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LncRNA NORAD stimulates proliferation and migration of renal cancer *via* activating the miR-144-3p/MYCN axis

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Abstract. – OBJECTIVE: The purpose of this study was to clarify the potential role of long non-coding RNA (lncRNA) NORAD in the development of renal cancer.

PATIENTS AND METHODS: Expression levels of NORAD, miR-144-3p, and MYCN in renal cancer tissues and cell lines were detected. After overexpression of NORAD, proliferative and migratory changes in ACHN and A498 cells were evaluated by Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. Thereafter, Luciferase assay was conducted to determine the interaction in the NORAD/miR-144-3p/MYCN axis. Besides, its biological function in influencing phenotype changes of renal cancer cells was finally demonstrated by rescue experiments.

RESULTS: The results manifested that NORAD and MYCN were upregulated, while miR-144-3p was downregulated in renal cancer tissues. Overexpression of NORAD stimulated proliferative and migratory potentials in ACHN and A498 cells, which were partially abolished by co-overexpression of miR-144-3p. Moreover, NORAD/miR-144-3p/MYCN axis was found to be responsible for stimulating the malignant development of renal cancer.

CONCLUSIONS: LncRNA NORAD stimulates proliferative and migratory potentials in renal cancer by sponging miR-144-3p to upregulate MYCN.

Key Words:
Renal cancer, NORAD, MiR-144-3p, MYCN, Proliferative and migratory potentials.

Introduction

Renal cancer, also known as renal cell carcinoma (RCC), is a malignant tumor originating in the renal urothelial, which accounts for about 4% of adult malignant tumors, and 90-95% of renal tumors[1]. With the advancement of imaging and surgical technologies, the 5-year survival of renal cancer has been greatly improved[2]. However, the cause of renal cancer remains largely unknown. Traditional therapeutic methods, such as chemotherapy and radiotherapy, are not ideal in renal cancer patients, and a small number of renal cancer patients are sensitive to immunotherapy[3], so it is necessary to clarify the molecular mechanism of renal cancer development[4,5].

Long non-coding RNAs (lncRNAs) are extensively involved in human diseases[6,7]. In particular, they are vital regulators in tumor diseases by mediating tumor cell behaviors[8,9]. Abnormally expressed lncRNAs in renal cancer are closely linked to disease development and prognosis[10-12]. For example, HOTAIR is upregulated in RCC tissues and cell lines. The knockdown of HOTAIR effectively suppresses proliferative, migratory, and invasive capacities, but induces apoptosis in RCC[13,14]. MALAT1 is significantly upregulated in RCC, and its level is linked to tumor size, tumor staging, and lymphatic metastasis in RCC patients[15].

In this paper, it was found that NORAD was upregulated in renal cancer tissues, indicating a potential role of NORAD in the development of renal cancer. Through biological analysis, the target gene of NORAD was identified. The findings of this study may provide a new direction in the clinical treatment of renal cancer.

Patients and Methods

Sample Collection

Renal cancer tissues and paired normal ones were surgically resected from 36 renal cancer patients treated in Tengzhou Central People's Hospital from May 2016 to December 2018 and stored at -80°C. None of included patients were treated with anti-tumor strategy before surgery.

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This study received the approval by the Ethics Committee of Tengzhou Central People's Hospital and was conducted after informed consent was obtained from each subject.

Cell Culture and Transfection

Human renal cancer cell lines (786-O, A498, ACHN, and OS-RC-2) and normal renal tubular epithelial cell line (HK-2) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Transfection plasmids were provided by Genechem, Co., Ltd. (Shanghai, China). Then, the cells were cultured to 60-70% confluence, and transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in the serum-free medium. 6 hours later, the original medium was replaced with complete medium.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated into 96-well plates with 2×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added in each well. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Transwell Assay

100 μL of suspension (1×10^5 cells/mL) was inoculated in the upper layer of a transwell chamber (Millipore, Billerica, MA, USA), which was inserted in a 24-well plate, with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, the bottom cells reacted with 15-min methanol, 20-min crystal violet, and were captured using a microscope. Finally, migratory cells were counted in 10 random fields per sample (magnification: 200×).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs in cells and tissues were extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) by the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) and, which were sent for qRT-PCR. Relative level of the target was calculated using $2^{-\Delta\Delta Ct}$ method. The primer sequences are as follows. NORAD: forward: 5'-AGCGAAGTCCCGAACGACGA-3' and reverse: 5'-TGGGCATTTCCAACGGGCCAA-3', MYCN: forward: 5'-ACCCGGACGAAGATGACTTCT-3' and reverse: 5'-CAGCTCGTTCTCAAGCAGCAT-3', glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-TCAAGATCATCAGCAATGCC-3' and reverse: 5'-CGATACCAAAGTTGTCATGGA-3', MiR-144-3p: forward: 5'-GGGAGATCAGAAGGTGATT-3' and reverse: 5'-GTGCAGGGTCCGAGGT-3', U6: forward: 5'-ATACAGAGAAAGTTAGCACGG-3' and reverse: 5'-GGAATGCTTCAAAGAGTTGTG-3'.

Luciferase Assay

Luciferase vectors of NORAD and MYCN were constructed based on the predicted binding sequences in the promoter regions. The cells were co-transfected with NC/miR-144-3p mimics and wild-type/mutant vector for 48 h, and then, lysed for measuring Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation (SD). The differences between the two groups were analyzed by the *t*-test. The relationship between the expression levels of two genes was analyzed by Pearson correlation test. $p<0.05$ suggested that the difference was statistically significant.

Results

Expression Levels of NORAD and MiR-144-3p in Renal Cancer

NORAD was found to be upregulated in renal cancer tissues compared with normal ones (Figure 1A). Its level in renal cancer cell lines was identically highly expressed (Figure 1B). On the contrary, miR-144-3p was downregulated in renal cancer tissues (Figure 1C), and negatively correlated with NORAD level (Figure 1D).

NORAD Stimulated Proliferative and Migratory Potentials In Renal Cancer

Firstly, the transfection efficacy of pcDNA-NORAD in ACHN and A498 cells was tested (Figure 2A, 2C). After overexpression of NORAD, cell viability was markedly enhanced in renal cancer cells (Figure 2B, 2D). Transwell assay showed

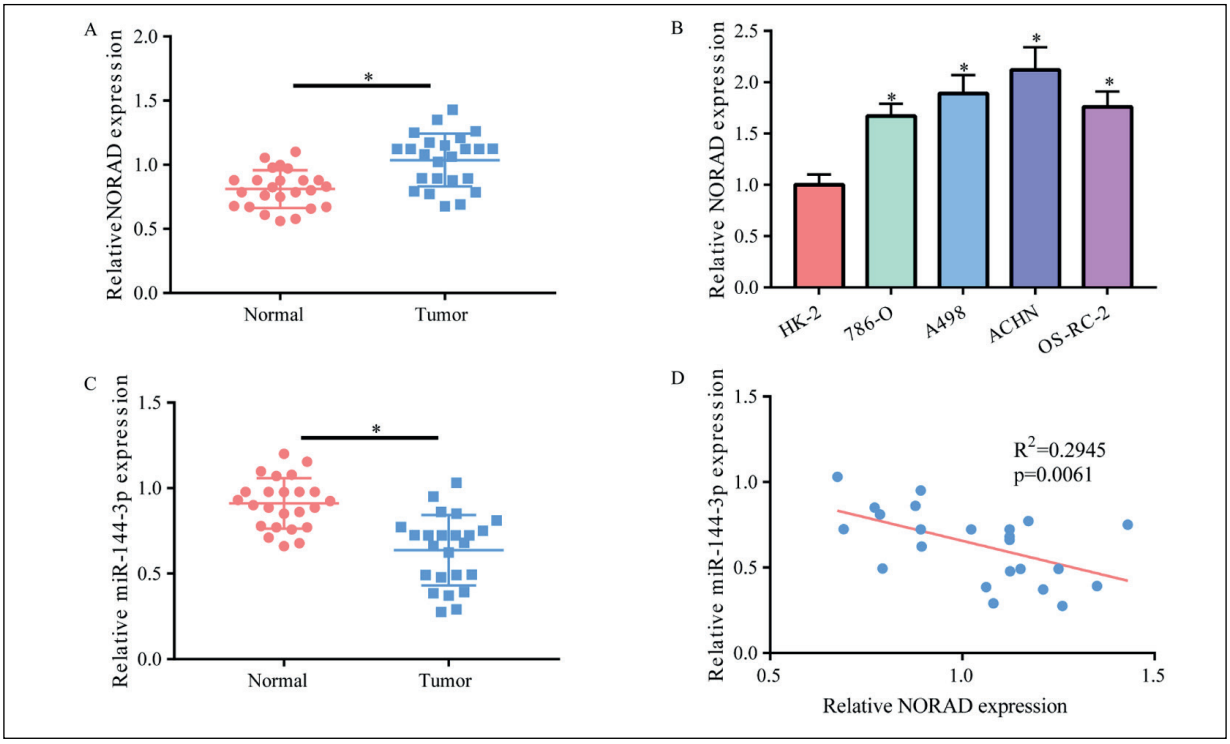

Figure 1. Expression levels of NORAD and miR-144-3p in renal cancer. **A,** NORAD expressions in 32 paired renal cancer tissues and normal tissues. **B,** NORAD expressions in renal cancer cell lines. **C,** MiR-144-3p expressions in 32 paired renal cancer tissues and normal tissues. **D,** A negative correlation between expression levels of NORAD and miR-144-3p in renal cancer tissues. *p<0.05.

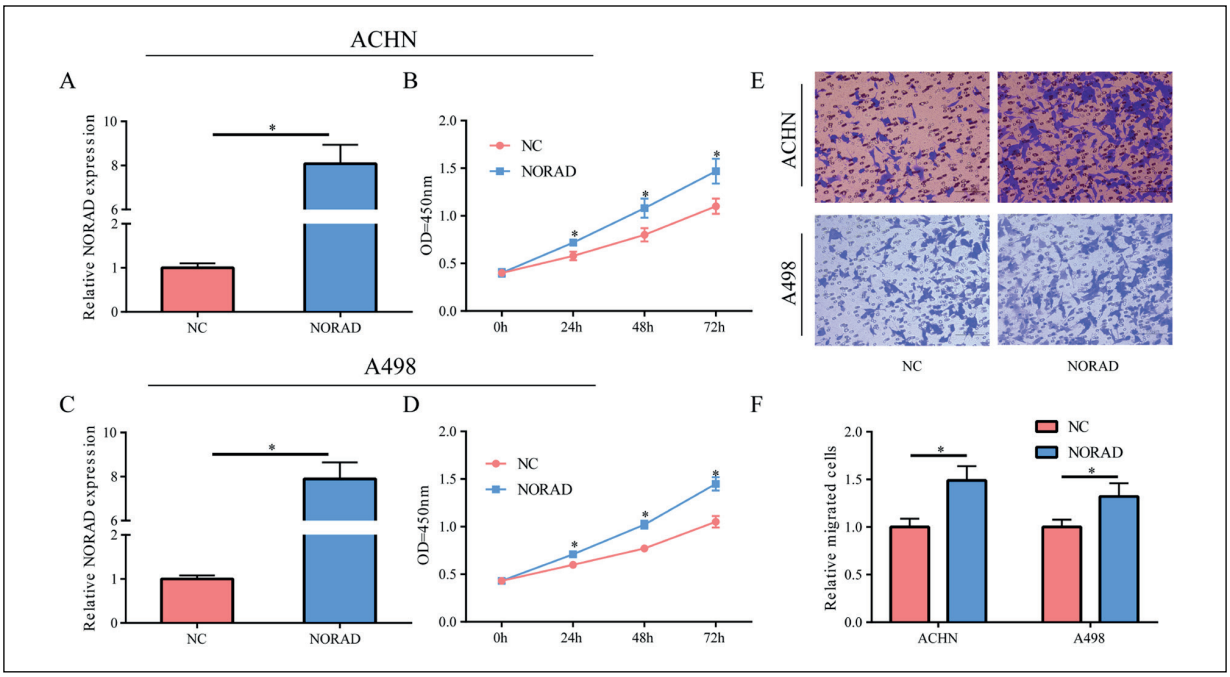

Figure 2. NORAD stimulates proliferative and migratory potentials in renal cancer. **A,** NORAD expression in ACHN cells transfected with NC or pcDNA-NORAD. **B,** Viability in ACHN cells transfected with NC or pcDNA-NORAD. **C,** NORAD expression in A498 cells transfected with NC or pcDNA-NORAD. **D,** Viability in A498 cells transfected with NC or pcDNA-NORAD. **E, F,** Migration in ACHN and A498 cells transfected with NC or pcDNA-NORAD (magnification: 400×) *p<0.05.

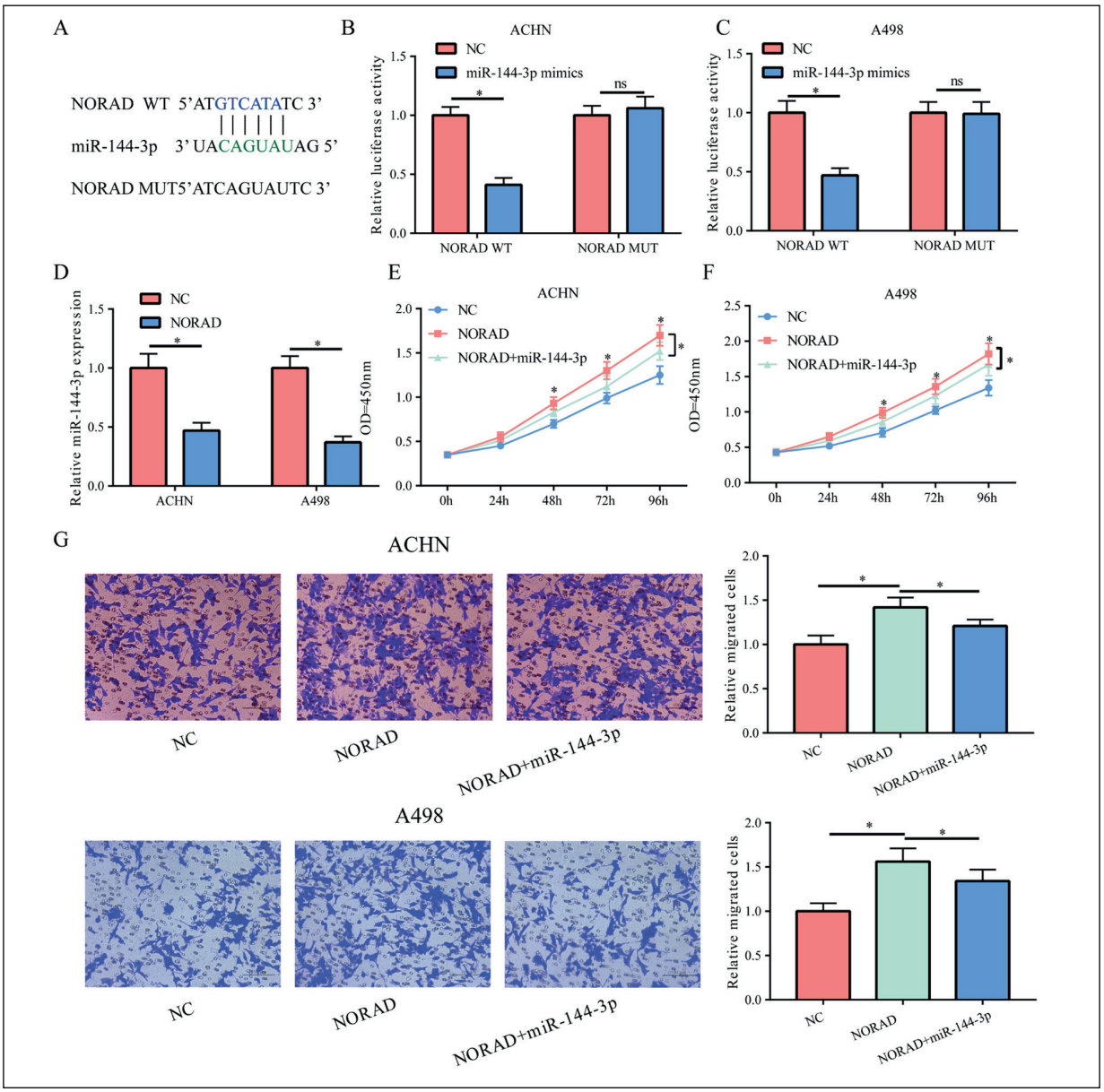

Figure 3. MiR-144-3p is the target gene of NORAD. **A,** Binding sequences in the 3'UTR of NORAD and miR-144-3p. **B, C,** Luciferase activity in ACHN (**B**) and A498 cells (**C**) co-transfected with NC/miR-144-3p mimics and NORAD-WT/NORAD-MUT. **D,** MiR-144-3p expression in ACHN and A498 cells transfected with NC or pcDNA-NORAD. **E, F,** Viability in ACHN (**E**) and A498 cells (**F**) transfected with NC, pcDNA-NORAD or pcDNA-NORAD+miR-144-3p mimics. **G,** Migration in ACHN and A498 cells transfected with NC, pcDNA-NORAD or pcDNA-NORAD+miR-144-3p mimics (magnification: 400×) *p<0.05, ns: no significant difference.

increased migratory potential in ACHN and A498 cells overexpressing NORAD (Figure 2E, 2F).

MiR-144-3p Was the Target Gene of NORAD

The binding sequences in the 3'UTR of NORAD and miR-144-3p were predicted by bioinformatics analysis (Figure 3A). Luciferase vectors of NORAD were constructed based on the binding sequences. The results showed that the overexpression of miR-144-3p markedly decreased the Luciferase activity in wild-type NORAD vector, confirming the binding between NORAD and miR-144-3p (Figure 3B, 3C). It is shown that miR-144-3p was downregulated in renal cancer cells overexpressing NORAD (Figure 3D). Notably, enhanced viability in ACHN and A498 cells overexpressing NORAD was partially

reduced by overexpression of miR-144-3p (Figure 3E, 3F). Besides, increased migratory cell number after overexpression of NORAD in renal cancer cells, as expected, was partially reversed by overexpression of miR-144-3p (Figure 3G).

MYCN Was the Target Gene of MiR-144-3p

In a similar way, MYCN was proved to be the target gene of miR-144-3p (Figure 4A-4C). Transfection efficacy of miR-144-3p mimics was tested in ACHN and A498 cells (Figure 4D, E). MYCN level was markedly downregulated in renal cancer cells overexpressing miR-144-3p (Figure 4F, 4G). In renal cancer tissues, MYCN was upregulated and negatively correlated to miR-144-3p level (Figure 4H, 4I).

Discussion

NORAD is identified to participate in DNA repair[16]. In addition, NORAD is considered as an oncogene that is upregulated in many types of solid tumors[17]. The findings of this study identically showed highly expressed NORAD in renal cancer tissues and cell lines. The overexpression of NORAD markedly stimulated renal cancer cells to proliferate and migrate. It is suggested that NORAD was a vital regulator in the malignant development of renal cancer.

By sponging corresponding miRNAs, lncRNAs serve as ceRNAs and thus regulate life activities[18,19]. The complicated post-transcriptional network lncRNA-miRNA-mRNA is formed by competitively binding to miRNA response

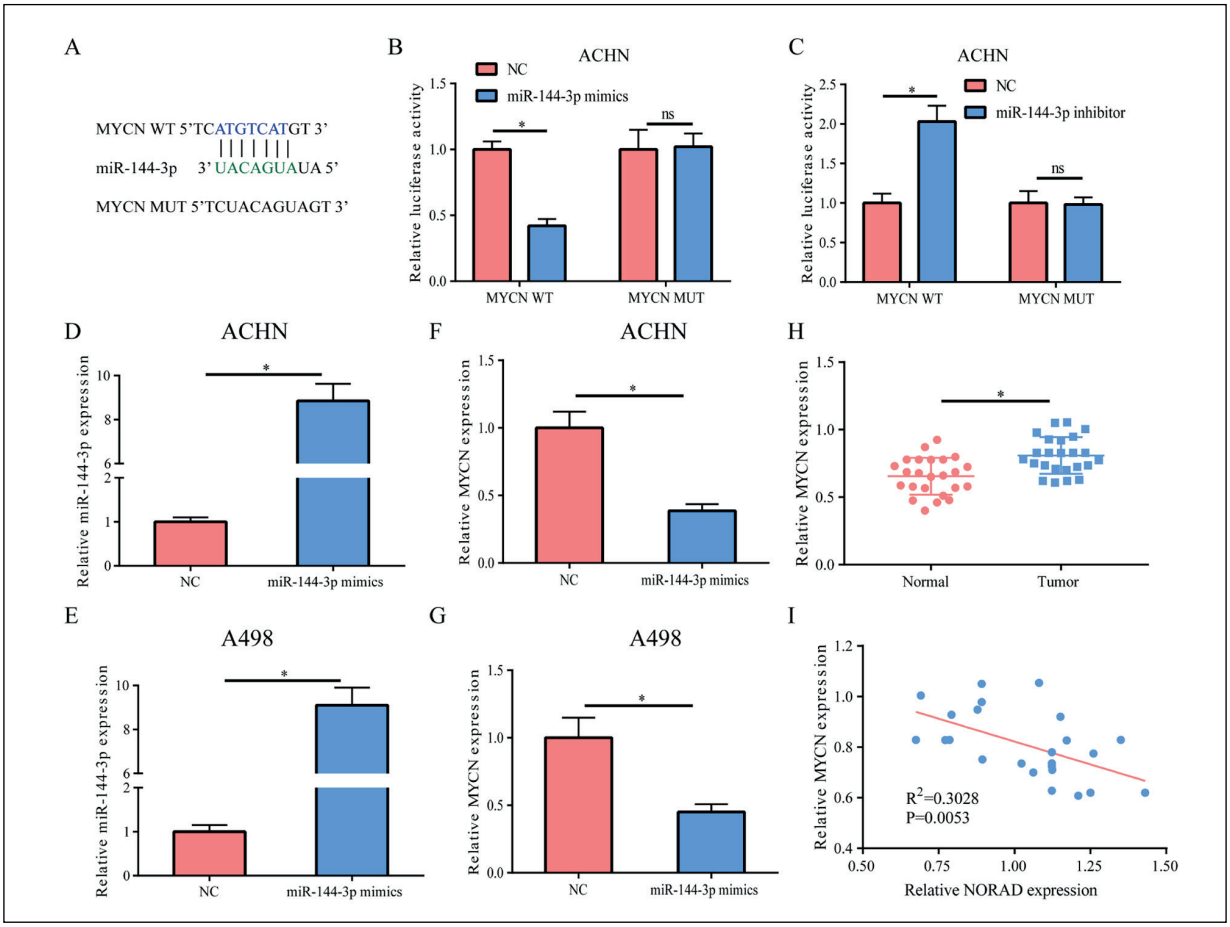

Figure 4. MYCN is the target gene of miR-144-3p. **A,** Binding sequences in the 3'UTR of miR-144-3p and MYCN. **B, C,** Luciferase activity in ACHN (**B**) and A498 cells (**C**) co-transfected with NC/miR-144-3p mimics and MYCN-WT/MYCN-MUT. **D, E,** MiR-144-3p expression in ACHN (**D**) and A498 cells (**E**) transfected with NC or miR-144-3p mimics. **F, G,** MYCN expression in ACHN (**F**) and A498 cells (**G**) transfected with NC or miR-144-3p mimics. **H,** MYCN expression in normal and tumor tissues. **I,** A negative correlation between expression levels of miR-144-3p and MYCN in renal cancer tissues. *p<0.05, ns: no significant difference.

elements, which is of significance in tumor development[20]. Through bioinformatics analysis and Luciferase assay, miR-144-3p was verified to be the target gene of NORAD. NORAD was able to negatively regulate miR-144-3p level and more importantly, miR-144-3p could abolish the regulatory effects of NORAD on proliferative and migratory potentials in renal cancer cells.

The MYC oncogene family is widely involved in almost every aspect of tumor cell behavior. As a family member, MYCN exists in haploid state at the end of the short arm of chromosome 2 in normal adult somatic cells. MYCN aims to shorten cell growth cycle, promote cell proliferation and inhibit cell differentiation, apoptosis or programmed death[21]. It is reported that the knockdown of MYCN suppresses growth and proliferation of blastoma[22]. By regulating HES1 level, MYCN mediates apoptosis and drug resistance in small cell lung cancer[23].

As analyzed, MYCN was detected to be the target gene of miR-144-3p. Compared with normal tissues, MYCN was upregulated in renal cancer tissues. A negative correlation was identified between the expression levels of MYCN and miR-144-3p. Collectively, it was demonstrated that NORAD competitively bound to MYCN with miR-144-3p, thus exerting the carcinogenic role in renal cancer.

Conclusions

In brief, lncRNA NORAD stimulates proliferative and migratory potentials in renal cancer by sponging miR-144-3p to upregulate MYCN. We explored the pro-cancer role of NORAD in renal cancer for the first time, which not only enriched the theoretical content of the development of renal cancer, but also provided a new therapeutic target for the prevention and treatment of renal cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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