

Silencing of KCNK15-AS1 inhibits lung cancer cell proliferation via upregulation of miR-202 and miR-370

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Abstract. Lung cancer is the most common cause of cancer-associated mortality globally. Long non-coding RNAs (lncRNAs) are transcripts with a length of >200 nucleotides, which are not translated into proteins. Growing evidence has indicated that certain lncRNAs are associated with various biological processes in cancer. However, the functions of KCNK15 and WISP2 antisense RNA 1 (KCNK15-AS1) in lung cancer carcinogenesis and progression have remained elusive. The present study indicated that KCNK15-AS1 was overexpressed in lung adenocarcinoma tissues compared with paracancerous normal tissues, and the high expression of KCNK15-AS1 was significantly associated with poor prognosis compared with the patients with low expression ($P < 0.001$). Furthermore, the knockdown of KCNK15-AS1 was performed in A549 and H460 lung cancer cells with small interfering RNA, resulting in a significant inhibition of the proliferation, a decrease in the mRNA and protein expression of cyclin D1 (CCND1) and epidermal growth factor receptor (EGFR), in addition to the phosphorylation of protein kinase B, with a concomitant increase in the expression of microRNA (miR)-202 and miR-370 compared with negative control group. Rescue experiments demonstrated that the inhibition of miR-202 or miR-370 partially recovered the EGFR and CCND1 expression and the proliferation rates, which were reduced by KCNK15-AS1 silencing. In conclusion, these results suggested that KCNK15-AS1 functions as an oncogene

via regulating the miR-202/miR-370/EGFR axis in lung cancer and may provide a potential target for lung cancer treatment.

Introduction

Lung cancer is the leading cause of cancer-associated mortality globally. Although progress has been made in the treatment of lung cancer, the survival of patients with lung cancer remains poor with a 5-year survival rate of only 17% (1,2). The characteristics of lung cancer are uncontrolled proliferation and metastasis of tumor cells. Therefore, understanding the regulatory mechanisms underlying lung cancer carcinogenesis and progression is necessary for tumor therapy.

Non-coding RNAs, including microRNAs (miRNAs/miRs) and long non-coding RNAs (lncRNAs) are considered to be potential biomarkers and candidate targets for the treatment of numerous cancer types (3). Certain lncRNAs have key functions in a variety of biological processes, including proliferation, apoptosis, stem cell properties, differentiation and metastasis (4,5). To date, numerous lncRNAs have been reported to be involved in the genesis of lung cancer. The lncRNA activated by transforming growth factor- β was identified to be overexpressed in lung cancer tissues and to promote the proliferation and metastasis of tumor cells by activating the p38 signaling pathway (6). Salt-inducible kinase (SIK)1-LNC, a type of lncRNA adjacent to SIK, was reported to be downregulated in lung cancer tissues and to repress the proliferation, migration and invasion of lung cancer cells (7). LncRNA KCNK15 and WISP2 antisense RNA 1 (KCNK15-AS1) was determined to be overexpressed in lung cancer tissues, and the higher expression of KCNK15-AS1 was associated with a shorter survival (8). However, the functional roles and underlying mechanisms of KCNK15-AS1 in the genesis of lung cancer remain largely elusive.

miR-202 and miR-370 have been previously reported to be decreased in lung cancer (9-11). miR-202 induces cell cycle arrest and apoptosis by targeting cyclin D1 (CCND1) and inhibits cell proliferation, migration and invasion via targeting signal transducer and activator of transcription (STAT3) in lung cancer (12,13). miR-370 has a tumor suppressive function in lung cancer by targeting tumor necrosis factor receptor-associated factor (TRAF4) and epidermal growth factor receptor (EGFR) (11,14).

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Abbreviations: lncRNA, long non-coding RNA; miRNA, microRNA; KCNK15-AS1, KCNK15 and WISP2 antisense RNA 1; EGFR, epidermal growth factor receptor

Key words: KCNK15 and WISP2 antisense RNA 1, epidermal growth factor receptor, microRNA-202, microRNA-370, proliferation, lung cancer

In the present study, the regulatory functions of KCN15-AS1 in lung cancer development, in addition to the associated molecular mechanisms, were investigated.

Materials and methods

Patients and samples. Fresh lung adenocarcinoma (LAD) and adjacent normal tissue samples from 40 patients were collected at the Department of Thoracic Surgery of the First People's Hospital of Yunnan (Kunming, China) between June 2014 and September 2015 and immediately stored at -70°C . All patients with LAD were treated using radical surgery and no patients received any pre-operative treatment. All samples were residual specimens following diagnostic sampling, and all patients provided written informed consent for sampling and molecular analysis separately. The present study was ethically approved by the Institutional Ethics Committee of the First People's Hospital of Yunnan Province (Kunming, China). Paracancerous tissue samples were collected at a 2-cm distance from the tumor edge as previously described (15), and the normal tissues were pathologically confirmed. The samples were graded by the AJCC staging classification system (8th edition) (16). The mean age of the patients was 62 years old (range, 38-79), and 65% of the patients ($n=40$) were male. Overall survival (OS) was defined as the time from surgery treatment to mortality or to the last follow-up. The clinical-pathological characteristics of the samples are presented in Table I.

Cell culture. A549 (cat. no. CCL-185) and H460 cells (cat. no. HTB-177) were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin in an incubator at 37°C with 5% CO_2 .

Small interfering (si)RNA transfection. Cancer cells (2×10^5) were seeded in six-well plates and cultured with DMEM. KCN15-AS1 siRNAs, miR-202 inhibitor, miR-370 inhibitor and a negative control were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). siRNAs or inhibitors (50 nM) were transfected into cells using Lipofectamine[®] RNAiMAX Transfection reagent for 48 h according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). The sequences of the KCN15-AS1 siRNAs and negative control were as follows: siRNA-1 sense, 5'-GUCAUCACUACCAUC GGUGATT-3' and antisense, 5'-UCACCGAUGGUAGUG AUGACTT-3'; siRNA-2 sense, 5'-GUCCGAGGCGGAAAG CGGTT-3' and antisense, 5'-CCGCUUUCGCGCUCGGAC TT-3'; negative control sense, 5'-UUCUCCGAACGUGAC ACUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGA ATT-3'; miR-202 inhibitor, 5'-CAAAGAAGUAUAUGCAA GGAA-3'; miR-370 inhibitor, 5'-GUAACUGCAGAGACG UGACCUG-3'; inhibitor negative control, 5'-CAGUACUUU UGUGUAGUACAA-3'. The miR-202 inhibitor and miR-370 inhibitor were used at a final concentration of 20 nM.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was

isolated from cells using RNAiso Plus (Takara Bio, Inc., Otsu, Japan). RT-qPCR was used to determine the expression levels of KCN15-AS1, CCND1, EGFR and GAPDH. PCR was performed in a total volume of 20 μl , including 10 μl PowerUp[™] SYBR[™] Green Mix (Thermo Fisher Scientific, Inc.), 2 μl complementary DNA and 1 μl primer mix (10 μM each). PCR was performed in an ABI 7300 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. The PCR data were normalized to GAPDH and the relative expression of each gene was calculated using the $2^{-\Delta\Delta\text{C}_q}$ method (17). The following primers were used: KCN15-AS1 forward, 5'-AGC AGATGCAGAGAACCCAAA-3' and reverse, 5'-TTGCAA GGCAGGTGTTTGTTC-3'; CCND1 forward, 5'-GCTGCG AAGTGGAAACCATC-3' and reverse, 5'-CCTCCTTCTGCA CACATTTGAA-3'; EGFR forward, 5'-AGGCACGAGTAA CAAGCTCAC-3' and reverse, 5'-ATGAGGACATAACCA GCCACC-3'; GAPDH forward, 5'-AAATCCCATCACCAT CTCCAG-3' and reverse, 5'-GAGTCCTTCCACGATACC AAAGTTG-3'.

Western blot analysis. Cells were lysed using radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 1% Nonidet P-40]. Following incubation on ice for 45 min, the homogenates were centrifuged at 13,000 \times g for 15 min at 4°C . The concentrations of samples were detected using the Bradford method (Beyotime Institute of Biotechnology, Haimen, China). Proteins (10-20 $\mu\text{g}/\text{lane}$) were separated by 12% SDS-PAGE and then transferred onto a 0.45- μm polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with 5% skimmed milk for 30 min at room temperature. The membranes were incubated with primary antibody at 4°C overnight. Prior to incubation with secondary antibodies [anti-mouse immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked antibody, cat no. 7075, 1:10,000 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA; anti-rabbit IgG, HRP-linked antibody, cat no. 7074, 1:10,000 dilution, Cell Signaling Technology, Inc.] at room temperature for 1 h, the membranes were washed with Tris-buffered saline containing 0.3% Tween-20. The protein signals were visualized using the Enhanced Chemiluminescence Detection reagent (cat no. PE0020; Beijing Solarbio Bioscience & Technology Co., Ltd., Beijing, China). The primary antibodies and dilution ratios were as follows: CCND1 (cat no. sc-450; 1:1,000 dilution; Santa Cruz Biotechnology, Inc.); EGFR (cat no. 4267, 1:1,000 dilution; Cell Signaling Technology, Inc., Dallas, TX, USA); phosphorylated protein kinase B (AKT; cat no. 4060, 1:1,000 dilution; Cell Signaling Technology, Inc.); AKT (cat no. 9272, 1:1,000 dilution; Cell Signaling Technology, Inc.) and β -actin (cat no. 4970, 1:5,000 dilution; Cell Signaling Technology, Inc.).

Cell proliferation assay. A Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to quantify the proliferation of A549 and H460 cells according to the manufacturer's protocol. Cells were cultured at 1,000 cells/well in 96-well plates and transfected for 24 h. Following incubation for 24, 48, 72, 96 or 120 h at 37°C , 10 μl CCK-8 reagent was added to each well, followed by incubation

Table I. Association between KCNK15-AS1 expression and clinicopathological characteristics.

Characteristic	n	KCNK15-AS1 expression		P-value
		High (n=19)	Low (n=21)	
Sex				0.7475
Female	14	6	8	
Male	26	13	13	
Age, years				0.5266
<50	15	6	9	
≥50	25	13	12	
Degree of differentiation				0.0309
Well or moderate	18	5	13	
Poor or undifferentiated	22	14	8	
Clinical stage				0.0270
I-II	16	4	12	
III-IV	24	15	9	
T-stage				0.7518
T1-T2	21	9	12	
T3-T4	19	10	9	
N classification				0.0309
N0-N1	18	5	13	
N2-N3	22	14	8	
M classification				0.5962
M0	37	17	20	
M1	3	2	1	

KCNK15-AS1, KCNK15 and WISP2 antisense RNA 1; T, tumor; N, nodes; M, metastasis.

at 37°C for 1 h. The absorbance of each well was read at 450 nm on a microplate spectrophotometer (SPECTRAMax 190; Molecular Devices, LLC, Sunnyvale, CA, USA). Three independent experiments were performed.

Statistical analysis. Data were presented as the mean ± standard deviation, and statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The expression levels of KCNK15-AS1 between LAD tissues and paracancerous normal tissues were analyzed using a Student's t-test (two-tailed, paired). Other data were analyzed using a Student's t-test (two-tailed, unpaired) between two groups and one-way analysis of variance followed by a Tukey's post-hoc test for multiple comparisons. The association between KCNK15-AS1 expression and clinicopathological characteristics were analyzed using a χ^2 test. Estimation of survival time distribution was performed using the Kaplan-Meier method. $P < 0.05$ was considered to indicate a statistically significant difference. All experiments were independently performed at least three times.

Results

Silencing of KCNK15-AS1 inhibits the proliferation and decreases the expression of CCND1 in lung cancer cells. A previous study indicated that KCNK15-AS1 was overexpressed

in lung cancer and that the high expression of KCNK15-AS1 was associated with poor prognosis (8). In the present study, 40 patients with lung cancer were assessed to confirm these results, and the analysis suggested that KCNK15-AS1 was significantly overexpressed in lung cancer compared with paracancerous tissues ($P < 0.001$) and that its high expression was associated with poor overall survival (Fig. 1A and B). The high expression of KCNK15-AS1 was significantly associated with a poor degree of differentiation, advanced clinical stage and lymph node metastasis ($P < 0.05$; Table I). In order to investigate the function of KCNK15-AS1, it was knocked down using siRNAs (Fig. 2A). The results indicated that the silencing of KCNK15-AS1 significantly inhibited the proliferation of A549 and H460 lung cancer cells ($P < 0.05$; Fig. 2B). CCND1 is involved in the regulation of the cell cycle (18). Notably, the knockdown of KCNK15-AS1 caused a significant downregulation of the expression of CCND1 at the mRNA levels ($P < 0.001$; Fig. 2C) and downregulation at the protein level (Fig. 2D). Furthermore, the expression of CCND1 in lung cancer tissues was significantly higher compared with that in paracancerous normal tissues ($P < 0.001$; Fig. 2E).

Silencing of KCNK15-AS1 decreases the expression of EGFR and inhibits the phosphorylation of AKT. The EGFR/AKT axis has important functions in cancer cell proliferation (19,20);

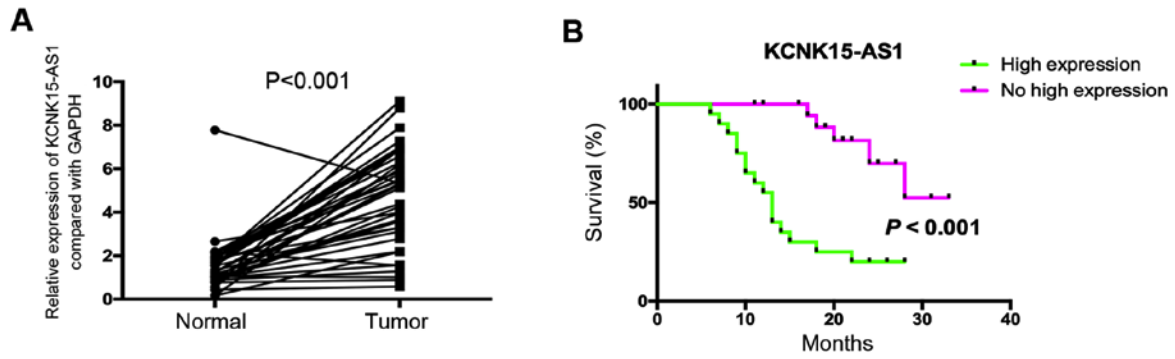


Figure 1. KCNK15-AS1 is overexpressed in lung cancer tissues and its overexpression is associated with poor survival. (A) Expression of KCNK15-AS1 was detected using reverse transcription-quantitative polymerase chain reaction in 40 lung cancer tissues. (B) Survival analysis was performed using the Kaplan-Meier method. KCNK15-AS1, KCNK15 and WISP2 antisense RNA 1.

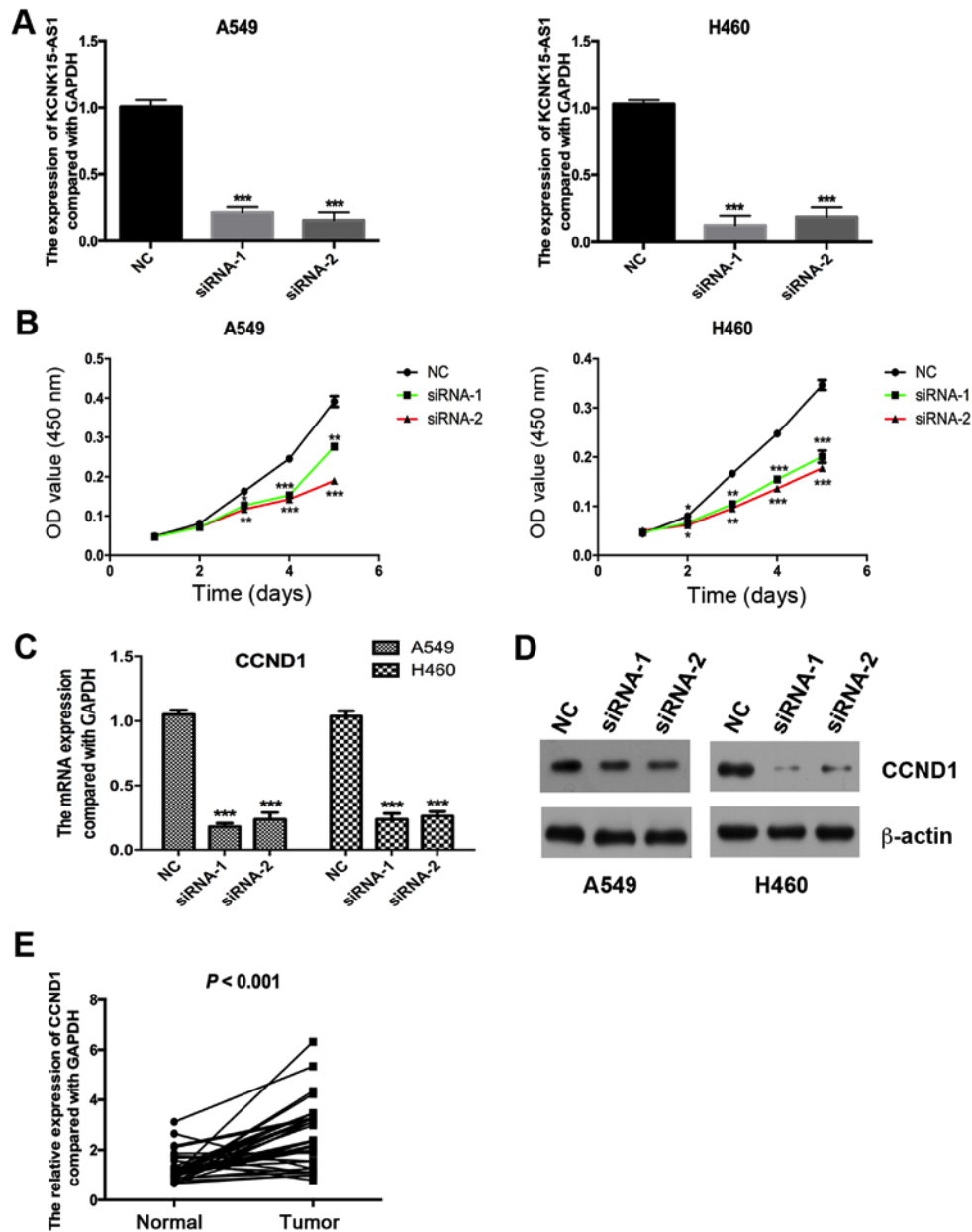


Figure 2. Silencing KCNK15-AS1 inhibits lung cancer cell proliferation and downregulates CCND1 expression. (A) Efficiency of KCNK15-AS1 knockdown was detected using RT-qPCR. (B) Proliferation of lung cancer cells was detected by a Cell Counting Kit-8 assay. (C) mRNA and (D) protein expression of CCND1 were analyzed using RT-qPCR and western blot assays. (E) mRNA expression of CCND1 in lung cancer tissues and paracancerous normal tissues was analyzed using RT-qPCR. Three independent experiments were performed. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. the NC. KCNK15-AS1, KCNK15 and WISP2 antisense RNA 1; RT-qPCR, reverse transcription-quantitative PCR; CCND1, cyclin D1; NC, negative control; siRNA, small interfering RNA.

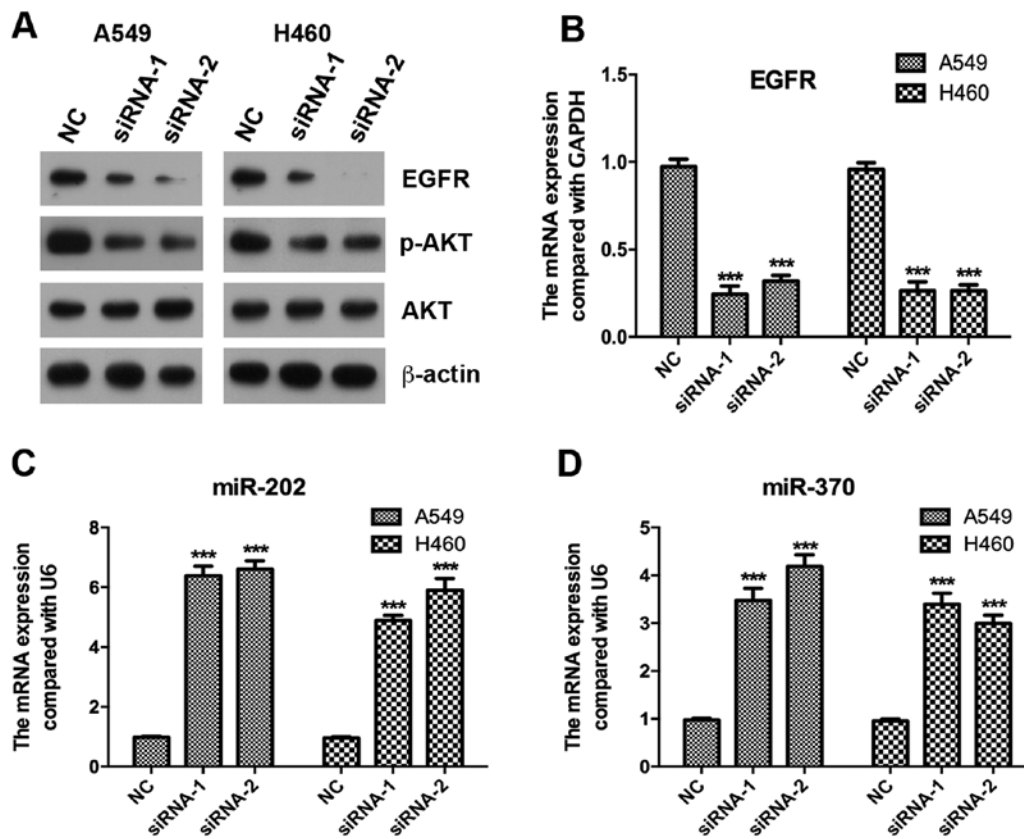


Figure 3. Silencing of KCNK15-AS1 decreases the expression of EGFR and inhibits the phosphorylation of AKT. (A) Protein levels of EGFR, p-AKT and AKT were detected using western blot analysis. mRNA expression of (B) EGFR, (C) miR-202 and (D) miR-370 were analyzed using reverse transcription-quantitative polymerase chain reaction. Three independent experiments were performed. *** $P < 0.001$ vs. NC. KCNK15-AS1, KCNK15 and WISP2 antisense RNA 1; EGFR, epidermal growth factor receptor; AKT, protein kinase B; p-AKT, phosphorylated AKT; miR, microRNA; NC, negative control; siRNA, small interfering RNA.

therefore, the present study assessed whether the silencing of KCNK15-AS1 affects EGFR/AKT signaling. RT-qPCR and western blot analyses indicated that the silencing of KCNK15-AS1 reduced the protein expression and significantly reduced the mRNA levels of EGFR ($P < 0.001$) and inhibited the phosphorylation of AKT (Fig. 3A and B).

In tumors, EGFR has been reported to be targeted and regulated by miR-202 and miR-370 (14,21,22). The results of the present study suggested that the silencing of KCNK15-AS1 caused a significant upregulation of the expression of miR-202 and miR-370 in A549 and H460 lung cancer cells ($P < 0.001$; Fig. 3C and D).

Inhibition of miR-202 or miR-370 partially recovers the inhibitory effect of KCNK15-AS1 knockdown on the proliferation of lung cancer cells. To confirm the involvement of miR-202 or miR-370 in the regulatory effect of KCNK15-AS1 on lung cancer cell proliferation, initially, miR-202 and miR-370 were knocked-down using inhibitors in A549 and H460 cells. A RT-qPCR assay confirmed the efficiency of miR-202 and miR-370 silencing (Fig. 4A and B). miR-202 and miR-370 inhibitors also significantly decreased the expression levels of miR-202 and miR-370 in the KCNK15-AS1-silenced lung cancer cells compared with the negative control ($P < 0.001$; Fig. 4C and D). Inhibition of miR-202 or miR-370 partially recovered the EGFR expression levels in the KCNK15-AS1 silenced lung cancer cells (Fig. 4E-H). Furthermore, the

knockdown of miR-202 or miR-370 partially recovered the cell proliferation ability and CCND1 expression in the KCNK15-AS1-silenced lung cancer cells (Fig. 5A-C).

Discussion

LncRNAs, a class of RNAs with a length of >200 nucleotides, have important functions in numerous biological processes, including tumor formation and development (23). A previous study indicated that KCNK15-AS1 was overexpressed in lung cancer tissues, and the higher expression of KCNK15-AS1 was associated with poor survival (8). On the other hand, KCNK15-AS1 was reported to be decreased in pancreatic cancer tissues, and to inhibit the migration and invasion of pancreatic cancer cells (24). Furthermore, the expression of KCNK15-AS1 was regulated by the m6A eraser AlkB homolog 5, RNA demethylase (24). Therefore, KCNK15-AS1 may have different functions in different types of cancer, and elucidation of the mechanisms associated with KCNK15-AS1 overexpression is urgently required.

The results of the present study initially confirmed the overexpression and association with the poor prognosis of KCNK15-AS1 in lung cancer. Furthermore, it was determined that the knockdown of KCNK15-AS1 significantly inhibited the proliferation of lung cancer cells and caused the down-regulation of the cell cycle-associated gene CCND1 at the mRNA and protein levels.

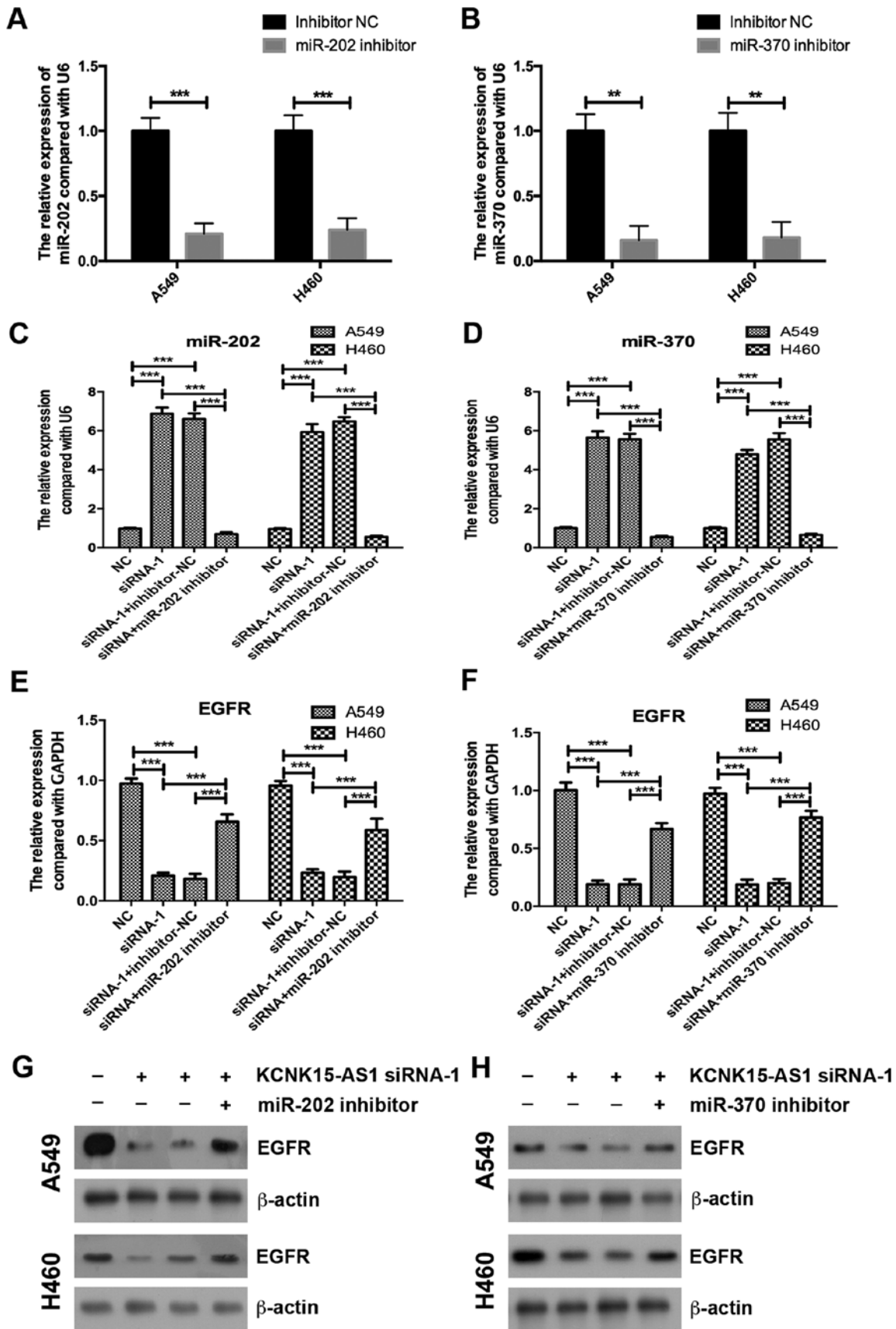


Figure 4. Downregulation of miR-202 or miR-370 partially recovered the inhibitory effects of KCNK15-AS1 knockdown on the EGFR/AKT axis. (A and B) The efficiencies of miR-202 inhibitor and miR-370 inhibitor were analyzed by qPCR. (C and D) The knockdown efficiencies of miR-202 or miR-370 using inhibitors were analyzed by qPCR. (E and F) Knockdown of miR-202 or miR-370 partially recovered the expression of EGFR determined by qPCR. Expression of EGFR was detected using western blot analysis following the inhibition of (G) miR-202 or (H) miR-370 in lung cancer cells with KCNK15-AS1 silencing. Three independent experiments were performed. **P<0.01 and ***P<0.001 with comparisons shown by lines. KCNK15-AS1, KCNK15 and WISP2 antisense RNA 1; EGFR, epidermal growth factor receptor; p-AKT, phosphorylated AKT; AKT, protein kinase B; miR, microRNA; NC, negative control; siRNA, small interfering RNA; qPCR, quantitative PCR.

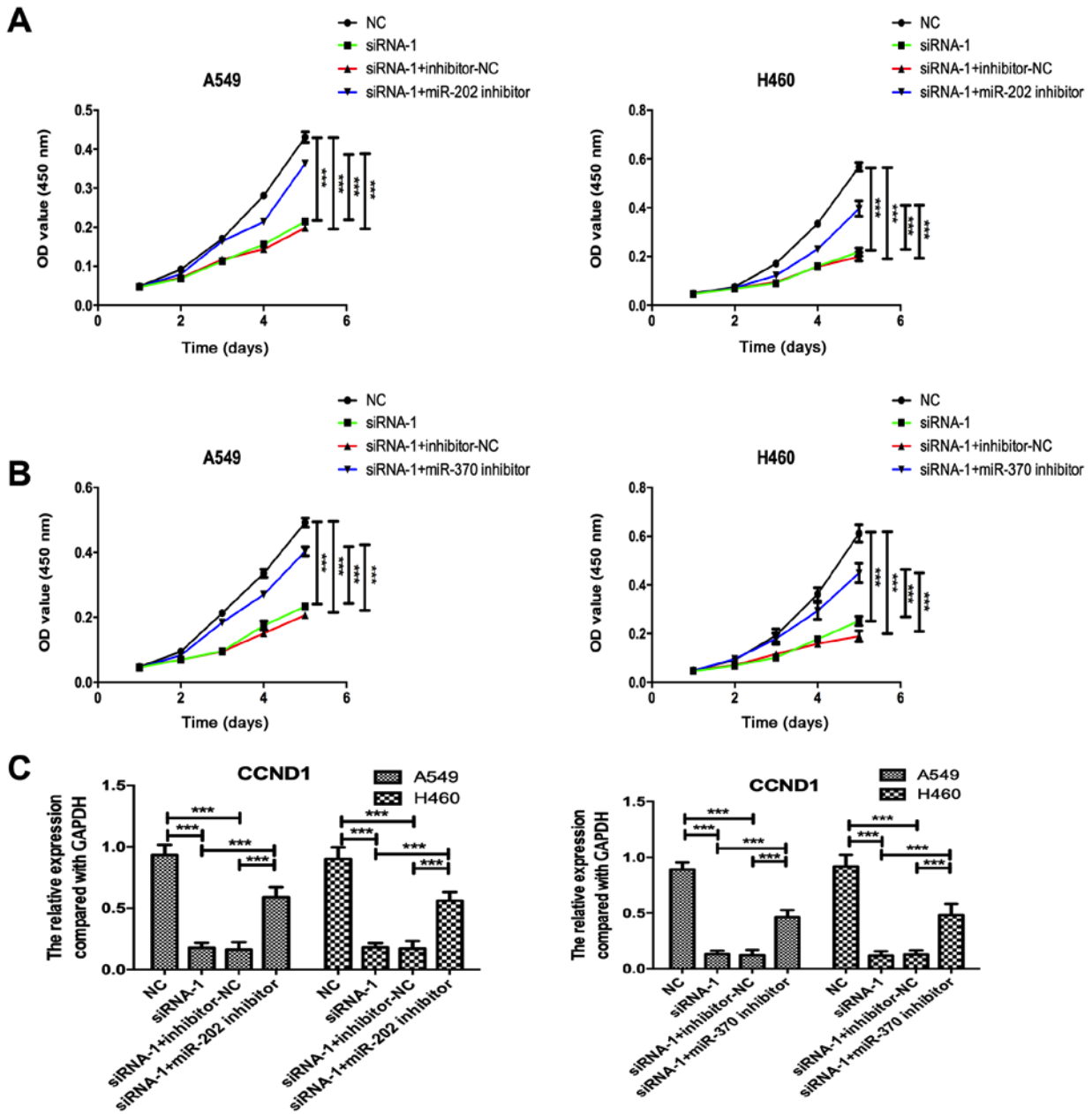


Figure 5. Downregulation of miR-202 or miR-370 partially recovered the inhibitory effects of KCN15-AS1 knockdown on lung cancer cell proliferation. Proliferation of lung cancer cells following the use of a (A) miR-202 inhibitor or a (B) miR-370 inhibitor was detected using a Cell Counting Kit-8 assay. (C) mRNA expression of CCND1 was analyzed using a reverse transcription-quantitative polymerase chain reaction. Three independent experiments were performed. ***P<0.001 with comparisons shown by lines. KCN15-AS1, KCN15 and WISP2 antisense RNA 1; CCND1, cyclin D1; miR, microRNA; siRNA, small interfering RNA; NC, negative control.

The EGFR/AKT signaling pathway has important functions in lung carcinogenesis and certain miRNAs are known to regulate this signaling pathway. miR-133a was reported to decrease the expression of EGFR and inhibit the phosphorylation of AKT in human non-small cell lung cancer cells (25). miR-145 was reported to induce apoptosis and inhibit the migratory ability of non-small cell lung cancer cells by inhibiting the EGFR/phosphoinositide-3-kinase/AKT signaling pathway (26). The present study indicated that KCN15-AS1 activated the EGFR/AKT signaling pathway via reducing miR-202 and miR-370.

Previous studies have indicated that miR-202 exhibits tumor suppressive functions and is downregulated in

numerous types of cancer, including prostate cancer, breast cancer, osteosarcoma and lung cancer (12,27-29). miR-202 has been reported to be significantly downregulated in bladder cancer tissues and cell lines, and the overexpression of miR-202 inhibited cell proliferation, colony formation, invasion and migration *in vitro*, as well as suppressed tumor growth *in vivo*; of note, miR-202 exerted its tumor suppressive functions via targeting EGFR (21). In lung cancer, miR-202 was decreased, and the overexpression of miR-202 enhanced the sensitivity of lung cancer cells to cisplatin by inactivating the Ras/mitogen-activated protein kinase signaling pathway (30). miR-202 was also demonstrated to have a tumor suppressive function in lung cancer by targeting

STAT3 and CCND1 (12,13). miR-370 was downregulated in lung cancer tissues and the overexpression of miR-370 inhibited the proliferation, colony formation, migration and invasion of lung cancer cells. Furthermore, miR-370 was indicated to target EGFR and regulate its expression, and also to inhibit AKT phosphorylation (14). miR-370 also inhibited the CCND1/CCND kinase (CDK)4/CDK6 pathway by increasing p21 expression in lung cancer cells, and suppressing the progression of non-small cell lung cancer via targeting and reducing the expression of TRAF4 (11,31). In gastric cancer, the expression of miR-370 was negatively associated with EGFR, and the overexpression of miR-370 suppressed the proliferation and migration of gastric cancer cells by targeting EGFR (22). The present study indicated that KCN15-AS1 knockdown inhibited lung cancer cell proliferation, reduced CCND1 expression and inactivated the EGFR/AKT axis via upregulating miR-202 and miR-370 by a rescue experiment.

In conclusion, the results of the present study suggested that KCN15-AS1 promoted lung cancer cell proliferation and inactivated the EGFR/AKT signaling pathway via down-regulating miR-202 and miR-370. Future studies should focus on the mechanisms of how KCN15-AS1 regulates miR-202 and miR-370, in addition to the diagnostic and therapeutic potential of KCN15-AS1 in lung cancer.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JP and HP designed the study. JP, XC, HC, ZX, HW, ZS, JL and XN performed the experiments. JP, XC and HP analyzed the data. JP and HP wrote the paper.

Ethics approval and consent to participate

The present study was ethically approved by the Institutional Ethics Committee of the First People's Hospital of Yunnan Province (Kunming, China). All samples were residual specimens following diagnostic sampling, and all patients provided written informed consent for sampling and molecular analysis separately.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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