RT-qPCR.

Table I. Correlation between NORAD levels and clinical features in osteosarcoma patients.

Parameter	Total number	Low NORAD expression	High NORAD expression	χ^2	P-value
Sex				0.5357	0.4642
Male	16	7	9		
Female	14	8	6		
Age (years)				0.1587	0.6903
<20	21	10	11		
≥20	9	5	4		
Histological classification				2.4000	0.3012
Osteoblastic	15	6	9		
Chondroblastic	10	5	5		
Fibroblastic	5	4	1		
Tumor grade				7.0335	0.0080[a]
Low	11	9	2		
High	19	6	13		
Enneking stage				6.7857	0.0336[a]
I	8	7	1		
II	14	6	8		
III	8	2	6		
Tumor size (cm)				4.8214	0.0281[a]
<8	16	11	5		
≥8	14	4	10		
Metastasis				9.6000	0.0019[a]
No	10	9	1		
Yes	20	6	14		

[a]P<0.05.

Cell proliferation. Cell proliferation was measured using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. In brief, the transfected cells (Saos-2 and U2OS cells were transfected with si-NC, si-NORAD, miR-NC, miR-155-5p mimics, inh-NC, miR-155-5p-in, si-NC + inh-NC, si-NORAD + inh-NC, si-NC + miR-155-5p-in, si-NORAD + miR-155-5p-in, respectivly) were seeded into individual wells of a 96-well plate at a density of $1x10^4$ per well and incubated for 0, 24, 48 and 72 h (37˚C, 5% CO_2), respectively. A 10 μl volume of CCK-8 solution was added to each well and incubated for a further 1 h. The OD values at 450 nm were measured using a microplate reader (Bio-Rad Laboratories, Inc.).

Transwell assay. Transfected cells (Saos-2 and U2OS cells were transfected with si-NC, si-NORAD, miR-NC, miR-155-5p mimics, inh-NC, miR-155-5p-in, si-NC + inh-NC, si-NORAD + inh-NC, si-NC + miR-155-5p-in, si-NORAD + miR-155-5p-in, respectivly) were seeded into a 24-well plate at a density of $5x10^4$ cells/well and cultivated at 37˚C, 5% CO_2 equipped with Transwell chambers (8 μm pore size; BD Biosciences). The cells were suspended in the upper chamber in a serum-free medium (~$1x10^5$ cells/well), and the lower chamber was loaded with a medium containing 10% FBS. After 24 h, the cells on the surface of the upper chamber were gently wiped off with a cotton swab, and the cells that migrated or invaded the lower chamber were fixed with methanol (37˚C 15 min) and then stained with crystal violet (37˚C, 30 min). Finally, the stained cells were visualized under an inverted microscope (magnification, x400) and counted in five random fields.

Bioinformatics analysis. In the present study, StarBase database (version 3.0; http://starbase.sysu.edu.cn/) was used to identify the binding sites between miR-155-5p and NORAD.

Statistical analysis. All experiments were repeated three times, with three replicates each time. All statistical analyses were conducted using SPSS 20.0 (SPSS, Inc.) and Graphpad Prism 7 software (GraphPad Software, Inc.). Measurement data are presented as the mean ± standard deviation. One-Sample Kolmogorov-Smirnov test was used to examine whether the data are normally distributed or not. For normally distributed data, comparisons between two groups were performed using unpaired t-test. The comparisons among three or more groups were performed with one-way ANOVA followed by Tukey's post hoc test. For data with a skewed distributed, the comparison between two groups was performed by paired sample Wilcoxon signed-rank test. The relationship between NORAD/miR-155-5p and clinicopathological characteristics

Table II. Correlation between miR-155-5p levels and clinical features in osteosarcoma patients.

Parameter	Total number	Low miR 155-5p expression	High miR-155-5p expression	χ^2	P-value
Sex				2.3295	0.1269
Male	16	7	9		
Female	14	10	4		
Age (years)				0.5236	0.4693
<20	21	11	10		
≥20	9	6	3		
Histological classification				1.3575	0.5073
Osteoblastic	15	7	8		
Chondroblastic	10	7	3		
Fibroblastic	5	3	2		
Tumor grade				8.2935	0.0040[a]
Low	11	10	1		
High	19	7	12		
Enneking stage				8.8244	0.0121[a]
I	9	7	2		
II	13	9	4		
III	8	1	7		
Tumor size (cm)				4.6930	0.0303[a]
<8	16	12	4		
≥8	14	5	9		
Metastasis				7.3692	0.0066[a]
No	10	9	1		
Yes	20	8	13		

[a]P<0.05.

was determined with the χ^2 test. P<0.05 indicated a statistically significant difference.

Results

NORAD expression is increased in OS tissues in comparison with controls and is closely associated with the clinicopathological characteristics of OS. To investigate whether NORAD was expressed at abnormal levels in OS, RT-qPCR was used to determine the expression level of NORAD in tumor tissues and adjacent tissues from 30 OS patients. The results showed that the expression level of NORAD in tumor tissues was significantly higher than that in matched control tissues (Fig. 1A; P<0.001). Additionally, the expression of NORAD was examined in 143B, HOS, MG63, Saos-2, U2OS and hFOB cells. It was found that the expression of NORAD in all OS cell lines was higher than that in hFOB cells (Fig. 1B, P<0.001). To understand the clinical significance of NORAD in OS, the relationship between the expression level of NORAD and the clinical characteristics of OS was evaluated. The above 30 patients were divided into a low expression group (n=15) and a high expression group (n=15) according to the expression level of NORAD in their tumor tissues. The results suggested that a high NORAD expression level was related to tumor grade, Enneking stage, tumor size and the status of metastasis (Table I; P<0.05). However, the expression level of NORAD was not associated with the sex, age or histological subtype of the patients. All results suggested that the expression level of NORAD was increased in OS vs. matched control tissues and the patients with a high expression level of NORAD had an unfavorable prognosis.

miR-155-5p expression is reduced in OS tissues and cell lines in comparison with controls. miR-155-5p was significantly reduced in both OS tissues and OS cell lines in comparison with controls (Fig. 1C and D; P<0.001). Furthermore, a reduced expression level of miR-155-5p was associated with tumor grade, Enneking stage, tumor size and the status of metastasis (Table II, P<0.05). These results supported the hypothesis that miR-155-5p can act as a tumor suppressor in OS. A correlation analysis was conducted between the expression levels of NORAD and miR-155-5p in the 30 OS tumor tissue samples. The results indicated a negative relationship between NORAD and miR-155-5p expressions (Fig. 1E; r=-0.6248; P<0.001), suggesting a potential regulatory relationship between NORAD and miR-155-5p.

NORAD knockout inhibits the proliferation, migration and invasion of OS cells. To explore the effect of NORAD on the malignant phenotypes of OS, NORAD siRNA and NC siRNA

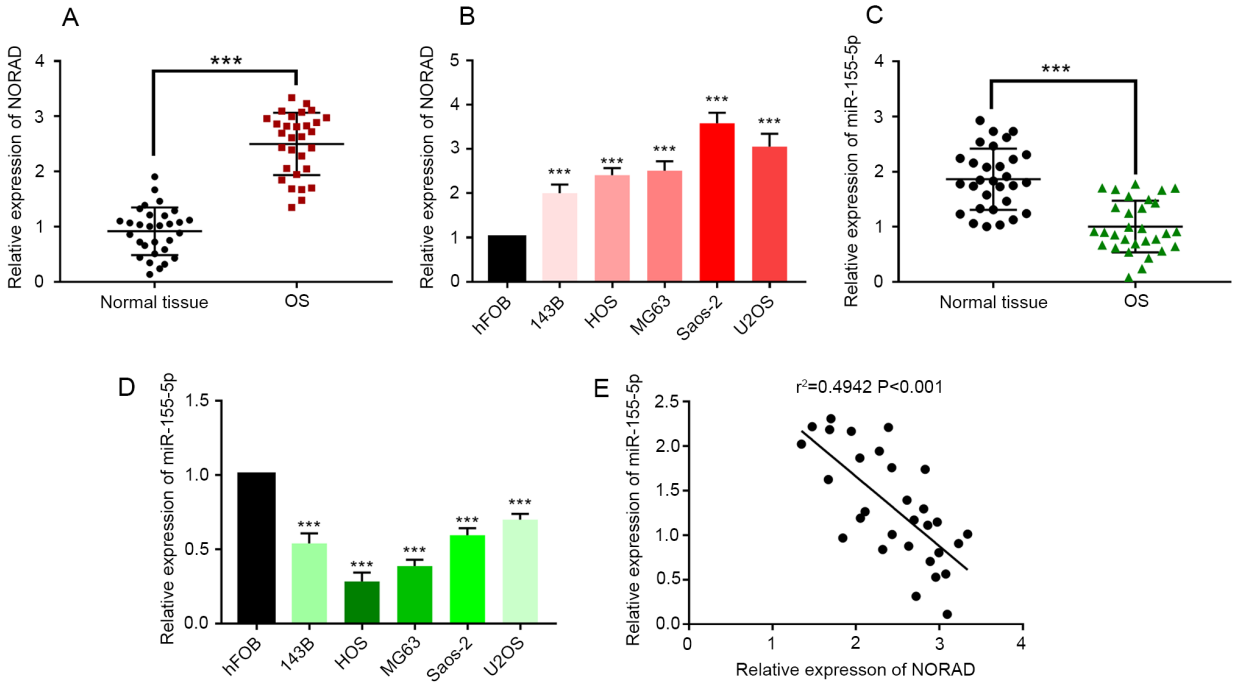

Figure 1. NORAD expression is increased and miR-155-5p expression reduced in OS tissues and cells in comparison with controls. (A) RT-qPCR was performed to measure the expression of NORAD in OS tumor and adjacent normal tissues from 30 patients. (B) RT-qPCR was performed to measure the expression of NORAD in 143B, HOS, MG63, Saos-2, U2OS and hFOB cells. (C) RT-qPCR was performed to measure the expression of miR-155-5p in OS tumor and adjacent normal tissues from 30 patients. (D) RT-qPCR was performed to measure the expression of miR-155-5p in 143B, HOS, MG63, Saos-2, U2OS and hFOB cells. (E) Correlation analysis between the expression levels of NORAD and miR-155-5p in 30 OS tumor tissues. ***P<0.001. NORAD, non-coding RNA activated by DNA damage; miR, microRNA; OS, osteosarcoma; RT-qPCR, reverse transcription quantitative PCR.

were transfected into Saos-2 and U2OS cells to observe their effects. The NORAD expression level in the si-NORAD group was significantly lower than that in the si-NC group (Fig. 2A; P<0.001). A CCK-8 assay was performed to investigate the effect of NORAD on cell proliferation, and the result showed that the proliferation of OS cells was significantly inhibited after NORAD knockdown (Fig. 2B). Furthermore, the effect of NORAD knockdown on the migration and invasion of OS cells was studied. Transwell assay showed that the count of migrating and invading cells in the si-NORAD group was significantly lower than that in the si-NC group (Fig. 2C; P<0.001). These results suggested that NORAD modulated the malignant phenotypes of OS cells.

miR-155-5p inhibits the proliferation, invasion and migration of OS cells. The function of miR-155-5p in OS was explored in the present study. After validating transfection efficiency (Fig. 3A; P<0.01), the CCK-8 assay was performed to investigate the effect of miR-155-5p on cell proliferation. The result indicated that the proliferation of OS cells was inhibited by miR-155-5p but promoted by miR-155-5p inhibitors in comparison with controls (Fig. 3B). Furthermore, the effects of miR-155-5p on the invasion and migration of OS cells were studied. The Transwell assay showed that the count of migrating and invading OS cells was reduced with treatment with miR-155-5p mimics but increased with treatment with miR-155-5p inhibitor in comparison with controls (Fig. 3C and D; P<0.001). Collectively, these results support the hypothesis that miR-155-5p acted as a tumor suppressor in OS.

NORAD acts as a sponge of miR-155-5p in OS. Using StarBase, a miR-155-5p binding site was identified in NORAD (Fig. 4A). RT-qPCR suggested that the knockdown of NORAD significantly up-regulated the expression level of miR-155-5p in both OS cell lines, while the transfection of miR-155-5p mimics did not change the expression level of NORAD (Fig. 4B and C). To investigate whether NORAD could directly interact with miR-155-5p, a dual luciferase reporter assay was performed by constructing NORAD-wt and NORAD-mut luciferase reporter plasmids. Subsequently, the NORAD-wt or NORAD-mut luciferase reporter plasmid was co-transfected into HEK-293T cells with miR-155-5p mimics or miR-NC. The results showed that the luciferase activity in the HEK-293T cells co-transfected with NORAD-wt and miR-155-5p mimics was significantly inhibited. However, the luciferase activity in the HEK-293T cells co-transfected with NORAD-mut and miR-155-5p mimics showed no statistically significant changes (Fig. 4D). A RIP experiment was also conducted and the results implied that NORAD and miR-155-5p formed a complex in OS cells (Fig. 4E). Collectively, these data suggested that miR-155-5p interacted with its binding site on NORAD to negatively regulate the expression of NORAD. Subsequently, a CCK-8 experiment showed that the knockdown of NORAD could inhibit the proliferation of OS cells, but this effect could be partially neutralized by the co-transfection with miR-155-5p inhibitors (Fig. 4F). The Transwell assay also suggested that the knockdown of NORAD inhibited the migration and invasion of Saos-2 cells, and this effect could also be partially reversed by miR-155-5p inhibitors (Fig. 4G and H). It was concluded that NORAD may regulate the malignant phenotypes of OS cells via regulating miR-155-5p expression.

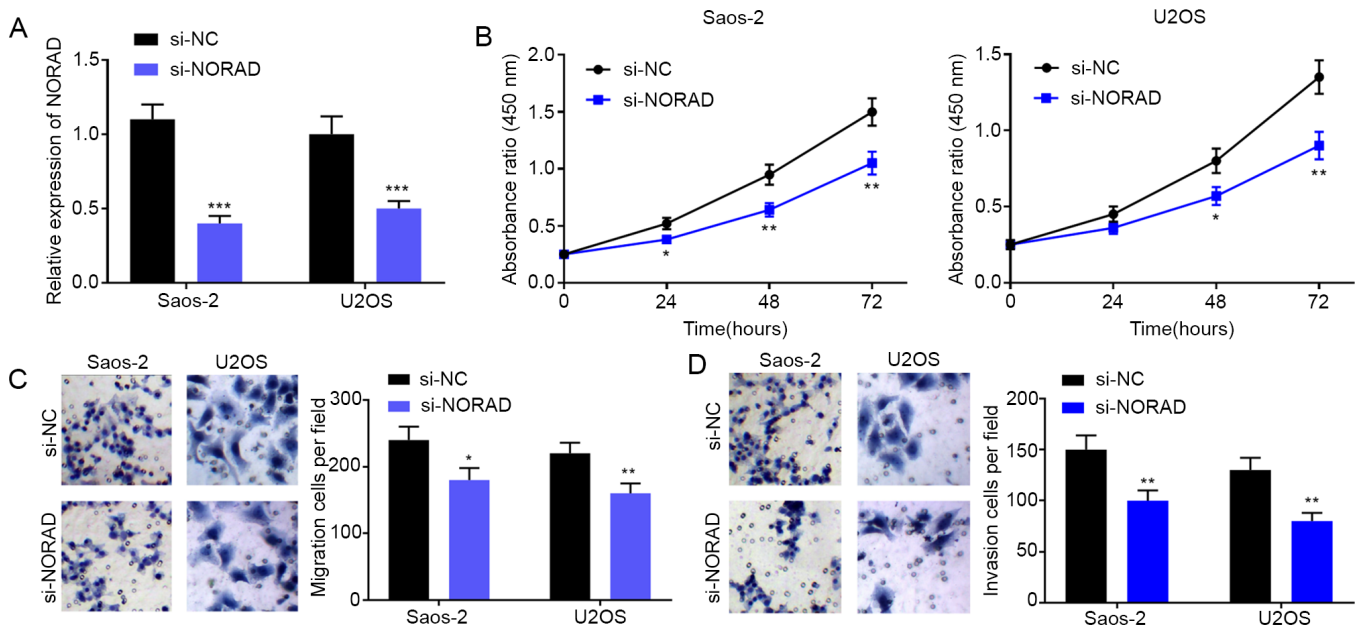

Figure 2. Knockdown of NORAD inhibits the proliferation, migration and invasion of OS cells. (A) NORAD knockdown was validated by RT-qPCR. (B) Proliferation of OS cells in the si-NORAD and si-NC groups was compared using the Cell Counting Kit-8 assay. (C) Migration of OS cells in the si-NORAD and si-NC groups was measured by the Transwell assay. (D) Invasion of OS cells in the si-NORAD and si-NC groups was measured by the Transwell assay. *P<0.05, **P<0.01, ***P<0.001 vs. si-NC. NORAD, non-coding RNA activated by DNA damage; OS, osteosarcoma; RT-qPCR, reverse transcription quantitative PCR; si, short interfering; NC, non-coding.

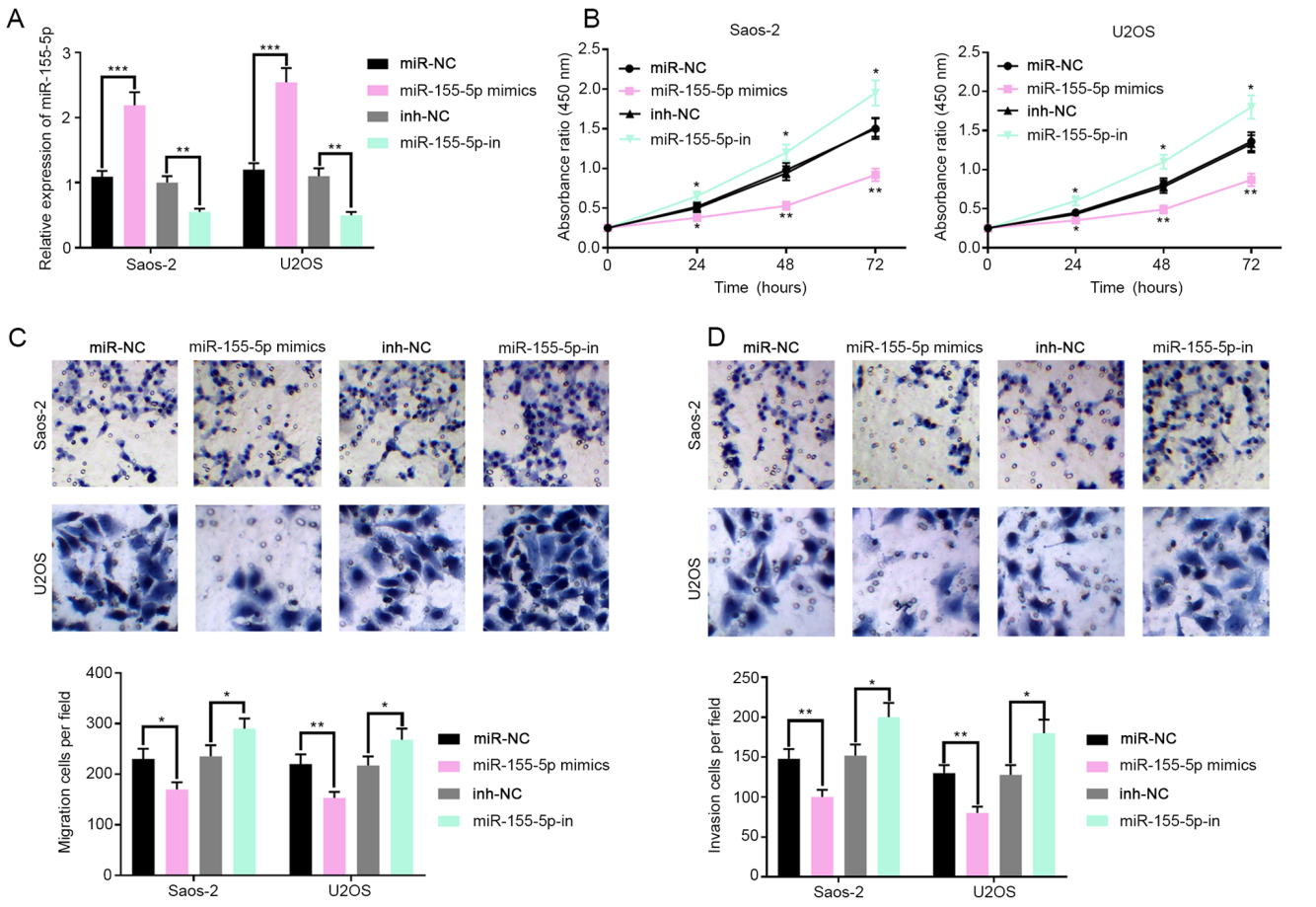

Figure 3. miR-155-5p regulates the proliferation, invasion and migration of OS cells. (A) Transfection efficiency of miR-155-5p mimics and inhibitors in OS cell lines was validated by RT-PCR. (B) After the transfection of miR-155-5p mimics or inhibitors, the proliferation of OS cells in different groups was compared with the CCK-8 assay. (C) After the transfection of miR-155-5p mimics or inhibitors, the migration of OS cells in different groups was compared with the Transwell assay. (D) After the transfection of miR-155-5p mimics or inhibitors, the invasion of OS cells in different groups was compared with the Transwell assay. *P<0.05, **P<0.01, ***P<0.001 vs. NC or NC-in. miR, microRNA; OS, osteosarcoma; RT-qPCR, reverse transcription quantitative PCR; NC, non-coding.

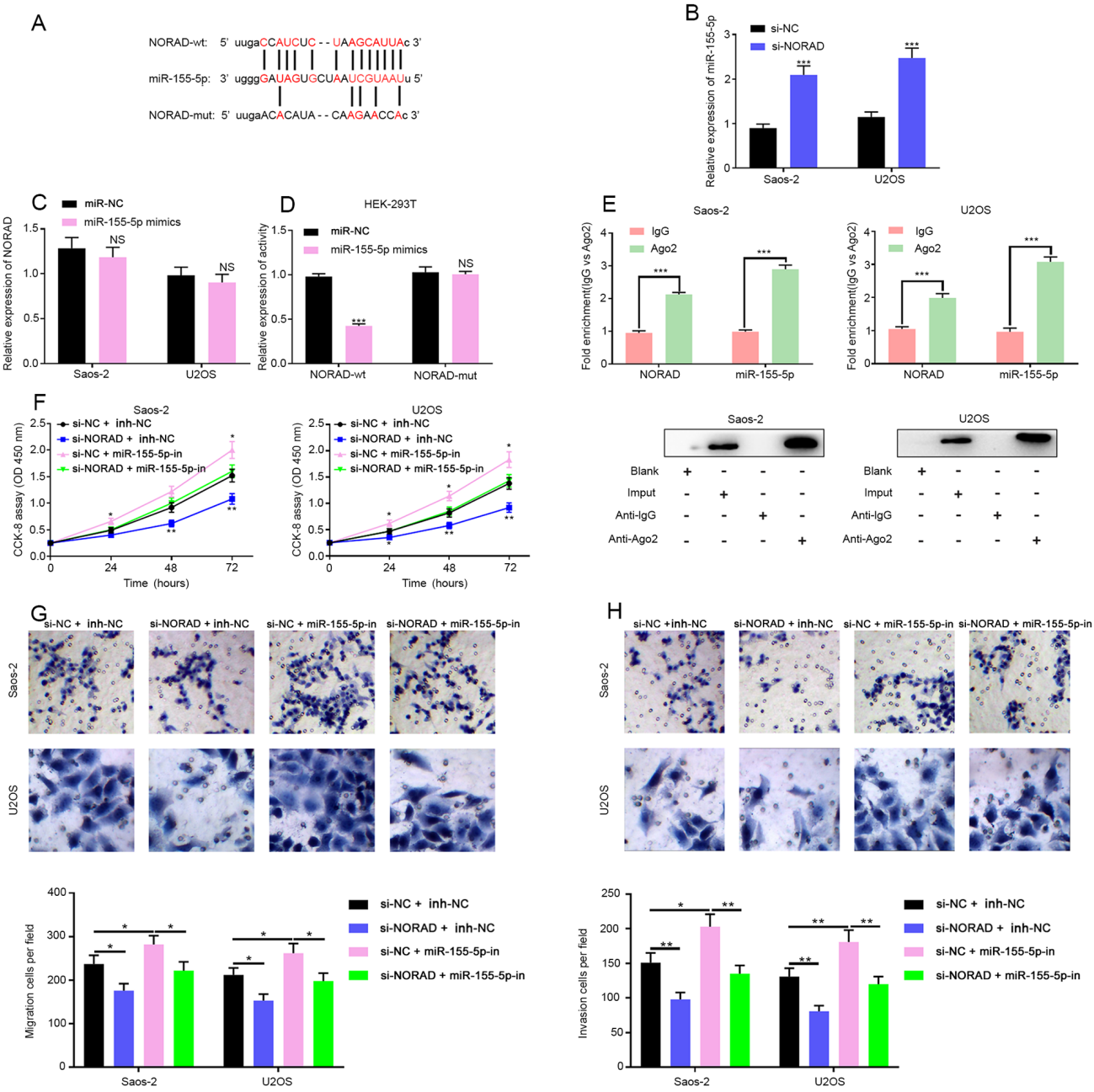

Figure 4. NORAD acts as a miR-155-5p sponge in OS. (A) StarBase predicted a possible binding site of miR-155-5p on NORAD. (B) The expression of miR-155-5p was examined by RT-qPCR after NORAD knockdown in OS cells. (C) The expression of NORAD was examined by RT-qPCR after transfection of miR-155-5p into OS cells. (D) Luciferase reporter assay was used to assess the binding between NORAD and miR-155-5p. (E) Direct binding between NORAD and miR-155-5p was detected by the RNA immunoprecipitation assay. (F) Proliferation of OS cells in si-NORAD + miR-NC, si-NORAD + miR-155-5p-in, si-NC + miR-155-5p-in and si-NC + miR-NC groups was measured with the Transwell assay. (G) Migration and (H) invasion of OS cells in si-NORAD + miR-NC, si-NORAD + miR-155-5p-in, si-NC + miR-155-5p-in and si-NC + miR-NC groups were measured with the Transwell assay. *P<0.05, **P<0.01, ***P<0.001 as indicated. NORAD, non-coding RNA activated by DNA damage; miR, microRNA; OS, osteosarcoma; RT-qPCR, reverse transcription quantitative PCR; si, short interfering.

Discussion

In recent years, studies have shown that lncRNAs play important roles in the progression of many diseases (24-26). According to previous reports, NORAD expression is increased in many tumors and contributes to their progression (27-31). One example is that the overexpression of NORAD promoted the invasion and migration of malignant melanoma cells by regulating the miR-205/EGLN2 pathway (31). The present study aimed to establish whether NORAD played a similar role in OS. The RT-qPCR experiment indicated that NORAD expression was increased in OS tissues and cell samples. The correlation between NORAD expression and the clinicopathological features of OS patients was then investigated, and the results suggested that a high expression level of NORAD was correlated with the tumor grade, Enneking stage, tumor size, and tumor metastasis, indicating that NORAD may play an oncogenic role in the pathological process of OS. In the present study, NORAD siRNA was also used to knockdown the expression level of NORAD in OS cell lines. The results

suggested that the down-regulation of NORAD could inhibit the proliferation, migration and invasion of OS cells. From these results, it was concluded that NORAD indeed acted as an oncogene in the progression of OS, which was consistent with its role in other malignancies.

miRs are important factors in tumorigenesis and tumor progression and the up-regulation or down-regulation of miR expression levels can affect the biological behavior of tumor cells. For example, miR-30a-5p can inhibit the metastasis of human colon cancer by targeting ITGB3 (32); the down-regulation of miR-362-3p and miR-329 can accelerate the progression of human breast cancer (33). In the present study, it was validated that miR-155-5p was down-regulated in OS tissues. The correlation between the expression level of miR-155-5p and the clinicopathological features of OS patients was also analyzed, and the results showed that a low miR-155-5p expression level was correlated with the tumor grade, Enneking stage, tumor size and tumor metastasis, indicating that miR-155-5p may act as a tumor suppressor in OS. In a previous study, miR-155-5p was shown to inhibit the proliferation of OS cells and induce cell apoptosis (34), which was consistent with the results of the present study.

Accumulating evidence suggests that lncRNAs can bind to miRs to act as an endogenous miR sponge, thus regulating the expression and biological function of miRs (35). lncRNA SNHG3, lncRNA SNH5 and lncRNA FBXL19-AS1 have been reported to interact with miRs to regulate the progression of OS (24,36,37). To further clarify the mechanism underlying the role of NORAD in the carcinogenesis of OS, a bioinformatics analysis was performed to identify miR-155-5p as a potential target of NORAD. RT-qPCR, luciferase assay and RIP experiment were used to confirm that NORAD could indeed bind to miR-155-5p. It was also confirmed that the miR-155-5p expression in OS cell lines was significantly increased after the NORAD expression was knocked down. The proliferation activity of OS cells with inhibited expressions of both NORAD and miR-155-5p was stronger than that of the OS cells in the si-NORAD group but weaker than that of the OS cells in the miR-155-5p-in group. Thereby, it was concluded that NORAD acted as an oncogene by sponging miR-155-5p.

There are several limitations to the present study. First of all, *in vivo* experiments are required to further confirm the findings of this study. Additionally, NORAD may act through other miRNA targets to affect the biological behaviors of OS, and these potential miRNA targets remain to be screened and validated. Additionally, the downstream genes and signaling pathways of miR-155-5p involved in OS progression remain to be validated. In addition to acting as ceRNA, lncRNA can also participate in cancer progression via other mechanisms, including chromatin modification, regulation of RNA processing (splicing, editing, localization, translation, degradation), post-translational regulation of protein activity and localization, facilitation of ribonucleoprotein complex formation, and gene silencing through the production of endogenous siRNA (38-40). It is also important to find out whether NORAD promotes OS progression via the above mechanisms.

In conclusion, NORAD expression is increased in OS tissues and may be used as a marker for the prognosis of patients with OS. NORAD directly targets miR-155-5p and inhibits its expression, thus promoting the progression of OS. Therefore, NORAD may be used as a new therapeutic target in OS management.

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Availability of data and materials

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

Authors' contributions

FX, YW and BZ conceived the study and performed the experiments. LY, JW, SZ and ZX analyzed and interpreted the data. FX, JW, LY, YW and SZ drafted and critically revised the manuscript for important intellectual content. YW and BZ confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Review Board of Ezhou Central Hospital and patients or their guardians provided their informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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