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# MicroRNA-345 suppresses cell invasion and migration in non-small cell lung cancer by directly targeting YAP1

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**Abstract. – OBJECTIVE: To investigate the roles and the underlying mechanism of miR-345 in regulating non-small cell lung cancer (NSCLC) cell migration and invasion.**

**PATIENTS AND METHODS: Fifty-two pairs of NSCLC tissues and matched normal lung tissue samples were collected from NSCLC patients who had undergone surgical resection between June 2015 and August 2017 in our hospital. Human NSCLC cell lines (NCI-H460, NCI-H1299, A549 and GLC-82) and normal human bronchial epithelium cell BEAS-2B were cultured. NSCLC cell migration and invasion capacities were determined by transwell assays. The relative protein and mRNA expression level of yes-associated protein 1 (YAP1) were detected by Western blot and Quantitative Real-time polymerase chain reaction (qRT-PCR) analysis, respectively.**

**RESULTS: MiR-345 was prominently downregulated in NSCLC tissue specimens. Results of transwell assays showed that miR-345 overexpression could dramatically inhibit NSCLC cell migration and invasion. Additionally, data in current study also identified YAP1 as a direct functional target of miR-345 in NSCLC cells. YAP1 expressions were negatively correlated with the miR-345 expressions in NSCLC tissue samples. Moreover, YAP1 was found to be involved in the functions of miR-345 in inhibiting NSCLC cell invasion and migration.**

**CONCLUSIONS: All the above results indicated that miR-345 inhibited NSCLC cell migration and invasion by targeting YAP1, suggesting that miR-345/YAP1 axis might be a promising biomarker for NSCLC treatments.**

*Key Words:*
microRNA-345, Non-small cell lung cancer (NSCLC), Migration, Invasion, YAP1.

## Introduction

Lung cancer is one of the most common frequently occurring aggressive cancers with a high incidence rate and poor prognosis, and has become one principal factor for human mortalities worldwide[1]. In addition, due to the high metastatic ability and recurrence, the 5-year survival rate of lung cancers remains poor, and is lower than most common cancers[2]. In particular, non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer types[3]. Although advancement has been made in NSCLC treatments, including surgery, radiotherapy chemotherapy, genetic therapies and immune therapies, most patients with NSCLC are still generally diagnosed at advanced stages accompanied by poorer prognosis[4,5]. The frequent relapses and poor outcomes of NSCLC highlight the urgent need for the development of novel early biomarkers and screening methods to accurately detect the metastasis and recurrence. MicroRNAs (miRNAs) have been reported to regulate the expressions of target genes in various cellular processes, *via* binding to the 3'-untranslated regions (3'UTRs) of mRNA for translation suppression or degradation[6]. Previous studies demonstrated that multiple miRNAs played vital functions in tumor progression. According to Xu et al[7], miR-532-5p exerted oncogenic functions in human gastric carcinoma via the regulation of runt-related transcription factor 3(RUNX3). Yan et al[8] showed that miR-495 repressed colorectal cancer cell proliferation and migration by regulating family with sequence similarity 83 member D (FAM83D).


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Yang et al[9] found that miR-1207-5p was associated with esophageal carcinoma progression via regulating stomatin-like protein 2. Recent studies have reported that miR-345 expressions are dramatically declined in NSCLC. However, the precise molecular mechanisms by which miR-345 affects NSCLC progression still remain largely unknown. Yes-associated protein 1 (YAP1), an oncogenic transcriptional coactivator, has recently been identified as a molecular target for tumor therapy. YAP1 is implicated in the regulation of multiple cellular processes, including cell apoptosis, proliferation, growth, tumorigenesis, stem cell differentiation and renewal. Sun et al[10] showed that YAP1 promoted cell proliferation, migration and invasion in gastric cancer; Other investigations by Sun et al[11] revealed that YAP1promoted the self-renewal and survival of breast cancer initiating cell by regulating mothers against decapentaplegic homolog 3 (Smad3) signaling. Li et al[12] reported that YAP1 contributed to bladder cancer cell proliferation and migration by regulation of the H19 long noncoding RNA. Taken together, previous findings demonstrated strong contributory functions of YAP1 in tumor progression. However, the functional roles of YAP1 in NSCLC invasion and migration remain unclear. Based on the previous studies, we speculated that YAP1 was involved in NSCLC migration and invasion. In current study, we aimed to identify the role of YAP1 and interpret the potential mechanisms.

## Patients and Methods

### Patients

Fifty-two pairs of NSCLC tissues and matched normal lung tissue samples were collected from NSCLC patients who had undergone surgical resections from June 2015 to August 2017 at the Affiliated Hospital of Weifang Medical University. All the tissue samples were snap-frozen immediately in liquid nitrogen and stored at 80°C. No patient had been treated with chemotherapy or radiotherapy before surgery resection. Signed written informed consent was also obtained from all participants prior to the study. This study was approved by the Ethics Committee of the Affiliated Hospital of Weifang Medical University.

### Cell Culture

Human NSCLC cell lines (NCI-H460, NCI-H1299, A549 and GLC-82) and normal human bronchial epithelium cell BEAS-2B were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified incubator at 37°C with 5% $CO_2$.

### Cell Transfection

MiR-345 mimics/inhibitors, YAP1 vector, siRNA and the corresponding negative controls (NC) were obtained from GenePharma (Shanghai, China). Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was utilized to transfect them into NSCLC cells in line with the manufactures' instruction. Cells were collected for further analysis at 48 h post-transfection.

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

RNA was extracted from NSCLC cells and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, Taqman MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to synthesize the complementary DNA (cDNA). qRT-PCR was carried out with the SYBR PCR Master Mix and ABI 7500 Fast (Applied Biosystems; Foster City, CA, USA). The relative expressions of miR-345 and YAP1 were determined using the $2^{-\Delta\Delta Ct}$ method, with U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous controls.

### Transwell Assays

NSCLC cell migration and invasion capacities were detected using transwell chambers (8 μm pore size; Corning, Corning, NY, USA) coated with or without Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), respectively. NSCLC cells treated with miR-345 mimics or inhibitor were added into the upper transwell chambers, while for the rescue assays, cells transfected with miR-345+YAP1 siRNA or miR-345+negative control siRNA were placed into the upper transwell chambers. On the other hand, medium containing 10% fetal bovine serum (FBS) was used as a chemoattractant, which was placed into the bottom chambers. After being cultured for 48 h at 37°C in a 5% $CO_2$ atmosphere, cells stayed in the upper chamber were carefully removed with a cotton swab while cells that had traversed the membrane were subsequently fixed and stained with formaldehyde (4%) and

crystal violet (0.1%), respectively. The invasive and migratory cells were measured and counted in five randomly selected fields under an inverted microscope (Olympus, Tokyo, Japan).

### Western Blot Analysis

Radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors and phosphatase inhibitors (Roche, Basel, Switzerland) were used to lyse NSCLC cells for total protein extractions. The protein concentration was quantified by a bicinchoninic acid protein (BCA) assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Next, protein was separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Little Chalfont, UK), which was blocked with 5%-skimmed milk in Tris-buffered saline and Tween-20 (TBST). After that, the membrane was incubated with appropriate primary antibodies overnight at 4°C, followed by the incubation with goat anti-rabbit secondary antibody (1:4000, ab7090; Abcam, Cambridge, MA, USA) for 2 h at room temperature. The primary anti-bodies were as follows: anti-YAP1 (1:1000; ab52771; Abcam, Cambridge, MA, USA); anti-GAPDH (1:1000; ab22555; Abcam, Cambridge, MA, USA). Enhanced Chemifluorescence Western blotting kit (Pierce; Rockford, IL, USA) was utilized to detect the protein bands. GAPDH was an internal control.

### DualLuciferase Reporter Analysis

The NSCLC cells were co-transfected with miR-345 mimics or miR-control (NC) and pmirGLO vectors (Promega, Madison, WI, USA) which contained YAP1 fragments with wild-type (WT) or mutant (MUT) binding sites of miR-345 by Lipofectamine 2000 following the manufacturer's proposals. Then, cells were collected at 48 h post-transfection to detect the luciferase activities using a dual luciferase reporter gene assay kit (Promega, Madison, WI, USA).

### Statistical Analysis

All the above assays were performed three times. Statistical Product and Service Solutions (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis. Correlation between mRNA and miRNA were estimated using the Spearman's correlation method. Student's *t*-test, one-way analysis of variance (ANOVA) and Scheffe's post-hoc analysis were applied, where appropriated. $p<0.05$ was identified as statistically significant difference.

## Results

### MiR-345 Expressions were Decreased and YAP1 Expressions were Increased in NSCLC

MiR-345 and YAP1 expressions in NSCLC tissues and cells were examined by performing qRT-PCR. Firstly, miR-345 expressions in NSCLC tissues were detected and results indicated that miR-345 was significantly downregulated in NSCLC tissue samples in comparison with the normal tissues (Figure 1A). Then, we measured the miR-345 expression in NSCLC cells and human normal bronchial epithelium cell BEAS-2B. As expected, we found that miR-345 expressions were remarkably reduced in NSCLC cells when compared to BEAS-2B (Figure 1B). Subsequently, the expressions of YAP1 in NSCLC cells were detected by qRT-PCR analysis and results indicated that YAP1 was markedly upregulated in NSCLC cells in contrast with BEAS-2B cells (Figure 1C). Furthermore, a prominent negative correlation between miR-345 and YAP1 expressions was observed in NSCLC tissues (Figure 1D).

### MiR-345 Restoration Inhibited NSCLC Cell Migration and Invasion

To investigate the functional effects of miR-345 on NSCLC migration and invasion, we performed miR-345 overexpression or knockdown assays by transfecting miR-345 mimics or inhibitor into A549 or GLC-82 cells, which had the relatively lowest and highest endogenous miR-345 expressions. qRT-PCR assays were conducted to confirm the successful overexpression or inhibition of miR-345 in A549 or GLC-82 cells (Figure 2A and 2B). Then, transwell assays were carried out to determine whether the migration and invasion abilities of A549 or GLC-82 cells were affected by miR-345 overexpression or inhibition. Data revealed that miR-345 overexpression significantly repressed A549 cell migration and invasion (Figure 2C). On the other hand, miR-345 inhibition was found to significantly promote GLC-82 invasion and migration (Figure 2D). Results showed that miR-345 exerted anti-tumor functions in NSCLC.

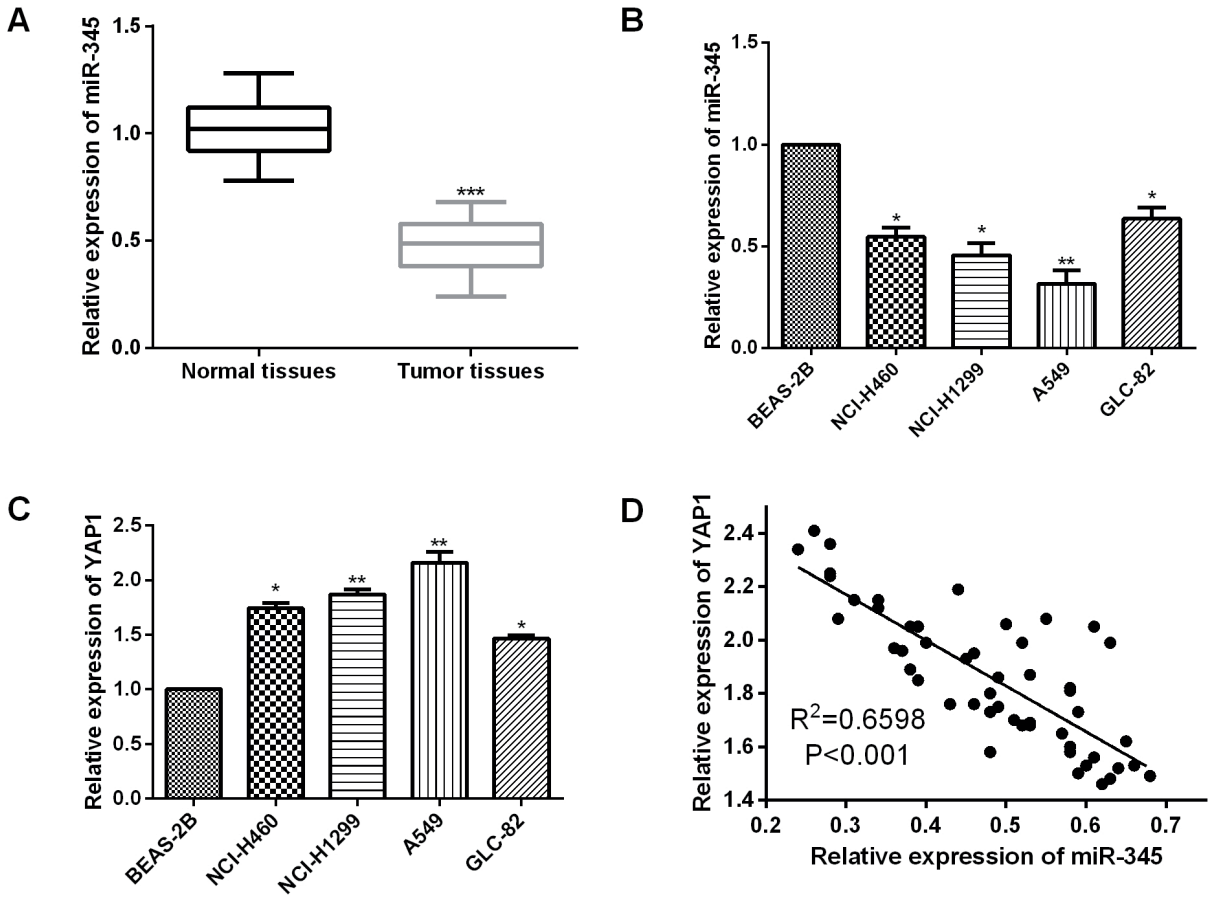


**Figure 1.** MiR-345 expression was decreased and YAP1 expression was increased in NSCLC. ***A,*** miR-345 expressions in NSCLC tissues and paracancerous tissues were detected by qRT-PCR. ***B,*** miR-345 expressions in NSCLC cells and normal bronchial epithelium cell BEAS-2B were measured using qRT-PCR. ***C,*** YAP1 expressions were significantly upregulated in NSCLC cells. ***D,*** miR-345 expressions were negatively correlated to YAP1 expressions in NSCLC tissues. *$p$<0.05, **$p$<0.01, ***$p$<0.001.

### *YAP1 was a Directed Target of miR-345 in NSCLC Cells*

Bioinformatics analysis by TargetScan revealed that YAP1 was a potential target of miR345 (Figure 3A) and luciferase reporter assays were conducted to confirm the combination between YAP1 and miR345. Luciferase reporter vectors containing wide type (WT) or mutant type (MUT) YAP1 3'UTR and miR-345 mimics were cotransfected into NSCLC cells. Results demonstrated that miR-345 restoration led to a prominent decrease in luciferase activity of YAP1-3'UTR-WT whereas there was no significant effect on the luciferase activity of YAP1-3'UTR-MUT in NSCLC cells (Figure 3B). Moreover, we investigated the regulatory functions of miR345 in YAP1 expressions in NSCLC cells, which were treated with miR-345 mimics or inhibitor. Results showed that YAP1 expressions were markedly reduced by miR-345 mimics in A549 cells (Figure 3C). On the other hand, YAP1 expressions were evidently increased in GLC-82 cells with transfection of miR-345 inhibitor (Figure 3D).

### *YAP1 was Implicated in the Functional Roles of miR345 in NSCLC Cells*

Having confirmed that YAP1 was a direct target of miR-345, we next analyzed whether YAP1 could regulate the inhibitory effects of miR-345 on NSCLC migration and invasion. YAP1 overexpression vector was transfected into miR-345-overexpressed A549 cells and qRT-PCR was performed to verify the transfection efficiencies (Figure 4A). Subsequently, transwell assays were carried out to confirm the biological functions of YAP1 in regulating the NSCLC cell migration and invasion abilities mediated by miR-345. We found that YAP1 restoration distinctly

promoted the cell migration and invasion capacities of miR-345 overexpressed A549 cells (Figure 4B). In addition, YAP1 expression in miR-345 suppressed GLC-82 cells was notably decreased by YAP1 siRNA (Figure 4C). Moreover, transwell assays showed that YAP1 knockdown in miR-345-suppressed GLC-82 cells prominently inhibited the invasion and migration abilities (Figure 4D). Taken together, these findings indicated that YAP1 mediated the functional effects of miR-345 on NSCLC cell invasion and migration.

## Discussion

NSCLC is one of the most common types of lung cancers and has been studied by researchers around the world. Most NSCLC patients present distant metastasis at the time of diagnosis[13]. Recently, despite the significant advancements in early diagnosis and treatment approaches for NSCLC patients, existing therapies remain insufficient to predict and diagnose the outcome of NSCLC patients due to the lack of potential biomarkers for molecular personalized or targeted therapies[14,15]. Therefore, there is an urgent need to identify targeted therapies to improve early diagnosis and prognosis prediction of NSCLC patients. Currently, miRNAs, such as miR-124[16], miR-940[17] and miR-26b[18], have attracted much attention for the diagnosis of NSCLC patients. Present study aimed to explore the functional effects and potential mechanisms of miR-345 in NSCLC cell invasion and migration. Recently, miR-345 has been found as a vital regulator of tumors, and the biological functions of miR-345 have been investigated in tumor progression. For instance, Yu et al[19] showed that miR-345 inhibi-

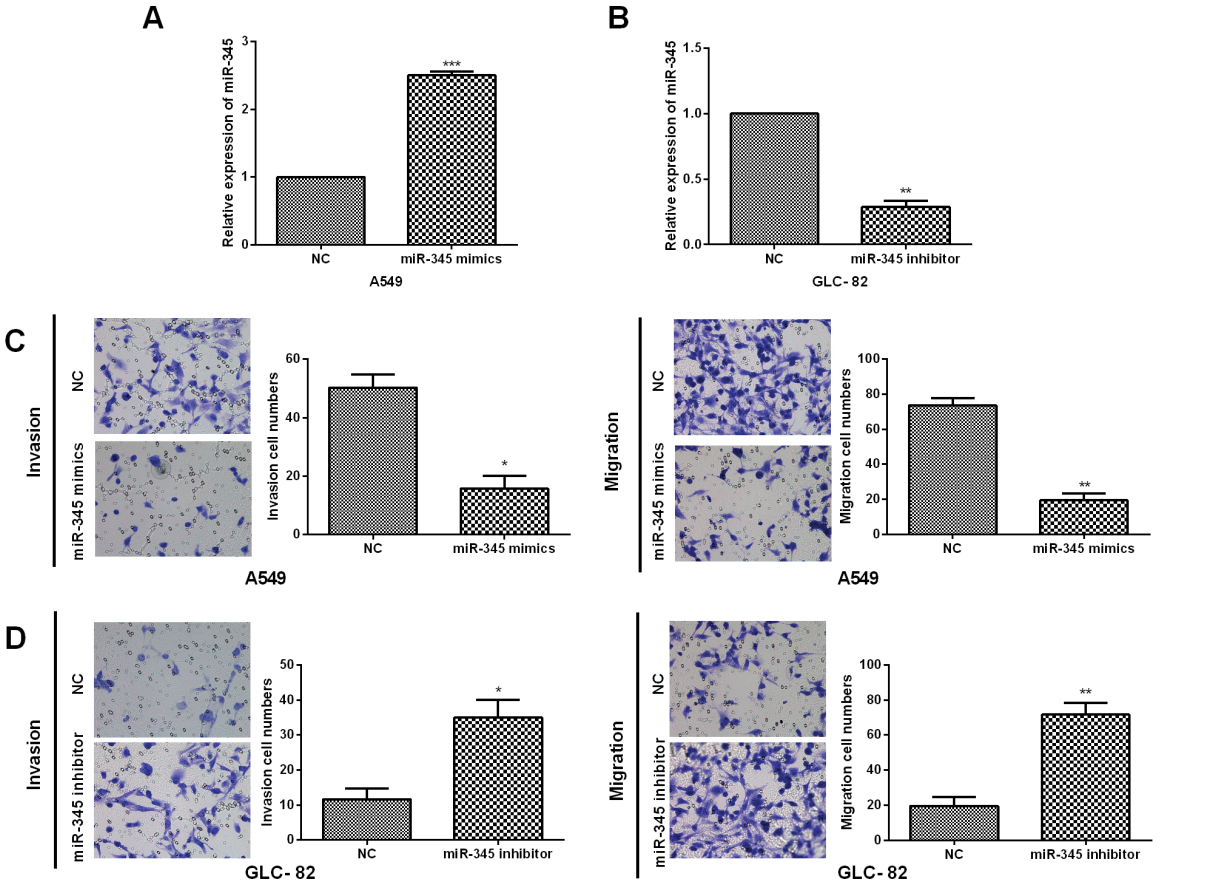


**Figure 2.** MiR-345 restoration significantly repressed NSCLC cell migration and invasion. ***A-B,*** qRT-PCR analysis of miR-345 expressions in A549 or GLC-82 cells treated with miR-345 mimics or inhibitor. ***C-D,*** Transwell assays were performed to determine the invasion and migration abilities of A549 or GLC-82 cells treated with miR-345 mimics or inhibitor. *$p$<0.05, **$p$<0.01, ***$p$<0.001.

ted hepatocellular carcinoma cell metastasis and epithelial-mesenchymal transition (EMT) via regulating interferon regulatory factor (IRF1)-mediated mammalian target of rapamycin (mTOR)/ signal transducer and activator of transcription 3 (STAT3)/ protein kinase B (AKT) pathway. Chen et al[20] proposed that miR-345 suppressed human prostate cancer cell proliferation, invasion and migration via the regulation of mothers against decapentaplegic homolog 1(Smad1). Feng et al[21] observed that miR-345 inhibited gastric cancer cell metastasis and EMT through regulating forkhead box Q1 (FOXQ1). In our study, we found that miR-345 was prominently downregulated in NSCLC. Functionally, miR-345 overexpression significantly repressed the migration and invasion abilities of NSCLC cells, suggesting that miR-345 exerted anti-tumor functions in NSCLC. There were several studies supported our results. According to Chen et al[22], miR-345 was dramatically downregulated in NSCLC and the declined miR-345 expressions were related to the poor prognosis of NSCLC patients. Previous studies demonstrated that YAP1 played critical roles in tumor progression of multiple cancers. For instance, Xu et al[23] found that miR-622 suppressed glioma cell proliferation via targeting YAP1. Yang et al[24] reported that knockdown of YAP1 inhibited osteosarcoma cell proliferation. Yokoyama et al[25] revealed that YAP1 was involved in the development of mesothelioma, which was negatively regulated by Merlin via the phosphorylation. In addition, YAP1 was found to play important roles in NSCLC. According to Yokoyama et al[25], YAP1 overexpression rescued the inhibitory functions of miR-138 in NSCLC cell proliferation[26]. Data in present study further confirmed that YAP1 functioned as an oncogene in NSCLC. We observed that YAP1 expressions were notably enhanced in NSCLC and acted as a direct functional target for miR-345. Moreover, YAP1 was verified to be implicated in the inhi-

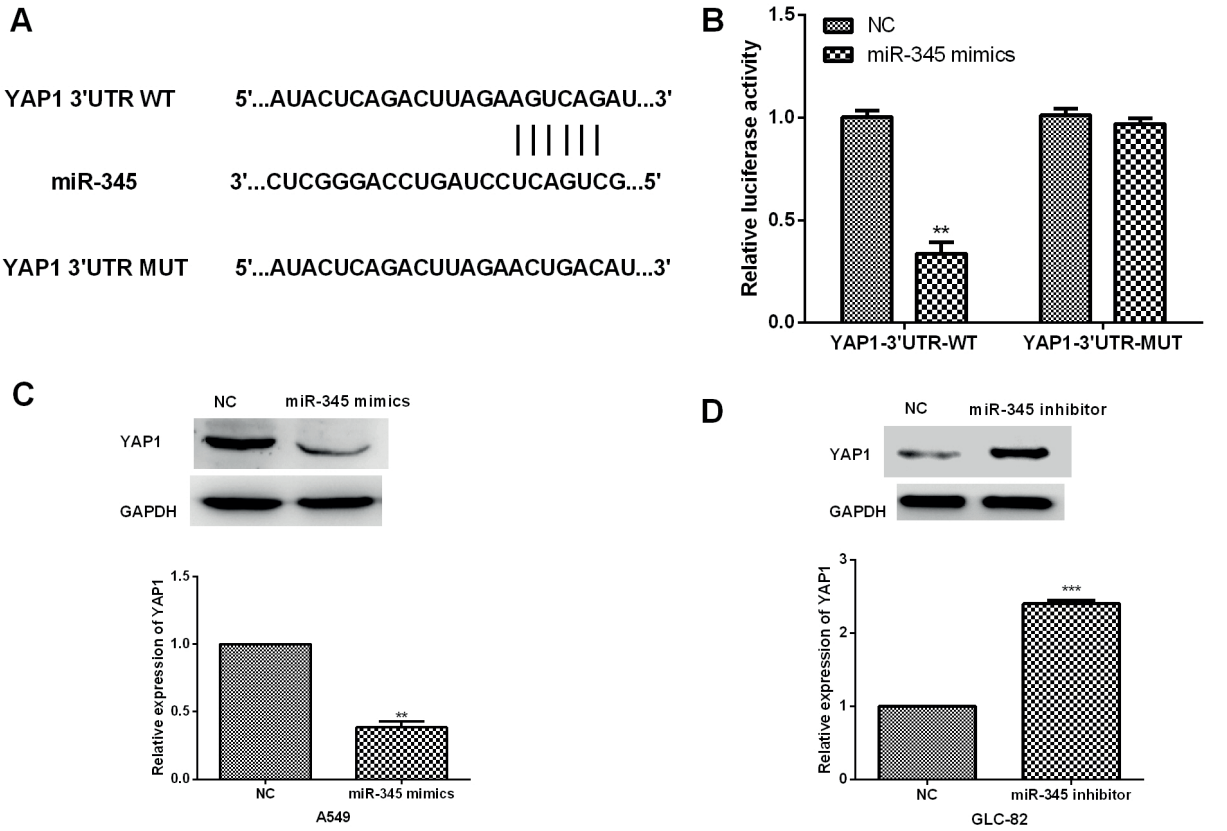


**Figure 3.** YAP1 was a direct target of miR-345 in NSCLC cells. ***A,*** The putative binding sites of miR-345 in YAP1 3'-UTR. ***B,*** Dual-luciferase reporter analysis of NSCLC cells cotransfected with WT or MUT YAP1 3'-UTR and miR-345 mimics. ***C, D,*** YAP1 expressions in A549 or GLC-82 cells treated with miR-345 mimics or inhibitor. $^{**}p<0.01$, $^{***}p<0.001$.

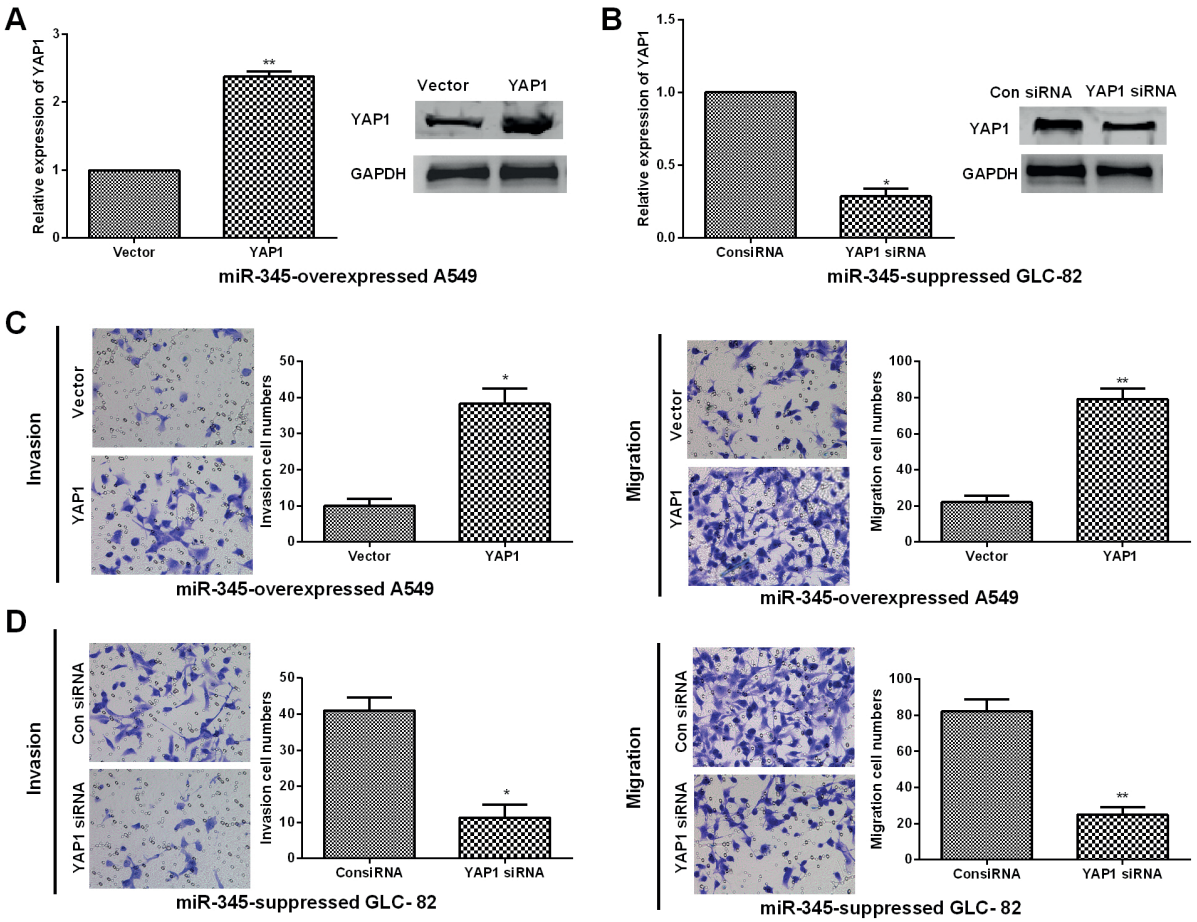


**Figure 4.** YAP1 regulated the functions of miR-345 in NSCLC cell migration and invasion. ***A,*** YAP1 expressions in miR-345-overexpressed A549 cells which were cotransfected with YAP1 overexpression vector. ***B,*** YAP1 expressions in miR-345-suppressed GLC-82 cells which were cotransfected with YAP1 siRNA. ***C,*** Cell migration and invasion abilities were detected in A549 cells cotransfected with miR-345 mimics and YAP1 overexpression vector using transwell assays. ***D,*** Cell migration and invasion abilities were detected by transwell assays in GLC-82 cells cotransfected with miR-345 inhibitor and YAP1 siRNA. **p*<0.05, ***p*<0.01.

bitory functions of miR-345 in NSCLC invasion and migration.

## Conclusions

We revealed that miR-345 was significantly downregulated in NSCLC. Furthermore, overexpression of miR-345 dramatically inhibited NSCLC cell migration and invasion. YAP1 was further identified as a direct functional target of miR-345. Moreover, YAP1 was required for the functional effects of miR-345 on NSCLC cell migration and invasion. Above data indicated that miR-345 acted as a tumor suppressor in NSCLC and miR-345/ YAP1 might be a promising biomarker for NSCLC therapies.

## Conflict of Interest

The Authors declare that they have no conflict of interest.

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