LncRNA NORAD Promotes Proliferation And Inhibits Apoptosis Of Gastric Cancer By Regulating miR-214/Akt/mTOR Axis

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Purpose: In previous studies, we confirmed that the overexpression of lncRNA NORAD was associated with the occurrence and development of gastric cancer (GC). The aim of the present study was to explore the molecular mechanism of lncRNA NORAD on GC cell proliferation and apoptosis in vitro and in vivo.

Patients and methods: The quantitative Real-Time PCR (qRT-PCR) was used to determine the expression levels of lncRNA NORAD and miR-214 in GC tissues and cells. The interaction between lncRNA NORAD and miR-214 was investigated by biological information and Dual-Luciferase gene reporter assay. Effect of lncRNA NORAD on GC tumor growth in vivo was studied in tumor xenograft model mice. The apoptosis of GC cells was determined by flow cytometry. The proliferation of GC cells was determined by 5-ethynyl-2’-deoxyuridine (EDU) and colony formation assays. Western Blot was used to determine the expressions of caspase-3, Akt and mTOR in GC tissues and cells.

Results: The qRT-PCR results showed that lncRNA NORAD was highly expressed in human GC tissues and cell lines, while miR-214 was significantly down-regulated. Meanwhile, there was a direct interaction between lncRNA NORAD and miR-214. In addition, lncRNA NORAD could promote the growth and proliferation of GC cells both in vivo and in vitro. NOARD could also inhibit the apoptosis of GC cells by down-regulating caspase-3; however, miR-214 overexpression attenuated this effect. Moreover, lncRNA NORAD promoted the phosphorylation of Akt and mTOR in mouse GC tissues and GC cell lines, while miR-214 mimics inhibited that promotion.

Conclusion: These results suggested that NORAD could promote the development of GC by inhibiting miR-214 expression and activating the Akt/mTOR signaling pathway.

Keywords: gastric cancer, miR-214, lncRNA NORAD, Akt/mTOR

Introduction

Gastric cancer (GC) is one of the malignant tumors of digestive tract, which has high morbidity and mortality.1 The early diagnosis rate of GC is low, once diagnosed, most patients are already in the late stage, and the cure rate is very low.2 Therefore, it is of great importance to reveal the molecular mechanism of the occurrence and development of GC for the treatment and improvement of prognosis of gastric cancer. The most obvious pathological feature of tumor cells is the imbalance of cell proliferation and apoptosis.3 The normal apoptosis process of tumor cells is inhibited, and the proliferation ability is significantly enhanced, which makes invasion and metastasis extremely easy.4 Thus, the development of GC is closely related to the proliferation...
and apoptosis of GC cells. In addition, the formation and development of GC is a complex pathological process, involving not only gene mutation but also changes in intracellular signaling pathways.\(^5,6\) Studies have found that the protein kinase B/rapamycin target protein (Akt/mTOR) pathway is a classical signaling pathway that promotes proliferation and anti-apoptosis.\(^7\) Excessive activation of Akt/mTOR pathway can promote the proliferation and metastasis of a variety of malignant tumor cells and cause the occurrence, development, and metastasis of tumors.\(^8-10\) Phosphorylated Akt can regulate downstream target proteins mTOR, Bcl-2, Bax, caspase-9 and caspase-3 to induce tumor cell proliferation and inhibit apoptosis.\(^11\) The cell apoptosis process is completed by activating the caspase cascade, in which the activated caspase-3 acts as the terminal protease and eventually causes cell apoptosis.\(^12\) Furthermore, the expression level of caspase-3 is also closely related to the apoptosis of GC cells.\(^13\)

Currently, studies have found that a variety of long non-coding RNAs (lncRNAs) are involved in the growth, proliferation and apoptosis of GC cells, and can be used as potential targets for GC therapy.\(^14,15\) LncRNA NORAD is a newly discovered lncRNA that is overexpressed in colorectal cancer and breast cancer.\(^16,17\) In a recent study, Z. Miao reported that NORAD promoted the growth of gastric cancer cells by sponging miR-608.\(^18\) In addition, Yu SY also reported that the silencing of NORAD inhibited gastric cancer cell proliferation and invasion by the RhoA/ROCK1 pathway.\(^19\) Micro RNAs (miRNAs) is a class of endogenous short-chain non-coding RNA molecules composed of 18–22 nucleotides, which are involved in tumorogenesis, cell growth, apoptosis, and signal transduction and so on.\(^20\) Studies have found that lncRNAs can be used as competitive endogenous RNAs (ceRNAs) to bind miRNAs and inhibit the function of miRNAs, thus affecting the occurrence and development of tumors.\(^21\) Zhang et al reported that lncRNA NORAD promoted the occurrence and development of colorectal cancer by targeting and binding with miR-202-5p.\(^22\) MiR-214 is a miRNA involved in the occurrence and development of ovarian cancer, cervical cancer, gastric cancer and pancreatic cancer.\(^23,24\) However, there were no studies on the effects of lncRNA NORAD and miR-214 on the proliferation and apoptosis of GC cells.

In this study, human GC tissues, GC cell lines and GC xenograft model mice were selected as subjects to explore the molecular mechanism of lncRNA NORAD and miR-214 in the proliferation and apoptosis of GC cells, aiming to provide a theoretical basis for GC targeted therapy.

**Materials And Methods**

**Tissues Collection**

Twenty-three GC patients diagnosed by histopathology in General Hospital of Ningxia Medical University from 2010 to 2011 were selected, and GC tissues and adjacent non-tumor tissues were obtained by surgery. All the tissue samples after liquid nitrogen refrigeration were saved in the -80°C cryogenic refrigerator. All patients received no other treatment before surgery and all patients provided written informed consent. This study was approved by the General Hospital of Ningxia Medical University and that this was conducted in accordance with the Declaration of Helsinki.

**Cell Culture**

Normal gastric cell line (GSE1) and human gastric adenocarcinoma cell lines (BSG823, MKN28, BGC803 and BGC823) were obtained from the cell bank of the Chinese academy of sciences. Cells were incubated in DMEM (Gibco) medium containing 10% FBS, and penicillin and streptomycin (HyClone) were selectively added. The pc-NORAD, sh-NORAD or miR-214 mimic were transfected into BGC803 and BGC823 cells using RNAiMax, Lipofectamine LTX and Plus reagents (Thermo).

**The qRT-PCR Assay**

Total RNA in tissues and cells was extracted using Trizol reagent (Invitrogen) and the purity of RNA was determined. The extracted RNA was reversely transcribed into cDNA using the GoScript reverse transcription system (Promega). Primers were amplified using the SYBR Premix Dimmer Eraser kit (TaKaRa). Using U6 or GADPH as internal reference standards, and through the 2^−ΔΔCt method to calculate the relative expression levels of genes.

**Luciferase Reporter Gene Assay**

The pc-NORAD or sh-NORAD were transfected into GC cell lines and the dual-luciferase assay kit (Promega) was used to detect the luciferase activity in the cells, following the instructions of the kit. The sequences of NORAD and miR-214 are listed here. NORAD (F: 5’TGATAGGATACATCTTGGACATGGA3’), (R: 5’AACCTAATGACAAGTCCTGACATAAC3’) miR-214 forward: 5’-ACAGCAAGGCACACGACAGAGG-3’ and reverse: 5’- GTGCAGGTTCCGAGGT-3’.
GAPDH forward: 5’-GGGAGCCAAAAGGTCAT-3’
reverse: 5’-GAGTCCFTTCCACGATACCAA-3’.

RNA Pulldown Assay
In short, 39-biotinylated miRNA mimic was transfected into BSG823 cells and lysed. Streptavidin-coupled beads were incubated with BSG823 cell lysis products to form biotin-miRNA-lncRNA complex. RNA digested by protease K was determined by PCR and miR-67 of caenorhabditis elegans was used as a negative control.

Mouse Model Of Tumor Xenotransplantation
BGC823 cells (1×10⁷) transfected with pcNORAD or shNORAD were subcutaneous inoculated into BALB/c nude mice without thymus at 4 weeks of age to establish a tumor xenograft mouse model. Monitor tumor growth every 3 days and measure tumor volume. This study was conducted in strict accordance with the institutional guidelines for the care and use of experimental animals and was approved by the animal experiment ethics committee of General Hospital of Ningxia Medical University.

Immunohistochemistry And Western Blot Assays
The immunohistochemistry and Western Blot analysis of tissues or cells were performed as described in the literature.²⁵

Cell Proliferation Assay
EdU Imaging Kit (RiboBio) was used to test the cell proliferation capacity, and the specific procedures were strictly in accordance with the instruction of the Kit. Meanwhile, colony formation assay was used to determine the ability of cell clone formation.

Cell Apoptosis Assay
Annexin V/PI staining and flow cytometry were used to determine the cell apoptosis. In short, the transfected cells were re-suspended in PBS buffers and stained with Annexin V-PI kit. The apoptosis rate was analyzed by flow cytometry.

Statistical Analysis
All data are expressed as mean ± standard deviation (SD). SPSS17.0 software was used for analysis in this study, and Student t-test was used for statistical significance analysis. P <0.05 indicated a significant difference.

Results
The mRNA Expressions Of IncRNA NORAD And miR-214 In GC Tissues And Cell Lines
First, the mRNA expressions of lncRNA NORAD and miR-214 in GC tissues (n = 36) and adjacent non-tumor tissues (n = 36) were determined by qRT-PCR. The mRNA expression of IncRNA NORAD in GC tissues was significantly higher than that in adjacent tissues (Figure 1A, P < 0.05). Meanwhile, the results of correlation analysis showed that the mRNA expression of IncRNA NORAD in GC tissues was negatively correlated with the mRNA expression of miR-214 (Figure 1B). In addition, the mRNA expressions of IncRNA NORAD and miR-214 in GC cell lines were determined by qRT-PCR. The results showed that mRNA expression of IncRNA NORAD in GC cell lines (BSG823, MKN28, BGC803, BGC823) were significantly higher than that in GSE1 cells (Figure 1C, P < 0.05), while the mRNA expression of miR-214 in GC cell lines were significantly lower than that in GSE1 cells (Figure 1D, P < 0.05). These results suggested that NORAD was highly expressed in GC tissues and cells, while miR-214 was down-regulated.

Identification Of miR214 As The Target Gene Of lncRNA NORAD In GC Cells
First, we transfected pc-NORAD or sh-NORAD into BGC823 and BGC803 cells to determine the mRNA expressions of IncRNA NORAD and miR214 in the cells. The results showed that transfected pc-NORAD significantly up-regulated the expression of IncRNA NORAD in GC cells (Figure 2A, P < 0.05, P < 0.01) and significantly inhibited the expression of miR214 (Figure 2C, P < 0.05). Meanwhile, we transfected miR-214 mimic or its control into BGC823 and BGC803 cells and found that the transfected with miR-214 mimic significantly up-regulated the expression of miR-214 in cells (Figure 2B, P < 0.05, P < 0.01), indicating the successful transfection of miR-214 mimic. In addition, we through the biological analysis (http://starbase.sysu.edu.cn) to predict miR-214 might exist the IncRNA NORAD binding sites (Figure 2D). Furthermore, wild-type NORAD (NORAD-WT) or mutant NORAD (NORAD-MUT) was transfected with miR-214 mimic or its control into BGC823 and BGC803 cells, and luciferase activity was measured. The results showed that, compared with the control group, the luciferase activity in BGC823 and BGC803 cells co-transfected by NORAD-WT and miR-214 mimic
were significantly decreased (Figure 2E, P < 0.01), which indicated that miR-214 was a direct target of lncRNA NORAD. Moreover, the RNA pulldown results found that, compared with the control group, the mRNA expression of lncRNA NORAD in the bio-miR-214 group was remarkably up-regulated (Figure 2F, P < 0.01), which further confirmed that miR-214 was a direct target of lncRNA NORAD.

**Effect Of lncRNA NORAD On GC Tumor Cell Growth In Vivo**

BGC823 cells transfected with pc-NORAD or sh-NORAD were subcutaneous injected into nude mice to establish a GC xenograft mouse model, and the effect of lncRNA NORAD on the growth of GC tumor cells in vivo was investigated. The results found that the GC tumor volume in pc-NORAD group was remarkably higher than that in pc-NC group (P < 0.01), while the GC tumor volume in sh-NORAD group was remarkably lower than that in sh-NC group (Figure 3A–C, P < 0.01), which indicated that NORAD overexpression significantly promoted the GC tumor growth. In addition, immunohistochemical results found that the number of Ki67-positive cells in pc-NORAD group was more than that in pc-NC group, while the number of Ki67-positive cells in sh-NORAD group was lower than that in sh-NC group (Figure 3D), indicating that NORAD overexpression observably promoted the proliferation of GC tumor cells. Furthermore, TUNEL staining showed that TUNEL positive cells in pc-NORAD group were lower than those in pc-NC group, while TUNEL positive cells in sh-NORAD group were more than those in sh-NC group (Figure 3E), suggesting that NORAD overexpression significantly inhibited apoptosis of GC tumor cells.
Effects Of IncRNA NORAD On Apoptosis And Proliferation Of GC Cells

First, the protein expression of caspase-3 in pc-NORAD or sh-NORAD transfected BGC823 and BGC803 cells were detected by Western Blot. It was found that protein expression of caspase-3 in sh-NORAD group was significantly higher than that in control group (P < 0.05), while there was no significant difference in caspase-3 expression between pc-NORAD group and control group (P > 0.05), which indicated that NORAD silencing significantly promoted the expression of caspase-3 in GC cells. Meanwhile, flow cytometry showed that the apoptosis rate of GC cells in the sh-NORAD group was significantly higher than that in the control group (P < 0.05), while the apoptosis rate of GC cells in the pc-NORAD group was not significantly different from that in the control group (Figure 4B and C, P > 0.05). It suggested that NORAD silencing significantly promoted the apoptosis of GC cells. In addition, colony formation and EDU assay were used to detect the proliferation of BGC823 and BGC803 cells transfected with pc-NORAD or sh-NORAD. The results showed that the number of clones and EDU positive cells in pc-NORAD group were markedly more than those in control group (P < 0.05), while the number of clones and EDU positive cells in shNORAD group were significantly lower than those in control group (Figure 4D and E, P < 0.05), indicating that NORAD overexpression significantly promoted the cell proliferation.

Effect Of miR-214 On Proliferation And Apoptosis Of GC Cells Mediated By IncRNA NORAD

The pc-NORAD or/and miR-214 mimic were transfected into BGC823 and BGC803 cells to determine
the effect of miR-214 on IncRNA NORAD-mediated GC cell proliferation and apoptosis. Western Blot results showed that transfection of miR-214 mimic significantly inhibited the expression caspase-3 of in GC cells induced by pc-NORAD (Figure 5A, P<0.01). Meanwhile, the flow cytometry results found that the apoptosis rate of GC cells in the pc-NORAD + miR-214 mimic group was significantly higher than that in the pc-NORAD group (Figure 5B, P<0.01), which indicated that the transfection of miR-214 mimic significantly promoted the apoptosis of GC cells inhibited by pc-NORAD. In addition, EDU and colony formation results found that the number of EDU positive cells and clone cells in the pc-NORAD + miR-214 mimic were significantly lower than those in the pc-NORAD group (Figure 5C and D, P<0.01), which suggested that the transfection of miR-214 mimic significantly inhibited the proliferation of GC cells promoted by pc-NORAD.

Effects Of NORAD And miR-214 Expressions On Akt/mTOR Pathway In GC Cells

Western Blot was used to determine whether the Akt/mTOR pathway in mouse GC tissues and GC cells were related to the proliferation and apoptosis of GC cells mediated by NORAD/miR-214. The results showed that p-Akt and p-mTOR were significantly up-regulated in pc-NORAD-transfected tumor tissues (P<0.05), while p-Akt and p-mTOR were significantly inhibited in sh-NORAD transfected tumor tissues (Figure 6A, P<0.05). In addition, pc-NORAD or/and miR-214 mimic were transfected into BGC823 and BGC803 cells and the expressions of p-Akt and p-mTOR in the cells were detected. It was found that the expressions of p-Akt and p-mTOR were significantly up-regulated in GC cells transfected with pc-NORAD (P<0.05), while miR-214 mimic significantly inhibited this promotion (Figure 6B, P<0.05,
These results suggested that NORAD/miR-214-mediated GC cell proliferation and apoptosis were related to the activation of Akt/mTOR signaling pathway.

**NORAD Expressions And Clinical Characteristics**

We also provided the information about the clinical characteristics of patients with GC including pathological stage, invasion, and metastasis state. Supplementary table 1 shows all the details. It was noted that the expressions of NORAD had little impact from age or gender. However, larger tumor size (>5 cm), lymph node metastasis, higher tumor grade, and advanced tumor stages would significantly increase the NORAD expressions. Overall, NORAD might be up-regulated in tumors with characteristics of large size, metastasis, high grade, or advanced stages.

**Discussion**

GC is a common malignant tumor, which has the characteristics of high incidence and low cure rate, and seriously threatens human health. Recent studies have found that various lncRNAs and miRNAs have regulatory effects on the growth, proliferation, differentiation, and apoptosis of GC cells. LncRNA NORAD has been confirmed to be overexpressed in breast cancer and colorectal cancer. In addition, miR-214 was proved to be involved in the pathogenesis of gastric cancer, ovarian cancer, cervical cancer, and pancreatic cancer. However, the mechanism of lncRNA NORAD and miR-214 in GC cell proliferation and apoptosis is still unclear. Studies have confirmed that lncRNAs can act as ceRNAs, bind to miRNAs and inhibit the function of miRNAs, thus affecting the occurrence and development of tumors. In this study, it was found that lncRNA NORAD was highly...
expressed in human GC tissues and cell lines, while the expression of miR-214 was significantly down-regulated. In addition, this study also predicted the existence of lncRNA NORAD binding sites in miR-214 through bioinformatics, and the lncRNA NORAD could directly regulate the expression of miR-214, confirming that miR-214 was a direct target of lncRNA NORAD. Zhang et al found that lncRNA NORAD was overexpressed in colorectal cancer (CRC) tissues and cell lines, and could be used as ceRNA of miR-202-5p to promote CRC cell proliferation and induce apoptosis. He et al reported that lncRNA NORAD could accelerate the proliferation and metastasis of thyroid cancer cells by targeting and binding miR-202-5p to promote the EMT process of cells, which had the role of an oncogene. Therefore, our results suggested that lncRNA NORAD played an oncogene role in GC progression by regulating the expression of miR-214. The imbalance of tumor cell proliferation and apoptosis is the main cause of tumor invasion and metastasis. Therefore, the development of GC is closely related to the proliferation and apoptosis of GC cells. At present, the GC subcutaneous tumor xenograft model is an effective animal model for preclinical study of gastric cancer. In this study, BGC823 cells transfected with pcNORAD or shNORAD were subcutaneous injected into nude mice, and a GC xenograft mouse model was established to investigate the effect of lncRNA NORAD on the growth of GC tumor cells in vivo. It was found in the mouse model of GC xenotransplantation that lncRNA NORAD could promote the growth and proliferation of GC cells and inhibit the apoptosis of tumor cells in vivo. In addition, this study also found that lncRNA NORAD enhanced the proliferation of GC cells in vitro and inhibited the apoptosis of GC cells, while miR-214 overexpression

Figure 5 Effect of miR-214 on proliferation and apoptosis of GC cells mediated by lncRNA NORAD. (A) Detection of the caspase-3 expression in GC cells transfected with pc-NORAD or/and miR-214 mimic by Western Blot. (B) Detection of the apoptosis rate of GC cells transfected with pc-NORAD or/and miR-214 mimic by flow cytometry. (C) Observation of the proliferation of GC cells transfected with pc-NORAD or/and miR-214 mimic by EDU staining. (D) Detection of the colony formation ability of GC cells transfected with pc-NORAD or/and miR-214 mimic by colony formation assay. *p < 0.05, **p < 0.01.
showed an opposite effect. Zhou et al found that lncRNA NORAD could promote the proliferation and metastasis of breast cancer cells by activating the TGF-β/RUNX2 pathway. Yang et al found that miR-214 could inhibit the proliferation and metastasis of GC cells by targeting PTEN. Therefore, the results of this study suggested that lncRNA NORAD could promote the proliferation of GC cells and inhibit apoptosis by targeting miR-214.

The development of GC involves not only gene mutation but also changes in intracellular signaling pathways. It has been found that excessive activation of the Akt/mTOR pathway can promote the proliferation and metastasis of tumor cells. Phosphorylated Akt induces tumor cell proliferation and inhibits apoptosis by regulating downstream target proteins mTOR and caspase-3, etc. To further investigate the mechanism of lncRNA NORAD/miR-214 in the proliferation and apoptosis of GC cells, we measured the phosphorylation levels of Akt and mTOR in mouse GC tissues and GC cell lines. The results showed that lncRNA NORAD significantly up-regulated the phosphorylation of Akt and mTOR in mouse GC tissues and GC cell lines, while miR-214 had the opposite effect. In addition, we also found that NORAD silencing significantly promoted the expression of caspase-3 in GC cells. Wu et al found that Sinularin could induce apoptosis of GC cells by inhibiting the activation of Akt/mTOR pathway and caspase-3. Wu et al reported that miR-5590-3p inhibited the activation of Akt/mTOR pathway by targeting DDX5, and ultimately suppressed the growth and proliferation of GC cells. Thus, the results of this study suggested that NORAD/miR-214-mediated GC cell

Figure 6 Effects of NORAD and miR-214 expressions on Akt/mTOR pathway in GC cells. (A) Determination of the expressions of Akt and mTOR in mouse tumor tissues by Western Blot. (B) Determination of the expressions of Akt and mTOR in BGC823 and BGC803 cells transfected with pc-NORAD or/and miR-214 mimic by Western Blot. *P < 0.05, **P < 0.01.
proliferation and apoptosis were related to the activation of Akt/mTOR signaling pathway.

We carried out the experiments in vivo and observed that lncRNA NORAD could promote the GC tumor cell growth. However, it is still unknown whether NORAD could modulate miR-214/Akt/mTOR axis in animal experiments. Further studies are necessary to fully understand the functions of NORAD, as well as miR-214/Akt/mTOR in clinical trials.

Conclusion
In conclusion, the present study assessed the molecular mechanism of lncRNA NORAD on GC cell proliferation and apoptosis in vitro and in vivo. These results suggested that NORAD could promote the development of GC by inhibiting miR-214 expression and activating the Akt/mTOR signaling pathway, which provided a potential target for the treatment of GC patients.

Disclosure
The authors report no conflicts of interest in this work.

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