

## Original Article

# Long non-coding RNA NEAT1 promotes human clear cell kidney carcinoma progression through negative regulation of miR-129-5p

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**Abstract:** Increasing evidence showed that long non-coding RNAs (lncRNAs) play important roles in tumor development and progression including clear cell kidney carcinoma (KIRC). However, the potential biological roles and regulatory mechanisms of lncRNANEAT1 (nuclear enriched abundant transcript 1) involved in KIRC are still unclear. In the present study, our results showed that NEAT1 expression was significantly increased in KIRC tissues and cell lines compared to adjacent non-tumor tissues and HK-2 cell line, high NEAT1 expression was associated with higher histological grade, lymph node metastasis, distant metastasis and poor overall survival of KIRC patients. In addition, functional experiments revealed that NEAT1 suppression inhibited cell proliferation, invasion and arrested cell cycle in G0/G1 phase *in vitro*. Furthermore, we found that miR-129-5p could act as a target of NEAT1 and the inhibition of KIRC progression induced by NEAT1 suppression required the activity of miR-129-5p. Overall, our results suggested that lncRNANEAT1 might promote progression of KIRC probably by negative modulation of miR-129-5p, which providing a new insight about the carcinogenesis mechanism of KIRC.

**Keywords:** Clear cell kidney carcinoma, long non-coding RNAs, NEAT1, miR-129-5p

## Introduction

Renal cell carcinoma (RCC) is the most common malignant neoplasm of the kidney, which accounts for 2-3% of all adult malignancies [1]. The incidence and mortality rate of RCC has increased in recent years, especially in young patients and those with high-grade disease [2]. Clear cell kidney carcinoma (KIRC) is the most common and most aggressive histologic subtype of RCC, which accounts for approximately 75%-80% of RCC [3]. Radical nephrectomy is the principal and most effective treatment for RCC. However, because of the lack of early detection and prognostic markers for kidney carcinoma, approximately 25%-30% of patients have already developed metastases at the time of diagnosis with poor prognosis [4]. Therefore, understanding of the molecular mechanisms of KIRC and to identify reliable biomarkers and novel therapeutic targets for KIRC are instantly required.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs which length is more than

200 nucleotides (nt) with no protein-coding capacity [5]. lncRNAs are increasingly recognized to play major regulatory roles in diverse biological processes and diseases by regulating gene expression at the chromatin organization, transcriptional and post-transcriptional levels including cancers [6, 7]. Recent studies showed that aberrant expression of lncRNAs were involved in RCC progression. For example, Zhang et al found that upregulation of lncRNA MALAT1 correlated with tumor progression and poor prognosis in clear cell renal cell carcinoma [8]. Xiong et al showed that high expression of the lncRNA HEIRCC promoted renal cell carcinoma metastasis by inducing epithelial mesenchymal transition [9]. Zhai et al showed that lncRNA SARCC suppressed VHL-mutant RCC cell proliferation yet promotes VHL-normal RCC cell proliferation via modulating androgen receptor/HIF-2 $\alpha$ /C-MYC axis under hypoxia [10]. Those studies suggested that lncRNAs play important roles in RCC's progression. However, the expression and function of lncRNANEAT1 in KIRC pathogenesis were still largely unclear.

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In the present study, our data showed that NEAT1 existed high fold change in KIRC among TCGA Data Portal. We also confirmed that the expression of NEAT1 was significantly upregulated in KIRC tissues and high NEAT1 expression was associated with advanced clinical features and poor overall survival. Moreover, we showed that NEAT1 inhibition suppressed renal cancer cell proliferation, invasion and arrested cell cycle in G0/G1 phase in vitro. In addition, we suggested that NEAT1 function as an oncogene by negative regulation of miR-129-5p in KIRC progression.

### Materials and method

#### *Patients and specimens*

From 2011 to 2012, 63 pairs of RCC tissue specimens and the corresponding adjacent non-tumor tissues were collected from renal cancer patients, who had undergone radical nephrectomy at the Department of Urologic Surgery, The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology. All the patients were diagnosed as clear cell kidney carcinoma by histopathological examination. The research was approved by the ethical committee of The First Affiliated Hospital, and College of Clinical Medicine of Henan University of Science and Technology, and written informed consent was provided by each patient before surgery.

#### *Cell lines and cell culture*

The human renal cancer cell lines 786-O, ACHN, Caki-1, and Caki-2 were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Immortalized normal human proximal tubule epithelial cell line HK-2 was purchased from the American Type Culture Collection (ATCC, USA). The cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in RPMI-1640 (Gibco) supplement with 10% FBS (Gibco) and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin).

#### *Total RNA extraction and quantitative real-time PCR*

Total RNA was extracted from tissues or cells using the RNAi so Plus (Takara) according to the manufacturers' protocol. RNA was reversed tr-

anscribed into cDNAs using the Primer-Script one step RT-PCR kit (TaKaRa). The cDNA template was amplified by real-time RT-PCR using the SYBR Premix Dimmer Eraser kit (TaKaRa). Gene expression in each sample was normalized to GAPDH expression. Real-time-PCR reactions were performed by the ABI7500 system. The relative expression fold change of mRNAs was calculated by the 2<sup>-ΔΔCt</sup> method. The primer pairs were listed as follows: 5'-TGGCTAGCTC-AGGGCTTCAG-3' (Forward) and 5'-TCTCCTTGC-CAAGCTTCCTT-3' (Reverse) for NEAT1, 5'-CATC-AAGAAGGTGGTGAAGCAG-3' (Forward) and 5'-CGTCAAAGGTGGAGGAGTGG -3' (Reverse) for GAPDH.

#### *Cell transfection*

Specific siRNAs targeting NEAT1 and a scrambled negative control (si-NC) were synthesized by Genepharma Co., Ltd. (Shanghai, China). Cells were seeded onto the plate and reached 40% confluence on the second day, the siRNAs were transfected into the cells using Lipofectamine 2000 reagents (Life Technologies) according to the manufacturer's instruction. The efficiency of RNA interference was determined by qRT-PCR after 48 h transfection.

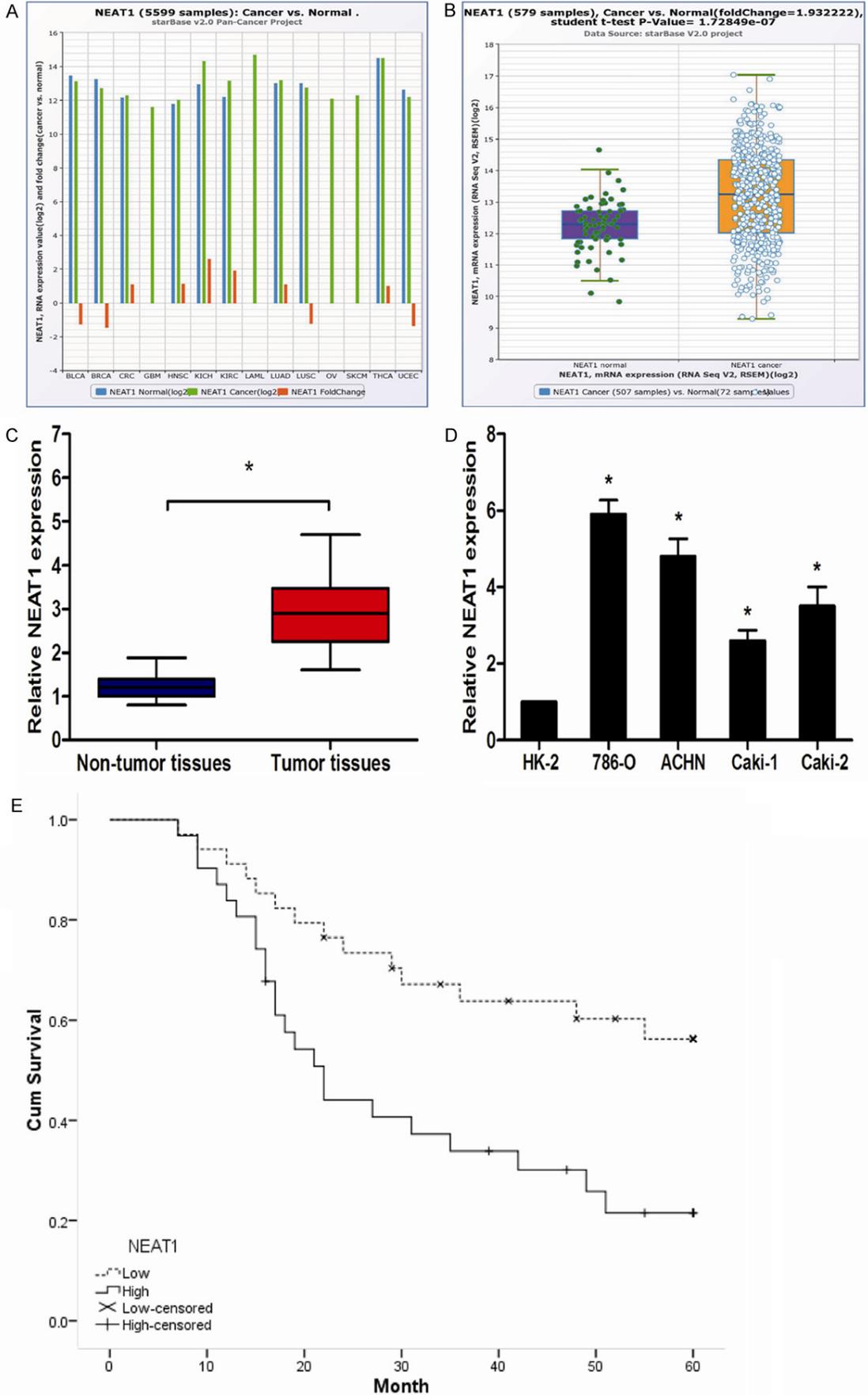
#### *Cell proliferation assay*

Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8, Beyotime) assay according to the manufacturer's protocol. Briefly, a total of approximately 10000 cells were plated in 96-well plates, treated with 10 µl/well of CCK-8 solution during the last 4 h of culture, and the cell proliferation curves were plotted using the 450 nm absorbance at each time point. All experiments were performed in triplicate.

#### *Cell cycle assay*

Cells were harvested after siRNAs transfection for 48 h, then washed twice with cold PBS and fixed in 70% alcohol at -20°C overnight. After fixation, cells were stained with propidium iodide (PI) at a final concentration of 50 ng/ml in the dark tubes at 37°C for 30 min to detect cell cycle. The stained cells were then analyzed by using flowcytometry BD FACS Canto II (BD Biosciences). All experiments were performed in triplicate.

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**Figure 1.** Relative expression of NEAT1 in KIRC tissues and its clinical significance. A. Relative expression of NEAT1 among Pan-Cancer including 14 cancer types from TCGA Data Portal from starBASE v2.0. B. Relative expression of NEAT1 in clear cell kidney carcinoma (KIRC) or normal from TCGA Data Portal. C. Relative expression of NEAT1 in 63 pairs of KIRC tissues and adjacent non-tumor tissues. NEAT1 expression was determined by qRT-PCR and normalized to GAPDH expression. D. Relative expression of NEAT1 in renal cancer cell lines (786-O, ACHN, Caki-1, and Caki-2) and normal human proximal tubule epithelial cell line HK-2. E. Kaplan-Meier analyses of the associations between NEAT1 expression and overall survival of patients with KIRC (log-rank test;  $P=0.005$ ). \* $P<0.05$ .

**Table 1.** Association between NEAT1 expression and clinicopathological features in KIRC patients

Parameters	Group	Total	NEAT1 expression		P value
			High	Low	
Gender	Male	28	15	13	0.693
	Female	35	17	18	
Age (years)	<60	33	19	14	0.259
	≥60	30	13	17	
Tumor size (cm)	<4 cm	26	16	10	0.153
	≥4 cm	37	16	21	
Histological grade	G1-G2	35	13	22	0.015
	G3-G4	28	19	9	
Tumor stage	T1-T2	36	15	21	0.094
	T3-T4	27	17	10	
Lymph nodes metastasis	Negative	47	19	28	0.005
	Positive	16	13	3	
Distant metastasis	Negative	48	21	27	0.045
	Positive	15	11	4	

### Transwell invasion assay

To determine cell invasion, Matrigel-coated invasion chambers (Invitrogen) were used according to the manufacturer's protocol. Cells transfected with siRNA were suspended in 200  $\mu$ l serum-free RPMI-1640 medium and seeded on the upper chamber. The lower chamber was filled with 10% FBS as the chemoattractant. After 48 h, cells on the upper side of the membrane were wiped off, cells on the lower side of the membrane were fixed and stained with crystal violet solution. The cells under the microscopic fields in each chamber were photographed and counted, values were expressed as fold induction. All experiments were performed in triplicate.

### Dual-luciferase reporter assay

Human HEK293T cells were co-transfected with pmirGLO-NC, pmirGLO-NEAT1-wt or pmirGLO-NEAT1-mut (Sangon biotech). 2 ng of pRL-TK (Promega) were also co-transfected with

miR-129-5p mimics or miR-NC into HEK293T cells by using Lipofectamine 2000 (Invitrogen). The relative luciferase activity was normalized to Renilla luciferase activity 48 h after transfection. All experiments were performed in triplicate.

### Statistical analysis

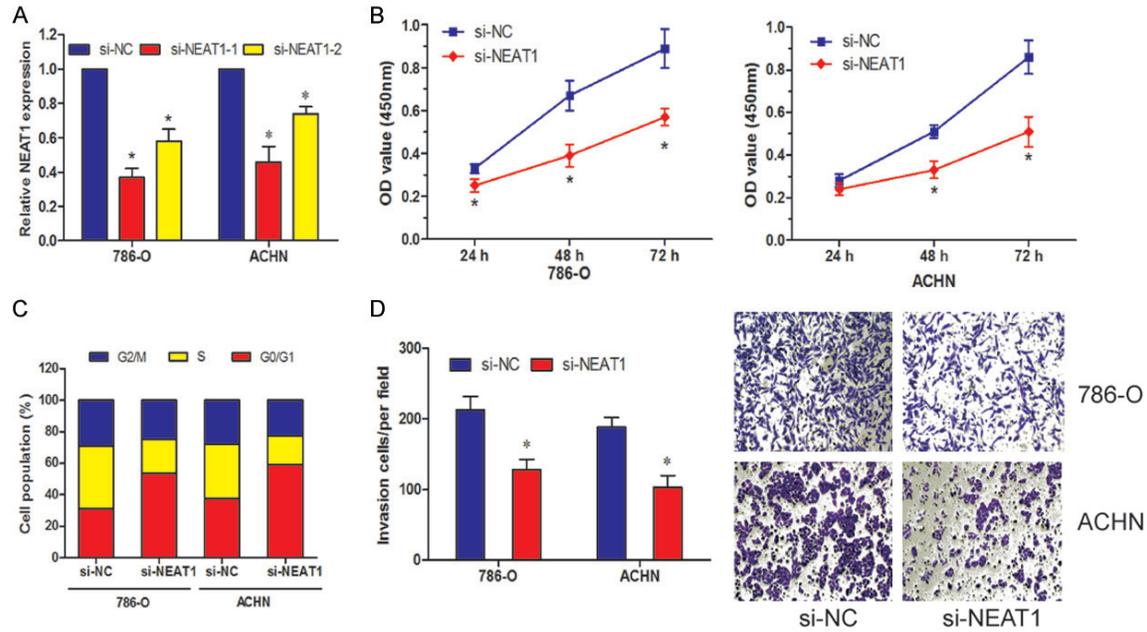
All statistical analyses were performed using SPSS version 18.0 software. The data are shown as the mean  $\pm$  SD from at least three independent experiments. The differences between groups were analyzed using Two-tail Student's t-test and ANOVA. Correlation between NEAT1 expression and miR-129-5p expression in KIRC tissues was analyzed using Pearson's correlation coefficient. A  $P$  value less than 0.05 was considered to be statistically significant.

## Results

### LncRNA NEAT1 was elevated in clear cell kidney carcinoma (KIRC)

To explore whether lncRNA NEAT1 was involved in the progression of KIRC, we determined NEAT1 expression levels in TCGA Data Portal from starBASE v2.0. We found that NEAT1 was significantly increased in tumor tissues than in normal tissues in chromophobe renal cell carcinoma (KICH) and clear cell kidney carcinoma (KIRC) (**Figure 1A**). Relative expression levels of NEAT1 was increased in cancer tissues compared to normal counterparts in KIRC (**Figure 1B**;  $P<0.05$ ). To further support this conclusion, we explored the relative expression levels of NEAT1 in renal cancer tissues and cell lines. qRT-PCR showed that NEAT1 expression was significantly higher in KIRC tissues compared with that in adjacent non-tumor tissues (**Figure 1C**;  $P<0.05$ ). In addition, our data showed that NEAT1 was upexpressed in RCC cell lines (786-O, ACHN, Caki-1 and Caki-2) in comparison to

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**Figure 2.** Knockdown of NEAT1 inhibited renal cancer cells proliferation and invasion. A. Relative expression of NEAT1 in 786-O and ACHN cells was reduced by si-NEAT1 compared to si-NC group. B. CCK-8 assay showed NEAT1 depletion suppressed the proliferation ability of 786-O and ACHN cells. C. Flow cytometry assay showed NEAT1 depletion arrested 786-O and ACHN cells in G1/G0 phase. D. Transwell invasion assay showed that NEAT1 suppression reduced 786-O and ACHN cells invasion ability. \* $P < 0.05$ .

that of in HK-2 cell lines (**Figure 1D**;  $P < 0.05$ ). Taken together, these data indicated that NEAT1 might play a critical role in KIRC development.

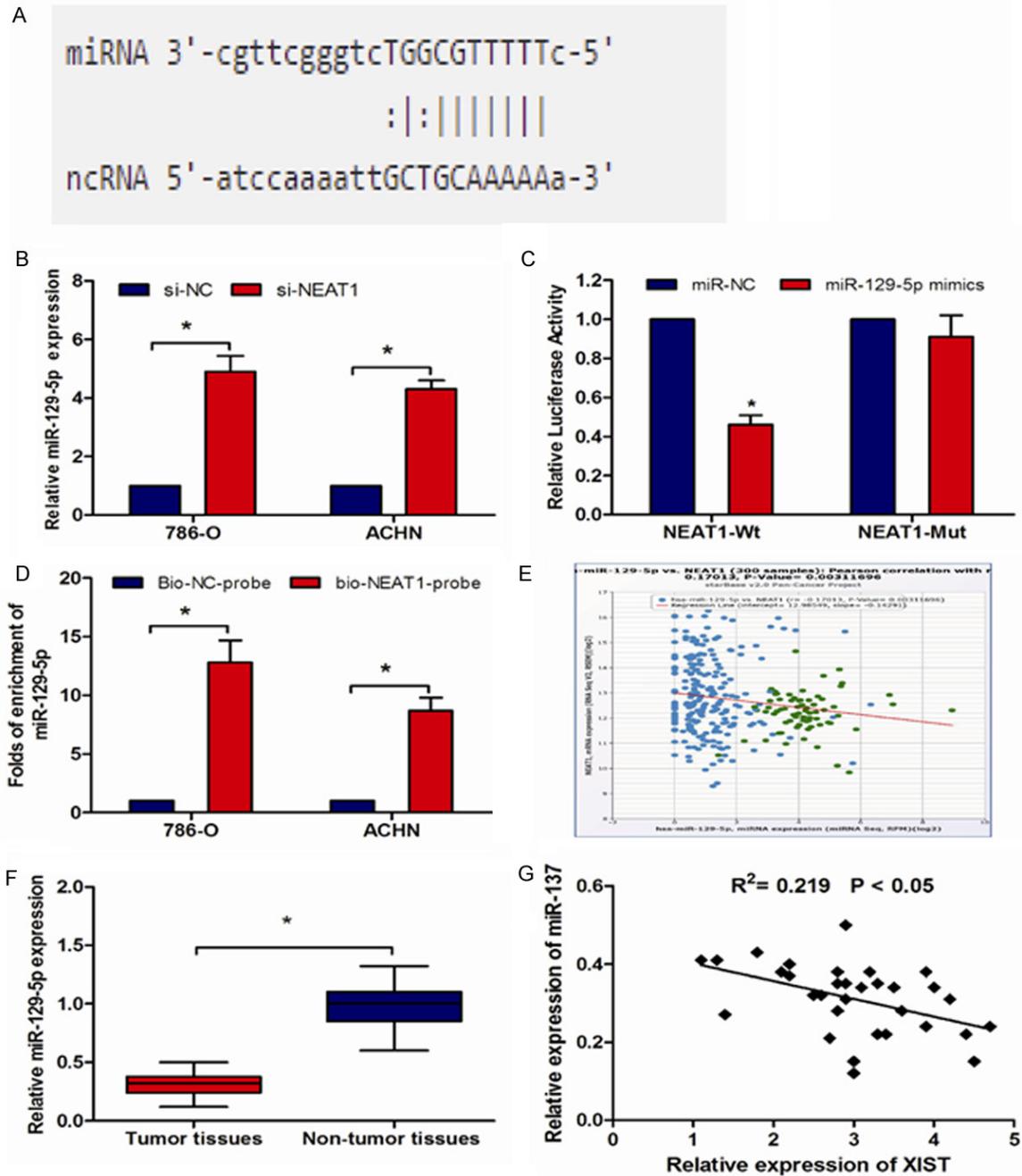
### Correlations between lncRNA NEAT1 expression and clinicopathological features

According to the median value of lncRNA NEAT1 expression in KIRC tissues, we divided the patients into two groups: high NEAT1 group ( $n=32$ ) and low NEAT1 group ( $n=31$ ). We found that high NEAT1 group was positively associated with higher histological grade, lymph node metastasis and distant metastasis (**Table 1**;  $P < 0.05$ ). However, there was no significant correlation between NEAT1 expression and other clinicopathologic features, such as age, gender, tumor size and tumor stage (**Table 1**;  $P > 0.05$ ). We further explored whether NEAT1 expression was associated with patients' overall survival. Kaplan-Meier survival curves showed that the overall survival rate was significantly lower in patients with high NEAT1 expression compared to those with low expression (**Figure 1E**;  $P < 0.05$ ).

### Knockdown of NEAT1 inhibited renal cancer cells proliferation and invasion

To further determine the function of NEAT1 in renal cancer, the NEAT1-specific si-NEAT1 was designed and transfected into 786-O and ACHN cells. qRT-PCR showed that cells transfected with si-NEAT1 showed a significantly reduced expression of NEAT1 compared with cells transfected with si-NC (**Figure 2A**;  $P < 0.05$ ). CCK-8 assay reported that NEAT1 inhibition obviously suppressed the proliferation rate of 786-O and ACHN cells compared with cells transfected with si-NC (**Figure 2B**;  $P < 0.05$ ). Then, we explored whether the effects of NEAT1 on cell proliferation was by altering cell-cycle progression. Flow cytometry showed that NEAT1 knockdown led a significant accumulation of cells in G0/G1 phase compared to cells transfected with si-NC (**Figure 2C**;  $P < 0.05$ ). In addition, transwell invasion assay revealed that cells transfected with si-NEAT1 significantly suppressed renal cancer cell invasion ability compared to the si-NC group (**Figure 2D**;  $P < 0.05$ ). These findings suggested that NEAT1 might act as a tumor oncogene in the progression of KIRC.

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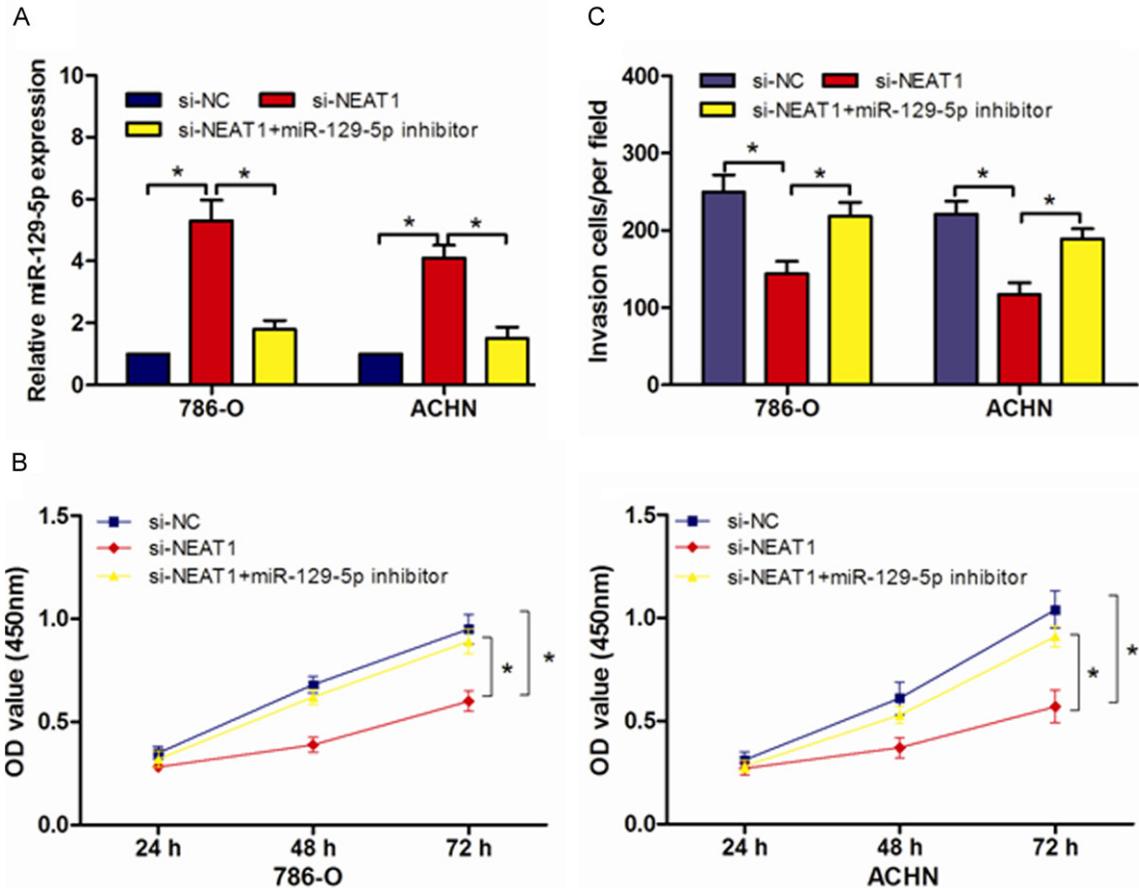


### miR-129-5p was a target of NEAT1

Increasing studies showed that lncRNA might act as a competing endogenous RNA (ceRNA)

or a molecular sponge in regulating the biological functions of miRNA. In the present study, we used starbase 2.0 (<http://starbase.sysu.edu.cn/>) to find that miR-129-5p is potentially bind

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**Figure 4.** The effects of NEAT1 inhibition on renal cancer cells were reversed by miR-129-5p inhibitor. A. Relative expression of miR-129-5p in renal cancer cells which transfected with si-NEAT1 or si-NEAT1+miR-129-5p inhibitors. B. CCK-8 assay indicated that NEAT1 inhibition could be reversed by miR-129-5p inhibitor. C. Transwell invasion assay showed cell invasion ability on NEAT1 suppression could be reversed by miR-129-5p inhibitor. \* $P < 0.05$ .

to NEAT1 (Figure 3A;  $P < 0.05$ ). qRT-PCR showed that miR-129-5p expression was significantly decreased in renal cancer cells transfected si-NEAT1 compared to si-NC group (Figure 3B;  $P < 0.05$ ). Dual-luciferase reporter assay showed that miR-129-5p mimics might reduce luciferase activity of pmirGLO-NEAT1-Wt, while it may not affect the luciferase activity of pmirGLO-NEAT1-Mut (Figure 3C;  $P < 0.05$ ). In addition, to examine whether NEAT1 could pull down miR-129-5p, we applied a pull down assay by a biotin-labeled specific NEAT1 probe. MiR-129-5p was precipitated as revealed by qRT-PCR (Figure 3D;  $P < 0.05$ ). Moreover, we explored the correlation between NEAT1 and miR-129-5p expression from TCGA Data Portal, our data showed that NEAT1 expression was associated with miR-129-5p in KIRC tissues (Figure 3E;  $P < 0.05$ ). To further support this conclusion, we further determine the expression of miR-

129-5p in 33 paired KIRC tissues by qRT-PCR. We found that miR-129-5p expression was decreased in KIRC tissues and negatively correlated with NEAT1 expression in KIRC tissues (Figure 3F and 3G;  $P < 0.05$ ). These data suggested that miR-129-5p was an inhibitory target for NEAT1 in KIRC progression.

### *Anti-miR-129-5p reversed the effect of NEAT1 knockdown in renal cancer cells in vitro*

To explore whether the tumor suppressive effects of NEAT1 inhibition were mediated by miR-129-5p, we co-transfected miR-129-5p inhibitor and si-NEAT1 into renal cancer cell lines, our data showed that miR-129-5p expression was higher in cell transfected with si-NEAT1 than si-NC or si-NEAT1+miR-129-5p inhibitor (Figure 4A;  $P < 0.05$ ). CCK-8 assay indicated that the inhibited proliferation induced by NEAT1 sup-

pression in renal cancer cells was partly abolished by the introduction of miR-129-5p inhibitor (**Figure 4B**;  $P < 0.05$ ). In addition, transwell invasion assay also showed that the suppressed invasion induced by NEAT1 inhibition in renal cancer cells could be partly abolished by the introduction of miR-129-5p inhibitor (**Figure 4C**;  $P < 0.05$ ). Those results suggested the miR-129-5p mediated the tumor suppressive effects of NEAT1 suppression in renal cancer cells.

### Discussion

In recent years, lncRNAs have become the research hotspot in cancer diagnosis and treatment [11]. lncRNAs are involved in the regulation of intracellular gene expression, and their abnormal expression has been known to be related to several human diseases especially cancer. For example, Lin et al found that increased expression of lncRNA ANRIL promoted lung cancer cell metastasis and correlated with poor prognosis [12]. Li et al showed that overexpression of lncRNA HOTTIP could increase chemoresistance of osteosarcoma cell by activating the Wnt/ $\beta$ -catenin pathway [13]. Lei et al suggested that lncRNA ATB contributed to gastric cancer growth through a MiR-141-3p/TGF $\beta$ 2 feedback loop [14]. However, the function and underlying mechanism of lncRNAs in the progression of cancer is still unclear.

NEAT1 (nuclear enriched abundant transcript 1) is a newly identified nuclear-restricted lncRNA, which has earned the reputation as a transcriptional regulator for numerous genes [15]. Previous papers showed that NEAT1 play important roles in tumor progression. For example, Zeng et al showed that inhibition of lncRNA NEAT1 impaired myeloid differentiation in acute promyelocytic leukemia cells [16]. Fu et al indicated that NEAT1 was an unfavorable prognostic factor and regulated migration and invasion in gastric cancer [17]. Zhen et al suggested that lncRNA NEAT1 promoted glioma pathogenesis by regulating miR-449b-5p/c-Met axis [18]. However, the clinical significance and biology functions of NEAT1 in KIRC is still unknown.

In the current study, our data showed that the expression of NEAT1 was significantly upregulated in KIRC tissues and cell lines compared to adjacent non-tumor tissues and normal human proximal tubule epithelial cell line HK-2.

High expression of NEAT1 was closely associated with higher histological grade, lymph node metastasis, distant metastasis and poor overall survival in KIRC patients. In vitro assay showed that NEAT1 inhibition significantly reduced cell proliferation, arrested the cell cycle in G0/G1 phase and suppressed cell invasion ability, suggesting that NEAT1 might act as a tumor oncogene in the development and progression of renal cancer.

In recent years, increasing studies showed that there is a novel regulatory mechanism between lncRNAs and miRNAs [19]. lncRNAs might function as a ceRNA or a molecular sponge in modulating miRNA [20]. For example, Lv et al suggested that lncRNA Unigene56159 promoted epithelial mesenchymal transition by acting as a ceRNA of miR-140-5p in hepatocellular carcinoma cells [21]. Luan et al showed that lncRNA MALAT1 acted as a ceRNA to promote malignant melanoma growth and metastasis by sponging miR-22 [22]. Huang et al found that lncRNA CASC2 functioned as a ceRNA by sponging miR-18a in colorectal cancer [23]. In the present study, we explored whether miR-129-5p mediated the suppressive effects of NEAT1 inhibition in renal cancer progression. We found that miR-129-5p was an inhibitory target for NEAT1 in KIRC progression. Furthermore, we found that the reduced renal cancer cell proliferation and invasion by suppressing NEAT1 expression could largely abolished by the introduction of miR-129-5p inhibitor. Thus, those data suggested that NEAT1 could promote renal cancer progression by acting as a molecular sponge to regulate miR-129-5p.

In conclusion, our results provided strong evidence that NEAT1 acted as an oncogenic lncRNA that promoted KIRC progression through miR-129-5p, suggesting NEAT1 could act as a potential biomarker and therapeutic target for the treatment of KIRC.

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### Disclosure of conflict of interest

None.

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