Original Article

The long non-coding RNA *CRNDE* promotes osteosarcoma proliferation and migration by sponging *miR-136-5p/MRP9* axis

Xiaomin Ding[1#], Yawen Zhang[1#], Jinrong Liang[1], Junyi Yin[2], Naveed Akbar[3], Verónica Miguel[4], Yan Zhou[1]

[1]Department of Oncology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China; [2]Department of Medical Oncology, Shanghai Tongji University Affiliated Tongji Hospital, Shanghai, China; [3]Division of Cardiovascular Medicine, Radcliffe Department of Medicine, University of Oxford, Oxford, UK; [4]Institute of Experimental Medicine and Systems Biology, RWTH Aachen University Hospital, Aachen, Germany

Contributions: (I) Conception and design: X Ding; (II) Administrative support: Y Zhou; (III) Provision of study materials or patients: Y Zhang; (IV) Collection and assembly of data: J Liang, J Yin; (V) Data analysis and interpretation: X Ding, Y Zhou; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Yan Zhou. Department of Oncology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Shanghai 200233, China. Email: 1987719zy@163.com.

Background: The long-noncoding RNA colorectal neoplasia differentially expressed (*CRNDE*) gene has been found to be upregulated in several solid tumors. Whether *CRNDE* affects osteosarcoma (OS) and its underling mechanism remains unknown.

Methods: Tumor tissues and corresponding normal tissues were collected from 45 patients with OS. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was applied to determine lncRNA *CRNDE* level in the tissues. Participants were divided into a high *CRNDE* group and a low *CRNDE* group according to the median value of lncRNA *CRNDE* expression detected by in situ hybridization (ISH). The differences between high and low expression of lncRNA *CRNDE* in patients were compared clinically by chi-square test. Kaplan-Meier survival analysis was applied to analyze the relationship between lncRNA *CRNDE* expression and patient survival. Subsequently, silencing or overexpression of lncRNA *CRNDE* were performed in MG63 and 143B cell lines, qRT-PCR was applied to verify the expression of lncRNA *CRNDE*, *miR-136-5p*, and *MRP9*; dual-luciferase reporter assay was used to evaluate the targeting relationship between *miR-136-5p*, lncRNA *CRNDE*, and Cell Counting Kit-8 (CCK8), wound-healing, and Transwell assays were used to analyze for cell proliferation, migration, and invasion, respectively, and western blot was used to detect expression in cells.

Results: The expression of *CRNDE* in OS tissues was higher than that in normal tissues. High lncRNA *CRNDE* expression was significantly associated with clinical stage, lung metastasis, and poor prognosis in OS patients. Additionally, overexpression of lncRNA *CRNDE* promoted proliferation and migration of OS cells. Bioinformatics analysis showed that lncRNA *CRNDE* competitively inhibited *miR-136-5p* through acting as a competitive endogenous RNA (ceRNA). It was also revealed that *miR-136-5p* is a binding target gene of lncRNA *CRNDE* and that *MRP9* is involved in this process as a downstream target gene of *miR-136-5p*.

Conclusions: The lncRNA *CRNDE* promotes the proliferation and migration of OS cells by regulating the *miR-136-5p/MRP9* pathway, and lncRNA *CRNDE* can be a significant marker of OS prognosis.

Keywords: Osteosarcoma (OS); lncRNA *CRNDE*; *miR-136-5p*; *MRP9*; proliferation

Submitted May 20, 2022. Accepted for publication Jul 29, 2022.
doi: 10.21037/atm-22-3602
View this article at: https://dx.doi.org/10.21037/atm-22-3602

Introduction

Osteosarcoma (OS) is a malignant bone tumor, which mostly occurs in children and adolescents. It is rare with an approximate incidence of 30 per million although occurs anywhere, about 75% are located in the extremities (most commonly in the thigh) and 10% each in the trunk wall and retroperitoneum (1). Previously, the 5-year survival rate of surgery was below 30%, but now, through the combination of neoadjuvant chemotherapy, surgical resection, adjuvant chemotherapy, targeted therapy, and immunotherapy, the 5-year survival rate of OS patients has increased up to 70%. However, lung metastasis is still a primary factor affecting the prognosis of OS patients (2,3). Moreover, heterogeneity of OS may partly influences patients' response to treatment drugs. And there is currently no standard salvage treatment as third-line chemotherapy for OS patients after the failure of second-line therapy. Thus, it is necessary to explore the mechanisms that mediate the invasion and metastasis of OS and more effective therapeutic regimens, which can inhibit its progress and improve the clinical prognosis.

The DNA able to encode proteins comprise only a small portion of the genome. 98% of the DNA transcription products are non-coding RNA (ncRNA), which are divided into short-chain (<200 nt) and long-chain ncRNA (>200 nt) based on their length. Since the discovery of lncRNA, its relevance to diseases, especially tumors, has received increasing attention (4). The lncRNA titin antisense RNA 1(*TTN-AS1*) regulates OS cell apoptosis and drug resistance through the *miR-134-5p/MBTD1* axis (5). The lncRNA *SNHG3* can regulate the *miRNA-151a-3p/RAB22A* axis to affect the invasion and migration of OS (6). Besides, lncRNA *SNHG4* can promote the proliferation and migration of OS by sponging and absorbing *miR-377-3p* (7). The above outcomes indicate that lncRNA is involved in the occurrence and development of OS. The *CRNDE* gene was originally found in colorectal cancer (CRC) tissue. It is located on chromosome 16 and has a length of 1,059 bp (8). A study showed that knockout of *CRNDE* upregulates miR-136-5p, inhibit cell proliferation, and promote apoptosis (9). It has been reported that lncRNA *CRNDE* can promote the proliferation, migration, and invasion of hepatocellular carcinoma cells by inhibiting *miR-384* (10). Another study reported that lncRNA *CRNDE* can serve as molecular sponge of *microRNA-136* in mammary cancer to activate the *Wnt/β-catenin* signaling pathway (11); knockdown of *CRNDE* inhibited cell proliferation, migration, and invasion of ovarian cancer (12). Moreover, lncRNA *CRNDE* can also be applied as a biomarker for clear cell renal cell carcinoma and glioma in patients (13,14). The above findings suggest the correlation between lncRNA *CRNDE* and tumors. However, the effect of lncRNA *CRNDE* on OS remains unclear.

Many studies have revealed that lncRNAs can directly bind to microRNAs (miRNAs) and regulate their activities as competitive endogenous RNAs (ceRNAs). Multitude of studies have confirmed the complicated cross-regulation among lncRNA, miRNA, and messenger RNA (mRNA), as well as the potential regulatory effect of lncRNA-miRNA-mRNA axis on OS (15). For example, Jing *et al.* found that *CRNDE* can promote the advance of non-small cell lung cancer by regulating *miRNA-338-3p* (16). Ma *et al.* revealed that *HOTAIR* has a carcinogenic effect on gallbladder carcinoma through the negative regulation of miR-130a (17). Nevertheless, the impact of lncRNA *CRNDE* targeting miRNA in OS requires further investigation. Osteosarcoma is a rare disease, which only scratches a little interest of researchers. The studies associated with lncRNAs are still less comparing with other common tumors. Here, we first proposed lncRNA *CRNDE* was the key factor to modulate the expression of *MRP9* through sponging *miR-136-5p*. As soon as this relationship was disturbed, the invasion and proliferation capacity of OS cells was decreased significantly. Furthermore, *MRP9*, a drug resistance-related protein, is rarely been regards as an oncogenic protein in research. But our study proved its key function in the OS progression, which provides a new underling target for OS treatment. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3602/rc).

Methods

Clinical samples

This study included 45 patients treated in our hospital from January 2015 to July 2018. Both OS tumor tissue samples and corresponding normal tissues were collected from these patients. All patients signed informed consent, and this study was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (No. 2017-049-1). The research was conducted in accordance with the World Medical Association Declaration of Helsinki (as revised in 2013).

Cell culture and cell transfection

The OS cells lines MG63 and 143B were obtained from the Chinese Academy of Sciences (Beijing, China). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) in a humidified atmosphere containing 5% CO_2 at 37 ℃. The full-length lncRNA *CRNDE* sequence was inserted into pHBLV-CMV-MCS-3FLAG-EF1-ZsGreen-T2A-PURO vector (*OE-CRNDE*, Hanbio, Shanghai, China); the short hairpin RNAs (shRNAs) for lncRNA *CRNDE* were ligated into pHBLV-U6-MCS-CMV-ZsGreen-PGK-PURO vector (*shCRNDE#1*, *shCRNDE#2*, *shCRNDE#3*, Hanbio, Shanghai, China); and corresponding negative controls were constructed (OE-NC, sh-NC). The miR-136-5p mimic and its negative control (NC) were purchased from Ruibo (Guangzhou, China). Cells were transfected according to the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) manufacturer's instructions. After 6 hours of transfection, supernatants were removed and fresh medium replaced, cells were subsequently rested for 48 hours. The stably transfected cells were selected with 2 μg/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA).

Dual-Luciferase reporter assay

The mutant vector was designed by mutating the putative binding site of *miR-136* on lncRNA *CRNDE* and *MRP9*. MG63 cells were co-transfected with wild-type vector or mutant vector along with miR-136 mimic or NC mimics. After 48 hours, we detected luciferase activity through dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Cell proliferation assay

Cells were seeded at a density of 5×10^3 cells/cm^2 into the 96-well plates. Cell Counting Kit-8 (CCK-8) was added and incubated with cells according to the instructions of the CCK-8 kit (Dojindo, Kumamoto, Japan). The absorbance was measured at 450 nm with a microplate reader (Biotek, Winooski, USA).

Wound-healing assay

Cells were seeded into 6-well plates and cultured overnight. The wounds were scratched with a 200 μL pipette tip. Subsequently, the cells were washed with phosphate-buffered saline (PBS) to remove the suspending cells and cultured continuously. After 24 hours, the wounds were imaged using a microscope (Olympus, Tokyo, Japan).

Transwell assay

Membranes of Transwell plates were covered with diluted Matrigel (Corning Inc., Corning, NY, USA), and in the upper chamber of each well 5×10^4 cells were seeded with serum-free DMEM. In the lower chamber of each well, DMEM with 10% FBS was added. After 24 hours of incubation, the cells on the upper chamber were removed, the membrane was fixed with 4% paraformaldehyde for 15 minutes, and stained with 0.1% crystal violet for 20 minutes. The invading cells were counted in 5 random fields under a microscope (Olympus, Tokyo, Japan).

Quantitative reverse transcription polymerase chain reaction

The RNA and miRNA of the cells was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA) or miRcute miRNA Isolation Kit (TIANGEN, Beijing, China). The RNA was reverse transcribed into complementary DNA (cDNA) by using the PrimeScript^{TM} RT reagent kit (Perfect Real Time, Takara, Shiga, Japan) or miRcute Plus miRNA First-Strand cDNA Kit (TIANGEN, China). Then, polymerase chain reaction (PCR) amplification of the synthesized cDNA was executed with SYBR Green qPCR Mix kit (Takara, Japan) or miRcute Plus miRNA qPCR Kit (SYBR Green, TIANGEN, China. The PCR analysis was performed to assess the levels of lncRNA *CRNDE*, *miR-136-5p*, and *MRP9*. Their relative expression was analyzed by the $2^{-\Delta\Delta CT}$ method and normalized to the control condition. Primers sequences are shown in the *Table 1*.

Western blot analysis

The protein of cells was extracted by using lysis buffer (Biyuntian, Ningxia, China) containing protease inhibitors. The concentration of protein was quantified with a bicinchoninic acid (BCA) protein assay kit (CoWin Biosciences (Cwbio), Jiangsu, China). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% skim milk and

Table 1 Primer sequences

Gene name	Primer sequences
CRNDE	F: 5'-AAATTCATCCCAAGGC-3'
	R: 5'-TTCCAGTGGCATCCTC-3'
miR-136-5p	F: 5'-ACTCCATTTGTTTTGATGATGGA-3'
	R: 5'-GTGCAGGGTCCGAGGTATTC-3'
	F: 5'-CAGGAAGGAACCGTGAC-3'
	R: 5'-TCCGTGAGCCCTTGTC-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3'
	R: 5'-AACGCTTCACGAATTTGCGT-3'
β-actin	F: 5'-CATGTACGTTGCTATCCAGGC-3'
	R: 5'-CTCCTTAATGTCACGCACGAT-3'

incubated with *MRP9* antibody (1:1,000, Biorbyt Ltd., Cambridgeshire, UK) at 4 ℃ overnight. After washing with tris-buffered saline with Tween 20 (TBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 hours at room temperature (1:1,000, Solarbio, Beijing, China). The bands were visualized by chemiluminescence reagent kit (Thermo Fisher Scientific, Waltham, MA, USA).

In situ hybridization

The matched OS and normal tissue samples were obtained from the Oncology Department of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. The expression of *CRNDE* was detected by *CRNDE* probe (tactggctattgg aaggaggagattctgaagataa) and evaluated with the POD Kit (Bioster, Hubei, China). All procedures were performed by following the manufacturer's instructions. Brownish yellow particles were considered as positive staining.

Statistical analysis

All data were presented as the means ± SD and calculated using SPSS 24.0 (IBM Corp., Armonk, NY, USA). The *t*-test was used to analyze the differences between two groups, while more than two groups were compared by one-way ANOVA. The relationship between lncRNA *CRNDE* expression and clinicopathological features of OS patients were evaluated by the chi-square test. An overall survival curve was constructed by Kaplan-Meier method and significance was assessed by the log-rank test. A P value <0.05 was considered statistically significant.

Results

LncRNA CRNDE is upregulated in OS tissues and associates with poor prognosis of patients with OS

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis demonstrated that lncRNA *CRNDE* level was higher in OS tissues than that in normal tissues (*Figure 1A*).

Then, we classified patients into a high *CRNDE* group and low *CRNDE* group based on the median value of lncRNA *CRNDE* expression. In situ hybridization (ISH) revealed low lncRNA *CRNDE* expression in the normal tissues. Whereas there was some positive expression in the low *CRNDE* group, and a higher number of positive cells in the high *CRNDE* group (*Figure 1B*). As shown in *Table 2*, high lncRNA *CRNDE* level were correlated with clinical stage and pulmonary metastasis, whereas it did not correlate with age, gender, tumor size, necrosis rate, pathological type, or local recurrence. Kaplan-Meier survival analysis revealed that OS patients with higher lncRNA *CRNDE* expression had a shorter overall survival than those with lower *CRNDE* expression (*Figure 1C*). All of these findings indicate that lncRNA *CRNDE* expression associate with poor prognosis in OS patients.

LncRNA CRNDE binds miR-136-5p directly

The potential lncRNAs-miRNAs interaction was predicted by ENCORI (https://starbase.sysu.edu.cn). We found that *miR-136-5p* complementarily combined with lncRNA *CRNDE* (*Figure 2A*). To confirm the interaction between lncRNA *CRNDE* and *miR-136-5p*, we designed dual-luciferase system containing the binding sequences of *miR-136-5p* with lncRNA *CRNDE*. The results showed that *miR-136-5p* overexpression decreased the luciferase activity of *CRNDE-WT* but not that of *CRNDE-MUT* (*Figure 2B*), which confirmed the potential interaction between lncRNA *CRNDE* and *miR-136-5p*. We transfected lentiviral vectors into MG63 cells and 143B cells. The down-regulation of lncRNA *CRNDE* was acquired by transiently transfecting lncRNA *CRNDE* shRNAs into the cells (*shCRNDE#1*,*2*,*3*). As *shCRNDE*#2 had the most efficacy in suppressing lncRNA *CRNDE* expression, and it was selected for the subsequent experiments (*Figure 2C*). Next, the *miR-136-5p* expression in OS cells transfected with vectors were

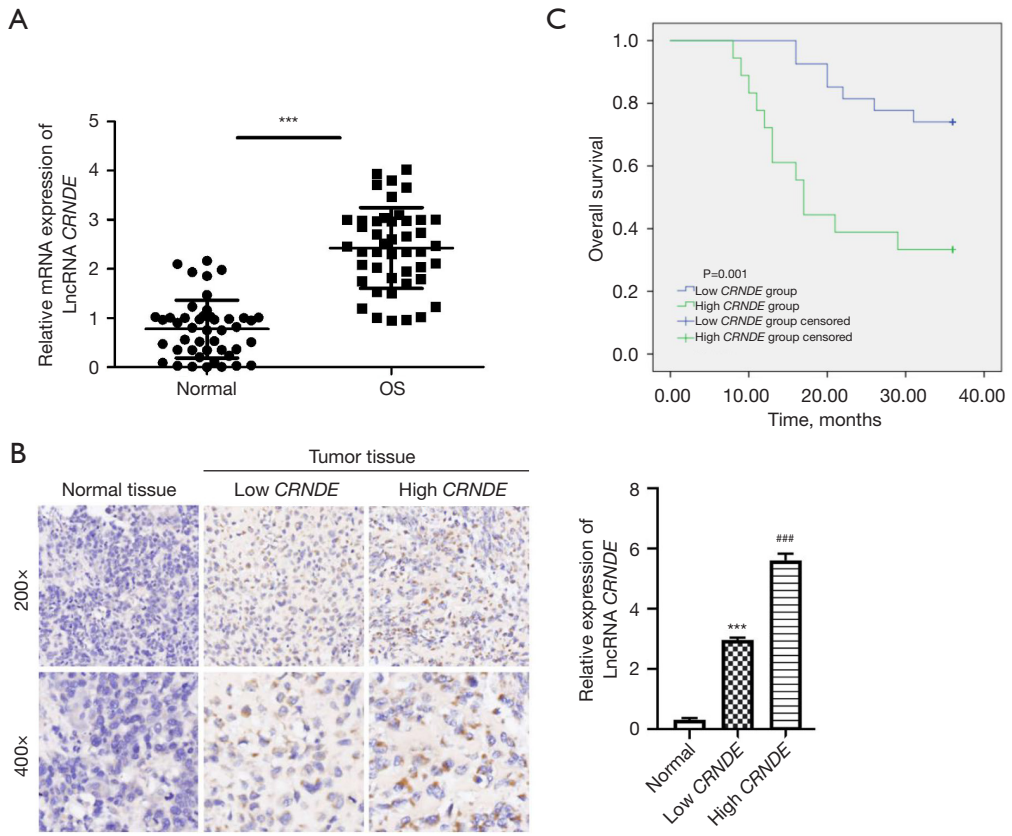

Figure 1 LncRNA *CRNDE* is over-expressed in OS tissue and is associated with OS progression. (A) The relative expression of lncRNA *CRNDE* in the tumor tissue and adjacent tissue of OS patients were detected by qRT-PCR, n=45, ***P<0.001 *vs.* Normal group. (B) ISH analysis of the lncRNA *CRNDE* in OS tissue and adjacent normal tissues. The positive particles are brown-yellow, ***P<0.001 *vs.* Normal group; ###P<0.001 *vs.* low *CRNDE* group. HE staining. (C) The overall survival of OS patients with high and low LncRNA *CRNDE* expression groups was analyzed by Kaplan-Meier survival analysis. Data were shown as mean ± SD. LncRNA, long non-coding RNA; OS, osteosarcoma; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation; HE, hematoxylin-eosin.

detected. Overexpression of lncRNA *CRNDE* could notably reduce the *miR-136-5p* level in cells (*Figure 2D*). The above results suggest that lncRNA *CRNDE* can target *miR-136-5p* expression in OS.

Overexpression of miR-136-5p can reduce the promotion of OS cells proliferation and migration by overexpression of lncRNA CRNDE

The effect of lncRNA *CRNDE* on OS cell proliferation and invasion and the role of *miR-136-5p* were further investigated. As shown in *Figure 3A-3C*, overexpression of lncRNA *CRNDE* could accelerate cell proliferation, migration, and invasion; silencing lncRNA *CRNDE* could suppress cell proliferation, migration, and invasion; while co-transfection with *miR-136* mimic reversed the promoting function of lncRNA *CRNDE* overexpressed cells. Globally, lncRNA *CRNDE* markedly promotes malignant progression of OS cells, while *miR-136-5p* blocks this effect.

LncRNA CRNDE upregulates MRP9 through inhibiting miR-136-5p

By using TargetScan 7.0 (www.targetscan.org/vert_70/), we found that *miR-136-5p* target *MRP9* has a binding site in the 3'-untranslated region (3'-UTR) region of the *MRP9* gene (*Figure 4A*). This observation was confirmed through a dual luciferase assay containing the *miR-136* binding sites on the *MRP9* 3'UTR (*Figure 4B*). Results showed that *miR-136-5p* overexpression decreased the luciferase activity

Table 2 Correlation between *CRNDE* expression and clinicopathological characteristics in 45 patients with OS

Characteristics	CRNDE expression		P value
	Low level [27]	High level [18]	
Gender			0.619
Male	10	8	
Female	17	10	
Age (years)			0.464
<15	7	3	
≥15	20	15	
Tumor size (cm)			0.465
≤10	15	8	
>10	12	10	
Clinical stage			0.001
II	23	6	
III	4	12	
Necrosis rate			0.519
≤90%	17	13	
>90%	10	5	
Pathological type			0.761
Conventional	22	14	
Non-conventional	5	4	
Pulmonary metastasis			0.001
Negative	23	7	
Positive	4	11	
Local recurrence			0.329
Negative	26	16	
Positive	1	2	

OS, osteosarcoma.

in the *MRP-WT* construct, but not in the case of *MRP-MUT*. As expected, the mRNA and protein levels of *MRP9* were increased after lncRNA *CRNDE* overexpression and decreased after the silencing on this lncRNA. However, co-transfection with the *miR-136* mimic reversed the high expression of *MRP9* in lncRNA *CRNDE*-overexpressed cells (*Figure 4C,4D*). In conclusion, lncRNA *CRNDE* targets *miR-136-5p* expression and regulates *MRP9* expression, thereby exerting a tumor-promoting effect.

Discussion

In recent years, the importance of lncRNA in numerous diseases has been gradually recognized; lncRNAs can also be used as markers of tumor prognosis. Many lncRNAs have been found to be abnormally expressed in OS. Multiple studies have verified the oncogenic function of lncRNA *CRNDE* in a variety of cancers (18). Wang *et al.* reported that *CRNDE* played an oncogenic role in cell proliferation and metastasis of pancreatic cancer through upregulating

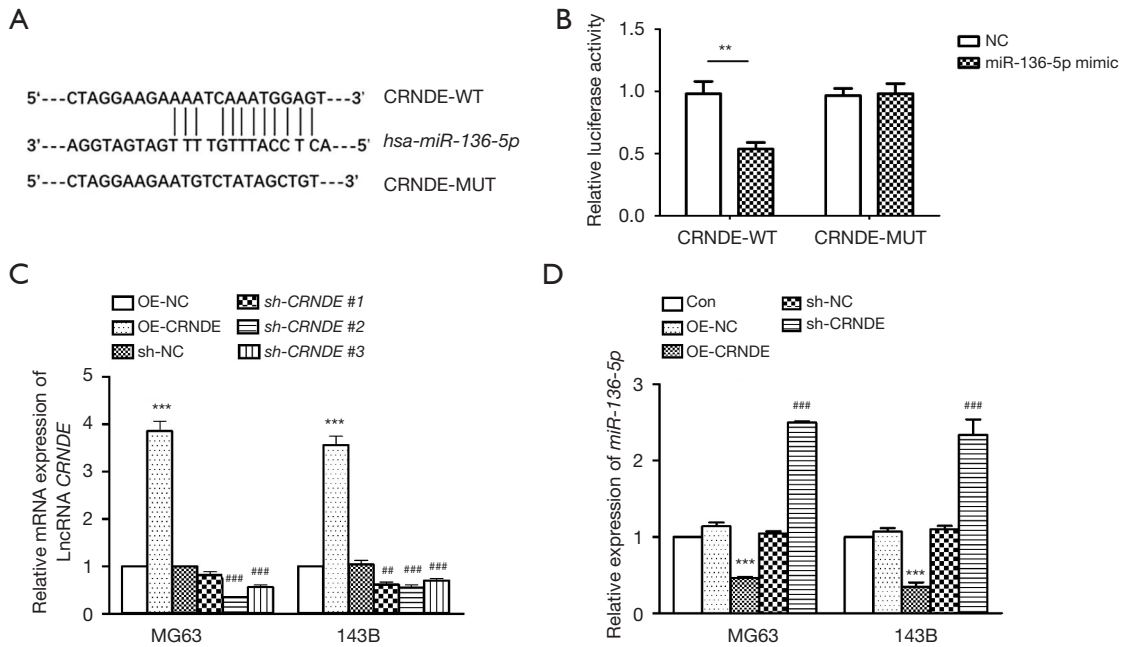

Figure 2 LncRNA *CRNDE* binds *miR-136-5p* directly. (A) ENCORI indicated that lncRNA *CRNDE* sequence contained the putative binding site of *miR-136-5p*. (B) Dual-luciferase reporter gene assays to verify the relationship between *miR-136-5p* and lncRNA *CRNDE*, **P<0.01 *vs.* NC group. (C) MG63 and 143B cells were transfected with lentivirus vector, *OE-CRNDE* and *sh-CRNDE* (*shCRNDE #1 #2 #3*). The expression level of lncRNA *CRNDE* was detected by qRT-PCR. (D) *miR-136-5p* expression level in response to lncRNA *CRNDE* overexpression and inhibition were measured by qRT-PCR. Data were shown as mean ± SD. ***P<0.001 *vs.* OE-NC group; ##P<0.01 *vs.* OE-CRNDE; ###P<0.001 *vs.* sh-NC group. lncRNA, long non-coding RNA; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation. NC, negative control.

IRS1 (19). In this study, we demonstrate that lncRNA *CRNDE* is significantly upregulated in OS patients, and its high expression is associated with poorer patient prognosis. Besides, overexpression of lncRNA *CRNDE* promotes the proliferation, migration, and invasion of OS cells.

Not only lncRNA can activate or inhibit target gene expression by directly binding to it, but it also participates in the regulation of gene expression by partaking in histone modification or recruiting transcription factors (20). In addition, lncRNA can be used as a ceRNA to bind to miRNA, and regulate the downstream genes (21). Hu *et al.* reported that *CRNDE* acted as a growth-promoting lncRNA in gastric cancer cells through targeting *miR-145* (22). Also, *CRNDE* was found to promote progression of glioma through regulating the *miR-384/PIWIL4/STAT3* axis (23). In CRC, *CRNDE* was particularly upregulated and interacted with *miR-181a-5p* to accelerate CRC cell proliferation and chemoresistance via activation of the *Wnt*/β-*catenin* signaling (24). In this study, lncRNA *CRNDE* targets *miR-136-5p* expression in OS, and overexpression of *miR-136-5p* reverses the promotion effect of lncRNA *CRNDE* overexpression on OS cell malignant process.

Many studies have shown that *miR-136-5p* is a tumor suppressor. Yan *et al.* (25) found that *miR-136* was low-expressed in triple-negative breast cancer tissues. After over-expression *miR-136* in cells, it could significantly inhibit tumor cell migration by inhibiting the *RASAL2* gene. Yang *et al.* (26) reported that *miR-136* inhibited glioma cell proliferation and induced apoptosis by regulating *AEG-1* and *Bcl-2* gene expression. Zhao *et al.* (27) showed that inhibiting *miR-136* expression would up-regulate its target *AEG-1* gene and could significantly improve the metastatic ability of liver cancer cells. The suppressive effect of *miR-136* in lung cancer cells has also been confirmed (28). In this study, we find that *miR-136-5p* targets and binds to *MRP9*, regulating its expression.

The member of the ATP-binding cassette (*ABC*) superfamily, *MRP9*, located at 16q12.1. It was first cloned in 2003 by Shimizu *et al.* (29). The *ABC* superfamily is one of the largest protein families. It involves the transmembrane

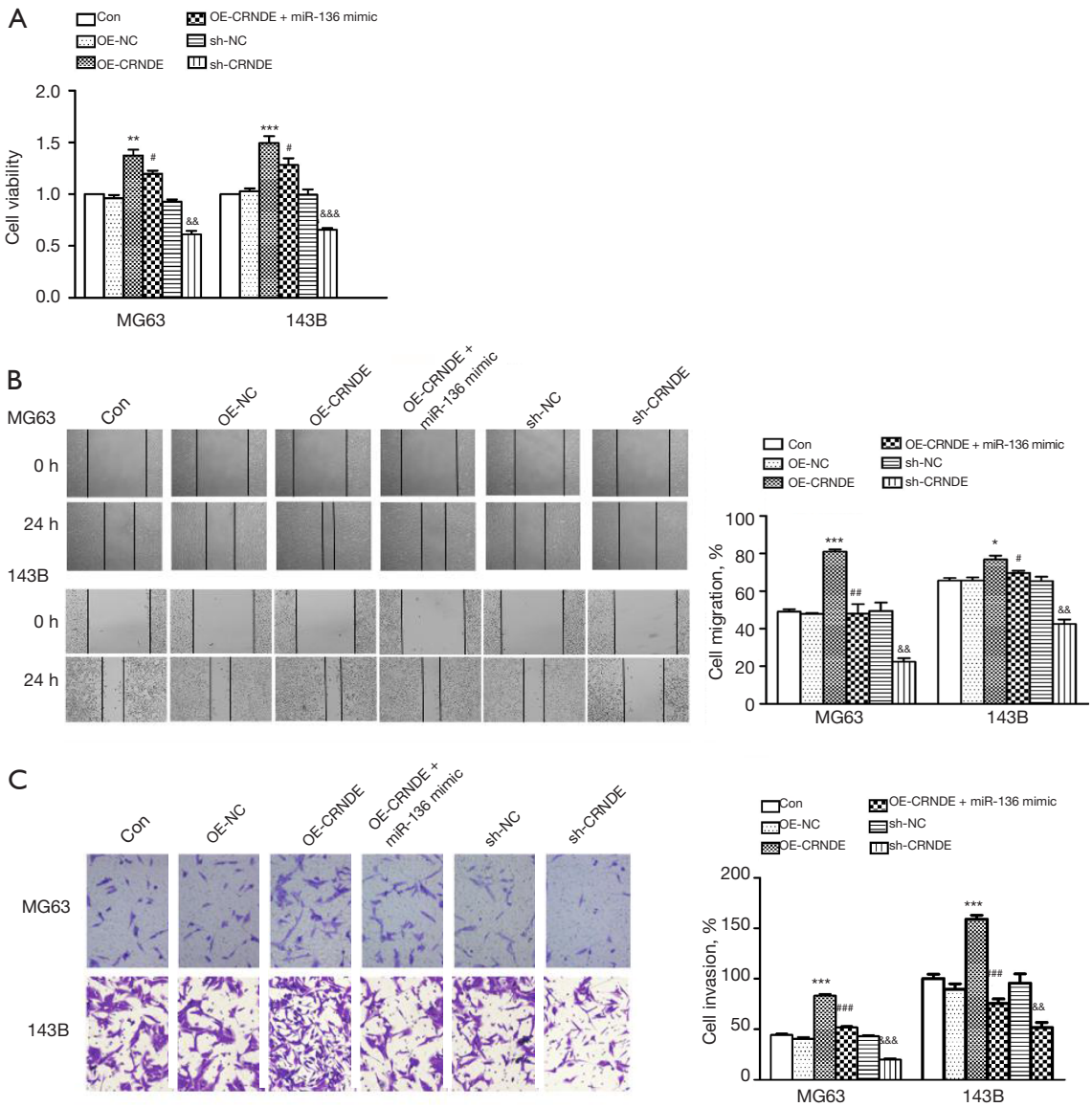

Figure 3 Effects of lncRNA *CRNDE* and *miR-136-5p* on the malignant progression of OS cells. (A) Cell proliferation was measured by CCK-8 assay at 24 h. (B) Migration ability was detected by the wound healing assay (observational method: inverse microscope ×100). (C) Transwell assay was performed to detect the migration ability (×200, 0.1% crystal violet staining). Data were shown as mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 *vs.* OE-NC group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 *vs. OE-CRNDE + miR-136* mimic group; $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 *vs.* sh-NC group. lncRNA, long non-coding RNA; OS, osteosarcoma; CCK-8, Cell Counting Kit-8; SD, standard deviation.

transport of various substrates, including the ability to use the energy of ATP hydrolysis to transport drugs from the cytoplasm to the outside of the cell (30). Therefore, most studies have been related to tumor treatment and drug resistance (31). However, a study pointed that *MRP9* is abnormally up-regulated in breast cancer tissues (32), while its expression in liver cancer tissues is also significantly higher than in adjacent tissues (33). In this study, we show that overexpression of lncRNA *CRNDE* increases *MRP9* level in OS cells, but lncRNA *CRNDE* overexpression combined with *miR-136-5p* mimic mostly reverses *MRP9* expression. Collectively, all data suggest that lncRNA *CRNDE* upregulates the expression of *MRP9* through binding to *miR-136-5p*.

Osteosarcoma is a heterogeneous mesenchymal malignancy. Despite combining surgery and chemotherapy

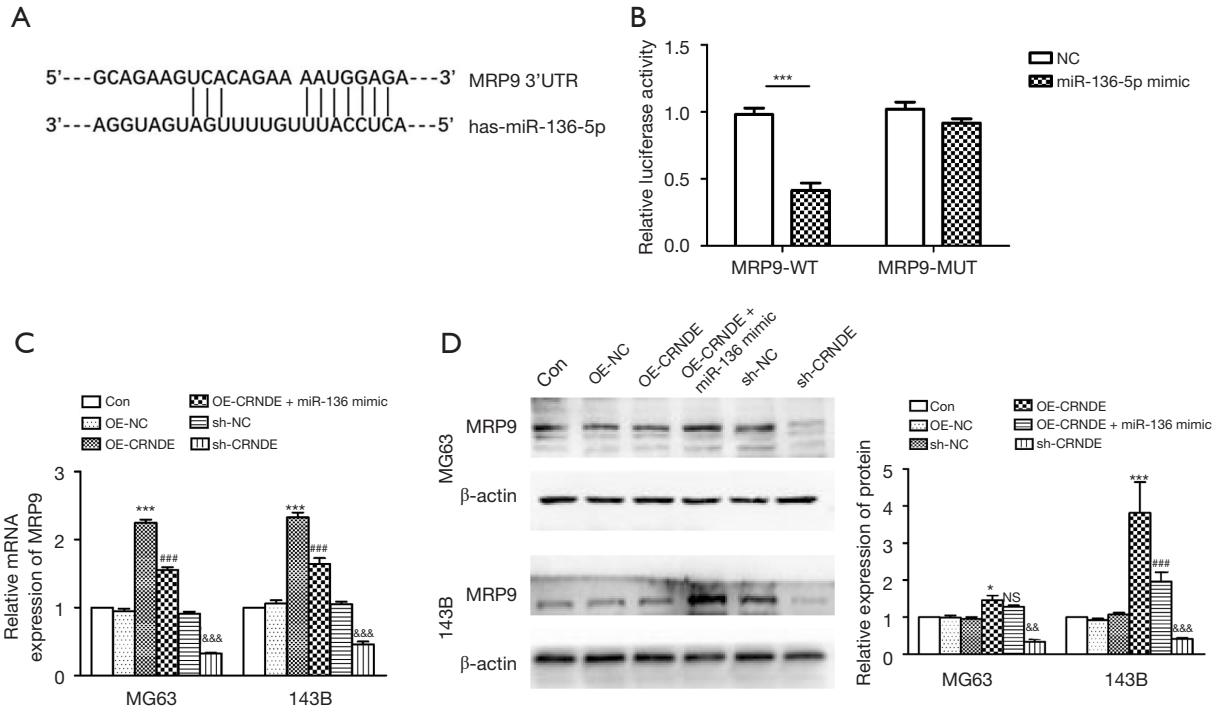

Figure 4 LncRNA *CRNDE* upregulates *MRP9* by inhibiting *miR-136-5p*. (A) TargetScan7.0 shows that there is a binding site between *miR-136-5p* and the 3'UTR of *MRP9*. (B) Dual-luciferase reporter gene assays to verify the relationship between *miR-136-5p* and *MRP9*, ***P<0.001 *vs.* NC group. (C) The mRNA expression levels of *MRP9* in MG63 and 143B cells were detected by qRT-PCR. (D) The protein expression levels were detected by western blot. *P<0.05 and ***P<0.001 *vs.* OE-NC group; ###P<0.001 *vs. OE-CRNDE + miR-136* mimic group; &&P<0.01, &&&P<0.001 *vs.* sh-NC group. Data were shown as mean ± SD. mRNA, messenger RNA; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation. NS: P>0.05, no significant.

as the standard of treatment, the overall survival rate of OS patients with recurrence or metastasis is still not encouraging, partly owing to heterogeneous histological and drug resistance. It is urgent to seek novel therapies to enhance the efficacy of the current treatment strategies. *CRNDE* is extremely abundant in the sections from 45 OS cases. Kapalan-Meier survival analysis showed that *CRNDE* was a significantly poorer prognosis. Our study suggests that *CRNDE* could be developed as a potential prognostic biomarker for OS. Moreover, we proved *CRNDE* served as an endogenous sponge to reduce *miR-136-5p* expression by binding to it directly, which regulating the expression of downstream targets *MRP9* indirectly. Regrettably, although current several studies have proved that lncRNAs play key role in the occurrence and progression of tumors, providing new directions for early diagnosis, prognosis, and therapeutic targets of tumors. Owing to its diagnostic specificity and actual clinical value, lncRNAs have not yet been included in real-world study.

Conclusions

In summary, this study demonstrates lncRNA *CRNDE* can play an oncogenic role in OS, and promote OS progression through competitively binding with *miR-136-5p* to regulate *MRP9* expression. Therefore, *CRNDE* is proposed to be a potential target for the OS therapy.

Acknowledgments

The authors appreciate the academic support from the AME Oncology Collaborative Group.
Funding: This work was supported by Talent Project of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital (No. ynlc201603), the Interdisciplinary Program of Shanghai Jiao Tong University (No. YG2017GN19),

research grants from the British Heart Foundation (BHF) Centre of Research Excellence, Oxford (N.A. RE/13/1/30181 and RE/18/3/34214, to Naveed Akbar).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3602/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-3602/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-3602/coif). NA received research grants from the British Heart Foundation (BHF) Centre of Research Excellence, Oxford (N.A. RE/13/1/30181 and RE/18/3/34214). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital approved the present study (No. 2017-049-1). The research was conducted in accordance with the World Medical Association Declaration of Helsinki (as revised in 2013). All patients provided written informed consent before their inclusion to the study.

References

1. Mehren MV, Kane JM, Bui MM, et al. NCCN Guidelines Insights: Soft Tissue Sarcoma, Version 1.2021: Featured Updates to the NCCN Guidelines. Journal of the National Comprehensive Cancer Network 2020;18:1604-12.
2. Ferrari S, Serra M. An update on chemotherapy for osteosarcoma. Expert Opin Pharmacother 2015;16:2727-36.
3. Bielack SS, Kempf-Bielack B, Delling G, et al. Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 2002;20:776-90.
4. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature 2004;431:931-45.
5. Fu D, Lu C, Qu X, et al. LncRNA TTN-AS1 regulates osteosarcoma cell apoptosis and drug resistance via the miR-134-5p/MBTD1 axis. Aging (Albany NY) 2019;11:8374-85.
6. Zheng S, Jiang F, Ge D, et al. LncRNA SNHG3/miRNA-151a-3p/RAB22A axis regulates invasion and migration of osteosarcoma. Biomed Pharmacother 2019;112:108695.
7. Huang YF, Lu L, Shen HL, et al. LncRNA SNHG4 promotes osteosarcoma proliferation and migration by sponging miR-377-3p. Mol Genet Genomic Med 2020;8:e1349.
8. Graham LD, Pedersen SK, Brown GS, et al. Colorectal Neoplasia Differentially Expressed (CRNDE), a Novel Gene with Elevated Expression in Colorectal Adenomas and Adenocarcinomas. Genes Cancer 2011;2:829-40.
9. Liu C, Liu B, Shen C, et al. lncRNA CRNDE promotes proliferation and inhibits apoptosis of U937 cells by downregulating miR-136-5p and upregulating MCM5. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2021;37:987-95.
10. Chen Z, Yu C, Zhan L, et al. LncRNA CRNDE promotes hepatic carcinoma cell proliferation, migration and invasion by suppressing miR-384. Am J Cancer Res 2016;6:2299-309.
11. Huan J, Xing L, Lin Q, et al. Long noncoding RNA CRNDE activates Wnt/β-catenin signaling pathway through acting as a molecular sponge of microRNA-136 in human breast cancer. Am J Transl Res 2017;9:1977-89.
12. Wang Q, Wang LX, Zhang CY, et al. LncRNA CRNDE promotes cell proliferation, migration and invasion of ovarian cancer via miR-423-5p/FSCN1 axis. Mol Cell Biochem 2022;477:1477-88.
13. Ding C, Han F, Xiang H, et al. LncRNA CRNDE is a biomarker for clinical progression and poor prognosis in clear cell renal cell carcinoma. J Cell Biochem 2018;119:10406-14.
14. Jing SY, Lu YY, Yang JK, et al. Expression of long non-coding RNA CRNDE in glioma and its correlation with

tumor progression and patient survival. Eur Rev Med Pharmacol Sci 2016;20:3992-6.
15. Wang JY, Yang Y, Ma Y, et al. Potential regulatory role of lncRNA-miRNA-mRNA axis in osteosarcoma. Biomed Pharmacother 2020;121:109627.
16. Jing H, Xia H, Qian M, et al. Long noncoding RNA CRNDE promotes non-small cell lung cancer progression via sponging microRNA-338-3p. Biomed Pharmacother 2019;110:825-33.
17. Ma MZ, Li CX, Zhang Y, et al. Long non-coding RNA HOTAIR, a c-Myc activated driver of malignancy, negatively regulates miRNA-130a in gallbladder cancer. Mol Cancer 2014;13:156.
18. Zhang J, Yin M, Peng G, et al. CRNDE: An important oncogenic long non-coding RNA in human cancers. Cell Prolif 2018;51:e12440.
19. Wang G, Pan J, Zhang L, et al. Long non-coding RNA CRNDE sponges miR-384 to promote proliferation and metastasis of pancreatic cancer cells through upregulating IRS1. Cell Prolif 2017;50:e12389.
20. Mengelbier LH, Karlsson J, Lindgren D, et al. Deletions of 16q in Wilms tumors localize to blastemal-anaplastic cells and are associated with reduced expression of the IRXB renal tubulogenesis gene cluster. Am J Pathol 2010;177:2609-21.
21. Das S, Ghosal S, Sen R, et al. lnCeDB: database of human long noncoding RNA acting as competing endogenous RNA. PLoS One 2014;9:e98965.
22. Hu CE, Du PZ, Zhang HD, et al. Long Noncoding RNA CRNDE Promotes Proliferation of Gastric Cancer Cells by Targeting miR-145. Cell Physiol Biochem 2017;42:13-21.
23. Zheng J, Liu X, Wang P, et al. CRNDE Promotes Malignant Progression of Glioma by Attenuating miR-384/PIWIL4/STAT3 Axis. Mol Ther 2016;24:1199-215.
24. Han P, Li JW, Zhang BM, et al. The lncRNA CRNDE promotes colorectal cancer cell proliferation and chemoresistance via miR-181a-5p-mediated regulation of Wnt/β-catenin signaling. Mol Cancer 2017;16:9.
25. Yan M, Li X, Tong D, et al. miR-136 suppresses tumor invasion and metastasis by targeting RASAL2 in triple-negative breast cancer. Oncol Rep 2016;36:65-71.
26. Yang Y, Wu J, Guan H, et al. MiR-136 promotes apoptosis of glioma cells by targeting AEG-1 and Bcl-2. FEBS Lett 2012;586:3608-12.
27. Zhao J, Wang W, Huang Y, et al. HBx elevates oncoprotein AEG-1 expression to promote cell migration by downregulating miR-375 and miR-136 in malignant hepatocytes. DNA Cell Biol 2014;33:715-22.
28. Yang Y, Liu L, Cai J, et al. Targeting Smad2 and Smad3 by miR-136 suppresses metastasis-associated traits of lung adenocarcinoma cells. Oncol Res 2013;21:345-52.
29. Shimizu H, Taniguchi H, Hippo Y, et al. Characterization of the mouse Abcc12 gene and its transcript encoding an ATP-binding cassette transporter, an orthologue of human ABCC12. Gene 2003;310:17-28.
30. Kaur H, Lakatos-Karoly A, Vogel R, et al. Coupled ATPase-adenylate kinase activity in ABC transporters. Nat Commun 2016;7:13864.
31. König J, Hartel M, Nies AT, et al. Expression and localization of human multidrug resistance protein (ABCC) family members in pancreatic carcinoma. Int J Cancer 2005;115:359-67.
32. Bera TK, Iavarone C, Kumar V, et al. MRP9, an unusual truncated member of the ABC transporter superfamily, is highly expressed in breast cancer. Proc Natl Acad Sci U S A 2002;99:6997-7002.
33. Slot AJ, Molinski SV, Cole SP. Mammalian multidrug-resistance proteins (MRPs). Essays Biochem 2011;50:179-207.

(English Language Editor: J. Jones)

Cite this article as: Ding X, Zhang Y, Liang J, Yin J, Akbar N, Miguel V, Zhou Y. The long non-coding RNA *CRNDE* promotes osteosarcoma proliferation and migration by sponging *miR-136-5p/MRP9* axis. Ann Transl Med 2022;10(15):835. doi: 10.21037/atm-22-3602

