

MiR-326 mediates malignant biological behaviors of lung adenocarcinoma by targeting ZEB1

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Abstract

MiR-326 functions as an antioncogene in the several types of cancer. However, the underling mechanisms through which miRNA-326 regulates the anti-carcinogenesis of lung adenocarcinoma have remained elusive. The aim of this study was to explore the role and regulatory mechanism of miR-326 in cell proliferation, invasion, migration and apoptosis in lung adenocarcinoma. Quantitative real-time PCR (qRT-PCR) was used to detect the expression pattern of miR-326 in human bronchial epithelial cells (HBES-2B), 4 kinds of lung adenocarcinoma cell lines (H23, H1975, H2228, H2085) and 20 lung adenocarcinoma tissues. Then, H23 cells were infected with miR-326 mimics, miR-326 inhibitors and si-ZEB1 to build up-regulated miR-326 cell lines, down-regulated ZEB1(zinc-finger-enhancer binding protein 1)cell lines, simultaneous down-regulated ZEB1 and miR-326 cell lines. Moreover, CCK-8 assay, transwell invasion assay, wound healing

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assay and flow cytometry assay were employed to examine the effects of miR-326 and ZEB1 on the proliferation, invasion, migration and apoptosis abilities of H23 cells. Western blot was performed to explore the effects of miR-326 and ZEB1 on the expression of invasion and migration related proteins N-cadherin, E-cadherin, MMP7, MMP13, SLUG and apoptotic proteins PARP, BAX. On the mechanism, a dual-luciferase reporter gene was used to measure the target relationship between miR-326 and ZEB1. MiR-326 expression was significantly downregulated in lung adenocarcinoma tissues and cells. Overexpression of miR-326 significantly inhibited the malignant behaviors of H23 cells. Mechanically, luciferase reporter assay showed that ZEB1 was a direct target of miR-326. MiR-326 mimic downregulated the expression of ZEB1. Furthermore, knocking down ZEB1 strongly inhibited the proliferation, invasion and migration of H23 cells but promoted apoptosis. MiR-326 could target ZEB1 to inhibit the proliferation, invasion and migration of lung adenocarcinoma cells and promote apoptosis, which is a potential therapeutic target for lung adenocarcinoma.

Keywords

Lung adenocarcinoma, miR-326, malignant biological behaviors, zinc-finger-enhancer binding protein 1, epithelial mesenchymal transition

Introduction

Lung cancer is one of the most common cancer and the leading cause of cancer-related death worldwide.^{1,2} This disease has caused broad concerns with its unique clinical and epidemiological characteristics and poor prognosis. The 5-year survival rate of lung cancer patients is still less than 20% due to its complex pathogenesis, lacking of early specific characteristics, and most people are diagnosed at terminal-stage of lung cancer. The main histopathologic type is lung adenocarcinoma,^{3,4} diagnosis of lung adenocarcinoma is difficult in the earlier period, and lack comprehension of the disease is the main reason leading to poor prognosis. Therefore, relevant molecular mechanisms of invasion and metastasis must be elucidated.⁵

MiRNA is a short non-coding RNA which is considered as an epigenetic factor.⁶ Notably, the ectopic expression of various miRNAs is associated with various malignancies, including lung cancer.⁷⁻⁹ In recent years, miRNA-326 in the invasion and metastasis of tumor has been extensively investigated and has represented a kind of tumor suppressor genes. However, the underlying mechanisms through which miRNA-326 regulates the anti-carcinogenesis of lung adenocarcinoma have remained elusive. So the aim of this study was to explore the role and regulatory mechanism of miR-326 in cell proliferation, invasion, migration and apoptosis in lung adenocarcinoma and provide a new research direction of searching novel targets of molecular targeted therapy of lung adenocarcinoma.¹⁰

Epithelial-mesenchymal transition (EMT), which occurs during critical phases of embryonic development in many animal species, indicates that epithelial cells gradually transform into mesenchymal-like cells that lose their epithelial functionality and characteristics.^{10,11} ZEB1, as an important transcription factor in EMT formation, is generally believed to foster migration, invasion and metastasis. A study found that miRNA-200 suppresses EMT by targeting ZEB factors.¹² However, whether the expression of miRNA-326 is associated with the EMT in patients with lung adenocarcinoma remains elusive.

In the present study, miRNA-326 was aberrantly low in lung adenocarcinoma compared to normal tissues and cells. The lower expression of miRNA-326 and higher expression of ZEB1 were proved to be significantly in connection with the malignant biological behaviors of lung adenocarcinoma cells. When miRNA-326 was overexpressed, the expression of ZEB1, which is one kind of EMT-associated genes, was decreased in the H23 lung adenocarcinoma cell line. Thus, miRNA-326 acted as an antioncogene in lung adenocarcinoma and the present results also suggested that the miRNA-326 may be related with EMT in lung adenocarcinoma.

Materials and methods

Clinical tissues

A total of 20 clinical lung adenocarcinoma surgical samples and corresponding normal tissues were obtained at the Sichuan cancer hospital from 2017 to 2018 (Table 1). The final diagnosis was established on pathological study which is the 'gold standard'. The patients had received no previous treatment before surgery.

Cell lines culture and transfection

Human lung adenocarcinoma cell lines (H23, H1975, H2228 and H2085) and human bronchial epithelial cells (BEAS-2B) were all bought in Chinese Academy

Table 1 Lung adenocarcinoma tissue specimens relevant patient information.

No.	Gender	Age	Smoking status	Size (cm)	Stage
1	Female	44	Never	2 × 2 × 2	T1N0M0,IA
2	Male	49	10 years	3 × 2.5 × 2	T3N2M0,IIIB
3	Male	47	30 years	1 × 2 × 1.5	T1N0M0,IA
4	Female	48	Never	2 × 2.1 × 1.8	T1N0M0,IA
5	Female	63	Never	4 × 4 × 2	T2N2M0,IIIA
6	Female	65	Never	2 × 2 × 2	T2N2M0,IIIA
7	Female	70	Never	3 × 3 × 3	T2N1M0,IB
8	Female	53	Never	2 × 2 × 1.5	T1N1M0,IB
9	Male	65	Never	1 × 0.8 × 1	T1N1M0,IB
10	Male	74	Never	3.5 × 3 × 3	T3N3M0,IIIC
11	Female	51	Never	3 × 3 × 3	T2N2M0,IIIA
12	Male	61	Never	3 × 3 × 2	T2N0M0,IB
13	Male	72	Never	5 × 4 × 3	T2N2M0,IIIA
14	Female	53	Never	3 × 3 × 2.5	T2N1M0,IB
15	Male	54	50 years	1.5 × 1.3 × 1.2	T2N0M0,IB
16	Female	59	Never	5 × 4 × 3	T3N2M0,IIIB
17	Male	62	30 years	1 × 1 × 2	T1N0M0,IA
18	Male	54	30 years	5.2 × 5 × 3.4	T3N2M0,IIIB
19	Female	44	Never	4 × 4 × 2	T2N0M0,IB
20	Female	62	Never	2 × 2 × 1.6	T1N1M0,IB

of Sciences. Roswell Park Memorial Institute 1640 (PRMI1640, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, BI, Israel) and 1% penicillin streptomycin (Invitrogen, USA) were used for all cells culture at 37°C and 5% CO₂ in a humidified incubator. The cells were transfected with plasmid DNA (si-ZEB1) (Genechem, Shanghai, China), miR-326 mimics or miR-326 inhibitor (Genepharma, Shanghai, China) through a Lipo6000™ transfection reagent (Beyotime, China) in accordance with the operation manual. The follow-up experiments were carried out 48 h later.

Cell proliferation assay

H23 cells were counted and inoculated into 96-well plates with 1000 cells per well. One hundred microliter fresh medium was added to each well and the cells were cultured in an incubator with four multiple Wells in each group. Then, 10 µl cck-8 reagent was added to each well 1 h before the test. The culture was continued in dark for 1–4 h. The absorbance at 450 nm was determined by microplate reader everyday for 4 days. This process was repeated thrice for each set of samples.

Transwell invasion assay

H23 cells were planted onto the top chambers with 100 µl (10⁶/ml) for each well and then incubated for 48 h. Then, the cells with invasive ability were fixed with 4% paraformaldehyde for 15 min. Finally, we placed the sample under an inverted microscope at 100× magnification. This experiment was repeated thrice.

Wound healing assay

In this experiment, 2 × 10⁵ H23 cells were routinely counted and inoculated in a 24-well plate with three multiple Wells. The cell density reached 100% after incubated overnight. Scratching was performed with 10ul sterile tips perpendicularly to the cell plane. At the appropriate time points: 0 h, 6 h, 12 h, 24 h and 48 h, the scratches width was observed under the microscope and take pictures; Image J was used to analyze the data. This experiment was repeated thrice.

Flow cytometry assay

H23 cells were mixed with 500 µl pre-cooled binding buffer after being digested by trypsin (Thermo Scientific, USA). Next, 5 µl Annexin V-FITC (Thermo Scientific, USA) was added, and the cells were incubated at room temperature and away from light for 15 min. Finally, 2.5 uL propidium iodide (PI) was added 5 min before the operation. This experiment was repeated thrice.

Quantitative real-time PCR (qRT-PCR)

The total RNA of human lung adenocarcinoma cell lines and tissues were extracted using the fast200 kit (Fastagen, China) in accordance with the manufacturer's

instructions and the purity was measured by ultraviolet spectrophotometer. The reverse transcription was operated using PrimeScript™ RTreagentKit (TAKARA, Japan) and SYBRPremixExTaq™ (TAKARA, Japan) was used for qRT-PCR. PCR amplification reaction conditions on machine: pre-denaturation at 95°C, denaturation at 95°C, annealing at 60°C and extension at 85°C followed by reaction for 30 s, 5 s, 30 s and 5 s, with 40 cycles. The primer sequences are miR-326-F 5'-CTCTGGGCCCTTCCTC-3', R 5'-GAACATGTCTGCGTATCTC-3'; U6-F 5'-CTTCGGCAGCACATATACT-3', R 5'-AAAATATGGAACGCTTCACG-3'; β -actin-F 5'-CACCATTGGCAATGAGCGGTTC-3', R 5'-AGGTCTTTGCGGATGTCCACGT-3'; ZEB1-F 5'-GGCATAACCTACTCAACTACGG-3', R 5'-TGGGCGGTGTAGAATCAGAGTC-3'.

Western blot

The primary antibodies against β -actin, ZEB1, N-cadherin, MMP7/13, SLUG, PARP and BAX were purchased from CST (Cell Signaling Technology, USA) and E-cadherin antibody was bought from ABclonal (China). The membranes were incubated with secondary antibodies (Bioworld Technology, USA) for 1.5 h at room temperature, and the signal was visualized using an HRP substrate (Millipore, Germany). The analysis was used the MD Image Quant Software.

Luciferase reporter assay

Wild-type, mutant and luciferase reporter plasmids of miR-326 and ZEB1 3'-UTR binding sequences were synthesized and provided by Guangzhou Ruibo Biotechnology. Over expression/NC miR-326 H23 cells were transfected with wild-type pmirGLO-ZEB1 vector (WT) or mutated-type pmirGLO-ZEB1 vector (MUT). The cells were collected, and the activities of firefly and kidney luciferases were detected using the dual-luciferase reporter gene assay system.

Statistical analysis

SPSS19.0 software, GraphPad Prism 7.0 and Image J were used for statistical analysis picture production. Furthermore, t^2 test was used for further pairwise comparison. p value <0.05 or <0.01 reach statistical significance.

Results

MiR-326 is expressed at a low level in lung adenocarcinoma

To specify the role of miR-326 in lung adenocarcinoma, we first tested the expression level of miR-326 in 20 lung adenocarcinoma tissues and the corresponding normal tissues using qRT-PCR. We found the level of miR-326 is obviously reduced in lung adenocarcinoma tissues compared with normal tissues ($p < 0.01$, Figure 1(a)). Secondly, we examined the expression of miR-326 in four types of

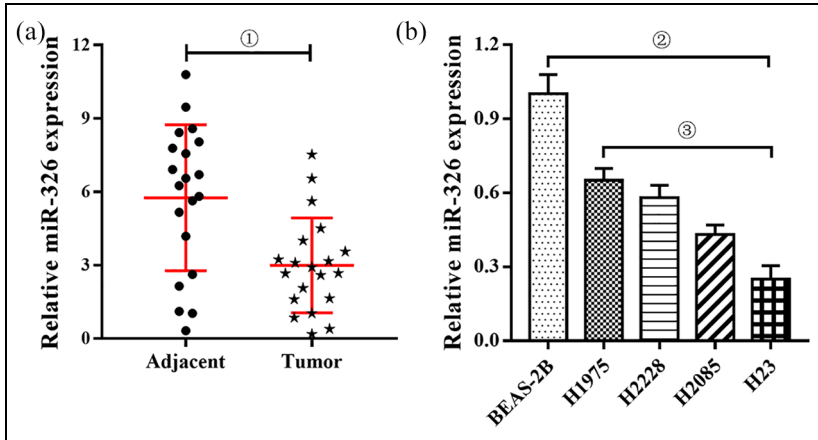


Figure 1. Biological effect of miR-326 in related tissues and cells: (a) expression of miR-326 in lung adenocarcinoma tissues and the corresponding normal tissues are shown as the mean \pm standard deviation (SD) data and (b) expression of miR-326 in human bronchial epithelial cells and four lung adenocarcinoma cell lines. All experiments were repeated three times. ①②③indicates $p < 0.01$.

lung adenocarcinoma cell lines (H23, H1975, H2228 and H2085) and human bronchial epithelial cells (BEAS-2B). And results showed that miR-326 expression levels in lung adenocarcinoma cells are significantly lower in lung adenocarcinoma cells than in BEAS-2B ($p < 0.01$, Figure 1(b)). Furthermore, the expression of miR-326 was lower in H23 cells than that in the other three lung adenocarcinoma cell lines ($p < 0.01$, Figure 1(b)). Therefore, we speculated that the low expression of miR-326 may be related to the occurrence and development of lung adenocarcinoma, and H23 cells were selected for the follow-up experiments.

MiR-326 upregulation suppresses proliferation, invasion and migration of H23 cell lines and induces apoptosis

The expression level of miR-326 of H23 cell line was the lowest in four kinds lung adenocarcinoma cell lines. Therefore, H23 cells were selected to measured the function of miR-326. MiR-326 mimics were used to build the over expressed miR-326 H23 cell lines, and miR-NC was set as the control ($p < 0.01$, Figure 2(a)). Proliferation assay results indicated that the overexpression of miR-326 significantly inhibited the proliferation activity of H23 cells in comparison with the control group ($p < 0.01$, Figure 2(b)). Transwell invasion assay results showed that the overexpression of miR-326 also caused a markedly invasive ability to decrease in H23 cells ($p < 0.01$, Figure 2(c) and (e)). In addition, miR-326 overexpressed H23 cells migrated more slowly than the control group in the wound healing assay ($p < 0.01$, Figure 2(d) and (f)). Flow cytometry analysis indicated that the

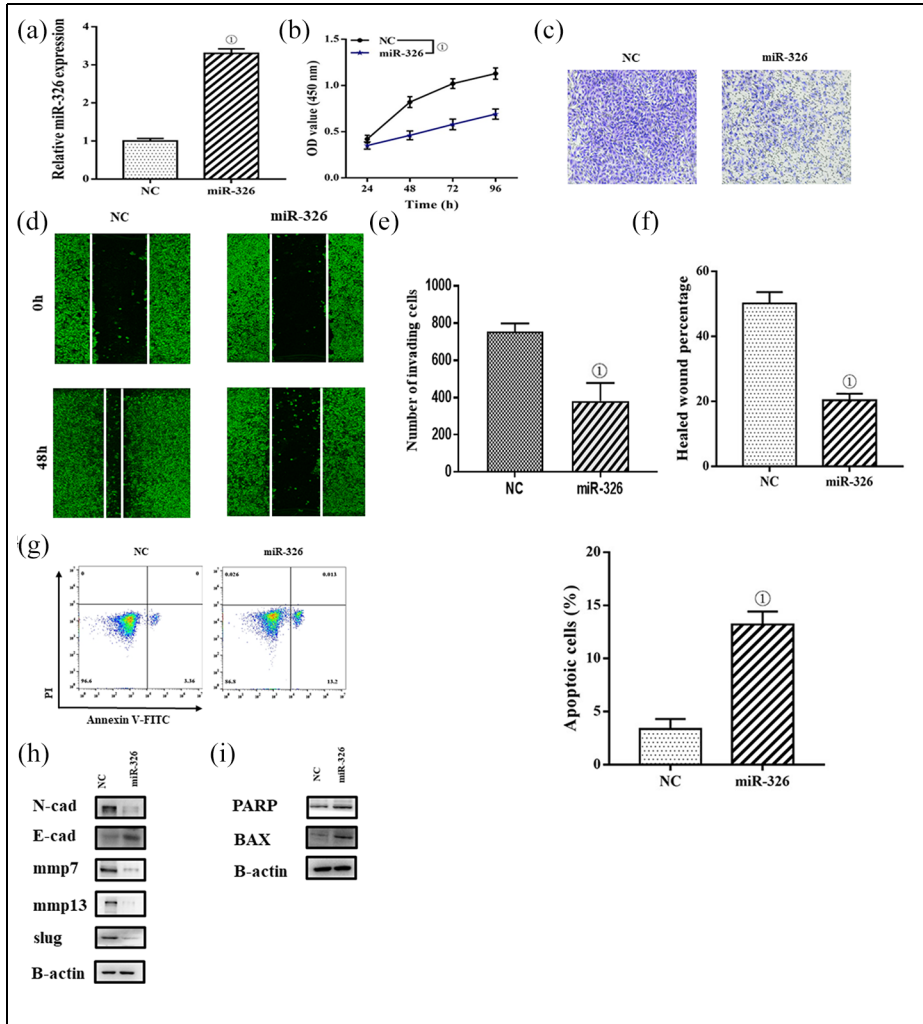


Figure 2. MiR-326 regulates H23 cell proliferation, migration, invasion and apoptosis: (a) qRT-PCR of miR-326 expression levels in H23 cells. Overexpression of miR-326 in H23 cells by miR-326 mimics (mimics), (b) Cck-8 assay of H23 cells transfected with miRNA negative control (miR-NC) and miR-326 mimics, (c) and (e) trans-well invasion assay for H23 cells transfected with miR-NC or miR-326 mimics, (d) and (f) wound healing assay for H23 cells transfected with miR-NC or miR-326 mimics, (g) flow cytometry assay for H23 cells transfected with miR-NC or miR-326 mimics, and (h) Western blot showing that overexpression of miR-326 decreased the expression of N-cadherin, MMP7, MMP13 and slug, while increased the expression of E-cadherin, PARP and BAX. All experiments were repeated three times.

① indicates $p < 0.01$.

overexpression of miR-326 enhanced the apoptotic rate of H23 cells ($p < 0.01$, Figure 2(g)). It was shown that the overexpression of miR-326 attenuated the expression of invasion- and migration-associated proteins (N-cadherin, MMP7, MMP13 and SLUG). Meanwhile, we also demonstrated that E-cadherin, PARP and BAX were increased in miR-326 overexpressed H23 cells (Figure 2(h) and (i)). These results showed that miR-326 might act by regulating EMT and the upregulation of miR-326 suppressed the proliferation, invasion and migration of H23 cell lines and induced apoptosis.

ZEB1 is a direct target of miR-326 and contributes to proliferation, invasion and migration of H23 cells and decline in apoptosis

As we all know, ZEB1 has a closely related function on EMT. In order to explore the targeting relationship between miR-326 and ZEB1, we selected RNA sequences with homologous binding sites with miR-326, including six base matching. Luciferase activity assay showed that, compared with the miR-NC group, the fluorescence activity of miR-326 transfected ZEB1-WT was significantly reduced. No significant change was observed in fluorescence activity after ZEB1-MUT transfection (MUT, ZEB 3'-UTR; $p < 0.01$, Figure 3(a) and (b)). Western blot showed that, after being transfected with miR-326 mimics, the expression level of ZEB1 in H23 cells was decreased ($p < 0.01$, Figure 3(c) and (d)). Moreover, the detection of 20 clinical samples of lung adenocarcinoma showed that miR-326 was negatively correlated with the expression of ZEB1 (Figure 3(e)). To further investigate whether miR-326 regulates the malignant biological behaviors of H23 by targeting ZEB1, we knocked-down ZEB1 by si-ZEB1 and transfected with miR-326 inhibitor simultaneously in H23. Western blot results showed that si-ZEB1 can significantly decrease ZEB1 expression and the transfection efficiency was about 54.94% ($p < 0.05$, Figure 4(a)). Although we transfected with plasmid DNA (si-ZEB1), we could not change total expression sequences of ZEB1 in H23 cells, thus ZEB1 expression was also showed in si-ZEB1 cells. Whereas the expression level was significantly upregulated in H23 cells transfected with miR-326 inhibitor and si-ZEB1 than in si-ZEB1 group ($p < 0.01$, Figure 4(a)). MiR-326 inhibitor had positive effects on other sequences which still expressed ZEB1 instead of the si-ZEB1 sequence. However, the difference between the control group and the miR-326 inhibitor and si-ZEB1 group was not statistically significant ($p > 0.05$). qRT-PCR showed that the expression of miR-326 was prominently down-regulated in H23 cells transfected with miR-326 inhibitor and si-ZEB1 than in si-ZEB1 group ($p < 0.01$, Figure 4(a)). Subsequently, the proliferation assay results jointly confirmed that knockdown ZEB1 significantly inhibited the proliferation activity of H23 cells in comparison with the control group ($p < 0.01$, Figure 4(b)). The results of the transwell and wound healing tests showed that the invasion and migration abilities by knockdown ZEB1 were significantly downregulated in H23 cells than in the control group ($p < 0.01$, Figure 4(c) and (d)). The flow cytometry results showed that knockdown ZEB1 significantly promoted the apoptosis level of H23

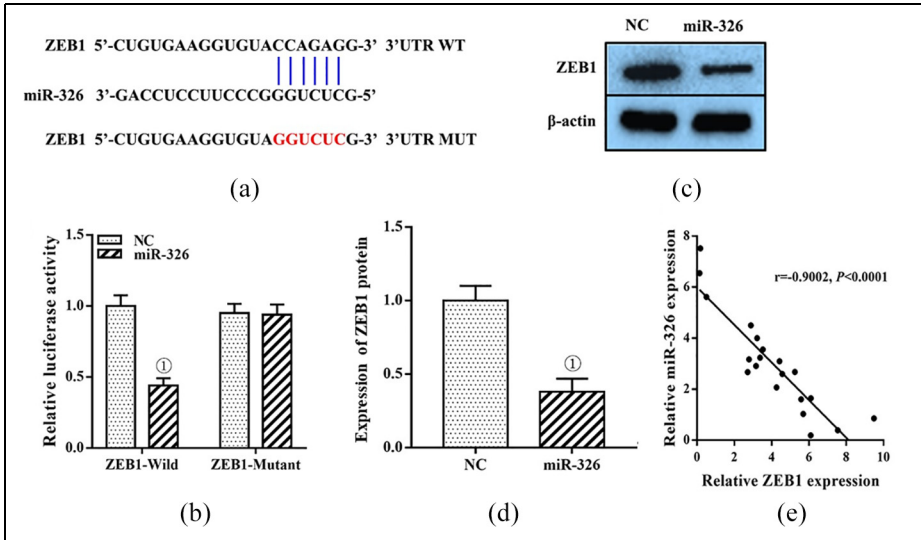


Figure 3. MiR-326 directly targets ZEB1 in lung adenocarcinoma cells and tissues: (a) sequence alignment of miR-326 and 3' UTR of ZEB1, (b) luciferase reporter assay of WT and MUT, (c and d) Western blot analyses of ZEB1 in H23 cells transfected with miR-NC or miR-326 mimics, and (e) correlation between miR-326 and ZEB1 expression in lung adenocarcinoma tissues. All experiments were repeated three times.

① indicates $p < 0.01$.

cells in comparison with the control group ($p < 0.01$, Figure 4(e)). In addition, in comparison with the group transfected with si-ZEB1 only, both knockdown of miR-326 and ZEB1 could significantly upregulated the proliferation, invasion and migration abilities of H23 cells ($p < 0.01$), whereas the level of apoptosis was down-regulated ($p < 0.01$). However, the differences were not statistically significant in comparison with the control group ($p > 0.05$). As shown in Figure 4(f) and (g), si-ZEB1 reduced the protein expression of N-cadherin and MMP13, while si-ZEB1 increased the expression level of E-cadherin, PARP and BAX. Western blot analysis also showed that protein expression of N-cadherin and MMP13 was increased and protein expression of E-cadherin, PARP and BAX was reduced after both knockdown of miR-326 and ZEB1. Therefore, miR-326 downregulated ZEB1 to inhibit H23 cell proliferation, invasion, migration and induce apoptosis.

Discussion

As a member of miRNA, miRNA-326 is a class of molecules related to tumor inhibition and is expected to be a new target for diagnosis and treatment of lung adenocarcinoma. The results of the present study reconfirmed that miRNA-326 inhibited tumor proliferation and metastasis but promoted apoptosis in lung

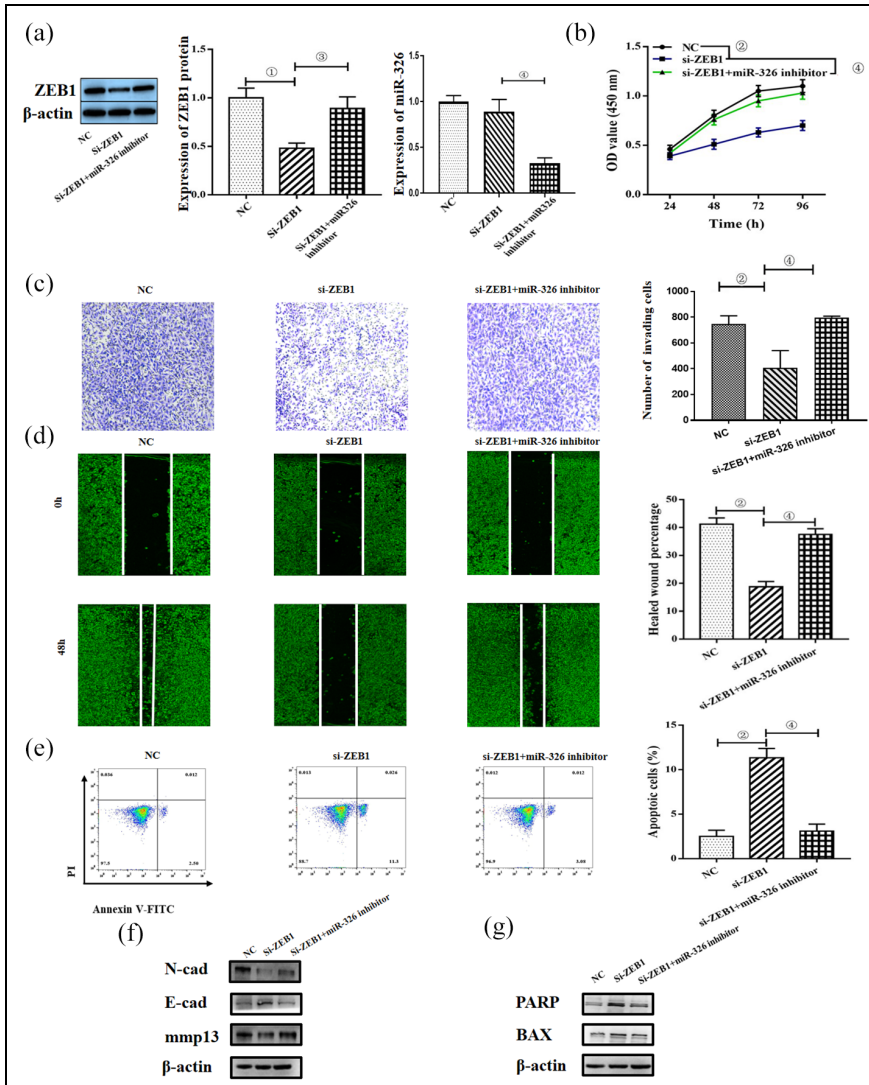


Figure 4. miR-326 contributes to the proliferation, invasion and migration of H23 cells and decline in apoptosis by targeting ZEB1: (a) Western blot analyses of ZEB1 and qRT-PCR of miR-326 expression levels in H23 cells transfected with NC and si-ZEB1 or si-ZEB1 + miR-326 inhibitor, (b) cck-8 assay of H23 cells transfected with NC and si-ZEB1 or si-ZEB1 + miR-326 inhibitor, (c and d) transwell invasion assay and wound healing assay for H23 cells transfected with NC and si-ZEB1 or si-ZEB1 + miR-326 inhibitor, (e) flow cytometry assay for H23 cells transfected with NC and si-ZEB1 or si-ZEB1 + miR-326 inhibitor, and (f and g) relative proteins abundances of N-cadherin, E-cadherin, MMP13, PARP and BAX in NC and si-ZEB1 or si-ZEB1 + miR-326 inhibitor were determined by Western bot. All experiments were repeated three times.

①③indicates $p < 0.05$; ②④indicates $p < 0.01$.

adenocarcinoma. Several studies have reported that miRNA-326 is an anti-oncogene which inhibited cell proliferation in the various types of malignancies including: endometrial cancer, hepatocellular carcinoma and cervical cancer.^{13–15} A recent study indicated that miR-326 was not only associated with stage (low stages I–II and high stages III–IV) and invasiveness (T2–T4) in primary colorectal cancer biopsies but also correlated with extended overall survival.^{16,17} However, how miR-326 influences the tumor initiation and progression of lung adenocarcinoma has not been elucidated clearly. Understanding and targeting miR-326 would find out a new index of diagnosis, treatment strategies or prognosis for lung adenocarcinoma. In a follow-up experiment, we will continue examining the relationship between miR-326 and the patients' clinical outcome or progress in lung adenocarcinoma and exploring the mechanism of it.

EMT plays an important role in the development of tumor, especially in the progress of tumor invasion and metastasis. Some markers related to metastasis must be determined and used to predict the prognosis of patients. Therefore, proteins related to EMT are extensively used to predict the outcomes of diseases.^{18,19} ZEB1, as a zinc-finger E homeobox-binding transcription factor, is best known for the role of a regulator in EMT.²⁰ ZEB1 has been confirmed to promote EMT through different signal paths: classic TGF- β (transforming growth factor β)/Smad signaling pathway and COX-2(cyclooxygenase-2) signal transduction pathway.^{21–23} On the other hand, there was also increasing evidences confirmed that the functions of ZEB proteins were not only limited to EMT regulation. Some studies have shown that ZEB1 expression was observed in noninvasive neoplastic lesions, such as in situ pancreas adenocarcinoma or pancreatic intraepithelial lesions, which means ZEB1 a key driver of early steps of pancreatic tumorigenesis may relate to the proliferation and apoptosis of tumors.^{24–26} Thus, ZEB1 may not only prompt the invasion, and migration of H23 cells but also alter proliferation and apoptosis. We speculated that ZEB1 was the target of miR-326 and luciferase reporter assay had also shown that ZEB1 was a direct target of miR-326. This study confirmed that miR-326 negatively regulated ZEB1, and the expression levels of the two are negatively correlated in lung adenocarcinoma tissues. About 50% of human cancer patients have P53 mutations. Except losing the function of tumor suppressive in wild-type P53, mutant P53 proteins could obtain novel oncogenic functions named mutant P53 gain-of-function (GOF).²⁷ Previous studies showed that there was a complex interaction between ZEB1 and P53 family members about proliferation and cell survival. Beyond that, ZEB1 has been proved to prevent tumor apoptosis by affect P53 and RB-dependent on suppressive pathways.^{26,28} Hence, it is meaningful to detect the interaction of ZEB1 and p53 mutation in lung adenocarcinoma or other kinds of cancer.

Nevertheless, the present study has several limitations despite the favorable application prospects of these results. First, a large sample size is required to further unravel the story of this newly identified mechanism. This study only used 20 samples, which may have led to selection bias and limited practicability. Meanwhile, we did not perform sample size calculation for tissue samples. Therefore, future studies

must include various lung adenocarcinoma cases. Second, only H23 cell lines were selected in this study. In the future study, the function of miRNA-326 must be identified in a different lung adenocarcinoma cell line. Furthermore, we studied only one siRNA to knockdown ZEB1 according to the laboratory conditions. Finally, the concept must be taken from the chalkboard to the clinics. A follow-up study will focus on the relationship between the expression of miRNA-326 and the prognosis of patients.

Conclusion

We demonstrated that miR-326 could target ZEB1 to inhibit the proliferation, invasion and migration of LADC cells and promote apoptosis, which is a potential therapeutic target for lung adenocarcinoma. These results support the notion that miRNA-326 acts as a tumor suppressor gene in lung adenocarcinoma and may act as an inhibitor in lung adenocarcinoma onset and progression and may participate in controlling epithelial mesenchymal transition.

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Author contributions

RZ, CX, JTH and QL designed this study. HW, MXL, RZ, JTH and YQL completed the luciferase reporter assay and western blot experiment. qRT-PCR and its relevant experiment were performed by MXL and QCL. YT and STW cultured the cells and completed relevant transfection and transwell experiments. JCL, RX and SHX collected the tissue samples. All authors took part in analysis of data and the writing of the manuscript. All authors have read and approved the manuscript and have agreed to take responsibility for all aspects of the study to ensure that the accuracy or completeness of any part of the work is properly investigated and resolved.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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Ethics approval

Ethical approval for this study was obtained from *Ethics Committee of Sichuan Cancer Hospital (NO. SCCHEC-02-2017-011)*.

Informed consent

Written informed consent was obtained from all subjects before the study.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

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