LncRNA RP11-805J14.5 functions as a ceRNA to regulate CCND2 by sponging miR-34b-3p and miR-139-5p in lung adenocarcinoma

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Abstract. Lung adenocarcinoma (LUAD) is the most common lung cancer with high incidence. The prognosis of LUAD is poor due to its aggressive behavior. Long non-coding RNAs (lncRNAs) have been reported as a key modulator on LUAD progression. Therefore, the present study aimed to clarify the molecular mechanism of lncRNAs in LUAD development. The expression of lncRNA RP11-805J14.5 (RP11-805J14.5) in LUAD tissues and cells was quantified based on the data in The Cancer Genome Atlas (TCGA). Cell viability was determined using Cell Counting Kit-8 method. Apoptotic cells were sorted and determined by flow cytometry. Cell migration and invasion abilities were detected by the Transwell assay. Luciferase reporter experiment and RNA pull-down assay were utilized to determine the interactions between RP11-805J14.5, microRNA (miR)-34b-3p, miR-139-5p, and cyclin D2 (CCND2). A xenograft tumor was established to determine tumor growth *in vivo*. RP11-805J14.5 was highly expressed in LUAD and associated with poor survival of LUAD patients. Knockdown of RP11-805J14.5 suppressed LUAD cell growth, invasion, migration and tumor growth, indicating that RP11-805J14.5 is an important regulator of LUAD. Our study demonstrated that the regulation of RP11-805J14.5 on LUAD was mediated by CCND2 whose expression was regulated by sponging miR-34b-3p and miR-139-5p. The expression of RP11-805J14.5 was increased in LUAD, and the knockdown of RP11-805J14.5 expression suppressed LUAD cell growth, invasion and migration by downregulating CCND2 by sponging miR-34b-3p and miR-139-5p, indicating that RP11-805J14.5 could be a prospective target for LUAD therapy.

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Introduction

Lung cancer is a frequent malignancy and a leading cause of cancer-related deaths worldwide (1,2). As a common type of lung cancer, lung adenocarcinoma (LUAD) accounts for approximately 50% of primary lung cancer cases (3,4). The prevalence of LUAD has been markedly increased in recent years, especially in women, which may be associated with smoking (4). At present, the treatment of LUAD mainly includes radical resection, radiotherapy, chemotherapy, molecular targeted therapy and immunotherapy (4). In particular, molecular targeted therapy and immunotherapy are important treatment methods for LUAD patients, which significantly prolong the survival time and improve the quality of life. However, due to tumor invasion and distant metastasis, the 5-year survival rate of LUAD remains <20% (1,5,6). Thus, LUAD is a great threat to global health. Hence, it is essential to investigate the underlying mechanism of LUAD progression and search for new therapeutic strategies.

Long non-coding RNAs (lncRNAs) are a category of non-coding RNAs (ncRNA) with a length normally longer than 200 nucleotides (7). Although lncRNAs cannot be translated into proteins, they effectively regulate gene expression by acting as competing endogenous RNAs (ceRNAs) that potently sponge microRNAs (miRNAs) (8,9). Accumulating evidence has revealed that lncRNAs play multiple roles in developing numerous diseases, especially in cancers (10-13). For example, lncRNA RP11-757G1.5 has been revealed to increase the proliferation and metastatic ability of colorectal cancer via sponging miR-139-5p that subsequently increases the expression of YAP1 (14). LINC00665 has been demonstrated to facilitate breast cancer development by regulating the expression of LIN28B via sponging miR-379-5p (15).

In LUAD, some lncRNAs play vital roles in cancer progression (16-18). For example, lncRNA ZFPM2-AS1 was demonstrated to promote the proliferation ability of LUAD via the miR-18b-5p/VMA21 axis (16). LncRNA LINC00511

accelerated the progression of LUAD through modulation of the expression level of PKM2 by sponging miR-625-5p (17). To provide improved explication of the regulatory role of lncRNAs in LUAD, the repertoire of genes dysregulated in LUAD from The Cancer Genome Atlas (TCGA) dataset were first analyzed and it was revealed that RP11-805J14.5 expression was significantly increased in LUAD tumor tissues. In the present study, it was revealed that RP11-805J14.5 could sponge miR-34b-3p and miR-139-5p, thereby regulating CCND2 expression. Interestingly, in bladder cancer, miR-34b-3p could directly bind to CCND2 and P2RY1 mRNAs to regulate their expression and mitigate the chemotherapy resistance of bladder cancer cells (19). However, the role of RP11-805J14.5 in LUAD progression has not been reported. Therefore, the present study aimed to investigate the effect of RP11-805J14.5 on LUAD progression and elucidate the underlying mechanism.

Materials and methods

Patient tissues and cell culture. Tumor tissues and adjacent normal tissues were collected from 60 LUAD patients (age range, 41-73 years; mean age, 58±2.2 years; male/female ratio, 6:7) who underwent surgical resection between August 2016 and June 2020 at Hwa Mei Hospital (Ningbo, China). The inclusion criteria was as follows: None of the patients underwent preoperative chemoradiotherapy and anticancer treatments before surgery. All the cases were confirmed by histological detection. All LUAD patients signed informed consent. The present study was approved by The Ethics Committee of Hwa Mei Hospital (approval no. IR-B-2021-3-18). Human lung epithelial cell line BEAS-2B (CRL-9609), large cell lung carcinoma (LCLC) cell line H460 (HTB-177), LUAD cell lines H1650 (CRL-5883), A549 (CCL-185) and H1975 (HTB-183), and LCLC cell line H1299 (CRL-5826) were purchased from American Type Culture Collection (ATCC). BEAS-2B cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in an atmosphere containing 5% CO_2. H460, H1650, A549, H1975 and H1299 cells were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C, in an atmosphere containing 5% CO_2. The cell lines were cultured for 6-8 passages for gene expression analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from tissues and cell lines were extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) and reversely transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) was employed to conduct the RT-qPCR assay. The reaction mixture was incubated at 95˚C for 2 min. The thermocycling program consisted of holding at 94˚C for 2 min, followed by 30 cycles of 30 sec at 94˚C, 30 sec at 56˚C, and 60 sec at 72˚C. The primers of RP11-805J14.5, miR-34b-3p, miR-139-5p and CCND2 were as follows: RP11-805J14.5 forward, 5'-GAGTAGATGAGAGCC GGCAG-3' and reverse, 5'-GGGGATCCCAGGACTCTTCT-3'; miR-34b-3p forward, 5'-TCTATTTGCCATCGTCTA-3' and reverse, 5'-CAGGCAGCTCATTTGGAC-3' (20); miR-139-5p forward, 5'-GCCTCTACAGTGCACGTGTCTC-3' and reverse, 5'-CGCTGTTCTCATCTGTCTCGC-3' (21); CCND2 forward, 5'-TACACCGACAACTCCATCAAGC-3' and reverse, 5'-GCC AGGTTCCACTTCAACTTC-3' (19). β-actin was used as an internal reference of RP11-805J14.5 and CCND2, and the primers were as follows: Forward, 5'-CCTGTACGCCAACAC AGTGC-3' and reverse, 5'-ATACTCCTGCTTGCTGATCC-3'. In addition, U6 was used as an internal reference of miR-34b-3p and miR-139-5p. The primers for U6 were: Forward, 5'-CTC GCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGA ATTTGCGT-3'. The analysis of the relative expression levels was conducted using the $2^{-\Delta\Delta Cq}$ method (22).

Cell transfection. The siRNAs targeting RP11-805J14.5, miR-34b-3p mimic and miR-139-5p mimic as well as miR-34b-3p inhibitor, miR-139-5p inhibitor were separately designed by Shanghai GeneChem Co., Ltd. The open reading frame of CCND2 was inserted into the expression vector pcDNA 3.1(+) (Sigma-Aldrich; Merck KGaA) to overexpress CCND2, which was also constructed by Shanghai GenePharma Co., Ltd. An empty vector [(pcDNA3.1(+)] was used as a negative control for CCND2 overexpression. A total of 20 nM of each construct was transfected into cells at 37˚C for 15 min using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. After 48 h, the transfected cells were harvested to perform subsequent experiments. The sequences were as follows: si-NC, 5'-TTCTCCGAACGT GTCACGT-3'; si-RNA-RP11-805J14.5#1, 5'-TTGTAGTAT GGAAGTTGTAAAGC-3'; si-RNA-RP11-805J14.5#2, 5'-TAG TATGGAAGTTGTAAAGCTGT-3'; mimics-NC, 5'-TTCTCC GAACGTGTCACGT-3'; miR-34b-3p mimics, 5'-UACCGU CACCUCAAUCACUAAC-3'; miR-139-5p mimics, 5'-UGA CCUCUGUGCACGUGACAUCU-3'; inhibitor-NC, 5'-UUC UCCGAACGUGUCACGUTT-3'; miR-34b-3p inhibitor, 5'-AUGGCAGUGGAGUUAGUGAUUG-3'; miR-139-5p inhibitor, 5'-ACUGGAGACACGUGCACUGUAGA-3'; and CCND2, 5'-AUCUGUUCAGUGUUAAGUGAUUU-3'.

Cell Counting Kit-8 (CCK-8) assay. Transfected LUAD cells were seeded into 96-well plates at a density of $1x10^3$ cells/well. Subsequently, 10 μl of CCK-8 (Dojindo Laboratories, Inc.) was mixed with cells in each well at indicated time-points (0, 24, 48 and 72 h), and incubated for 1 h at 37˚C in an atmosphere containing 5% CO_2. The absorbance at 450 nm was recorded to analyze cell viability.

Flow cytometry. Annexin V-FITC Apoptosis Detection Kit (cat. no. 331200; Thermo Fisher Scientific, Inc.) was utilized to analyze cell apoptosis according to the manufacturer's instructions. Briefly, transfected $2x10^5$ LUAD cells were collected, washed using PBS, and suspended in 500 μl binding buffer, followed by treatment with 5 μl Annexin V-FITC and 3 μl propidium iodide (PI) at room temperature for 15 min in darkness. The proportion of apoptotic cells was determined by flow cytometry (FACSCalibur; BD Biosciences). Cell apoptosis was determined using FlowJo 7.6.1 software (FlowJo LLC).

Transwell migration assay. Transfected LUAD cells ($1x10^5$) were seeded into the upper Transwell chamber (8-μm pore

size) and cultured in the DMEM without FBS. The complete medium (DMEM containing 10% FBS) was added to the lower chamber. Subsequently, cells were incubated at 37˚C for 24 h, the migrating cells were fixed in 4% paraformaldehyde for 15 min at 37˚C and subjected to 0.1% crystal violet staining for 30 min at room temperature. Five areas were randomly selected to determine the number of migrating cells under a light microscope (Nikon Corporation) at a magnification of x200.

Transwell invasion assay. Transfected LUAD cells ($1x10^5$) were seeded into the upper Transwell chamber, which was precoated with Matrigel for 24 h at 37˚C, and cultured in DMEM without FBS. DMEM containing 10% FBS was added to the lower chamber. After cells were incubated at 37˚C for 24 h, the invading cells were fixed in 4% paraformaldehyde for 15 min at 37˚C and subjected to 0.1% crystal violet staining for 30 min at room temperature. Five areas were randomly selected to determine the number of invading cells under a light microscope (Nikon Corporation) at a magnification of x200.

Dual-luciferase reporter assay. The wild-type (WT) and mutant (MUT) sequences of RP11-805J14.5 targeted to miR-34b-3p and miR-139-5p, and the WT and MUT sequences of the 3'-untranslated region (UTR) of CCND2 were subcloned into the psiCHECK-2 vector (Promega Corporation) respectively, followed by co-transfecting with miR-34b-3p mimic or miR-139-5p mimic into $lx10^6$ 293T cells (CRL-3216; ATCC) using Lipofectamine 3000 (Thermo Fisher Scientific, Inc.). After 48 h, the transfected cells were harvested to record the relative luciferase activity using the Dual-luciferase reporter assay system (Promega Corporation) and normalized to *Renilla* luciferase activity.

Nuclear-cytoplasmic fractionation. Cell fractionation buffer (Thermo Fisher Scientific, Inc.) was used to isolate nuclear and cytoplasmic fractions. Then, the extraction of cytoplasmic and nuclear RNAs was accomplished using PARIS™ Kit (cat. no. AM1921; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and was followed by RT-qPCR to assess RP11-805J14.5 expression.

RNA pull-down assay. Biotinylated-miR-34b-3p probes and biotinylated-miR-139-5p were obtained from Guangzhou RiboBio Co., Ltd. The probes were incubated at 4˚C for 2 h with 50 μl C-1 magnetic beads (Thermo Fisher Scientific, Inc.) to generate probe-coated beads and then incubated at 4˚C for 4 h with 0.7 ml RIPA lysis buffer (Thermo Fisher Scientific, Inc.) to lyse LUAD cells. After washing, RNA complexes were subjected to centrifugation at 11,100 x g for 10 min and then eluted by denaturation in 1X protein loading buffer for 10 min at 100˚C. Subsequently, RT-qPCR was used to determine the expression of absorbed RP11-805J14.5.

Western blot analysis. LUAD cell lysates were obtained utilizing RIPA lysis buffer (Sigma-Aldrich; Merck KGaA) and quantified using the BCA method. Lysate samples (40 μg) were resolved in 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk for nonspecific binding for 2 h at 4˚C. The membrane was then probed with anti-CCND2 (1:1,000 dilution; cat. no. 3741) antibody and anti-GAPDH (1:1,000 dilution; cat. no. 8884; both from Cell Signaling Technology, Inc.) antibody at 4˚C for 12 h. Subsequently, the membrane was incubated with IgG H&L (HRP-conjugated; 1:1,000; cat. no. 7076S; Cell Signaling Technology, Inc.) for 2 h at 24˚C. GAPDH was used as the internal control protein. The protein bands were visualized using the ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.).

Xenograft tumor establishment. A total of 12 male BALB/c nude mice aged 4 weeks (weight, 15-20 g) were purchased from Charles River Laboratories, Inc. and kept under standard laboratory conditions: temperature at 22˚C, humidity 55% and a 12-h light/dark cycle. Food and water were provided *ad libitum*. A549 cells that were stably transfected with the siRNA targeting RP11-805J14.5 or negative control were selected using puromycin at 0.5 mg/ml. To establish xenograft tumors, stably transfected A549 cells ($5x10^6$) in 100 μl PBS were subcutaneously injected into the right flank of the mice. The tumor size was measured based on the formula (length x $width^2/2$) every 7 days until 28 days post injection. The humane endpoint of this experiment was set to the maximum size the tumors (2,000 mm^3) allowed to grow in the mice before euthanasia. Both the research team and the veterinary staff monitored animals twice daily. At the end of animal experiment, the maximum percentage of tumor weight/mice weight observed was 5.9%. The maximum tumor diameter observed in the present study was 17.2 mm. The duration of the experiment was 4 weeks. Subsequently, 12 mice were sacrificed under anesthesia induced by mask inhalation of vaporized isoflurane (concentration was maintained at 1.5%) and sacrificed by cervical dislocation. Sacrifice was confirmed by cessation of breathing and heartbeat. Then the tumor volume and RP11-805J14.5 expression were determined. The expression of RP11-805J14.5 was determined using RT-qPCR. All experiments abided by the Institutional Animal Care of Hwa Mei Hospital and were approved by the Experimental Animal Ethics Committee of Hwa Mei Hospital (approval no. IR-D-2021-5-25).

Bioinformatics analysis. The TCGA dataset of RP11-805J14.5 expression in LUAD patients was acquired from the Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) database. The target miRNAs that bound RP11-805J14.5 were studied by screening analysis using the LncBase v2.0 (http://carolina.imis.athena-innovation.gr/diana_tools/web/) database. The target mRNAs that bound miRNA were studied by screening TargetScan Release 7.2 (http://www.targetscan.org/vert_72/).

Statistical analysis. Data were presented as the mean ± standard deviation (SD) and analyzed using SPSS version 22.0 (IBM Corp.). The normality test was performed by Kolmogorov-Smirnov method. The statistical difference between two groups was analyzed by unpaired Student's t-test and paired Student's t-test was used for Figs. 1C and 3E analysis. Pearson's correlation analysis was performed to determine the

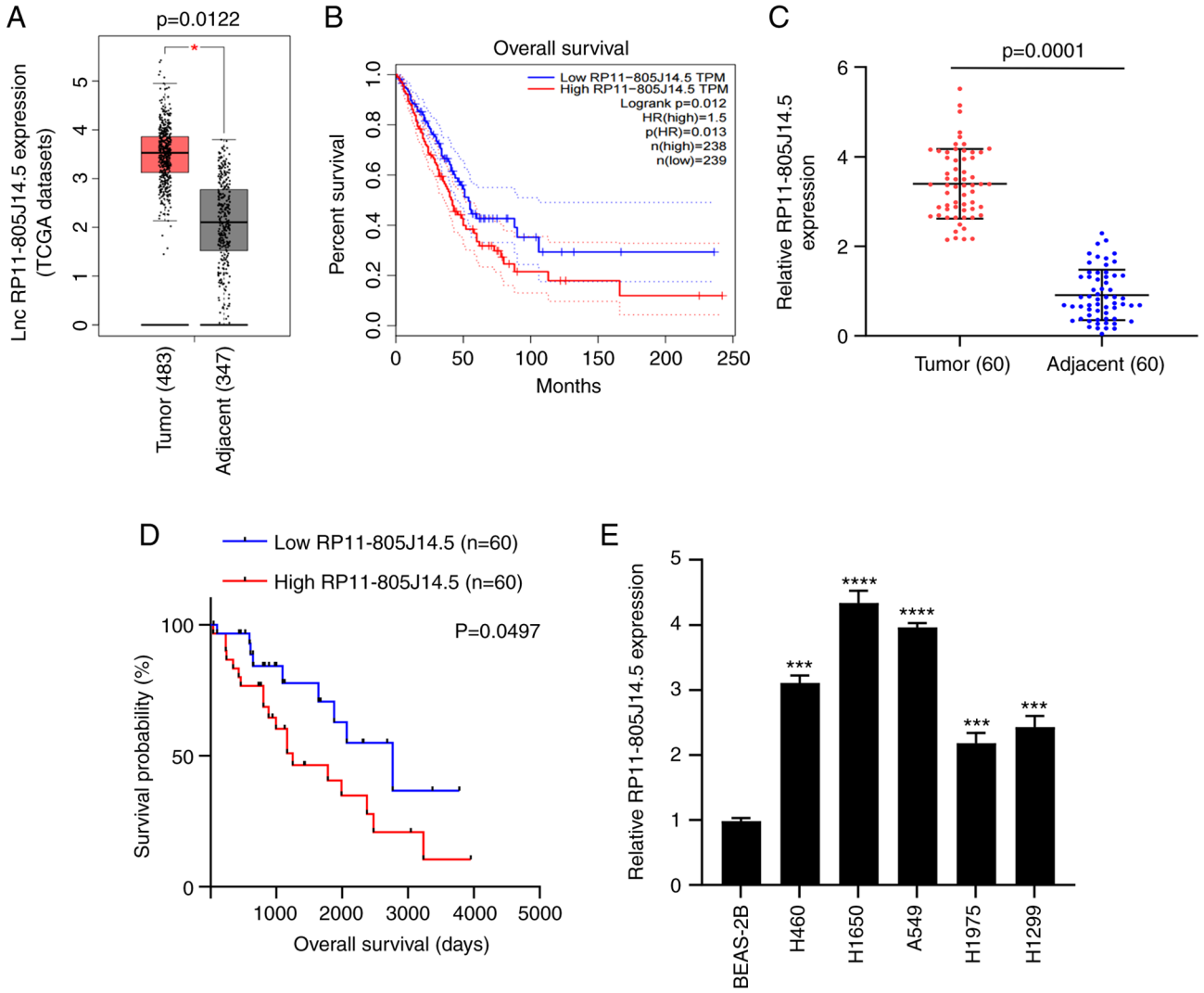

Figure 1. RP11-805J14.5 is highly expressed in LUAD. (A) The expression of RP11-805J14.5 in LUAD was defined based on the data from the TCGA dataset utilizing GEPIA. (B) The association of RP11-805J14.5 and overall survival of LUAD patients was presented based on the data from the TCGA dataset. (C) RP11-805J14.5 expression in tumor tissues and adjacent normal tissues of 60 LUAD patients was assessed using RT-qPCR. (D) The association of the expression level of RP11-805J14.5 and overall survival of 60 LUAD patients was analyzed using Kaplan-Meier analysis. (E) The expression levels of RP11-805J14.5 in human lung epithelial cell line BEAS-2B and LUAD cell lines H460, H1650, A549, H1975, and H1299 were detected using RT-qPCR. All experiments were conducted at least three times. *P<0.05, ***P<0.001 and ****P<0.0001. LUAD, lung adenocarcinoma; TCGA, The Cancer Genome Atlas; RT-qPCR, reverse transcription-quantitative PCR.

correlation between the expression level of RP11-805J14.5 and miR-34b-3p or miR-139-5p. Survival analysis was conducted using Kaplan-Meier analysis with the log-rank testing. Group differences were compared by one-way ANOVA with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

RP11-805J14.5 is highly expressed in LUAD. To investigate the role of lncRNAs in LUAD progression, the TCGA dataset was first analyzed using GEPIA (23) which contains RNA sequencing expression data of lung cancer samples of patients worldwide. The results revealed that the expression of RP11-805J14.5 was significantly elevated in LUAD tumor tissues (P<0.05; Fig. 1A) and some overlap in tumor and non-tumor tissues was revealed. The LUAD patients with high expression of RP11-805J14.5 had poor survival (P=0.013; Fig. 1B). Next, RT-qPCR was used to verify the expression of RP11-805J14.5 in tumor tissues and adjacent normal tissues from 60 LUAD patients. It was revealed that RP11-805J14.5 expression was significantly increased in tumor tissues (P=0.0001; Fig. 1C). Furthermore, Kaplan-Meier analysis revealed that the high expression of RP11-805J14.5 was associated with poor survival of LUAD patients (P=0.0497; Fig. 1D). Due to the amount of data, sample source and tumor heterogeneity, the median survival time was also different, but this did not affect the effect of RP11-805J14.5 expression on survival rate. Subsequently, the RP11-805J14.5 expression in LUAD cells was determined. Consistent with the expression in LUAD tissues, RP11-805J14.5 expression was significantly increased in LUAD cell lines H460, H1650, A549, H1975, and H1299 compared with BEAS-2B cells (P<0.001; Fig. 1E). Therefore, it was concluded that RP11-805J14.5 was highly expressed in LUAD and was associated with poor overall survival of LUAD patients.

Knockdown of RP11-805J14.5 inhibits LUAD cell growth, invasion and migration, as well as tumor growth. To elucidate the function of RP11-805J14.5 in LUAD, two siRNAs targeting RP11-805J14.5 were transfected into A549 and H1650 cells that exhibited the highest expression of RP11-805J14.5. RP11-805J14.5 was significantly downregulated by these two siRNAs in A549 and H1650 cells (P<0.01; Fig. 2A). Then, functional assays revealed that knockdown of RP11-805J14.5 suppressed cell viability (P<0.01; Fig. 2B), induced cell apoptosis (P<0.01; Fig. 2C) and inhibited cell migration (P<0.01; Fig. 2D) and invasion abilities (P<0.01; Fig. 2E). To study the role of RP11-805J14.5 in tumor growth, A549 cells transfected with siRNA targeting RP11-805J14.5 were injected into the mice to establish a xenograft tumor model. Results revealed that RP11-805J14.5 knockdown significantly inhibited tumor size (P<0.01; Fig. 2F). Moreover, the expression level of RP11-805J14.5 was significantly decreased in mice tumor tissues (P<0.001; Fig. 2G). Thus, RP11-805J14.5 knockdown inhibited the cell growth, invasion and migration of LUAD cells as well as tumor growth.

RP11-805J14.5 serves as miR-34b-3p and miR-139-5p sponges in LUAD. To investigate the underlying mechanism of RP11-805J14.5 in LUAD, the subcellular location of RP11-805J14.5 in LUAD cells was identified. It was revealed that RP11-805J14.5 was highly expressed in the cytoplasm compared with the nucleus in A549 and H1650 cells (P<0.001; Fig. 3A). Reasonably, the lncRNA was produced in the nucleus, and subsequently transported to the cytoplasm to play its biological function. Accumulating evidence has revealed that lncRNAs sponge miRNAs and regulate miRNA expression (24,25). Therefore, the target miRNAs that bound RP11-805J14.5 were studied. The screening analysis using LncBase revealed that RP11-805J14.5 may bind to miR-34b-3p and miR-139-5p. The predicted binding motifs of RP11-805J14.5, miR-34b-3p and miR-139-5p are presented in Fig. 3B. To confirm the prediction, luciferase and RNA pull-down assays were conducted. miR-34b-3p mimics and miR-139-5p mimics significantly decreased the activity of the WT of RP11-805J14.5 reporter while they did not affect the activity of MUT (P<0.001; Fig. 3B). An RNA pull-down assay revealed that biotinylated-miR-34b-3p probe and biotinylated-miR-139-5p probe enriched more RP11-805J14.5 compared with the control probe in A549 and H1650 cells (P<0.001; Fig. 3C). In addition, RP11-805J14.5 knockdown significantly increased the expression of miR-34b-3p and miR-139-5p in A549 and H1650 cells (P<0.001; Fig. 3D). The expression of miR-34b-3p and miR-139-5p was significantly decreased in tumor tissues compared with that in adjacent normal tissues (P<0.001; Fig. 3E). Pearson's correlation analysis revealed a negative correlation between the expression of miR-34b-3p and RP11-805J14.5 in LUAD (P<0.0001; Fig. 3F). Moreover, a negative correlation was also identified between the expression of miR-139-5p and RP11-805J14.5 in LUAD (P<0.0001; Fig. 3F). Collectively, these results indicated that RP11-805J14.5 served as a miR-34b-3p and miR-139-5p sponge in LUAD. The expression of miR-34b-3p and miR-139-5p in A549 and H1650 cells transfected with miR-34b-3p and miR-139-5p mimics or inhibitor was then detected (Fig. S1A and B) using RT-qPCR. The results revealed that in cells transfected with miR-34b-3p or miR-139-5p mimics, the level of miR-34b-3p or miR-139-5p was significantly increased but significantly decreased when transfected with miR-34b-3p or miR-139-5p inhibitor, respectively.

RP11-805J14.5 regulates CCND2 expression via sponging miR-34b-3p and miR-139-5p. Usually, miRNAs directly bind target mRNAs and regulate their expression. Through screening TargetScan Release 7.2 (26), it was revealed that CCND2 was a potential target gene of miR-34b-3p and miR-139-5p. The predicted binding sequences of CCND2, miR-34b-3p, and miR-139-5p are presented in Fig. 4A. Luciferase assays revealed that miR-34b-3p mimics and miR-139-5p mimics significantly reduced the activity of the 3'-UTR of CCND2 WT but did not affect the activity of the 3'-UTR of CCND2 MUT in 293T cells (P<0.001; Fig. 4A). Moreover, miR-34b-3p mimics decreased the mRNA and protein levels of CCND2 while miR-139-5p inhibitor increased these levels in A549 and H1650 cells (P<0.001; Fig. 4B and C). Furthermore, knockdown of RP11-805J14.5 decreased the mRNA and protein levels of CCND2 (P<0.001), however this decrease was abolished by either miR-34b-3p inhibitor or miR-139-5p inhibitor in A549 and H1650 cells (P<0.01; Fig. 4D and E). Collectively, these results indicated that RP11-805J14.5 served as a sponge of miR-34b-3p and miR-139-5p, thereby modulating the expression of CCND2.

RP11-805J14.5 regulates LUAD progression by modulating CCND2 expression via sponging miR-34b-3p and miR-139-5p. Given the demonstrated interactions between RP11-805J14.5 and miR-34b-3p/miR-139-5p, and between these two miRs and CCND2, it was investigated whether miR-34b-3p, miR-139-5p and CCND2 mediated the regulation of RP11-805J14.5 on LUAD progression. To this end, the overexpression plasmid of CCND2 was transfected into A549 and H1650 cells, and transfection efficiency was verified using RT-qPCR and western blotting (P<0.01; Fig. 5A). Then, the functional assays of RP11-805J14.5, miR-34b-3p, miR-139-5p and CCND2 on LUAD progression were conducted. Results revealed that knockdown of RP11-805J14.5 suppressed cell viability (P<0.001, Fig. 5B), but this effect was reversed by downregulation of miR-34b-3p and miR-139-5p or overexpression of CCND2 in A549 and H1650 cells (P<0.01; Fig. 5B). Moreover, cell apoptosis induced by RP11-805J14.5 knockdown was inhibited by decreasing miR-34b-3p and miR-139-5p expression or by CCND2 overexpression in A549 and H1650 cells (P<0.01; Fig. 5C). Furthermore, RP11-805J14.5 knockdown suppressed cell migration and invasion (P<0.001), while this effect was abolished by either the downregulation of miR-34b-3p or miR-139-5p or CCND2 overexpression in A549 and H1650 cells (all P<0.01; Fig. 5D and E). Thus, our results indicated that RP11-805J14.5 regulated LUAD progression by modulating CCND2 expression via acting as a miR-34b-3p and miR-139-5p sponge.

Discussion

LUAD is a major type of primary lung cancer, and its incidence has been increased in recent years (3,4). However, the prognosis of LUAD patients is low due to the aggressive behavior

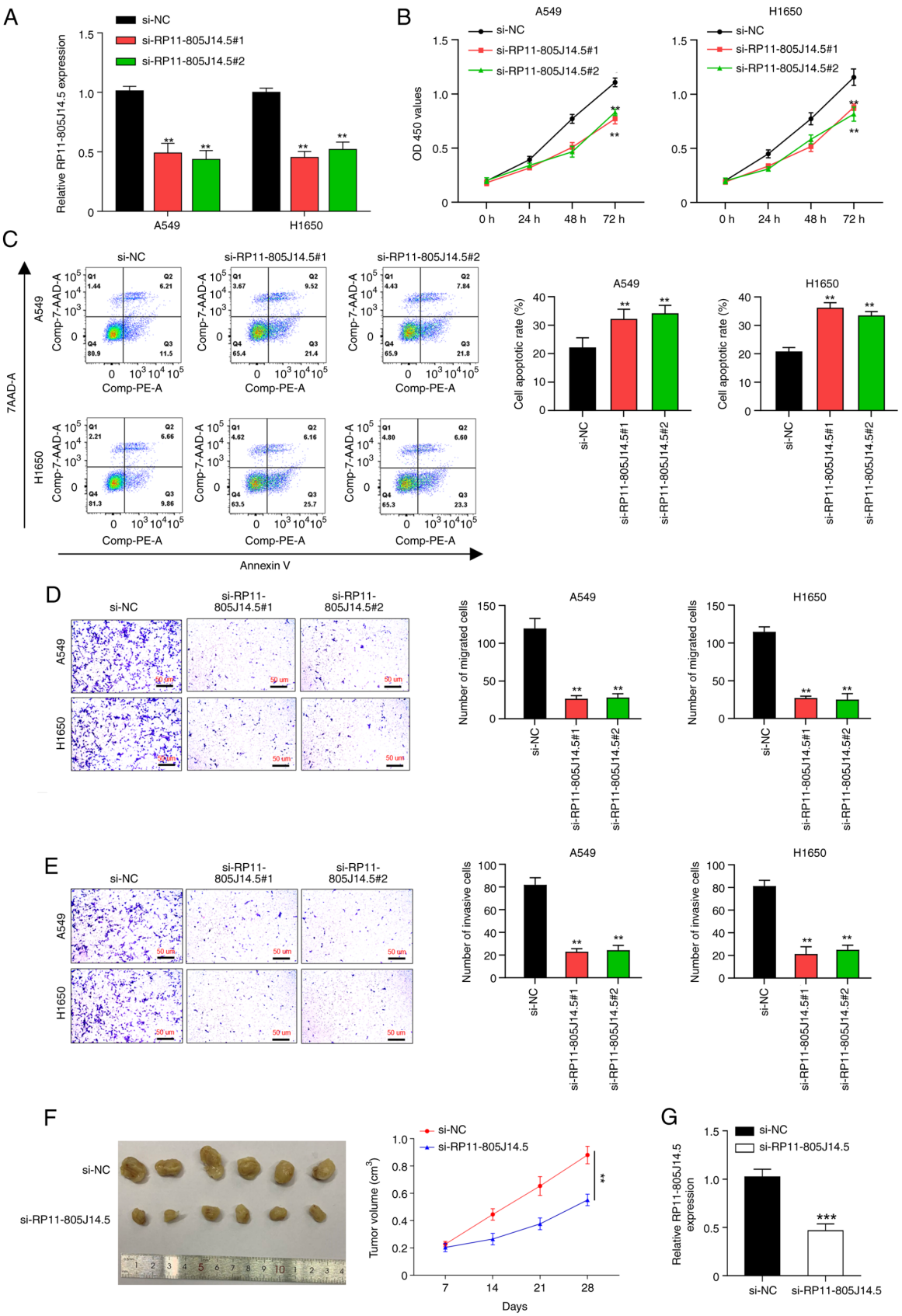

Figure 2. Knockdown of RP11-805J14.5 inhibits LUAD cell growth, invasion, migration and tumor growth. (A) The transfection efficiency of two siRNAs targeting RP11-805J14.5 in A549 and H1650 cells was determined using RT-qPCR. (B) Cell viability of A549 and H1650 cells with RP11-805J14.5 knockdown was determined using Cell Counting Kit-8 assay. (C) Cell apoptosis of A549 and H1650 cells with RP11-805J14.5 knockdown was determined using flow cytometry. (D) Transwell migration assays evaluated the migration abilities of A549 and H1650 cells with RP11-805J14.5 knockdown. (E) Transwell invasion assays assessed the invasion abilities of A549 and H1650 cells with RP11-805J14.5 knockdown. (F) Tumor sizes in xenograft tumor models were measured every 7 days until 28 days after establishment. (G) The expression of RP11-805J14.5 in xenograft tumors was determined using RT-qPCR. All experiments were conducted at least three times. **P<0.01 and ***P<0.001. LUAD, lung adenocarcinoma; si-, small interfering; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

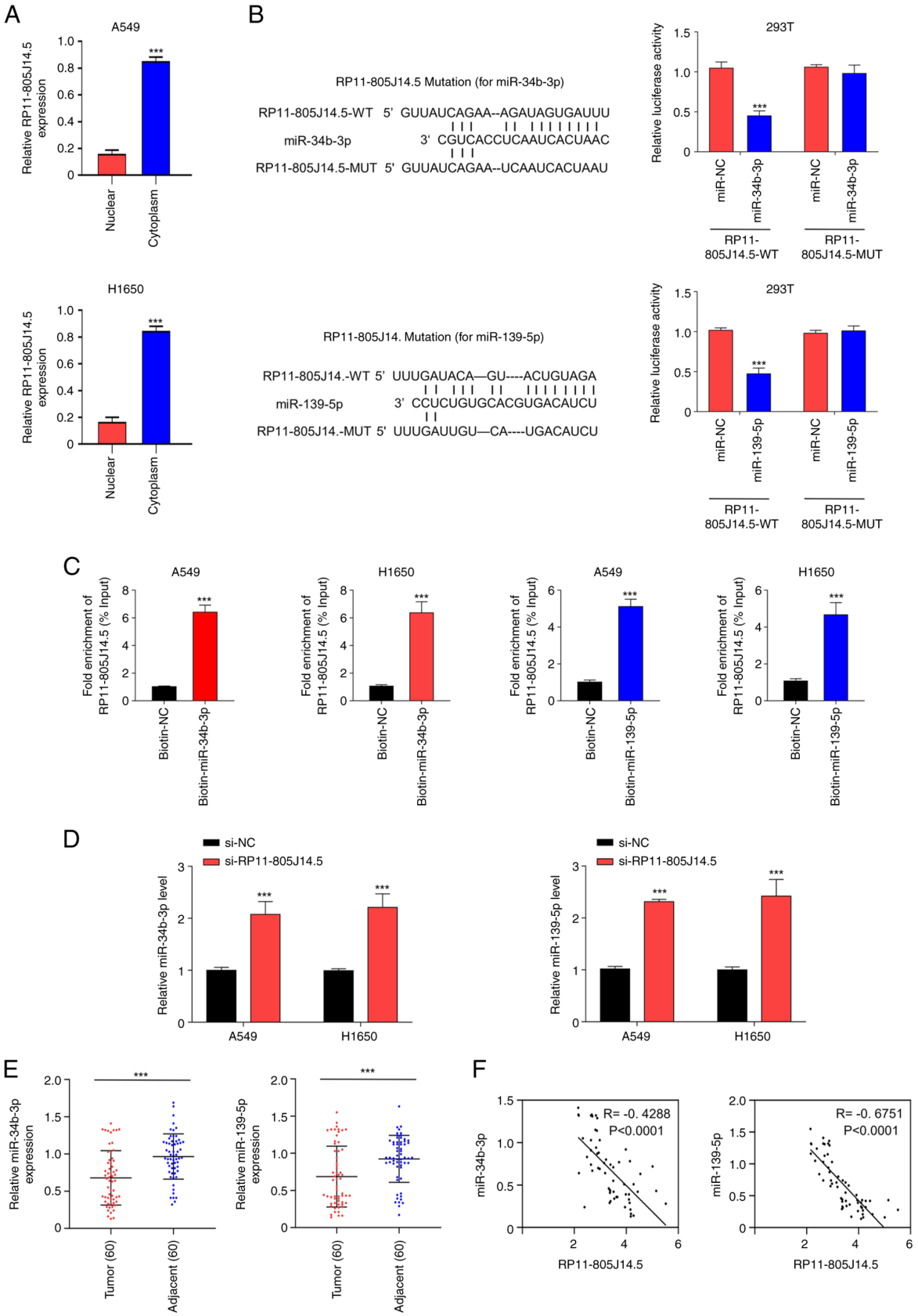

Figure 3. RP11-805J14.5 serves as a miR-34b-3p and miR-139-5p sponge in LUAD. (A) RT-qPCR was used to determine the levels of RP11-805J14.5 in the nucleus and cytoplasm of A549 and H1650 cells. (B) Luciferase assays were used to explore whether RP11-805J14.5 directly targeted miR-34b-3p and miR-139-5p in 293T cells. (C) RNA pull-down assays were conducted to further demonstrate the binding of RP11-805J14.5 and miR-34b-3p or miR-139-5p in A549 and H1650 cells. (D) The levels of miR-34b-3p and miR-139-5p were explored using RT-qPCR in A549 and H1650 cells with knockdown of RP11-805J14.5. (E) The levels of miR-34b-3p and miR-139-5p in tumor tissues and adjacent normal tissues of 60 LUAD patients were assessed using RT-qPCR. (F) The association of the levels of miR-34b-3p or miR-139-5p and RP11-805J14.5 was analyzed using Pearson's correlation analysis. All experiments were conducted at least three times. ***P<0.001. miR, microRNA; LUAD, lung adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR; si-, small interfering; NC, negative control; WT, wild-type; MUT, mutant.

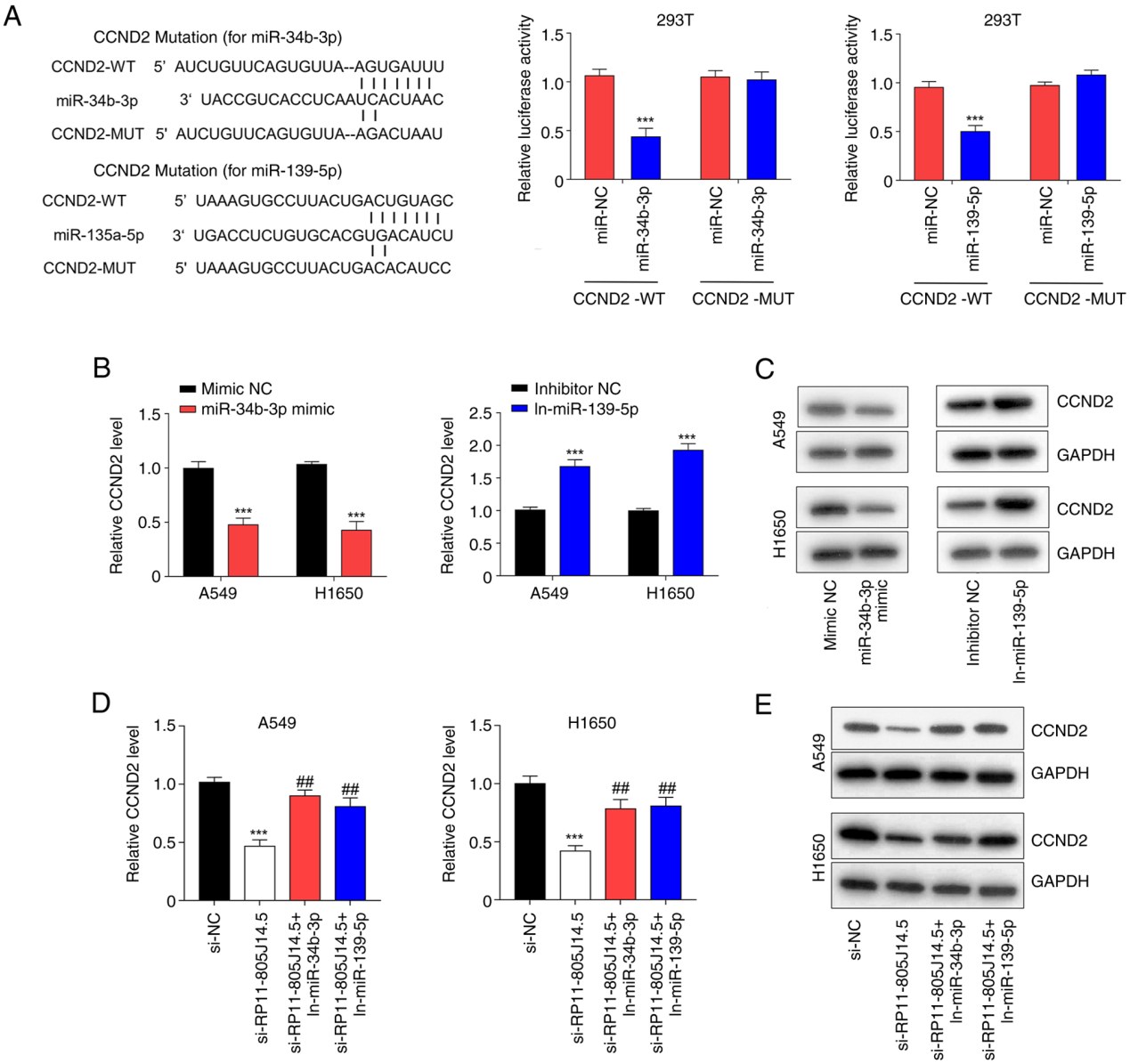

Figure 4. RP11-805J14.5 regulates CCND2 expression via sponging miR-34b-3p and miR-139-5p. (A) Luciferase assays were used to confirm the binding of miR-34b-3p and miR-139-5p to CCND2 in 293T cells. ***P<0.001 vs. miR-NC. (B) The mRNA levels of CCND2 were determined using RT-qPCR after the overexpression of miR-34b-3p and inhibition of miR-139-5p. ***P<0.001 vs. mimic NC or inhibitor NC. (C) The protein levels of CCND2 were determined using western blotting after the overexpression of miR-34b-3p and inhibition of miR-139-5p. (D) The mRNA levels of CCND2 were determined using RT-qPCR in A549 and H1650 cells co-transfected with siRNAs targeting RP11-805J14.5 and miR-34b-3p inhibitor or miR-139-5p inhibitor. (E) The protein levels of CCND2 were determined using western blotting in A549 and H1650 cells after co-transfection with siRNAs targeting RP11-805J14.5 and miR-34b-3p inhibitor or miR-139-5p inhibitor. All experiments were conducted at least three times. ***P<0.001 vs. si-NC; and ##P<0.01 vs. si-RP11-805J14.5. CCND2, cyclin D2; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; si-, small interfering; NC, negative control; WT, wild-type; MUT, mutant; In, inhibitor.

of this cancer (6). Therefore, it is imperative to elucidate the molecular mechanism of LUAD development and explore new therapeutic strategies. LncRNAs play a pivotal role in LUAD progression (16-18). However, the biological function of numerous lncRNAs has not been fully understood.

To define the role of lncRNAs in LUAD progression, the data of LUAD from TCGA dataset were screened, which revealed that RP11-805J14.5 expression was significantly elevated in LUAD tumor tissues even though RP11-805J14.5 expression revealed some overlap in tumor and non-tumor tissues. Next, the elevated expression of RP11-805J14.5 in LUAD tissues (which were collected from Hwa Mei Hospital) and cells was confirmed using RT-qPCR. The results of the present study explicitly demonstrated that RP11-805J14.5 was increased in LUAD tissues and cells. Moreover, the increased expression of RP11-805J14.5 was associated with poor survival of LUAD patients. Although the median survival time in TCGA datasets was not exactly the same as that in the LUAD samples collected, it still indicated that lncRNA RP11-805J14.5 is a potential prognostic marker. Therefore, it is necessary to further expand the sample size to confirm that lncRNA RP11-805J14.5 is a valuable prognostic indicator. Furthermore, it was revealed that knockdown of RP11-805J14.5 inhibited LUAD cell growth, invasion, migration and tumor growth. The present study is, to the best of our knowledge, the first study on the role of RP11-805J14.5 in LUAD progression.

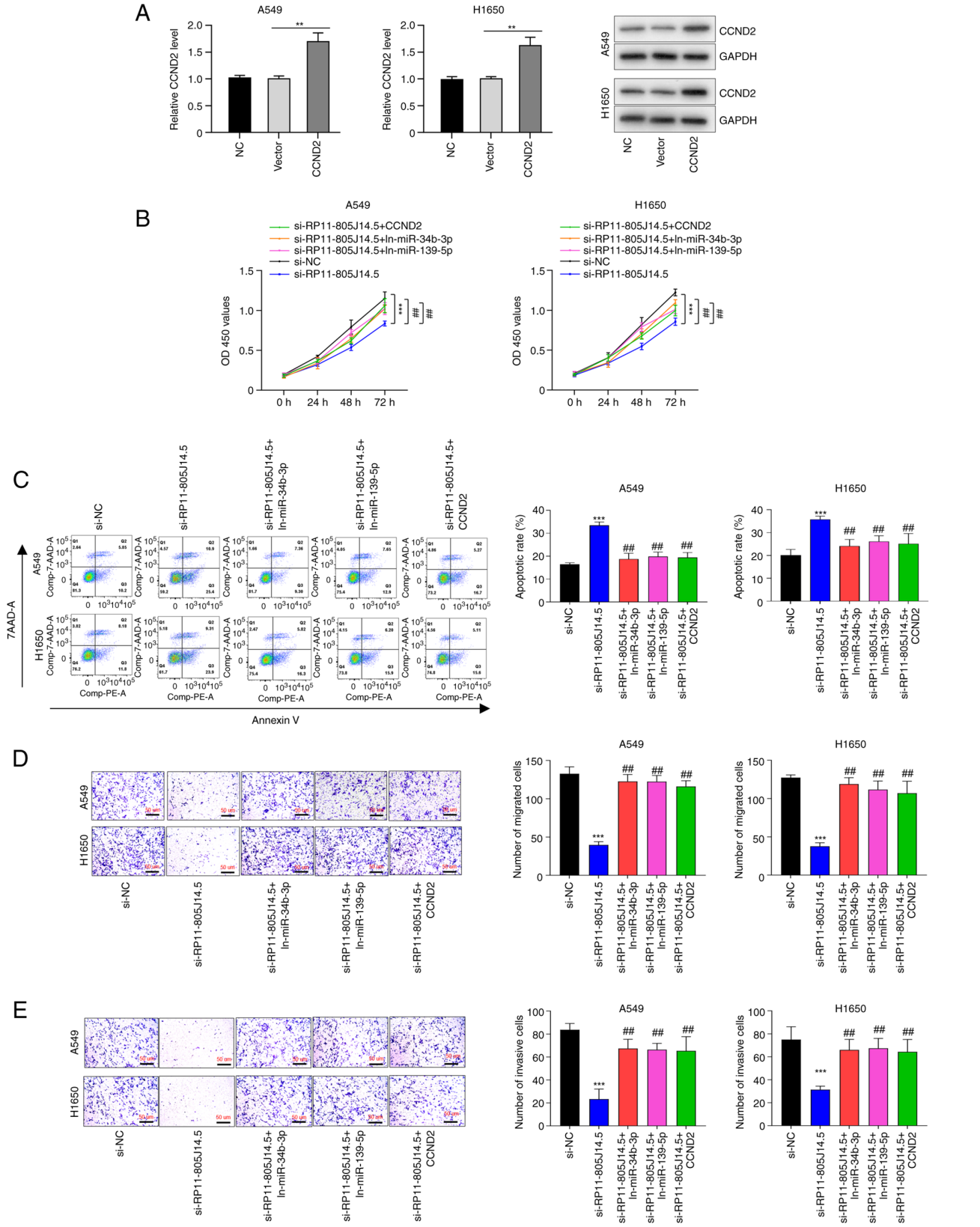

Figure 5. RP11-805J14.5 regulates LUAD progression by modulating CCND2 expression via sponging miR-34b-3p and miR-139-5p. (A) The transfection efficiency of the overexpression plasmid of CCND2 in A549 and H1650 cells was determined using reverse transcription-quantitative PCR and western blot analysis. (B) Cell viability of A549 and H1650 cells co-transfected with siRNAs targeting RP11-805J14.5 and miR-34b-3p inhibitor or miR-139-5p inhibitor or CCND2-overexpressing plasmid was determined using Cell Counting Kit-8 assays. (C) Cell apoptosis of A549 and H1650 cells co-transfected with siRNAs targeting RP11-805J14.5 and miR-34b-3p inhibitor, miR-139-5p inhibitor or CCND2-overexpressing plasmid was determined using flow cytometry. (D) Transwell migration assays determined the migration abilities of A549 and H1650 cells after co-transfection with siRNAs targeting RP11-805J14.5 and miR-34b-3p inhibitor or miR-139-5p inhibitor or CCND2-overexpressing plasmid. (E) Transwell invasion assays assessed the invasion abilities of A549 and H1650 cells after co-transfection with siRNAs targeting RP11-805J14.5 and miR-34b-3p inhibitor or miR-139-5p inhibitor or CCND2-overexpressing plasmid. All experiments were conducted at least three times. ***P<0.001 vs. si-NC; and $^{\#\#}$P<0.01 vs. si-RP11-805J14.5. LUAD, lung adenocarcinoma; CCND2, cyclin D2; miR, microRNA; small interfering; NC, negative control; In, inhibitor.

LncRNAs regulate gene expression via acting as sponges of miRNAs, thereby modulating disease development (27). In order to elucidate the mechanism of RP11-805J14.5 in LUAD, it was first determined that RP11-805J14.5 mainly exists in the cytoplasm. The putative target miRNAs were then screened and confirmed. miR-34b-3p and miR-139-5p were identified as the miRNAs that bound to RP11-805J14.5, and which were negatively regulated by RP11-805J14.5. Both miR-34b-3p and miR-139-5p were decreased in LUAD tissues, which was consistent with recent studies (28,29). Specifically, miR-34b-3p and miR-139-5p have been revealed as key regulators in numerous diseases. For example, miR-34b-3p inhibited the chemoresistance of bladder cancer cells (19). miR-34b-3p may promote antiplatelet efficiency of aspirin by inhibiting thromboxane synthase expression (29) and miR-34b-3p impaired HUVEC viability and migration via targeting PDK1 in an *in vitro* model of gestational diabetes mellitus (30). LncRNA GAS5 silencing was revealed to attenuate oxygen-glucose deprivation/reperfusion-induced injury in brain microvascular endothelial cells via miR-34b-3p-dependent regulation of EPHA4 (31). However, miR-34b-3p played an important role in non-small cell lung cancer (NSCLC), which suppressed cell proliferation and the cell cycle via binding to CDK4 (32). Piperlongumine inhibited the growth of NSCLC cells via the miR-34b-3p/TGFBR1 pathway (33). In addition, miR-139-5p suppressed the epithelial-mesenchymal transition and increased the chemotherapeutic sensitivity of colorectal cancer cells via targeting BCL2 (34). LncRNA AFAP1-AS1 suppressed miR-139-5p and promoted cell proliferation and chemotherapy resistance of NSCLC by competitively upregulating RRM2 (35). Furthermore, miR-139-5p expression was decreased in lung cancer patients with lytic bone metastasis, which indicated that miR-139-5p may be a biomarker and target in monitoring and controlling bone metastasis of lung cancer (28). All aforementioned studies have revealed that miR-34b-3p and miR-139-5p play an indispensable role in NSCLC, which further supports our research.

MiRNAs can suppress the expression of target genes via binding to the 3'-UTR of target genes (36). In the present study, it was revealed that CCND2 could be targeted by miR-34b-3p and miR-139-5p and negatively regulated by these two miRNAs. Furthermore, it was also demonstrated that RP11-805J14.5 served as the sponge of miR-34b-3p and miR-139-5p to modulate the expression of CCND2. Previous studies have analyzed the pathogenesis and progression of NSCLC at the molecular level. With the development of NGS technology, RNA-seq gene expression profiles have also been drawn (37-39). In addition, Tao *et al* provided a systematic view of the functional alterations during tumorigenesis that may help to elucidate the mechanisms of lung cancer and lead to improved treatments for patients (37). A computational method was built to identify new potential candidate cancer driver genes. The analyses indicated that some of the obtained genes have the potential to drive tumorigenesis on multiple differentiation levels. It is hopeful that the findings of the present study will promote the study of cancer driver genes and provide new insights into the investigation of tumor initiation. To achieve this purpose, Chen *et al* used protein information (protein-protein interaction) to investigate cancer driver genes (40). Liu *et al* detected a series of genes and the identification of the transcription factors provided a new insight into its oncogenic role in tumor initiation and progression, and benefited the discovery of a functional core set that may reverse malignant transformation and reprogram cancer cells (41). In the present study, it was also demonstrated that the aberrant expression level of CCND2 which was triggered by RP11-805J14.5 was the underlying cause of LUAD. However, most of the genes in these expression profiles have not been verified by functional experiments. CCND2 belongs to the D-type cyclin family, and it plays a critical role in the cell cycle (42). The function of CCND2 in cancer progression regulation has been demonstrated in prostate (43), ovarian (44), thyroid cancer (45), and NSCLC (46). Accumulating evidence revealed that the imbalance of circular RNAs plays a key role in multiple solid tumors. Circ-RAD23B that is overexpressed in NSCLC, has been revealed to be associated with lymph node invasion, poor differentiation and short overall survival (OS). In addition, circ-RAD23B has been demonstrated to act as an oncogene in NSCLC cells. Mechanistically, circ-RAD23B can bind miR-593-3p and miR-653-5p to increase the expression of CCND2 and TIAM1. Rescue experiments revealed that circ-RAD23B promoted cell growth through the miR-593-3p/CCND2 axis, and promoted cell invasion through the miR-653-5p/TIAM1 pathway. In conclusion, it is considered that circ-RAD23B is a promising biomarker and therapeutic target for NSCLC (46). The present study demonstrated the role of CCND2 in promoting cell growth in NSCLC. In our study, RP11-805J14.5 regulated LUAD cell growth, invasion and migration through modulation of CCND2 expression via sponging miR-34b-3p and miR-139-5p, which also indicated the function of CCND2 in LUAD. Collectively, lncRNA RP11-805J14.5 functions as a ceRNA to regulate CCND2 expression by sponging miR-34b-3p and miR-139-5p in LUAD. However, our research still has some shortcomings, such as the small sample size which will be expanded in a future study. In addition, the prognosis analysis of lung cancer patients will be further improved in order to more accurately determine whether lncRNAs can be used as prognostic indicators of NSCLC. In conclusion, RP11-805J14.5 was decreased in LUAD, and its knockdown suppressed LUAD cell growth, invasion and migration by decreasing CCND2 via sponging miR-34b-3p and miR-139-5p, rendering RP11-805J14.5 a prospective target for LUAD therapy.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HZ and GZ made substantial contributions to conception and design of the study. XX, EZ and MY performed the experiments and acquired the data. JN and XJ analyzed the data. HZ and XX drafted the manuscript. GZ and XX critically revised the study for important intellectual content. EZ and MY confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the work.

Ethics approval and consent to participate

The present study was approved (approval no. IR-B-2021-3-18) by The Ethics Committee of Hwa Mei Hospital (Ningbo, China) and written informed consent was obtained from patients in all cases. All animal experiments abided by the National and International regulations and policies and were approved (approval no. IR-D-2021-5-25) by the Experimental Animal Ethics Committee of Hwa Mei Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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