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ORIGINAL RESEARCH

Long Noncoding RNA CDKN2B-AS1 Facilitates Lung Cancer Development Through Regulating miR-378b/NR2C2

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Aim: Long noncoding RNA (lncRNA) have proved to be important regulators in various diseases. CDKN2B-AS1 was a newly identified tumor-related lncRNA, and previous studies have reported its function in laryngeal squamous cancer and osteosarcoma. However, the function and mechanism of lncRNA CDKN2B-AS1 in lung cancer are still unknown.

Methods: Cell proliferation, invasion, migration and apoptosis were detected via CCK-8, transwell assay and Western blot. Bioinformatics analysis was used to predict the potential target of CDKN2B-AS1. A rescue experiment was performed to identify the relationship between CDKN2B-AS1 and miR-378b.

Results: The expression of lncRNA CDKN2B-AS1 was significantly upregulated in lung cancer tissues and cell lines. Overexpression of CDKN2B-AS1 promoted cell proliferation, invasion and reduced cell apoptosis. Knockdown of CDKN2B-AS1 inhibited cell proliferation, invasion and increased cell apoptosis. Bioinformatics analysis predicted that miR-378b was the direct target. We also provided evidence that NR2C2 was the target of miR-378b. The expression of NR2C2 was significantly upregulated in lung cancer tissues and cell lines. The rescue experiment further confirmed the relationship between CDKN2B-AS1 and miR-378b. Overexpression of miR-378b completely reversed the function of CDKN2B-AS1.

Conclusion: Taken together, our results comprehensively analyzed the function of CDKN2B-AS1 in lung cancer and provided a possible mechanism that CDKN2B-AS1 facilitates lung cancer development by regulating miR-378b and NR2C2. Thus, our study offers a potential therapeutic target for treating lung cancer.

Keywords: lncRNA, lung cancer, CDKN2B-AS1

Introduction

Lung cancer is the most common malignant tumor worldwide and the second leading cause in cancer.[1] Many efforts and studies have been done to explore the molecular basis of lung cancer and improve the clinical diagnosis and cure rate.[2] It is reported that one-quarter of all cancer deaths are due to lung cancer.[3] There is no doubt the investigation of lung cancer is of great clinical value and social benefit.

Long noncoding RNA (lncRNA) is one type of noncoding RNA, which is longer than 200nt and without coding potential.[4] LncRNA has been widely studied in various diseases, including diabetes,[5] cardiovascular disease[6] and bone disease.[7] LncRNA can be classified as intronic lncRNA, intergenic lncRNA, promoter associated lncRNA, antisense lncRNA, and untranslated region (UTR) associated lncRNA.[8] LncRNA serve important functions, including structural or

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trafficking roles, cell differentiation and apoptosis.[9] LncRNA also have a broad range of mechanisms, including regulating the neighboring gene, miRNA-sponge action and others. Some lncRNAs have been indicated as playing a critical role in different types of malignant tumors. For example, overexpression of lncRNA IGFBP4-1 reprograms energy metabolism to promote lung cancer progression.[10] Long noncoding RNA linc00673 regulates non-small cell lung cancer proliferation, migration, invasion and epithelial mesenchymal transition by sponging miR-150-5p.[11] Thus, further research about lncRNA in lung cancer will deepen understanding of cancer development.

In previous studies, researchers have reported lncRNA CDKN2B-AS1 in different diseases. For example, long noncoding RNA CDKN2B-AS1 facilitates laryngeal squamous cell cancer through regulating the miR-497/CDK6 pathway.[12] CDKN2B-AS1 exerts oncogenic role in osteosarcoma by promoting cell proliferation and EMT.[13] However, the function and mechanism of lncRNA CDKN2B-AS1 in lung cancer remains unclear. In this study, we performed comprehensive functional analysis of lncRNA CDKN2B-AS1 and verified in two different lung cancer cell lines. Our results showed that overexpression of lncRNA CDKN2B-AS1 could promote cell proliferation and invasion. We identified miR-378b as the direct target of lncRNA CDKN2B-AS1. We also proved the antitumor role of miR-378b in lung cancer. Furthermore, we found that miR-378b played its role through regulating NR2C2 negatively. Based on the above results, lncRNA CDKN2B-AS1 induced cell proliferation and invasion, which could be reversed by the reintroduction of miR-378b. Our research unmasked the association between lncRNA CDKN2B-AS1 and miR-378b through targeting 3′-UTR of NR2C2 mRNA.

Materials and Methods

Cell Culture

Human lung cancer cell lines A549 and H1299, H226, H446 and human bronchial epithelial cells BEAS-2B were obtained from the Shanghai cell bank of the Chinese Academy of Sciences. Cells were cultured in DMEM medium or in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Life Technologies, Waltham, MA, USA) in 5% CO_2 incubator at 37 °C. The cell lines were tested for mycoplasma contamination using the Universal Mycoplasma Detection Kit (ATCC, 30–1012K) before use to ensure that they were mycoplasma-free.

Sample Collection

Lung cancer tissues and normal tissues were collected from lung cancer patients who received pulmonary lobe resection in our hospital as previously described.[14] Tissues of lung cancer were collected from patients who received surgical treatment for lung cancer and tissues of normal lung were collected from non-tumor benign diseases. None of them had experienced chemotherapy or radiotherapy before the surgery. Both histological classifications and tumor-node-metastasis (TNM) stages met the criteria of the National Comprehensive Cancer Network (NCCN). The study was approved by the Institute Research Ethics Committee of Henan chest hospital and every patient had agreed and signed the informed consent. These tissues were stored at −80 °C for further research. Detailed patient information is listed in Supplementary Table 1.

Cell Transfection

siRNA of CDKN2B-AS1 was purchased from Gene Pharma (Shanghai, China). Negative control was purchased from Santa Cruz (USA). Vectors of TUSC7 with the wild-type or mutant binding sites for miR-378b were constructed, vectors of 3′-UTR of NR2C2, with wild-type or mutant binding sites for miR-378b were constructed by Gene Pharma. The miR-378b mimics and mimics control were purchased from Gene Pharma (Shanghai, China). In brief, cell lines A549 and H1299 were transfected with different vectors with lipo2000 reagent (Invitrogen) according to the manufacturer's protocol when the cell confluence reached 30%. Detailed transfection vectors can be found in figure legends. Cells were incubated for 48h before further study.

Cell Proliferation Assay

Lung cancer cell lines H1299 and A549 were transfected with si-NC, si-CDKN2B-AS1, NC, CDKN2B-AS1, miR-378b mimics and mimics NC. Cells were seeded into 96-well plates with density of 3×10^4 cells/mL. After 48h transfection, 10μL CCK-8 reagent was added to each well at 0h, 24h, 48h, 72h and 96 h and then the optical density was measured at 450 nm after 2h incubation.

Cell Invasion Assay

A549 and H1299 cell lines' invasion ability was tested by transwell invasion assay. In brief, transwell insert chamber, coated with Matrigel (BD Biosciences, USA), was used according to the manufacturer's instruction. si-NC, si-CDKN2B-AS1, NC, CDKN2B-AS1 transfected cells were incubated in insert chambers for 24h and invaded cells were dyed with crystal violet and observed under a microscope.

Dual-Luciferase Reporter Assay

A549 and H1299 cell lines were co-transfected with wide-type CDKN2B-AS1 or mutant CDKN2B-AS1 with binding sites for miR-378b. Dual-luciferase reporter assay were conducted using a Dual Luciferase Reporter Assay System (Promega, USA) following the manufacture's protocol. Luciferase activity was measured with Multiskan Spectrum (Thermo Fisher, USA). Similarly, the vectors of 3′-UTR of NR2C2 mRNA, with wild-type or mutant binding sites for miR-387b and miR-387b mimic or mimic NC were co-transfected into A549 and H1299 cells.

Real-Time PCR

Total RNA was extracted and lysed by TRIzol reagent (Thermo Fisher, USA). RNA reverse transcription was performed using a PrimeScript™ RT reagent kit with gDNA eraser (Takara, Japan) according to the manufacturer's instructions, and cDNA was performed by qRT-PCR using SYBR® Premix Ex Taq™ (Takara, Japan). The data were normalized using β-actin levels and further analyzed by the 2−ΔΔCT method.

Western Blot

A549 and H1299 cell lines were harvested and lysed by RIPA lysis buffer containing proteinase inhibitor (Roche, USA). Total protein was quantified using BCA protein assay kit (Pierce, USA). Protein samples were resolved by 10% SDS-PAGE gel (80 V, 120 mins) and transferred to polyvinylidene difluoride (PVDF) membrane (300 mA, 90 mins). And membranes were blocked with 5% pure milk for 1 hour. Then they were incubated with primary antibodies against Ki67 (1:1000, CST, USA), PCNA (1:1000, CST, USA), Bax (1:1000, CST, USA), Bcl-2 (1:1000, CST, USA), NR2C2 (1:1000, CST, USA), β-actin (1:5000, CST, USA) at 4 °C overnight, followed by incubation with a peroxidase-conjugated goat anti-rabbit (or mouse) IgG antibody. Immunopositive bands were analyzed using a FluorChem M system (ProteinSimple, USA).

Data Analysis

We used SPSS 21.0 to calculate the values (means ± standard error of the mean (SEM). And statistical analyses were analyzed using two-sided Student's *t*-test or one-way ANOVA. The statistical significance was P<0.05.

Results

Biological Features of lncRNA CDKN2B-AS1

To study the biological features of lncRNA CDKN2B-AS1, first, we verified the expression of lncRNA CDKN2B-AS1 in lung cancer tissues and cell lines. The expression of lncRNA CDKN2B-AS1 was significantly upregulated in tumor tissues and cell lines (Figure 1A and C). Through bioinformatics analysis, we predicted that miR-378b is the direct target of lncRNA CDKN2B-AS1. The expression of miR-378b was significantly down-regulated in tumor tissues and cell lines (Figure 1B and D).

Overexpression of CDKN2B-AS1 Promoted Cell Proliferation and Invasion

To study the function of lncRNA CDKN2B-AS1, first, we constructed an overexpression vector of lncRNA

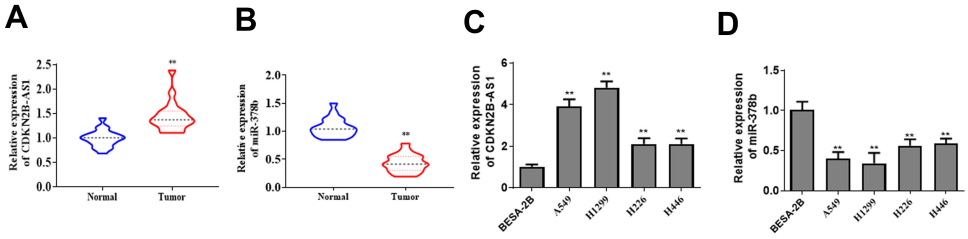

Figure 1 Biological features of lncRNA CDKN2B-AS1. (**A**) The expression of lncRNA CDKN2B-AS1 was increased in lung cancer tissues (N=20). (**B**) The expression of miR-378b was decreased in lung cancer tissues (N=20). (**C**) The expression of lncRNA CDKN2B-AS1 was increased in lung cancer cell lines. (**D**) The expression of miR-378b was decreased in lung cancer cell lines. **P<0.01.

CDKN2B-AS1. The overexpression efficiency was confirmed by real-time PCR. The expression of lncRNA CDKN2B-AS1 was upregulated in A549 and H1299 cell lines (Figure 2A). Next, invasion, proliferation and apoptosis were evaluated by transwell, CCK8 and Western blot. Our results showed that overexpression of lncRNA CDKN2B-AS1 significantly promoted cell invasion in A549 and H1299 cell lines (Figure 2B). Similarly, overexpression of lncRNA CDKN2B-AS1 promoted cell proliferation via CCK8 assay (Figure 2C). Moreover, we also provide evidence that lncRNA CDKN2B-AS1 can increase the protein level of Ki67 and PCNA, while decrease the protein level of Bax, suggesting that overexpression could also inhibit cell apoptosis (Figure 2D). Thus, our results demonstrated that lncRNA CDKN2B-AS1 could promote cancer development.

Knockdown of CDKN2B-AS1 Inhibited Cell Proliferation and Invasion

To further study the function of lncRNA CDKN2B-AS1, we also constructed a knockdown vector. The knockdown efficiency was confirmed by real-time PCR. The expression of lncRNA CDKN2B-AS1 was significantly downregulated in A549 and H1299 cell lines (Figure 3A). Transwell assay showed that knockdown of lncRNA CDKN2B-AS1 reduced the cell invasion in different cell lines (Figure 3B). Knockdown of lncRNA CDKN2B-AS1 inhibited lung cancer development, as indicated, reduced cell proliferation and increased cell apoptosis (Figure 3C). Knockdown of lncRNA CDKN2B-AS1 significantly increased the protein level of Bax, suggesting that knockdown of lncRNA CDKN2B-AS1 could promote cell apoptosis (Figure 3D). Thus, our results demonstrated that knockdown of lncRNA CDKN2B-AS1 could inhibit the progress of lung cancer.

miR-378b is the Direct Target of CDKN2B-AS1

To study the mechanism associated with lncRNA CDKN2B-AS1, we first performed a location experiment. Our results showed that lncRNA CDKN2B-AS1 and miR-378b located in cytoplasm, and GAPDH and U6 served as positive control (Figure 4A). Next, we used bioinformatics analysis to predict the potential binding target. As shown in Figure 4B, we predicted that miR-378b is the direct target of lncRNA CDKN2B-AS1. The potential binding sequence is shown in Figure 4B. Next, we constructed

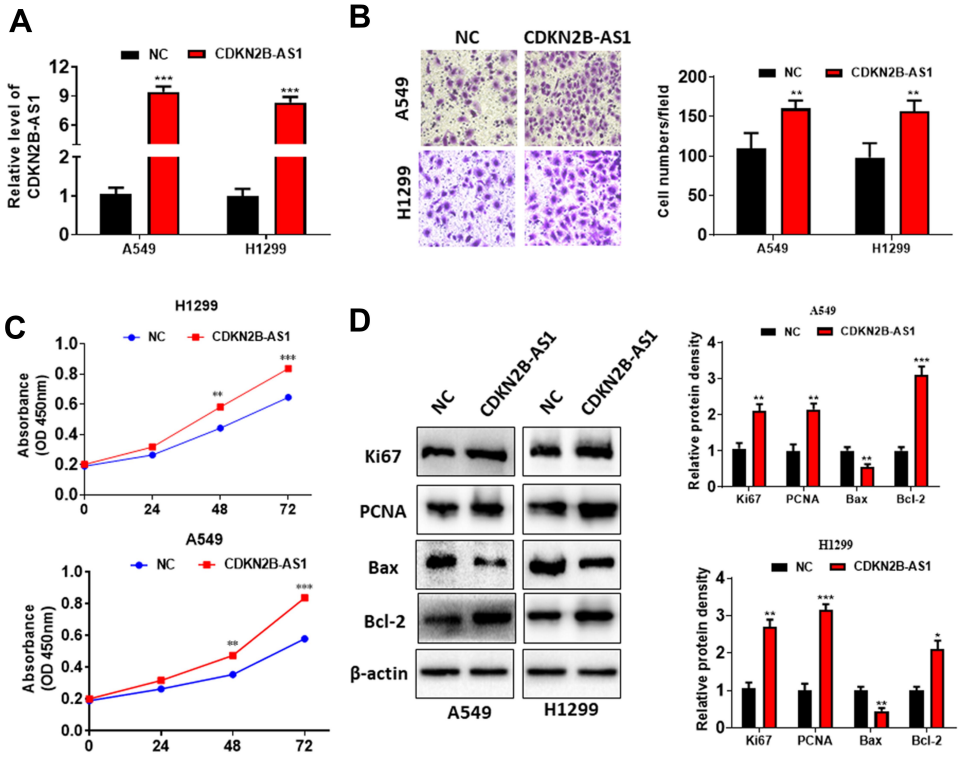

Figure 2 Overexpression of lncRNA CDKN2B-AS1 promoted lung cancer development. (**A**) Overexpression was verified by real-time PCR. (**B**) Cell invasion was assessed by transwell. (**C**) Overexpression of lncRNA CDKN2B-AS1 promoted cell proliferation in A549 and H1299 cells. (**D**) Cell proliferation and apoptosis markers were measured by Western blot. *P<0.05, **P<0.01, ***P<0.001.

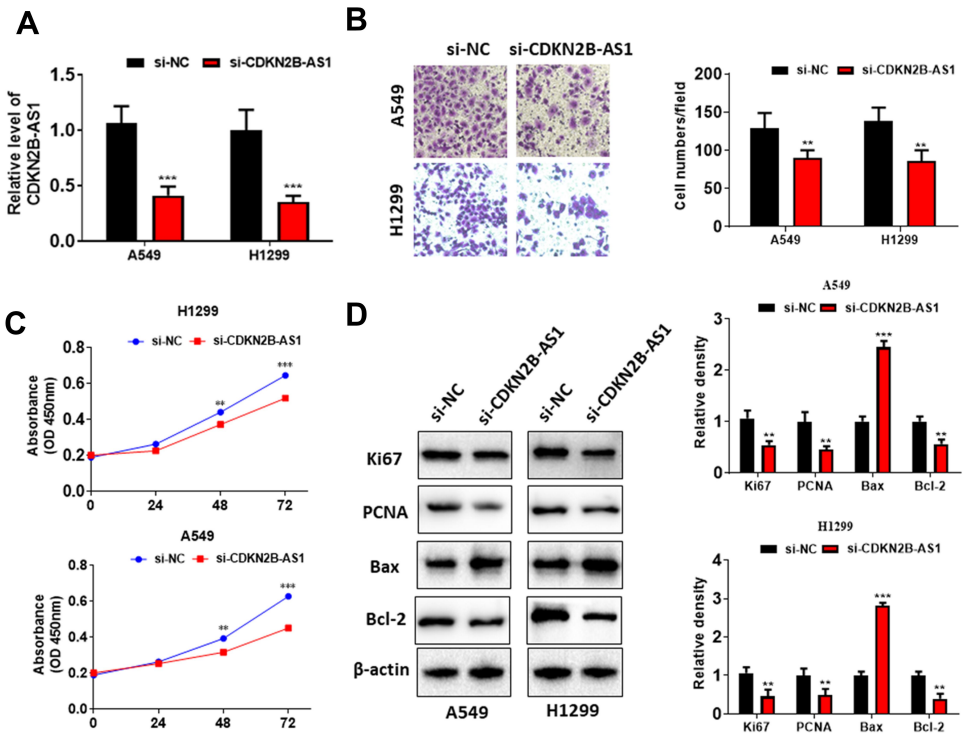

Figure 3 Knockdown of lncRNA CDKN2B-AS1 inhibited lung cancer development. (**A**) Knockdown was verified by real-time PCR. (**B**) Cell invasion was assessed by transwell. (**C**) Knockdown of lncRNA CDKN2B-AS1 inhibited cell proliferation in A549 and H1299 cells. (**D**) Cell proliferation and apoptosis markers were measured by Western blot. **P<0.01, ***P<0.001.

miR-378b overexpression mimics and NC mimics. The overexpression efficiency was confirmed by real-time PCR. The expression of miR-378b was significantly upregulated in A549 and H1299 cell lines (Figure 4C). Thus, we wondered whether miR-378b could be regulated by lncRNA CDKN2B-AS1. We performed dual-luciferase reporter assay and found that the wide type activity of lncRNA CDKN2B-AS1 was manifestly reduced after miR-378b overexpression, whereas no difference can be observed in mutant group (Figure 4D). Overexpression of lncRNA CDKN2B-AS1 could reduce the expression of miR-378b and knockdown of CDKN2B-AS1 increased the expression of miR-378b (Figure 4E). Our results also revealed that lncRNA CDKN2B-AS1 can negatively regulate the expression of miR-378b (Figure 4F). In addition, we also checked the function of miR-378b in lung cancer cell lines. Overexpression of miR-378b can inhibit the cell proliferation in A549 and H1299 cell lines (Figure 4G). Transwell assay showed that upregulation of miR-378b inhibited the cell invasion (Figure 4H)

NR2C2 is the Direct Target of miR-378b

To further explore the mechanism of miR-378b, we used bioinformatics software to predict the potential binding target of miR-378b. We noticed that NR2C2 may be the potential target of miR-378b. The potential binding sequence is shown in Figure 5A. We used dual-luciferase reporter assay to verify the relationship between miR-378b and NR2C2. The relative activity of wide type NR2C2 was significantly downregulated in miR-378b overexpression group in A549 and H1299 cell lines (Figure 5B). Overexpression of miR-378b could significantly reduce the expression of NR2C2 by real-time PCR (Figure 5C). Similar results can be achieved by Western blot. The protein level of NR2C2 was significantly downregulated in miR-378b overexpression group (Figure 5D). The expression of NR2C2 was increased in lung cancer tissues compared with normal lung tissues (Figure 5E). Next, we considered the relationship between miR-378b and lncRNA CDKN2B-AS1 and NR2C2. Overexpression of lncRNA CDKN2B-AS1 significantly increased the protein level of NR2C2, while re-introduction of miR-378b alleviated the above effect. The protein level of NR2C2 was significantly downregulated in co-transfection group of lncRNA CDKN2B-AS1 and miR-378b (Figure 5F). Similar results can be achieved by real-time PCR (Figure 5G). Lastly, we verified the relationship between lncRNA CDKN2B-AS1 and NR2C2. Our results suggested that

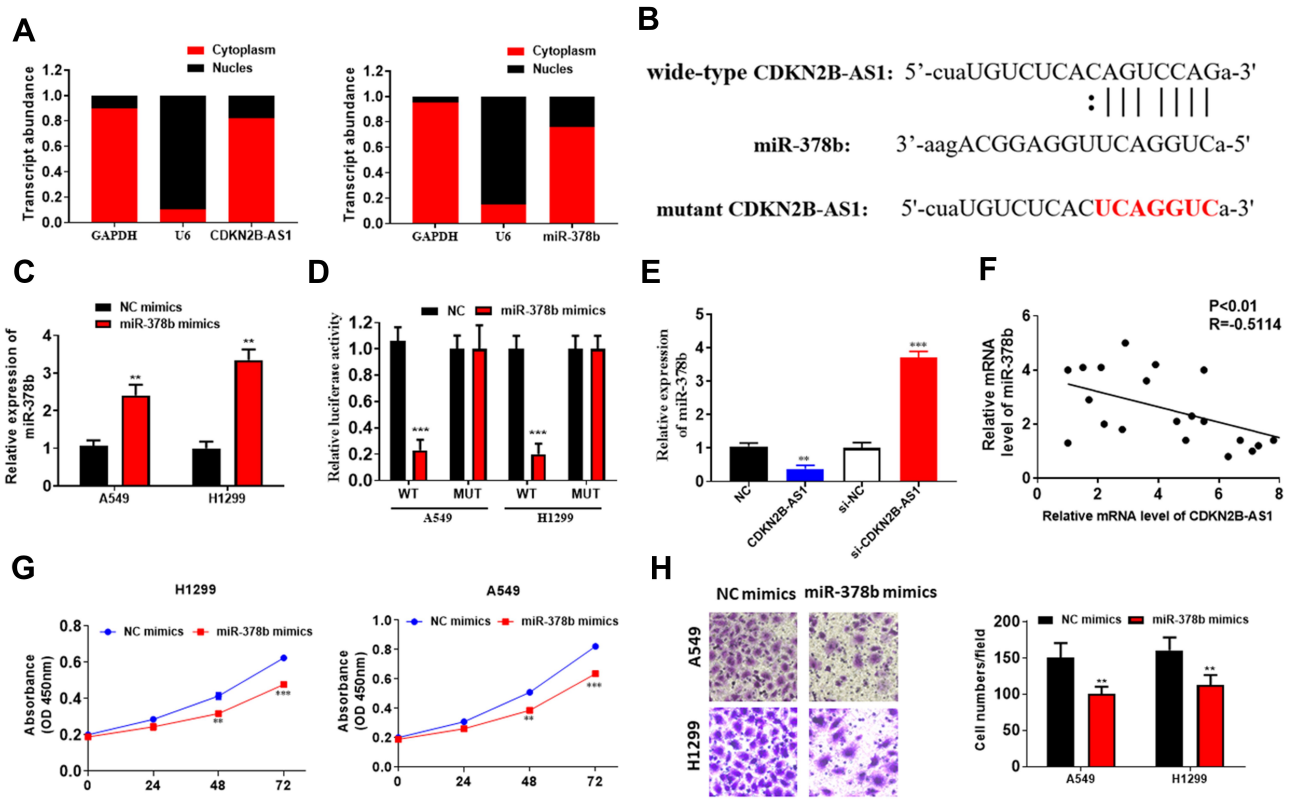

Figure 4 miR-378b was the direct target of CDKN2B-AS1. (**A**) CDKN2B-AS1 and miR-378b located in cytoplasm. (**B**) The potential binding sequence between CDKN2B-AS1 and miR-378b was shown. (**C**) Overexpression of miR-378b was verified by real-time PCR. (**D**) Dual-luciferase reporter was performed to identify the relationship between miR-378b and CDKN2B-AS1. (**E**) Overexpression of CDKN2B-AS1 reduced the expression of miR-378b and vice versa. (**F**) CDKN2B-AS1 negatively regulated the expression of miR-378b. (**G**) Overexpression of miR-378b inhibited cell proliferation in A549 and H1299 cells. (**H**) Overexpression of miR-378b reduced the cell invasion. **P<0.01, ***P<0.001.

lncRNA CDKN2B-AS1 can positively regulate the expression of NR2C2 (Figure 5H).

CDKN2B-AS1 Facilitates Cancer Development by Regulating miR-378b

Although we proved that lncRNA CDKN2B-AS1 can regulate miR-378b and NR2C2, whether the cancer development was influenced by the above relationship remains unknown. We performed a comprehensive rescue experiment to confirm the hypothesis. Overexpression of lncRNA CDKN2B-AS1 significantly increased the cell invasion number, however, co-transfection of lncRNA CDKN2B-AS1 and miR-378b blocked such an effect in A549 and H1299 cell lines (Figure 6A and B). Re-introduction of miR-378b completely reversed the pro-proliferation and anti-apoptosis effect induced by overexpression of lncRNA CDKN2B-AS1 (Figure 6C). Western blot also revealed the expression of cell proliferation markers such as PCNA, Ki-67 were also rescued by transfecting with miR-378b in A549 and H1299 which had been transfected with lncRNA CDKN2B-AS1 (Figure 6D and E).

Discussion

In this study, we investigated the function and mechanism of lncRNA CDKN2B-AS1 in lung cancer. Our results showed that the expression of lncRNA CDKN2B-AS1 was significantly upregulated in lung cancer tissues and cell lines. Overexpression of CDKN2B-AS1 was associated with increased cell proliferation activities and invasion activities. Overexpression of miR-378b completely reversed the function of CDKN2B-AS1. Taken together, our results provided a possible mechanism that CDKN2B-AS1 facilitates lung cancer development by regulating miR-378b and NR2C2. Thus, our study offers a potential therapeutic target for treating lung cancer.

The mechanisms of lncRNA can be diverse, including regulation of gene transcription, post-transcriptional regulation, and epigenetic regulation.[15] In the epigenetic regulation, ZNF667-AS1 interacts with UTX to decrease histone H3K27 tri-methylation to activate ZNF667 and

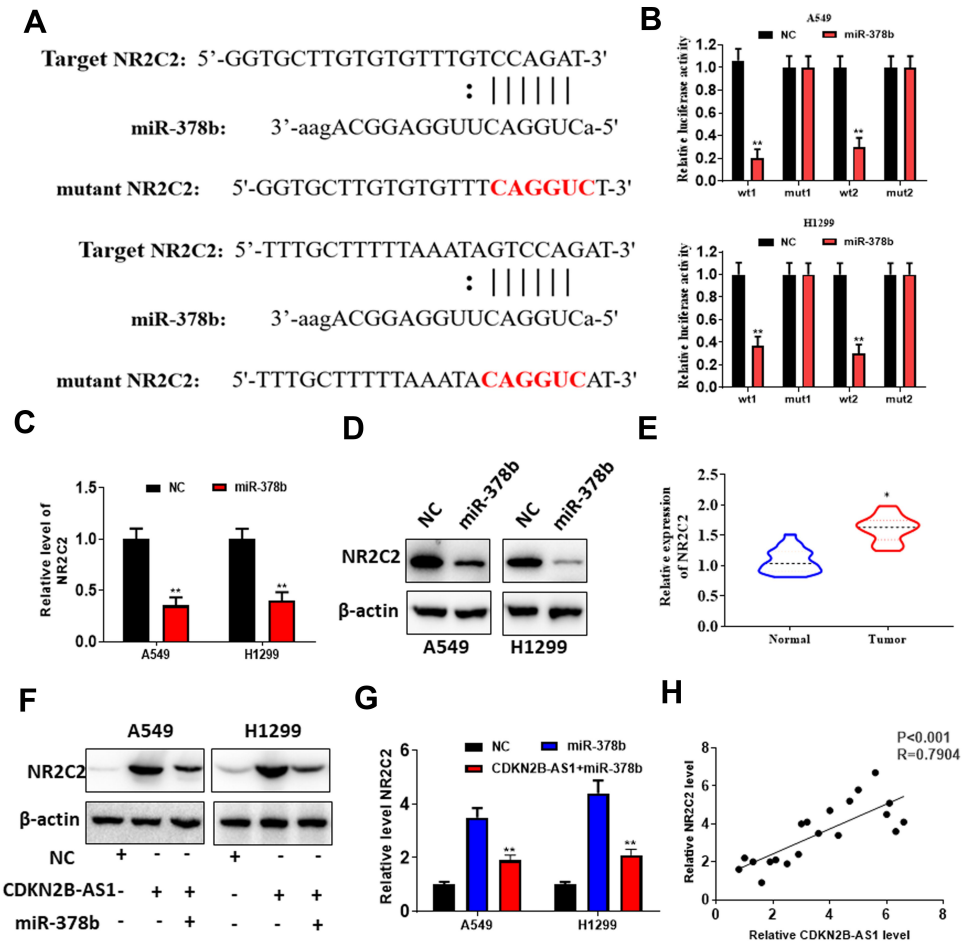

Figure 5 NR2C2 was the direct target of miR-378b. (**A**) The potential binding sequence between NR2C2 and miR-378b was shown. (**B**) Dual-luciferase reporter was performed to identify the relationship between miR-378b and NR2C2. (**C**) Overexpression of miR-378b reduced the expression of NR2C2 by real-time PCR. (**D**) Overexpression of miR-378b reduced the expression of NR2C2 by Western blot. (**E**) The expression of NR2C2 was increased in lung cancer tissues. (N=20) (**F**) Overexpression of miR-378b blocked the effect of CDKN2B-AS1 by Western blot. (**G**) Overexpression of miR-378b blocked the effect of CDKN2B-AS1 by real-time PCR. (**H**) CDKN2B-AS1 positively regulated the expression of NR2C2. *P<0.05, **P<0.01.

E-cadherin expression.[16] LncRNA LINRIS stabilizes IGF2BP2 and promotes the aerobic glycolysis in colorectal cancer.[17] In post-transcriptional regulation, lncRNAs may control various aspects of post-transcriptional mRNA processing by pairing with microRNAs and snoRNAs.[18] One of the most widely investigated mechanism of lncRNAs is that they can regulate post-transcriptional mRNA by sponging microRNAs which can target and degrade the targeted mRNAs.[19,20] For example, LncRNA ELFN1-AS1 promotes esophageal cancer progression by up-regulating GFPT1 via sponging miR-183-3p.[21] LSAMP-AS1 binds to microRNA-183-5p to suppress the progression of prostate cancer by up-regulating the tumor suppressor DCN.[22] In order to reveal the inner mechanism of lncRNA CDKN2B-AS1 in lung cancer, we detected the subcellular localization of lncRNA CDKN2B-AS1 and we found lncRNA CDKN2B-AS1 mainly located in cell cytoplasm in A549 and H1299 cells. Considering the cytoplasm lncRNA can sponge miRNA to exert its function, we used online bioinformatics analysis to predict the potential target miRNAs. We found the miR-387b may be the target according to the principle of complementary base pairing. Further analysis showed that lncRNA CDKN2B-AS1 promotes lung cancer development by negatively regulating miR-387b.

MiRNAs have been widely studied in various diseases. The function and mechanism of miRNA can be diverse. For example, miR-21 regulates vascular smooth muscle cell function in arteriosclerosis obliterans of lower extremities through AKT and ERK1/2 pathways.[23] Mir-26b inhibits growth and resistance to paclitaxel chemotherapy by silencing the CDC6 gene in gastric cancer.[24] Manipulation of miR-378b may afford a new strategy to clinical treatment of skin injury and repair.[25] In this study,

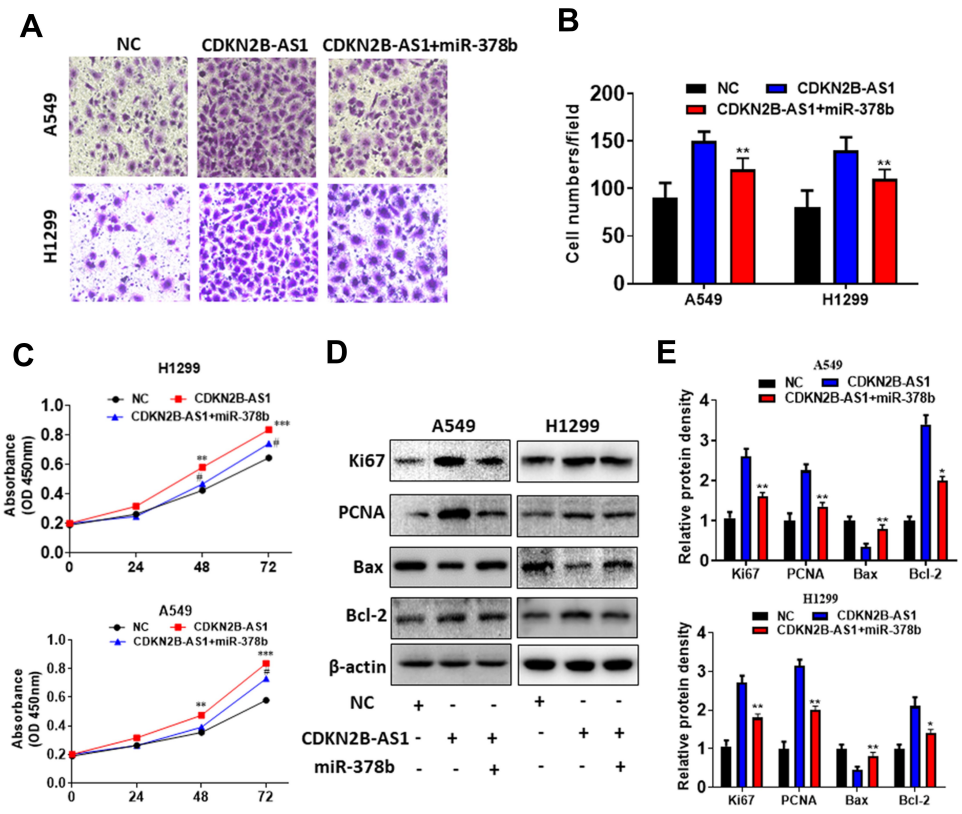

Figure 6 lncRNA CDKN2B-AS1 facilitates cancer progress through regulating miR-378b. (**A**) Overexpression of miR-378b blocked the cell invasion induced by lncRNA CDKN2B-AS1. (**B**) Quantification data were shown. (**C**) Overexpression of miR-378b rescued the pro-proliferation effect of CDKN2B-AS1 in H1299 and A549 cells. (**D**) Proliferation and apoptosis markers were measured via Western blot. (**E**) Quantification data were shown. *P<0.05, **P<0.01, ***P<0.001, #CDKN2B-AS1 VS CDKN2B-AS1+miR-378b, P<0.05.

we found that miR-378b may be the potential target of lncRNA CDKN2B-AS1 through bioinformatics analysis. Among those predicted miRNAs, we used real-time PCR to detect the expression of targets, and we found that the expression of miR-378b was significantly downregulated upon overexpression of lncRNA CDKN2B-AS1. By dual-luciferase reporter assay, we found that the relative luciferase activity was significantly reduced in the group co-transfected with miR-378b mimics and NR2C2 vectors with the wild type binding site for miR-181a mimics when compared with the NC group or mutant group which indicated the direct combination of miR-378b and 3′-UTR of NR2C2 mRNA. We also proved that NR2C2 was positively correlated with the expression of lncRNA CDKN2B-AS1 both in mRNA level and in protein level.

However, our study still has limitations. NR2C2 together with NR2C1 forms the core of the DRED (direct repeat erythroid-definitive) complex that represses embryonic and fetal globin transcription. LncRNA CDKN2B-AS1 facilitates NR2C2 expression through regulating miR-378b. Whether the formation of such complexes has also been reduced remains unknown. Hence, further study about the downstream mechanism are clearly warranted.

To sum up, we performed comprehensive analysis of lncRNA CDKN2B-AS1 and verified the function in lung cancer. We also provided complete evidence that lncRNA CDKN2B-AS1 can promote lung cancer development by regulating miR-378b and NR2C2. Our studies provide new targets for lung cancer investigation.

Data Sharing Statement

The data used to support the findings of this study are included within the article.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas. They all took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to

which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing financial or non-financial interests for this work.

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