LncRNA NRON Inhibits Osteosarcoma Cell Proliferation, Invasion by Regulating MVB12B

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Abstract

**Aims:** Long noncoding RNA have been proved as important regulator in various diseases. NRON was a newly identified tumor-related IncRNA, and previous studies have reported its function in hepatocellular carcinoma and heart failure. However, the function and mechanism of IncRNA NRON in osteosarcoma still unknown.

**Methods:** Cell proliferation, invasion, migration and apoptosis were detected via CCK-8, transwell assay and Western. Bioinformatics analysis was used to predict the potential target of NRON. Rescue experiment was performed to identify the relationship between NRON and MVB12B.

**Results:** The expression of lncRNA NRON was significantly downregulate in osteosarcoma tissues and cell lines. Knockdown of NRON promoted cell proliferation, invasion and EMT. Overexpression of NRON inhibited cell proliferation, invasion and EMT. Bioinformatics analysis predicted that MVB12B was the direct target. The expression of MVB12B was significantly upregulated in osteosarcoma tissues and cell lines. Rescue experiment further confirmed the relationship between NRON and MVB12B. Overexpression of MVB12B completely reversed the function of NRON.

**Conclusion:** Taken together, our results comprehensively analyzed the function of NRON in osteosarcoma and provided possible mechanism that NRON inhibited osteosarcoma development by regulating MVB12B. Thus, our study may offer a potential therapeutic target for treating osteosarcoma.

Introduction

Osteosarcoma is the most prevalent primary malignant bone cancer and is most common in children and teenagers[1]. It is most common that people often die due to distant metastasis[2]. The pathological development of osteosarcoma involved various process, including invasion[3], EMT[4] and angiogenesis[5]. Despite remarkable advancement in the biomarkers and treatment of osteosarcoma, there still numerous questions waiting for answered[6]. Thus, exploring new targets and effective drugs are clearly warranted.

Long noncoding RNA is a kind of noncoding RNA that longer than 200 nt with limited coding potential[7]. Accumulating studies have reported the various role of IncRNA in different biological and pathological process[8, 9]. For example, IncRNA PANDAR dictates the chemoresistance of ovarian cancer via regulating SFRS2-mediated p53 phosphorylation[10]. Over-expressed IncRNA HOTAIRM1 promotes tumor growth and invasion through up-regulating HOXA1 in glioblastoma multiforme[11]. LncRNA XIST mediates bovine mammary epithelial cell inflammatory response via NF-κB/NLRP3 inflammasome pathway[12]. Importantly, increasing evidences have demonstrated that dysregulated expression of IncRNA will influence the development of various human cancers[13, 14]. There is no doubt that exploring the function and mechanism of IncRNAs will provide new choice for drug administration[15].
Previous studies have revealed the function of lncRNA NRON, a newly identified tumor-related lncRNA, in hepatocellular carcinoma and heart failure\[16\]. However, the function and mechanism of lncRNA NRON in osteosarcoma still unknown. In this study, we found that knockdown of NRON promoted cell proliferation, invasion and EMT. Bioinformatics analysis predicted that MVB12B is the potential target. The expression of MVB12B was significantly upregulated in osteosarcoma tissues and cell lines. Rescue experiment further confirmed the relationship between NRON and MVB12B. Overexpression of MVB12B completely reversed the function of NRON. Taken together, our results comprehensively analyzed the function of NRON in osteosarcoma and provided possible mechanism that NRON inhibited osteosarcoma development by regulating MVB12B.

To be brief, by conducting these comprehensive experiments, we want to explore the inner association between lncRNA NRON and MVB12B and demonstrate that lncRNA NRON could influence the progress of osteosarcoma by regulating MVB12B. The current research may explore the new target for inhibiting osteosarcoma.

**Materials And Methods**

**Cell culture**

The cell lines, KHOS, U2OS, HOS, Saos-2, MG63 and HFOB1.19 used in this study were purchased from American Type Culture Collection (ATCC) or maintained in our lab. U2OS and HOS cells were cultured in DMEM medium (Gibco, USA) containing 10% FBS (Gibco, USA). Cells were maintained at 37°C in 5% CO$_2$ incubator.

**Cell transfection**

siRNA-NRON, negative control, overexpression plasmid and NC plasmid were purchased from Gene Pharma (Shanghai, China). Briefly, Cell lines U2OS and HOS were transfected with different vector with lipo3000 reagent (Invitrogen) following the manufacturer's protocol when cell confluence reached 40%. Cells were cultured for 72h before harvesting for further research.

**Cell proliferation**

HOS and U2OS cells were transfected with si-NC, si-NRON, NC, NRON, MVB12B and NC as mentioned above. Cells were seeded (2000 cells per well) into 96-well plates. After 48h transfection, 10μL CCK-8 reagent was added to each well at 0h, 24h, 48h, 72h and then the optical density was measured at 450nm after 2h incubation.

**Transwell assay**

U2OS and HOS cells were re-suspended in 100 μL serum-free medium and were plated in the top chamber of each insert (8-μm pore size, Corning, USA) with a Matrigel-coated membrane (BD Bioscience, San Jose, USA) for the transwell assay. Lower chambers of the inserts were filled with DMEM medium with 10%
FBS. Twenty-four hours later, cells invaded to the lower surface of the insert were fixed, stained with crystal violet and counted under a light microscope.

**Real-time PCR**

Total RNA was extracted and lysed by TRIzol reagent (Thermo Fisher, USA). RNA reverse transcription was performed using a PrimeScript™ RT reagent Kit with gDNA eraser (Takara, Japan) according to the manufacturer’s instructions, and cDNA was performed by qRT-PCR using SYBR® Premix Ex Taq™ (Takara, Japan). The data were normalized using β-actin levels and further analyzed by the 2−ΔΔCT method.

**Western blotting**

Proteins from HOS and U2OS cells were extracted by using the RIPA buffer. Total protein was quantified using BCA protein assay kit (Pierce, USA). Protein samples were resolved by 10% SDS-PAGE gel (80 V, 120mins) and transferred to polyvinylidene difluoride (PVDF) membrane (300mA, 90 mins). And membranes were blocked with 5% pure milk for 1 hour. Then they were incubated with primary antibodies against E-cadherin (1:1000, CST, USA), N-cadherin (1:1000, CST, USA), Vimentin (1:1000, CST, USA), MVB12B(1:1000, Santa Cruz, CA), β-actin(1:5000, CST, USA) at 4 °C overnight, followed by incubation with HRP conjugated secondary antibodies (Abcam, CA, USA) for 1h at room temperature. The bands were visualized by using the ECL kit (Thermo Fisher Scientific, Rockford, USA).

**Statistical analysis**

We used SPSS 21.0 to calculate the values (means ± standard error of the mean (SEM). And statistical analyses were analyzed using two-sided Student’s t-test or one-way ANOVA. The statistical significance was P<0.05.

**Results**

**Biological features of lncRNA NRON**

To study the function of lncRNA NRON, we firstly analyzed the biological features of lncRNA NRON in osteosarcoma. Our results showed that the expression of lncRNA NRON was significantly downregulated in osteosarcoma tissues (Fig.1A). Similar, we also analyzed the expression of lncRNA NRON in osteosarcoma cell lines (Fig.1B). Real-time PCR results showed that the expression of lncRNA NRON was significantly downregulated in osteosarcoma cell lines. In addition, we analyzed the expression of lncRNA NRON in different mouse organs. Our results showed that the expression of lncRNA NRON was highly in muscle tissues, and expressed in various tissues, suggesting that lncRNA NRON may also possess function in other disease (Fig.1C). Lastly, we analyzed the conservation level of lncRNA NRON in different animals. Bioinformatics analysis showed that lncRNA NRON is highly conserved in mouse, dog, elephant and chicken (Fig.1D), suggesting lncRNA NRON may play an important role in the biological process.

**Knockdown of lncRNA NRON promoted cell proliferation and invasion**
To study the function of IncRNA NRON, we constructed knockdown siRNA. The knockdown efficiency was confirmed via real-time PCR. The expression of IncRNA NRON was significantly reduced in HOS and U2OS cell lines (Fig.2A). We performed CCK-8, transwell and western blot to evaluate the proliferation, invasion and EMT. CCK-8 results showed that knockdown of IncRNA NRON promoted cell proliferation in HOS and U2OS cell lines (Fig.2B). Transwell assay showed that downregulated of IncRNA NRON increased the cell invasion number (Fig.2C). Lastly, we examined the EMT markers, E-cadherin, N-cadherin and Vimentin, to evaluate the influence of IncRNA NRON. Our results showed that knockdown of IncRNA NRON reduced the protein level of E-cadherin, while increased the protein level of N-cadherin and Vimentin in HOS and U2OS cell lines (Fig.2D). Thus, we proved that knockdown of IncRNA NRON can promote the development of osteosarcoma.

**Overexpression of IncRNA NRON inhibited cell proliferation and invasion**

To further study the function of IncRNA NRON, we constructed overexpression vector. The overexpression efficiency was verified via real-time PCR. The expression of IncRNA NRON was significantly increased in HOS and U2OS cells (Fig.3A). We performed CCK-8, transwell and western blot to evaluate the proliferation, invasion and EMT. CCK-8 results showed that overexpression of IncRNA NRON inhibited cell proliferation in HOS and U2OS cell lines (Fig.3B). Transwell assay showed that upregulated of IncRNA NRON reduced the cell invasion number (Fig.3C). Lastly, we examined the EMT markers, E-cadherin, N-cadherin and Vimentin, to evaluate the influence of IncRNA NRON. Our results showed that overexpression of IncRNA NRON increased the protein level of E-cadherin, while decreased the protein level of N-cadherin and Vimentin in HOS and U2OS cell lines (Fig.3D). Thus, we proved that overexpression of IncRNA NRON can inhibit the development of osteosarcoma.

**Biological features of MVB12B**

To explore the mechanism of IncRNA NRON involved in osteosarcoma, we used bioinformatics analysis to predict the potential target of IncRNA NRON. As shown in Fig.4A, MVB12B is the neighboring gene of IncRNA NRON. The expression of MVB12B in different tissues was showed in Fig.4A, suggesting that MVB12B may possess function in the tumor development. Next, we analyzed the biological features of MVB12B in osteosarcoma. The expression of MVB12B was significantly increased in osteosarcoma tissues (Fig.4B). We also analyzed the expression of MVB12B in cancer cell lines, which is upregulated in osteosarcoma cell lines (Fig.4C). We wondered the relationship between IncRNA NRON and MVB12B. Overexpression of NRON reduced the expression of MVB12B and vice versa (Fig.4D). Similar results can be observed by western blot, suggesting that IncRNA NRON can negatively regulate the expression of MVB12B at mRNA and protein level (Fig.4E). Next, we performed experiments to assess the function of MVB12B in osteosarcoma. Firstly, we constructed overexpression vector of MVB12B. The expression of MVB12B was significantly upregulated in HOS and U2OS cells (Fig.4F). CCK-8 assay showed that overexpression of MVB12B significantly promoted cell proliferation in HOS and U2OS cells, as indicated by CCK-8 assay (Fig.4G). Thus, our results showed that MVB12B is the potential target of IncRNA NRON.

**Reintroduction of MVB12B rescued the function of IncRNA NRON**
To verify the relationship between lncRNA NRON and MVB12B, we performed comprehensive rescue experiment to examine whether lncRNA NRON inhibit the osteosarcoma development by regulating MVB12B. Co-transfection group of lncRNA NRON and MVB12B significantly increased the cell invasion number compared with lncRNA NRON overexpression group in HOS and U2OS cells (Fig.5A). CCK-8 assay showed that reintroduction of MVB12B completely blocked the inhibit effect induced by overexpression of lncRNA NRON (Fig.5B). Western blot showed that reintroduction of MVB12B reversed the effect of lncRNA NRON, as indicated by decreased protein level of E-cadherin and increased protein level of N-cadherin and Vimentin (Fig.5C, Fig.5D). Thus, our results showed that lncRNA NRON can inhibit cell proliferation and invasion by negatively regulating MVB12B.

**Discussion**

Previous studies have revealed the function of lncRNA NRON, a newly identified tumor-related lncRNA, in hepatocellular carcinoma and heart failure. However, the function and mechanism of lncRNA NRON in osteosarcoma still unknown. In this study, we found that knockdown of NRON promoted cell proliferation, invasion and EMT. Bioinformatics analysis predicted that MVB12B is the direct target. The expression of MVB12B was significantly upregulated in osteosarcoma tissues and cell lines. Rescue experiment further confirmed the relationship between NRON and MVB12B. Overexpression of MVB12B completely reversed the function of NRON. Taken together, our results comprehensively analyzed the function of NRON in osteosarcoma and provided possible mechanism that NRON inhibited osteosarcoma development by regulating MVB12B.

Long noncoding RNA has been showed to play important roles in various disease[17]. For example, Long noncoding RNA LINC00511 contributes to breast cancer tumourigenesis and stemness by inducing the miR-185-3p/E2F1/Nanog axis[18]. LncRNA UCA1 promotes tumor metastasis by inducing miR-203/ZEB2 axis in gastric cancer[19]. IncRNA NRON has been reported in numerous diseases, which is highly expressed in resting CD4(+) T lymphocytes, could be involved in HIV-1 latency by specifically inducing Tat protein degradation[20]. NRON IncRNA potently suppresses the viral transcription by decreasing the cellular abundance of viral transactivator protein Tat. However, little is known about the function of NRON in osteosarcoma. In our study, we utilized overexpression and knockdown vector of NRON in osteosarcoma cell lines to study the invasion, proliferation and EMT indexes. Our results showed that NRON possessed an anti-tumor function, as indicated by inhibited proliferation and cell invasion number.

The mechanism of lncRNA can be diverse, including regulation of gene transcription, post-translational regulation, epigenetic regulation[21]. In post-translational regulation, for example, IncRNA RMST Suppressed GBM Cell Mitophagy through Enhancing FUS SUMOylation[22]. Lnc-CCDST promotes DHX9 degradation by serving as a scaffold to facilitate the formation of MDM2 and DHX9 complexes[23]. In epigenetic regulation, SWINGN influences the ability of the SWI/SNF complexes to drive epigenetic activation of specific promoters, suggesting a SWI/SNF-RNA cooperation to achieve optimal transcriptional activation[24]. Previous studies have shown that LncRNA NRON alleviates atrial fibrosis via promoting NFATc3 phosphorylation[16]. Long noncoding RNA NRON contributes to HIV-1 latency by
specifically inducing tat protein degradation[20]. NRON can function through epigenetic regulation and post-translational regulation. In our study, we found the neighboring gene MVB12B was significantly changed upon IncRNA NRON treatment. Overexpression of IncRNA reduced the expression of MVB12B at mRNA and protein level. Thus, we speculate that NRON may influence the level of MVB12B at transcription level. Further rescue experiment revealed that re-introduction of MVB12B can completely restored the function of IncRNA NRON.

NRON has been reported that directly bind with Tat to the ubiquitin/proteasome component to facilitate Tat protein degradation[20]. There still another report about NRON in atrial fibrosis, which can inhibit fibroblast proliferation and downstream targets, including collagen I and collagen III, through promoting NFATc3 phosphorylation. To date, our results was the first study of function and mechanism of NRON in osteosarcoma. Interestingly, the mechanism of NRON in osteosarcoma was different with HIV infection and atrial fibrosis, suggesting that NRON may associated with numerous mechanisms and involved in different pathological process. Thus, further study of NRON in other disease is clearly warranted.

To sum up, our study uncovered the role of IncRNA NRON in osteosarcoma, which possessed an anti-tumor role in the development of osteosarcoma. Further studies of IncRNA undoubtedly deepen understanding of the occur and pathology of osteosarcoma. Thus, our study may offer a potential therapeutic target for treating osteosarcoma.

**Declarations**

**Acknowledgments**

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**Conflict of Interest:** The authors declare no competing financial interests.

**Data availability:**

The data used to support the findings of this study are included within the article.

**Author contribution:**

Xinlong Ma, Yongcheng Hu designed the research. Yancheng Liu, Jingyu Zhang, Yongqiang Jiao performed the experiments and wrote the manuscript. Yongqiang Jiao and Shuwei Ma analyzed the data.

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Figures
**Figure 1**

Biological features of lncRNA NRON. A. The expression of NRON was downregulated in tumor tissues. B. The expression of IncRNA NRON was downregulated in cell lines. C. Multi-tissue analysis revealed the expression of IncRNA NRON in different tissues. D. IncRNA NRON is highly conserved in different animals.

**Figure 2**

Knockdown of IncRNA NRON promoted cell proliferation. A. Knockdown efficiency was confirmed via real-time PCR. B. Knockdown of IncRNA NRON significantly promoted cell proliferation in HOS and U2OS cells. C. Knockdown of IncRNA NRON promoted cell invasion. D. Knockdown of IncRNA NRON decreased the protein level of E-cadherin, while increased the level of N-cadherin and Vimentin.
Figure 3

Overexpression of lncRNA NRON inhibited cell proliferation. A. Overexpression efficiency was confirmed via real-time PCR. B. Overexpression of lncRNA NRON inhibited cell proliferation. C. Overexpression of lncRNA NRON inhibited cell invasion. D. Overexpression of lncRNA NRON increased the protein level of E-cadherin and decreased the level of N-cadherin and Vimentin.
Figure 4

Biological features of MVB12B. A. Biological features of MVB12B in different tissues. B. The expression of MVB12B was significantly increased in tumor tissues. C. The expression of MVB12B was significantly increased in cell lines. D. IncRNA NRON negatively regulate the expression of MVB12B via real-time PCR. E. IncRNA NRON negatively regulate the protein level of MVB12B via western blot. F. Overexpression efficiency of MVB12B was confirmed via real-time PCR. G. Overexpression of MVB12B promoted cell proliferation in HOS and U2OS cells.
Figure 5

Reintroduction of MVB12B rescued the function of IncRNA NRON. A. Co-transfection of MVB12B and IncRNA NRON rescued the invasion effect of IncRNA NRON. B. Proliferation rates were measured by CCK8. C. Co-transfection of MVB12B and IncRNA NRON decreased the protein level of E-cadherin compared with IncRNA NRON group. D. Quantification data of western blot.