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Original Article

MicroRNA-146 inhibits epithelial-mesenchymal transition by regulating FOXM1 in lung cancer cells

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Abstract: Aims: Aberrant expression of microRNA-146 (miR-146) has been reported to be involved in the progression of non-small cell lung cancer (NSCLC). However, its role in epithelial-mesenchymal transition (EMT) has not yet to be elucidated. The present study was aimed to clarify the role of miR-146 in EMT phenotype of NSCLC cells. Methods: Four human NSCLC cell lines A549, Calu3, Calu1 and H1299 were used in the study. MiR-146 mimic, inhibitor and miR-control were transfected into H1299 cells, respectively. Further, the effects of overexpressing Forkhead box M1 (FOXM1) in H1299-miR-146 mimic cells and silencing FOXM1 in H1299-anti-miR-146 cells were measured. Functionally, the transwell assay was used to assess NSCLC cell migration and invasion. The mRNA expressions of miR-146, E-cadherin and vimentin were evaluated by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The protein expressions of E-cadherin, vimentin and FOXM1 were measured by western blot. Results: MiR-146 negatively correlated with invasion and EMT phenotype of A549, Calu3, Calu1 and H1299 cells. Overexpressing miR-146 suppressed cell migration, invasion, EMT phenotype and expressions of FOXM1 in H1299 cells, while silencing miR-146 exerted opposite effects ($P < 0.05$). In addition, upregulating FOXM1 could restore the miR-146 inhibitory effect on EMT phenotype, cell migration and invasion; and FOXM1 knockout possessed opposite effects in H1299 cells ($P < 0.05$). Conclusion: MiR-146 inhibits EMT in NSCLC cells via regulating FOXM1. We newly provide a potential miR-146-FOXM1 mechanism for regulating EMT in lung cancer, which sheds further light on clinical therapy of NSCLC.

Keywords: MicroRNA-146, epithelial-mesenchymal transition, FOXM1, non-small cell lung cancer

Introduction

Lung cancer is one of the most widespread cancers worldwide, accounting for the highest mortality of nearly 30% [1]. The non-small cell lung cancer (NSCLC) is the main lung cancer subtype (approximately 80%), which has been widely prevalent in the past two decades [2, 3]. NSCLC is a debilitating disease that brings about a heavy load of symptoms and poor living quality. It was reported that the 5-year survival of NSCLC patients following the curative resection was only 30-60% [4, 5]. Although clinical therapies have been improved, to our current knowledge of the pathophysiology, there remains no established strategy of NSCLC for clinical use. Currently a promising therapeutic option for cancers is to explore specific mediators in genes that encode key signaling pathways related to cellular survival and tumor metastasis [1]. Thus, elucidating the potential mechanism which regulates the progression of NSCLC is urgently needed and of great interest.

MicroRNAs (miRNAs) are a highly conserved class of small non-coding RNAs that are 20-25 nucleotides in length [6]. There is overwhelming evidence corroborating that miRNAs are involved in a host of biological processes, especially human cancers, by regulating the expression of target genes at the post-transcriptional level [7-9]. A growing body of reports serve as the basis for the regulatory effect of miRNAs on growth, invasion and diagnosis of NSCLC, such as miR-222, miR-181a, miR-21, miR-205, miR-449a, miR-143, miR-221 and miR-10b [10-15].

The miR-146 family, including miR-146a/b in human, plays a significant role in tumorigenesis

of breast cancer [16-18], pancreatic cancer [19] and prostate cancer [20, 21]. MiR-146a was considered to suppress cell growth, migration and promote apoptosis as a lung tumor suppressor [22], which was consistent with a previous study [23]. Recently, studies of cancer progression have highlighted the importance of epithelial-mesenchymal transition (EMT), which potently drives tumor metastasis as well as cell invasion [24, 25]. One literature offered cursory examination of miR-146-5p regulating papillary thyroid carcinoma (PTC) proliferation and invasion during EMT [26]. Hitherto, the effects of miR-146 on EMT phenotype in NSCLC cells, which contributes to lung cancer progression, are not addressed.

The present study was aimed to clarify the role of miR-146 in EMT phenotype of NSCLC cells. We detected whether aberrant miR-146 played a role in cell migration, invasion and EMT phenotype of NSCLC cell line, as well as its underlying mechanism. This study was expected to offer a novel miR-146-based therapeutic target of NSCLC.

Materials and methods

Cell culture

Four NSCLC cell lines A549, Calu3, Calu1 and H1299 (American Type Culture Collection, Manassas, USA) were grown in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 1 × antibiotic-antimycotic mixture and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). The four lung cancer cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All cells were cultured to 80% confluency as judged under a phase contrast microscopy (Olympus Optical Co., Tokyo, Japan).

Plasmids and transfection

H1299 cells were incubated at 1×10^5 cells/well of six-well plates overnight. The miR-146 mimic, inhibitor and miR-control (GenePharma Co, Shanghai, China) were transfected into H1299 cells on the next day, respectively. Cell transfections were carried out using Lipofectamine 3000 reagent (Invitrogen, CA, USA) based on manufacturer's introductions. Thereafter, we fused the FLAG gene 3' to the Forkhead box M1 (FOXM1) gene to generate FLAG-tagged FOXM1, which was transfected into H1299-miR-146 mimic cells. H1299-anti-miR-146 cells were transfected with lentiviruses encoding FOXM1 small hairpin RNA (shRNA).

Migration and invasion assays

A Transwell system containing a polycarbonate filter membrane with a 8 μm-size pore (Costar, Cambridge, USA) was employed to evaluate lung cancer cell migration and invasion. First, the original invasive capacities of four NSCLC cell lines A549, Calu3, Calu1 and H1299 were measured; second, the cell migration and invasion in H1299 cells with miR-146 mimic, anti-miR-146 and miR-control were assessed; last, the cell migration and invasion in H1299-miR-146 mimic cells with FLAG-FOXM1 and H1299-anti-miR-146 cells with FOXM1 shRNA were assessed. For cell invasion assays, briefly, after cells were trypsinized (0.25% trypsin; Sigma, USA) and suspended, they were seeded on the upper chamber at a density of 3.0×10^5 cells/well. The 0.8 mL of Dulbecco's modified Eagle's medium (DMEM, Lonza, Walkersville, USA) containing 10% FBS was applied to the bottom chamber as chemoattractant. Following incubation in 5% CO_2 at 37°C for 1 d, adherent cells on the bottom surface of the filter were fixed with 4% methanol (Sigma, USA), stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China), and counted under a light microscope (Pharmacia Biotech, USA). Cell migration assay was performed based on the above procedure except that the transwell was without matrix gel pre-coated.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA of lung cancer A549, Calu3, Calu1 and H1299 cells were isolated respectively using Trizol reagent (Invitrogen, CA, USA) and treated with DNaseI (Promega Biotec., Madison, USA). A total of 2 μg RNA was used to synthesize poly-oligo (dT) primed complementary DNA (cDNA) with the RevertAid H Minus First strand Cdna Synthesis Kit (Fermentas, Hanover, Germany). The relative expression of miR-146 was normalized to the internal control (U6) via the equation $2^{-\Delta\Delta Ct}$. Primers for miR-146 and U6 were produced by the miScript Primer Assay kit (Qiagen, Dusseldorf, Germany). QRT-PCR reactions for E-cadherin and Vimentin were performed using RiboMAX Large Scale RNA Pro-

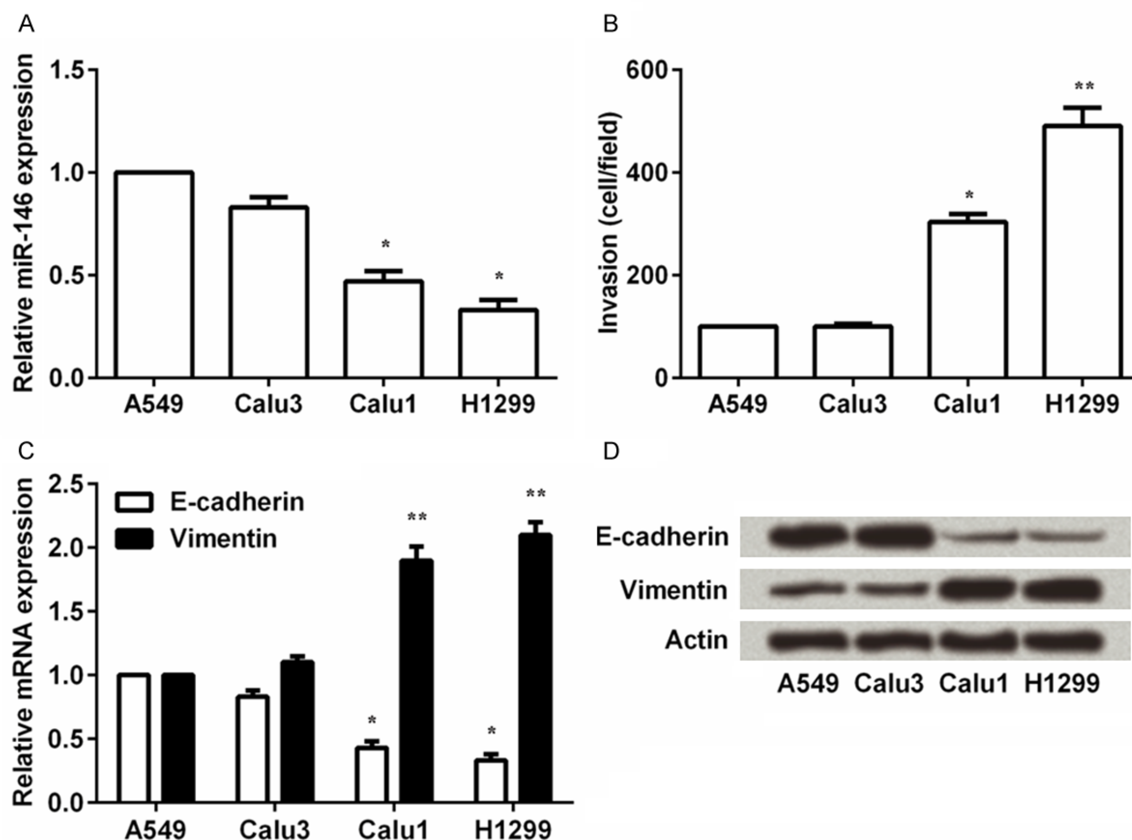

Figure 1. MiR-146 negatively correlated with NSCLC cell invasion and EMT phenotype. A. Relative miR-146 expressions in A549, Calu3, Calu1 and H1299 cell lines; B. Cell invasion in A549, Calu3, Calu1 and H1299 cell lines; C. The mRNA expressions of E-cadherin and vimentin in A549, Calu3, Calu1 and H1299 cell lines; D. The protein expressions of E-cadherin and vimentin in A549, Calu3, Calu1 and H1299 cell lines. MiR, microRNA; EMT, epithelial-mesenchymal transition, NSCLC, non-small cell lung cancer. *, $P < 0.05$; **, $P < 0.01$.

duction System T7 (Promega, Karlsruhe, Germany). The primer sequences were: E-cadherin, forward 5'GAGCCTGAGTCCTGCAGTCC'3, reverse 5'TGTATTGCTGCTTGGCCTCA'3 [27]; vimentin, forward 5'AAAGGATCCATGTCTACCAGGTCTGTGTC'3, reverse 5'ACTTCTCAGCATCACGATGACTCTAGATTT'3 [28]; Actin, forward 5'ATCTGGCACCACACCTTCTAC'3, reverse 5'CAGCCAGGTCCAGACGCAGG'3.

Western blot

The protein used for western blot assay was extracted from A549, Calu3, Calu1 and H1299 cells respectively with RIPA lysis buffer containing 1 mg protease inhibitors (Applygen Technologies Inc., Beijing, China). Proteins concentration was quantified by Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Bonn, Germany). We constructed the Bio-Rad Bis-Tris Gel western blotting system, in which primary antibodies E-cadherin (ab1416), vimentin (ab8978), FOXM1 (ab175798) and the internal control Actin were from Abcam (Cambridge, United Kingdom). Each membrane was incubated with the indicated primary antibodies at 4°C overnight and then secondary antibodies were marked by horseradish peroxidase for 2 h at 37°C. Moreover, protein samples were boiled in the polyvinylidenefluoride (PVDF) membrane and antibodies was transferred. The bands were visualized with the WEST-ZOL plus system (Intron Biotechnology, Seongnam, Korea).

Statistical analysis

Each test group was assayed in triplicate and the results were presented as mean ± standard deviation (SD). All data were performed by one-way analysis of variance (ANOVA) with Statistical Package for the Social Sciences (SPSS) version

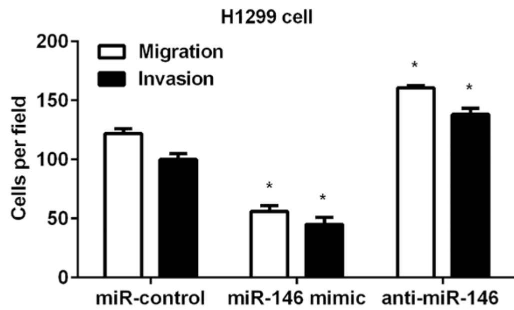

Figure 2. MiR-146 suppressed lung cancer H1299 cell migration and invasion. MiR, microRNA. *, $P < 0.05$.

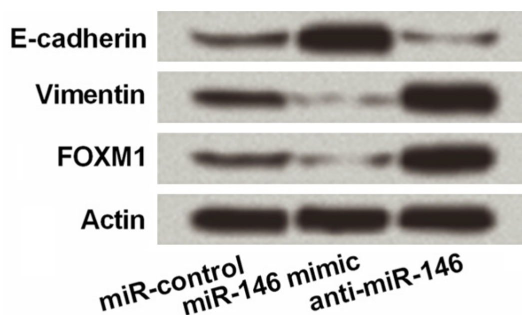

Figure 3. MiR-146 inhibited EMT phenotype in H1299 cells. MiR, microRNA; FOXM1, Forkhead box M1; EMT, epithelial-mesenchymal transition.

19.0 (IBM Corporation, New York, USA). A statistical significance was defined when $P < 0.05$.

Results

The miR-146 expression profile and EMT phenotype in invasive NSCLC cells

To investigate the role of miR-146 in the invasive capability and EMT phenotype of four NSCLC cell lines A549, Calu3, Calu1 and H1299, firstly the base line expression of miR-146, cell invasion and EMT molecular markers were assessed in all cell lines. We found a negative correlation between the level of miR-146 and invasion in four NSCLC cells (**Figure 1A** and **1B**). Consistent with the invasive potential, higher levels of E-cadherin were expressed in low invasive cells (A549 and Calu3), while highly invasive cell lines (Calu1 and H1299) expressed higher levels of vimentin (**Figure 1C** and **1D**). Thus, miR-146 negatively correlated with invasion and EMT phenotype of A549, Calu3, Calu1 and H1299 cells, suggesting that downregulating miR-146 might lead to EMT derived invasive phenotype.

MiR-146 suppressed lung cancer H1299 cell migration and invasion

The highest invasive capacity and lowest level of EMT phenotype of H1299 cells accounted for the optimum option in further study. After miR-146 mimic, inhibitor and miR-control were transferred into H1299 cells, we observed the effects of aberrant miR-146 on cell migration and invasion. Using transwell assay, H1299 cell migration and invasion were moderately enhanced by the miR-146 inhibitor with striking difference compared to mock controls ($P < 0.05$, **Figure 2**). The H1299 cell line stably transfected with miR-146 mimic displayed decreased migratory and invasive capacity when compared with the miR-control ($P < 0.05$). Taken together, miR-146 suppressed cell migration and invasion in lung cancer H1299 cell line.

MiR-146 inhibited EMT phenotype in H1299 cells

The process of EMT is commonly characterized by down-regulation of E-cadherin and up-regulation of vimentin [29-31]. To verify whether miR-146 is able to affect EMT phenotype in NSCLC cells, we next detected the effects of ectopic transfection of miR-146 on expressions of two important EMT molecular markers E-cadherin and vimentin in H1299 cell line. The miR-146 mimic in H1299 cells raised epithelial marker E-cadherin expression and decreased mesenchymal marker vimentin expression; while miR-146-silencing cells showed a decrease in E-cadherin expression and an increase in vimentin expression (**Figure 3**). Besides, the presupposed targeted gene FOXM1 was downregulated by overexpressing miR-146 and upregulated by miR-146 inhibitor. Collectively, miR-146 inhibited EMT phenotype in lung cancer H1299 cells, which might be associated with the expressions of FOXM1.

Ectopic expressions of FOXM1 regulated EMT phenotype in H1299 cells

To further investigate the inhibitory mechanism of miR-146 on EMT process, we evaluated ectopic expressions of FOXM1 on EMT phenotype in H1299-miR-146 mimic cells and H1299-anti-

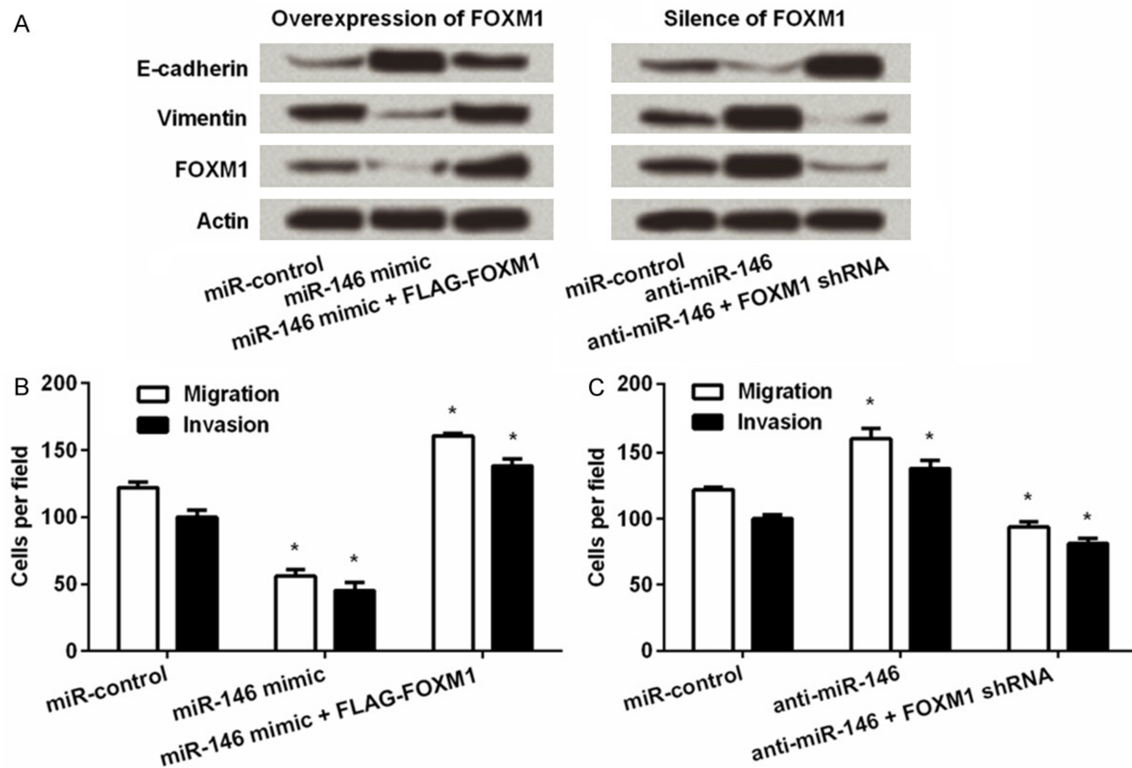

Figure 4. Ectopic expressions of FOXM1 regulated EMT phenotype in H1299 cells. A. The protein expressions of E-cadherin, vimentin and FOXM1 in H1299 cells; B. The effects of FOXM1 overexpression on cell migration and invasion in miR-146-overexpressing H1299 cells; C. The effects of FOXM1 knockout on cell migration and invasion in miR-146-silencing H1299 cells. MiR, microRNA; shRNA, small hairpin RNA; FOXM1, Forkhead box M1; EMT, epithelial-mesenchymal transition. *, $P < 0.05$.

miR-146 cells, respectively. **Figure 4A** and **4B** showed that FLAG FOXM1 remarkably promoted EMT phenotype, cell migration and invasion as compared to miR-146-overexpressing cells (both $P < 0.05$). Further, FOXM1 shRNA observably suppressed EMT phenotype, cell migration and invasion compared with miR-146-silencing cells (both $P < 0.05$; **Figure 4A** and **4C**). These data claimed that upregulated FOXM1 could restore the miR-146 inhibitory effect on EMT phenotype in H1299 cells, which indicated that miR-146 inhibited EMT phenotype by regulating FOXM1.

Discussion

In the current work we have explored the role of miR-146 in EMT phenotype of human NSCLC cells. Multiple evidences have hinted miR-146 as a tumor inhibitor. However, its molecular mechanism on lung tumor EMT has not yet to be elucidated. First we identified the relative expressions of miR-146 inversely correlated with invasive capability and EMT phenotype of A549, Calu3, Calu1 and H1299 cell lines. Then transwell assays reflected that miR-146 strongly attenuated cell migration and invasion in H1299 cells. We observed that miR-146 upregulated the expression of E-cadherin but suppressed the expression of vimentin. Finally, the FLAG-mediated overexpression of FOXM1 in miR-146 mimic H1299 cells upregualted the inhibited EMT phenotype; while the shRNA-mediated knockout of FOXM1 in anti-miR-146 H1299 cells neutralized the promoted EMT phenotype. Collectively, our study demonstrated that miR-146 exerted inhibitor effects on EMT by regulating FOXM1 in NSCLC cells.

In the four NSCLC cell lines, H1299 cells possessed highest invasive capacity and lowest EMT phenotype, which agreed with a preceding report [32]. Resulting from the downregulation of miR-146 contributing to EMT derived invasive phenotype, we seleceted H1299 cell line to verify the role of miR-146 in NSCLC cells.

Functionally, overexpressing miR-146 in H1299 cells showed significantly impaired invasion and migration relative to miR-control cells, which was in line with pancreatic cancer [19] and breast cancer [33]. This finding supported the propose that miR-146 had therapeutic potential to inhibit lung cancer migration and invasion.

Next, the mechanism underlying miR-146 regulating cell migrationa and invasion was highly anticipated. As previously mentioned, diminished adhesive and migratory capacity of ovarian cancer cells was attributed to the inhibition of EMT [34], which prompted the current investigation to confirm the effects of aberrant miR-146 on NSCLC EMT phenotype. The EMT process in which cancer cells acquire a mesenchymal phenotype has been considered to be a critical mechanism for the development of cancers [26, 35]. The absence of epithelial E-cadherin expression and gain of mesenchymal vimentin expression are known as the dominating markers of EMT [32]. Our data uncovered that miR-146 resisted EMT by upregulating E-cadhrin and downregulating vimentin expressions in H1299 cells. Likewise, both miR-149 and miR-134 were found to inhibit NSCLS cells EMT [32, 36]. Thereby, the results suggested that miR-146 acted as a novel EMT suppressor in NSCLC cells. The results implied that miR-146 suppressed lung cancer cell invasion and EMT, contributing to the protection against lung tumor metastasis.

Studies have identified many miR-146 targets are involved in cell proliferation, differentiation and migration of cancers, including epidermal growth factor receptor (EGFR) [19, 20], chemokine receptor-4 (CXCR4) [18], NOTCH1 [37], ROCK1 [21] and PRKCE [38]. FOXM1 is a member of Fox transcriptional factors, which is commonly expressed in numerous tumor cells. Emerging research manifested that overexpressing FOXM1 resided in a variety of aggressive tumors including brain, liver, breast, colon and lung, acting as a proto-oncogene underlying human carcinogenesis [39, 40]. Additionally, overexpressing FOXM1 coincided with oncocytes metastasis [32, 36]. In the current study, western blot assay revealed that expressions of FOXM1 protein were dysregulated by ectopic miR-146 in H1299 cells, indicating that FOXM1 was a direct regulating gene of miR-146 in NSCLC. Then we observed that silencing FOXM1 by shRNA suppressed EMT in H1299 cells through neutralizing the effects of anti-miR-146. It was tempting to speculate that miR-146-mediated FOXM1 silence might contribute to suppressing cell metastasis, and overexpressing miR-146 and silencing FOXM1 were promising lung cancer suppressors.

In summary, these results demonstrate that miR-146 inhibits EMT in NSCLC cells via regulating FOXM1. We newly provide a potential miR-146-FOXM1 mechanism for regulating EMT in lung cancer, which sheds further light on clinical therapy of NSCLC.

Disclosure of conflict of interest

None.

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