

# Long Non-Coding RNA CDKN2B-AS1 Facilitates Laryngeal Squamous Cell Cancer Through Regulating miR-497/CDK6 Pathway

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**Background:** CDKN2B antisense RNA 1 (CDKN2B-AS1), a long noncoding RNA, was reported to play crucial roles in the progression of multiple cancers. However, the functional roles and regulatory mechanism of CDKN2B-AS1 in human laryngeal squamous cell cancer (LSCC) remain unclear. The goals of this study were to investigate biological roles and underlying mechanisms of CDKN2B-AS1 in LSCC.

**Methods:** The expressions of CDKN2B-AS1, miR-497 and cyclin-dependent kinase 6 (CDK6) were detected in LSCC tissues and cell lines by real-time quantitative PCR (qRT-PCR). The effects of CDKN2B-AS1 on LSCC cell proliferation, apoptosis, migration and invasion were examined by corresponding experiments. Bioinformatics analysis and luciferase activity assay were applied to analyze the interaction between CDKN2B-AS1 and miR-497.

**Results:** The expression of CDKN2B-AS1 was significantly higher in LSCC tissues than in adjacent normal tissues. Higher CDKN2B-AS1 was closely associated with lymph node metastasis and advanced clinical stage. Moreover, CDKN2B-AS1 knockdown by siRNA significantly inhibited the proliferation, induced cell apoptosis, and suppressed migration and invasion in LSCC cells. Mechanically, CDKN2B functions as an oncogenic lncRNA in LSCC via regulating miR-497/CDK6 axis.

**Conclusion:** The observations in this study identify CDKN2B-AS1 an oncogenic role in the tumorigenesis of LSCC by regulating miR-497/CDK6 axis and indicate that it may serve as a potential target for LSCC treatment.

**Keywords:** laryngeal squamous cell cancer, long noncoding RNA, CDKN2B-AS1, CDK6, miR-497

## Introduction

Laryngeal squamous cell carcinoma (LSCC) is the second highest incidence among the head and neck malignancies in the world.<sup>1</sup> Despite the improvement in the presently available treatments, such as surgical intervention, radiotherapy and chemotherapy, patients with LSCC have a poor prognosis because of its uncontrolled recurrence and metastasis.<sup>2,3</sup> Therefore, there is an urgent need to probe molecular mechanisms underlying the pathogenesis of LSCC for ameliorating its diagnosis and treatment.

Long noncoding RNA (lncRNAs) are class of RNA transcripts longer than 200 nucleotides in length and without protein-coding ability.<sup>4</sup> Growing evidence has suggested that lncRNAs play crucial roles in regulation of cell proliferation,

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apoptosis, differentiation and chromosome inactivation.<sup>5,6</sup> Moreover, many lncRNAs are identified to be closely associated with initiation and progression of various cancers.<sup>7,8</sup> A number of lncRNAs have emerged as crucial regulators in the physiopathology of LSCC, specifically, as potential diagnostic biomarkers or therapeutic targets for this disease.<sup>9,10</sup>

CDKN2B antisense RNA 1 (CDKN2B-AS1 also named ANRIL), a 3.8-kb lncRNA, was reported to be upregulated in tumor tissues and function as an oncogenic lncRNA in multiple cancers, such as hepatocellular carcinoma,<sup>11</sup> non-small cell lung cancer,<sup>12</sup> ovarian cancer,<sup>13</sup> cervical cancer,<sup>14</sup> pancreatic cancer,<sup>15</sup> and bladder cancer.<sup>16</sup> However, the biological function and underlying mechanism of CDKN2B-AS1 in LSCC remain unclear.

In the present study, the expression and biological function of CDKN2B-AS1 in LSCC were investigated by corresponding experiments. Then, we explored the molecular mechanism involved in regulating tumorigenesis in LSCC. The results suggested that CDKN2B-AS1 might be a novel therapeutic strategy for LSCC.

## Materials And Methods

### Patients And Specimens

This study was approved by the Research Ethics Committee of the First Hospital of Jilin University (Changchun, China), and informed consent was signed by all patients. Fifty-four pairs of LSCC tissues and matched adjacent normal tissue specimens were harvested from patients who underwent surgery in the First Hospital of Jilin University from March 2015 to March 2016. All tissues were pathologically confirmed by two pathologists. None of the patients received chemo/radiotherapy or biotherapy prior to surgery. All samples were frozen in liquid nitrogen within 5 mins after resection and then sorted at  $-80^{\circ}\text{C}$  until further use for the mRNA analysis. The clinical data of the patients are listed in Table 1.

### Cell Culture And Transfection

TU212 cells (a LSCC cell line) were purchased from American Type Culture Collection (ATCC; Manassas, VA) and were grown in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA) containing with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/mL penicillin, and 100  $\mu\text{g/mL}$  streptomycin in humidified air with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

**Table 1** Correlation Between Clinicopathological Features And CDKN2B-AS1 Expression In LSCC Tissues

Variables	No. Of Cases	CDKN2B-AS1		P-Value
		High	Low	
Age (years)				$P=0.7827$
<55	21	11	10	
$\geq 55$	33	19	14	
Gender				$P=0.2703$
Female	22	10	12	
Male	32	20	12	
Clinical stage				$P=0.0147$
I-II	43	20	23	
III-IV	11	10	1	
Primary location				$P=0.2732$
Supraglottic	26	12	14	
Glottic	28	18	10	
Lymph node metastasis				$P=0.0013$
No	40	17	23	
Yes	14	13	1	

Small interfering RNA against CDKN2B-AS1 (si-CDKN2B-AS1) and scramble siRNA (si-NC, as negative control) were designed and synthesized by GenePharma (Shanghai, China). The mimic and inhibitor of miR-497 and the negative control (NC) were synthesized by GenePharma (Shanghai, China). Overexpression CDKN2B-AS1 plasmid (pCDNA3.1-CDK6) was granted Dr. Jun Wang (Jilin University). Transient transfection of TU212 cells was done with one of the aforementioned mimics, plasmid, inhibitor or siRNA with Lipofectamine<sup>®</sup> 3000 (Invitrogen, USA) according to the manufacturer's protocol. The transfection efficiency was determined by qRT-PCR after transfection for 48 h.

### Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) was employed to extract total RNA from tissues and cultured cells following the manufacturer's instructions. The miR-497 expression level was detected by using the Taqman MicroRNA Reverse Transcription Kit and the miRNA qPCR Assay Kit (Applied Biosystems, Foster City, CA, USA). For detection of CDKN2B-AS1 or cyclin-dependent kinase 6 (CDK6) mRNA expression, complementary

DNA was synthesized from 2 µg of total RNA using cDNA Synthesis SuperMix kit (TaKaRa, Dalian, China) and then was quantified by using SYBR-Green Real-Time Master Mix (TaKaRa). All reactions were conducted using 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The expressions of CDKN2B-AS1/CDK6 and miR-497 were calculated using  $2^{-\Delta\Delta C_t}$  method and normalized to *GAPDH* and *U6*, respectively.

## Cell Fractionation

Nuclei EZ Lysis Buffer (Sigma) was used to fractionate nuclear and cytoplasmic from TU212 cells according to the manufacturer's instructions. Then, the expression of CDKN2B-AS1 in nuclear and cytoplasmic was examined using qRT-PCR. *U6* was used as the nuclear control and *GAPDH* was used as the cytoplasmic control.

## Cell Proliferation Assay

Cell proliferation was measured at 24 hrs post-transfection by a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) based on the manufacturer's instructions. Briefly, transfected cells ( $1 \times 10^3$  cells per well) were seeded into 96-well plates. At the indicated time points (24 hrs, 48 hrs and 72 hrs), 10 µL of CCK-8 solution was added to each well and cultured another 4 hrs at 37°C. The absorbance at 450 nm was measured with a Microplate Reader (Bio-Rad, Hercules, CA, USA).

## Cell Apoptosis Assay

Cells were harvested 48 hrs after transfection. The percentage of apoptotic cells was determined using Annexin-V-Phycoerythrin (PE) Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) under FACS Calibur (BD Biosciences) according to the manual. The data were assessed with Flowjo software (Tree Star Corp, San Carlos, CA, USA).

## Wound Healing Assay

The abilities of cell migration of TU212 cells were examined using a wound healing assay. Briefly, transfected cells were seeded into 6-well plates at a density of  $1 \times 10^5$  cells/well and grown to full confluence. An artificial homogeneous wound was made using a sterile plastic micropipette tip. The cells were washed with PBS and then cultured for 24 hrs, at free-serum medium. Images were taken at 0 and 24 hrs after the wounding using under a microscope (Olympus, Tokyo, Japan; 100× magnification).

## Transwell Invasion Assay

Twenty-four-well transwell chambers with 8 µL pore size (BD Biosciences) were applied to determine cell invasion ability. Briefly, the transfected cells ( $1 \times 10^5$  cells/well in 200 µL serum-free medium) were placed into the upper chamber precoated with Matrigel. The lower chamber was filled with 600 µL medium containing 10% FBS. After incubation for 48 hrs, the invaded cells were fixed with 70% ethanol for 10 mins and stained with 0.1% crystal violet (Merck, Darmstadt, Germany) for 15 mins. These cells were imaged and counted in 5 randomly selected fields under a microscope (Olympus, 100× magnification).

## Western Blot Assay

The total protein isolation and Western blot analyses were performed as previously described.<sup>17</sup> The membranes were probed with primary antibodies: mouse monoclonal anti-human CDK6 (1:1000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and mouse monoclonal anti-human GAPDH (1:5000; Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were incubated with secondary antibody of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10,000; Santa Cruz Biotechnology, Inc.). GAPDH was used as an internal control. The protein bands were observed using enhanced chemiluminescence (ECL) reagents (Super Signal Dura kit, Pierce, IL, USA) and were quantified using Image Lab™ Software (Bio-Rad).

## Bioinformatics And Luciferase Assay

Starbase2.0 (<http://starbase.sysu.edu.cn/starbase2/index.php>) was used to predict miRNAs that could bind to CDKN2B-AS1. The 3' untranslated region (UTR) of CDKN2B-AS1 containing a potential binding site for miR-497 was synthesized by GenePharma Co., Ltd., and inserted into a luciferase-reporter vector psiCHECK2 (Promega Corporation, Madison, WI, USA) and yield as WT-CDKN2B-AS1. Mutant sequence of CDKN2B (WT-CDKN2B-AS1) was constructed by a QuikChange XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). For the luciferase assay, TU212 cells were cultured to 70–80% confluence in 24-well plates and co-transfected with a miR-497 mimic or miR-NC and WT-CDKN2B-AS1 or MT-CDKN2B-AS1 reporter plasmids using Lipofectamine 3000. At 48 hrs post-transfection, dual-luciferase reporter assay (Promega Corporation) was

employed to measure firefly luciferase and Renilla luciferase activities. Renilla luciferase activity was normalized to firefly luciferase activity.

## RNA Immunoprecipitation (RIP) Assay

RIP assay was done to verify the binding between CDKN2B-AS1 and miR-497 by the EZ-Magna RIP kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Briefly, cells were lysed in complete RIP lysis buffer. Subsequently, cell lysate was incubated with RIP buffer containing magnetic beads conjugated with human anti-Ago2 antibody (1:100 dilution, Millipore) and negative control normal mouse IgG (1:100 dilution, Millipore), followed by incubation with Proteinase K buffer to remove protein. The RNA was extracted for detection miR-497 and CDKN2B-AS1 by qRT-PCR.

## Statistical Analysis

All results presented as the mean  $\pm$ SD (standard deviation) from at least three independent experiments with similar results and were analyzed using SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA). Continuous data were compared using the Student's two-tailed *t*-test (2 groups data) or one-way analysis of variance ( $>2$  groups' data). Correlations between CDKN2B-AS1 expression and miR-497 or CDK6 expression were determined by Pearson's correlation analysis. In all cases,  $P < 0.05$  was considered a significant difference.

## Results

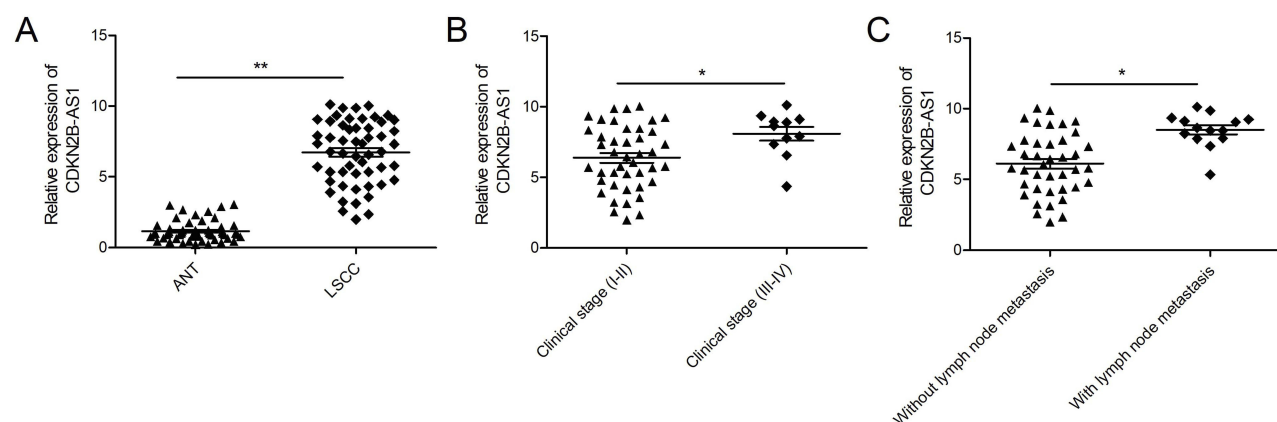
### CDKN2B-AS1 Expression Levels In LSCC And Its Clinical Significance

The expressions of CDKN2B-AS1 in primary LSCC and adjacent normal tissues (ANT) were detected by qRT-PCR. Our results demonstrated that the CDKN2B-AS1 expression was higher in LSCC tissues than that in adjacent normal tissues (Figure 1A). The expression of CDKN2B-AS1 was also elevated in samples with advanced clinical stages (III–IV) and lymph node metastasis compared with sample with a clinical stage (I–II) and without lymph node metastasis in LSCC tissues, respectively (Figure 1B and C).

We determined whether CDKN2B-AS1 expression was associated with the clinicopathological features of LSCC patients. As shown in Table 1, CDKN2B-AS1 expression was associated with lymph node metastasis and clinical stage. But gender, age, and primary location had no associations with CDKN2B-AS1 expression (Table 1). These results suggested that CDKN2B-AS1 might be involved in LSCC progression.

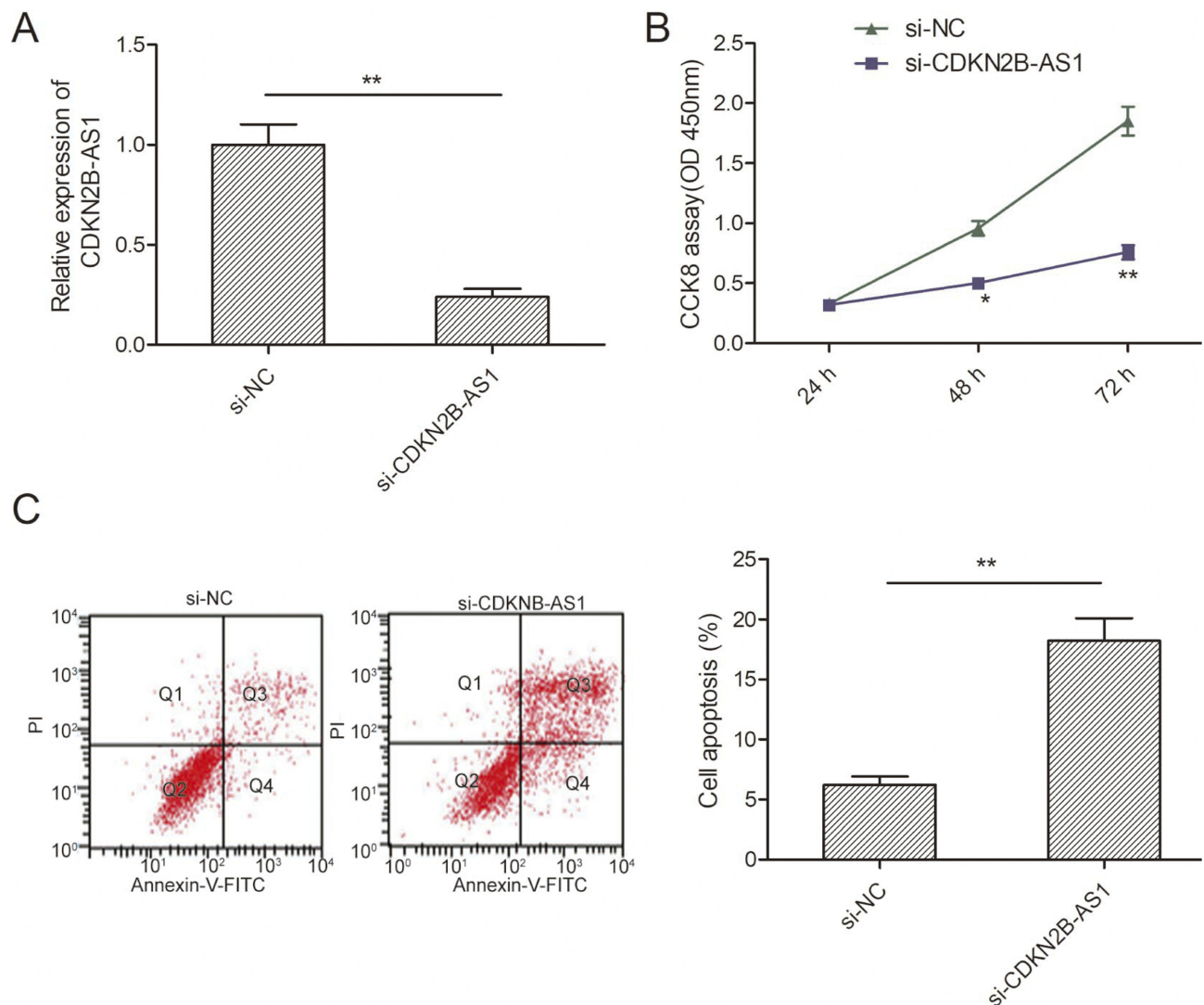
### CDKN2B-AS1 Depletion Inhibits LSCC Proliferation And Induces Apoptosis

To investigate the functional role of CDKN2B-AS1 in LSCC progression, we knocked down the CDKN2B-AS1 in TU212 cells by transfection with si-CDKN2B-AS1, and the efficacies of CDKN2B-AS1 knockdown were determined using qRT-PCR. A significantly lower level of CDKN2B-AS1 was observed in CDKN2B-AS1 knockdown TU212 cells than that in the control (si-NC) (Figure 2A). CCK-8 assay



**Figure 1** CDKN2B-AS1 expression levels in LSCC and its clinical significance. (A) The expression of CDKN2B-AS1 was determined in LSCC tissues and adjacent normal tissues (ANT). The CDKN2B-AS1 expression was normalized to GAPDH. (B) The expression of CDKN2B-AS1 in LSCC tissues with different clinical stages. The CDKN2B-AS1 expression was normalized to GAPDH. (C) The expression of CDKN2B-AS1 in LSCC tissues with or without lymph node metastasis. The CDKN2B-AS1 expression was normalized to GAPDH. \* $P < 0.05$ ; \*\* $P < 0.01$ .





**Figure 2** CDKN2B-AS1 depletion inhibits LSCC proliferation and induces apoptosis. **(A)** The expression of CDKN2B-AS1 in TU212 cells transfection with si-CDKN2B-AS1 or si-NC by qRT-PCR. CDKN2B-AS1 expression was normalized to GAPDH. **(B)** Cell proliferation was examined in TU212 cells transfection with si-CDKN2B-AS1 or si-NC by CCK8 assay. **(C)** Cell apoptosis was examined in TU212 cells transfection with si-CDKN2B-AS1 or si-NC by flow cytometry assay. \* $P < 0.05$ ; \*\* $P < 0.01$ .

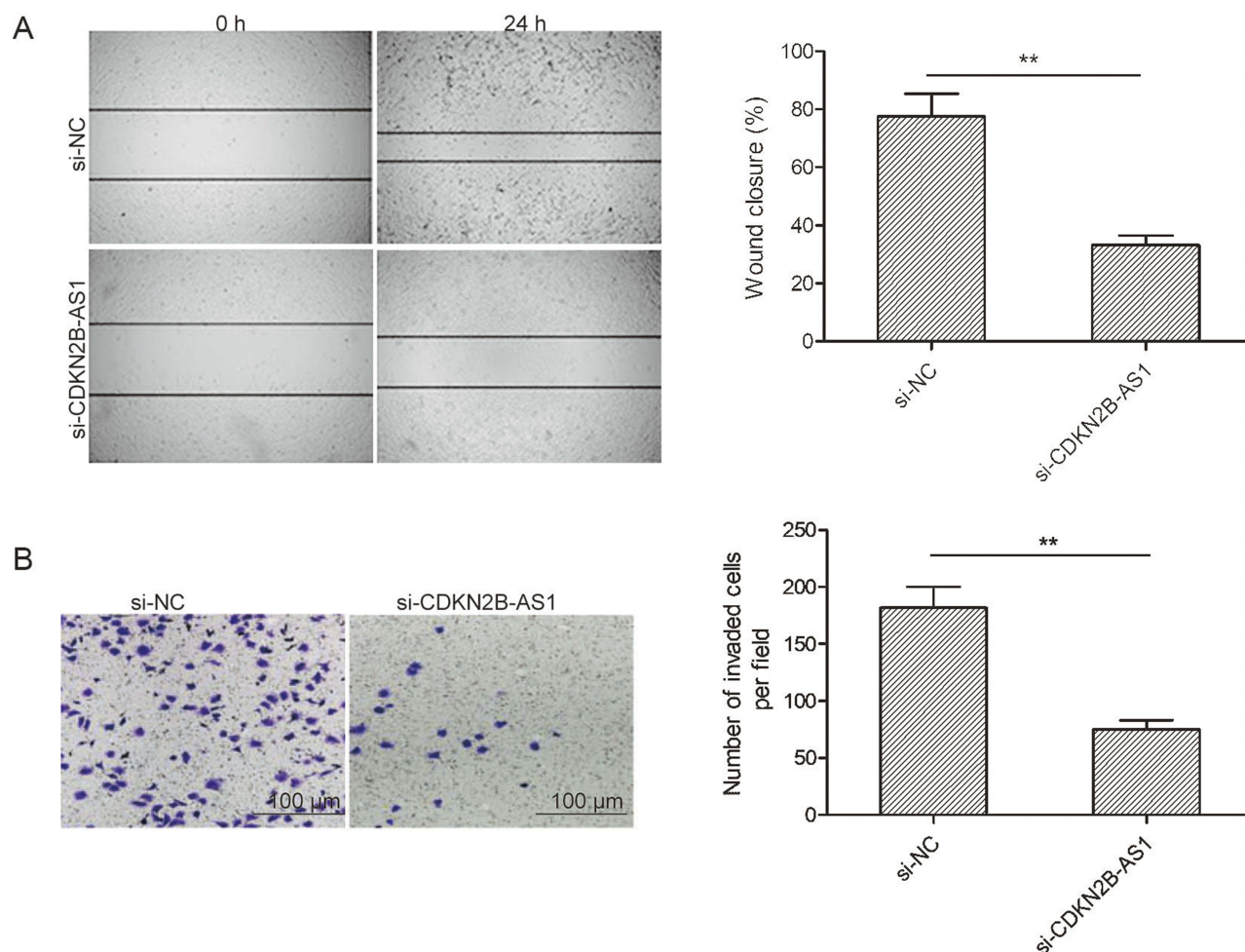
demonstrated that CDKN2B-AS1 depletion decreased the proliferation of TU212 cells according to the OD450 measured at 48-hr and 72-hr time points (Figure 2B). Flow cytometry assay revealed that knockdown of CDKN2B-AS1 significantly induced apoptosis in TU212 cells (Figure 2C).

### CDKN2B-AS1 Depletion Inhibits LSCC Migration And Invasion

The effects of CDKN2B-AS1 on LSCC cell migration and invasion were tested by wound healing and transwell invasion assays, respectively. The results illustrated that knockdown of CDKN2B-AS1 significantly decreased migratory and invasive capabilities of TU212 cells (Figure 3A and B).

### CDKN2B-AS1 Acted As A Sponge Of miR-497 In LSCC Cells

It was well known that lncRNAs could act as competing endogenous RNAs (ceRNAs) to sponge miRNAs and thus regulate cancer progression.<sup>18,19</sup> We hypothesized that CDKN2B-AS1 might be a ceRNA to sponge miRNA. To test this hypothesis, we first measured the expression of CDKN2B-AS1 in the cytoplasm and nucleus of TU212 cells by qRT-PCR. As shown in Figure 4A, CDKN2B-AS1 expression in cytoplasm was higher than that in the nucleus, suggesting that CDKN2B-AS1 could act as an endogenous sponge for miRNAs in the cytoplasm. Starbase2.0 predicted that there were complementary binding sites between miR-497 and CDKN2B-AS1



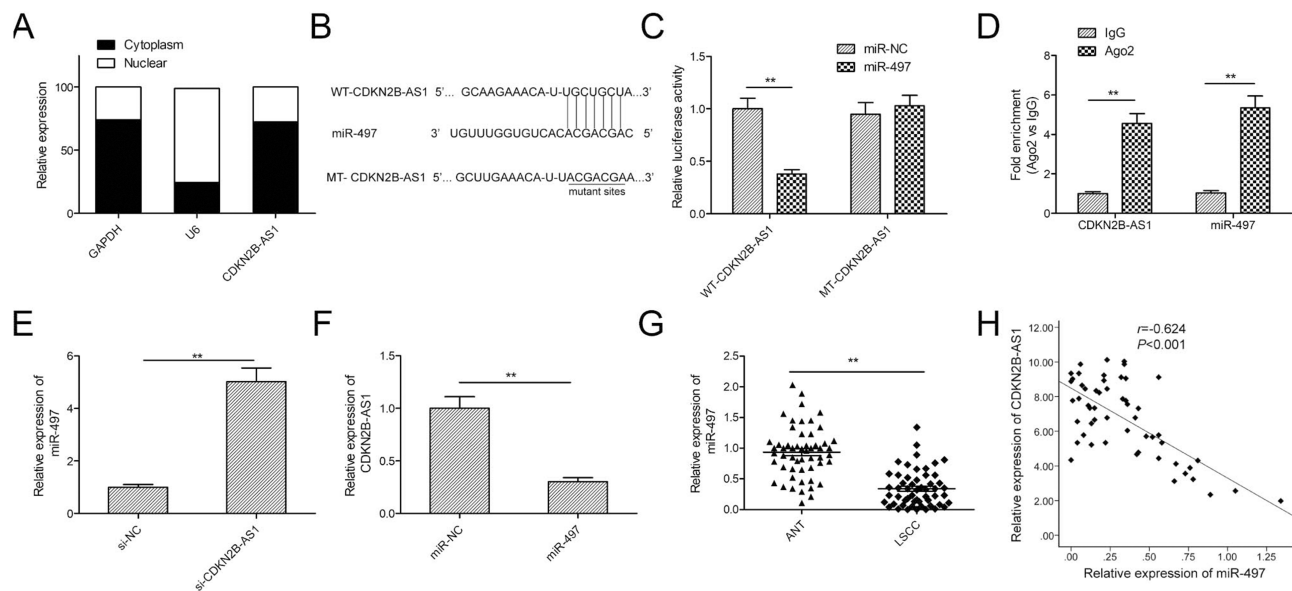
**Figure 3** CDKN2B-AS1 depletion inhibits LSCC cell migration and invasion. **(A)** Cell migration was determined in TU212 cells transfection with si-CDKN2B-AS1 or si-NC by wound healing assay. **(B)** Cell invasion was detected in TU212 cells transfection with si-CDKN2B-AS1 or si-NC by transwell invasion assay. \*\* $P < 0.01$ .

(Figure 4B). To confirm this predication, the luciferase reporter assay was carried out and found that miR-497 overexpression obviously suppressed the luciferase activity of WT-CDKN2B-AS1 in TU212 cell, but not of MT-CDKN2B-AS1 (Figure 4C). RIP assay showed that CDKN2B-AS1 and miR-497 were enriched in TU212 cells following immunoprecipitation using the anti-Ago2 antibody compared to control (IgG) (Figure 4D), suggesting that CDKN2B-AS1 could directly bind to miR-497 in LSCC cells through an Ago2-dependent manner. In addition, CDKN2B-AS1 knockdown increased the expression of miR-497 in TU212 cells (Figure 4E), while overexpression of miR-497 decreased the expression of CDKN2B-AS1 in TU212 cells (Figure 4F). In addition, we also examined the expression of miR-497 in LSCC tissues and adjacent normal tissues and found that the expression of miR-497 was reduced in LSCC tissues compared to

adjacent normal tissues (Figure 4G), and its expression was negatively correlated with CDKN2B-AS1 in LSCC tissues (Figure 4H). These results suggested that CDKN2B-AS1 might be a sponge of miR-497 in LSCC cells.

### CDKN2B-AS1 Promotes LSCC Progression Via Regulating miR-497/CDK6 Axis

CDK6 was confirmed a target of miR-497 in LSCC cells.<sup>20</sup> Thus, we investigated the association CDKN2B-AS1 with miR-497 and CDK6 in LSCC. We found that overexpression of CDK6 or inhibition of miR-497 could reserve the increased miR-497 expression in TU212 cells mediated by CDKN2B-AS1 knockdown (Figure 5A). Moreover, CDKN2B-AS1 depletion significantly decreased the mRNA and protein expression levels of CDK6 in TU212



**Figure 4** CDKN2B-AS1 acted as a sponge of miR-497 in LSCC cells. **(A)** The expression of CDKN2B-AS1 was examined in cytoplasm and nucleus of TU212 cells. U6 was used as the nuclear control and GAPDH was used as the cytoplasmic control. **(B)** The predicted miR-497 binding sites in the region of CDKN2B-AS1 and the corresponding mutant sequence were shown. **(C)** Effect of miR-497 on the luciferase activity of WT-CDKN2B-AS1 and MT-CDKN2B-AS1 reporter systems was detected via luciferase reporter assay. **(D)** RIP assay revealed that the expressions of miR-497 and CDKN2B-AS1 were enriched in Ago2-containing beads. **(E)** The expression of miR-497 was examined by qRT-PCR in TU212 cells transfected with si-CDKN2B-AS1 or si-NC. MiR-497 expression was normalized to U6. **(F)** The expression of CDKN2B-AS1 was examined by qRT-PCR in TU212 cells transfected with miR-497 mimic or miR-NC. CDKN2B-AS1 expression was normalized to GAPDH. **(G)** The expression of miR-497 was examined by qRT-PCR in LSCC tissues and adjacent normal tissues (ANT). MiR-497 expression was normalized to U6. **(H)** Correlation between CDKN2B-AS1 and miR-497 expression in LSCC tissues was analyzed by Pearson's correlation analysis. \*\* $P < 0.01$ .

cells, while overexpression of CDK6 or downregulation of miR-497 reserved this trend (Figure 5B and C). In addition, we measured the mRNA expression of *CDK6* in LSCC tissues and adjacent normal tissues by qRT-PCR. We found that the expression of *CDK6* was increased in LSCC tissues compared to adjacent normal tissues (Figure 5D), and its expression was positively correlated with CDKN2B-AS1 in LSCC tissues (Figure 5E).

To investigate whether CDKN2B-AS1 knockdown exerts an anti-cancer effect in LSCC cells via regulation miR-497/CDK6 axis, we performed rescue experiments by inhibiting miR-497 expression or overexpression of CDK6 in CDKN2B-AS1-depletion TU212 cells. The result revealed that inhibition of miR-497 or overexpression of CDK6 could partially reverse the effect of CDKN2B-AS1 knockdown on cell proliferation, apoptosis, migration and invasion of TU212 cells (Figure 5F–I). These above findings suggested that CDKN2B-AS1 promoted LSCC progression by regulating miR-497/CDK6 axis.

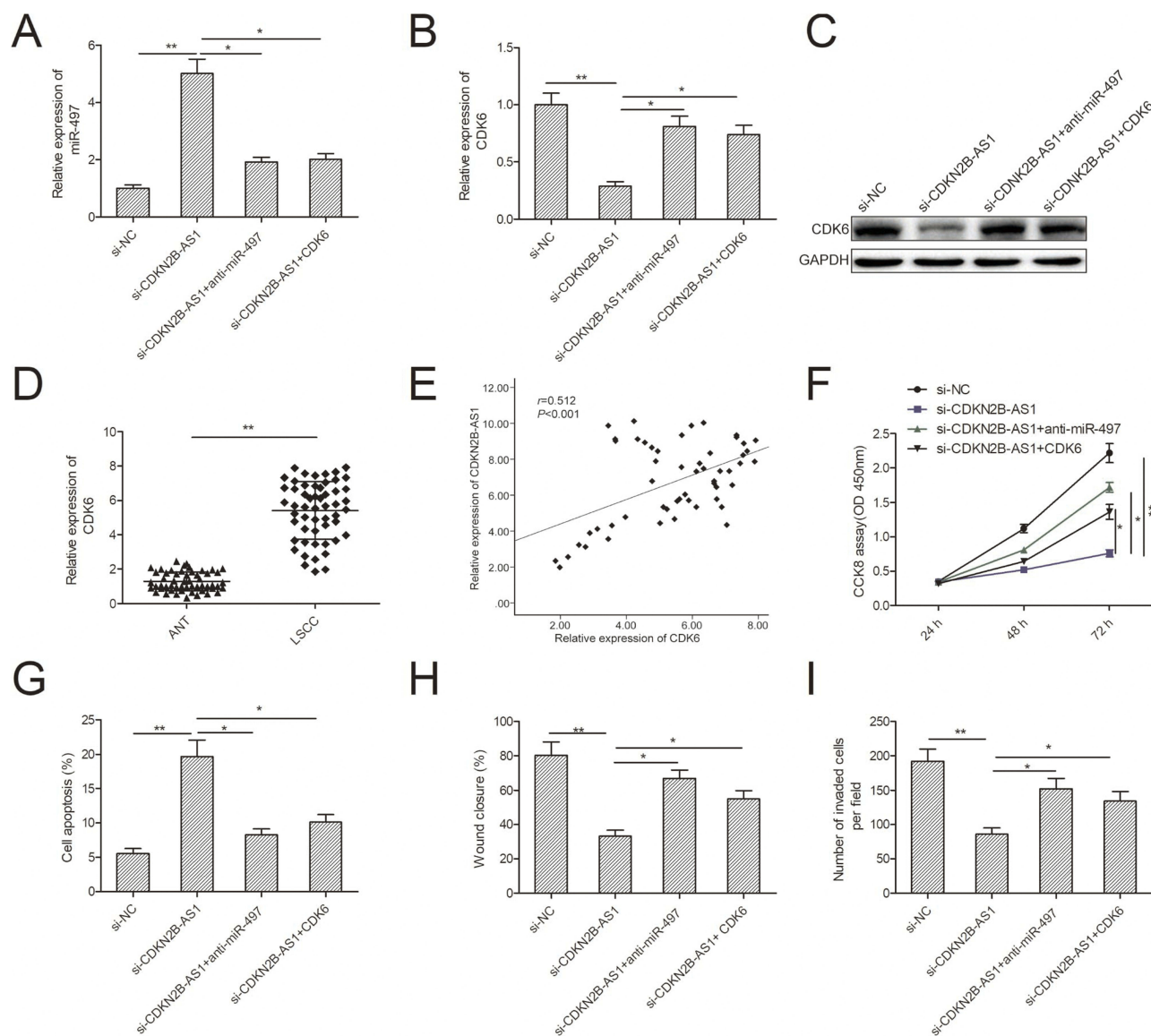
## Discussion

Many lncRNAs were reported to be involved in tumorigenesis and metastasis of LSCC.<sup>9,10</sup> For example, Tai et al revealed that lncRNA SOX2OT promoted LSCC cell

growth in vitro and in vivo via epigenetically inhibiting PTEN via methyltransferase EZH2.<sup>21</sup> Sun et al reported that lncRNA UCA1 promoted LSCC cell proliferation, invasion and migration in vitro by activating Wnt/ $\beta$ -catenin signaling pathway.<sup>22</sup> Liu et al showed that knockdown of LINC00339 inhibited the proliferation, invasion, and epithelial–mesenchymal transition (EMT) progression of LSCC cells by regulating the miR-145 expression.<sup>23</sup> Thus, more efforts should be done to clarify the molecular mechanisms of lncRNAs in LSCC, which contribute to find novel diagnosis and therapy markers for LSCC.

LncRNA CDKN2B-AS1 was found to be closely related to tumorigenesis, metastasis, prognosis and diagnosis of multiple cancers and function as an oncogenic lncRNA.<sup>11–16</sup> For example, overexpression of CDKN2B-AS1 resulted in the promotion of cell proliferation, invasion, migration and inhibition of apoptosis and senescence of cervical cancer cells by regulating miR-181a-5p/TGF $\beta$ 1 axis.<sup>24</sup> CDKN2B-AS1 promoted hepatocellular carcinoma growth and metastasis by promoting NAP1L1-mediated PI3K/AKT/mTOR signaling via acting as a molecular sponge of let-7c-5p.<sup>11</sup> In this research, we revealed that CDKN2B-AS1 was highly expressed in LSCC tissues. Increased CDKN2B-AS1 was closely correlated with advanced TNM stage and lymph





**Figure 5** CDKN2B-AS1 promotes LSCC progression via regulating miR-497/CDK6 axis. **(A)** The expression of miR-497 was examined in TU12 cells transfected with si-NC, si-CDKN2B-AS1, si-CDKN2B-AS1+miR-497 inhibitor (anti-miR-497) or si-CDKN2B-AS1+CDK6 overexpression plasmid (CDK6). miR-497 expression was normalized to U6. **(B, C)** The expression of CDK6 on mRNA and protein levels were determined in TU12 cells transfected with si-NC, si-CDKN2B-AS1, si-CDKN2B-AS1+anti-miR-497, or si-CDKN2B-AS1+CDK6. GAPDH was used as control. **(D)** The mRNA expression of CDK6 was examined by qRT-PCR in LSCC tissues and adjacent normal tissues (ANT). GAPDH was used as control. **(E)** Correlation between CDKN2B-AS1 and CDK6 expression in LSCC tissues was analyzed by Pearson's correlation analysis. **(F–I)** Cell proliferation, apoptosis, migration and invasion were detected in TU12 cells transfected with si-NC, si-CDKN2B-AS1, si-CDKN2B-AS1+anti-miR-497 or si-CDKN2B-AS1+CDK6. \* $P < 0.05$ ; \*\* $P < 0.01$ .

node metastasis. CDKN2B-AS1 silencing in LSCC cells inhibited the viability, migration and invasion, as well as induced apoptosis. These results suggested that CDKN2B-AS1 might play an oncogenic role in LSCC progression.

Growing evidence demonstrated that lncRNAs exerted tumor oncogenic or suppressor roles in cancer progression via regulating the expression of miRNAs.<sup>18,19</sup> Several studies demonstrated that CDKN2B-AS1 sponges different miRNAs to modulate tumor progression, such as miR-181a-5p, miR-125a-3p, miR-24, miR-191, miR-122-5p, miR-34a,

and miR-199a.<sup>24–30</sup> In our study, we identified that miR-497 could bind with CDKN2B-AS1 through a public database (Starbase 2.0). Then, luciferase reporter and RIP assays confirmed that CDKN2B-AS1 could specifically associate with miR-497 in LSCC cells. miR-497, a known tumor-suppressor microRNA, was reported to be involved in the initiation and development of various cancers including LSCC.<sup>20</sup> In the present study, we found that the expression of miR-497 was reduced in LSCC tissues compared to adjacent normal tissues, which was consistent with the previous result.<sup>20</sup>



Moreover, the expression of miR-497 was negatively correlated with CDKN2B-AS1 in LSCC tissues. We also showed that inhibition of miR-497 could partially reversed the effect of CDKN2B-AS1 knockdown on cell proliferation, apoptosis, migration and invasion of LSCC cells. These results implied that CDKN2B-AS1 exerted an oncogenic role in LSCC partially targeting miR-497.

A new posttranscriptional regulatory mechanism revealed that lncRNAs could sponge miRNAs to regulate the target gene of miRNAs.<sup>19</sup> CDK6 was identified as a direct target of miR-497 in LSCC.<sup>20</sup> CDK6, a member of cyclin-dependent kinases (CDK) family, was reported to play key roles in various cellular processions, including cell proliferation, cycle, differentiation and metastasis.<sup>31,32</sup> It has been shown that CDK6 expression was upregulated and functioned as an oncogene in LSCC.<sup>20,33</sup> Here, we found that knockdown of CDKN2B-AS1 could decrease CDK6 expression in LSCC cells, while miR-497 inhibitor reserved this trend. In addition, we found that the expression of CDK6 was positively correlated with CDKN2B-AS1 expression in LSCC tissues. We also demonstrated that overexpression of CDK6 could partially reverse the effect caused by CDKN2B-AS1 knockdown in LSCC cells. These results suggested that CDKN2B-AS1 positively regulated CDK6 expression in LSCC cells by sponging CDK6.

In summary, the present study demonstrated that CDKN2B-AS1 expression was upregulated in LSCC tissues and was closely associated with clinical stage and lymph node metastasis. Moreover, CDKN2B-AS1 promoted LSCC progression by targeting miR-497/CDK6 axis. These findings provide us insights into the molecular mechanism underlying the effect of CDKN2B-AS1 on LSCC and suggest CDKN2B-AS1 as a therapeutic target for LSCC.

## Acknowledgments

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## Disclosure

The authors report no conflicts of interest in this work.

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