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NCK1-AS1 promotes the proliferation, migration, invasion, and EMT of non-small cell lung cancer by regulating the miR-361-5p/ADAM10 axis

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Summary. Lung cancer, one of the most frequently diagnosed cancers, causes a huge number of mortalities globally. Among lung cancers, non-small cell lung cancer (NSCLC) is the most recorded. Despite accumulating research, the molecular basis of NSCLC progression remains poorly known. Therefore, we aim to assess the function of NCK1-AS1 in NSCLC and elucidate the molecular mechanism. Firstly, we quantified the NCK1-AS1 level in tumors and adjacent healthy tissues. NCK1-AS1 was significantly upregulated in NSCLC tumors, which was associated with poor prognosis in patients. Silencing NCK1-AS1 significantly inhibited the proliferation, migration, and invasion, as well as the EMT of NSCLC cell lines. Starbase bioinformatic prediction revealed that NCK1-AS1 targets miR-361-5p which acts to regulate ADAM10 gene expression. Our result showed that NCK1-AS1 upregulation markedly reduced miR-361-5p mRNA expression, while increasing ADAM10 expression. For the first time, we demonstrated that NCK1-AS1 regulates the miR-361-5p/ADAM10 axis, thereby promoting NSCLC progression. NCK1-AS1 might be developed as a therapeutic target for treating NSCLC.

Key words: Non-small cell lung cancer, NCK1-AS1, miR-361-5p, ADAM10

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Introduction

Lung cancer is a major cancer type, representing a leading cause of cancer mortality globally (Siegel et al., 2017; Bray et al., 2018). Less than 20 percent of overall survival cases of lung cancer were recorded in the last 5 years, making it a significant challenge for humanity (Zappa and Mousa, 2016). Basically, lung cancer has two primary forms dependent on its histological features: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (Herbst et al., 2008) with 85% of cases being NSCLC (Zakaria et al., 2017). The primary feature of cancer cells is metastasis, causing about 90% of death in patients with cancer. Several cellular pathways are involved in cancer metastasis including epithelial-mesenchymal transition (EMT), mostly described as the classical cancer metastasis theory (Ombrato and Malanchi, 2014). During this process, loss of apical-basal polarity, adhesion, alteration in cytoskeleton composition and acquiring fibroblasts or phenotypes of mesenchymal cells was observed in epithelial cells (Son and Moon, 2010). Treatment strategies for NSCLC including chemotherapy, radiation, surgery, and new-targeted therapy, which is primarily based on the investigation of NSCLC's molecular mechanism of development and progression, have been significantly enhanced (Antonia et al., 2016; Kazandjian et al., 2016; Lo Russo et al., 2016). Generally, the overall survival rate of NSCLC individuals remains relatively poor (Fenchel et al., 2016). Hence, NSCLC patients urgently need improved treatment strategies. Long non-coding RNA (lncRNA) are RNAs class with over 200 nucleotides in length, involved in several biological mechanisms in cancer cells, including migration, invasion, metastasis, proliferation, and apoptosis (Quinn and Chang, 2016). Recent studies have shown that lncRNAs are crucial molecular substances associated with cancer progression (Schmitt and Chang, 2016). High expression of small nucleolar RNA host

gene 1 (SNHG1) was revealed to enhance NSCLC progression (Li et al., 2018c). Also, Li et al. (2018b) reported that MALAT1 might enhance NSCLC colony formation and proliferation, while Fang et al. (2016) and Tantai et al. (2015) showed that XIST (lncRNA x-inactive specific transcript) was aberrantly expressed and plays an oncogenic role in NSCLC. Several studies have reported that microRNAs (miRNAs), non-coding RNAs comprising approximately 22 nucleotides, play a role as an oncogene or tumor suppressors (Georges et al., 2007; Nitu et al., 2017). It was revealed that miR-212-3p acts as a tumor suppressor in several cancers including intrahepatic cholangiocarcinoma, osteosarcoma, and glioblastoma (Distler et al., 1988; Ju et al., 2018). Downregulation of miR-212 was observed in NSCLC, while its overexpression was shown to inhibit NSCLC cell metastasis (Tang et al., 2017). Moreover, the low expression of the NFIB gene has been implicated in the progression of NSCLC (Becker-Santos et al., 2016). Yet, the involvement of NCK1-AS1 in NSCLC has not been validated.

This study, therefore, aims to molecularly dissect the role of NCK-AS1 in NSCLC. Starbase predicted NCK1-AS1 as a target for miR-361-5p, which acts as a tumor suppressor gene in gastric cancer, liver cancer, neuroblastoma, and lung cancer. MiR-361-5p was predicted to target the ADAM10 gene. Our result demonstrated that NCK1-AS1 promotes NSCLC development by modulating the miR-361-5p/ADAM10 axis. Hence, NCK1-AS1 might serve as a suitable biomarker for the timely diagnosis of NSCLC.

Materials and methods

Human NSCLC samples and cell lines

In total, 68 NSCLC tissues together with adjacent normal ones were acquired from patients admitted to Chinese PLA General Hospital in China. Specifically, after the surgery, NSCLC tissues were confirmed through pathological diagnosis prior to final selection. After, the samples were retrieved and stored in liquid nitrogen to snap freeze for subsequent differential expression analysis and molecular experiments. All the 68 patients (40 Males and 28 Females) involved in this study have never received chemotherapy treatment, radiotherapy or immunotherapy before the surgical operation was conducted. Also, the individual patient's consent was sought prior to the experiments, and after the approval by the Research Ethics Committee of Chinese PLA General Hospital.

Cell culture and transient transfection

For further molecular experiments, the NSCLC cell lines A549, H1229, CALU3, and CALU6 were purchased from the Shanghai Cell Bank, China, and the normal human bronchial epithelial cell line (HBE) was acquired from CHI Scientific, Inc., (Shanghai, China). Incubation of all cells was done in the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, USA) in a well-humidified environment with 5% CO_2 and a temperature of about 37°C. The mimic, inhibitor, and NC of miR-361-5p and two small interfering RNAs, si-NC and si-NCK1-AS1, were acquired from Genepharma (Shanghai, China). The vectors, pcDNA3.1-ADAM10, and pcDNA3.1, were obtained from Santa Cruz (USA). Cell transfections were done through the use of Lipofectamine 2000 (Invitrogen, USA). Following transfection, cells were collected at 72h for protein or RNA extraction.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNAs were extracted by RNAiso Plus (TaKaRa Bio, China). After quantification, 2 μg of RNA was used for reverse-transcription with Prime Script RT reagent kit (Promega, USA). QRT-PCR was run on the ABI 7500 Real-time PCR System (Applied Biosystems, USA) with SYBR Premix PrimerEraser kit (Thermo Fisher Scientific, USA). The GAPDH gene was employed as the internal control of the NCK1-AS1 and ADAM10 mRNA, while U6 served as miRNA endogenous control. After, the $2^{-\Delta\Delta Ct}$ method was employed in calculating the relative expression levels in different experimental groups and under different conditions.

Subcellular fractionation

First, RNAs from the cytoplasm and nuclear were isolated by PARIS Kit (Life Technologies, MA, USA). Then, qRT-PCR was performed to quantify NCK1 AS1 using GAPDH and U6 as controls for the amplification of NCK1 AS1 from the cytoplasm and nucleus, respectively.

Dual luciferase reporter assay

NCK1-AS1 and ADAM10 wild and mutant type sequences were subcloned into the pmirGLO luciferase reporter vector (Promega, USA) for NCK1-AS1 and ADAM10 WT and MUT establishment. Subsequently, A549 and H1229 cells were transfected with these reporter vectors with either miR-361-5p or miR-NC. After 48 hours of co-transfection, the luciferase activities in A549 and H1229 cell lines were subsequently examined using the dual Luciferase Reporter Assay Kit (Promega, USA).

RNA immunoprecipitation (RIP) assay

The Magna RNA-binding protein immuno-precipitation kit (Millipore, USA) was used for RIP to determine NCK1-AS1 enrichment in different fractions. Briefly, A549 and H1229 cells were lysed in RIP buffer and then incubated with anti-Ago2- or anti-IgG-

conjugated magnetic beads (Sigma-Aldrich, USA). Afterward, immunoprecipitated RNAs were isolated by proteinase K digestion, and qRT-PCR was carried out to evaluate NCK1-AS1 levels.

Cell counting kit 8 (CCK 8) assay

The CCK-8 assay kit (Dojindo, Japan) was employed for assessing cell proliferation following the producer's guide. Concisely, following cell seeding into a 96-well plate, CCK-8 solution (10 μL) was added at 0h, 24h, 48h, 72h, and 96h. Afterward, the 450 nm absorbance was measured. All measurements were repeated twice.

Colony formation assay

A549 and H1229 cells were placed into a 96-well plate and then cultured in RPMI medium. Following fixation in paraformaldehyde (4%) for 15 minutes, the cells were stained by crystal violet (0.2%) for 1h. Finally, the colony number was counted.

Western blot

To analyze various protein expressions in the NSCLC cell lines, the cell lines were lysed in RIPA buffer, after which the lysates were quantified by the BCA protein assay kit (Thermo Fisher Scientific, USA). The extracted proteins were applied to SDS-PAGE and then were transferred onto PVDF membranes, which were followed by incubation with primary antibodies against E-cadherin, N-cadherin, Vimentin, and ADAM10 (Cell signaling Technology, USA) overnight at 4°C and with treatment with horseradish peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific, USA) for 1h at room temperature. GAPDH was adopted as the internal control. Protein visualization was done with the use of a chemiluminescence detection kit (GE Healthcare, USA).

Transwell assay

Cell migration and invasion capabilities were examined in a 24-well transwell chamber (8 μm pore size; Coaster, USA). The cell migration assay was done by transfecting A549 and H1229 cells into the chamber (upper part) with a serum-free RPMI-1640 medium (Invitrogen, USA) while the other part of the chamber (lower) contained the RPMI-1640 medium plus FBS (10%, Hyclone USA). Following 24 hours of culture, the cells in the upper chamber were collected; while the cells migrated to the lower chamber were fixed in methanol (95%), stained with crystal violet (0.1%, Sigma-Aldrich) at room temperature, and counted under the light microscope (Olympus Corporation, Japan). Notably, for the invasion assay, the upper chamber was pre-coated with Matrigel (BD Bioscience, USA).

Biotin RNA pull down assay

Cell lysates of A549 and H1229 cells were incubated with synthesized sense and anti-sense DNA probes labeled by biotin for 3h. After, RNA complexes were isolated by streptavidin-coupled agarose beads (Invitrogen, USA) followed by the quantification by qRT-PCR.

In vivo tumor model

Animal administration was approved by the Institutional Animal Care and Use Committee of the First Medical Center, Chinese PLA General Hospital. Xenograft mouse models were established from five-week-old BALB/c nude mice (n=5 per group) obtained from Shanghai Experimental Animal Center (Shanghai, China). A549 and H1229 cells ($2x10^6$) stably transfected with sh-NCK1-AS1 or scramble were placed in PBS (150 μL) and were administered subcutaneously to the mice through the left flank for NSCLC tumor growth. Tumor growth was assessed and tumor volumes were estimated ($0.5\times length\times width^2$) every week. To evaluate tumor foci in the lungs, bioluminescent flux (photons/s/sr/cm^2) was assessed every two weeks by the IVIS spectrum imaging system (Caliper, USA) coupled with the Living Image software (Caliper, USA). After four weeks, the mice were euthanized.

Statistical analysis

The data from the experiment were analyzed using SPSS (version 18.0) software and expressed as means ± standard deviation (SD). Data from two groups and from multiple groups were compared by Student's t-test and one-way ANOVA with Tukey's post hoc test, respectively. Pearson correlation analysis was performed to assess the correlations between NCK1-AS1 and miR-361-5p. All experiments were repeated three times. *p*-values <0.05 were considered significant statistically.

Results

Upregulation of NCK1-AS1 in lung cancer tissues and cells associates with poor prognosis

This study was initiated by analyzing the differential expression of NCK1-AS1 in 68 NSCLC tumor tissues and 68 adjacent normal ones through qRT-PCR analysis. The result showed a significantly overexpressed NCK1-AS1 in NSCLC cancer tissues ($p<0.01$, Fig. 1A). For further experiments, the expression level of NCK1-AS1 in NSCLC cell lines (CALU3, CALU6, A549, and H1229) as well as the human bronchial epithelial cells (HBE) was tested. The result revealed a significantly higher expression of NCK1-AS1 in NSCLC cell lines than in HBE, with the levels of A549 and H1229 being the highest. These two cell lines were subsequently used

for various molecular experimental works (p<0.01, Fig. 1B). Taking the median expression value of NCK1-AS1 in NSCLC tissues in Figure 1A as the cut-off value, the 68 NSCLC patients were assigned to the high (n=34) and low NCK1-AS1 expression groups (n=34). Furthermore, using KM-plotter, we analyzed the prognosis of NSCLC patients in these two groups. As displayed in the survival curve plot in Figure 1C, we found that patients with a high NCK1-AS1 expression level had a significantly lower overall survival rate when compared to the survival rate of patients with low NCK1-AS1 low levels (p=0.032). Further, using the chi-square test, we assessed the relationship between NCK1-AS1 expression and NSCLC clinicopathological data. We observed that NCK1-AS1 expression was significantly associated with tumor size, TNM staging, and lymph node metastasis but not with the age, tumor differentiation and gender of patients (p<0.05, Table 1).

Knockdown NCK1-AS1 inhibits the proliferation, migration, invasion and EMT transformation of non-small cell lung cancer cells

To understand the role of NCK1-AS1 in NSCLC, we conducted a functional study by silencing NCK1-AS1 in the NSCLC cell lines using si-NCK1-AS1#1 and si-NCK1-AS1#2. As shown in Figure 2A, the si-NCK1-AS1 effectively downregulated NCK1-AS1 expression in both A549 and H1229 cell lines compared to the negative control (si-NC) group (p<0.01). Through CCK-8 assay, we observed that NCK1-AS1 knockdown significantly inhibited the viability of the two cell lines (p<0.01, Fig. 2B). Moreover, cell proliferation was significantly reduced when NCK1-AS1 expression was silenced (p<0.01, Fig. 2C). Also, the cell migrative and invasive abilities were significantly inhibited by NCK1 AS1 knockdown (p<0.01, Fig. 2D,E). Besides, we confirmed that NCK1-AS1 knockdown significantly affected the expression level of EMT-related proteins, with E-cadherin expression being upregulated while N-cadherin and Vimentin expressions were inhibited (Fig. 2F).

NCK1-AS1 endogenously targets miR-361-5p

We also carried out a nuclear and cytoplasmic separation experiment in the A549 and H1229 cells to define the subcellular localization of NCK1-AS1 in the NSCLC cells. Using qRT-PCR, we detected that NCK1-AS1 is significantly enriched in the cytoplasm (p<0.01, Fig. 3A). Furthermore, to understand the mechanism through which NCK1-AS1 exerts its regulatory role in

Table 1. Clinicopathological data of 68 NSCLC patients.

Variable	NCK1-AS1 expression		*P* value
	Low (34)	High (34)	
Age			
<60	18	15	0.467
≥60	16	19	
Gender			
Male	21	19	0.622
Female	13	15	
Tumor size (cm)			
<3	22	8	0.001
≥3	12	26	
Differentiation			
Well	14	16	0.625
Moderate-poor	20	18	
Lymph node metastasis			
Negative	21	10	0.007
Positive	13	24	
TNM Stage			
I+II	20	11	0.028
III+VI	14	23	

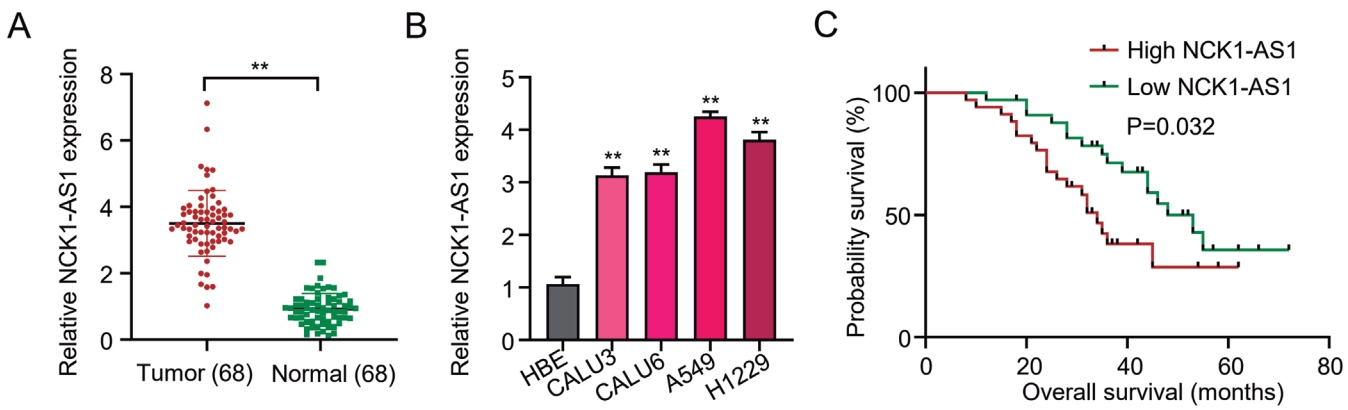

Fig. 1. NCK1-AS1 is highly expressed in lung cancer tissues and cells, and is associated with poor prognosis. The differential expression of NCK1-AS1 in lung cancer cells evaluated through qRT-PCR analysis. NCK1-AS1 was significantly overexpressed in NSCLC tumor tissues compared to the adjacent normal ones **(A)**. The significant expression of NCK1-AS1 in NSCLC cell lines assessed in comparison with HBE **(B)**. Using KM- plotter, NSCLC prognosis rate was determined. High NCK1-AS1 expression was associated with lower patient survival rate **(C)**. **p<0.01.

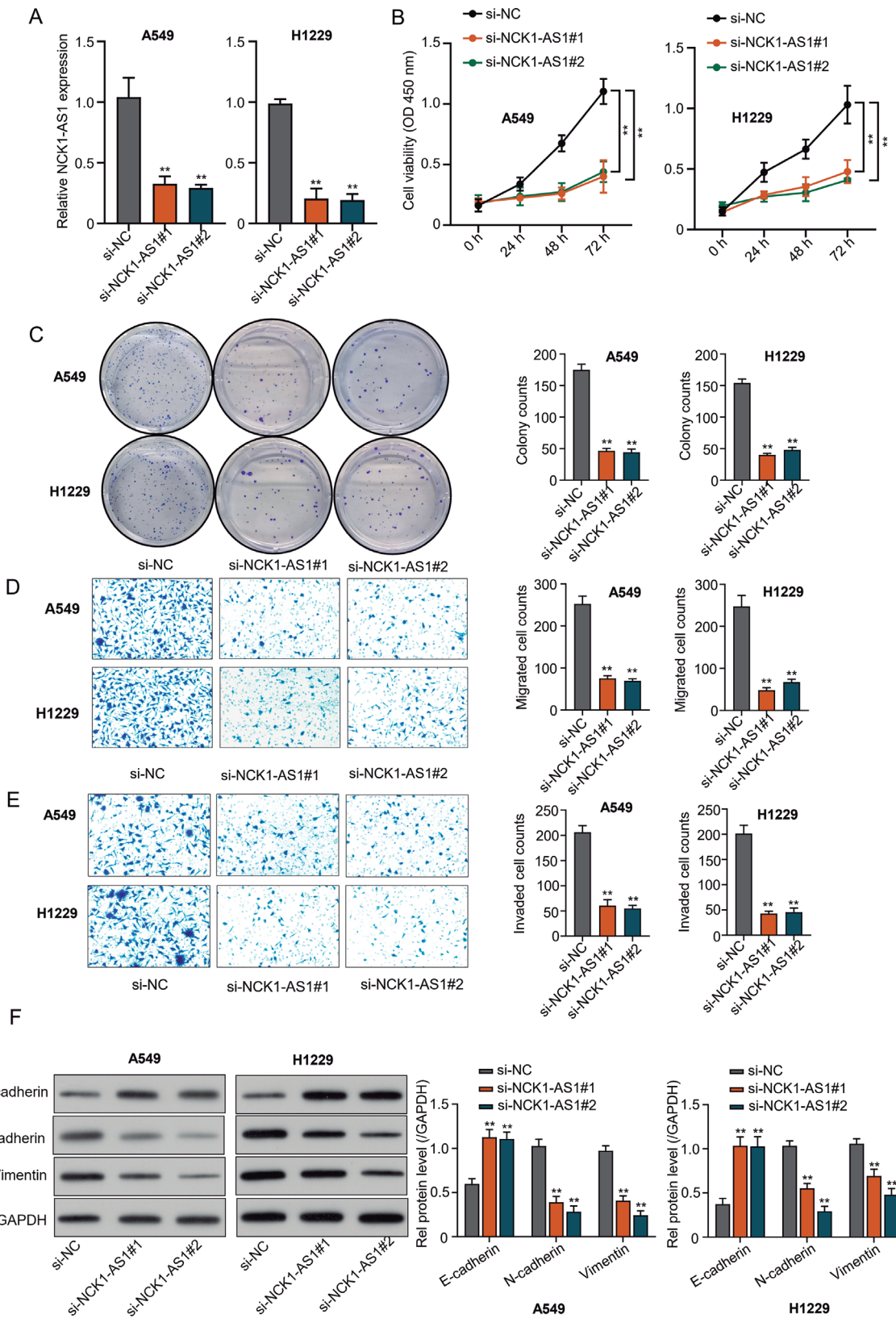

Fig. 2. NCK1-AS1 Knockdown inhibits the proliferation, migration, invasion and EMT transformation of non-small cell lung cancer cells. NCK1-AS1 expression was remarkably decreased in A549 and H1229 cell lines transfected with si-NCK1-AS **(A)**. CCK-8 method was used to detect the viability of A549 and H1229 cells after NCK1-AS1 knockdown **(B)**. Colony formation assay was done to detect the number of colonies formed in the A549 and H1229 cells showing that silencing NCK1-AS1 significantly reduced NCK1-AS1 proliferation **(C)**. si-NCK1-AS1 significantly inhibited the migration and invasion ability of A549 and H1229 cells as assessed by the transwell assay (**D, E**). Western Blot analysis confirmed that silencing NCK1-AS1 significantly upregulated E-cadherin expression in the NSCLC cell lines and repressed N-cadherin and Vimentin protein expression **(F)**. $^{**}p<0.01$.

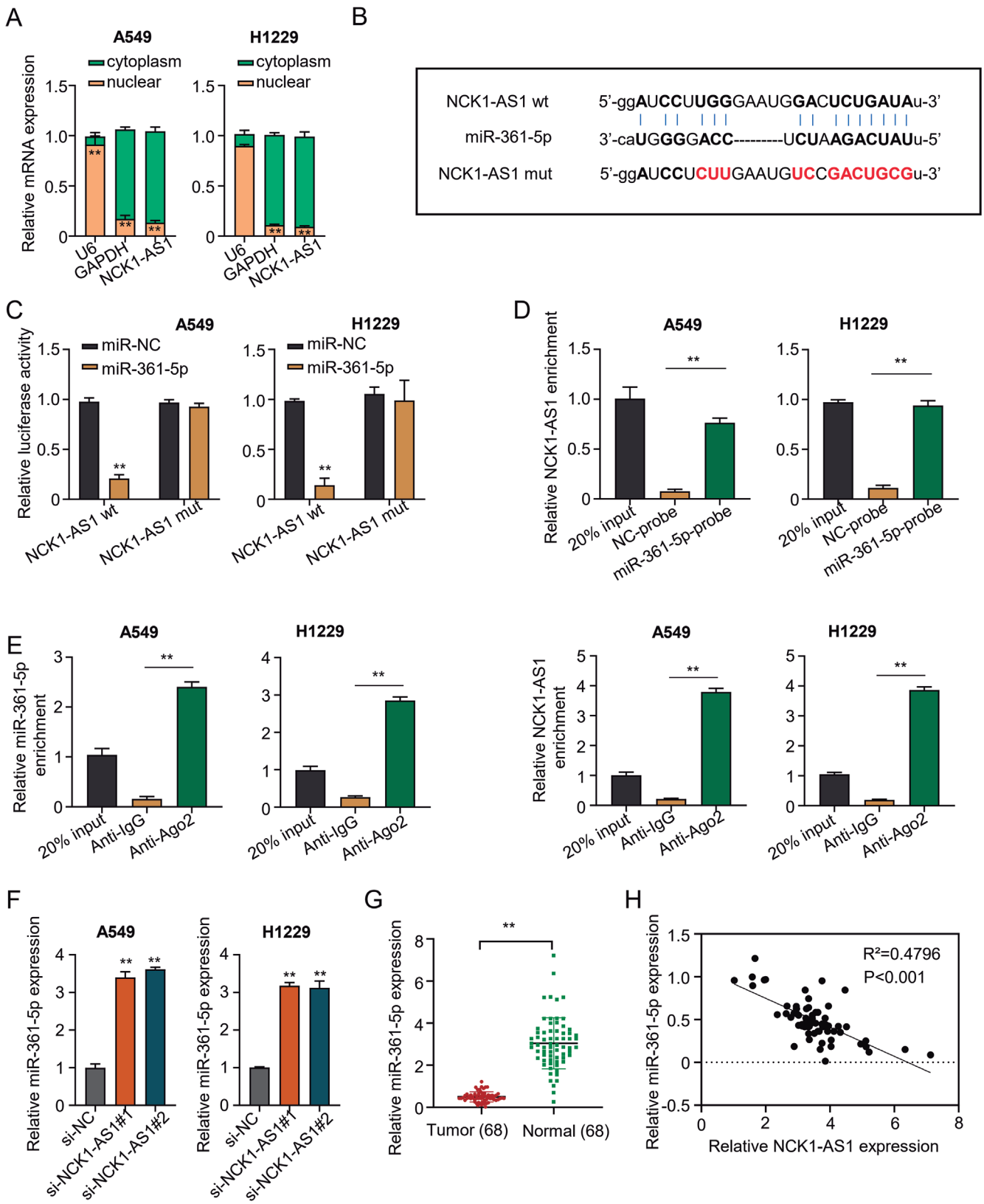

Fig. 3. NCK1-AS1 endogenously targets miR-361-5p. Subcellular localization of NCK1-AS1 in the A549 and H1229 cells was done; qRT-PCR showed that NCK1-AS1 was significantly enriched in the cytoplasm of the NSCLC cell lines **(A)**. Starbase database was used for bioinformatic prediction analysis. NCK1-AS1 can bind to miR-361-5p binding site **(B)**. Dual-luciferase reporter assay showed that miR-361-5p mimics can significantly reduce the luciferase activity of NCK1-AS1 compared to miR-NC **(C)**. Biotinylated-RNA pull down assay showed that miR-361-5p probe significantly pull down more NCK1-AS1 in the A549 and H1229 cell lines compared to the negative control group **(D)**. RIP assay confirmed the interaction between NCK1-AS1 and miR-361-5p; they were enriched in the anti-Ago2 group compared to anti-IgG **(E)**. Silencing NCK1-AS1 significantly upregulated miR-361-5p expression in NSCLC cell lines **(F)**. qRT-PCR analysis showed that miR-361-5p was significantly overexpressed in NSCLC tumor samples compared to the normal ones **(G)**. The miR-361-5p expression was negatively correlated to NCK1-AS1 expression **(H)**. $^{**}p<0.01$.

NSCLC, the Starbase database was used for bioinformatics prediction analysis. We found that NCK1-AS1 contains nucleotide sequences similar to those on the miR-361-5p binding sites (Fig. 3B). Further, we attempted to verify the prediction through a dual-luciferase reporter assay. MiR-361-5p mimics, not miR-NC, significantly inhibited the luciferase activity of the cells transfected with the NCK1-AS1 wild type (wt), whereas the level of luciferase activity of the cells was not reduced after the miR-361-5p binding site in the NCK1-AS1 was mutated ($p<0.01$, Fig. 3C). We also conducted a biotinylated-RNA pulldown assay which showed that miR-361-5p probe can significantly pull down more NCK1-AS1 in the A549 and H1229 cell

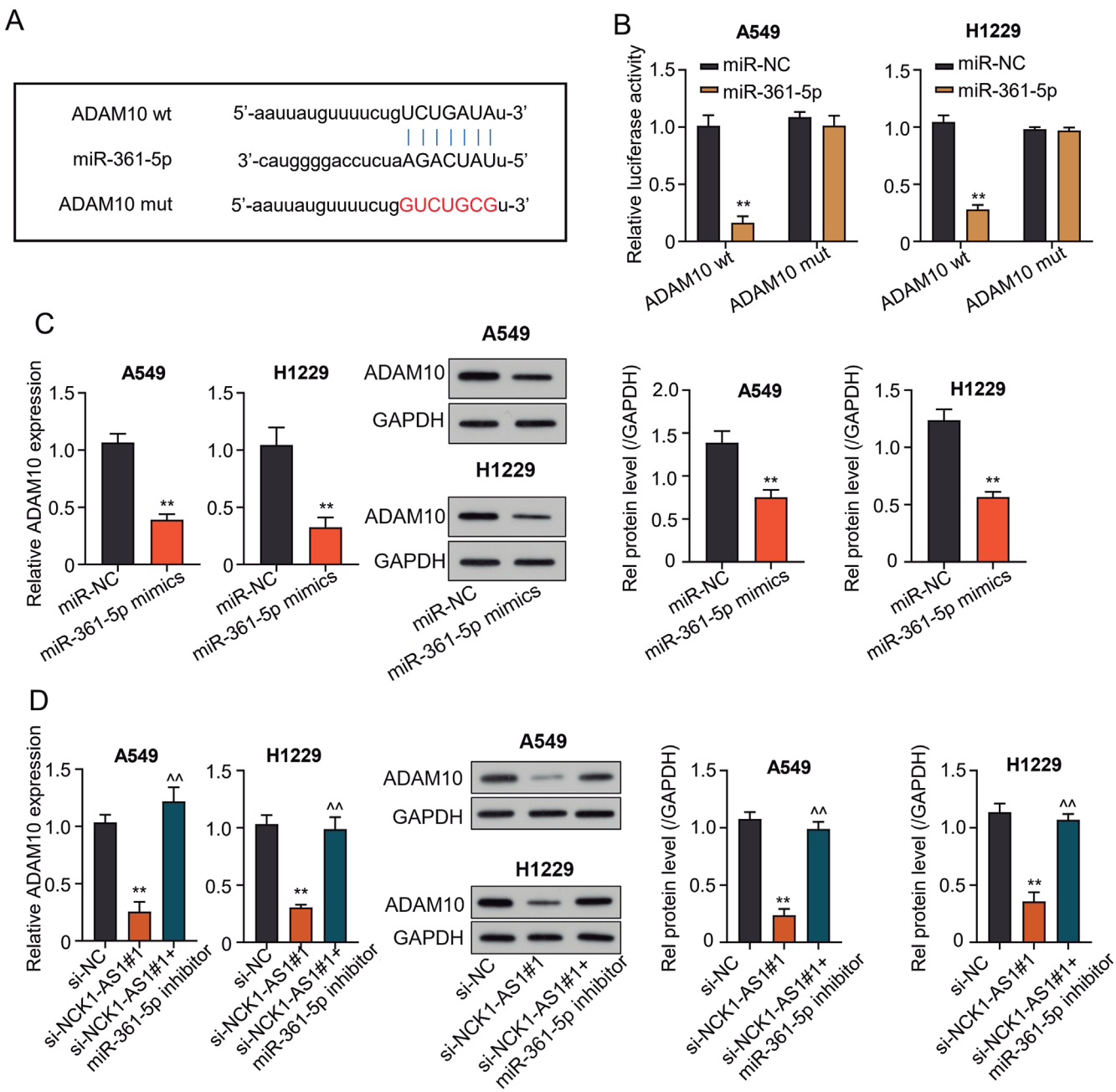

Fig. 4. MiR-361-5p targets ADAM10 in NSCLC cells. Prediction of miR-3611-5p targets gene through Starbase. MiR-3611-5p targets ADAM10 **(A)**. Dual-luciferase reporter assay showed that miR-361-5p mimics significantly inhibit the luciferase activity of ADAM10 wt cell group but had no significant effect on the ADAM10 mut cell group **(B)**. miR-361-5p mimics significantly inhibit ADAM10 mRNA and protein expression **(C)**. si-NCK1-AS1 significantly inhibits ADAM10 mRNA and protein expression level while miR-361-5p inhibitor significantly upregulated ADAM10 expression level **(D)**. $^{**}p<0.01$.

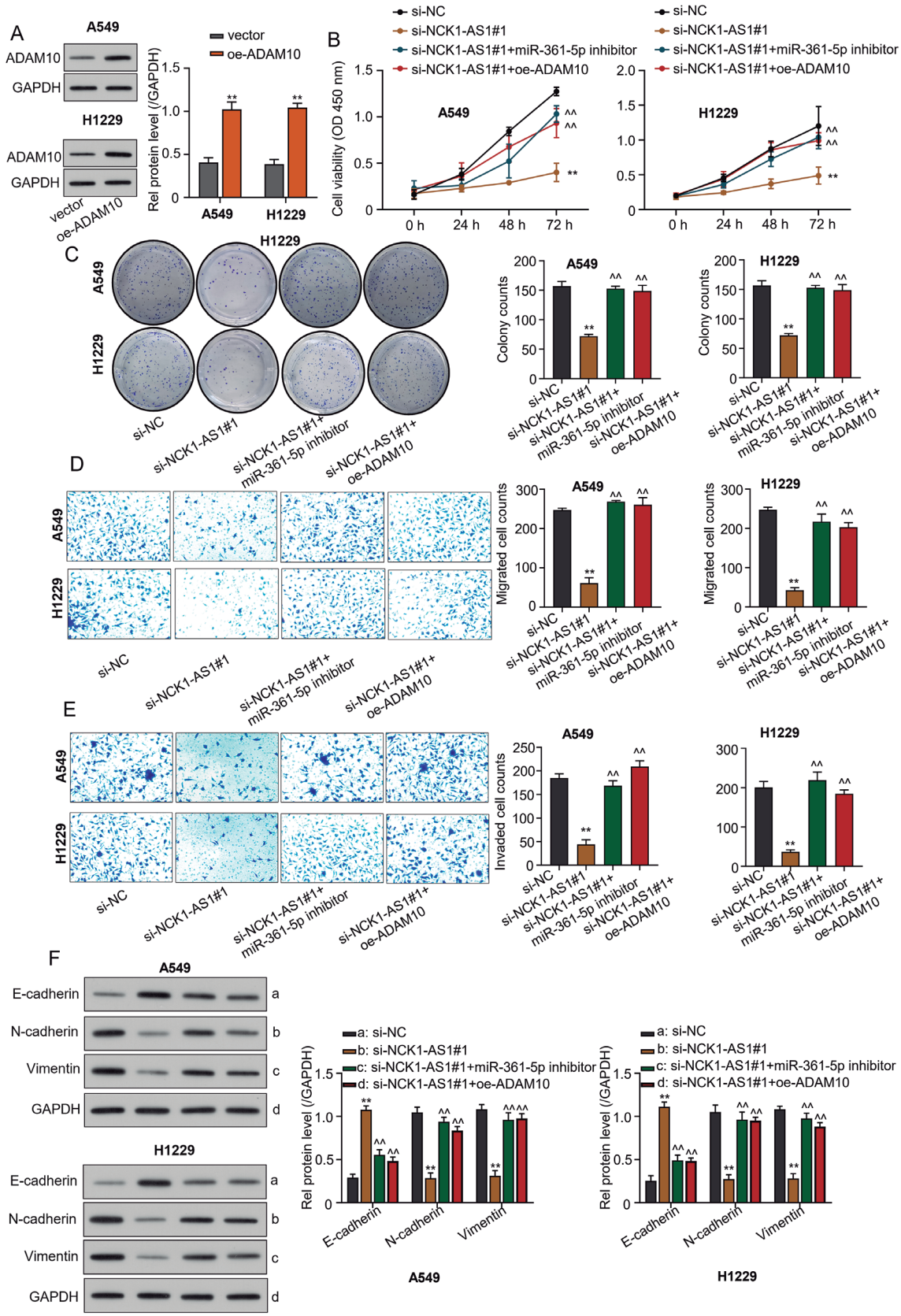

Fig. 5. NCK1-AS1 regulates the progression of non-small cell lung cancer through the miR-361-5p/ADAM10 axis. Transfection of A549 and H1229 cell lines with ADAM10 overexpression plasmid (oe-ADAM10) shows significant upregulation in ADAM10 protein expression level in the cell group as compared to the empty vector group **(A)**. CCK-8 assay revealed that overexpression of ADAM10 or the inhibition of miR-361-5p increased the viability of si-NCK1-AS-silenced NSCLC cell lines **(B)**. Overexpression of ADAM10 or inhibition of miR-361-5p in si-NCK1-AS1-knockdown cell lines restored the colony formation ability of NSCLC cell lines **(C)**. Cotransfection of si-NCK1-AS1-knockdown cell lines with either miR-361-5p inhibitor or oe-ADAM10 plasmid significantly restored the migration and invasion ability of NSCLC cell lines **(D, E)**. Western blot analysis showed that miR-361-5p inhibition and ADAM10 overexpression repressed E-cadherin expression and upregulated N-cadherin and Vimentin expression in the A549 and H1229 cell lines **(F)**. $^{**}p<0.01$; $^{\wedge\wedge}p<0.01$.

lines than the negative control (NC) probe ($p<0.01$, Fig. 3D). Besides, the RIP assay confirmed the direct interaction between NCK1-AS1 and miR-361-5p, as the enrichment of NCK1-AS1 and miR-361-5p was found in the anti-Ago2 cell group but not in the anti-IgG group ($p<0.01$, Fig. 3E). Further experiments confirmed that silencing NCK1-AS1 dramatically upregulated miR-361-5p expression in the NSCLC cells ($p<0.01$, Fig. 3F). Also, through qRT-PCR analysis, we observed that miR-361-5p was evidently upregulated in the NSCLC tumor samples relative to the adjacent normal samples ($p<0.01$, Fig. 3G). Moreover, miR-361-5p expression levels were found to be negatively correlated with NCK1-AS1 expression levels in NSCLC tumor samples ($p<0.001$, Fig. 3H).

miR-361-5p targets ADAM10 in NSCLC cells

Using Starbase, we predicted that the miR-361-5p could target the ADAM10 gene, which was subsequently confirmed by the dual-luciferase reporter assay (Fig. 4A). MiR-361-5p mimics, not miR-NC, significantly inhibited the luciferase activity of the cells transfected with the ADAM10 wild type (wt), whereas the level of luciferase activity of the cells was not reduced after the miR-361-5p binding site in the ADAM10 was mutated ($p<0.01$, Fig. 4B). In line with the prediction result, miR-361-5p mimics significantly inhibited ADAM10 expression in both A549 and H1229 cell lines ($p<0.01$, Fig. 4C). Notably, silencing NCK1-AS1 significantly decreased the ADAM10 mRNA and protein levels, while the inhibition of miR-361-5p significantly upregulated ADAM10 expression levels ($p<0.01$, Fig. 4D).

NCK1-AS1 regulates NSCLC progression through the miR-361-5p/ADAM10 axis

Subsequently, we tried to elucidate the mechanism of NSCLC progression by overexpressing ADAM10 in the NSCLC cell lines. As shown in figure 5A, the presence of ADAM10 overexpression plasmid (oe-ADAM10) markedly upregulated ADAM10 protein abundance as compared to the transfection with empty vectors. Further, the CCK-8 assay revealed that

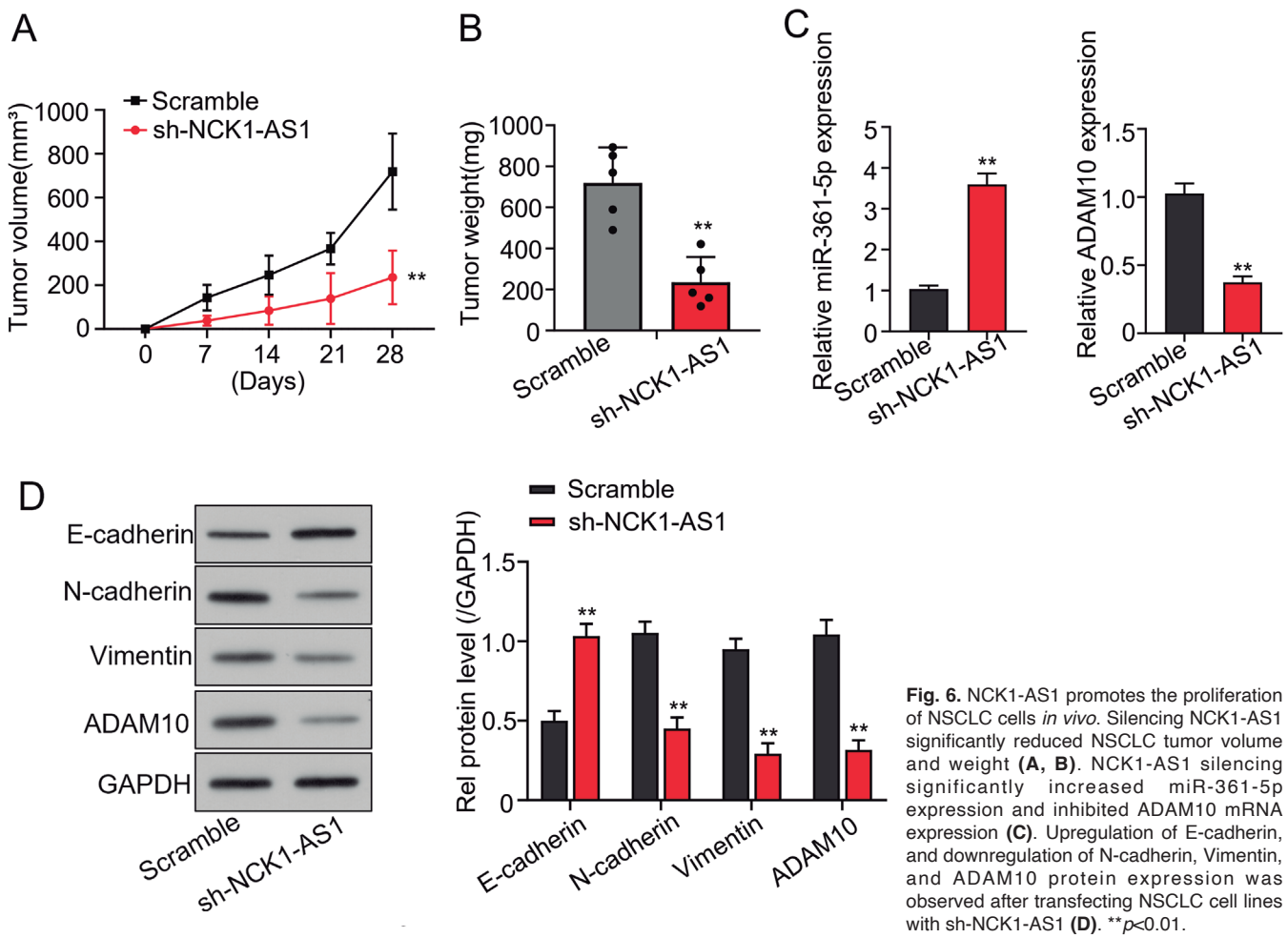

Fig. 6. NCK1-AS1 promotes the proliferation of NSCLC cells *in vivo*. Silencing NCK1-AS1 significantly reduced NSCLC tumor volume and weight **(A, B)**. NCK1-AS1 silencing significantly increased miR-361-5p expression and inhibited ADAM10 mRNA expression **(C)**. Upregulation of E-cadherin, and downregulation of N-cadherin, Vimentin, and ADAM10 protein expression was observed after transfecting NSCLC cell lines with sh-NCK1-AS1 **(D)**. $^{**}p<0.01$.

overexpressing ADAM10 and inhibiting miR-361-5p could remove the inhibition on the NSCLC cell viability by si-NCK1-AS1 ($p<0.01$: compared to si-NCK1-AS1#1 cell group, Fig. 5B). Moreover, cell proliferation was also restored by overexpressing ADAM10 and inhibiting miR-361-5p in the si-NCK1-AS1-knockdown cell lines ($p<0.01$, Fig. 5C). Furthermore, the impaired cell migration and invasion caused by si-NCK1-AS1-knockdown were significantly restored by co-transfecting with either miR-361-5p inhibitor or oe-ADAM10 plasmid ($p<0.01$, Fig. 5D,E). E-cadherin protein levels were also repressed either by miR-361-5p inhibition or ADAM10 overexpression, while the reverse was observed when the expression level of N-cadherin and Vimentin protein was measured in the A549 and H1229 cell lines (Fig. 5F). Altogether, these data suggest that NCK1-AS1 significantly regulates the proliferation, migration, invasion, and EMT of NSCLC by inhibiting miR-361-5p expression and upregulating ADAM10 gene expression in NSCLC cells.

NCK1-AS1 promotes proliferation of NSCLC cells in vivo

Finally, we developed an *in vivo* NSCLC mouse model through sh-NCK1-AS1 injection. The tumor growth (volume and weight) was measured over 28 days. The result revealed a significant decrease in the volume and weight of tumor in the group injected with the sh-NCK1-AS1 compared to that injected with scramble RNA (control group), suggesting that silencing NCK1-AS1 can significantly reduce NSCLC tumor growth ($p<0.01$, Fig. 6A,B). Interestingly, silencing NCK1-AS1 lead to a notable increase in miR-361-5p expression levels, and repression of ADAM10 mRNA expression growth ($p<0.01$, Fig. 6C). Besides, E-cadherin protein levels were markedly increased, whereas the levels of N-cadherin, Vimentin, and ADAM10 were decreased (Fig. 6D).

Discussion

The continuous increase in lung cancer-related death and its lower rate of survival (less than 20%), has created growing concern and interest in elucidating the biomarkers for early prognosis, therapeutic targets, and treatments, in order to promote the longevity of lung cancer (LC) patients (Wolters-Everhardt et al., 1987; Leakey et al., 1988). To this end, several studies have been carried out to identify putative genes or regulatory ncRNA, whose function and mechanism of regulation remain largely unknown (Peng et al., 2017; Xu et al., 2019). These ncRNAs could serve as functional biomarkers for LC detection and as therapeutic targets for drug development. Our result revealed that there was a remarkably high expression of NCK1-AS1 in NSCLC tissues, which was found to be associated with tumorigenesis, metastasis, as well as poor prognosis in NSCLC patients. This observation is identical to a recent study by Luo et al. (2020) which also observed that NCK1-AS1 was markedly upregulated in NSCLC tissues and cell lines and was significantly associated with adverse pathological signatures and poor prognosis in patients with lung cancer.

Recently, the identification of NCK1-AS1 as an oncogene has mostly been done in other cancer cells. It has been shown in cervical cancer that lncRNA upregulation of lncRNA NCK1 AS1 accelerates cervical cancer cell proliferation and induces cell cycle progression (Luo et al., 2020). Other reports showed that NCK1-AS1 upregulation in cervical cancer cells enhances cell proliferation through the induction of cell phase transition (Li et al., 2018a). Apart from acting as gene expression regulators, various lncRNAs can also reciprocate with each other to function (Blazquez Encinar et al., 1989). Interestingly, Blazquez Encinar et al. (1989) observed that the lncRNA FEZF1-AS1 promotes EMT of NSCLC by downregulating E-cadherin and regulating the WNT pathway. Another study by Qiao et al. (2020) reported that NCK1-AS1 drives cancer cell proliferation, thus increasing cell stemness in patients with urinary bladder cancer by downregulating miR-143. As a highly dynamic process, EMT has been implicated in tumor progression, metastasis, and the generation of stem cell properties which significantly contribute to the drug resistance of cancer cells (Grayzel, 1989; Nieto et al., 2016; Moustakas and de Herreros, 2017). Suppression of cell adhesion molecules such as E-cadherin and ZO-1 and upregulation of the essential components in EMT, like N-cadherin and Vimentin, have been suggested as the molecular mechanism responsible for this event (Larue and Bellacosa, 2005). In this study, we revealed that NCK1-AS1 knockdown notably increased the expression of E-cadherin, while the expression of N-cadherin and Vimentin were inhibited, suggesting that silencing NCK1-AS1 may partially regulate the abundance of EMT-induced proteins and the consequent NSCLC tumor development and progression.

More and more evidence showed that lncRNA can play a role of competitive endogenous RNA (ceRNA) by binding to miRNA, thus regulating mRNA expression and biological functions in NSCLC (Wang et al., 2021a). For instance, PVT1 promotes the proliferation, migration and invasion of NSCLC cells by indirectly mediating FGFR1 via targeting miR-551b (Wang et al., 2021b). LncRNA MEG8 promotes NSCLC progression by modulating the miR-15a-5p-miR-15b-5p/PSAT1 axis (Guo et al., 2021). Previous studies revealed that NCK1-AS1 may act as ceRNA to exert its function (Cheng et al., 2019; Qiao et al., 2020). For example, as reported, NCK1-AS1 competitively binds to miR-137 to regulate NUP43 suppression, thus, the cell proliferation was significantly decreased and the migration and invasion were suppressed by NCK1-AS1 silence (Oud et al., 1987). Moreover, NCK1-AS1 facilitates cell proliferation and migration in melanoma via targeting the miR-526b-5p/ADAM15 axis (Lin et al., 2021). Non-coding miRNAs have been observed to bind to mRNAs

through specific binding sequences to promote their degradation, through which miRNAs regulate the epithelial phenotype and EMT (Ru et al., 2012). Zhang et al. (2012) reported that miR29b and miR30a repress SNAIL1 expression, thereby reversing EMT and decreasing cell invasion. We observed in this study that the knockdown of NCK-AS1 significantly upregulated miR-361-5p expression, suggesting that miR-361-5p is a potential tumor suppressor.

Our investigation further showed that in the absence of NCK1-AS1, miR-361-5p expression is significantly increased, while ADAM10 expression levels are evidently inhibited. Oppositely, the inhibition of miR-361-5p significantly upregulated the ADAM10 mRNA and protein levels and silencing NCK1-AS1 significantly reduced NSCLC tumor growth. NCK1-AS1 has been found to upregulate TGF-β1 in prostate cancer (PC) cells and accounts for NCK1-AS1 mediation in regulating cell migration and invasion in PC (Guan et al., 2019). In sum, this study demonstrated that NCK1-AS1 acts as an oncogene to promote the tumorigenesis of NSCLC by regulating the miR-361-5p/ADAM10 axis.

Conclusion

We demonstrated that NCK1-AS1 sponges miR-361-5p and upregulates ADAM10 expression, promoting the proliferation, migration, invasion, and EMT of NSCLC cells. The critical function of NCK1-AS1 in NSCLC progression makes it a promising therapeutic target for NSCLC treatment.

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Authors' contributions. BC W, ZZ W and QW J designed the project and collected data. BC W and HL X analyzed the data and drafted the manuscript. XY C did almost all the experiments and were involved in data collection and analysis. All the authors revised and corrected the manuscript.

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