European Review for Medical and Pharmacological Sciences 2021; 25: 6187-6195

LncRNA NORAD promotes thyroid carcinoma progression by targeting miR-451

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Abstract. – OBJECTIVE: Long non-coding RNA (lncRNA) NORAD plays an essential role in the development and progress of papillary thyroid carcinoma (PTC). MicroRNA-451 (MiR-451) has been identified as playing an inhibitory role in some types of cancer. However, the molecular mechanism of lncRNA NORAD regulating metastasis of PTC cells by miR-451 has not been fully elucidated.

MATERIALS AND METHODS: Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) or Western blot analysis of the expression of NORAD, miR-451, and interleukin-6 receptor (IL-6R) in PTC cell lines were carried out. The detection of Luciferase reporter gene showed the relationship between lncRNA NORAD, miR-451 and IL-6R. Cell Counting Kit-8 (CCK-8) assay and transwell assay were performed to detect the influence of lncRNA NORAD, miR-451 on the proliferation, migration, and invasion of PTC cells.

RESULTS: The results of RT-qPCR and Western blotting suggested that the expression of lncRNA NORAD and IL-6R were higher than that of the control group, while the expression of miR-451 was lower. Transwell assay indicated that the knockdown of lncRNA NORAD or overexpression of miR-451 significantly inhibited cell proliferation, migration and invasion in PTC cell lines. In addition, lncRNA NORAD negatively controls the expression of miR-451, resulting in the upregulation of IL-6R. IL-6R overexpression can reverse the inhibitory effect of lncRNA NORAD knockdown or miR-451 on PTC cell proliferation and metastasis.

CONCLUSIONS: Our results indicated that the cell migration and invasion were inhibited by knockdown of lncRNA NORAD or overexpression of miR-451, suggesting that the axis of lncRNA NORAD -miR-451- IL-6R was involved in the development of PTC.

Key Words:
LncRNA NORAD, IL-6R, MiR-451, Thyroid cancer.

Introduction

Papillary thyroid carcinoma (PTC) is slow-growing epithelial malignancy. It is the most common endocrine system malignancy and accounts for the ninth most common cancer overall. Thyroid carcinoma is the most common endocrine malignancy[1]. Although the survival is generally good, but the mortality rate is higher than that of all other endocrine organ cancers[2].

Wu et al[3] showed that NORAD expression in esophageal squamous cell carcinoma was upregulated compared to that in adjacent normal tissue samples. Li et al[4] indicated that NORAD expression was upregulated in pancreatic cancer tissue samples, while NORAD over expression was related to a shorter OS (total survival). At the same time, the study indicated that ectopic expression of NORAD increased the invasion and migration of pancreatic cancer cells[4]. Zhang et al[5] showed that NORAD expression was upregulated in colorectal cancer (CRC) tissue samples, while the high expression of NORAD was related to prognosis and CRC metastasis. However, the expression and function of NORAD are not clear in PTC.

MicroRNA (miRNA) is a kind of small non-coding RNAs that degrade mRNA or target mRNA by inhibiting the expression of negatively regulated genes[6]. In particular, Lee et al[7] and Wang et al[8] showed that the expression of miR-222 and miR-146b has been independently demonstrated to be related with poor prognosis and lymph node involvement in thyroid cancer. The role of miR-451 in bladder cancer was originally reported in 2014. Low levels of miR-451 have been reported in bladder cancer tissues, where miR-451 has been shown to be a tumor suppressor through epithelial mesenchymal transformation[9]. MiR-451 can inhibit the expression of pro-inflammatory molecules in mice during the development of diabetes and nephropathy by targeting LMP7/NF/kB and reduce glomerular damage[10]. Studies in non-small cell lung cancer (NSCLC) have demonstrated the tumor inhibitory role of miR-451. The upregulation of miR-451 inhibited the growth of NSCLC cell line A549

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and enhanced its apoptosis[11,12]. MicroRNA-451 is used as a prognostic marker for the diagnosis of thyroid papillary carcinoma and lymph node metastasis[13].

Interleukin-6 (IL-6) is a heterogeneous cytokine. IL-6 signaling through IL-6 receptor (IL-6R) plays a pivotal part in the inflammation and immune response[14,15]. IL-6 exerts its function by combining to its receptor and further activating various signaling pathways, such as Janus kinase/signal and transcription activator (JAK/STAT) and mitogen activated protein kinase pathway[16]. A great deal of studies have shown that IL-6 and its related signals contribute to the pathway of cell proliferation, migration and invasion of various tumor cells[17-20]. In addition, the physiological function of IL-6 has shown that it not only promotes tumor proliferation, but also promotes metastasis and symptoms[18,21,22]. There are reports of increased IL-6 expression, cancer type is associated with high serum IL-6 level metastasis and poor prognosis[23,24]. Some related studies have shown that compared to healthy individuals, the patients with thyroid benign diseases and PTC have significantly higher serum IL-6. In addition, the level of IL-6 in PTC patients was significantly higher than that of patients with thyroid benign diseases[25]. There is evidence that IL-6 signaling pathway contributes to the development of NSCLC[26]. There is evidence that the high expression polymorphism of IL-6 gene is associated with the susceptibility of papillary thyroid cancer[27]. In our study, we attempted to measure the change of lncRNA NORAD in PTC cell lines. We demonstrated a significant increase in NORAD expression in TPC1 and FTC133 cell lines compared to normal cells. LncRNA NORAD can inhibit the progression of PTC cells by targeting miR-451.

Materials and Methods

Cell Lines and Cell Transfection

Two PTC cell lines (TPC1 and FTC133) and a normal cell line (Nthy-ori3-1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) medium containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) and were used for all cell lines used in this study. The humidity was 100% and the temperature was 37°C with 5% carbon dioxide. ShRNA NORAD is built in a specific order for NORAD. MiR-451 mimic/inhibitor were transfected into cells (GenePharma, Shanghai, China). PcDNA3.1 (+) vector was used to construct pcDNA-IL-6R vector. The transfection was completed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Extraction of total RNA from PTC cell lines with TRIzol solution (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNA PrimeScript™ RT Reagent kit using 1 μg total RNA. RT-qPCR was performed using SYBR premix Ex Taq™ II PCR Kit (TaKaRa, Ostu, Shiga, Japan). All RT-qPCR primers were provided from Invitrogen (Carlsbad, CA, USA). The relative gene expression level was calculated by $2^{-\Delta\Delta CT}$ method.

Transwell Assay

The number of PTC cell migration and invasion was measured by transwell assay. In short, cells were cultured in the upper chamber and cultured in serum-free DMEM medium. Moreover, the lower chamber was supplied with a complete growth medium. After 24 hours, the cells were fully incubated, and then, the cells were removed with cotton swabs and left in the upper chamber. After migration, the cytomembrane was stained with crystal violet.

Identification of Luciferase Activity

Luciferase activity was analyzed by Promega (Madison, WI, USA). After transfection, according to the experimental requirements, TPC1 and FTC133 cells were lysed in a dish with lysate buffer. The activity relative to Luciferase was determined by Varioskan LUX detection system (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blot Analysis

In short, radioimmunoprecipitation assay (RIPA) was used to prepare the cell lysate buffer (Beyotime, Shanghai, China). Then, the bicinchoninic acid (BCA) Kit (Solarbio, Beijing, China) was used for quantitative analysis of protein concentration. The samples were isolated from the concentration of 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Solarbio, Beijing, China) and transferred to methanol activated polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Then, immunoblotting and anti-E-cadherin, anti-IL-6R, anti-N-cadherin, anti-Vimentin,

anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were placed overnight at 4°C. First and second antibodies used in Western blot were both purchased from Abcam (Cambridge, MA, USA). Then, electrochemiluminescence (ECL; Millipore, Billerica, MA, USA) was used for chemiluminescence detection.

Cell Viability Test

Cell viability was measured through cells Counting Kit (CCK)-8. In short, cells were inoculated on 96 well plate with a density of 5×10^3 cells/pore. Then, the cells were incubated with miR-451 expression at the specified time (0, 12, 24 and 36 hours). Then, we added 100 μL CCK-8 reagent and incubated at 90°C for another 2 hours at 37°C. Measure absorbance at 450 nm to evaluate Safire 2 microplate reader. Relative cell activity expressed in absorbance percentage of the treatment group compared to the control group.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA). Data were represented as mean ± Standard Deviation (SD). The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $p<0.05$ indicated the significant difference.

Results

Down Regulation of NORAD Inhibits Cell Metastasis

Here, we used RT-qPCR to analyze the expression of NORAD in PTC cell lines. As expected, NORAD was upregulated in PTC cell lines (Figure 1A). In order to detect the potential function and mechanism of NORAD in pathogenesis of PTC, the function of NORAD was designed and measured in PTC cell line. Silent NORAD significantly inhibited the proliferation of PTC cells (Figure 1B). CCK-8 and transwell were used to determine the effect of NORAD on cell proliferation, migration and invasion. The results of CCK-8 showed that the proliferation of TPC1 and FTC133 cells transfected with sh-NORAD was significantly inhibited (Figure 1C and 1D). In addition, we further examine the effect of silent NORAD on cells migration and invasion. According to the results of transwell analysis, we found that the cells metastasis TPC1 and FTC133 cells were inhibited by sh-NORAD (Figure 1E and 1F). In addition, Western blot was used to detect the effect of NORAD knockdown on the EMT progression. As shown in Figure 1F, epithelial markers (E-cadherin) were increased, while interstitial markers (N-cadherin, Vimentin) were significantly reduced (Figure 1G and 1H). Therefore, we found that NORAD can improve cell migration and EMT progress.

MiR-451 Inhibited PTC Cells Proliferation and Metastasis

At first, the binding sites between wild-type NORAD (NORAD-wt) or mutant NORAD (NORAD-mut) and miR-451 were predicted by bioinformatics analysis (Figure 2A). Subsequently, we used RT-qPCR to know the expression level of miR-451 in PTC cell lines. As expected, miR-451 was downregulated in PTC cells (Figure 2B). In order to detect the potential function and mechanism of miR-451 in pathogenesis of PTC, the function of miR-451 was designed and measured in PTC cell line. We detected the expression of miR-451 when transfected miR-451 mimic with PTC cells, miR-451 mimic groups were increased significantly than NC groups (Figure 2C). It was found that the decrease of Luciferase activity of NORAD-wt was induced by miR-451 mimic (Figure 2D). However, the Luciferase activity of the NORAD-mut was almost unchanged (Figure 2E). CCK-8 and transwell were used to determine the effect of miR-451 on cell proliferation, migration and invasion. The results of CCK-8 displayed that the proliferation of TPC1 and FTC133 cells transfected with miR-451 mimic was significantly inhibited (Figure 2F and 2G). In the meanwhile, we further examine the effect of miR-451 mimic on cells migration and invasion. According to the results of transwell analysis, we found that the cells metastasis TPC1 and FTC133 cells were inhibited by miR-451 mimic (Figure 2H and 2I). Finally, we measured the EMT protein. When we transfected the miR-451 mimic, the E-cadherin was induced obviously. And the N-cadherin and Vimentin were increased (Figure 2J and 2K). Therefore, our experimental results prove that miR-451 mimic can indeed play a key role in the proliferation and metastasis of PTC cells.

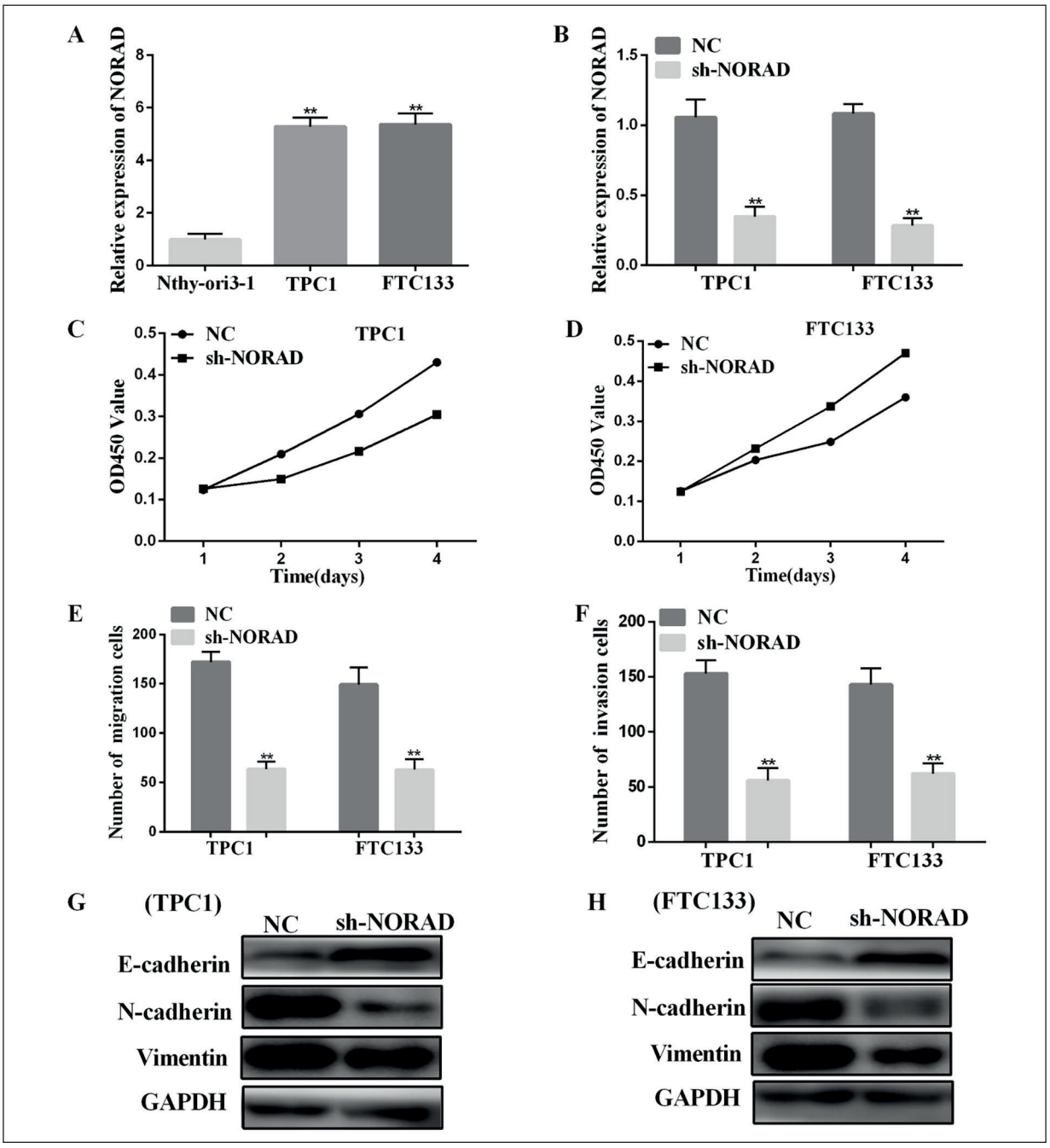

Figure 1. LncRNA NORAD was knocked down which can suppress TPC1 and FTC133 cell metastasis. **A,** Expression of lncRNA NORAD in two PTC cells and normal cell. **B,** Expression of lncRNA NORAD transfected with sh-NORAD or NC in PTC cells. **C, D,** CCK-8 detected the effect of lncRNA NORAD when knockdown lncRNA NORAD on cell proliferation in TPC1 and FTC133 cells. **E,** Knockdown lncRNA NORAD suppressing PTC cell migration. **F,** Knockdown lncRNA NORAD suppressing PTC cell invasion. **G, H,** The protein level of EMT-related marker protein in TPC cell transfected with sh-NC and sh-NORAD. $^{**}p<0.01$, $^{*}p<0.05$.

NORAD Upregulated IL-6R by Targeting MiR-451

In order to support the ceRNA hypothesis, it is necessary to find the target mRNA of miR-451. According to the prediction of bioinformatics tools, the binding sites between 3'UTR and miR-451 of IL-6R were predicted (Figure 3A). Similarly, the construction of Luciferase report

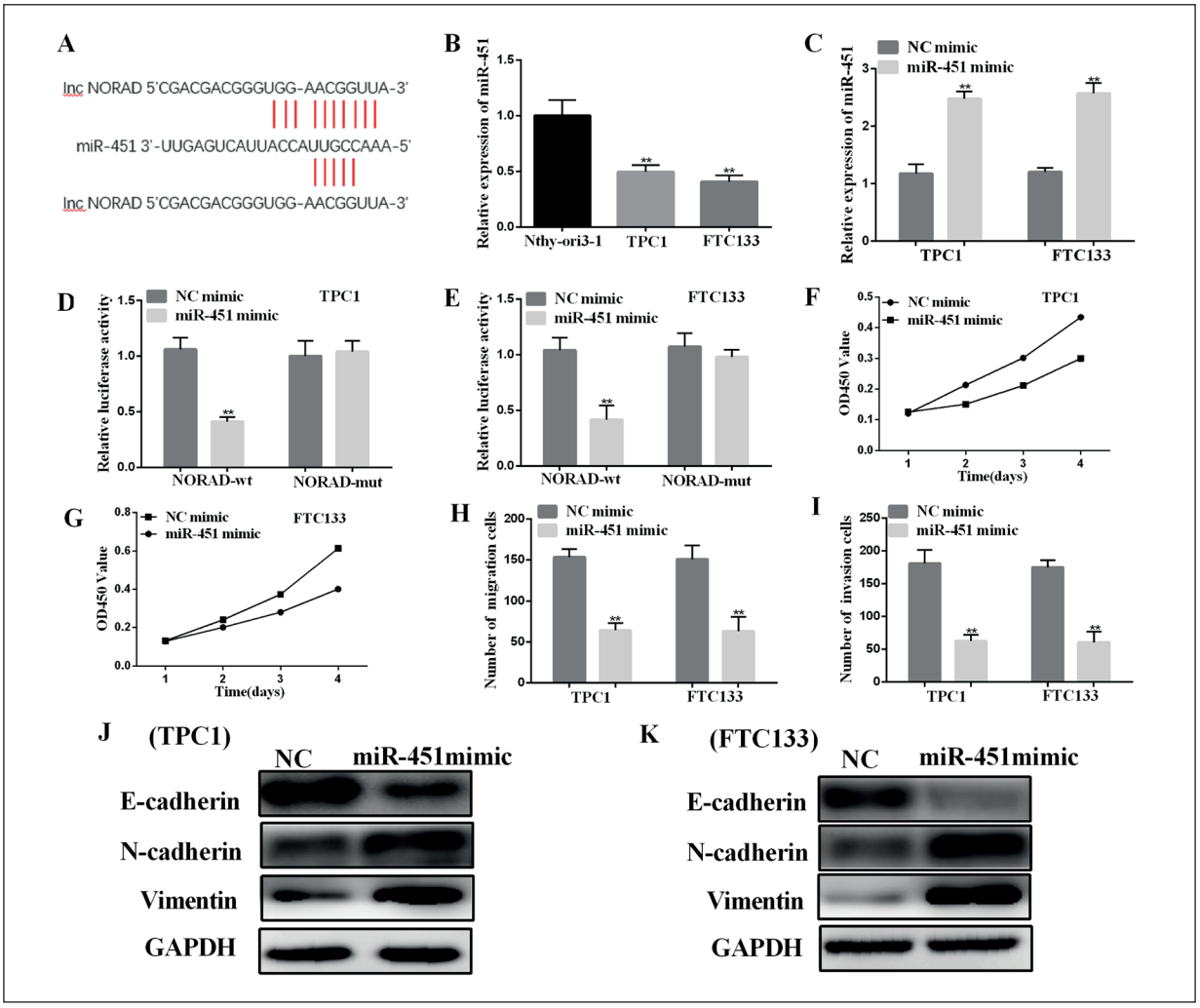

Figure 2. Expressions of miR-451 was examined by RT-qPCR in cells. **A,** Putative binding sites between lncRNA NORAD and miR-451. **B,** Expression of miR-451 in two PTC cells and normal cells. **C,** Expression of miR-451 transfected with miR-451 mimic or NC in PTC cells. **D, E,** The activity of luciferase was determined by dual-luciferase reporter gene assay. **F, G,** CCK-8 checked the effect of miR-451 on the proliferation of TPC1 and FTC133 cells. **H,** MiR-451 mimic suppressing PTC cell migration. **I,** MiR-451 mimic suppressing PTC cell invasion. **J, K,** The protein level of EMT-related marker protein in TPC cell transfected with NC mimic and miR-451 mimic.

vector showed that the Luciferase activity of the combination of miR-451 and IL-6R wild-type IL-6R (IL-6R-wt) decreased significantly, while the Luciferase activity of IL-6R mutant (IL-6R-mut) remained unchanged (Figure 3B and 3C). The expression of IL-6R was detected in different groups NC, sh-NORAD, sh-NORAD+NC and sh-NORAD+miR-451 inhibitor in TPC1 and FTC133 cells. When NORAD was knocked down, the expression of IL-6R was significantly reduced. Interestingly, when NORAD was knocked down and miR-451 mimic was transfected, most of the expression of IL-6R could be saved. So, we think that NORAD-miR-451 can play a role in the cell by regulating the expression level of IL-6R. In order to verify the results of this experiment, we used Western blot to verify the protein expression level of IL-6R. It was consistent with mRNA level. (Figure 3D and 3E). At last, we detected the role of NORAD-miR-451-IL-6R axis in the metastasis of PTC cell lines.

The Role of NORAD-miR-451-IL-6R Axis in the Development of PTC

In order to analyze the regulatory relationship among NORAD, miR-451 and IL-6R, the expression level of IL-6R in PTC cells co-transfected

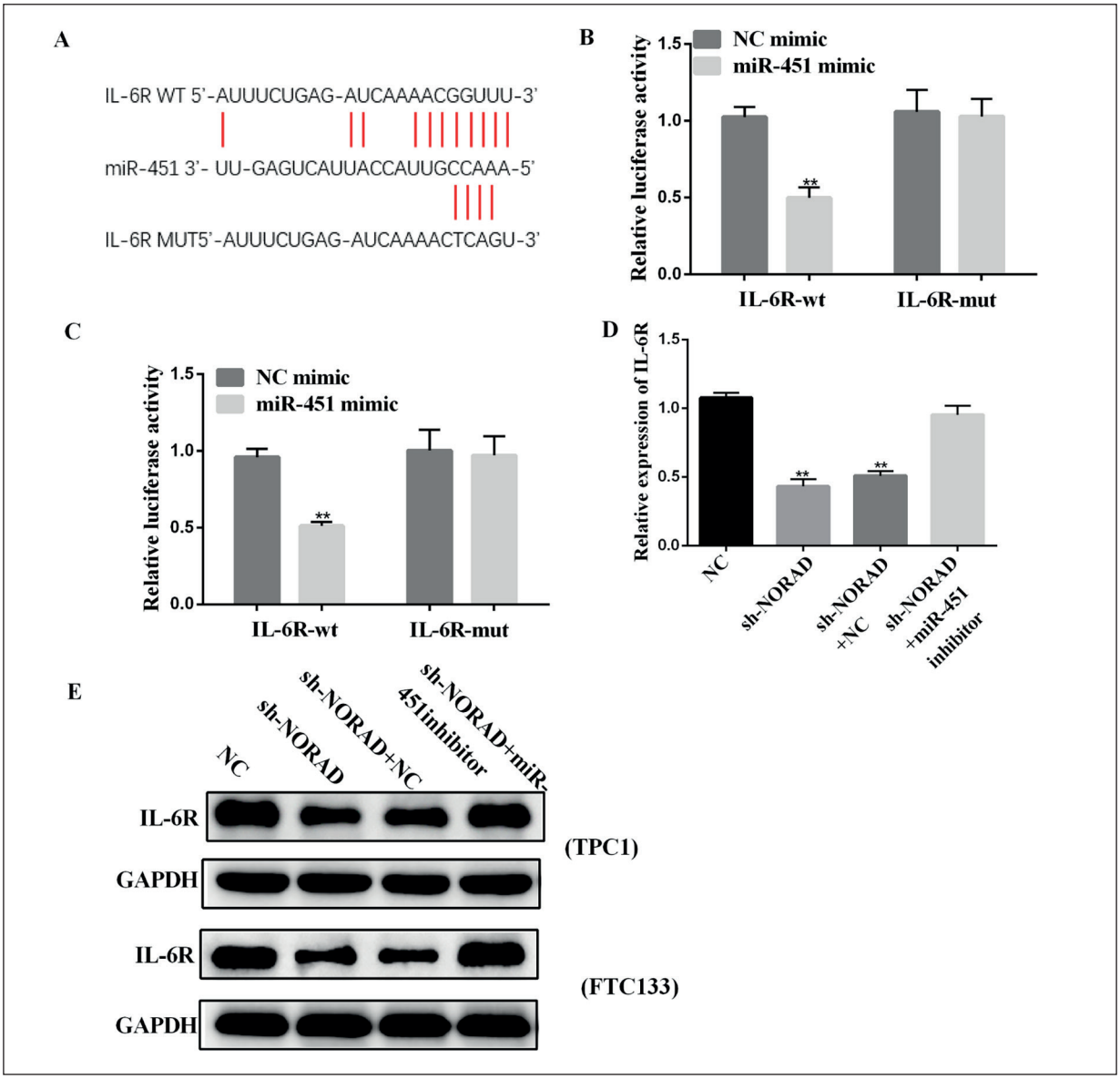

Figure 3. Putative binding sites between miR-451 and IL-6R. **A,** Display of the IL-6R 3'UTR-wt or -mut sequences with miR-451 sequences. **B, C,** Luciferase activity of IL-6R 3'UTR-wt or -mut in TPC1 and FTC133 cells after increasing or decreasing miR-451. **D,** The expression of IL-6R in sh-NC, sh-NORAD, sh-NORAD + NC, sh-NORAD + miR-451 inhibitor. **E,** The protein expression of IL-6R in sh-NC, sh-NORAD, sh-NORAD + NC and sh-NORAD + miR-451 inhibitor in TPC1 and FTC133 cells.

with miR-451 and sh-NORAD was measured at mRNA and protein levels respectively. As shown in Figure 4A and 4B, the migration and invasion of IL-6R level caused by sh-NORAD was reversed by miR-451 inhibitor or pcDNA-IL-6R. In order to determine the biological function of NORAD-miR-451-IL-6R axis in the development of PTC, a rescue test was designed and carried out in TPC1 and FTC133 cells. According to the results of Transwell assay, sh-NORAD induced cell invasion reduction was recovered by transfection with miR-451 inhibitor or pcDNA-IL-6R. At the same time, we used Western blotting to detect the proteins related to invasion and migration of PTC cells. Which is consistent with our expectation, the results at the protein level also show that miR-451 inhibitor or pcDNA-IL-6R reversed the inhibition of sh-NORAD can reverse the invasion and migration of cells inhibited by NORAD (Figure 4C and 4D). Based on the above

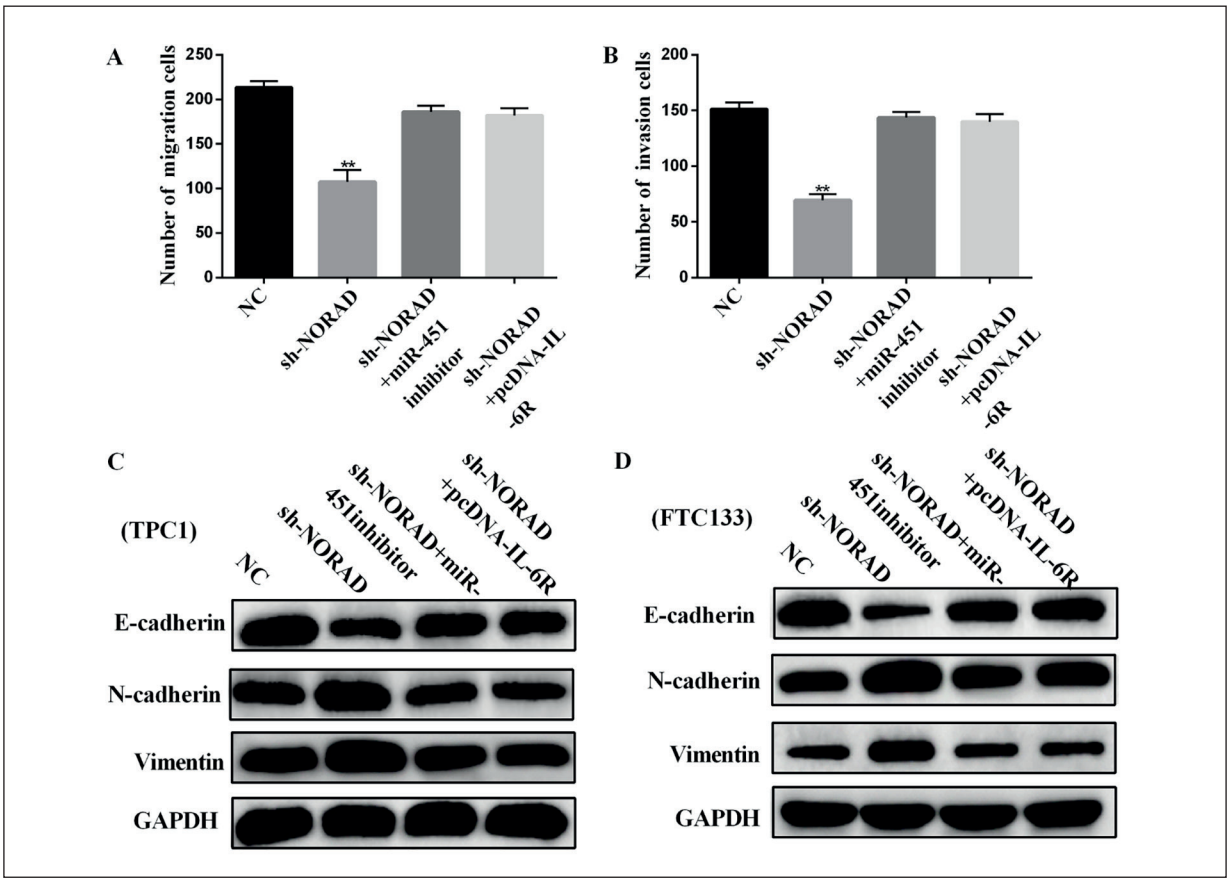

Figure 4. The role of NORAD-miR-451-IL-6R axis in the development of PTC. **A,** TPC1 and FTC133 cells migration after overexpressed IL-6R in NC, sh-NORAD, sh-NORAD and miR-451 inhibitor or sh-NORAD + pcDNA-IL-6 groups. **B,** TPC1 and FTC133 cells invasion after transfected with IL-6R in NC, sh-NORAD, sh-NORAD and miR-451 inhibitor or sh-NORAD + pcDNA-IL-6. **C, D,** The protein level of EMT-related marker protein in TPC1 cell overexpressed IL-6R in NC, sh-NORAD, sh-NORAD and miR-451 inhibitor or sh-NORAD + pcDNA-IL-6.

data, we confirm that NORAD can regulate the occurrence and development of PTC through miR-451/IL-6R axis.

Discussion

According to previous studies, lncRNAs have been shown to play important roles in various human cancers. LncRNA can regulate tumorigenesis and contribute to development of cancer by regulating various molecular mechanisms. Tan et al[28] published in 2019, showed that the expression level of NORAD in lung cancer and breast cancer was down regulated, while the low expression of NORAD in these cancer types was related to lymph node metastasis and poor prognosis. Wang et al[29] showed that NORAD may play an important role in regulating the function of osteosarcoma cells through endogenous competition with hsa-miR-199a-3p. The purpose of this study was to detect the specific mechanism and function of NORAD in PTC. RT-qPCR showed that NORAD was highly expressed in PTC cells compared with normal cells. Therefore, we confirmed the significance of NORAD in PTC. To further study the influence of NORAD on the activity of PTC cells, a loss of function test was carried out in two PTC cell lines. According to our experimental results, we found that inhibition of NORAD expression can inhibit cell proliferation and metastasis. Therefore, NORAD showed carcinogenicity in PTC.

The biological function of lncRNA is closely associated with microRNA and their interactions usually play crucial role in regulating the progression of multiple cancers. Lots of researches have shown that miRNA plays a crucial role in

the tumorigenesis of various cancers, and microRNA plays a crucial role in cell type specific tumorigenesis. MicroRNA expression may lead to the identification of biomarkers for diagnosis and prognosis. Of note, disorder of miRNA is related to the pathogenesis of thyroid cancer. Comprehensive miRNA analysis showed that compared with normal patients, a few miRNAs in PTC patients tissues were out of control[8,30]. Especially, Lee et al[7] and Wang et al[8] proved that the high expression of miR-222 and miR-146b is related to the poor prognosis and lymph node involvement of thyroid cancer. Other team studies have shown that the expression of miR-451 is negatively correlated with the invasiveness of HCC[31], glioblastoma[32] and non-small cell lung cancer[33]. *In vitro* study indicated that over expression of miR-451 inhibited cell proliferation and migration of c-myc mediated by targeting AKT/mTOR signalling pathway. In this study, we retrospectively analyzed the expression of miR-451 in PTC malignant tumors, which was analyzed in two most common specimens. Through bioinformatics analysis and luciferase reporter gene analysis, NORAD target miRNA in PTC cells was found. Our experimental results show that NORAD uses miR-451 as a target to regulate the progress of GC. On this basis, we analyzed and identified the negative correlation between miR-451 and NORAD. Therefore, we detected that NORAD can negatively regulate the invasion and migration of PTC cells by binding to miR-451. The results showed that the expression level of miR-451 in PTC cell lines was lower than that in normal cells. It was also found that IL-6R was the target gene of miR-451. IL-6R is positively regulated by NORAD in PTC cells. At last, rescue experiments were performed in TPC1 and FTC133 cells to verify the role of NORAD-miR-451-IL-6R in PTC. The novelty of this study was that this is the first report that NORAD showed carcinogenicity in PTC by regulating miR-451/IL-6R pathway, suggesting that it may provide novel potential therapeutic approaches for PTC.

Conclusions

Briefly, our results indicated that the cell migration and invasion were inhibited by knockdown of lncRNA NORAD or overexpression of miR-451, suggesting that the axis of lncRNA NORAD -miR-451- IL-6R was involved in the development of PTC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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