

ORIGINAL ARTICLE

MicroRNA-133 inhibits the growth and metastasis of the human lung cancer cells by targeting epidermal growth factor receptor

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Summary

Purpose: Accounting for 25% of all cancers and 20% of the cancer related-mortality, lung cancer is one of the devastating types of malignancies. The main obstacles for successful lung cancer manipulation include late diagnosis, dearth of safer chemotherapy and lack of potent therapeutic targets. Evidence indicates that microRNAs (miRs) may prove essential therapeutic targets for the management of deadly diseases, including cancer. Herein the role of miR-133 was investigated in lung cancer and the therapeutic potential of miR-133 was explored.

Methods: qRT-PCR was used for expression profiling of miR-133 and epidermal growth factor receptor (EGFR) in normal lung cell line MRC5 and lung cancer cell lines SK-MES-1, A549, A427 and DMS-53. Cell Counting Kit-8 (CCK8) 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-133 and western blot analysis was done to estimate the proteins' expression.

Results: miR-133 was significantly ($p < 0.05$) downregulated in lung cancer cell lines. Overexpression of miR-133

in A549 lung cancer cells caused significant ($p < 0.05$) inhibition of their proliferation via activation of apoptotic cell death, suggestive of the tumor suppressive role of miR-133. In addition, miR-133 overexpression also resulted in significant ($p < 0.05$) suppression of A549 cell migration and invasion. TargetScan analysis indicated EGFR to be the potential target of miR-133 in A549 cells. Analysis of EGFR expression in lung cancer cell lines showed up to 4.6 fold upregulation of EGFR. However, miR-133 overexpression resulted in downregulation of EGFR expression. Furthermore, silencing of EGFR also resulted in inhibition of proliferation, migration and invasion of A549 cells. However, overexpression of EGFR could nullify the tumor suppressive effects of miR-133, indicating EGFR inhibition is essential for the miR-133-mediated inhibitory effects on A549 cell proliferation.

Conclusion: Taken together, miR-133 acts as a tumor suppressor and may prove essential in the management of lung cancer.

Key words: lung cancer, tumor suppressor, miR-133, migration, apoptosis

Introduction

Lung cancer causes significant morbidity and mortality across the globe. It has been reported that more than one and a half million people died of lung cancer worldwide in 2012 [1]. The incidence of lung cancer is increasing at an alarming rate and it has been estimated that the deaths in lung can-

cer will increase to 3 million in 2013 [2]. The lack of potent chemotherapy, therapeutic targets and late diagnosis are the major obstacles in the treatment of lung cancer [3]. MicroRNAs (miRs) include around 23 RNA nucleotides that regulate the expression of a number of genes in human and other

organisms by binding to the mRNAs to enforce their post transcriptional suppression [4]. Since miRs modulate the expression of about 30% of the human protein coding genes, they are involved in different cellular and physiological processes, such as cell cycle, proliferation and apoptosis to name a few [5]. Accumulating evidence suggests that several miRs are aberrantly expressed in cancer cells and are considered to be prospective therapeutic targets/agents for the management of cancer [6]. Among miRs, miR-133 has been shown to be involved in the proliferation and metastasis of a number of cancers. For example miR-133 has been reported to inhibit the growth and metastasis of prostate cancer cells [7] and bladder cancer cells [8]. However the role of miR-133 in lung cancer is yet to be thoroughly investigated.

This study was designed to explore the role and therapeutic potential of miR-135 in lung cancer.

Methods

Cell lines and culture conditions

The normal lung cell line MRC5 and lung cancer cell lines SK-MES-1, A549, A427 and DMS-53 were procured from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium 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 a CO₂ incubator (Thermo Fisher Scientific, Inc.) at 37°C with 98% humidity and 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from the human lung cancer cell lines and normal cell line using RNeasy kits (Qiagen, Inc., Valencia, CA, USA). To reverse transcribe the cDNA, the Omniscript RT (Qiagen, Inc.) was employed using 1 µg of the extracted RNA. The cDNA was then used as a template for RT-qPCR analysis using the Taq PCR Master Mix kit (Qiagen, Inc.) according to the manufacturer's protocol. 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. GAPDH was used as an internal control and the relative quantification (2^{-ΔΔC_q}) method was used to evaluate the quantitative variation between the samples as described previously [14].

Cell transfection

When the lung cancer A549 cells reached 80% confluence, they were transfected with mimics-negative control (NC) miR-133, miR-133 inhibitor, Si-EGFR and pcDNA-EGFR (Shanghai GenePharma) with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as per the manufacturer's protocol.

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Cell proliferation assay

After 24 h of transfection, the lung cancer A549 cells were grown in 96-well plates in three replicates at the density of 1.0×10⁵ cells/well. The proliferation rate and the viability of the A549 cells were determined by Cell Counting Kit-8 (CCK8) following the guidelines of the manufacturer. Around 10 µL CCK-8 reagent were added into the wells at specific time intervals (12, 24, 48, 72 and 96 h) and the plates were then incubated for about 2 h at 37°C. The cell viability was then measured by taking the absorbance at 450 nm using spectrophotometer.

Cell cycle analysis

For cell cycle analysis, the transfected lung cancer A549 cells were incubated at 37°C for 24 h. The cells were then washed with phosphate buffered saline (PBS). Afterwards, the A549 cells were stained with propidium iodide (PI) and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Detection of apoptosis

For DAPI staining, the transfected lung cancer A549 cells (0.6×10⁶) were grown in 6-well plates. Following an incubation period of around 12 h, the A549 cells were subjected to incubation for 24 h at 37°C. As the cells sloughed off, 25 µl cell cultures were put onto glass slides and subjected to staining with DAPI. The slides were covered with a cover slip and examined with a fluorescence microscope. Annexin V/PI staining of the A549 cells was performed as described previously [10].

Cell migration and invasion assay

The migration and invasion abilities of the transfected lung cancer A549 cells were examined by transwell chamber assay. In brief, 1×10⁴ A549 cells were seeded in upper chamber of the transwell (8 µm pore size polycarbonate filters). This was followed by the placement of the chambers into 24-well plates and subjected to incubation at 37°C for 48 h. The inserts were coated with extracellular matrix gel (50 µl) (ECM, Sigma, USA) to monitor cell invasion. Swabbing was performed to remove the non-migrated and non-invaded cells from the upper surface. However, the migrated and the invaded cells on the lower surface were subjected to fixation with methanol for about 35 min, followed by staining with crystal violet (0.5%) for about 50 min, subjected to washing with PBS and finally counted under light microscope (5 fields, 200x magnification).

Western blotting

The A549 cells were firstly subjected to washing with ice-cold PBS and suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About 40 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then

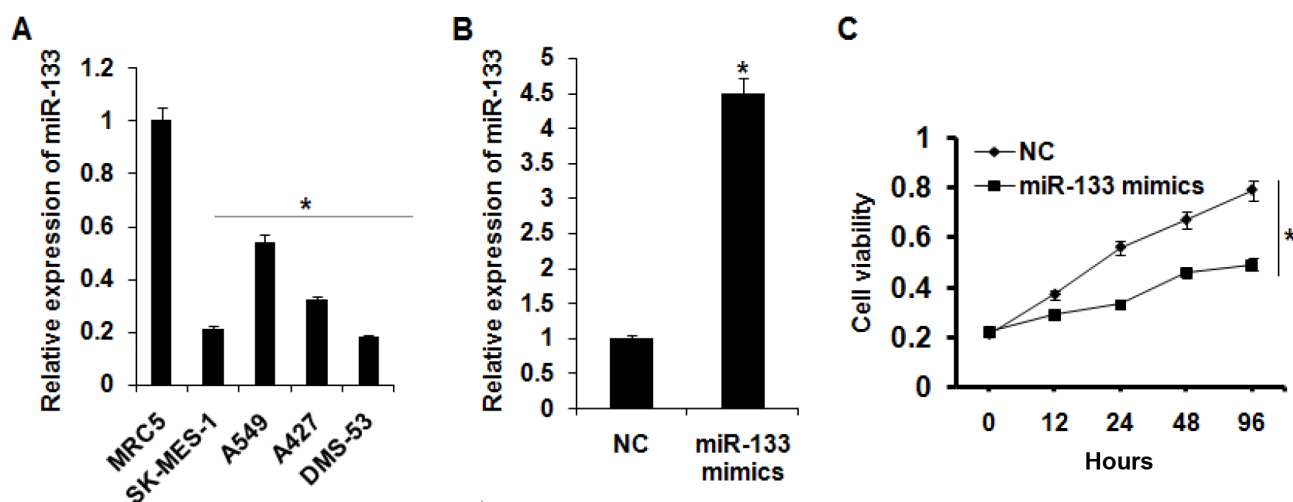


Figure 1. Overexpression of miR-133 inhibits the proliferation of lung cancer cells. **A:** Expression of miR-133 in MRC-5 normal and four different lung cancer cell lines. **B:** Expression of miR-133 in NC or miR-133 mimics-transfected A549 lung cancer cells. **C:** CCK-8 assay showing the percent viability of NC or miR-133 mimics-transfected A549 lung cancer cells at the indicated time intervals. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

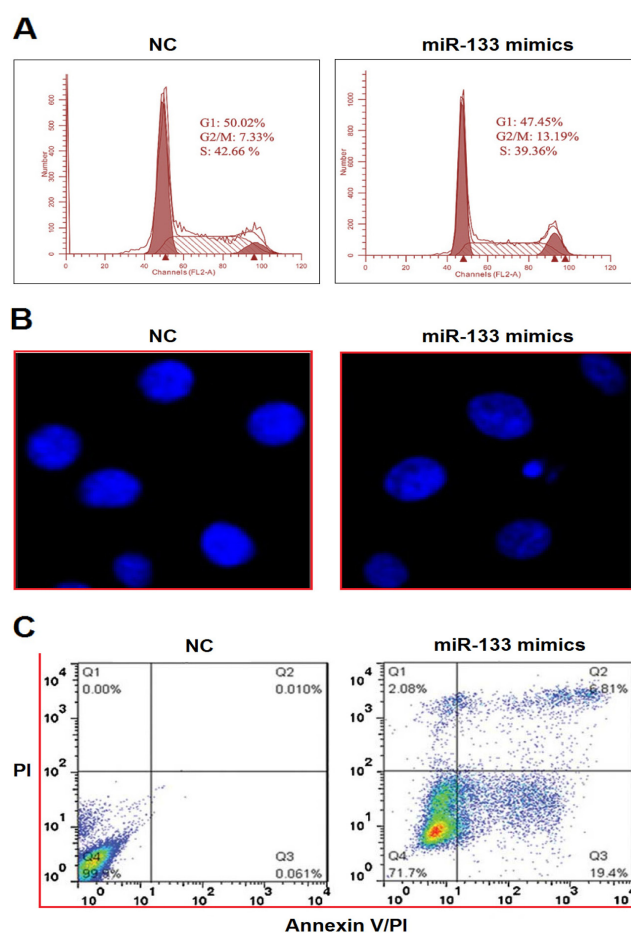


Figure 2. miR-133 overexpression triggers cell cycle arrest and apoptosis in A549 lung cancer cells. **A:** Flow cytometric analysis showing G2/M cell cycle arrest in A549 cells. **B:** DAPI staining showing induction of apoptosis in NC or miR-133 mimics-transfected A549 lung cancer cells. **C:** Annexin V/PI staining showing percentage of apoptosis in NC or miR-133 mimics-transfected A549 lung cancer cells. The experiments were performed in triplicate.

subjected to treatment with tris-buffered saline (TBS) and then exposed to primary antibodies at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

The experiments were performed in triplicate and expressed as mean \pm SD. Statistical analyses were done using Students *t*-test with GraphPad prism 7 software. Values of $p < 0.05$ were indicative of significant difference.

Results

miR-133 is significantly downregulated in lung cancer cell lines

The expression profile of miR-133 was investigated in normal (MRC5) and lung cancer cell lines (SK-MES-1, A549, A427 and DMS-53) by qRT-PCR (Figure 1A). It was found that miR-133 exhibited aberrant expression and was significantly down-regulated ($p < 0.05$) in all the lung cancer cell lines. Also miR-133 was downregulated in lung cancer lines by up to 5.5 fold relative to the normal cell MRC5 line. The highest downregulation was found in case of the A549 cell line and this study was taken forward for further experimentation.

miR-133 suppresses the growth of lung cancer cells

The overexpression of miR-133 in A549 cells was confirmed by the qRT-PCR which showed that transfection of the A549 cells with miR-133 mimics caused around 4.4 fold increase in the expression of miR-133 relative to negative control (NC) transfected cells (Figure 1B). The proliferation rate of

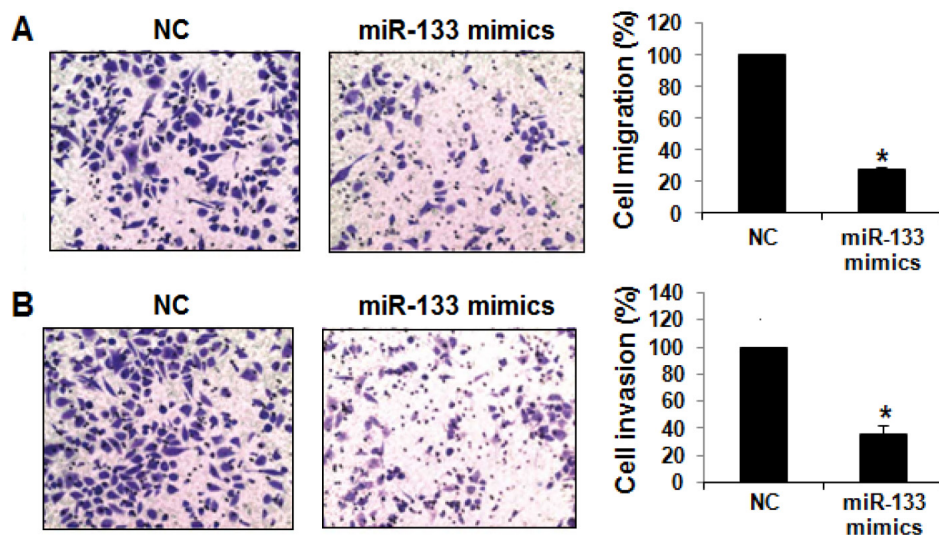


Figure 3. Overexpression of miR-133 inhibits the migration and invasion of lung cancer cells. Transwell assay showing **A:** percent migration and **B:** invasion in NC or miR-133 mimics transfected A549 cells. The Figure shows that miR-133 overexpression causes decrease in migration and invasion of A549 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

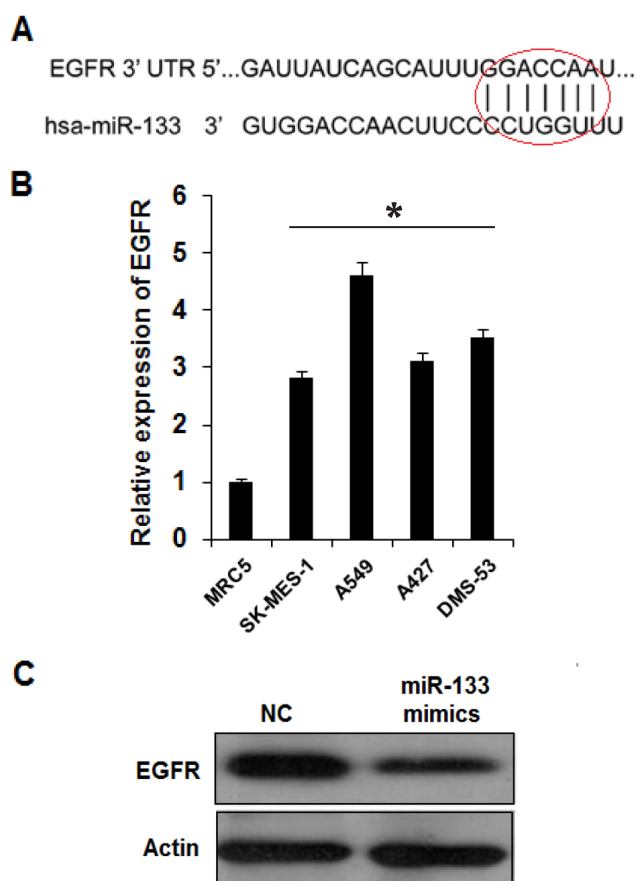


Figure 4. miR-133 exerts its effects by targeting EGFR. **A:** TargetScan analysis showing EGFR as the target of miR-133. **B:** Expression of EGFR in normal MRC5 and four different lung cancer cell lines showing upregulated expression of EGFR. **C:** Western blot analysis showing the expression of EGFR in NC or miR-133 transfected lung cancer cells which was overexpressed causing reduction in the expression of EGFR. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

the NC and miR-133 mimics A549 transfected cells was monitored at different time intervals by CCK-8 assay and it was revealed that the transfection of miR-133 mimics in the A549 cells caused significant decline in the proliferation rate (Figure 1C). Cell cycle analysis was then performed to check whether miR-133 overexpression triggers cell cycle arrest; what was found was that miR-133 overexpression had no effects on the cell cycle distribution (Figure 2A). However, DAPI staining of the NC and miR-133 transfected A549 cells revealed that miR-133 mimics transfection (ie., miR-133 overexpression) activated apoptotic cell death of the A549 cells (Figure 2B). Annexin V/PI staining clearly showed that the apoptotic cell percentage increased from 0.06 % in NC transfected cells to about 19.4% in the miR-133 mimics transfected A549 cells (Figure 2C). Taken together, these experiments indicate that miR-133 induced inhibition of A549 cell proliferation via activation of apoptotic cell death.

miR-133 inhibits the migration and invasion of lung cancer cells

The effects of miR-133 were also examined on the migration and invasion of the A549 lung cancer cells by transwell chamber assay. The results showed that the migration and invasion of the A549 lung cancer was significantly ($p < 0.05$) reduced in the miR-133 mimics transfected cells (Figure 3A and B). The A549 cell migration was inhibited by 73% and the invasion was inhibited by 65% in miR-133-transfected cells relative to the NC-transfected A549 cells. Taken together, these results show that miR-133 overexpression results

in inhibition of the migration and invasion of A549 lung cancer cells.

miR-133 targets EGFR in lung cancer cells

The target of miR-133 in lung cancer cells was identified by online TargetScan analysis. STAT-3 was identified as the potential target of miR-133 in A549 lung cancer cells (Figure 4A) and therefore the expression levels of EGFR were investigated in all the lung cancer cell lines as well as the normal cell line. It was found that, relative to the expression of EGFR, it was significantly upregulated (up to 4.6 fold) in the lung cancer cell lines (Figure 4B). However, as the A549 cells were transfected with the miR-133 mimics, the expression of EGFR was considerably downregulated as depicted by the western blot analysis (Figure 4C).

In order to know the effects of the EGFR silencing on the proliferation, migration and invasion of the lung cancer A549 cells, these cells were transfected with NC or Si-EGFR and the silencing of EGFR expression was confirmed by qRT-PCR anal-

ysis which showed 8 fold downregulation of the EGFR expression in Si-EGFR transfected A549 cells relative to the Si-NC transfected cells (Figure 5A). Furthermore, the results of the CCK-8 showed that Si-EGFR transfection (ie., EGFR silencing) caused significant decline ($p < 0.05$) in the proliferation, migration and invasion of the A549 lung cancer cells (Figure 5B-D).

EGFR rescues the effects of miR-133 overexpression in A549 cells

Since miR-133 overexpression and EGFR silencing exhibited similar effects on the proliferation, migration and invasion of the A549 cells, we sought to know if EGFR overexpression could nullify the effects of miR-133 overexpression in A549 cells. Interestingly, it was found that EGFR overexpression in the miR-133 mimics-transfected A549 cells promoted the proliferation of these cells, indicating that the inhibitory effects of the miR-133 overexpression are mainly due to EGFR suppression (Figure 6).

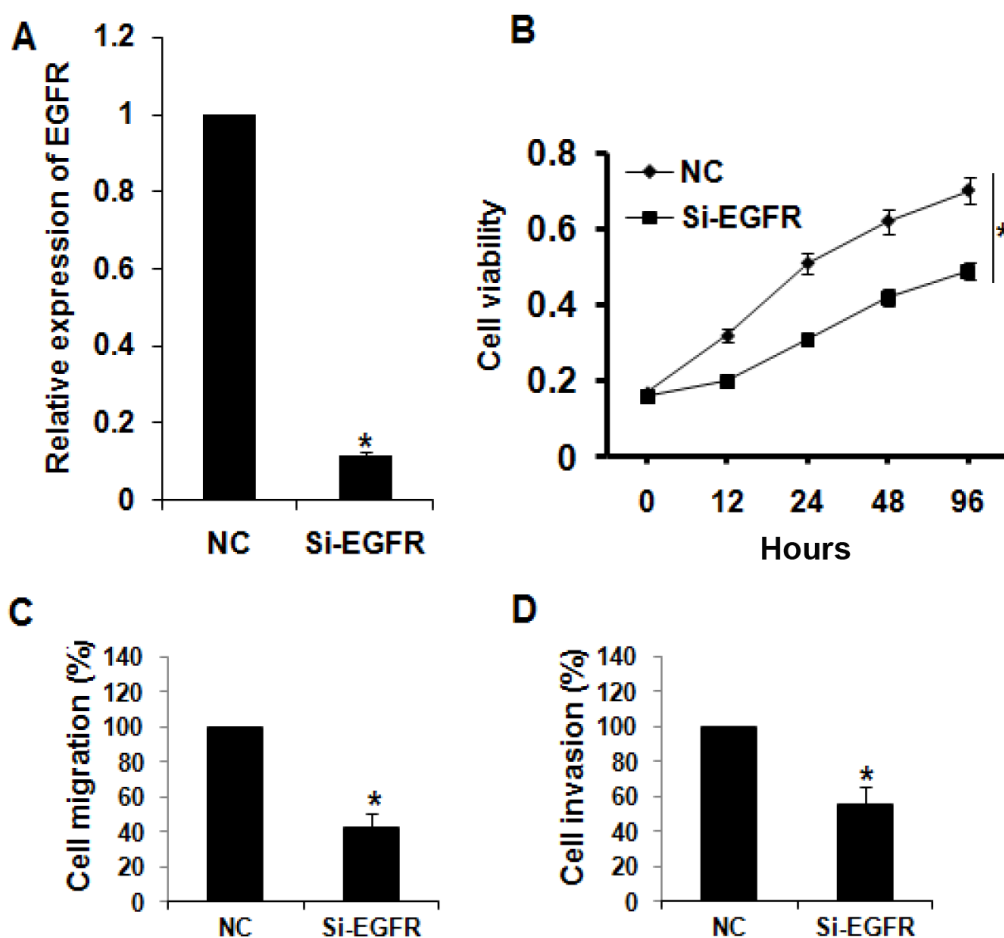


Figure 5. Silencing of EGFR inhibits the proliferation, migration and invasion of lung cancer cells. **A:** Expression of EGFR in Si-NC or Si-EGFR transfected A549 cells. **B:** CCK-8 assay showing percent viability of the Si-NC or Si-EGFR transfected A549 cells. **C:** Transwell assay showing percent migration. **D:** percent invasion of the Si-NC or Si-EGFR transfected A549 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* $p < 0.05$).

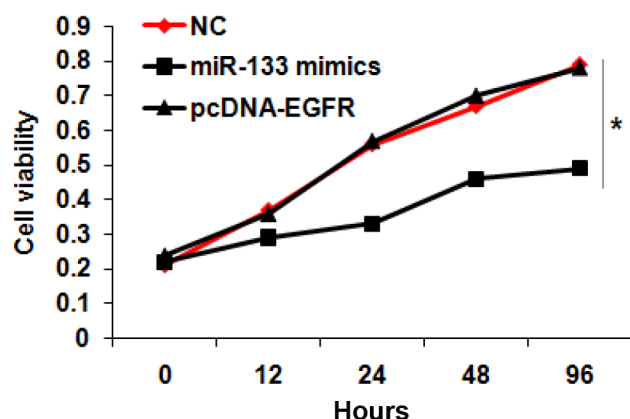


Figure 6. The effect of EGFR overexpression on the inhibitory effects of miR-133 overexpression on the proliferation of the A549 cells. The Figure shows that EGFR overexpression nullifies the effect of miR-133 overexpression on the proliferation of A549 cells. The experiments were performed in triplicate and expressed as mean \pm SD (* p <0.05).

Discussion

Lung cancer is one of the devastating malignancies that imposes huge disease burden worldwide. Accounting for about 25% of all the cancers, lung cancer is the most prevalently detected cancer across the world and is responsible for about 20% of cancer-related mortality [11]. Besides, the adverse effects of the anticancer agents used for the treatment of lung cancer affect the overall health of the patients [11]. Therefore, there is urgent need to explore new drugs or to identify effective therapeutic targets. miRs control the expression of the majority of the human genes and are involved in a wide array of cellular processes [12]. Because of the importance of the miRs in cellular physiological processes, several studies have revealed the potential of miRs as therapeutic targets [13]. Herein, the role and therapeutic potential of the miR-133 was investigated in lung cancer.

It was found that miR-133 was aberrantly down-regulated in the lung cancer cells. Previous studies have also shown the downregulation of miR-133a in ileal carcinoid tumors, prostate and breast cancer [7,8,14]. Overexpression of miR-133 in the A549 lung cancer cells caused significant reduction in the proliferation rate of the A549 lung cancer cells via induction of apoptotic cell death. Besides, it also resulted in suppression of cell migration and invasion of the A549 cells. A number of studies carried out earlier on miR-133 have also shown similar results. For example, miR-133 could suppress the growth and metastasis of prostate cancer cells [7]. In addition, it also regulates the survival of glioma cells [15]. Bioinformatic analysis together with dual luciferase indicated EGFR to be the potential target of miR-133. EGFR has shown to be an essential factor regulating the hormone sensitivity transition of several types of cancer cells [16]. Herein, we observed that EGFR was highly upregulated in the lung cancer and miR-133 overexpression could suppress the expression of EGFR. Additionally, EGFR silencing could also inhibit the growth of A549 lung cancer cells similar to that of miR-133 overexpression. Furthermore, EGFR inhibition was found to be essential for the tumor suppressive effects of miR-133 on lung cancer cells. Taken together, miR-133 acts a tumor suppressor in lung cancer and may prove as an essential therapeutic target for lung cancer.

Acknowledgement

We appreciate the support of this study by Pearl River Science and Technology special project in 2016 (no.201610010121).

Conflict of interests

The authors declare no conflict of interests.

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