European Review for Medical and Pharmacological Sciences 2020; 24: 704-711

LncRNA HANR aggravates the progression of non-small cell lung cancer *via* mediating miRNA-140-5p

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Abstract. – OBJECTIVE: The aim of this study was to elucidate the function of long non-coding ribonucleic acids (lncRNAs) HANR in aggravating non-small cell lung cancer (NSCLC) progression *via* targeting microRNA-140-5p (miRNA-140-5p).

PATIENTS AND METHODS: The relative expression level of HANR in NSCLC tissues and cell lines was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between HANR expression and the prognosis of NSCLC was analyzed. The regulatory effects of HANR on cellular behaviors of NSCLC cells were evaluated by Cell Counting Kit-8 (CCK-8), transwell and wound healing assay. Meanwhile, the relative expression of miRNA-140-5p in NSCLC tissues and cell lines was determined by qRT-PCR. In addition, rescue experiments were carried out to evaluate the potential influence of HANR/miRNA-140-5p on the progression of NSCLC.

RESULTS: HANR expression was significantly up-regulated in NSCLC tissues and cell lines. HANR expression was positively correlated with lymphatic metastasis and distant metastasis of NSCLC patients, whereas it was negatively correlated with the overall survival of NSCLC patients. Knockdown of HANR markedly suppressed the proliferative, migratory and invasive abilities of NSCLC cells. In NSCLC tissues, the miRNA-140-5p level was negatively associated with HANR level. Furthermore, inhibited cellular behaviors of NSCLC cells transfected with sh-HANR were partially reversed after miRNA-140-5p knockdown.

CONCLUSIONS: LncRNA HANR accelerates the proliferative, migratory and invasive abilities of NSCLC *via* negatively mediating miRNA-140-5p. Furthermore, HANR is closely correlated with lymphatic metastasis, distant metastasis and poor prognosis of NSCLC.

Key Words
LncRNA HANR, MiRNA-140-5p, NSCLC, Proliferation.

Introduction

Lung cancer is one of the most common malignancies in the world, whose incidence has increased significantly. In 2018, the mortality of lung cancer accounted for 26% in females and 28% in males of all tumor deaths[1,2]. Statistics have shown that the incidence of lung cancer is rapidly increasing in China. Meanwhile, the mortality of lung cancer has increased by 465% in the past decades, ranking first in countries and second in rural areas[3,4]. Non-small cell lung cancer (NSCLC) accounts for 75-80% of all lung cancer cases[5,6]. The prognosis of NSCLC is far away from satisfaction, with a 5-year survival of only 11%[5,7]. The major reasons for poor prognosis of NSCLC are tumor invasion and metastasis at the first time of diagnosis[8-10]. Currently, there is still a lack of effective strategies for controlling the metastasis of NSCLC. Therefore, it is urgent to search for early diagnostic and prognostic hallmark of NSCLC, thereby improving the survival of patients[11,12].

Genome-wide analysis has proposed that only 2% of genes can encode proteins. Over 90% of genes are transcribed into non-coding RNAs (ncRNAs)[12-14]. According to the length, ncRNAs are divided into short ncRNAs and long non-coding ribonucleic acids (lncRNAs)[15,16]. LncRNAs are a kind of RNAs with over than 200 nt in length[16]. It is well known that lncRNAs exert crucial functions in tumor biology[17,18]. A great number of studies have identified the potential of lncRNAs as therapeutic and prognostic markers in tumors[19,20]. Moreover, their levels may be related to the pathological characteristics of tumor patients. However, the exact role of lncRNA HANR in tumor progression has not been fully elucidated[21]. Therefore, the aim of this study was to mainly elucidate the potential function of HANR in the progression of NSCLC and its underlying mechanism.

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With the in-depth research on ncRNA, ceRNA theory has been proposed as a novel gene regulation network[22,23]. Regulatory interaction involving both lncRNA and microRNA (miRNA) has been pronounced in tumor biology[24]. Previous studies have indicated that this novel regulatory network is of great significance in clarifying tumor pathogenesis, and can be utilized as promising therapeutic strategies for tumors[24,25]. In this work, the function of lncRNA HANR/miRNA-140-5p in the progression of NSCLC was investigated.

Patients and Methods

Patients and NSCLC Samples

A total of 36 paired NSCLC tissues and adjacent normal tissues were surgically resected from NSCLC patients. All enrolled patients were diagnosed as NSCLC based on the guideline proposed by the Union for International Cancer Control (UICC). No patient underwent preoperative anti-tumor therapy. Informed consents were obtained from patients and their families before the study. This study was approved by the Ethics Committee of the Affiliated Hospital of Guangdong Medical University.

Cell Lines and Reagents

Bronchial epithelial cell line (BEAS-2B) and NSCLC cell lines (H358, PC-9, H1299, A549 and SPC-A1) were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle 's Medium (DMEM; Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and maintained in a 37°C, 5% CO_2 incubator.

Cell Transfection

Negative control (NC) and sh-HANR were provided by GenePharma, (Shanghai, China). The cells were first seeded into 6-well plates (Corning, Lowell, MA, USA). When cell confluence reached 40%, cell transfection was performed. At 48 h, the cells were harvested for subsequent experiments.

Cell Proliferation Assay

Cells were first seeded into 96-well plates (Corning, Lowell, MA, USA) with 2×10^3 cells per well. Cell proliferation was detected according to the instructions of Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Absorbance (A) at 450 nm was recorded at the appointed time points, and viability curve was finally plotted.

Transwell Cell Migration and Invasion Assay

Transfected cells for 48 h were digested and adjusted to the concentration 5.0×10^5/mL. 200 µL/well suspension was applied in the upper chamber of Matrigel-coated transwell inserts (Millipore, Billerica, MA, USA). Meanwhile, 700 µL of medium containing 10% FBS was added to the lower chamber. After 48 h of incubation, invasive cells were fixed with methanol for 15 min and stained with crystal violet for 20 min. The number of invading cells was counted under a microscope (Nikon, Tokyo, Japan). 5 fields were randomly selected for each sample. Transwell migration assay was conducted with the same procedures except for Matrigel pre-coating.

Wound Healing Assay

Cells were first seeded into 6-well plates at a density of 5.0×10^5/well. Until 90% of confluence, a 1 mL pipette tip was used to create an artificial wound in the confluent cell monolayer. The percentage of wound closure at 0 and 24 h was calculated, respectively.

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

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and purified by DNase I treatment. Extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) in strict accordance with the Primescript Reverse Transcription (RT) Reagent (TaKaRa, Otsu, Shiga, Japan). Subsequently, obtained cDNA was subjected to quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references for mRNA and miRNA, respectively. Each sample was performed in triplicate. The relative level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method, and was analyzed by iQ5 2.0 (Bio-Rad, Hercules, CA, USA). Primer sequences used in this study were as follows: HANR, F: 5'-CGGAGGATCCGGTCCTGACTC-3', R: 5'-GGCTACCTGTGTCTTAATCGGA-3'; miR-140-5p, F: 5'-TGCCGGGTAAACATCCCTCGACTG-3', R: 5'-GATTGCGTCGTCGTGGCAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Statistical Analysis

GraphPad Prism 5 V5.01 (Version X; La Jolla, CA, USA) software was used for all statistical analyses. Experimental data were expressed as mean ± standard deviation. Intergroup differences were analyzed by *t*-test. Differences among multiple groups were analyzed using one-way analysis of variance (ANOVA), followed by post-hoc test. $p<0.05$ was considered statistically significant.

Results

HANR Was Highly Expressed in NSCLC Tissues and Cell Lines

In this study, 36 pairs of NSCLC tissues and adjacent normal tissues were harvested. QRT-PCR data revealed that HANR was highly expressed in NSCLC tissues when compared with the adjacent normal tissues (Figure 1A). Identically, HANR

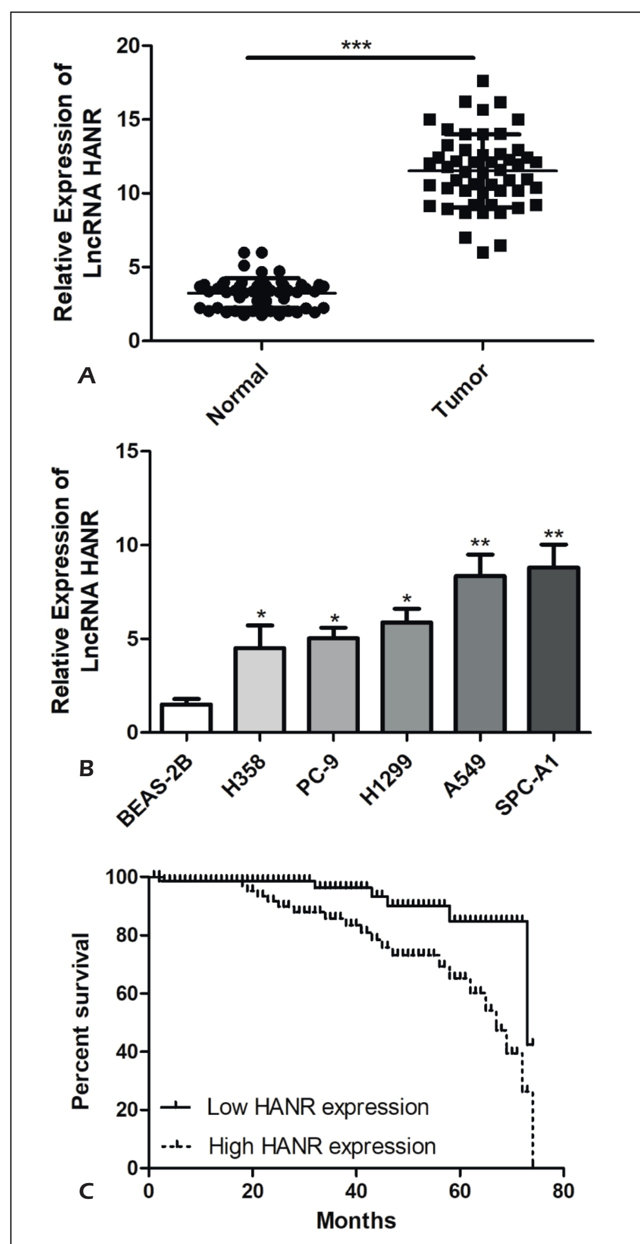

Figure 1. HANR was highly expressed in NSCLC tissues and cell lines. **A**, Relative level of HANR in NSCLC tissues and adjacent normal tissues detected by qRT-PCR. **B**, Relative level of HANR in bronchial epithelial cell line (BEAS-2B) and NSCLC cell lines (H358, PC-9, H1299, A549 and SPC-A1) detected by qRT-PCR. **C**, Kaplan-Meier curve was conducted based on HANR level in NSCLC patients. Data were expressed as mean±SD. $*p<0.05$, $**p<0.01$, $***p<0.001$.

expression in NSCLC cell lines was significantly higher than that of bronchial epithelial cell line BEAS-2B (Figure 1B). The results indicated that HANR might play a carcinogenic role in NSCLC.

HANR Expression Was Correlated with Pathological Staging and Overall Survival of NSCLC Patients

Based on the expression of HANR, enrolled NSCLC patients were divided into the high-level and low-level group. The correlation between HANR expression with age, gender, tumor grade, lymphatic metastasis and distant metastasis of NSCLC patients was evaluated. The results showed that the expression level of HANR was positively correlated with lymphatic metastasis and distant metastasis of NSCLC patients rather than the other indexes (Table I). Subsequently, survival analysis was conducted based on collected follow-up data. The Kaplan-Meier curve revealed that the survival of NSCLC patients with higher level HANR was significantly worse than that of those with a lower level (Figure 1C).

Knockdown of HANR Inhibited the Proliferative, Migratory and Invasive Abilities of NSCLC Cells

To elucidate the biological function of HANR NSCLC progression, the sh-HANR vector was first constructed. Transfection of sh-HANR markedly downregulated HANR expression in A549 and SPC-A1 cells (Figure 2A). Knockdown of HANR remarkably suppressed the viability of NSCLC cells, indicating inhibited proliferative ability (Figure 2B). The transwell assay revealed that transfection of sh-HANR markedly inhibited the migratory and invasive abilities of A549 and SPC-A1 cells (Figure 2C). Furthermore, would closure was remarkably reduced in NSCLC cells with HANR knockdown (Figure 2D). The above results indicated that HANR knockdown inhibited the proliferative, migratory and invasive abilities of NSCLC cells.

Downregulated MiRNA-140-5p in NSCLC

Previous research has predicted that miRNA-140-5p was the target gene of HANR. Here, miRNA-140-5p was lowly expressed in NSCLC tissues and cell lines (Figure 3A, 3B). Meanwhile, miRNA-140-5p expression was negatively correlated with HANR expression in NSCLC tissues (Figure 3C). Transfection of sh-HANR in A549 and SPC-A1 cells significantly upregulated miRNA-140-5p level, further confirming their negative correlation (Figure 3D). Subsequently, we explored the correlation between the miRNA-140-5p level and pathological indexes of NSCLC patients. Consistent with HANR level, the miRNA-140-5p level was markedly associated with lymphatic metastasis and distant metastasis of NSCLC (Table I).

HANR Modulated MiRNA-140-5p Expression in NSCLC Tissues and Cell Lines

We speculated that HANR might regulate the progression of NSCLC *via* mediating miRNA-

Table I. Association of lncRNA HANR and miR-140-5p expression with clinicopathologic characteristics of non-small cell lung cancer.

Parameters	No. of cases	HANR expression		*p*-value	miR-140-5p expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.298			0.221
<60	13	8	5		3	10	
≥60	23	10	13		10	13	
Gender				0.182			0.298
Male	18	11	7		5	13	
Female	18	7	11		8	10	
T stage				0.298			0.096
T1-T2	23	13	10		6	17	
T3-T4	13	5	8		7	6	
Lymph node metastasis				0.026			0.009
No	26	16	10		6	20	
Yes	10	2	8		7	3	
Distance metastasis				0.002			0.002
No	23	16	7		4	19	
Yes	13	2	11		9	4	

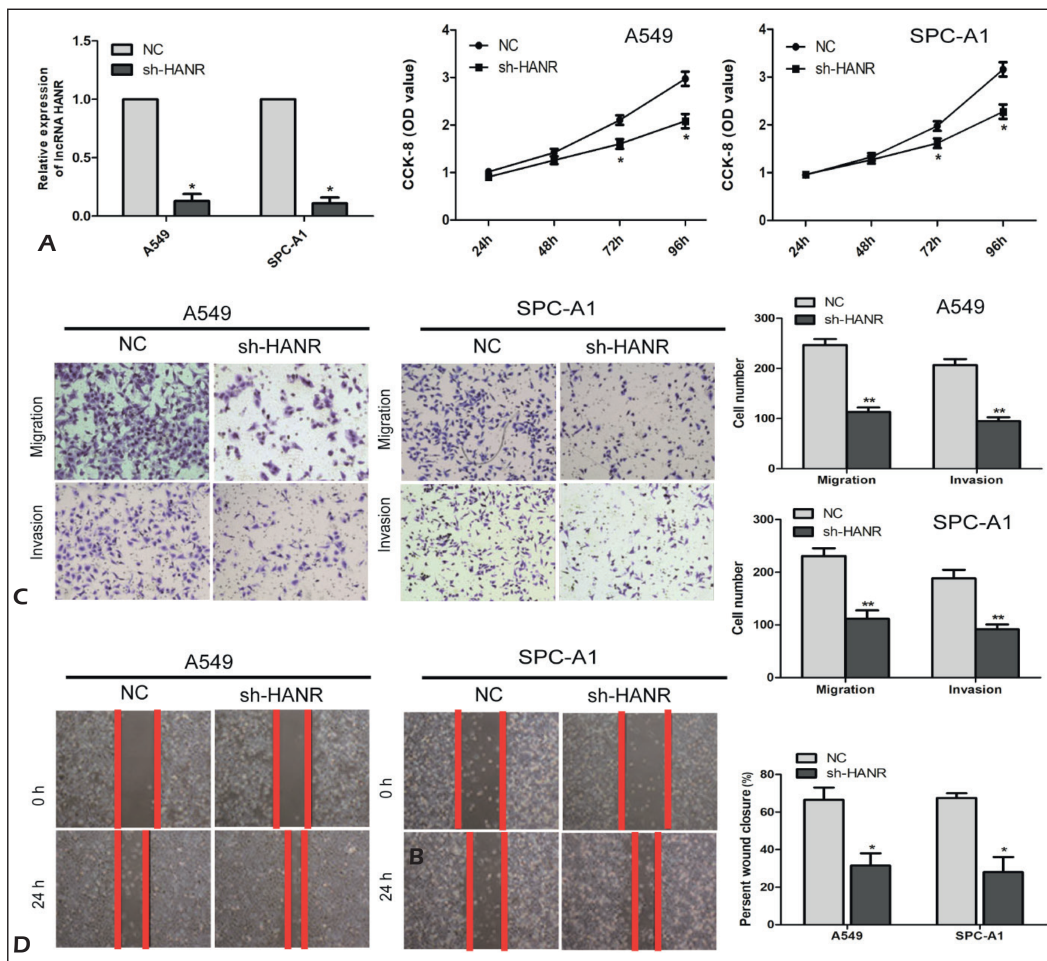

Figure 2. Knockdown of HANR inhibited the proliferative, migratory and invasive abilities of NSCLC cells. **A**, Transfection efficacy of sh-HANR in A549 and SPC-A1 cells determined by qRT-PCR. **B**, CCK-8 assay revealed the viability of A549 and SPC-A1 cells transfected with NC or sh-HANR. **C**, Transwell assay revealed the migration and invasion of A549 and SPC-A1 cells transfected with NC or sh-HANR (magnification 20×). **D**, Wound healing assay revealed wound closure of A549 and SPC-A1 cells transfected with NC or sh-HANR (magnification 20×). Data were expressed as mean ± SD. *p<0.05, **p<0.01.

140-5p. The miRNA-140-5p inhibitor was then constructed. Transfection of miRNA-140-5p inhibitor markedly upregulated HANR level in NSCLC cells. Moreover, downregulated level of HANR in cells transfected with sh-HANR could be partially reversed after miRNA-140-5p knockdown (Figure 4A). Notably, the inhibited proliferative, migratory and invasive abilities of NSCLC cells transfected with sh-HANR were partially reversed after miRNA-140-5p knockdown (Figure 4B-4D).

Discussion

Lung cancer is the most common malignancy with the highest morbidity and mortality worldwide[1,2]. Tumor invasion and metastasis are the major causes of poor prognosis of lung cancer. However, the diagnosis of lung cancer at the early stage is insufficient, with a lack of metastatic hallmarks[1,2,11,12]. In this study, HANR was highly expressed in NSCLC tissues relative to normal controls. Besides, HANR level was closely related to lymphatic and distant metastasis of NSCLC, suggesting the potential of HANR as a hallmark for NSCLC. The improvement of early-stage diagnostic efficacy and development of novel tumor hallmark are crucial challenges in NSCLC treatment[9-11]. This work demonstrated that a high level of HANR was correlated with poor prognosis of NSCLC.

Abnormally expressed lncRNAs in tumors have been well concerned[13-15]. Alterations of lncRNA level

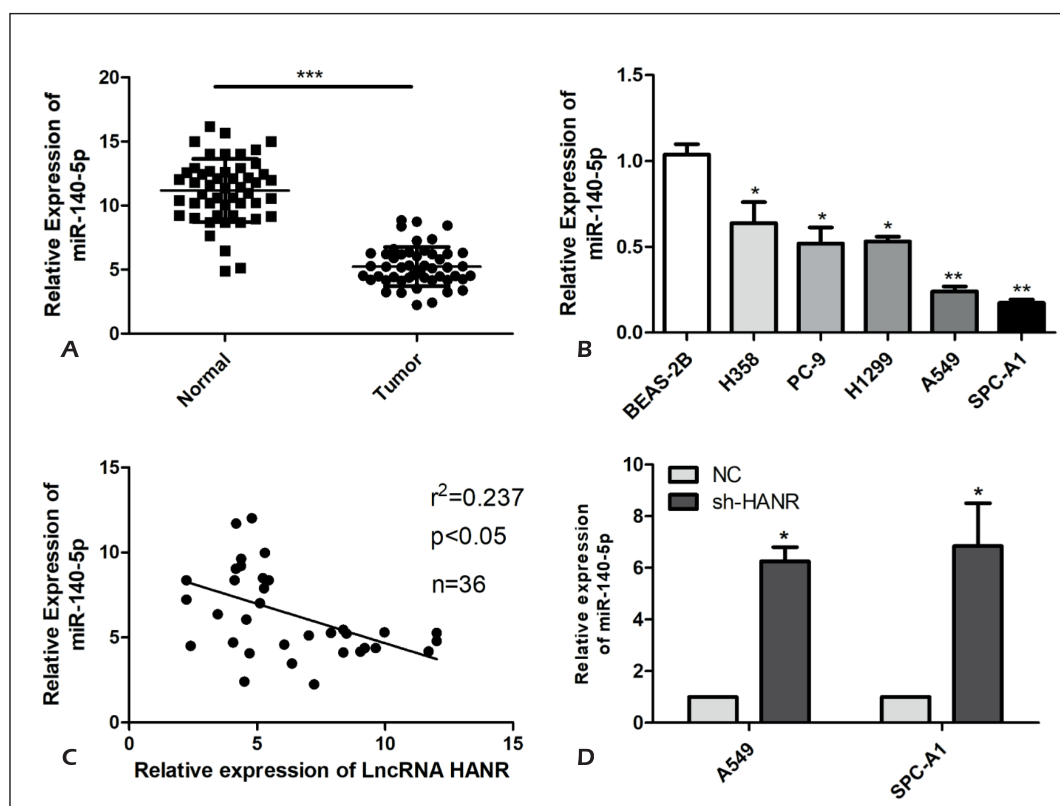

Figure 3. MiR-140-5p was lowly expressed in NSCLC tissues and cell lines. **A**, Relative level of miR-140-5p in NSCLC tissues and adjacent normal tissues detected by qRT-PCR. **B**, Relative level of miR-140-5p in bronchial epithelial cell line (BEAS-2B) and NSCLC cell lines (H358, PC-9, H1299, A549 and SPC-A1) detected by qRT-PCR. **C**, Correlation analysis on relative levels of HANR and miR-140-5p in NSCLC tissues. **D**, Relative level of miR-140-5p in A549 and SPC-A1 cells transfected with NC or sh-HANR. Data were expressed as mean ± SD. $*p<0.05$, $**p<0.01$, $***p<0.001$.

can influence the activity and expression of oncogenes or tumor-suppressor genes, thereafter affecting cellular behaviors of tumor cells[16,17]. Tumor cells are characterized by abnormal proliferation, inhibited apoptosis, and enhanced invasive and migratory abilities[17,18]. It is believed that genetic changes during transcription or translation may mediate tumor cell behaviors[19]. Here, transfection of sh-HANR markedly suppressed the proliferative, migratory and invasive abilities of A549 and SPC-A1 cells.

Current studies[20-25] have proposed that lncRNA can competitively bind to miRNAs, thereafter regulating target mRNAs. The previous research has already predicted the potential binding relationship between miRNA-140-5p and HANR through bioinformatics method. MiRNA-140-5p was lowly expressed in NSCLC tissues and cell lines. Furthermore, a negative correlation was identified between miRNA-140-5p level and HANR level in NSCLC tissues. Moreover, inhibited cellular behaviors of NSCLC cells transfected with sh-HANR were partially reversed after miRNA-140-5p knockdown. The above results concluded that HANR aggravated the progression of NSCLC *via* competitively binding to miRNA-140-5p and downregulating its level.

Conclusions

In sum, lncRNA HANR accelerates the proliferative, migratory and invasive abilities of NSCLC by negatively mediating miRNA-140-5p. Furthermore, it is closely correlated with lymphatic metastasis, distant metastasis and poor prognosis of NSCLC.

Funding Acknowledgments

State Natural Sciences Foundation (81871883).

Conflict of Interests

The authors declare that they have no conflict of interest.

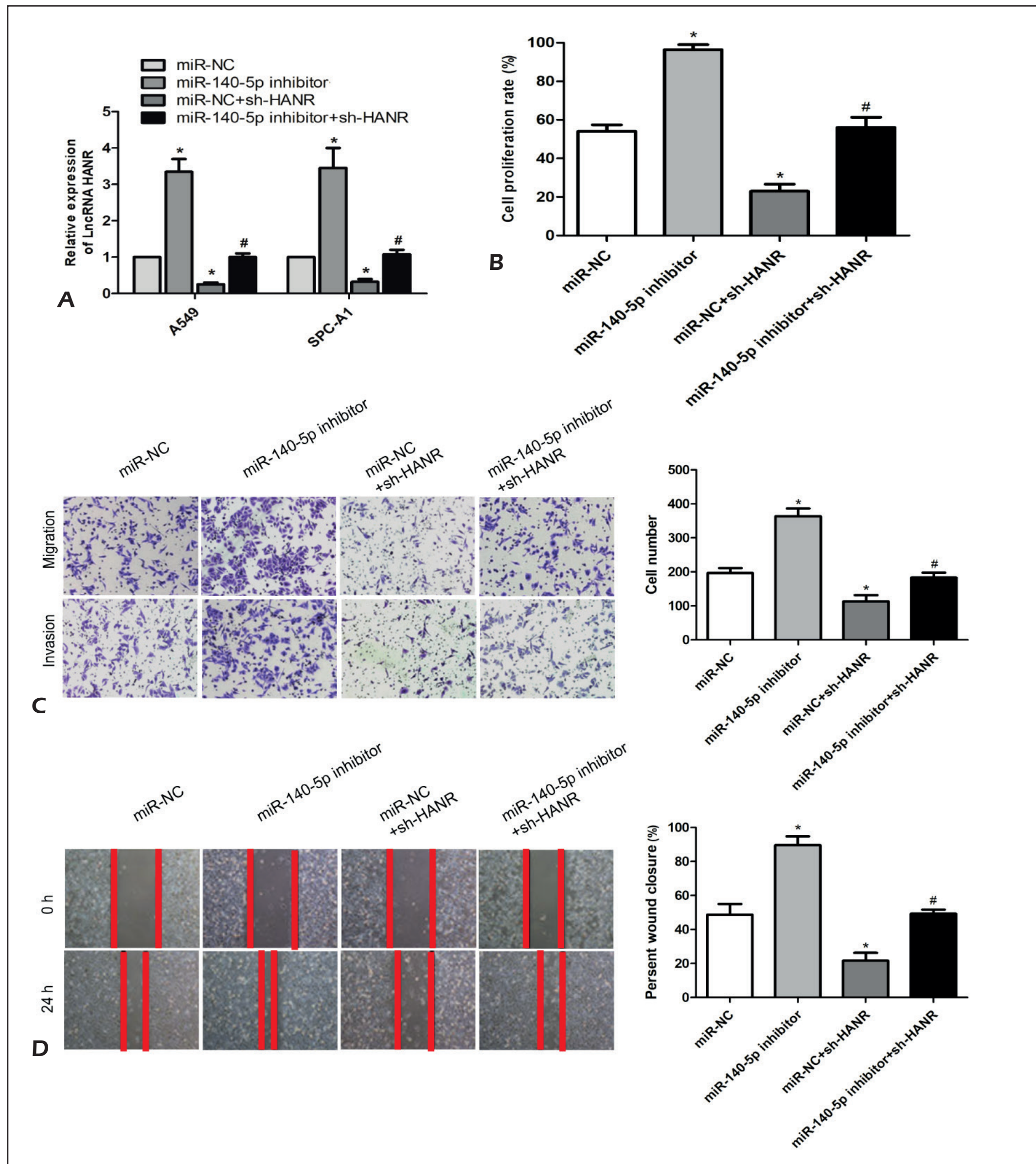

Figure 4. HANR modulated miR-140-5p expression in NSCLC tissues and cell lines. A549 and SPC-A1 cells were transfected with miR-NC, miR-140-5p inhibitor, miR-NC + sh-HANR, or miR-140-5p inhibitor + sh-HANR. **A**, Relative level of HANR in each group detected by qRT-PCR. **B**, Cell proliferation rate of each group. **C**, Migratory and invasive abilities of each group (magnification 20×). **D**, Percentage of wound closure in each group (magnification 20×). Data were expressed as mean ± SD. $^{*\#}p<0.05$.

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