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ORIGINAL ARTICLE

MicroRNA-16 inhibits the proliferation and metastasis of human lung cancer cells by modulating the expression of YAP1

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Summary

Purpose: *Accounting for significant human morbidity and mortality across the globe, lung cancer is the most prevalent type of cancer as far as incidence and mortality is concerned. MicroRNAs (miRs) have shown an amazing potential to act as therapeutic agents for the management of several human diseases. This study investigated the function of miR-16 in lung cancer.*

Methods: *The normal lung cancer cell line MRC3 and lung cancer cell lines SK-MES-1, A549, MS-53 and SK-LU-1 were used in the present study. The qRT-PCR was used for expression profiling of miR-16 and yes associated protein 1 (YAP1). WST-1 assay was used to monitor the proliferation rate. Flow cytometry was used for cell cycle analysis. Apoptosis was examined by DAPI and annexin V/propidium iodide (PI) staining. TargetScan analysis was performed to identify the potential target of miR-16 and western blot analysis was done to estimate the protein expression.*

Results: *The gene expression analysis showed miR-16 to be suppressed in lung cancer tissues and cell lines. Overexpression of miR-16 inhibited the growth and metastasis of the DMS-53 lung cancer cells via induction of the apoptotic cell death. Bioinformatic approaches revealed miR-16 exerts its effects by targeting YAP1. YAPI expression was found elevated in lung cancer tissues and its silencing halted the growth of the DMS-53 lung cancer cells. Nonetheless, YAP1 overexpression could reverse the growth inhibitory effects of miR-16.*

Conclusion: *Taken together, miR-16 may serve as novel therapeutic target for the treatment of lung cancer.*

Key words: *lung cancer, microRNA, apoptosis, proliferation, invasion*

Introduction

Lung cancer is the leading cause of cancer related morbidity and mortality across the world. Responsible for 1.35 million new cases (ie., about 12.4% of all the new cancer cases) and 1.18 million deaths (ie 15.6% of cancer-related deaths) annually across the globe, lung cancer is the most prevalent type of cancer as far as incidence and mortality are concerned [1]. It has been reported that 0.24 million new cases of lung cancer and 0.16 million deaths due to lung cancer in United States occurred in 2010. The 15.6% of 5-year survival rate of lung cancer in United State indicates that there has been very little improvement in the survival of the lung cancer patients in comparison to other cancer types [2]. Recent reports suggest that the incidence of lung cancer has almost doubled since 1985 [3]. All these studies point towards the need for identification of new therapeutic strategies and develop-

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ment of potent treatment regimes for lung cancer management. The utilisation of microRNAs (miRs) for cancer therapy is currently one of the promising fields in cancer research [4]. MiRs are around 18-25 nucleotides long endogenous molecules that control the expression of the target genes by binding to 3´UTR causing degradation of mRNA or repression of translation [5]. MiRs play vital roles in fundamental cellular processes which include but are not limited to proliferation, development, differentiation, apoptosis and autophagy [6]. There is strong evidence showing that many miRs show dysregulation in cancer tissues and play important part in the development of cancer [7]. Hence, it is believed that miRs may serve as therapeutic targets and will allow targeted therapy for the treatment of cancer [8]. MiR-16 has been shown to act as a tumor suppressor in different types of cancer [9].

This study investigated the function of miR-16 in lung cancer. The outcomes of this investigation revealed that miR-16 is suppressed in lung cancer tissues and cell lines and its overexpression inhibits the growth and metastasis via suppression of YAP1 (yes-associated protein 1). The outcomes of this investigation point towards miR-16 as a novel target in lung cancer and warrants further investigation.

Methods

All the experiments were performed in triplicate.

Cell lines and culture conditions

The normal lung cell line (MRC5) and lung cancer cell lines (SK-MES-1, A549, DMS-53, SK-LU-1) were purchased from American Type Culture Collection (Manassas, VA, USA). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The cells were cultured in an incubator (Thermo Fisher Scientific, Inc. Waltham, MA, USA) at 37°C with 98% humidity and 5% CO_2. All transfections were carried out by Lipofectamine 2000 (Invitrogen Carlsbad, California, United States) as per the manufacturer's protocol.

The qRT-PCR analysis

The total RNA from the normal and the lung cancer cell lines was isolated by TRIzol Reagent (Invitrogen) following the manufacturer's instruction. The cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and amplified with Platinum SYBR Green qRT-PCR SuperMix-UDG reagents (Invitrogen Carlsbad, California, United States) using the CFX96 sequence detection system (Bio-Rad, Hercules, CA, USA). The reaction mixture consisted of 20 µl containing 1.5 mM MgCl2, 2.5 units Taq DNA Polymerase, 200 µM dNTP, 0.2 µM of each primer and 0.5 µg DNA. The cycling conditions were as follows: 95°C for 20 sec, followed by 40 cycles of 95°C for 15 sec, and 58°C for 1 min. The expression was estimated by 2-ΔΔCt method and actin was used as an internal control.

Cell transfection

The miR-16 mimics and miR-negative control (NC) were synthesized by RiboBio (Guangzhou, China). Transfection was then carried out by the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions. As the DMS-53 cells reached 80% confluence, the appropriate concentrations of miR-29 mimics or NC were transfected into these cells.

WST-1 assay

The proliferation rate of DMS-53 cells was monitored by WST-1 assay. Briefly, DMS-53 cells were cultured in 96-well plates at a density of 2×10^5 cells/well. The cells were then transfected with miR-NC or miR-16 mimics and again incubated for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for 4 h. The absorbance was then taken at 450 nm using a victor 3 microplate reader to determine the proliferation rate at 0, 12, 24, 48 and 96 h time intervals.

DAPI and Annexin V/PI staining

The DMS-53 cells were transfected with suitable constructs and cultured for 24 h at 37°C and then fixed with ethanol (70%) for 20 min. The cells were then washed with phosphate buffered saline (PBS) and subsequently stained with DAPI. Finally, the cells were examined under microscope to detect the induction of apoptosis. The DMS-53 cells were transfected with appropriate constructs and then incubated for 48 h at 37°C. The cells were then dissociated with trypsin, PBS-washed and resuspended in 1× binding buffer which was followed by the addition of 5 µL of annexin V-FITC/PI. The cell culture was then placed in the dark for 15 min. The apoptosis percentage was evaluated by a flow cytometer.

Cell migration and invasion assay

Transwell chambers with Matrigel were employed to monitor the DMS-53 cell invasion. In brief, the cells were transfected with appropriate constructs and 48 h post-transfection the cells were harvested and suspended in fresh Dulbecco's modified Eagle's medium (DMEM). While 200 µL of the cell suspension containing approximately 5×10^4 cells was placed onto the upper compartment, a fresh 500 µ DMEM was placed in the lower compartment. After 24 h cells present at the upper compartment were removed by swabbing while cells that invaded to the lower surface were fixed with 2% paraformaldehyde solution and then subsequently stained with 0.05% crystal violet. Finally, 10 random fields (magnification ×200) were selected to determine the invasion under light microscope. The cell migration was determined by a similar procedure except that Martigel was not used.

Western blotting

The lung cancer tissues and cell lines were lysed with RIPA lysis buffer and the protein concentration in each sample was measured by Bradford assy. Equal concentrations of the proteins from each sample were loaded on 10% SDS polyacrylamide gels which was followed by shifting to polyvinylidene fluoride membranes. Blocking of the membrane was then performed by fat-free milk (5%) in tris-buffered saline (TBS) and polysorbate 20 mix. This was followed by incubation with a primary antibody for 24 h at 4°C. Subsequently, a secondary antibody was added at 25°C for about 2 h. The bands of interest were finally observed by chemiluminescence.

Statistics

Statistical analyses were done by applying one way analysis of variance (ANOVA), followed by Tukeys test using SPSS software package, version 9.05. The values are mean±SD of 3 independent experiments and $p<0.05$ was considered as significant difference.

Results

Downregulated expression of miR-16 in lung cancer

The determination of the expression of miR-16 in lung cancer and normal adjacent tissues was performed by qRT-PCR. We found that miR-16 expression was remarkably suppressed in lung cancer tissues. The downregulation of miR-16 in lung cancer tissues was up to 5.5-fold in comparison to the normal adjacent tissues (Figure 1A). Gene expression analysis of miR-16 in lung cancer cells showed miR-16 to be significantly (up to 6.3-fold) downregulated in lung cancer cell lines (Figure 1B). The highest downregulation was observed in DMS-53 cells. This cell line was therefore used for further studies.

Suppression of DMS-53 growth by miR-16 overexpression

The role of miR-16 in lung cancer was determined by overexpressing miR-16 in DMS-53 lung cancer cells (Figure 1C). The WST-1 assay showed a remarkable decline in the proliferation rate of the lung DMS-53 cancer cells upon miR-16 overexpression (Figure 1D). To ascertain the reasons for decline in the proliferation of the DMS-53 cells brought about by miR-16 overexpression, we performed DAPI staining and found that DAPI-positive DMS-53 cells increased upon miR-16 overexpression, suggestive of apoptosis (Figure 2A). The annexin

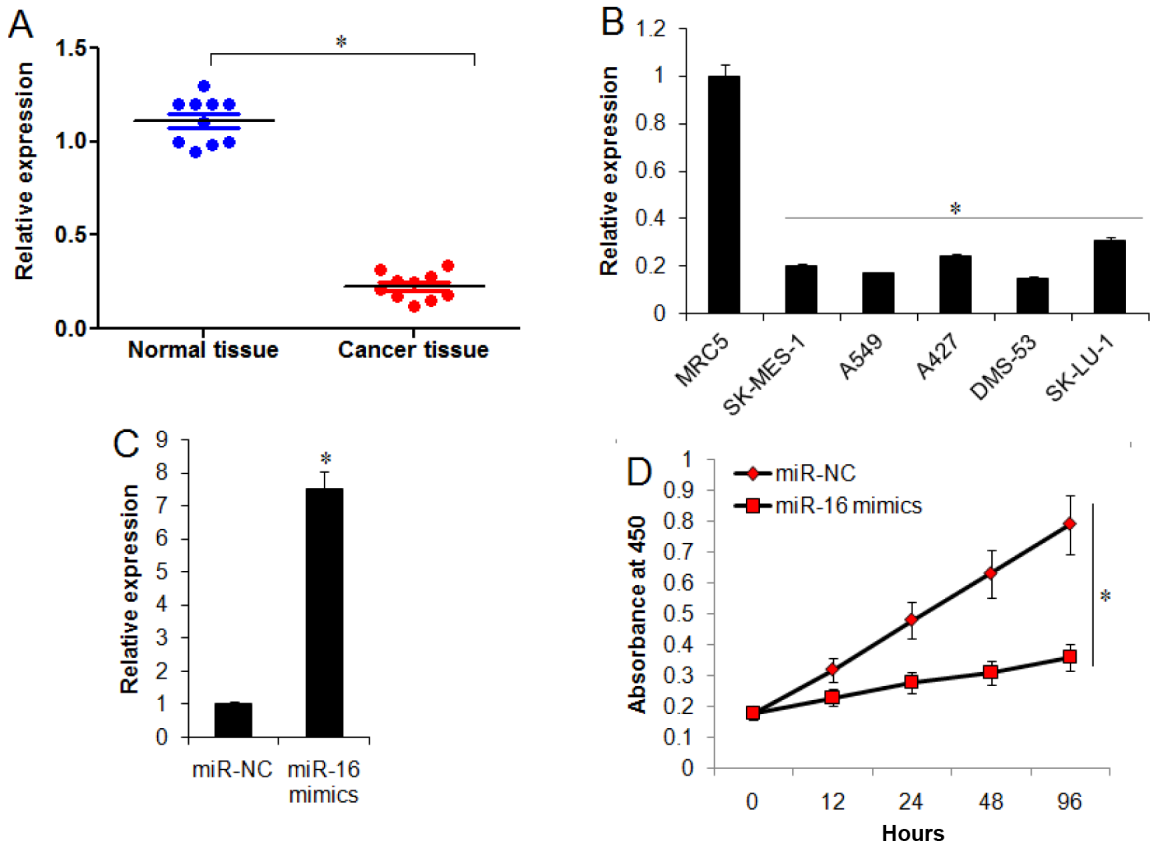

Figure 1. A: Expression of miR-16 in lung cancer and normal adjacent tissues. **B:** Expression of miR-16 in normal MRC5 and different lung cancer cell lines. **C:** Expression of miR-16 in miR-NC or miR-16 mimics transfected DMS-53 lung cancer cells. **D:** WST-1 assay showing the viability of the miR-NC or miR-16 mimics transfected DMS-53 lung cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*$p<0.05$).

V/PI assay showed that miR-16 overexpression caused increase in the percentage of the apoptotic DMS-53 cells (Figure 2B). The induction of apoptosis in DM-53 cells was also accompanied by upsurge of Bax and suppression of Bcl-2 (Figure 2C).

Suppression of metastasis of DMS-53 cells by miR-16

The transwell assay was used assess the effects of miR-16 on the metastasis of DMS-53 cells. We found that the migratory and invasive potential

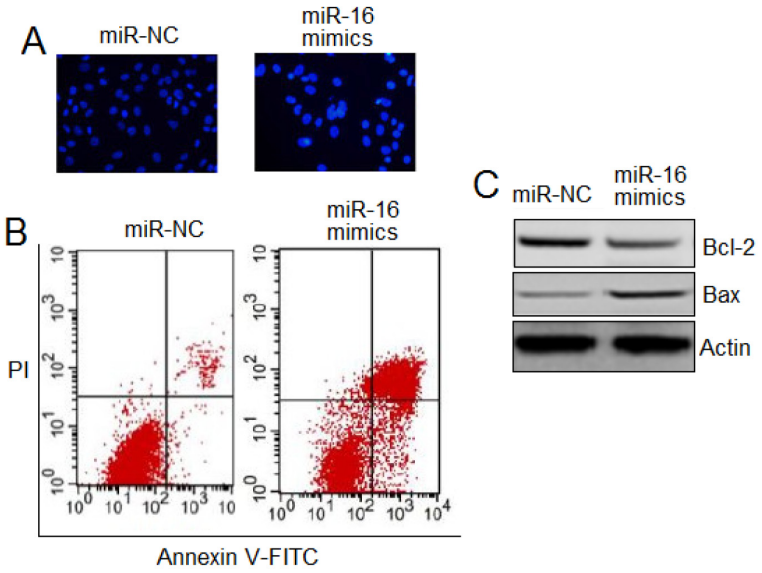

Figure 2. A: DAPI staining of the miR-NC or miR-16 mimics transfected DMS-53 lung cancer cells showing induction of apoptosis. **B:** Annexin V/PI staining of miR-NC or miR-16 mimics transfected DMS-53 lung cancer cells showing the extent of apoptosis. **C:** Western blot analysis showing the expression in miR-NC or miR-16 mimics transfected DMS-53 lung cancer cells. The experiments were performed in triplicate and expressed as mean ± SD.

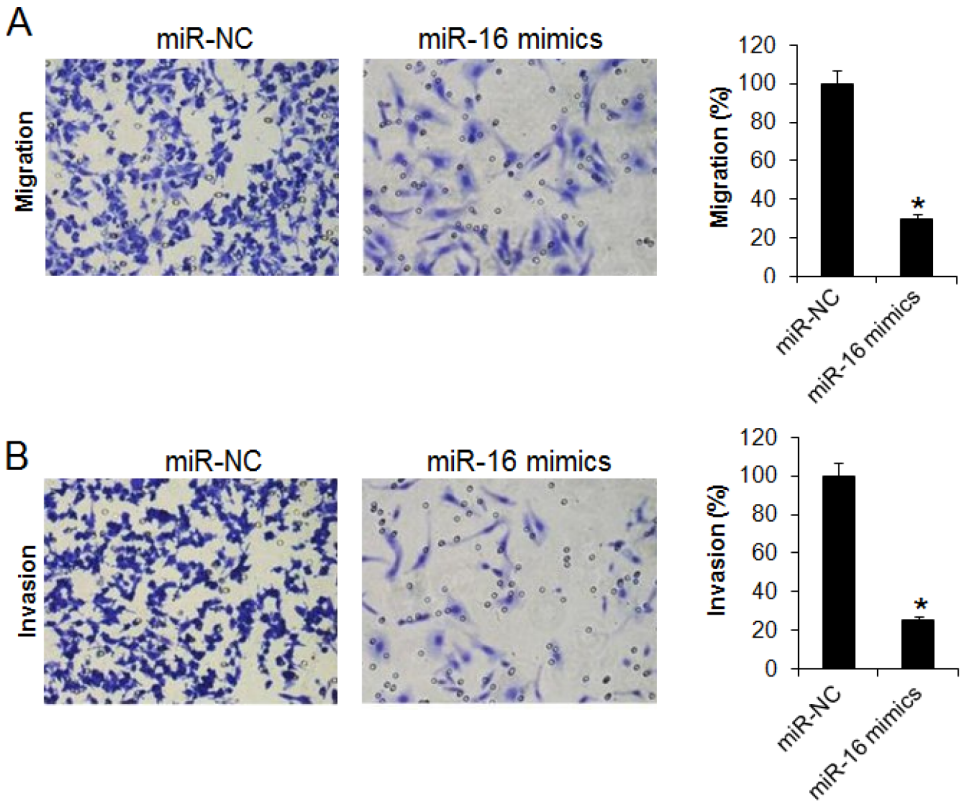

Figure 3. A: Transwell assay showing the migration of the miR-NC or miR-16 mimics transfected DMS-53 lung cancer cells. **B:** Transwell assay showing the invasion of the miR-NC or miR-16 mimics transfected DMS-53 lung cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

of the DMS-53 cells was suppressed upon miR-16 overexpression. The invasion and migration of the DMS-53 cells was suppressed by 75 and 70% relative to the control respectively (Figure 3A and 3B).

miR-16 targets YAP1 in lung cancer cells

TargetScan was used for identification of miR-16 targets. The TargetScan showed YAP1 to be the potential target of miR-16 (Figure 4A). Dual luciferase assay also validated the interaction between miR-16 and YAP1 (Figure 4B). We also investigated the expression of YAP1 in all lung cancer tissues and cell lines and the qRT-PCR revealed YAP1 to be aberrantly elevated in all the cell lines (Figure 4C and 4D). However, miR-16 overexpression could cause decline in YAP1 expression in the DMS-53 cells (Figure 4E). We also sought to ascertain if silencing of YAP1 caused similar effects on DMS-53 cells. What we found was that YAP1 silencing caused a remarkable decrease in the proliferation of DMS-53 cells (Figure 5A and 5B). The impact of YAP1 overexpression was also investigated on the proliferation of the DMS-53 cells overexpressing miR-16 and we found that overexpression of YAP1 in the DMS-53 cells overexpressing miR-16 promoted their growth thereby avoiding the tumor suppressive effects of miR-16 (Figure 5C).

Discussion

Ranked as the most prevalent type of cancer, lung cancer is responsible for excessive mortality world over. The prevalence of lung cancer is increasing at an alarming rate and the lung cancer 5-year survival rate is one of the poorest among all cancers [10]. There is pressing need to identify therapeutic targets and to develop potent treatment regimes for lung cancer. Recent studies have shown that miRs may serve as vital biomarkers and therapeutic agents for the treatment of deadly diseases such as cancer. Human miRs are highly conserved and exhibit multiple isoforms [11]. They negatively control the gene expression and it has been found that around 60% of the human

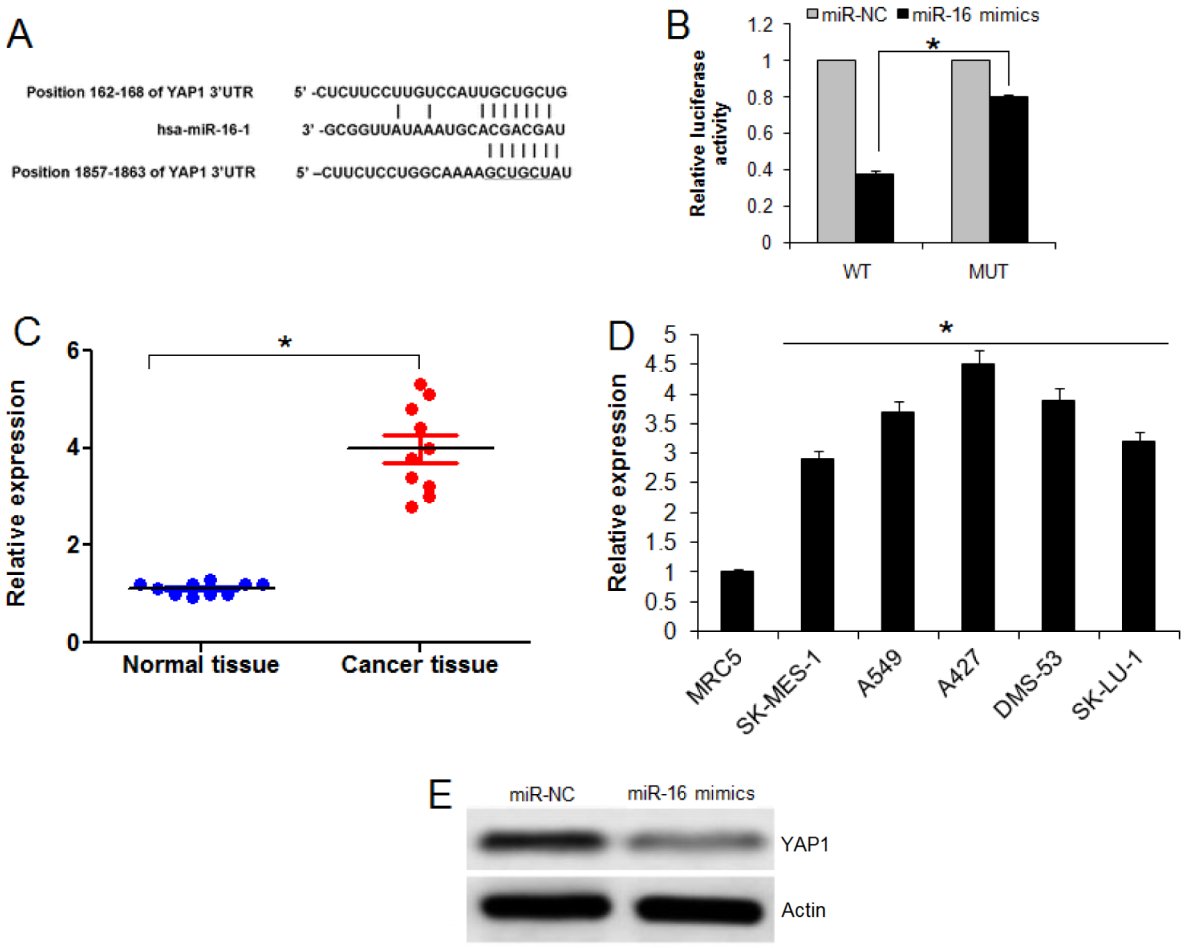

Figure 4. A: TargetScan analysis showing YAP1 expression of miR-16. **B:** Dual luciferase assay. **C:** Expression of YAP1 in cancer and normal adjacent tissues. **D:** Expression of YAPI in normal MRC5 and different lung cancer tissues. **E:** Western blot showing that overexpression of miR-16 inhibits the expression of YAP1 in lung cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

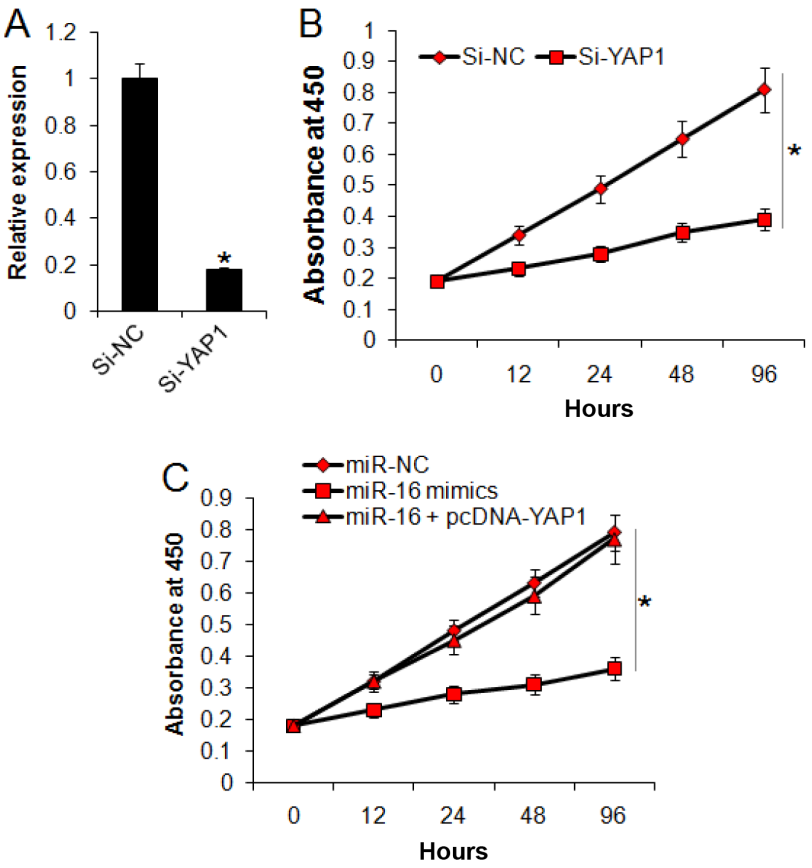

Figure 5. A: Expression of YAP1 in Si-NC and Si-YAP1 in DMS-53 cells. **B:** Viability of si-NC or si-YAP1 transfected DMS-53 lung cancer cells. **C:** Rescue assay showing the effect of YAP1 overexpression on the miR-16 mimics transfected DMS-53 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

protein-coding genes harbour miR-binding sites [12]. Since, miRs control vital cellular processes in humans, it is not surprising that they control the development of diseases such as cancer [13]. This study investigated the prospective of miR-16 in lung cancer treatment. We report that miR-16 is suppressed in lung cancer. A study carried out earlier has also shown miR-16 to be suppressed in gastric cancer [14]. The WST-1 assay showed that miR-16 overexpression resulted in decline in the growth of the DMS-53 lung cancer cells. Previous studies have also shown that miR-16 suppresses the growth of the pituitary tumor cells [15]. The DAPI and annexin V/PI assays showed that miR-16 overexpression promotes apoptosis in the DMS-53 cells which was also associated with enhancement of Bax and depletion of Bcl-2 expression. These observations are in agreement with a previous study wherein Cai et al reported that miR-16 causes apoptosis of osteosarcoma cells [16]. The miR-16 overexpression also resulted in the inhibition of the migratory and invasive potential of DMS-53 lung cancer cells. This is also in concordance with a previous research study wherein miR-16 has been found to inhibit the metastasis of the glioma cells [17]. The *in silico* analysis showed that miR-16 targets YAP1 in DMS-53 lung cancer cells which was also supported by the dual luciferase assay. YAPI expression was elevated in lung cancer tissues and cell lines and its expression was suppressed upon overexpression of miR-16 in DMS-53 lung cancer cells. Silencing of YAP1 also inhibited the growth of the DMS-53 cells while overexpression of YAP1 could avoid the tumor suppressive effects of miR-16. These findings are in good agreement with a previous study wherein suppression of YAP1 has been shown to be associated with inhibition of the growth of gastric adenocarcinoma cells [18].

Conclusion

The outcomes of the present study revealed that miR-16 is downregulated in lung cancer and controls the growth and metastasis of lung cancer cells via suppression of YAP1. The present study points towards miR-16 as novel target in lung cancer.

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Conflict of interests

The authors declare no conflict of interests.

References

1. Cruz CS, Tanoue LT, Matthay RA. Lung cancer: epidemiology, etiology, and prevention. Clin Chest Med 2011;32:605-44.
2. Molina JR, Yang P, Cassivi SD, Schild SE, Adjei AA. Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clinic Proceed 2008;83:584-94.
3. Alberg AJ, Samet JM. Epidemiology of lung cancer. Chest 2003;123:21S-49.
4. Garzon R, Calin GA, Croce CM. MicroRNAs in cancer. Ann Rev Med 2009;60:167-79.
5. Garzon R, Marcucci G, Croce CM. Targeting microRNAs in cancer: rationale, strategies and challenges. Nat Rev Drug Discov 2010;9:775.
6. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. Trends Mol Med 2014;20:460-9.
7. Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Ann Rev Pathol Mechan Disease 2014;9:287-314.
8. Lee YS, Dutta A. MicroRNAs in cancer. Ann Rev Pathol Mechan Dis 2009;4:199-227.
9. Aqeilan RI, Calin GA, Croce CM. miR-15a and miR-16-1 in cancer: discovery, function and future perspectives. Cell Death Differ 2010;17:215.
10. Alberg AJ, Brock MV, Samet JM. Epidemiology of lung cancer: looking to the future. J Clin Oncol 2005;23:3175-85.
11. Kong YW, Ferland-McCollough D, Jackson TJ, Bushell M. microRNAs in cancer management. Lancet Oncol 2012;13:e249-58.
12. Iorio MV, Croce CM. MicroRNAs in cancer: small molecules with a huge impact. J Clin Oncol 2009;27:5848.
13. Ventura A, Jacks T. MicroRNAs and cancer: short RNAs go a long way. Cell 2009;136:586-91.
14. Wang T, Hou J, Li Z et al. miR-15a-3p and miR-16-1-3p negatively regulate Twist1 to repress gastric cancer cell invasion and metastasis. Int J Biol Sci 2017;13:122.
15. Renjie W, Haiqian L. MiR-132, miR-15a and miR-16 synergistically inhibit pituitary tumor cell proliferation, invasion and migration by targeting Sox5. Cancer Lett 2015;356:568-78.
16. Cai CK, Zhao GY, Tian LY et al. miR-15a and miR-16-1 downregulate CCND1 and induce apoptosis and cell cycle arrest in osteosarcoma. Oncol Rep 2012;28:1764-70.
17. Li X, Ling N, Bai Y et al. MiR-16-1 plays a role in reducing migration and invasion of glioma cells. Anatom Record 2013;296:427-32.
18. Kang W, Tong JH, Lung RW et al. Targeting of YAP1 by microRNA-15a and microRNA-16-1 exerts tumor suppressor function in gastric adenocarcinoma. Mol Cancer 2015;14:52.