European Review for Medical and Pharmacological Sciences 2020; 24: 238-248

LncRNA NNT-AS1 regulates the progression of lung cancer through the NNT-AS1/miR-3666/E2F2 axis

J.-W. HUANG[1], X.-Y. LUO[2], Z.-H. LI[3], B.-P. LANG[4]

[1]Department of Pathology, Luoyang Central Hospital, Luoyang, China
[2]Department of Oncology, Luoyang Central Hospital, Luoyang, China
[3]Department of Gastrointestinal Surgery, Luoyang Central Hospital, Luoyang, China
[4]Department of Thoracic Surgery, Luoyang Central Hospital, Luoyang, China

Abstract. – OBJECTIVE: Lung cancer is the main burden on human health, with high mortality and poor prognosis. The involvement of long non-coding RNAs (lncRNAs) in the development of cancer has attracted wide attention. This study aimed to investigate the role and novel mechanisms of lncRNA nicotinamide nucleotide transhydrogenase antisense RNA 1 (NNT-AS1) in the progression of lung cancer.

MATERIALS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to detect the expression of NNT-AS1, microRNA-3666 (miR-3666), and E2F transcription factor 2 (E2F2). 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay was used to analyze cell proliferation. Flow cytometry was carried out to investigate cell apoptosis. Transwell assay was conducted to observe cell invasion. The interaction between miR-3666 and NNT-AS1 or E2F2 was predicted by bioinformatics tool starBase v2.0 and verified by Dual-Luciferase reporter assay. The protein level of E2F2 was quantified by Western blot.

RESULTS: NNT-AS1 and E2F2 were upregulated, but miR-3666 was downregulated in lung cancer tissues and cells. NNT-AS1 knockdown attenuated proliferation and invasion but enhanced apoptosis of lung cancer cells, while miR-3666 inhibition reversed these effects. It was confirmed that miR-3666 was a target of NNT-AS1 and it directly interacted with E2F2. The inhibitory proliferation and invasion, and acceleratory apoptosis of lung cancer cells, caused by miR-3666 enrichment, were overturned by E2F2 overexpression. Furthermore, E2F2 was regulated by NNT-AS1 through miR-3666.

CONCLUSIONS: NNT-AS1 participated in the progression of lung cancer through NNT-AS1/miR-3666/E2F2 regulatory axis at least in part. Our study supplied a promising strategy for the treatment of lung cancer.

Key Words:
NNT-AS1, MiR-3666, E2F2, Lung cancer.

Introduction

Lung cancer, mainly including non-small cell lung cancer (NSCLC) and small cell lung (SCLC) cancer, remains the primary cause of cancer-related deaths around the world[1]. In 2012, 1.8 million people were diagnosed with lung cancer, and the number of cases diagnosed with lung cancer reached 3 million in 2015[2,3]. The high mortality rate of lung cancer is mainly due to the difficulty in being diagnosed in the early stages because there are no apparent symptoms. As a matter of fact, more than 70% of lung cancers are diagnosed after the onset of late local symptoms or metastatic disease[4-7]. Therefore, the search for novel biological targets and related mechanisms is of great significance for the therapy and diagnosis of lung cancer.

The advance of transcriptomics reveals that over 90% of the human genomes transcribe non-coding RNAs (ncRNAs)[8]. Long non-coding RNAs (lncRNAs) are a kind of RNA molecules that have more than 200 nucleotides in length, without the capacity of protein-coding[9]. LncRNAs function in multiple biological progress, and their dysregulation has been identified to be associated with the development of diverse human cancers[10,11]. In lung cancer, several lncRNAs have been reported to be implicated into the occurrence, metastasis, and drug resistance. LncRNA LINC01186 was lowly expressed in lung cancer, and it regulated epithelial mesenchymal transition (EMT) through TGF-β signaling pathway[12]. LncRNA CPS1-IT1 functioned as a tumor inhibitor in lung cancer to block cell migration and invasion[13]. LncRNA Sox2ot knockdown led to G2/M arrest and inhibited cell proliferation by modulating the expression of EZH2 in lung cancer[14]. The regulation of cellular activities by lncRNA nicotinamide nucleotide transhydrogenase

Corresponding Author: Xiaoyong Luo, MD; e-mail: 13698887863@163.com

antisense RNA 1 (NNT-AS1) was documented in various cancers, such as osteosarcoma, gastric cancer, and ovarian cancer[15-17]. Unfortunately, the function of NNT-AS1 in lung cancer is blurry because of limited research.

MicroRNAs (miRNAs) are a cluster of ncRNAs with 18-22 nucleotides in length. Generally, miRNAs can be targeted by lncRNAs, leading to the inhibition of the expression and activity of miRNAs[18]. Besides, miRNAs could directly bind to the 3' untranslated region (3' UTR) of mRNAs to accelerate degradation and translation inhibition of mRNAs[19]. Following this regulatory manner, miR-3666 has been described in several cancers. For instance, miR-3666, a target of ATRA, directly interacted with E2H7 in colorectal cancer tissues, affecting the progression of colorectal cancer[20]. The potential role and regulatory axis of miR-3666 are deserved to explore in lung cancer.

E2F transcription factor 2 (E2F2) is a member of the E2F protein family. E2F proteins are key regulators of cell proliferation, differentiation, and apoptosis[21]. Recently, E2F2 has been referred to be linked to the progression of numerous cancers. Likewise, miR-638 blocked the characteristics and behaviors of breast cancer cells by targeting E2F2, suggesting that E2F2 was a tumor-promoter in breast cancer[22]. Similarly, in pancreatic cancer and osteosarcoma, E2F2 acted as an oncogene to promote the malignant activities of cancer cells. Nevertheless, the research of E2F2 in lung cancer is inadequate and its novel regulatory mechanism needs further investigation.

In this study, the expression level of NNT-AS1 was determined in lung cancer tissues and cells. The interaction between miR-3666 and NNT-AS1 or E2F2 was verified. Gain-function or loss-function experiments ensured the function of NNT-AS1, miR-3666, and E2F2 in lung cancer cells. Our study attempted to provide a novel mechanism of lung cancer development and ideal biomarkers for the improvement of lung cancer.

Materials and Methods

Specimen Collection

A total of 30 paired lung cancer tumor tissues (T) and adjacent normal tissues (N) were obtained from Luoyang Central Hospital. All samples were frozen in liquid nitrogen at once after resection and then stored at 80°C. All subjects signed informed consents before surgery. Our research was reviewed and approved by the Ethics Committee of Luoyang Central Hospital.

Cell Lines and Culture

Lung cancer cell lines, including H1650, H1975, H1299, and A549, and bronchial epithelioid cells (HBE) were purchased from BeNa Culture Collection (Suzhou, China). Based on the instructions, H1975 and HBE were cultured in 90% Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). H1650, H1299, and A549 were kept in 90% Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). All cells were maintained at 37°C in a humidified incubator with 5% CO_2.

Cell Transfection

Lentiviral vector encoding a short hairpin RNA targeting NNT-AS1 (sh-NNT-AS1) and negative control (sh-NC) were purchased from GenePharma (Shanghai, China). Inhibition of miR-3666 (anti-miR-3666), miR-3666 mimic (miR-3666), and their several controls (anti-miR-NC and miR-NC) were synthesized by Ribobio (Guangzhou, China). NNT-AS1 overexpression vector pcDNA3.1-NNT-AS1 (NNT-AS1), E2F2 overexpression vector pcDNA3.1-E2F2 (E2F2), and pcDNA3.1 empty vector (pcDNA) were constructed by GenePharma (Shanghai, China). All transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were harvested at 48 h after transfection for the following experiments.

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

TRIzol reagent (Beyotime, Shanghai, China) was used to isolate the total RNA from tissues and cells. RNA was reverse-transcribed into complementary DNA (cDNA) using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) for miR-3666 and the PrimeScript RT reagent kit (TaKaRa, Dalian, China) for NNT-AS1 and E2F2. Then, qRT-PCR was carried out using SYBR Green Master PCR mix (Applied Biosystems, Foster City, CA, USA) through the ABI 7900 system (Applied Biosystems, Foster City, CA, USA). The expression levels, normalized by Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or small nuclear RNA U6, were calculated using $2^{-\Delta\Delta Ct}$ method. The primers were listed as below: NNT-AS1, forward (F): 5'-ACGTGCAGACAACATCTACCT-3' and reverse (R): 5'-TACAACACCTTCCCGCAT-3';

E2F2, F: 5'-GTCCTTCCGAGGAGCCTCT-3' and R: 5'-GGGACAGGAACTGGTCCTC-3'; GAPDH, F: 5'-ACCACAGTCCATGCCATCAC-3' and R: 5'-TCCACCACCCT GTTGCTGTA-3'; miR-3666, F: 5'-ACGAGACGACGACAGAC-3' and R: 5'-CAGTGCAAGTGTAGATGCCGA-3'; U6, F: 5'-GCUUCGGCAGCACAUAUACUAAAAU-3' and R: 5'-CGCUUCACGAAUUUGCGUGUCAU-3'.

3-(4, 5-Dimethyl-2-Thiazolyl)-2, 5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

H1299 and A549 cells with different transfection were plated into 96-well plates (2×10^3) and cultured for 48 h. Next, cells were dealt with 10 μL MTT solution (Beyotime; 5 mg/mL) for 4 h. Then, 100 μL formazan solvent (Beyotime, Shanghai, China) was pipetted into each well for other 3 h to dissolve formazan. Finally, the absorbance at 490 nm was measured at a specific time by a microplate reader (Bio-Rad, Hercules, CA, USA). Five replicate wells were used for each group.

Flow Cytometry Assay

Annexin V-Fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Beyotime, Shanghai, China) was adapted for apoptosis analysis. In brief, H1299 and A549 cells with different transfection were collected, trypsinized, and re-suspended with phosphate-buffered saline (PBS; Beyotime, Shanghai, China) (5×10^4). Then, 195 μL Annexin V-FITC binding buffer was added to treat cells. Subsequently, 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) were added into each tube. Finally, the apoptotic cells were distinguished using flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell Assay

Transwell was conducted to detect cell invasion. H1299 and A549 cells with different transfection were re-suspended in serum-free medium and transferred into the top of 24-well transwell chambers (10 μm pore size; BD Biosciences, Franklin Lakes, NJ, USA) treated with Matrigel previously. The bottom of chambers was supplied with RPMI-1640 medium or DMEM medium containing 10% FBS. After 24 h incubation, the invaded cells at the lower surface were fixed with 4% paraformaldehyde (PFA), stained with crystal violet and photographed using the microscope (Olympus, Tokyo, Japan) in 5 random areas.

Bioinformatics Analysis and Dual-Luciferase Reporter Assay

Online tool starBase v2.0 was used to identify the putative target genes and analyze the binding sites between miR-3666 and NNT-AS1 or E2F2 3' UTR.

The wild type (WT) and mutant (MUT) sequences of NNT-AS1 (WT-NNT-AS1 and MUT-NNT-AS1) containing the binding sites of miR-3666 were amplified and inserted into the Dual-Luciferase reporter vector pGL3 (Promega, Madison, WI, USA), named as WT-NNT-AS1 and MUT-NNT-AS1. Later, H1299 and A549 cells were co-transfected with miR-3666 and WT-NNT-AS1 or MUT-NNT-AS1. After 48 h, the Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega, Madison, WI, USA). As for E2F2, E2F2 3' UTR-WT and E2F2 3' UTR-MUT were also constructed and conducted as the above description.

Western Blot

Total proteins were extracted from tissues and cells using RIPA lysis buffer (Beyotime, Shanghai, China). The proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after measurement of the concentration and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Subsequently, the membranes were blocked with tris-buffered saline (TBS) buffer containing 5% nonfat milk for 2 h and incubated with the primary antibodies against E2F2 (ab138515; 1:1,000; Abcam, Cambridge, MA, USA) or GAPDH (ab9485; 1:2,500; Abcam, Cambridge, MA. USA) overnight at 4°C. Next day, the membranes were washed with Tris-Buffered Saline and Tween-20 (TBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (ab205718; 1:5,000; Abcam, Cambridge, MA. USA). Finally, the enhanced chemiluminescence (ECL) kit (Beyotime, Shanghai, Chin) was used to visualize the protein signals on the membrane.

Statistical Analysis

Data were processed by SPSS (Version X; IBM Corp., Armonk, NY, USA). The data from at least 3 times for all experiments were presented as the mean ± standard deviation (SD). Student's *t*-test or One-way analysis of variance (ANOVA) followed by Tukey's test was used to identify the differences between the two groups or among multiple groups of variables. The correlation of the expression between two groups was identified by Spearman's correlation analysis. $p < 0.05$ was considered to be statistically significant.

Results

NNT-AS1 was Upregulated, while MiR-3666 was Downregulated in Lung Cancer Tissues and Cell Lines

To monitor the expression levels of NNT-AS1 and miR-3666, qRT-PCR analysis was performed. The expression of NNT-AS1 was increased in lung cancer tumor tissues relative to normal tissues (Figure 1A). Likewise, the expression of NNT-AS1 was also enhanced in lung cancer cell lines, including H1650, H1975, H1299, and A549, compared with bronchial epithelioid cell HBE (Figure 1B). On the contrary, miR-3666 was lowly expressed in lung cancer tumor tissues and lung cancer cell lines compared with normal tissues and normal cells, respectively (Figure 1C and 1D). Besides, Spearman's correlation coefficient revealed that the miR-3666 expression was negatively correlated with the NNT-AS1 expression (Figure 1E) in lung cancer tissues. The data suggested that NNT-AS1 and miR-3666 might play roles in lung cancer.

Knockdown of NNT-AS1 Inhibited Cell Proliferation and Invasion but Promoted Cell Apoptosis in Lung Cancer

To ascertain the role of NNT-AS1, the expression of endogenous NNT-AS1 was reduced in H1299 and A549 cells. Firstly, the expression level of NNT-AS1 was detected by qRT-PCR and the result showed that NNT-AS1 expression was significantly decreased after cells were transfected with sh-NNT-AS1 (Figure 2A). Next, MTT assay claimed that the ability of proliferation in H1299 and A549 cells was notably frustrated with sh-NNT-AS1 transfection (Figure 2B and 2C). Flow cytometry manifested that the apoptosis rate was significantly enhanced in H1299 and A549 cells transfected with sh-NNT-AS1 (Figure 2D). Transwell assay concluded that the number of invaded cells was substantially reduced in H1299 and A549 cells transfected with sh-NNT-AS1 (Figure 2E). These analyses indicated that NNT-AS1 knockdown contributed to the inhibition of lung cancer development.

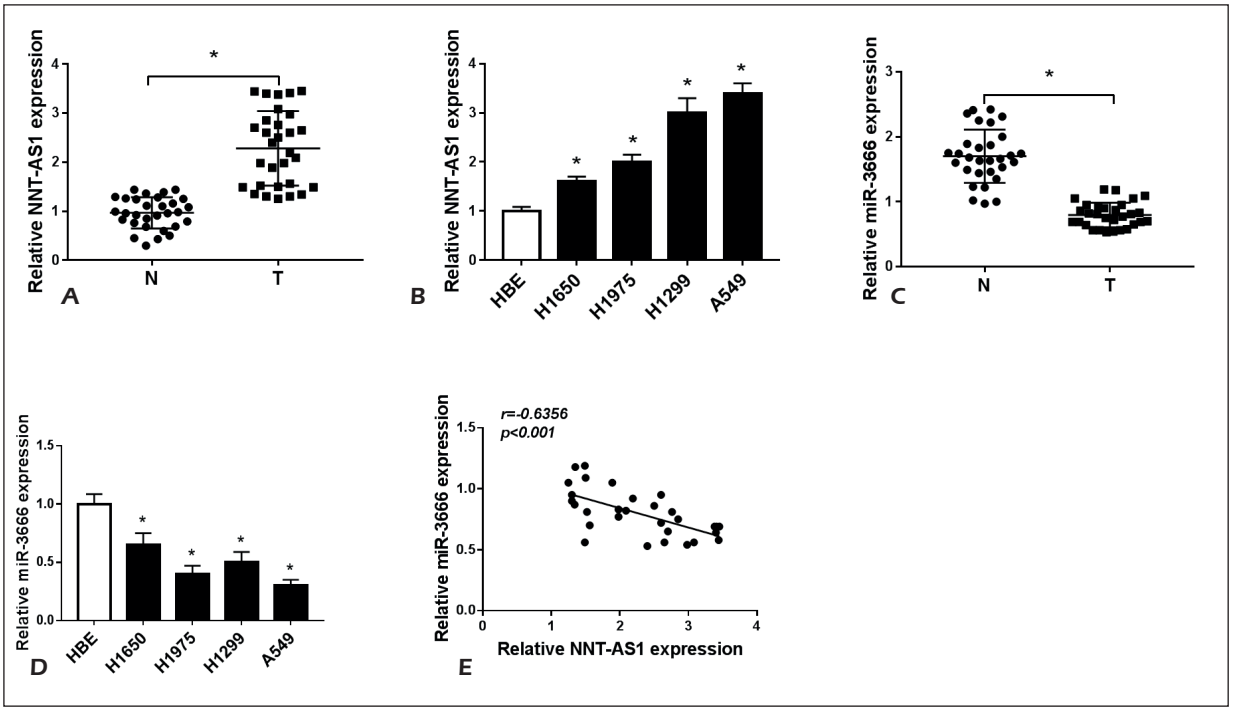

Figure 1. NNT-AS1 was up-regulated but miR-3666 was down-regulated in lung cancer tissues and cells. **A**-**B**, The expression of NNT-AS1 was measured by qRT-PCR in tumor tissues and lung cancer cell lines (H1650, H1975, H1299 and A549) relative to normal tissues and healthy cells, respectively. **C**-**D**, The expression of miR-3666 was also detected by qRT-PCR in tumor tissues and lung cancer cells. **E**, The correlation between miR-3666 and NNT-AS1 was analyzed by Spearman's correlation coefficient. *p<0.05.

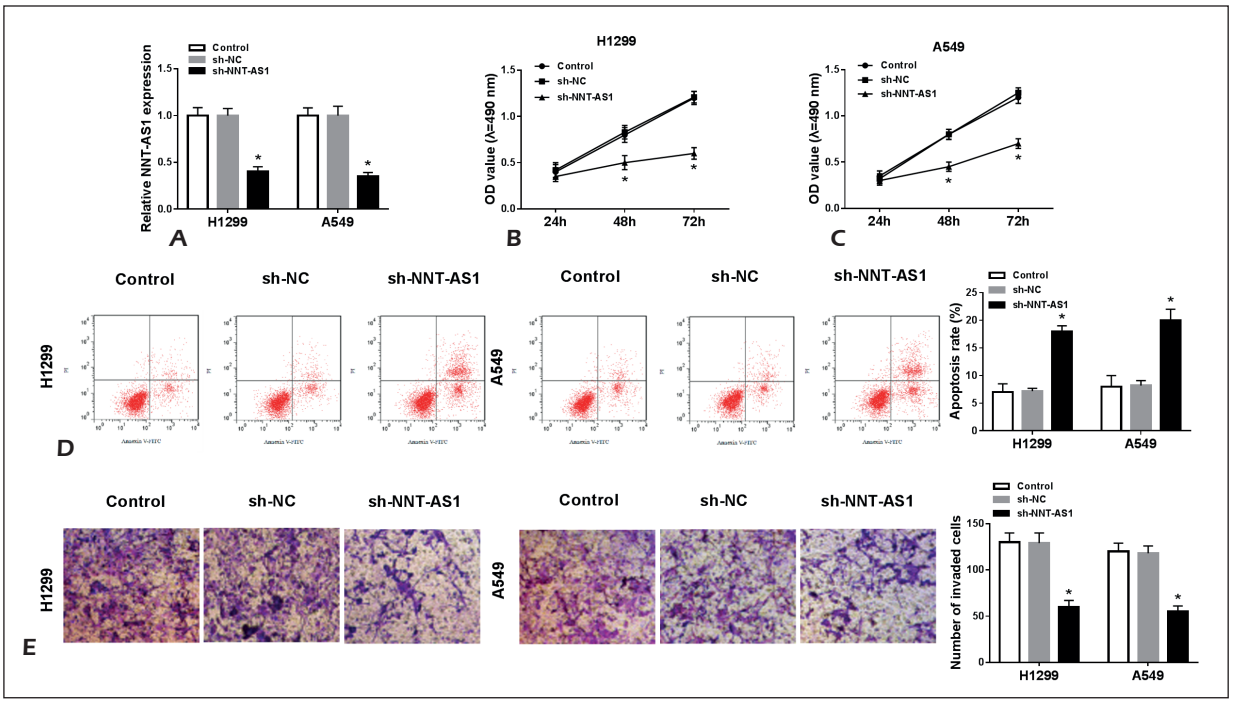

Figure 2. NNT-AS1 knockdown inhibited proliferation and invasion but promoted apoptosis of lung cancer cells. **A**, The efficiency of NNT-AS1 knockdown was assessed by qRT-PCR. **B-C**, The proliferation of H1299 and A549 cells was investigated by MTT assay. **D**, The apoptosis of H1299 and A549 cells was evaluated by flow cytometry. E, The invasion of H1299 and A549 cells was described by transwell assay (200x). *p<0.05.

NNT-AS1 Directly Interacted with MiR-3666, and MiR-3666 Inhibition Reversed the Effects of NNT-AS1 Knockdown in Lung Cancer Cells

To explore the underlying action mechanism of NNT-AS1 in lung cancer, the relationship between NNT-AS1 and miR-3666 was predicted and verified. As shown in Figure 3A, the binding sites between NNT-AS1 and miR-3666 were analyzed by bioinformatics tool starBase v2.0. Dual-Luciferase reporter assay presented that the Luciferase activity was prominently declined in H1299 and A549 cells transfected with WT-NNT-AS1 and miR-3666, while the Luciferase activity had no difference in cells transfected with MUT-NNT-AS1 and miR-3666 or miR-NC (Figure 3B and 3C). The analysis of qRT-PCR showed that the expression of miR-3666 was considerably strengthened in H1299 and A549 cells transfected with sh-NNT-AS1 but sharply dwindled in cells with NNT-AS1 transfection (Figure 3D). Then, H1299 and A549 cells were introduced with sh-NNT-AS1, sh-NC, sh-NNT-AS1+anti-miR-5666, and sh-NNT-AS1+miR-NC, respectively. It could be observed that the expression of miR-5666, induced by sh-NNT-AS1, was suppressed by sh-NNT-AS1+anti-miR-3666 (Figure 3E). The ability of cell proliferation was blocked by sh-NNT-AS1 but rescued by sh-NNT-AS1+anti-miR-3666 (Figure 3F and 3G). The cell apoptosis rate, promoted by sh-NNT-AS1, was depleted by sh-NNT-AS1+anti-miR-3666 (Figure 3H). The number of invaded cells was decreased in the sh-NNT-AS1 group but restored in the sh-NNT-AS1+anti-miR-3666 group (Figure 3I). These data proved that NNT-AS1 knockdown inhibited the malignant activities of lung cancer cells *via* up-regulating miR-3666.

E2F2 Was a Target of MiR-3666

To further explore the action mechanism of NNT-AS1 in lung cancer, the target mRNAs of miR-3666 were identified. As exhibited in Figure 4A, some binding sites between miR-3666 and E2F2 3' UTR were analyzed by online tool starBase v2.0. Next, we found that the Luciferase activity was pronouncedly declined in H1299 and A549 cells transfected with E2F2 3' UTR-WT and miR-3666 compared to miR-NC. However, the Luciferase activity did not alter in cells transfected with E2F2 3' UTR-MUT and miR-3666 or miR-NC (Figure 4B and 4C). Western blot analysis elucidated that the expression of E2F2 was reinforced with the decrease of miR-3666 expression but weakened with the increased of miR-

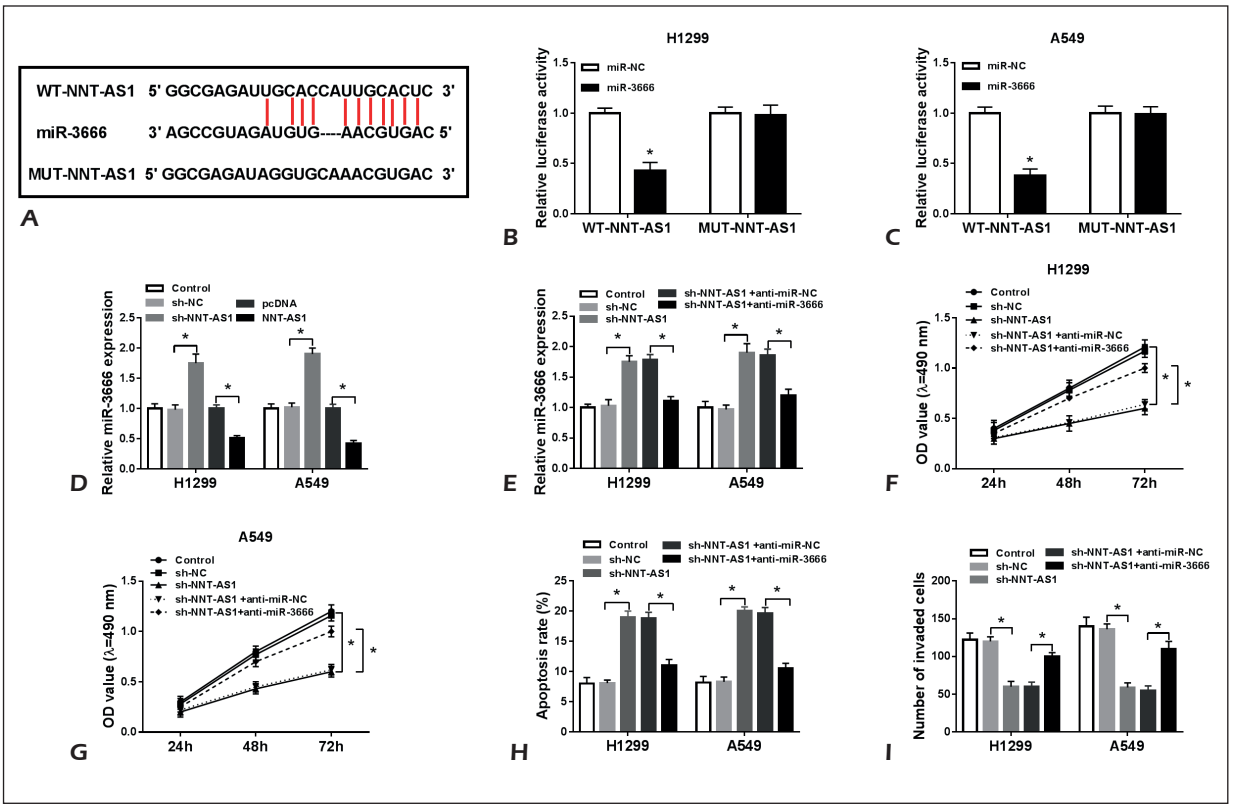

Figure 3. MiR-3666 was a target of NNT-AS1, and its inhibition reversed the effects of NNT-AS1 knockdown on lung cancer cells. A, The binding sites between NNT-AS1 and miR-3666 were predicted by bioinformatics tool starBase v2.0. **B**-**C**, The relationship between NNT-AS1 and miR-3666 was verified by Dual-Luciferase reporter assay. **D**, The expression of miR-3666 was measured by qRT-PCR in H1299 and A549 cells with NNT-AS1 knockdown or overexpression. H1299 and A549 cells were introduced with sh-NNT-AS1 or sh-NNT-AS1+anti-miR-3666, sh-NC or sh-NNT-AS1+anti-miR-NC as the control. The (**E**) expression of miR-3666, (**F**-**G**) proliferation, (**H**) apoptosis and (**H**) invasion were detected by qRT-PCR, MTT assay, flow cytometry and transwell assay, respectively. *p<0.05.

3666 expression in H1299 and A549 cells (Figure 4D). Besides, the expression of E2F2 was markedly enhanced in lung cancer tissues relative to normal tissues at both mRNA and protein levels (Figure 4E and 4F). Spearman's correlation coefficient deemed that E2F2 expression was negatively correlated with miR-3666 expression in lung cancer tissues (Figure 4G). Additionally, the expression of E2F2 was also accelerated in H1299 and A549 cells compared to HBE cell at both mRNA and protein levels (Figure 4H and 4I). Collectively, E2F2, a target of miR-3666, was upregulated in lung cancer tissues and cells.

E2F2 Overexpression Overturned the Impacts of MiR-3666 Enrichment in Lung Cancer Cells

To detect the function of the interaction between miR-3666 and E2F2, miR-3666 and miR-3666+E2F2 were introduced into H1299 and A549 cells, respectively, miR-NC or miR-3666+pcDNA as the control. The expression of E2F2 was checked at first and we observed that E2F2 was conspicuously downregulated in H1299 and A549 cells with miR-3666 transfection from the protein level, while it was swiftly upregulated in cells transfected with miR-3666+E2F2 (Figure 5A). Afterwards, MTT assay showed that the cell proliferation, suppressed by miR-3666 overexpression, was stimulated by miR-3666+E2F2 transfection in H1299 and A549 cells (Figure 5B and 5C). Flow cytometry assay showed that the apoptosis rate was elevated in the miR-3666 group but blocked in the miR-3666+E2F2 group (Figure 5D). Transwell assay demonstrated that the number of invaded cells was decreased with the overexpression of miR-3666. However, E2F2 overexpression strengthened the number of invaded cells together with miR-3666 overexpression (Figure 5E). These explorations established that miR-3666 enrichment alleviated lung cancer development by inhibiting the expression of E2F2.

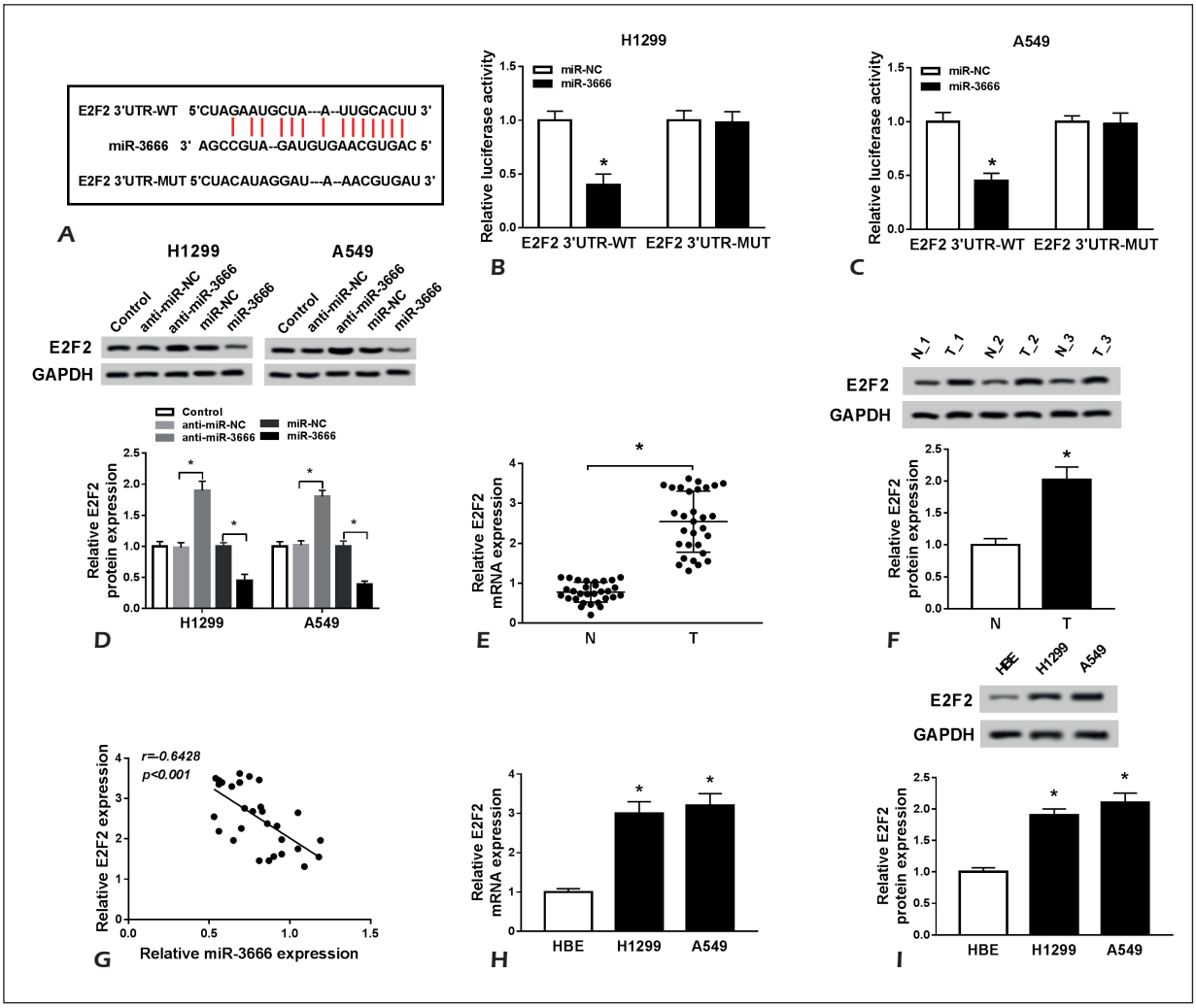

Figure 4. E2F2 was a target of miR-3666, and it was highly expressed in lung cancer tissues and cells. **A**, The binding sites between E2F2 3' UTR and miR-3666 were analyzed by starBase v2.0. **B**-**C**, The relationship between E2F2 and miR-3666 was confirmed by Dual-Luciferase reporter assay. **D**, The expression of E2F2 at the protein level was quantified by Western blot in H1299 and A549 cells transfected with anti-miR-3666 or miR-3666. **E**-**F**, The expression of E2F2 at mRNA and protein levels was assessed by qRT-PCR and Western blot in lung cancer tissues. **G**, The correlation of the expression between miR-3666 and E2F2 was analyzed by Spearman's correlation coefficient in lung cancer tissues. **H**-**I**, The expression of E2F2 at mRNA and protein levels was monitored by qRT-PCR and Western blot in lung cancer cells. *$p<0.05$.

NNT-AS1 Regulated E2F2 Expression by Sponging MiR-3666

To investigate whether E2F2 was regulated by NNT-AS1 *via* miR-3666, the expression of E2F2 was examined in H1299 and A549 cells transfected with miR-3666 and miR-3666+NNT-AS1, respectively, miR-NC or miR-3666+pcDNA as the control. As depicted in Figure 6A and Figure 6B, the expression of E2F2 at the protein level was strikingly reduced in cells with miR-3666 transfection, while E2F2 expression was restored in cells transfected with miR-3666+NNT-AS1. The data indicated that NNT-AS1 promoted the expression of E2F2 by adsorbing miR-3666 in lung cancer cells.

Discussion

With the improvement and development of diagnostic imaging, staging classification, and chemotherapy, the surgical mortality of lung cancer has been effectively controlled. However, the overall survival rate within five years after surgery is still weak[4]. Hence, the exploration of the mechanism of the occurrence and metastasis in lung cancer is valuable for the safety of human lives. Here, we found that NNT-AS1 was upregulated in lung cancer tissues and cells. NNT-AS1 knockdown inhibited proliferation and invasion but induced apoptosis of lung cancer cells. It was

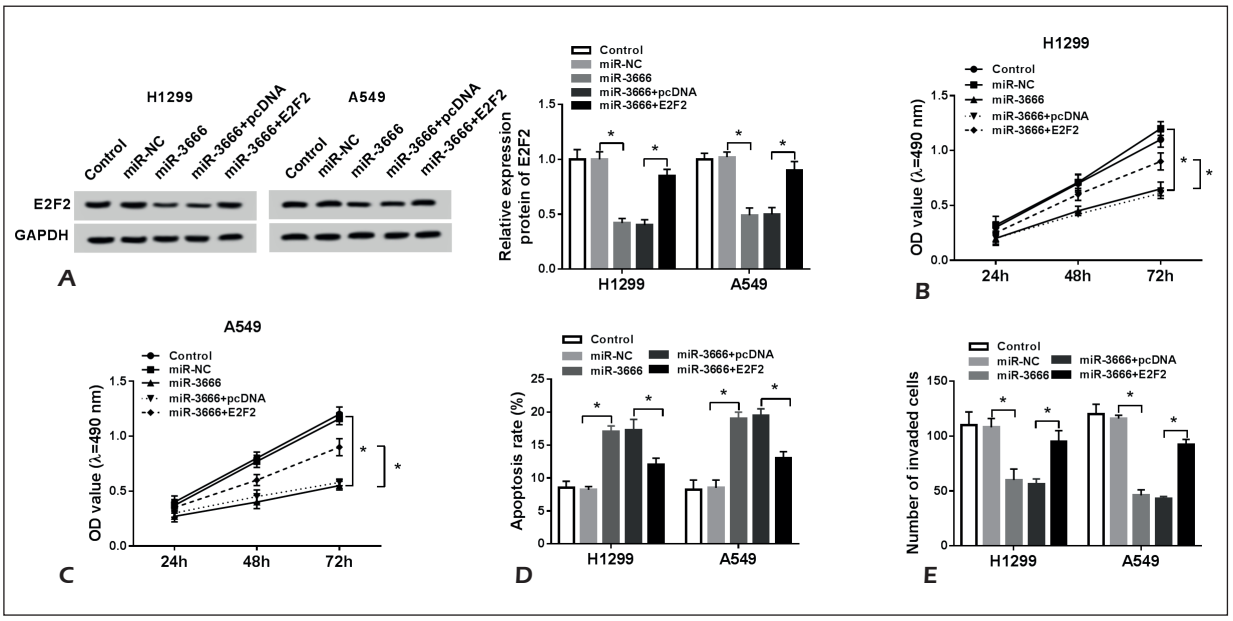

Figure 5. E2F2 overexpression reversed the impacts of miR-3666 enrichment in lung cancer cells. H1299 and A549 cells were introduced with miR-3666 and miR-3666+E2F2, respectively, miR-NC or miR-3666+pcDNA as the control. The (**A**) expression of E2F2 at the protein level, (**B**-**C**) cell proliferation, (**D**) cell apoptosis and (**E**) cell invasion were detected by Western blot, MTT assay, flow cytometry and transwell assay, respectively. *$p<0.05$.

confirmed that miR-3666 was a target of NNT-AS1, and E2F2 was a target of miR-3666. The inhibition of miR-3666 reversed the effects of NNT-AS1 knockdown, and E2F2 overexpression reversed the impacts of miR-3666 enrichment. We also proved that NNT-AS1 regulated the expression of E2F2 by mediating miR-3666. Our study put forward the role of NNT-AS1/miR-3666/E2F2 axis in the progression of lung cancer.

NNT-AS1 was frequently reported to be overexpressed in osteosarcoma[16], gastric cancer[17,23], and breast cancer[24]. NNT-AS1 exerted its role by acting as an oncogene to promote proliferation, migration, invasion, colony formation, and epithelial-mesenchymal transition (EMT) of cancer cells[24]. EMT was a process by which epithelial cells got rid of numerous epithelial traits and acquire various characteristics of mesenchymal cells[25]. Cancer cells became motorized and invasive by EMT procedure, and EMT is a key process during the onset of cancer metastasis[26]. An existing study illustrated that NNT-AS1 was expressed with a high level in tissues and cells of NSCLC, and its knockdown depleted proliferation and invasion of cancer cells[27]. Consistent with this report, the abundance of NNT-AS1 was also enhanced in cancer tissues and cells. Loss-function of NNT-AS1 suppressed proliferation and invasion but induced apoptosis of lung cancer cells. Besides, another report claimed that NNT-AS1 was upregulated in NSCLC cells with drug-resistance. NNT-AS1 downregulation accelerated apoptosis and cycle arrest but blocked drug-resistance and proliferation of NSCLC cells[28]. These data concluded that NNT-AS1 contributed to the growth, development, and drug-resistance of lung cancer cells, suggesting that NNT-AS1 was a key indicator to measure the progression and prognosis of lung cancer.

Many studies indicate that lncRNA develops its biological function *via* competitive suppression of miRNAs by acting as a competing endogenous RNA (ceRNA), including NNT-AS1. LncRNA, NNT-AS1 participated in the development of osteosarcoma[16], gastric cancer[17], and breast cancer[24] by acting as ceRNAs of miR-320a, miR-363, and miR-142-3p, respectively. Here, miR-3666 was screened and identified as a target of NNT-AS1. Interestingly, a previous study[29] observed that miR-3666 was downregulated in tissues and cells of lung cancer. Reintroduction of miR-3666 attenuated cell proliferation, migration, and invasion by regulating EMT and PI3K-AKT signaling pathway. Shi et al[30] viewed that miR-3666 overexpression impeded the cell growth of NSCLC and induced the expression of pro-apoptotic genes. In

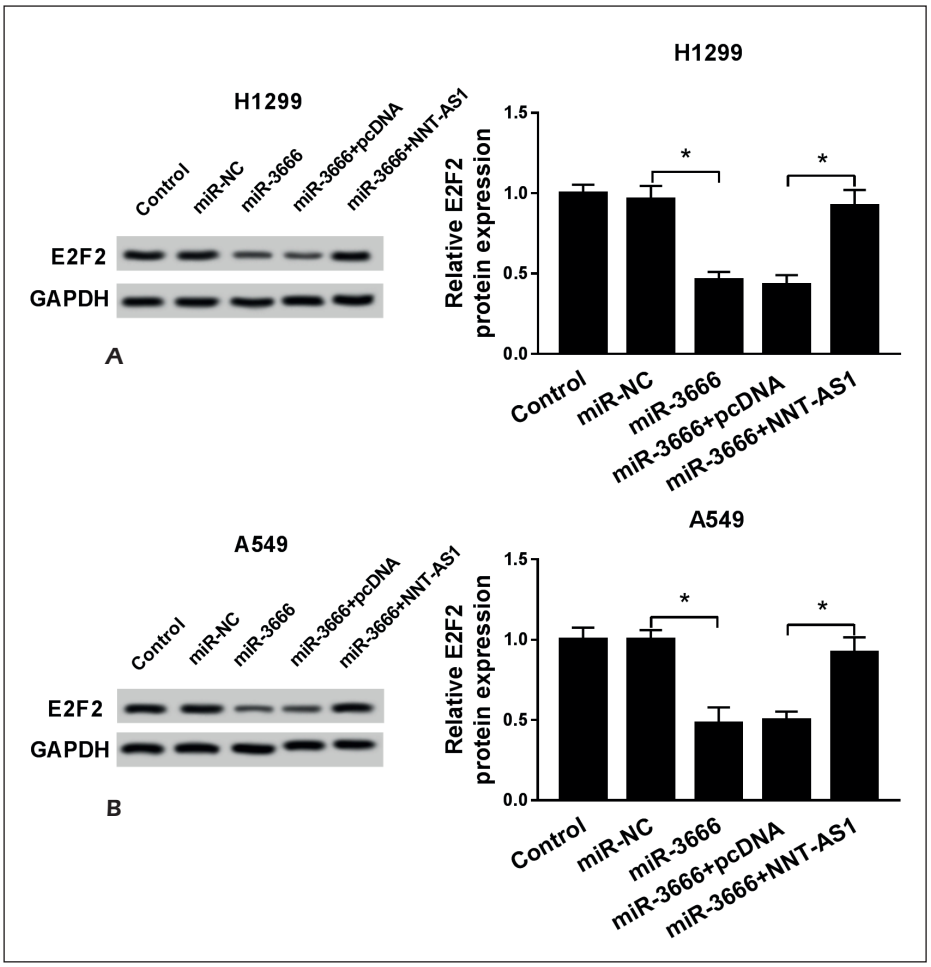

Figure 6. E2F2 was regulated by NNT-AS1 through miR-3666. H1299 and A549 cells were transfected with miR-3666 and miR-3666+NNT-AS1, respectively, miR-NC or miR-3666+pcDNA as the control. (**A**-**B**) The expression of E2F2 at the protein level was determined by Western blot after transfection. *$p<0.05$.

agreement with these prior researches, the expression of miR-3666 was diminished in lung cancer tissues and cells in our investigation, and miR-3666 inhibition overturned the impacts of NNT-AS1 knockdown in lung cancer cells. Above data hinted that miR-3666 was a promising tumor suppressor at least in lung cancer.

Generally, miRNAs exert their role *via* binding to the 3' UTR of target mRNAs. Following this manner, E2F2 was confirmed as a target of miR-3666. E2F2 has been mentioned in the progression of lung cancer by acting as a target gene of the upstream miRNAs. E2F2, targeted by miR-936, was upregulated in NSCLC, and its overexpression reversed the effects of miR-936 mimic in the development of lung cancer[31]. Similarly, E2F2 served as an oncogene to promote the progress of lung cancer by acting as a target of miR-let-7a or miR-99a[32,33]. In accordance with these findings, E2F2 was modulated by miR-3666, and its overexpression could rescue the influences of miR-3666 enrichment in lung cancer. These data implied that E2F2 was a tumor-promoter in the development process of lung cancer.

Conclusions

To sum up, the level of NNT-AS1 was aberrantly increased in lung cancer tissues and cells. NNT-AS1 knockdown was beneficial to control the proliferation and invasion of cancer cells but promote apoptosis. MiR-3666 was a target of NNT-AS1, and its inhibition overturned the effects of NNT-AS1 knockdown on the development of lung cancer. Furthermore, E2F2 was a target of miR-3666, and its expression was modulated by NNT-AS1 through miR-3666. In conclusion, our study demonstrated that NNT-AS1 regulated the progression of lung cancer through the NNT-AS1/miR-3666/E2F2 axis, providing a promising theoretical strategy for the treatment of lung cancer.

Conflict of Interests

The authors declare that they have no financial conflicts of interest.

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