

Original Paper

MicroRNA-124 Functions as a Tumor Suppressor by Regulating CDH2 and Epithelial-Mesenchymal Transition in Non-Small Cell Lung Cancer

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Key Words

MicroRNA-124 • CDH2 • Epithelial-mesenchymal transition • Non-small-cell lung cancer

Abstract

Background/Aims: Abnormal expression of microRNA-124 (miR-124) was found in non-small cell lung cancer (NSCLC). However, the association between miR-124 and CDH2 has not been reported yet. This study aims to reveal the inhibiting effects of miR-124 on the expression of CDH2 in NSCLC. **Methods:** Quantitative real-time polymerase chain reaction was used to evaluate the expression of miR-124 and CDH2 in NSCLC tissues. Cell viability, apoptosis and invasion assays were carried out in NSCLC cell lines after transfection. The regulation mechanism was confirmed by luciferase report assay and western blot (WB). **Results:** Significantly decreased expression of miR-124 was found in NSCLC specimens and cell lines. Overexpression of miR-124 apparently suppressed the proliferation and invasion of NSCLC cell lines *in vitro*. Luciferase report assay and WB revealed that CDH2 was a target gene of miR-124. Furthermore, results of WB showed that epithelial-mesenchymal transition (EMT) could be inhibited by up-regulation of miR-124. **Conclusions:** Taken together, our findings present the first evidence that miR-124 could suppress the expression of CDH2 and regulate EMT, which might lead to a potential therapeutic strategy focusing on miR-124 and CDH2 for human lung cancer.

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Introduction

Lung cancer is one of the most common causes of cancer-related mortality worldwide, among which non-small cell lung cancer (NSCLC) accounts for over 70% [1]. The population of NSCLC has grown fast over the past decades in China [2]. Although there have been numerous related studies and outcomes, most patients with NSCLC still have poor prognosis. The most common feature of malignancy is metastasis, which is most responsible for the low 5-year survival rate [3]. Therefore, looking for potential mechanisms of metastasis will help a lot in dealing with NSCLC.

MicroRNAs (miRNAs) are a class of small non-coding RNAs including 19 to 25 nucleotides. Results of previous studies showed that mature miRNAs could suppress the translation of target mRNA by binding to the 3'-untranslated region (3'-UTR) [4]. MiRNAs play pivotal roles in a wide range of cellular processes including proliferation, differentiation, apoptosis and metastasis [5-9]. Recent studies have already found aberrant alteration of miRNAs expressions in many diseases, especially in cancer [10-13].

Cadherin-2 (CDH2), encoding a protein called N-cadherin, is a member of cadherin family that regulates lots of biological processes. CDH2 is commonly up-regulated in various cancers, including colorectal cancer [14], prostate cancer [15], gastric cancer [16], bladder cancer [17] and lung cancer [18]. In addition, it is reported that CDH2 plays a significant part in epithelial-mesenchymal transition (EMT) [19].

EMT is a process by which epithelial cells switch from the epithelial phenotype to the mesenchymal phenotype, accompanied with the ectopic expression of EMT related genes [20]. EMT is recognized as a pivotal event for cancer cells to acquire metastasis ability [21].

Material and Methods

Clinical samples

A total of 77 patients involved in this study had received lung tumor resection at The First Affiliated Hospital of Nanjing Medical University in 2014. NSCLC samples and the adjacent tissues taken from the 77 patients were immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction. The tumors were classified according to World Health Organization classification. Every patient had written informed consent, and this study was approved by the Ethical Committee of The First Affiliated Hospital of Nanjing Medical University.

Cell culture

A549, H1299, SPCA1, H358, H1650 and BEAS-2B cell lines were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in an incubator containing 5% CO_2 .

Isolation of total RNA and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from fresh tissues or cells using TRIzol (Invitrogen). MiR-124 and CDH2 expression was evaluated by performing qRT-PCR, using ABI 7900 fast Real-time PCR system (ABI, CA, USA). The specific primers are as follows: for hsa-miR-124, forward, 5'-GGA CTT TCT TCA TTC ACA CCG- 3' and reverse, 5'-GAC CAC TGA GGT TAG AGC CA- 3'; for U6, forward, 5'-CTC GCT TCG GCA GCA CAT ATA CT- 3' and reverse, 5'-ACG CTT CAC GAA TTT GCG TGTC- 3'; for CDH2, forward, 5'-GTC AGC AGA AGT TGA AGA AAT AGTG-3' and reverse, 5'-GCA AGT TGA TTG GAG GGA TG-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward, 5'-TGG GTG TGA ACC ATG AGA AGT-3' and reverse, 5'-TGA GTC CTT CCA CGA TAC CAA-3'.

Transient transfection

Small interfering RNAs (siRNA) specific for CDH2 were purchased from GenPharma (Shanghai, China). The sequence used for si-CDH2-1 was 5'-CUA ACA GGG AGU CAU AUG GUG GAG C-TdT-3'; sequence for si-CDH2-2 was 5'-UGC AUA AUG CGA UUU CAC CAG-TdT-3'. To minimize nonspecific effects of interfering RNAs, non-targeting control siRNA was used as negative control. The siRNA transfection reagent (Invitrogen) was

used according to the manufacturer's instructions. Oligonucleotides negative control (NC), miR-124 mimics (mimics), inhibitor negative control (inhibitor NC) and miR-124 inhibitor (inhibitor) were purchased from GenePharma (Shanghai, China). Transfection of cells with oligonucleotides was performed using Lipofectamine 2000 Reagent (Invitrogen) at a final concentration of 100 nM. Transfection efficiency was monitored by qRT-PCR. The experiments were repeated at least three times, independently.

Cell-proliferation assay (cell-counting kit-8)

Cells were seeded into 96-well plates (6.0×10^3 cells per well). Cell viability was assessed by cell-counting kit-8 (CCK-8) assay (Beyotime, Shanghai, China). The absorbance of each well was read on a spectrophotometer (Thermo Scientific, Rockford, IL, USA) at 450 nm (A450). Three independent experiments were performed.

Apoptosis Assay

After cells were transfected for 48 hours, apoptosis of the cells was evaluated with annexin V labeling. An annexin V-FITC labeled Apoptosis Detection Kit (Abcam Cambridge, MA, USA) was used according to the manufacturer's protocol.

Transwell invasion assay

Transwell invasion assays were measured using 8 mm membrane pores transwell chambers (Corning, New York, USA). 1×10^5 cells in serum-free medium were seeded into the upper chamber which was pre-coated with Matrigel (BD, Bedford, MA, USA). After incubation for 24 hours, cells that migrated onto the lower surface of the membrane were fixed with 100% methanol and stained with 0.1% crystal violet. Then the non-invading cells on the upper membrane surface were removed with cotton swabs. Cells on the lower surface were counted and photographed, respectively.

Bioinformatics analysis

Bioinformatics method was used to predict the potential targeting genes of miR-124 in this study. The results of TargetScan (release 5.1, <http://www.targetscan.org/>), miRWalk, PICTAR 5 and miRanda indicated that 3'-UTR of CDH2 binds to miR-124 with the high score.

Western blot

Total protein was extracted from cells, measured using a protein assay (bicinchoninic acid method; Beyotime). Proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) transferred to polyvinylidene fluoride (PVDF) membrane, blocked in 5% dry milk at room temperature for 1 hour and immunostained overnight at 4°C using rabbit anti-N-cadherin (1:1000, Cell Signaling Technology, CST, USA), rabbit anti-E-cadherin (1:1000, CST), rabbit anti-β-catenin (1:1000, CST) and rabbit anti-Vimentin (1:1000, CST). Rabbit anti-GAPDH (1:5000, CST) was used as a loading control. The membrane was washed with tris-buffered saline-0.1% Tween 20 and incubated with a goat anti-rabbit secondary antibody (CST) for 1 hour at room temperature. Relative protein levels were quantified by Image J software.

Plasmid construction and cell transduction

The 3'-UTR sequence of CDH2 or a mutant sequence with the predicted target sites was inserted into the KpnI and SacI sites of pGL3 promoter vector (Genscript, Nanjing, China). They were named pGL3-CDH2 and pGL3-CDH2-mut. The cells were planted onto 6-well plates and were transfected with 100 ng of pGL3-CDH2 or pGL3-CDH2-mut, and miR-124 mimics (50nM) by using Lipofectamine 2000 (Invitrogen). The differences in transfection efficiency were normalized by co-transfecting a Renilla luciferase vector pRL-SV40 (5 ng).

Luciferase report assay

Luciferase report assay was measured in Victor 1420 Multilabel Counter (Wallac, Finland) using Luciferase Assay System (Promega, USA) according to the manufacturer's protocol, 48 hours after cell transfection.

Table 1. Expression levels of miR-124 and CDH2 in NSCLC tissues

Factors	miR-124 expression		p value	CDH2 expression		p value
	Low	High		Low	High	
Gender			0.56			0.21
Male	19	16		15	20	
Female	20	22		24	18	
Age (years)			0.74			0.12
<60	15	16		19	12	
≥60	24	22		20	26	
Smoker						0.92
Yes	21	22	0.72	22	21	
No	18	16		17	17	
Histology			0.19			0.57
Squamous carcinoma	18	12		14	16	
Adenocarcinoma	21	26		25	22	
Tumor size			0.003*			0.027*
T1/T2	18	30		29	19	
T3/T4	21	8		10	19	
Lymph node metastasis			0.030*			0.007*
No	13	22		24	11	
Yes	26	16		15	25	
Tumor stage			0.016*			0.002*
I - II	15	25		27	13	
III-IV	24	13		12	25	

*indicates P<0.05 (Chi-square test)

Statistical analysis

The chi-squared test was used to test the significance of differences in the data of Table 1. Pearson correlation was used for correlation analysis in Fig. 1C. Student's unpaired t-test was used to determine the significant differences of other results. P<0.05 was considered to be statistically significant.

Results

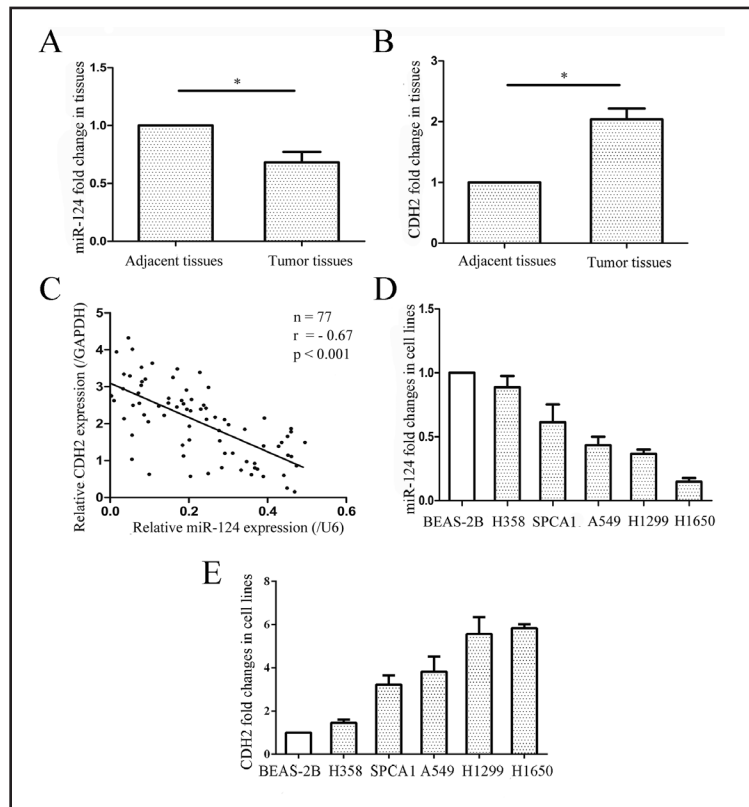
MiR-124 expression is down-regulated and has a negative correlation with CDH2 expression in human NSCLC tissues

Firstly, we analyzed the expression of miR-124 and CDH2 in human NSCLC samples and corresponding adjacent tissues using qRT-PCR. Significantly lower expression of miR-124 and higher expression of CDH2 were found in tumor specimens compared with corresponding adjacent areas (Fig. 1A, B). The inverse correlation between miR-124 and CDH2 was illustrated using Pearson correlation scatter plots (Fig. 1C). Then, we categorized all those patients into two groups by the expression levels of miR-124 or CDH2: one with more than median of miR-124 (or CDH2) expression level and another with less than median of miR-124 (or CDH2) expression level. After analyzing the clinical information of those patients, we found a correlation between miR-124 (or CDH2) expression levels and the pathological characteristics (Table 1). Gender, age, smoke and histology were not associated with the expression of miR-124 or CDH2. However, the tumor size, lymph node metastasis rate and tumor stage were certified to be significantly associated with miR-124 and CDH2 expression. These results were consistent with previous correlation analyses, suggesting that miR-124 and CDH2 might play important roles in the progression of NSCLC.

MiR-124 inhibits cell proliferation in vitro

To examine whether miR-124 could regulate proliferation and apoptosis of NSCLC *in vitro*, we carried out CCK-8 assay and apoptosis assay. In this study, the expression of miR-

Fig. 1. Expression of miR-124 and CDH2 in NSCLC clinical samples (N=77) and cell lines. A: The expression levels of miR-124 relative to U6 were determined in human NSCLC tissues and corresponding adjacent tissues by using qRT-PCR (n=77, $p < 0.05$). B: The mRNA expression level of CDH2 relative to GAPDH in human NSCLC tissues and corresponding adjacent tissues were detected by using qRT-PCR. C: A negative correlation was found between RNA expression of miR-124 and CDH2 in tumor samples. D and E: Expression levels of miR-124 and CDH2 in normal lung cell (BEAS-2B) and 5 lung cancer cell lines (H358, SPCA-1, A549, H1299, H1650). Data are represented as mean \pm S.E.M. *indicates $p < 0.05$.



miR-124 and CDH2 was examined in five lung cancer cell lines (A549, H1299, SPCA1, H358 and H1650) and a normal lung cell line (BEAS-2B) by using qRT-PCR. As shown in Fig. 1D and E, compared with BEAS-2B cell line, miR-124 expression was decreased and CDH2 expression was increased in all five cancer cell lines. What's more, the expression of miR-124 and CDH2 was moderate in A549 and H1299 cell lines. Hence, those two cell lines were chosen to further investigate the effects of miR-124 in NSCLC. Then, cells were transfected with miR-124 mimics, negative control (NC), miR-124 inhibitor and inhibitor negative control (inhibitor NC), respectively. The transfection efficiency was validated by using qRT-PCR (Fig. 2A). The CCK-8 assay showed distinct difference on proliferation after manipulation of miR-124 at 48-h and 72-h time points in both A549 and H1299 cells, while no significant difference was found at 24-h time point (Fig. 2B). Besides, we also performed apoptosis assays which showed no significant difference (Fig. 2C). These results suggested that miR-124 could inhibit proliferation of NSCLC cells *in vitro*.

MiR-124 suppresses the invasive ability of NSCLC cell lines *in vitro*

To explore the role of miR-124 in NSCLC invasion, we carried out transwell invasion assay. Our study showed that ectopic expression of miR-124 inhibited cell invasion, and vice versa (Fig. 3). It was obviously that miR-124 suppressed the invasive ability of NSCLC cell lines *in vitro*.

MiR-124 regulates CDH2 expression by directly binding to its 3'-UTR

Bioinformatics analyses (microRNA.org, miRDB and TargetScan database) led to a surprising prediction that 3'-UTR of CDH2 binds to miR-124 with a high score (mirSVR score: -1.1391). According to the results of prediction, the 3'-UTR fragment containing the predicted site was cloned into pGL3 luciferase reporter vector (pGL3-CDH2) to further determine the association between miR-124 and CDH2 expression. The 3'-UTR fragment with mutant sequence in the predicted target site was also cloned as a control group (pGL3-

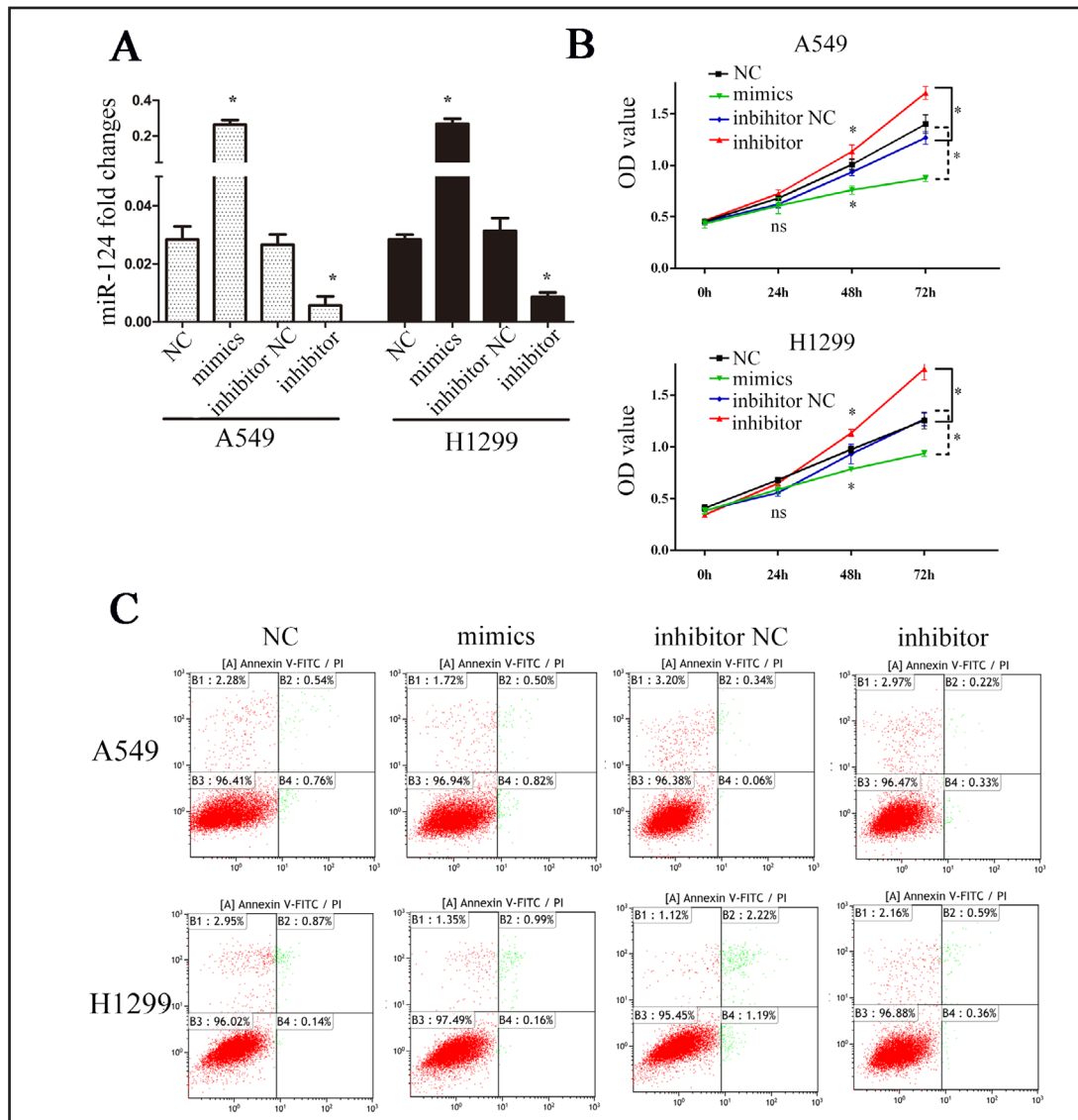


Fig. 2. miR-124 inhibited cell proliferation, but had no effects on NSCLC cell apoptosis *in vitro*. **A:** The results of miR-124 expression in cell lines transfected with miR-124 mimics, negative control (NC), inhibitor and negative control for inhibitor (inhibitor NC) were validated by using qRT-PCR. **B:** By cck-8 assay, we found distinct differences on proliferation after manipulation of miR-124 mimic and inhibitor in both A549 and H1299 cells at 48-h and 72-h time points. NC and inhibitor NC were used as controls. **C:** Apoptosis of cells were measured using flow cytometry assay with Annexin V and propidium iodide staining. *indicates $p < 0.05$, ns means not significant.

CDH2-MUT). The luciferase activity decreased in both A549 and H1299 cells co-transfected by mimics and pGL3-CDH2 vectors. However, miR-124 mimics did not have any effect on luciferase activity when target cells were transfected with pGL3-CDH2-MUT vector (Fig. 4A). In addition, qRT-PCR and western blot assay were performed to evaluate the expression levels of CDH2 in transfected A549 and H1299 cells. The results showed that up-regulated miR-124 suppressed the expression of CDH2 on both mRNA and protein levels, vice versa (Fig. 4B, C). These findings showed that miR-124 could suppress CDH2 expression, probably by binding to its 3'-UTR and conducting mRNA degradation.

Fig. 3. miR-124 regulated the invasive ability of NSCLC cell lines *in vitro*. The transwell assay was performed to assess the invasive capability of cells. A549 and H1299 cells were transfected with miR-124 mimics, negative control (NC), inhibitor and negative control for inhibitor (inhibitor NC), respectively. The invasive cells at the bottom of the membrane stained with crystal violet were visualized. The quantifications of cell invasion were presented as invasive cell numbers. All experiments were performed 3 times independently and shown as mean \pm S.E.M. *Indicates $p < 0.05$.

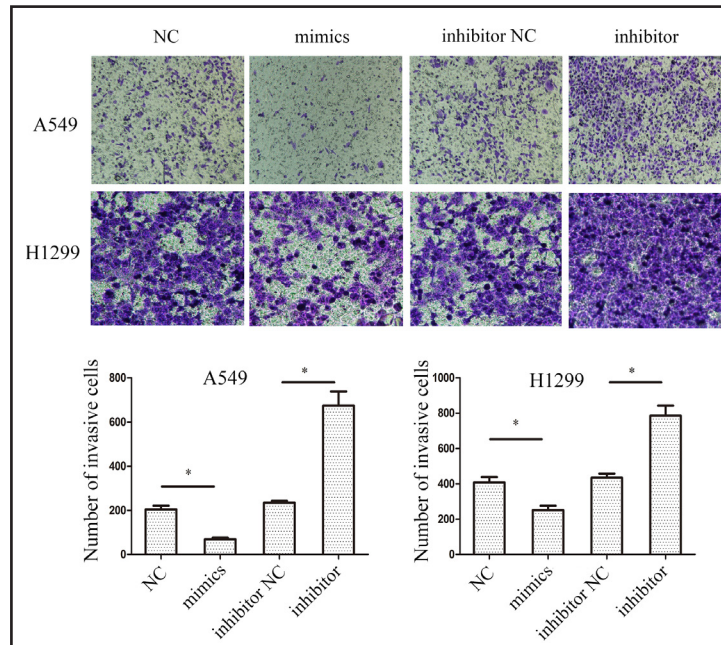
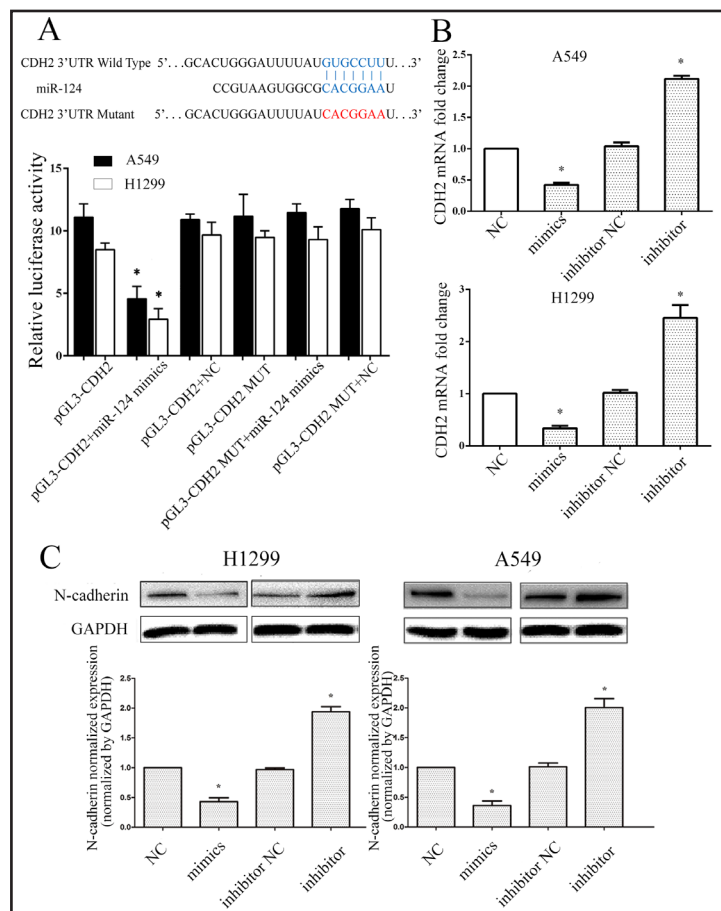


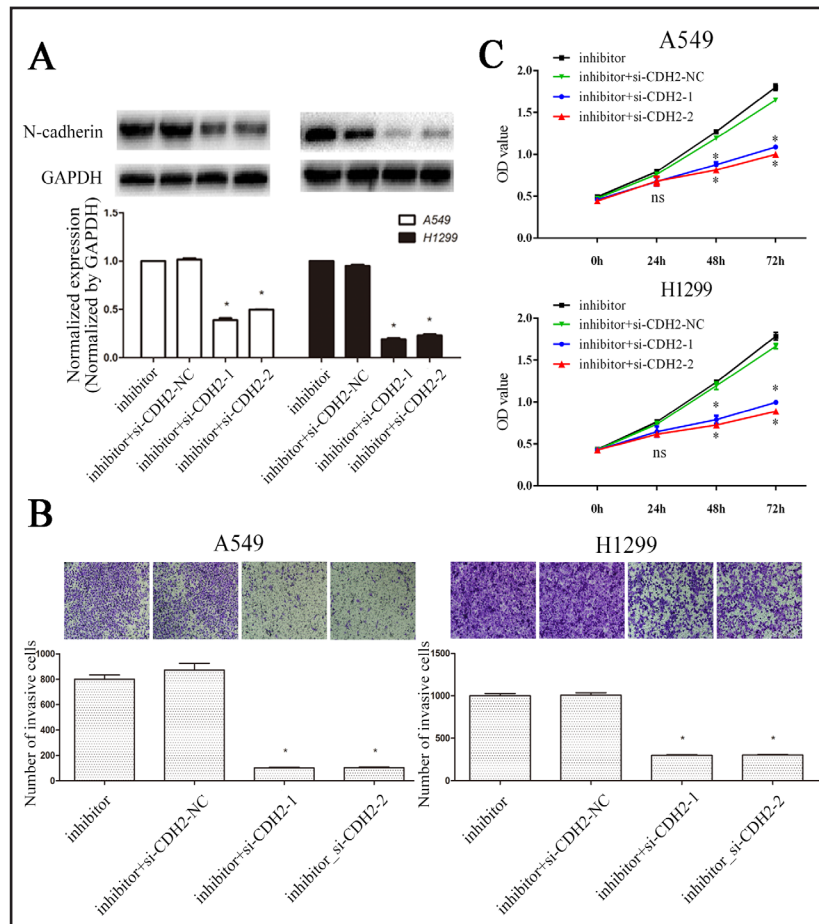
Fig. 4. miR-124 regulates CDH2 expression by directly binding its 3'-UTR. A: The potential miR-124 seed region at the 3'-UTR of CDH2 mRNA was computationally predicted by using TargetScan. A549 and H1299 cells were co-transfected with miR-124 mimics (or NC) with pGL3-CDH2 (or pGL3-CDH2-MUT) vector. Luciferase activity was normalized by the ratio of firefly and Renilla luciferase signals. B: CDH2 mRNA expression levels in transfected A549 and H1299 cells were analyzed by using qRT-PCR. GAPDH was used as a control. C: CDH2 protein expression levels in transfected A549 and H1299 cells were analyzed by western blot. GAPDH was used as a control. Data are represented as mean \pm S.E.M. *Indicates $P < 0.05$.



Silencing of CDH2 by small interfering RNA (siRNA) could abolish the effects of down-regulated miR-124

To investigate whether the functional effects of miR-124 on NSCLC cell lines relied on the expression level of CDH2, we carried out a rescue assay. In our study, A549 and H1299

Fig. 5. Silencing of CDH2 abolished the effects of down-regulated miR-124. A: Both A549 and H1299 cells were co-transfected with miR-124 inhibitor and siRNAs (si-CDH2-1 and si-CDH2-2). The transfection cells of miR-124 inhibitor and the co-transfection cells of miR-124 inhibitor and si-CDH2-NC were used as the control groups. The transfection efficiency was confirmed by using western blot assays. B: The representative images of transwell assay. C: The results of cck-8 assay. All experiments were performed in triplicate and the band intensity values were analyzed by using Image J. Data are represented as mean \pm S.E.M. *Indicates $P < 0.05$, ns means not significant.



cells were transfected with miR-124 inhibitor and siRNAs for CDH2 (si-CDH2-1 and si-CDH2-2). Meanwhile, cells transfected with miR-124 inhibitor and cells co-transfected with miR-124 inhibitor and si-CDH2-control were used as control groups. Compared with controls, cells transfected with miR-124 inhibitor and siRNAs showed an obviously lower expression level of N-cadherin (Fig. 5A). Then, transwell invasion assay was performed in miR-124 inhibitor-transfected A549 and H1299 cells. Compared with controls, the invasion ability of si-CDH2 groups was significantly suppressed (Fig. 5B). Similar results were also found in proliferation assay (Fig. 5C). Here we can draw a preliminary conclusion that the effects of miR-124 on NSCLC cells were at least partly dependent on CDH2.

MiR-124 regulates the expression of EMT hallmark genes

EMT has been proved a key process in the metastasis of various cancers. Tumor cells get EMT by transforming from adherent epithelial cells into motile mesenchymal cells. To gain further insight into the effects of miR-124 on EMT, western blot was performed to check expression levels of EMT regulatory proteins in cell lines. As shown in Fig. 6, transfection of miR-124 resulted in increased expression of E-cadherin, stable expression of total β -catenin and decreased expression of N-cadherin and Vimentin in A549 cells and H1299 cells. These results suggested that miR-124 was involved in the regulation of EMT in NSCLC *in vitro*.

Discussion

Lung cancer is the most common cause of cancer-related death in the world [1]. Metastasis is the major cause of death for patients with lung cancer [3]. Finding out potential

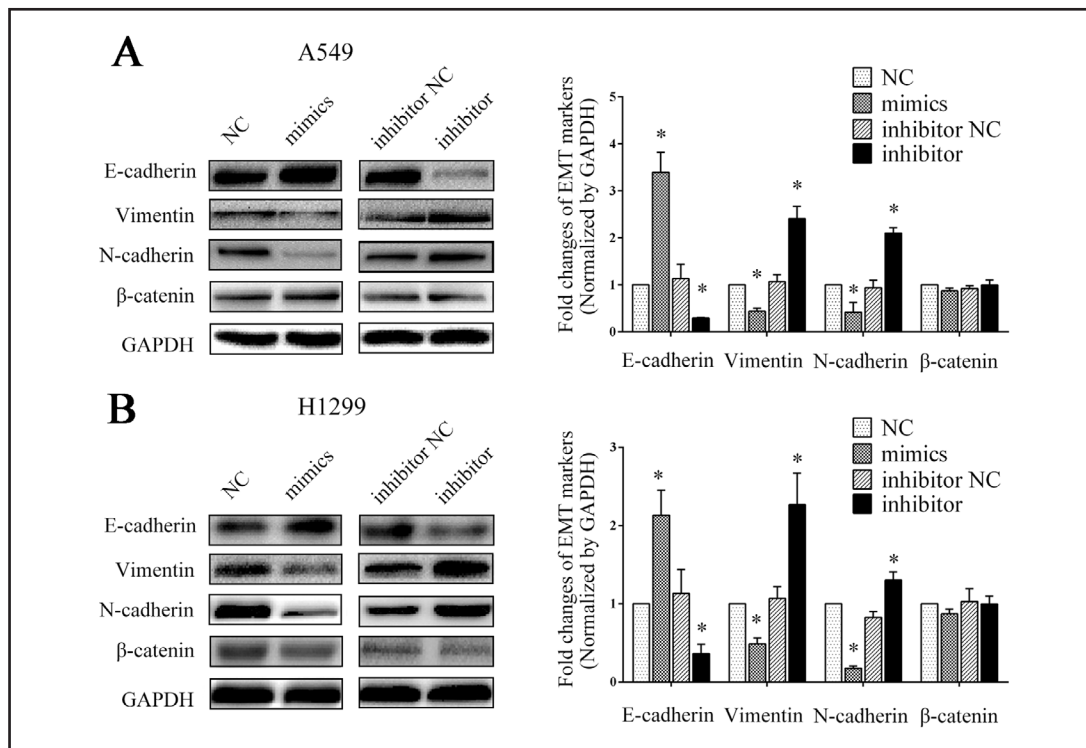


Fig. 6. Ectopic miR-124 expression contributed to EMT of NSCLC cells. A: E-cadherin, N-cadherin, Vimentin and β-catenin protein expression levels in A549 cells transfected with NC, miR-124 mimics, inhibitor NC, and miR-124 inhibitor. B: E-cadherin, N-cadherin, Vimentin and β-catenin protein expression levels in H1299 cells transfected with NC, miR-124 mimics, inhibitor NC, and miR-124 inhibitor. GAPDH was used as a control * Indicates $P < 0.05$.

mechanisms underlying the metastasis is crucial for improving surgery and treatment outcome.

MiRNAs have been widely recognized as powerful regulators for various kinds of cancers by changing the biological characters of tumor cells [22, 23]. For instance, Yoda et al. claimed that miR-375 suppressed cell migration by targeting Claudin-1 in lung cancer [24]. Li et al. reported that miR-21 was involved in acquired resistance of EGFR-TKI in NSCLC [25]. MiR-124 was firstly reported as a brain-enriched miRNA, which was proved to have deep-rooted relations to lots of malignances, including colorectal cancer, renal cancer, prostate cancer, esophageal cancer and breast cancer [26-30]. In addition, miR-124 has been studied for several times by different researchers in NSCLC. Berghmans et al. identified the significant difference of overall survival in NSCLC patients with different expression levels of miR-124 [31]. What's more, prior studies found that miR-124 suppressed the metastasis, inhibited the proliferation and functioned as a poor prognosis in NSCLC [32-34].

However, the underlying connection between miR-124 and CDH2 has not been elucidated in literature. In the present study, we confirmed that the expression of miR-124 was dramatically decreased in NSCLC tissue samples and cells. Aberrant expression of miR-124 was found to be related to the tumor stage characterized by tumor size and lymph node metastasis. Most especially, we revealed for the first time that miR-124 inhibited cell proliferation and suppressed cell invasion by directly binding to the 3'-UTR of CDH2 in NSCLC cell lines. Furthermore, we carried out the luciferase assay and a rescue assay, which greatly underpinned the results we had got.

CDH2 has been proved to be closely related to kinds of cancers including NSCLC. It was reported that overexpression of CDH2 promoted the invasion and induced EMT in erlotinib-resistant lung cancer cell lines [35]. Regulation of PI-3 kinase/Akt survival pathway by

CDH2 was found associated with the gefitinib-resistance of lung cancer cells [36]. Clinical study has confirmed the close association between high expression of CDH2 and to the brain metastases of NSCLC [37]. What's more, CDH2 is not only the target of miR-124, but also a significant mesenchymal phenotype marker. Along with decreased CDH1 and elevated Vimentin expression, over-expression of CDH2 is also a hallmark of EMT which has been regarded as a crucial progress in the cancer metastasis [38]. β -catenin is known as a key element of canonical Wnt signaling pathway, which participates a lot in EMT [39]. CDH1/ β -catenin complex plays an important role in maintaining epithelial integrity [40]. Mainstream opinion about β -catenin in EMT is that along with the reduction of CDH1, β -catenin transfers into cell nucleus and regulates the expression of downstream genes [41]. In our study, the total expression of β -catenin remained stable in cells with different expression levels of miR-124. Whether there was a nuclear translocation of β -catenin needs to be elucidated detailed by the following researchers.

Taken together, our study showed that miR-124 played a crucial role in NSCLC proliferation and invasion by regulating CDH2 expression and EMT progress. These findings may not only increase our knowledge of NSCLC development, but also help with therapeutic strategies for NSCLC.

Abbreviation

NSCLC (non-small cell lung cancer); miRNA (microRNA); CDH2 (Cadherin-2); PCR (Polymerase chain reaction); EMT (epithelial to mesenchymal transition); 3'-UTR (3'-untranslated region); siRNA (small interfering RNA); CCK-8 (cell-counting kit-8 assay).

Acknowledgements

This work was supported by a grant from the Project Funded by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions (No.JX10231801).

Disclosure Statement

None.

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