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microRNA-1205 promotes cell growth by targeting APC2 in lung adenocarcinoma

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Abstract. – OBJECTIVE: An increasing amount of evidence indicates that microRNAs (miRNAs) can be potential diagnostic and prognostic markers for various cancers. In this study, a novel miRNA, miR-1205, was identified in lung adenocarcinoma (LUAD).

PATIENTS AND METHODS: First, the expression of miR-1205 in tissues was determined and verified to be correlated with the prognosis of patients. Overexpression and knockdown in LUAD cells were chosen to evaluate the effect of miR-1205 on cell growth *in vitro*. Luciferase assays, Western blot and rescue assays were performed to screen and confirm potential targets of miR-1205.

RESULTS: We demonstrated that miR-1205 was down-regulated in the tissues of LUAD, and that miR-1205 may be a predictor of overall survival of LUAD. The overexpression of miR-1205 promoted cell proliferation and colony formation. Our results indicated that miR-1205 targeted APC2 directly, serving as a vital part in accelerating LUAD cell proliferation.

CONCLUSIONS: We showed that miR-1205 could promote LUAD cell growth by targeting APC2 protein expression and provided further proof of miR-1205 as a potential non-invasive biomarker and therapeutic target for LUAD.

Key Words
MiR-1205, LUAD, Proliferation, APC2.

Introduction

As one of the commonest malignant tumors, lung cancer has become the major cause of cancer-related deaths worldwide[1,2]. Approximately 80% of all lung cancer types belong to non-small cell lung cancer (NSCLC)[3]. Non-small cell lung cancer can be divided into two major subtypes: lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD)[4]. At present, the incidence of LUAD has exceeded that of LUSC, which has been the main cause of death in patients with NSCLC[5]. In recent years, precision medical and molecular targeted therapy technologies have made great progress, but the 5-year survival rate of patients with LUAD is still not optimistic[6]. Therefore, it is critical to find the diagnostic markers and effective therapeutic targets for LUAD. MiRNAs (microRNAs) are small RNAs consisting of 21-25 nucleotides and provide negative post-transcriptional regulation[7]. An increasing amount of evidence indicates that miRNAs are frequently dysregulated in diverse cancers and can be a crucial factor in regulating cancer progression and metastasis[8-10]. MiRNAs have been established as players with a relevant role in lung cancer progression, epithelial-mesenchymal transition and response to therapy[11]. MiR-4735-3p suppresses cell proliferation and migration by modulating FBXL3 in NSCLC[12], while miRNA-19 promotes NSCLC cell proliferation *via* inhibiting CBX7 expression[13]. MicroRNA-1205 (miR-1205), a member of the PVT1 region, hasn't been well explored[14]. Scholars[15] have indicated that circular RNA circ_0002052 suppresses osteosarcoma progression *via* modulating the Wnt/β-catenin pathway by miR-1205. However, studies on whether miR-1205 participates in the progression of LUAD have not been reported. Adenomatous polyposis coli 2 (APC2), a well-known homolog of the adenomatous polyposis coli (APC) tumor suppressor gene, regulates the Wnt/β-catenin signaling pathway negatively[16-18]. Zhang et al[19] have found that G9a inhibits growth and Wnt signaling pathway by targeting HP1α and APC2 gene expression in NSCLC. Despite this, little has been known about APC2 and its correlation with miRNA in LUAD. The relation between miR-1205 and APC2, as well as their expression

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and biological functions, have not been explored in LUAD. In this work, it was substantiated that miR-1205 is an oncogene of LUAD and can be used as a molecular marker for therapy.

Patients and Methods

Patients

We collected 54 pairs of tumors and adjacent tissues that were resected from LUAD patients in the First Affiliated Hospital of China Medical University general surgery since 2012. This study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University. Signed informed consents were obtained from all patients before the study. Samples were stored at -80°C after excision and prior to RNA extraction.

Cell Culture

A549, H1299, PC9, H460 cells and normal lung tissue cells (16HBE) were obtained from Shanghai Academy of Sciences (Shanghai, China). LUAD and normal cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA). Both were supplemented with 5% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) and 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were cultured in a 37°C incubator containing 5% CO_2.

Complementary Deoxyribose Nucleic Acid (cDNA) Synthesis and Quantitative Real Time-Polymerase Chain Reaction

Total RNA was extracted by RNA extraction kit (TaKaRa, Otsu, Shiga, Japan). The reverse transcription reaction was performed by PrimeScript RT kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was conducted by SYBR Premix ExTaq RR420A reagent (TaKaRa, Otsu, Shiga, Japan). The primers used in the experiment were purchased from BioEngineering (Shanghai, China). Specific primers and internal controls for mRNA are as follows: APC2: F: CTGTACCGGGTCTCTGCAGTGTTA R: TACGCCGGACAGATGGCTTTA; GAPDH: F: AAGGTGAAGGTCGGAGTCA R: GGAAGATGGTGATGGGATTT. GAPDH was used for internal reference of APC2 mRNA. The amplification results were detected by ABI 7300 Real Time-Polymerase Chain Reaction system (BD Biosciences, Franklin Lakes, NJ, USA). The results were shown in relative expressions calculated by the $2^{-\Delta\Delta CT}$ method.

Cell Transfection

MiR-1205 mimics, miR-1205 inhibitor, NC, APC2 plasmid and siAPC2 were synthesized and purchased by RiboBio (Guangzhou, China). In a six-well plate, cells were plated at a number of 20×10^4 per well. The next day, Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was conducted to transfect the experimental and control cells in accordance with the manufacturer's instructions, separately. After 2 days, all transfected cells were collected for the next step.

Cell Counting Kit-8 (CCK-8) Assay

The transfected cells were seeded in 96-well plates at a number of 1×10^3 per well. 10 μL of Cell Counting Kit-8 solution (CCK-8; Dojindo, Kumamoto, Japan) and 100 μL of the culture solution were mixed and added to each well. After 2 h, we measured optical density (OD) 450 with a microplate reader. The results of four days were measured continuously and plotted as a line graph.

Colony Formation

The transfected cells were seeded in six-well plates at a number of 1000 per well. The cells were incubated for 12 days. Finally, each well was stained with 0.1% crystal violet staining solution (Invitrogen, Carlsbad, CA, USA). Plates were washed with water and then photographed. Colonies which contained at least 50 cells were counted.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The transfected cells were seeded in a six-well plate at a number of 4×10^4 per well. The next day, according to the instructions, the cells were stained by EdU kit (RiboBio, Guangzhou, China). The results were photographed by fluorescence microscopy and merged.

Bioinformatic Analysis

TargetScan, miRWalk and miRPathDB were chosen to identify miR-1205 target genes.

Luciferase Reporter Assay

The 3′-UTR sequence of APC2 predicted to bind with miR-1205 or a mutated sequence within the predicted target sites was synthesized and inserted into the XbaI and FseI sites of the pGL3 control vector (Promega, Madison, WI, USA). H1299 and H460 cells were seeded in 24-well plates and were then co-transfected with miR-1205 mimics or NC and Luciferase reporter vec-

tors by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Reporter activities were measured on a SpectraMax L luminescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western Blot

Protein extraction was performed by adding radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and protease inhibitor to the cell pellet. The lysate was centrifuged at 12000 r/min for 15 min, and the supernatant was taken as the total protein. The extracted protein was mixed with a loading buffer sodium dodecyl sulphate (SDS)-Loading Buffer (CWBIO, Beijing, China), and then placed in a boiling water bath for 10 min. The sample was added to the prepared electrophoresis gel for electrophoresis. After electrophoresis, the protein bands in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were incubated with 1:1 000 diluted anti-APC2 (ab76452, Abcam, Cambridge, MA, USA) and anti-GAPDH (2118, Cell Signaling Technology, Danvers, MA, USA) primary antibodies at 4°C. After 12 h, horseradish peroxidase conjugated with IgG antibody was added and shaken at low speed for 90 min. An enhanced hypersensitive chemiluminescent substrate reagent (BD Biosciences, Franklin Lakes, NJ, USA) was added dropwise to the membrane and imaged using a Quant LAS 4010 imaging system (Tokyo, Japan).

Statistical Analysis

All values are expressed as means ± SD (Standard Deviation). The effects of different treatments were compared by an unpaired Student's *t*-test. A difference was considered of statistically significant if the probability was <0.05 ($p<0.05$). All analyses were carried out with GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Results

Upregulation of MiR-1205 in LUAD

The results of qRT-PCR showed that the content of miR-1205 in LUAD tissues was higher than that in adjacent tissues, and the difference between groups was of significance (Figure 1A). Similarly, in LUAD cell lines, the expression level of miR-1205 is generally higher than that in 16HBE (Figure 1B). In addition, based on the median level, we divided the 54 patients into high and low expression groups to explore correlations between clinicopathological features and miR-1205 expression (Figure 1C). The miR-1205 expression was markedly associated with tumor size and TNM stage, while no differences were found with gender, age, distant metastasis or lymph node metastasis (Table I). At the same time, the prognosis of patients with low expression of miR-1205 was better than that of patients with high expression (Figure 1D). The above results valitated that miR-1205 could function as a tumor-promoting factor in LUAD.

Overexpression of MiR-1205 can Induce Increased Cell Proliferation in LUAD Cells

As suggested in Figure 2A, the content of miR-1205 in the mimics group was markedly higher than that in the control group in H1299 cells. In the inhibitor group, the content of miR-1205 was only half of that in the control group in H460 cells. The results of the CCK-8 assay showed that high expression of miR-1205 resulted in an increased rate of cell proliferation, whereas the inhibition of miR-1205 expression reversed this phenomenon (Figure 2B). In the aspect of colony forming ability, the colonies of H1299 cells were significantly increased after overexpression of miR-1205, while the ability of colonies formed by low expression of miR-1205 was dramatically weaker than that of the control group (Figure 2C). The Edu experiment again validated the effect of miR-1205 on proliferation. As seen in Figure 2D, the overexpression of miR-1205 increased the percentage of Edu positive cells. Likewise, the knockdown of miR-1205 expression markedly decreased Edu positive cells in the miR-1205 inhibitor group. From the above results, we concluded that miR-1205 had an ability to enhance cell proliferation activity.

APC2 Was a Direct Target of MiR-1205

Previous studies have shown that miRNAs could degrade mRNA by binding to the 3'-UTR of mRNA to achieve post-transcriptional regulation. We found possible target genes through TargetScan, miRWalk and miRPathDB. It was predicted that miRNA-1205 has a 9-base binding site with the 3'-UTR region of APC2 mRNA. APC2 may be regulated by 3-UTR binding (Figure 3A). The expression of APC2 in tumor tissues was remarkably lower than that in adjacent tissues (Figure 3B). The expression of APC2 was

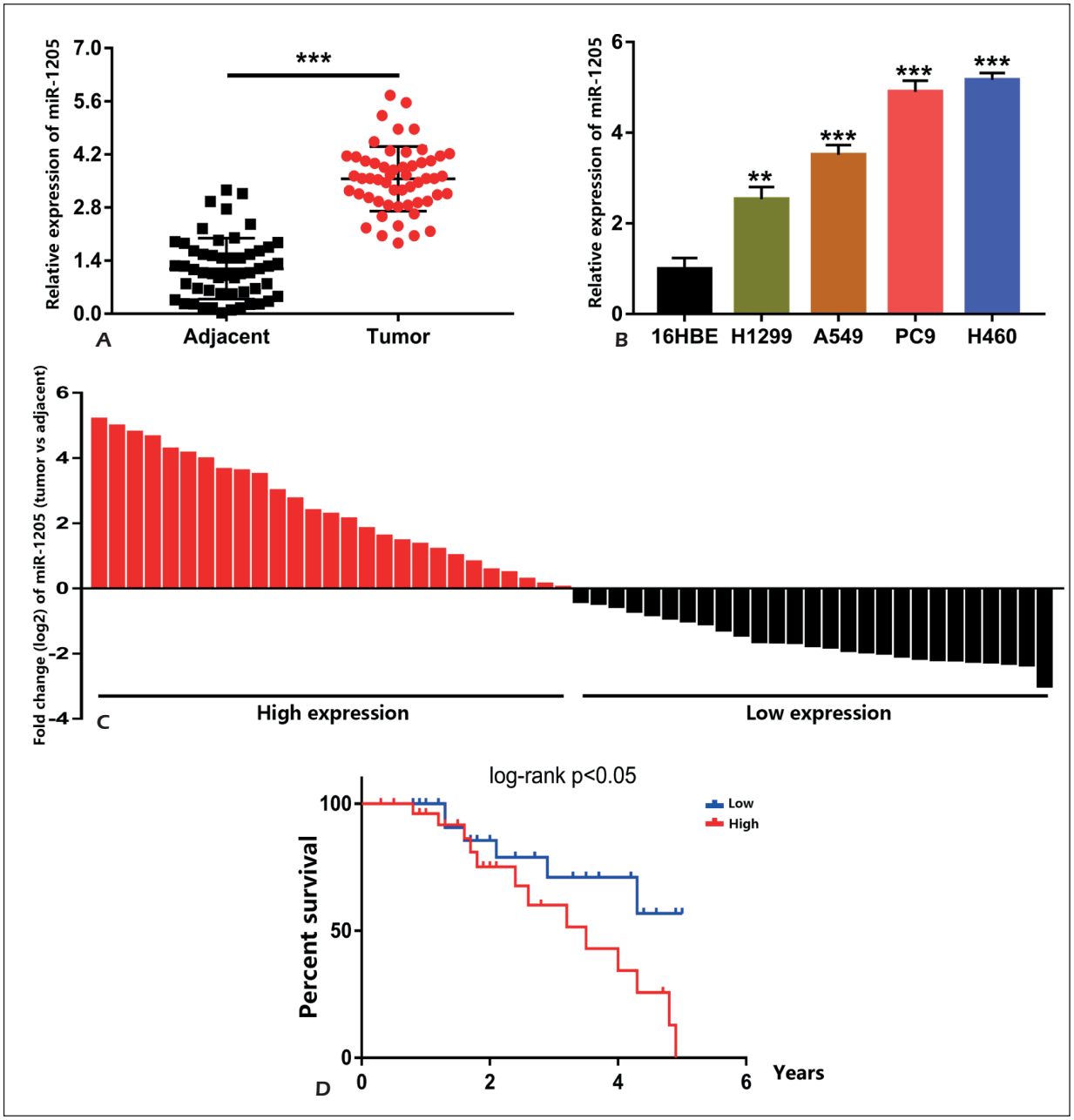

Figure 1. MiR-1205 was upregulated in LUAD tissues and cells. **A**, The miR-1205 expression in LUAD patients was detected by qRT-PCR. **B**, The miR-1205 expression in LUAD cell lines and 16HBE. **C**, The fold change (log2 change) in miR-1205 levels between 54 pairs of LUAD and adjacent normal tissues was measured and divided into low expression group (27 pairs) and high expression group (27 pairs) according to the median miR-1205 level. **D**, Percent survival of LUAD patients between high and low group. Data are represented as mean ± SD. (**p<0.01; ***p<0.001).

also markedly related with tumor size, lymph node metastasis and TNM stage, while no differences were found with gender, age or distant metastasis (Table I). At the same time, we also observed that the expression level of APC2 in 16HBE cells was also remarkably less than in lung cancer cell lines (Figure 3C). Moreover, the patient's miR-1205 and APC2 expression profiles showed a negative correlation (Figure 3D). To further validate our hypothesis, Dual-Luciferase report assay was executed. As seen in Figure 3E, Luciferase activity of APC2-wt was dramatically inhibited after co-transfection with miR-1205 mimics in comparison with the

Table I. Expression of miR-1205 and APC2 according to patients' clinical features.

Factors	miR-1205 expression		*p*-value	APC2 expression		*p*-value
	High	Low		High	Low	
Gender						
Male	10	15	0.274	16	9	0.101
Female	17	12		11	18	
Age (years)						
≥50	18	11	0.101	12	16	0.190
< 50	9	16		15	8	
Distant metastasis						
Yes	15	10	0.274	12	13	0.784
No	12	17		15	14	
Tumour size (cm)						
>5	19	10	0.029*	8	21	0.001*
≤ 5	8	17		19	6	
Lymph node metastasis						
Yes	20	12	0.052	10	22	0.002*
No	7	15		17	5	
TNM stage						
I-II	6	18	0.002*	7	17	0.013*
III- IV	21	9		20	10	

*indicates $p<0.05$ (Chi-square test).

APC2-mutin the H1299 and H460 cell lines. By qRT-PCR and Western blot, we found that the mRNA and protein expression of APC2 were evidently decreased after overexpression of miR-1205, which were markedly increased in cells with low expression of miR-1205 (Figure 3F, 3G and 3H). This result indicated that APC2 was the direct target gene of miR-1205.

MiR-1205 Promoted LUAD Proliferation by Targeting APC2

Since miR-1205 accelerated LUAD proliferation and APC2 was a direct target gene, we performed rescue assays to verify whether APC2 attenuated the miR-1205 effect on LUAD proliferation. In the miR-1205 mimics group, we transfected the APC2 plasmid to enhance APC2 expression. In the miR-1205 inhibitor group, we transfected siAPC2 to decrease the APC2 expression. Then, APC2 expression was detected by qRT-PCR and Western blot (Figure 4A and 4B). CCK-8 assay showed that the up-regulation of APC2 effectively reversed the promotion of the proliferation of H1299 cells by miR-1205 overexpression. The down-regulation of APC2 effectively reversed the inhibitory effect of miR-1205 down-regulation on H460 cell proliferation (Figure 4C). These findings indicated that miR-1205 promotes cell growth by modulating APC2 *in vitro*.

Discussion

As one of the deadliest tumors, the incidence and mortality of NSCLC are on the rise[20]. In recent years, precision medicine and targeted therapy have become the focus of individualized treatment research for lung cancer[21]. Numerous studies have shown that miRNAs have a vital function in tumor cell proliferation, differentiation, apoptosis, invasion and metastasis[22]. MiRNA-1205 (miR-1205), a member of the PVT1 region, has been found to play a part in the Wnt/β-catenin pathway[15]. In this work, it was first determined the overexpression of miR-1205 in LUAD tissues and cells. *In vitro* gain- and loss-of-function assays suggested that miR-1205 promoted LUAD cell growth and proliferation.

MiRNAs binding to the 3′-UTR of target mRNAs and in return leading to translational repression or degradation of mRNA have been extensively researched in recent years, which may be a significant way to examine their effects on tumors[23]. Some studies[24] have found that miR-582-5p functions as a tumor suppressor by targeting MAP3K2 in NSCLC, and the overexpression of microRNA-758 inhibits proliferation, migration, invasion of NSCLC cells by negatively regulating HMGB[25]. To explore the mechanism of miR-1205 in cell proliferation, we identified potential targets through online sites (TargetScan,

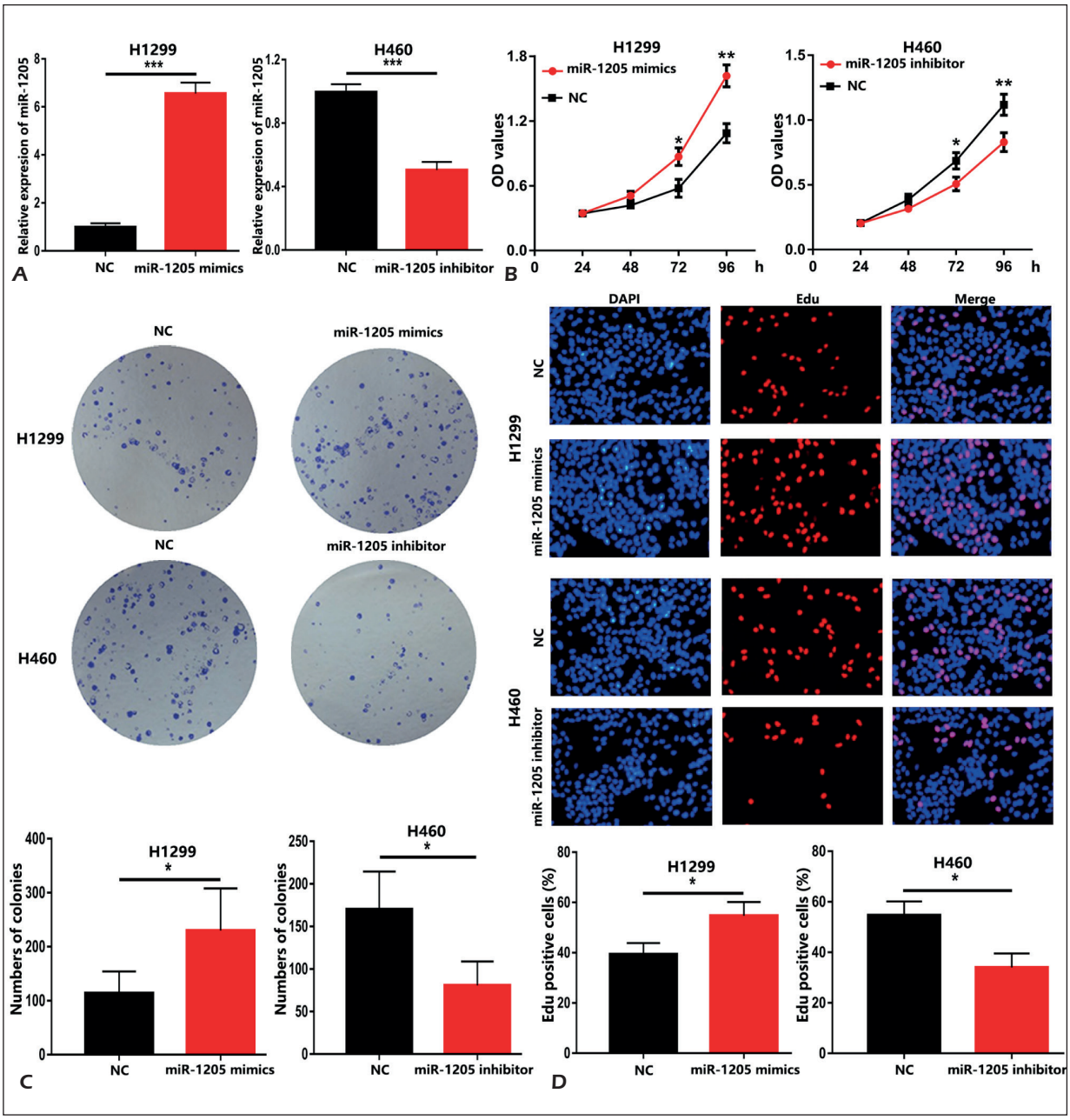

Figure 2. MiR-1205 promoted cell proliferation. **A**, H1299 cells were transfected with miR-1205 mimics or NC, H460 cells were transfected with miR-1205 inhibitor or NC, and the miR-1205 expression was detected by qRT-PCR. **B**, Using the CCK-8 assay, we found distinct differences in proliferation after manipulation of miR-1205 mimics or inhibitor at 96 h. **C**, Colony formation assays comparing the miR-1205 mimics, NC, miR-1205 inhibitor and inhibitor NC groups (magnification: 40×). **D**, Compared with the control, EdU cell growth profiles in H1299 cells and H460 cells after transfection with miR-1205 mimics and miR-1205 inhibitor, respectively (magnification: 100×). The data are represented as mean ± SD. (*p<0.05; **p<0.01; ***p<0.001).

miRWalk and miRPathDB). We also found a negative correlation between APC2 and miR-1205 expression. Thus, we suggested that miR-1205 may reduce protein expression by directly targeting the 3'-UTR of APC2. We observed that the mRNA and protein levels of APC2 were decreased in H1299 cells with overexpression of miR-1205 and increased in H460 cells with miR-1205 knockdown. The positive effect of miR-1205-induced cell proliferation could be reversed by overexpression of APC2. As far as we know, it is the first to link miR-1205 to tumor progression potential and prognosis in LUAD.

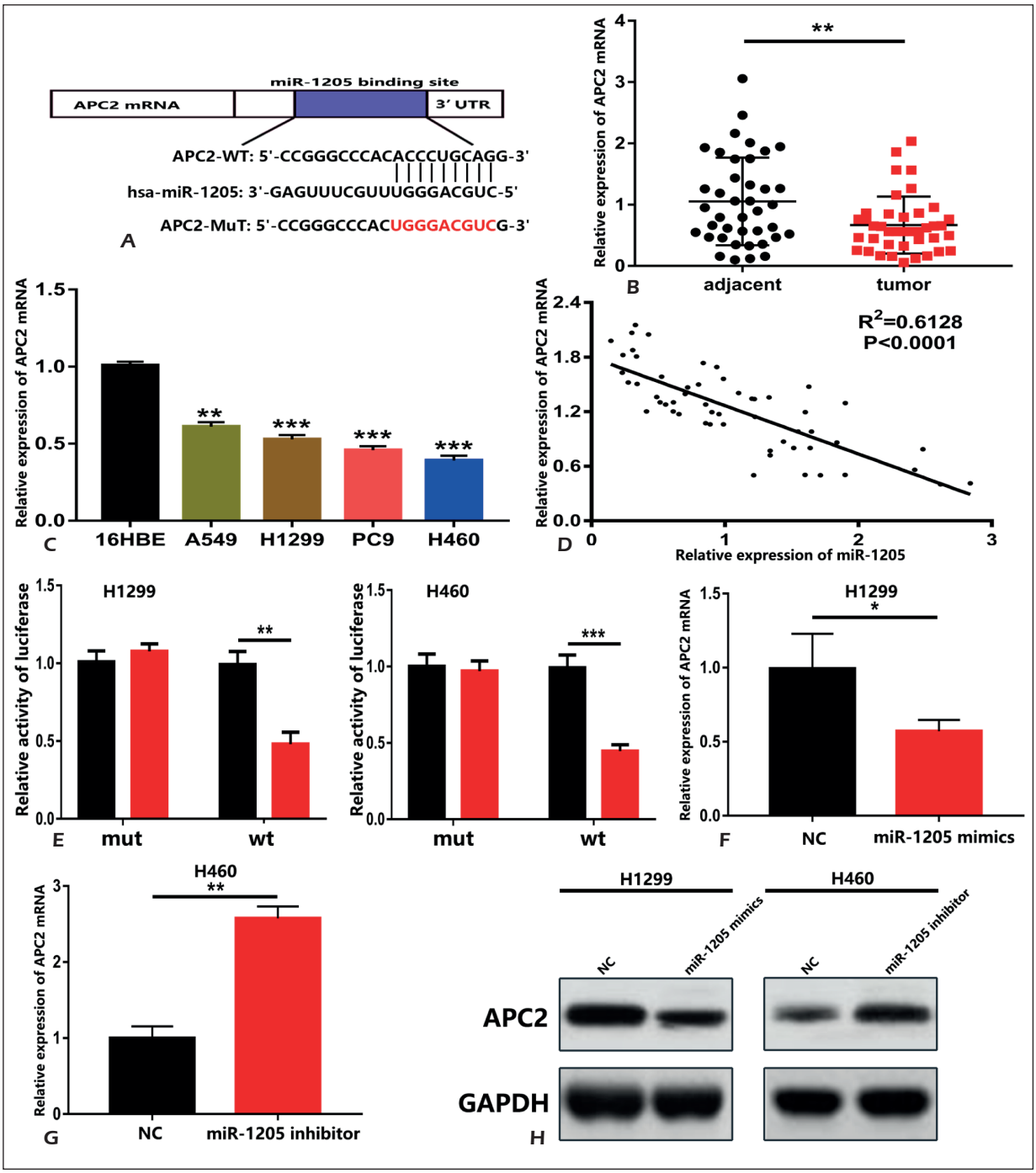

Figure 3. MiR-1205 suppressed APC2 expression by directly binding its 3'-UTR. **A**, A Luciferase reporter assay was conducted to verify that miR-1205 directly bound to the 3'-UTR sequences of APC2. **B**, The APC2 mRNA expression level in 54LUAD tissues and adjacent tissues was detected by qRT-PCR. **C**, The RNA expression levels of APC2 in LUAD cell lines and 16HBE were detected by qRT-PCR. **D**, A negative correlation was found between RNA expression of miR-1205 and APC2in tumor samples. **E**, Luciferase activity was analyzed in cells co-transfected with miR-1205 mimics or NC with APC2-wt or APC2-mut. **F**, and **G**, APC2 mRNA expression levels in transfected H1299 and H460 cells were analyzed by qRT-PCR. **H**, APC2 protein expression levels were analyzed by Western blot in transfected H1299 and H460 cells. GAPDH was used as a control. The data are expressed as the mean ± SD. (*p<0.05; **p<0.01; ***p<0.001).

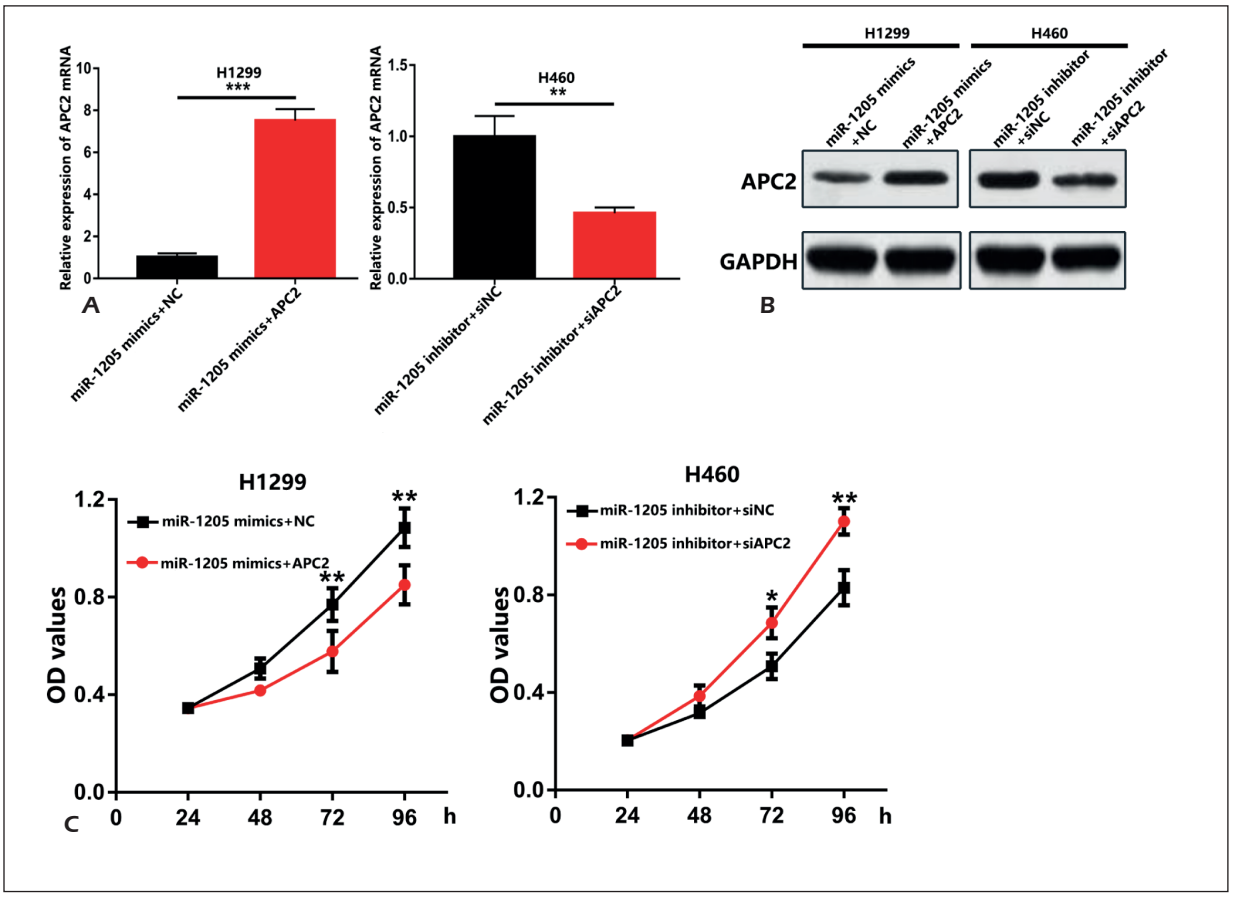

Figure 4. MiR-1205 regulated cell proliferation by targeting APC2. **A**, The expression of APC2 was verified by qRT-PCR in co-transfected cell lines. **B**, Western blot was used to verify the expression of APC2. **C**, and **D**, The roles of miR-1205 and APC2 in the regulation of LUAD cell proliferation were examined by CCK-8 assay. Data are expressed as the mean ± SD. (*p<0.05; **p<0.01; ***p<0.001).

APC2 functioned as a tumor suppressor in various cancers. MiR-4326 could promote cell proliferation by regulating APC2 in lung cancer[26]. MiR-939 promotes the proliferation of human ovarian cancer cells by repressing APC2 expression[27]. APC2 is closely related to multiple aspects of lung development and lung tumor progression[28]. Nevertheless, the probability that other downstream target genes may also be modulated by miR-1205 would not be eliminated from consideration by the results of our study. In this work, we first discovered that miR-1205 can be involved in LUAD progression by negatively regulating APC2.

Conclusions

We demonstrated that miR-1205 was upregulated in LUAD tissues, which may serve as an independent predictor of overall survival of LUAD. Furthermore, we found that miR-1205 has the ability to promote the growth and proliferation of LUAD cells by modulating APC2. Our results suggested that miR-1205 could perform as a tumor-promoting factor in LUAD and has potential as a prognostic biomarker and a potential therapeutic target for LUAD.

Conflict of Interests

The authors declare that they have no conflict of interest.

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