miR-129-2 upregulation induces apoptosis and promotes NSCLC chemosensitivity by targeting SOX4

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Abstract
Background: As one of the main causes of death worldwide, the treatment of non-small-cell lung cancer (NSCLC) is still unsatisfactory. This study aimed to explore the role of miR-129-2 in cell apoptosis and NSCLC chemosensitivity.

Methods: The effect of miR-129-2 on NSCLC was investigated using lung cancer cell lines (A549, NCI-H23, and HCC827), a normal lung cell line (BEAS-2B), and NSCLC tissues and adjacent healthy tissues. The oncogene SOX4 was verified as the target gene of miR-129-2 by luciferase reporter assay and real-time polymerase chain reaction.

Results: miR-129-2 expression was downregulated in NSCLC tissues, NCI-H23 cells, and A549 cells. miR-129-2 upregulation induced apoptosis in NCI-H23 and A549 cells. miR-129-2 upregulation also inhibited NSCLC in a xenograft mouse model, which was related to downregulation of SOX4 expression. Furthermore, miR-129-2 and SOX4 were aberrantly expressed in the cisplatin-resistant lung cancer cell line A549/DDP, and upregulation of miR-129-2 expression promoted cisplatin sensitivity in A549/DDP cells.

Conclusions: In conclusion, miR-129-2 expression was downregulated in NSCLC tissues and cell lines, and its upregulation induced cell apoptosis and promoted NSCLC chemosensitivity by regulating SOX4. Therefore, miR-129-2 can serve as a potential diagnostic and therapeutic target in NSCLC.

KEYWORDS
cisplatin, miR-129-2, NSCLC, SOX4, target

INTRODUCTION

As a type of lung cancer, non-small-cell lung cancer (NSCLC) represents one of the most common malignant diseases, with an increasing incidence, it comprises about 85% of all cases.1 The 5-year survival rate for metastatic NSCLC is only 5%. However, the diagnosis and treatment of NSCLC remain unsatisfactory, and the annual patient mortality rate is very high.3

microRNAs (miRNAs) are noncoding RNAs that are 20–24 nucleotides in length. They have been identified in various species and are post-transcriptional regulators of gene expression. The expression of several miRNAs has been reported in NSCLC tissue specimens.4 The effect and mechanism of some miRNAs in NSCLC have been determined in previous studies,5–8 for example when comparing to an adjacent normal tissue group, NSCLC tissues showed decreased miR-186 level. By cdc42 inhibition, miR-186 upregulation suppressed the growth and migration of NSCLC cells.9 However, the diagnosis and treatment of NSCLC remain unsatisfactory, and the annual patient mortality rate is very high.3

Weizheng Zhou and Chengliang Cai contributed equally to this work and should be considered as equal first coauthors.

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for instance miR-708 expression was upregulated in NSCLC tissues and was associated with poor survival.12 However, the effect and mechanism of miRNAs in NSCLC have not been fully elucidated. miR-129-2 is a functional miRNA that exerts multiple effects in various cancers, including hematologic cancer, esophageal carcinoma, and glioma.13–15 miR-129-2 expression is lower in lung cancer tissues than in healthy lung tissues. miR-129-2 upregulation was previously shown to inhibit the proliferation and invasion of lung cancer cells.16 Moreover, miR-129-2 expression was positively correlated with prognosis of NSCLC patients.17 In addition, enhanced miR-129-2 expression could inhibit proliferation and promote apoptosis of liver cancer cells.18 However, the role of miR-129-2 in apoptosis of NSCLC cells still poorly understood. Thus, the present study aimed to explore the role of miR-129-2 in cell apoptosis and chemosensitivity of NSCLC. A better understanding of the effect of miRNAs would aid in the development of more effective treatments for NSCLC.

MATERIALS AND METHODS

Clinical samples

Ethical Committee from the Changhai Hospital, The Second Military Medical University approved this research (approved number 2017LSE-09) on December 01, 2016. All study participants provided written informed consent before participating in the study. From January 2017 to December 2019 in the Changhai Hospital, The Second Military Medical University, the study population included patients diagnosed with NSCLC according to histopathological evaluation. Six pairs of NSCLC tissues and adjacent healthy tissues were excised when the patients underwent surgical resection. The samples were rinsed with phosphate-buffered saline (PBS), frozen in liquid nitrogen, and stored at −80°C for future study.

Establishment of a xenograft mouse model

A xenograft mouse model was established using 6-week-old male Bagg Albino (BALB)/c nude mice. They were stored in a pathogen-free environment under a 12-h/12-h light/dark cycle with free access to standard rodent chow. The A549 cultured in serum-free culture medium (5 × 10^6/100 μl) was subcutaneously injected into mice back. One week after injection, the mice were randomly divided into three groups. Then, lentiviral vectors expressing miR-129-2 were injected into mice in the experimental group through the tail vein, and negative-control lentivirus was injected into mice in the control group. Tumor tissues were collected after 2 weeks.

Cell culture

BEAS-2B, a normal lung cell line, and human lung cancer cell lines (NCI-H23, A549, and HCC827) were purchased from the Shanghai Institute of Biochemistry and Cell Biology. All cell lines were cultured at 37°C in a 5% CO2 incubator, and 10% Fetal Bovine Serum (FBS) was added to Roswell Park Memorial Institute-1640 medium.

Western blot

Total protein was obtained from tissues or cells then the suspension was loaded to wells for SDS-PAGE, then electrically transferred to nitrocellulose filter (NC) membrane, followed by 2 h blocking in skimmed milk with a concentration of 5%. Thereafter, membranes were incubated with anti-Bax antibody (ZSGB-BIO), anti-SOX4 antibody (ZSGB-BIO), and anti-cleaved Caspase-3 (Cell Signaling) at 4°C overnight. Thereafter, incubation with a second antibody for 1 h was carried out for the membrane. Membranes were imaged and then quantification was carried out.

Flow cytometry assay

A BD Pharmingen Annexin V-FITC Apoptosis Kit (BD Biosciences) was used to assess cell apoptosis. A previously described procedure was used.19

Cell transfection

All miR-129-2 mimics and corresponding negative control (RiBoBio) and transfection was carried out with X-tremeGENE siRNA Transfection Reagent (Roche) in accordance with protocol.

TUNEL staining

NSCLC tissues and adjacent healthy tissues were sectioned, dewaxed, and rinsed with water. They were then incubated in 3% hydrogen peroxide solution for 10 min and rinsed with PBS three times. This experiment was performed strictly according to the terminal deoxynucleotidyl transferase 2′-Deoxyuridine, 5′-Triphosphate (dUTP) nick end labeling (TUNEL) kit protocol.20

Real-time PCR

Total RNA from cells was extracted using TRIzol reagent (Roche). DNA was prepared with 300 ng RNA following kit instructions (Toyobo). Thereafter, real-time PCR was performed using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as the internal reference. The primers sequences were SOX4, 5′-GACCTGTGACGTGAAGCCTGGA-3′ (forward) and 5′-CCGGCTCAGTTAAAATGCC-3′ (reverse), and Gapdh, 5′-AAGAAGGTGTTAGGACAGGC-3′ (forward) and 5′-TCCACCACCTCTGTGTGTA-3′ (reverse). The
A primer for miR-129-2 was obtained from RiboBio Co., Ltd. The $2^{-\Delta\Delta CT}$ method was used for quantification.

**CCK-8 cell viability assay**

Cell viability was determined using a cell counting kit 8 (CCK-8) kit (Beyotime).

**Luciferase reporter assay**

NCI-H23 and A549 cells were cultured in 12-well plates for 24 h. Following transient transfection with a constructed plasmid, thymidine kinase promoter-Renilla luciferase reporter plasmid (Promega) was used to normalize luciferase activities. A dual-luciferase assay kit (Promega) and a luminometer were used to determine luciferase activity.21

**Data analysis**

Means ± SD are used to express the data. Differences among multiple groups or two groups were compared with ANOVA or Student’s t-test. A two-tailed $p < 0.05$ was statistically significant.

**RESULTS**

miR-129-2 expression was downregulated in human NSCLC cells and tissues

The miR-129-2 expression level was determined in human NSCLC cells and tissues. NSCLC tissues showed downregulated miR-129-2 expression levels compared with those in adjacent healthy tissues (Figure 1a). Similarly, the miR-129-2 level was also decreased in NSCLC cell line A549 and NCI-H23 NSCLC cell lines. The miR-129-2 level was also decreased in NSCLC cell line HCC827 compared with BEAS-2B, but had no statistical differences (Figure 1b). All human NSCLC cell lines and tissues showed downregulated miR-129-2 expression levels. Because miR-129-2 expression levels were more downregulated in A549 and NCI-H23 cells than in HCC827 cells, NCI-H23 and A549 cells were selected for subsequent experiments.

miR-129-2 upregulation induced cell apoptosis in NSCLC

To explore the effect of miR-129-2 on apoptosis, A549 and NCI-H23 cells were transfected with miR-129-2 mimics to upregulate miR-129-2 expression. miR-129-2 expression was significantly upregulated in miR-129-2 mimic-transfected A549 and NCI-H23 cells (Figure 2a,b). Flow cytometry analysis showed that miR-129-2 upregulation induced apoptosis of A549 and NCI-H23 cells (Figure 2c–f). In accordance with these results, Bcl-2-associated X protein (Bax), an apoptosis-related protein, was significantly upregulated in the presence of miR-129-2 (Figure 2g,h). These results suggest that upregulation of miR-129-2 expression induced apoptosis of NSCLC cells.

Oncogene SOX4 is the target gene of miR-129-2

To further explore the underlying mechanisms of miR-129-2, we predicted the targets of miR-129-2 using ENCORI (http://starbase.sysu.edu.cn/).22 SOX4 was determined as the target of miR-129-2, and the binding potential between miR-129-2 and SOX4 was predicted by PITA, miRanda, and TargetScan (Figure 3a). To study the direct binding effect of SOX4 and miR-129-2, Luciferase reporter assay was applied. In Figure 3b, miR-129-2 overexpressing cells A549 and NCI-H23 show reduced reporter activity after transfection of wild-type SOX4 3’-UTR reporter. Reporter
miR-129-2 upregulation promoted apoptosis of non-small-cell lung cancer cells.
(a) miR-129-2 expression in A549 cells. (b) miR-129-2 expression in NCI-H23 cells. (c) Representative flow cytometry images of A549 cells. (d) Representative flow cytometry images of NCI-H23 cells. (e) Apoptosis rate of A549 cells. (f) Apoptosis rate of A549 cells. (g) Bcl-2-associated X protein (Bax) expression in A549 cells. (h) Bax expression in NCI-H23 cells. Gapdh serves as an internal control. *p < 0.05, **p < 0.01, ***p < 0.001 versus miR-NC; n = 3. NC, negative control.
activity was unaffected by SOX4 3’-UTR mutant constructs (Figure 3b,c). The upregulated miR-129-2 expression level suppressed SOX4 expression at both the mRNA and protein levels (Figure 3d–g). These results indicate that miR-129-2 directly acts on SOX4.

**miR-129-2 upregulation inhibited NSCLC growth in the xenograft mouse model**

The effects of miR-129-2 on NSCLC were further examined by establishing a xenograft mouse model using A549 cells. Lentivirus was used as a carrier to upregulate miR-129-2 expression. Tumor growth was significantly suppressed through miR-129-2 upregulation (Figure 4a). The expression of the pro-apoptotic protein Bax and cleaved Caspase-3 was considerably higher than that in the miR-129-2-upregulated group (Figure 4b,c). In accordance with this result, TUNEL staining showed that apoptosis of NSCLC cells was induced by miR-129-2 upregulation (Figure 4d). Therefore, miR-129-2 upregulation induced an inhibitory effect on NSCLC in our xenograft mouse model.

**miR-129-2 upregulation inhibited SOX4 expression in the xenograft mouse model**

In our in vitro experiment, we observed that SOX4 was the target gene of miR-129-2, consequently the effect of miR-129-2 on SOX expression in xenograft mouse model was further studied. The results showed that in cells with transfection of miR-129-2, the protein as well as mRNA levels of SOX4 were both downregulated (Figure 5a–c). In contrast, the negative control exerted a negligible effect on SOX4 expression. These findings demonstrate that miR-129-2 upregulation suppressed SOX4 expression in our xenograft mouse model.

**The miR-129-2/SOX4 axis is involved in the inhibitory effect of cisplatin on NSCLC cell growth**

We demonstrated that the miR-129-2/SOX4 signaling pathway exerted effects on the development of NSCLC, but its therapeutic potential was unknown. Therefore, we investigated the involvement of the miR-129-2/SOX4 signaling pathway via the inhibitory effect of cisplatin. miR-129-2 expression was upregulated and SOX4 expression was downregulated in the cisplatin-resistant NSCLC cell line A549/DDP compared with that in A549 cells (Figure 6a–c).

**Promotion of cisplatin sensitivity in A549/DDP cells by miR-129-2 upregulation**

SOX and miR-129-2 contents were altered in A549/DDP cells. Whether miR-129-2 upregulation affected cisplatin sensitivity in A549/DDP was detected subsequently. The results showed that miR-129-2 upregulation inhibited A549/DDP cell viability (Figure 7a). Moreover, the miR-129-2-upregulated group had an increased number of apoptotic cells (Figure 7b,c). Similarly, Bax and cleaved Caspase-3 protein expression were also upregulated in this group (Figure 7d,e), and SOX4 protein and mRNA expression were downregulated (Figure 7f,g). The above results demonstrate that miR-129-2 upregulation enhanced cisplatin sensitivity in A549/DDP cells, which was at least partially related to SOX4 inhibition.

**DISCUSSION**

Cancer is the leading cause of death worldwide. Although noncoding RNAs have been identified as critical regulators in the initiation of various cancers and as having therapeutic
**Figure 4** miR-129-2 upregulation inhibited non-small-cell lung cancer (NSCLC) growth in the xenograft mouse model. (a) Representative tumor images and statistical results of tumor volume. (b) Expression level of Bcl-2-associated X (Bax) protein. (c) Expression level of cleaved Caspase-3 protein. (d) Terminal deoxynucleotidyl transferase dUTP nick end labeling-stained images. Gapdh serves as internal control. *p < 0.05, **p < 0.01 versus miR-NC; n = 3. NC, negative control.

**Figure 5** miR-129-2 upregulation inhibited SRY-box transcription factor (SOX) expression in the xenograft mouse model. (a) Representative images of immunohistochemistry staining. (b) SOX4 mRNA expression level. (c) SOX4 protein expression level. *p < 0.05, **p < 0.01 versus miR-NC; n = 3. NC, negative control.
potential,24–26 their underlying mechanisms of action have not been fully elucidated.

We obtained cell lines and tissues of NSCLC, detected the miR-129-2 level, and observed that, in comparison to adjacent normal tissues, miR-129-2 level was greatly reduced in NSCLC tissues. Correspondingly, compared with BEAS-2B, the normal lung cell line, miR-129-2 expression was lower in NCI-H23 and A549 cells, the NSCLC cell line. These results suggested that miR-129-2 content was decreased in tissues and cell lines of NSCLC.

The aberrant expression of miR-129-2 was reported to have a regulatory effect in other types of cancer. The loss of miR-129-2 expression was observed in glioblastoma patient samples and glioblastoma cell lines.15 Similarly, miR-129-2 expression was reduced in breast cancer; its downregulation promoted proliferation while inhibited cell apoptosis in breast cancer disease, which was related to upregulation of BCL2L2.27 miR-129-2 is reported to participate in cell invasion and proliferation in lung cancer.16 However, its effect on apoptosis in NSCLC is unknown. To observe the effect of miR-129-2 on NSCLC cells, we transfected miR-129-2 mimics into A549 and NCI-H23 cells. Flow cytometry analyses suggested that upregulated miR-129-2 expression induced apoptosis in both NCI-H23 and A549 cells.

We predicted the targets of miR-129-2 using bioinformatic methods to reveal the mechanism of miR-129-2. PITA, miRanda, and TargetScan software predicted that miR-129-2 had the potential to bind to SOX4. In addition, luciferase reporter assay results indicated that miR-129-2 targeted SOX4 directly. The real-time PCR results were
in agreement with the luciferase reporter assay results. miR-129-2 upregulation inhibited SOX4 expression at both the mRNA and protein levels. miR-129-2 silencing was related to SOX4 overexpression in gastric cancer, and this signaling pathway was related to the apoptosis of gastric cancer cells. SOX4 was also shown to be upregulated in clinical samples of NSCLC, which is related to the enhanced migration and invasion abilities of NSCLC cells. Similarly, SOX4 downregulation inhibited the migration and invasion of NSCLC cells, and high SOX4 expression levels were correlated with poor overall survival of patients with NSCLC and could be a clinical potential prognosis biomarker. Recently, Huang et al. found that a high level of SOX4 was also involved in the cisplatin-resistance of NSCLC. Li et al. further confirmed that enhanced expression of SOX4 promoted the malignant behaviors of NSCLC.

We then explored the in vivo effect of miR-129-2 on NSCLC by establishing a xenograft mouse model using A549 cells. Lentivirus was used to upregulate miR-129-2 in the mouse model. Our findings suggest that miR-129-2 overexpression significantly suppresses tumor growth, and the negative controls exerted a negligible effect on tumor growth. Furthermore, miR-129-2 treatment increased apoptosis of NSCLC cells. To examine whether this effect was related to altered SOX4 expression, immunohistochemistry staining, western blot analysis, and real-time PCR were conducted; the results revealed that SOX4 expression was downregulated following upregulation of miR-129-2 expression. The in vivo and in vitro findings were consistent with clinical observations that downregulation of SOX4 expression promoted metastasis and proliferation as well as induced cell apoptosis in patients with lung cancer.

SOX4 is also related to the chemoresistance of cancers. Yoon et al. showed that increased SOX4 expression was correlated with chemoresistance as well as therapy failure in patients with oral squamous cell carcinoma. SOX4 was also found to be involved in the chemoresistance of NSCLC. We then investigated whether the upregulation of miR-129-2 could attenuate the resistance of A549/DDP. Comparing to A549 cells, our conclusion demonstrated miR-129-2 downregulation and SOX4 upregulation in A549/DDP cells. Furthermore, upregulated miR-129-2 expression promoted cisplatin sensitivity in A549/DDP cells, which was correlated with inhibition of SOX4 expression.

Our study has clarified the expression, effect, and molecular mechanism of miR-129-2 in NSCLC. However, additional clinical evidence is necessary to prove the therapeutic potential of miR-129-2. Moreover, in recent years, long noncoding RNAs (lncRNAs) have been widely investigated in NSCLC, and these are shown to bind with miRNAs. Therefore, the interactions between miR-129-2 and lncRNAs require further investigation.

We conclude that the miR-129-2 expression levels were reduced in NSCLC cells and tissues. Furthermore, upregulation of miR-129-2 expression induces cell apoptosis and promotes the chemosensitivity of NSCLC by targeting SOX4, therefore miR-129-2 may be a potential diagnostic and therapeutic target in NSCLC.

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CONFLICT OF INTEREST
None.

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