Original Article
Inhibition of miR-130a expression promotes metastasis of lung squamous carcinoma cells

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Abstract: Aims: In this study, we aim to investigate the expression of miR-130a in human lung cancer tissues, to examine the association between miR-130a expressions and the clinicopathologic features of squamous carcinoma, and to determine the biological function of miR-130a in lung cancer. Methods: The expression of miR-130a in lung cancer tissues and their matched adjacent para-cancerous tissues was analyzed by Realtime PCR. A549 and Calu-3 cells were transfected with miR-130a mimics or scramble (NC), and the proliferation and migration of these cells were tested by MTT and CCK8 analysis. Cell cycle was detected by flow cytometry, and the expression of cell cycle-related proteins as well as epithelial mesenchymal transition (EMT)-related proteins was analyzed by Western blot. Results: The expression levels of miR-130a were significantly reduced in lung cancer tissues compared with adjacent para-cancerous tissues, and the expression levels of miR-130a were decreased in patients with lymph node metastasis compared with that without lymph node metastasis. However, changes of miR-130a expression were independent of sex, age, the size of tumor, lesion location, degrees of differentiation and the depth of tumor invasion. In *in vitro* experiments, the proliferation and migration of A549 and Calu-3 transfected with miR-130a mimics were significantly inhibited compared with NC. MiR-130a overexpression induced a G1/S arrest. CyclinD1, CyclinE1 and EMT-related protein E-Cadherin was upregulated while Vimentin was down regulated in miR-130a mimic transfected cells. Conclusions: These findings suggest that marked downregulation of the miR-130a may play a role in the development and invasion and metastasis of lung cancers. MiR-130a may be a potential therapeutic target of lung cancer.

Keywords: Lung squamous carcinoma, lung adenocarcinoma, miR-130a, cell cycle, metastasis

Introduction

Lung cancer, including non-small cell lung cancer (NSCLC) and small cell lung cancer, is one of the common malignant tumors [1]. Squamous cell carcinoma and adenocarcinoma are two major histological types of NSCLC, accounting for 80% of lung cancer [2]. Recently, with an increasing incidence rate, lung cancer has become the leading cause of cancer death worldwide [3]. Despite improvements in molecular diagnosis and targeted therapies, the overall 5-year survival rate remains about 10% [4]. Metastasis and recurrence are major reasons for the poor curative effect, and the majority of lung tumors lack effective treatment [5]. Thus, investigating the molecular mechanism of lung cancer development and identifying lung cancer related biomarkers and drug targets are significant for both the early diagnosis and the treatment of patients.

MicroRNAs (miRNAs), 18-25 nucleotides in length and highly conservative in evolution, are endogenous short noncoding RNAs that have important roles in almost all biological pathways [6]. Most miRNAs bind to target sequences located within the 3'-untranslated region (3'UTR) of mRNAs, repressing their translation to modulate the expression of target genes [7].

Many studies have reported that miRNAs could function as oncogenes or tumor suppressors, and miRNAs play critical roles in the invasion and metastasis of tumor [8, 9]. In body fluids miRNAs are abundant and more stable than mRNAs [10], indicating the potential use of tumor-specific miRNAs as diagnostic markers for cancer.

MiR-130a was reported recently to function as a tumor suppressor to suppress the proliferation and metastasis of prostate cancer cells by regulating mitogen activated protein kinases (MAPK) and Androgen receptor (AR) signal pathways [11]. Further studies also demonstrated its role in suppressing the proliferation and metastasis of hepatocellular carcinoma cells and breast cancer cells [12, 13]. At present, there is limited evidence of miR-130a's role in tumorigenesis of lung cancer and its putative target genes. Here in this study we investigated the expression levels of miR-130a in lung cancer tissues compared with adjacent para-cancerous tissues by real-time quantitative-PCR. We also reported the functional role of miR-130a in lung cancer development by Western blot, migration and invasion assay and cell cycle analysis in lung squamous carcinoma cells transfected with miR-130a mimics.

Materials and methods

Lung cancer tissue specimens

A total of 60 paired samples of human lung cancer and their matched adjacent noncancerous tissues were collected at the time of radical or palliative section between Dec, 2012 and Jan, 2014 at the People's hospital of Lishui, which were further confirmed by two pathologists after H&E staining according to the 2003 WHO tumor classification standard. A total of 35 cases of patients with NSCLC, including 27 cases of adenocarcinoma and 8 cases of squamous cell carcinoma, and 25 cases of small cell lung cancer were collected. The clinical stages of these cases are as follows: N0: 24; N1: 36; I/II: 37 and III/IV: 23. The matched normal tissues were obtained at least 5 cm distant from the tumor margin. Clinical information and medical records of patients were also collected.Prior written and informed consent were obtained from every patient and the study was approved by the ethics review board of Shandong University.

Cell culture and miRNA transfection

Human lung cancer cell lines, including A549 and Calu-3, were cultured in RPMI 1640 (Gibco, Thermo Fisher Scientific Inc, Vilnius, Lithuania) medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cultures were incubated in a humidified atmosphere with 5% CO_2 at 37°C. Cells were transfected with 20 mM of miR-130a mimics (RIOBIO, Guangzhou, China) or scramble (negative control, NC) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were collected at 24 h or 48 h post-transfection for further experiments.

RNA extraction and quantitative real-time PCR

For analysis the expression of miR-130a in lung cancer tissues, total RNAs were isolated using TRIzol® isolation reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Following gel electrophoresis verification of RNA integrity and quantification using UV spectrophotometer, 1 mg of total RNA was reverse transcribed using a PrimeScript RT Regent Kit (Takara Biotechnology (Dalian) Co.,Ltd., Dalian, China) with specific primers. The expression of small nuclear U6 was used as internal control. Quantitative real-time PCR was performed using the SYBR PrimeScript RT-PCR Kit (Takara). The relative expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method.

CCK-8 assay

For proliferation assay, A549 and Calu-3 cells were seeded into a 96-well plate at 1×10^3 cells in triplicate. The Cell Counting Kit-8 (Beyotime Institute of Biotechology, Haimen, Jiangsu, China) was added to the wells at every 24 h, 48 h, 72 h and cells were incubated for 30 min. The absorbance values in each well were measured with a microplate reader (Biorad, CA, USA) set at 490 nm.

Migration and invasion assay

For migration assay, 2×10^5 lung cancer cells in 200 mL of RPMI-1640 without FBS were plated on the top chamber of 8 µm pore filter Transwell chamber (Corning, NY, USA) inserts in 24-well plates. RPMI plus 10% FBS (500 mL) was added to the bottom chamber and incubated at 37°C and 5% CO_2. For analysis of inva-
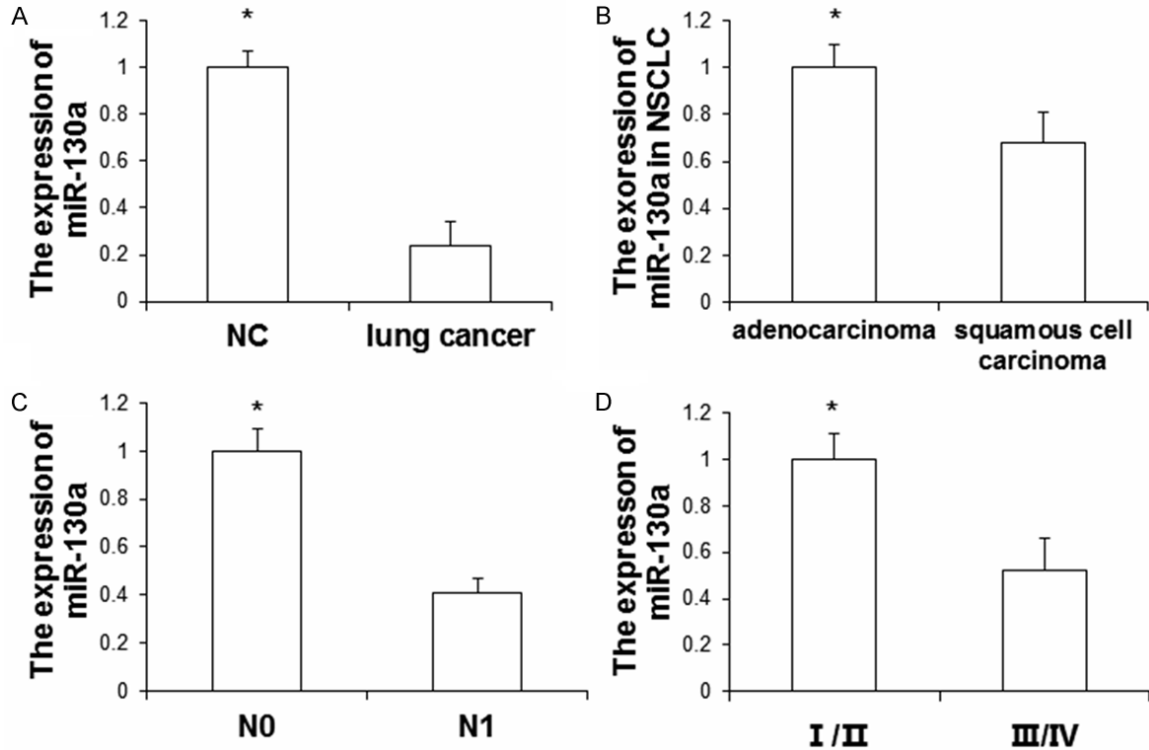
Figure 1. Expression levels of miR-130a in lung cancer specimens. A. The expression of miR-130a in 60 pairs of lung cancer tissues and compared normal tissues was detected by quantitative RT-PCR. B. Relative miR-130a expression levels in non-small cell lung squamous carcinoma tissues and small cell lung squamous carcinoma tissues. C. Relative miR-130a expression levels in lung tissues with (N1) or without lymph node (N0) metastasis. D. Relative miR-130a expression levels in lung cancer tissues of stage I/II and stage III/IV. *P < 0.05, paired t test.
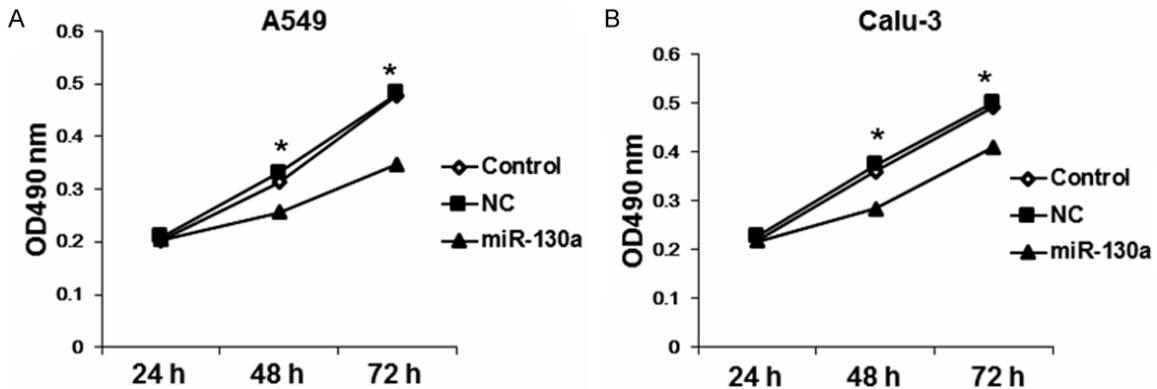
Figure 2. miR-130a suppresses A549 and Calu-3 cell proliferation. A549 and Calu-3 cells were transfected with miR-130a or scramble (NC). CCK-8 was performed at 24 h, 48 h and 72 h after transfection to analyze the effect of miR-130a on cell proliferation of A549 (A) and Calu-3 (B) cells.*P < 0.05, paired t test.

sive capacity, the transwell migration chambers were coated with Matrigel (BD Biosciences, San Jose, CA, USA) and incubated at 37°C for 60 min, allowing it to solidify. After 24 h, cells that did not pass through the chambers were

gently removed with a cotton swab, while the passed cells which located on the lower side of the chamber were fixed with formaldehyde, stained by Giemsa, and counted using Olympus BX53 microscopy (Olympus, Tokyo, Japan) at
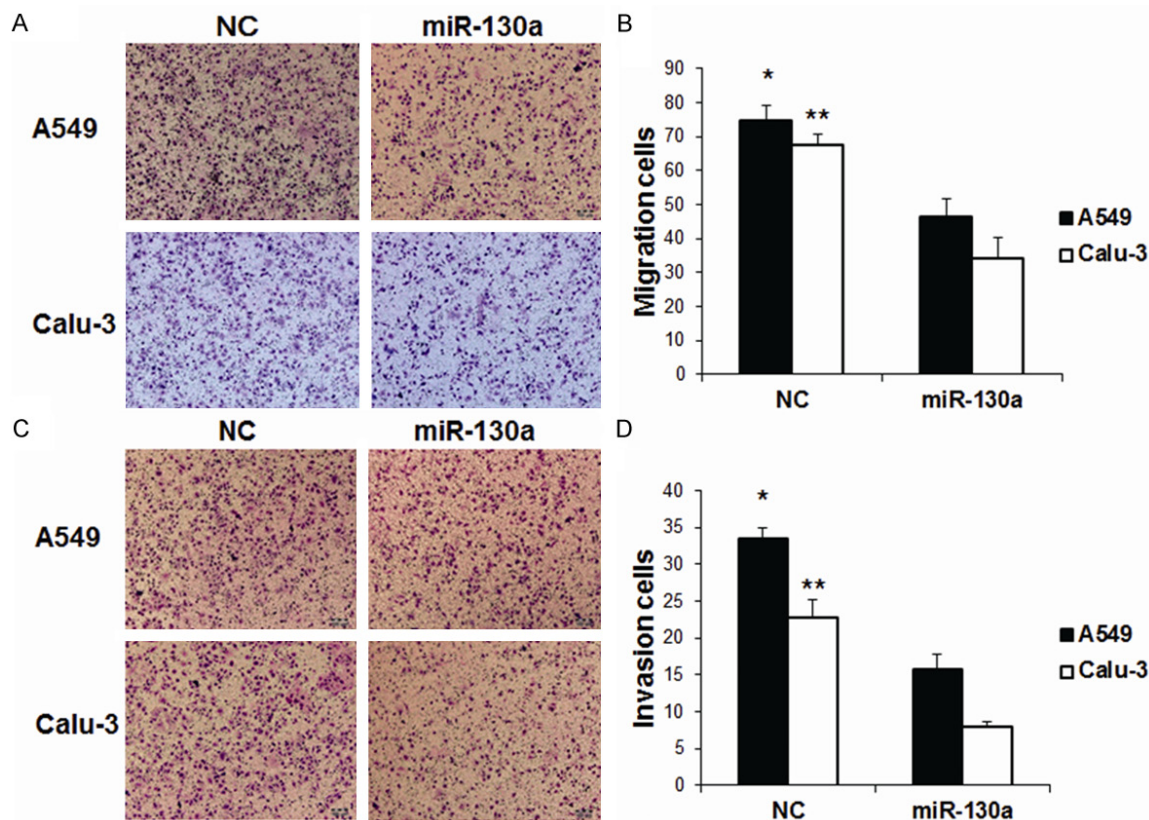
Figure 3. miR-130a inhibits A549 and Calu-3 cell migration and invasion *in vitro*. A549 and Calu-3 cells were transfected with miR-130a or scramble (NC). The effects of miR-130a on cell migration and invasion were detected using Transwell chamber assays. (A and B) showed the results on migration (× 200); (C and D) showed the results on invasion (× 200). *P < 0.05; **P < 0.01.

the magnification of 200 ×. Migration and invasion data were calculated by counting migrated cells in 5 fields.

Flow cytometry analysis of cell cycle

At 24 h after transfection, 1×10^5 cells were washed with cold PBS for two times and stained with Cell Cycle Assay Kit (BD Biosciences) according to the manufacturer's instructions. Cells were analyzed by flow cytometry using BD FACSVerse™ (BD Biosciences, NJ, USA) and Modfit software (Verity Software House, BD Biosciences, NJ, USA).

Western blot

Proteins were extracted by incubation with RIPA buffer and protease inhibitor PMSF. Then proteins were separated on SDS-Page and transferred to polyvinylidene difluoride membranes (PVDF, GE Healthcare Life Science, Piscataway,

NJ, USA). After blocking, the membranes were probed with the following antibodies: rabbit anti-CyclinD1 (1:1000); rabbit anti-CyclinE1 (1:1000), rabbit anti-Vimentin (1:1000), rabbit anti-E-Cadherin (1:1000), and mouse anti-GAPDH (1:5000). All of the above antibodies were purchased from Bioworld (St Louis Park, MN, USA). For detection, goat anti-rabbit (1:2000) or goat anti-mouse (1:5000) secondary antibodies conjugated to HRP (Bioworld, MN, USA) were used. Signal detection was performed using chemiluminescence reaction (ECL) (Millipore, Germany).

Statistical analysis

Data was expressed as mean ± SD. Statistical significance was determined with paired *t*-tests using SPSS 17.0 (SPSS Statistics/IBM Corp, Chicago, IL, USA). *P*-values < 0.05 were considered statistically significant.
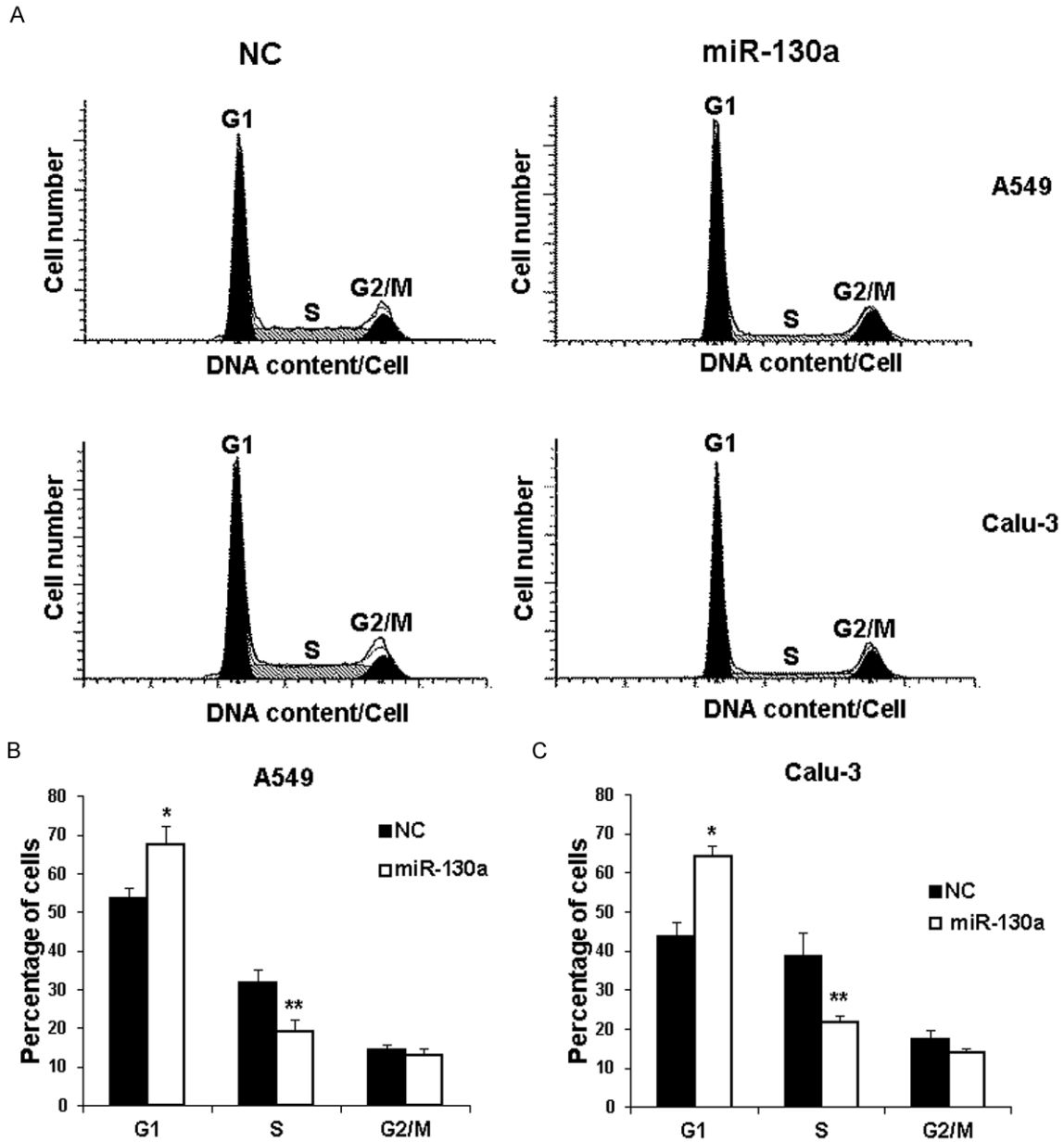
Figure 4. miR-130a induce cell cycle arrest. A549 and Calu-3 cells were transfected with miR-130a or scramble (NC). At 24 h after transfection the DNA contents of each sample was analyzed by flow cytometry. A. Representative flow cytometry results. B. Cell phase distribution of A549 cells. C. Cell phase distribution of Calu-3 cells. *P < 0.05; **P < 0.01.

Results

Expression of miR-130a in lung cancer tissues

We first determined the expression of miR-130a in lung cancer tissues by realtime PCR. Expression of miR-130a was significantly deregulated in lung cancer tissues (0.24±0.10) compared with normal ones (**Figure 1A**). Among different histological types, miR-130a expression was unchanged between tissues from small cell lung cancer and NSCLC (P > 0.05, data not shown), and miR-130a was significantly downregulated in patients with non-small cell lung squamous carcinoma (0.68±0.13) compared with that with lung adenocarcinoma (**Figure 1B**). In addition, we found that miR-130a was significantly decreased in patients
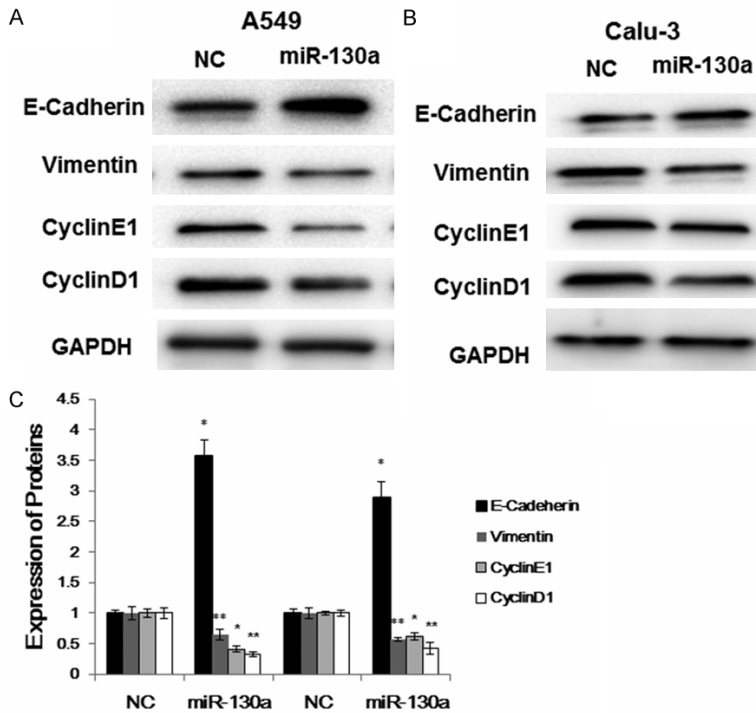
Figure 5. Western blot analysis of cell cycle-related and EMT-related proteins. A549 (A) and Calu-3 cells (B) were transfected with miR-130a or scramble (NC). Cell lysates were prepared and analyzed by Western blot for CyclinE1, CyclinD1, Vimentin, E-Cadherin and GAPDH. Representative Western blot results were shown. (C) Relative expression of proteins were calculated. *P < 0.05; **P < 0.01.

Ectopic expression of miR-130a suppresses the invasion and metastasis of lung cancer cells

Transwell assays without Matrigel showed that ectopic expression of miR-130a in A549 and Calu-3 cells resulted in a significant reduction of cells passed through the chambers compared with scramble group (46.5±5.30 vs. 74.6±4.37 cells in A549 and 34.0±6.3 vs. 67.5±3.10 cells in Calu-3, respectively) (both P < 0.05) (**Figure 3A** and **3B**). The similar result was observed in Matrigel invasion assays. The number of A549 and Calu-3 cells transfected with miR-130a mimic passed through the chambers decreased significantly compared with cells transfected with scramble (15.8±2.1 vs. 33.5± 1.50 cells in A549 and 8.00± 0.62 vs. 22.8±2.47 cells in Calu-3, respectively) (both P < 0.05) (**Figure 3C** and **3D**). These results indicate that miR-130a could efficiently repress cell motility and invasiveness of lung cancer cells *in vitro*.

Effect of miR-130a on cell cycle distribution

The cell cycle distribution was then analyzed by flow cytometry. Ectopic expression of miR-130a induced an accumulation of lung cancer cells A549 and Calu-3 in the G1/S phase compared to cells transfected with scramble (**Figure 4**). This result suggests that the G1/S arrest is induced by elevated expression of miR-130a, which may inhibit proliferation of lung cancer cells.

Cell cycle-related and epithelial mesenchymal transition (EMT)-related proteins are involved in miR-130a mediated suppression of tumor metastasis

To determine the putative proteins involved in the tumor suppression functions of miR-130a, A549 and Calu-3 cells were transfected with miR-130a mimic or scramble. Western blot analysis demonstrated that miR-130a overex-

with lymph node metastasis (N1, 0.41±0.06, **Figure 1C**), and was progressively down regulated in patients with increased clinical stages (0.52±0.14 in stages III, *versus* 1 in stages I 1, P < 0.05, **Figure 1D**). These results suggest that deregulation of miR-130a may play a role in the development and invasion and metastasis of lung cancers.

Ectopic expression of miR-130a affects the proliferation of lung cancer cells

To investigate the effect of miR-130a on the development of lung cancer, we established miR-130a overexpression models in lung cancer cell lines, A549 and Calu-3, by transiently transfection with miR-130a mimics. The proliferation was detected by CCK-8 assay. Overexpression of miR-130a significantly suppressed the proliferation of lung cancer cells compared with cells transfected with scramble at 48 h and 72 h post transfection in both A549 (**Figure 2A**) and Calu-3 cells (**Figure 2B**). This data indicates that miR-130a may act as a tumor suppressor gene to suppresses the proliferation of lung cancer cells.

pression resulted in significant decreases of cell cycle protein CyclinE1 and CyclinD1 and epithelial EMT-related protein Vimentin (**Figure 5**). However, expression of E-Cadherin was elevated in miR-130a overexpressed lung cancer cells (**Figure 5**). These results indicate that ectopic expression of miR-130a suppressesEMT, which further inhibits the invasion and metastasis lung cancer cells.

Discussion

Lung cancer is one of the malignant tumors that with worst prognosis [14], and lung cancer is the leading cause of cancer death [15]. The incidence and mortality of lung cancer in China is increasing in recent years, which threatens people's health greatly [16]. Lung tumors are often discovered as locally advanced or metastatic disease in 80% of patients at initial diagnosis, which results in the loss of optimal operation opportunity [17]. Therefore, prognosis will be improved if lung cancer could be detected at early stage and treated in time. To date, no available early diagnosis indicators for lung cancer are applied clinically. Currently, pattern recognition for exfoliative cells and bronchofibroscope are two methods for early screening of lung cancer, however, these methods are hardly applied clinically in large-scale due to their inherent drawbacks [18]. Recently, much attention has been paid to the screen of lung cancer biomarkers since molecular biomarkers are increasingly applied in the treatment of tumors [19]. Researches on the molecular mechanisms of the development of lung cancer and screenings for key biological molecules are of great value to both the early diagnosis and clinical treatment of lung cancer [20]. Many miRNAs are dysregulated in tumors and multiple miRNAs play important roles in the development of tumors. For example, miR-25 functions as an oncogene, and its ectopic expression could accelerate the metastasis of many tumors, such as gastric cancer, prostate cancer and non-small cell lung cancer [21-23]. MiR-17-92 family could induce the development of tumors, help cancer cells to resist the effect of chemotherapeutic drugs, and play an important role in the proliferation of tumor stem cells [24, 25]. In addition, the relative stability of miRNAs in peripheral blood makes them great biomarkers for tumors. Therefore, it is significant to find out important miRNAs in the development of lung cancer.

In this study, we investigated the expression of miR-130a and its functional role in lung cancer. Expression of miR-130a was significantly deregulated in lung cancer tissues compared with normal ones. Among different histological types, miR-130a expression was unchanged between tissues from small cell lung cancer and NSCLC but miR-130a was significantly downregulated in non-small cell lung squamous carcinoma compared with lung adenocarcinoma. Comparison of patients with different clinical stages demonstrated that the expression of miR-130a was down regulated in patients with stages III ncompared with that with stages Ist. Overall, these results indicate a correlation of miR-130a deregulation and the development of lung cancer.

We further studied the biological function of miR-130a in lung squamous carcinoma cell A549 and lung adenocarcinoma cell Calu-3. Ectopic expression of miR-130a resulted in the significant decrease of proliferation of A549 and Calu-3 cells. Analysis on cell cycle distribution showed that ectopic expression of miR-130a led to G1/S arrest. In addition, transwell assays demonstrated that overexpressed miR-130a suppresses the invasion and metastasis of lung cancer cells. The observations above intrigued us to investigate the changes of related protein levels downstream of miR-130a. Among cell cycle-related proteins, expression of CyclinE1 and CyclinD1 were downregulated upon miR-130a overexpression, and among EMT-related proteins, ectopic expression of miR-130a led to increased expression of E-Cadherin and deregulated expression of Vimentin. Overall, our data indicated that epithelial mesenchymal transition of lung cancer, which is the key step to the invasion and metastasis of tumors, is suppressed due to ectopic miR-130a expression. Changes in the expression of these proteins can also partially explain the results of cell cycle analysis and Transwell assay.

The role of miR-130a in tumorigenesis is still not fully understood. Although many studies have demonstrated its role as a tumor suppressor [11-13], it can also function as an oncogene to promote the proliferation, invasion and metastasis of gastric cancer by targeting Runx3 [26]. In addition, miR-130a promotes ovarian cancer cells resistant to chemotherapy, result-

ing in the survival of ovarian cancer cells [27]. As far as our study is concerned, miR-130a functions as a tumor suppressor in lung cancer, which may contradict to some previous studies.

In conclusion, our results provide evidence that miR-130a plays a vital role in the development and invasion and metastasis of lung cancers. Its marked depression in lung cancers promotes tumor metastasis. Therefore, research on miR-130a has great clinical value as a potential biomarker for lung cancer.

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Disclosure of conflict of interest

None.

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