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ORIGINAL ARTICLE

microRNA-100 functions as a tumor suppressor in non-small cell lung cancer via regulating epithelial-mesenchymal transition and Wnt/β-catenin by targeting HOXA1

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Keywords
Epithelial-mesenchymal transition; HOXA1; miR-100; non-small cell lung cancer; Wnt/β-catenin.

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Abstract

Background: Non-small cell lung cancer (NSCLC) is a leading subtype in lung cancer, with high morbidities and mortalities worldwide. microRNA (miRNA) has appeared to play indispensable roles in a variety of solid carcinomas. The current study focused on the functions of miR-100 in NSCLC.

Methods: qRT-PCR was performed to detect miR-100 and HOXA1 expressions in NSCLC tissues and cells. MTT and transwell assays were used to determine the functions of miR-100 in NSCLC cell proliferation, invasion and migration abilities. Western blot was used to measure related protein expressions.

Results: qRT-PCR results showed that miR-100 expressions were dramatically decreased in NSCLC tissues. MTT assays indicated that miR-100 restoration inhibited NSCLC cell proliferation. Furthermore, transwell assay was performed to determine the impacts of miR-100 on NSCLC invasion and migration abilities. As expected, the invasion and migration capacities were significantly repressed. Direct interactions between HOXA1 and miR-100 were also verified via dual-luciferase reporter assays. Western blot analysis demonstrated that miR-100 exerted suppressive functions via regulating EMT and Wnt/β-catenin in NSCLC cells.

Conclusions: Our results showed that miR-100 served antitumor roles in NSCLC, providing new evidence of miR-100 as a promising therapeutic biomarker in NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) remains one of the major factors for tumor-associated deaths globally despite significant advances in surgical treatments and drug development.[1] However, due to the high rate of drug resistance, metastases and relapses, the prognosis of NSCLC is still poor.[2] The frequent relapse and poor outcome in NSCLC patients highlight the imperative need to develop early biomarkers and novel screening strategies for the accurate detection of metastases and recurrence.[3,4] Therefore, it is essential to fully understand the tumorigenesis mechanisms of NSCLC and to develop new therapeutic approaches for NSCLC patients. Epithelial-mesenchymal transition (EMT) is considered as a crucial step in malignancy progression, in which progress the epithelial cells are conversed into mesenchymal cells in cell morphology.[5] EMT has been reported to exert pivotal functions in tumor progression, including metastases.[6] Wnt/β-catenin signaling pathway is also implicated in development of multiple tumors.[7] The Wnt/β-catenin is frequently aberrantly activated in human cancers, including gastric cancer,[8] osteosarcoma[9] and hepatocellular carcinoma.[10] Therefore, the mechanism

underlying EMT and Wnt/β-catenin signaling pathway in NSCLC are the focus of the current study.

The dysfunctions of microRNAs (miRNAs) have been identified as key regulators of various biological processes in numerous carcinomas.[11,12] miRNAs may modulate the expressions of different coding genes by inducing translational repression or the degradation of target mRNAs via base pairing with the specific sequences in the 3′UTRs.[13] In fact, the deregulations of many carcinogenic or antitumor miRNA have been verified to be associated with tumor initiation and progression.[14,15] For example, miR-133b/135a induced human renal carcinoma cell apoptosis via the JAK2/STAT3 signaling pathway[16]; miR-21 promoted triple-negative breast cancer cell proliferation and invasion via targeting PTEN[17]; and miR-4317 suppressed human gastric cancer cell proliferation via the regulation of ZNF322.[18] However, the exact mechanism by which miR-100 exerts its functions in NSCLC progression is not well elucidated.

Homeobox (HOX) genes often present different expression patterns during development, and changes in the expressions have been related to a variety of diseases, including neoplasia.[19] HOX are implicated in development through modulating cell angiogenesis, migration, survival and differentiation.[20] Homeobox A1 (HOXA1) is one of the important members of the HOX family and overexpression of HOXA1 has been confirmed in various cancers, which is associated with tumor development and poor prognosis.[21] Some studies have demonstrated that HOXA1 plays vital roles in tumorigenesis. For instance, miR-99a suppressed nasopharyngeal carcinoma cell metastases and invasion via regulation of HOXA1.[22] Another study indicated that HOXA1 promoted prostate cancer cell progression.[23]Additionally, overexpressed HOXA1 in oral squamous cell carcinomas was confirmed to be correlated with poor prognosis.[24] However, the exact functions of HOXA1 in NSCLC are not well understood.

Table 1 Primer sequences for qRT-PCR

Primer	Sequence
miR-100 forward	5′- GCGGCAACCCGTAGATCCGAA-3′
miR-100 reverse	5′- GTGCAGGGTCCGAGGT-3′
U6 forward	5′-CTCGCTTCGGCAGCACA-3′
U6 reverse	5′- AACGCTTCACGAATTTGCGT-3′
HOXA1 forward	5′- -CGGCTTCCTGTGCTAAGTCT -3′
HOXA1 reverse	5′- TAGCCCAGCCAAATACACGG -3′
GAPDH forward	5′-ACCTGACCTGCCGTCTAGAA-3′
GAPDH reverse	5′-TCCACCACCCTGTTGCTGTA-3′

U6: small nuclear RNA, snRNA. HOXA1, homeobox A1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Methods

Patients and tumor samples

A total of 52 pairs of NSCLC tissue samples and adjacent normal tissue samples were obtained from NSCLC patients who underwent surgical resections at The Affiliated Hospital of Qingdao University from September 2015 to November 2017, without having prior chemotherapy or radiation therapy. All tissue samples were instantly ice-covered in liquid nitrogen and stockpiled at −80°C. Informed consent was obtained from all participants. All tissue specimens were obtained with approval of the Medical Ethics Committee of The Affiliated Hospital of Qingdao University.

Cell lines

Human normal bronchial epithelium cell BEAS-2B and NSCLC cells (NCI-H460, NCI-H1299, SPC-A1 and A549) were purchased from ATCC and maintained in DMEM (Gibco; Thermo Fisher Scientifc, Inc., Waltham, MA, USA) which contained 10% FBS (Gibco; Thermo Fisher Scientifc, Inc.) in a humidified atmosphere with 5% CO_2 at 37°C.

Cell transfections

miR-100 mimics, inhibitor as well as the corresponding negative controls were obtained from GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was applied for miRNA transfections in accordance with the manufacturer's instructions.

qRT-PCR

TRIzol reagent (Invitrogen) was applied to extract the total RNAs from the tissues or cells in line with the manufacturer's proposals. After that, the PrimeScript reverse transcription reagent kit (Thermo Fisher Scientific) was utilized for cDNA syntheses. Quantitative RT-PCR was performed using SYBR Premix Ex Taq II (Takara, Dalian, China) with the 7500 real-time RT-PCR system (Applied Biosystems, Foster City). The $2^{-\Delta\Delta Ct}$ method was used to calculate expression levels. U6 and GAPDH served as internal controls for miR-100 and HOXA1, respectively. The primer sequences are listed in Table 1.

MTT assays

MTT assays were employed to determine the impacts of miR-100 on NSCLC cell proliferation ability. In brief, transfected NSCLC cells were plated into 96-well plates and incubated for indicated times (0, 24, 48 and 72 hours).

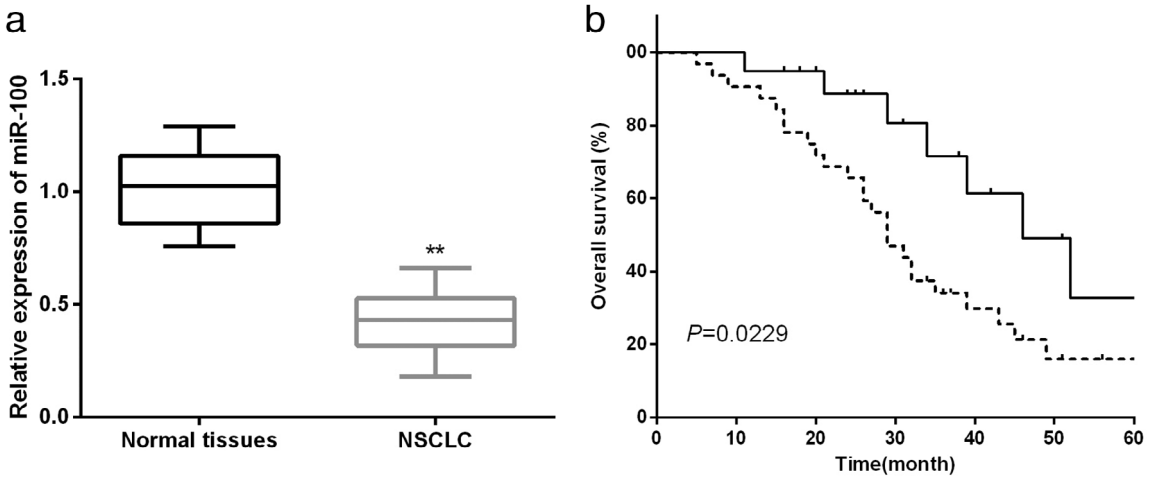

Figure 1 miR-100 was downregulated in NSCLC tissues and associated with a poor prognosis of NSCLC patients. (**a**) miR-100 expressions in NSCLC tissues were detected using qRT-PCR. (**b**) Kaplan-Meier analysis of different miR-100 expressions in NSCLC patients. $^{**}P < 0.01$ (—— miR-100(+) and – – – miR-100(-)).

Table 2 Correlation of miR-100 expression with the clinicopathological characteristics of the NSCLC patients

		miR-100† expression		
Clinicopathological features	Cases (n = 52)	High (n = 21)	Low (n = 31)	P-value
Age (years)				0.3363
>60	30	13	17	
≤60	22	8	14	
Gender				0.3612
Male	28	10	18	
Female	24	11	13	
Tumor size (cm)				0.1534
≥ 5.0	24	6	18	
< 5.0	28	15	13	
Lymph node metastasis				0.0026*
Yes	23	18	5	
No	29	3	26	
Histology				0.2867
Squamous cell carcinoma	26	12	14	
Adenocarcinoma	26	9	17	
TNM stage				0.0030*
I + II	24	16	8	
III + IV	28	5	23	
Smoker				0.4056
Yes	29	13	16	
No	23	8	15	

*Statistically significant. †The mean expression level of miR-100 was used as the cutoff. NSCLC, non-small cell lung cancer; TNM, tumor-node-metastasis.

Then, MTT (5 mg/mL) solution was appended into each well and incubated for another 4 hours. After that, the culture medium was removed and DMSO was added to dissolve the MTT-formazan crystals. The absorbance values were determined using a microplate reader (BioTek, Winooski, VT, USA) at 490 nm.

Transwell assays

Transwell assay was conducted to determine the functions of miR-100 overexpression or knockdown in NSCLC cell invasion and migration using 8 μm pore sized transwell chamber (BD Biosciences, San Jose, CA, USA) coated with

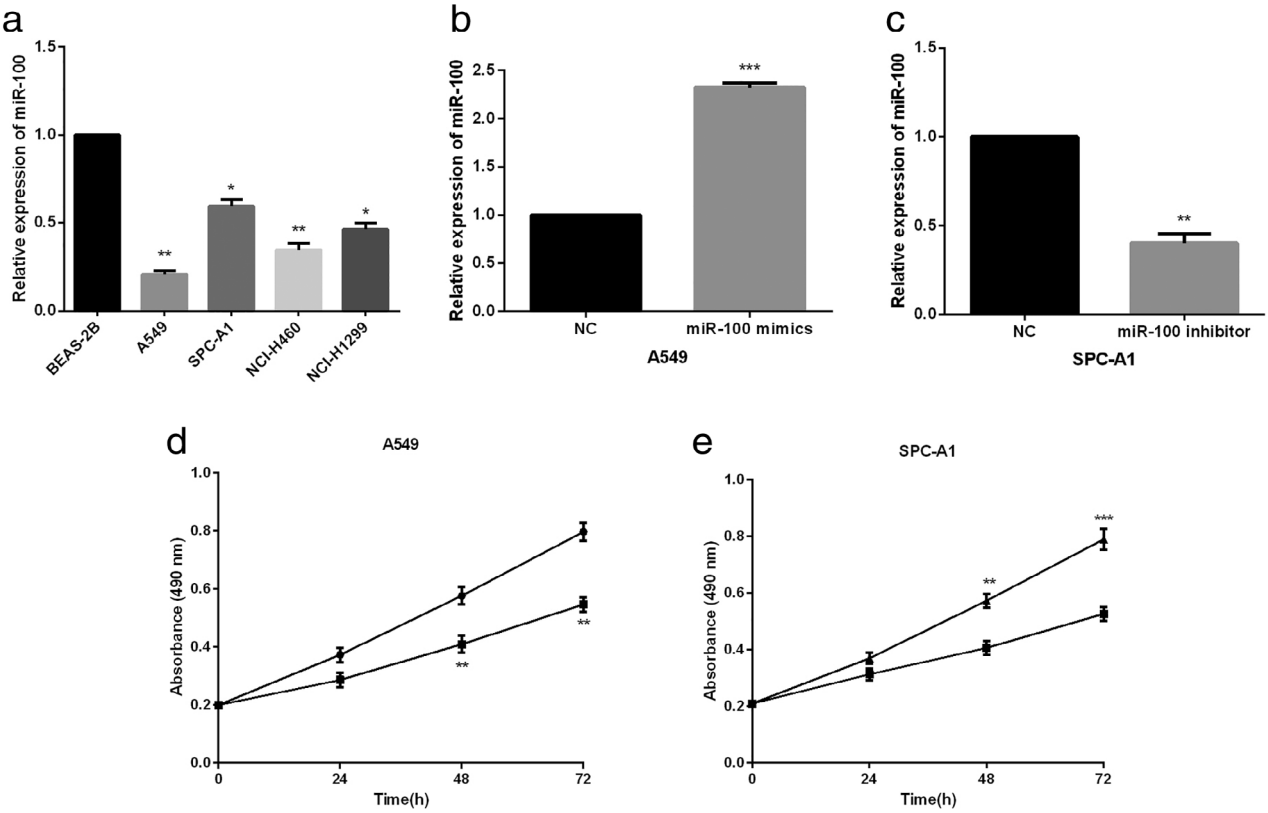

Figure 2 miR-100 inhibited NSCLC cell proliferation. (**a**) qRT-PCR was used to measure miR-100 expressions in NSCLC cells. (**b**, **c**) miR-100 overexpression or inhibition was confirmed by qRT-PCR. (**d**, **e**) MTT assays were performed to detect the functions of miR-100 in NSCLC cell proliferation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (d: —●— NC and —■— miR-100 mimics; e: —■— NC and —▲— miR-100 inhibitor).

or without Matrigel. Transfected NSCLC cells in serum-free medium were plated into the top chambers. In the meantime, medium containing 10% FBS was placed into the bottom chamber as a chemoattractant. Following incubation at 37°C in a 5% CO_2 atmosphere for 48 hours, cells which had not invaded or migrated through the membrane were wiped with a cotton swab, whereas cells which had adhered to the undersurface of the membrane were fixed with methanol and stained with crystal violet. Invasion or migration capacities were then quantified by counting five independent visual fields under the microscope (Olympus).

Western blot

Total cellular proteins were isolated using RIPA buffer (Thermo Scientific) and quantified with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein lysate was then separated by 10% SDS-PAGE, followed by transfer onto PVDF membrane (Invitrogen). After blocking with 5% skim milk in TBST at room temperature for 2 hours, the membrane was incubated at 4°C overnight with specific primary antibodies: HOXA1 (1:1000, Abcam), cyclin D1 (1:1000, Abcam), c-Myc (1:1000, Abcam), β-catenin(1:1000, Abcam), p-GSK3β (1:1000, Abcam), E-cadherin (1:2000, Abcam), N-cadherin(1:2000, Abcam), Vimentin (1:1000, Abcam) and GAPDH (1:1000, Abcam). After washing with TBST, the membrane was incubated with HRP-labeled secondary antibody (1:3000, Abcam) at room temperature for 2 hours. GAPDH was used as an internal control. Finally, the bands for proteins were visualized using ECL western blot detection reagents (Beyotime).

Luciferase activity assay

The full-length 3′-UTR of HOXA1 with miR-100-5p binding sites were inserted into the downstream of firefly luciferase gene of the pGL3 promoter vector (Invitrogen; Thermo Fisher Scientific, Inc.) by Genepharma (Shanghai, China) to construct the wild-type (WT) or mutant (MUT) HOXA1 3′-UTR for luciferase reporter experiments. NSCLC cells were cotransfected with miR-100 mimics and WT or MUT HOXA1-3′UTR using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 hours, cells were

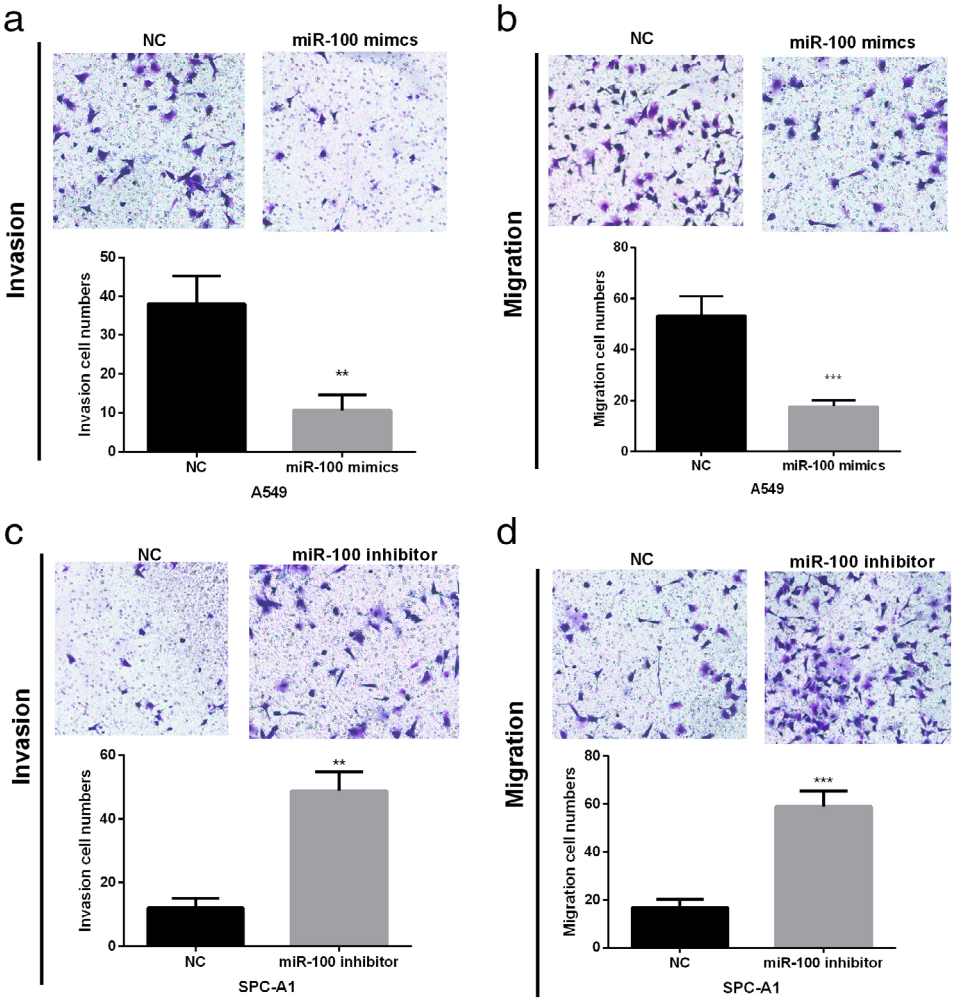

Figure 3 miR-100 suppressed the cell invasion and migration abilities of NSCLC cells. (**a**, **b**) The impacts of miR-100 restoration on NSCLC cell invasion and migration were determined using transwell assays. (**c**, **d**) Transwell assay was performed to determine invasion and migration capacities of miR-100 suppressed NSCLC cells. $^{**}P < 0.01$, $^{***}P < 0.001$.

collected and analyzed with the dual-luciferase reporter assay system (Promega) following the manufacturer's instructions.

Xenograft mouse model

All animal assays were approved by the Research Ethics Committee of The Affiliated Hospital of Qingdao University. A549 cells stably transfected with miR-100 (lenti-miR-100) or a control (lenti-control) were injected subcutaneously into nude mice (4–6 weeks old). Tumor growths were monitored every three days via measuring tumor volumes with the following formula: tumor volume = 1/2 × (length × width^2).

Statistical analysis

All data in the current study were derived from at least three independent experiments. SPSS software version 17.0 (SPSS Inc., Chicago, IL) was used to perform statistical analysis. Multiple comparisons were conducted using ANOVA followed by Scheffe's post-hoc analysis, whereas comparisons between the two groups were performed using Student's *t*-test. Kaplan-Meier method and log-rank test were applied to estimate the survival rates and compare the survival curves, respectively. $P < 0.05$ indicated statistically significant differences.

Results

miR-100 was expressed at low levels in NSCLC and correlated with poor prognosis

To explore the functions of miR-100 in NSCLC, we first measured miR-100 expression levels in NSCLC tissues. qRT-PCR analysis demonstrated that miR-100 expressions were prominently downregulated in NSCLC tissues (Fig 1a). According to the mean level of miR-100, the NSCLC patients were assigned into low and high miR-100 groups. Results showed that low miR-100 in NSCLC tissues were associated

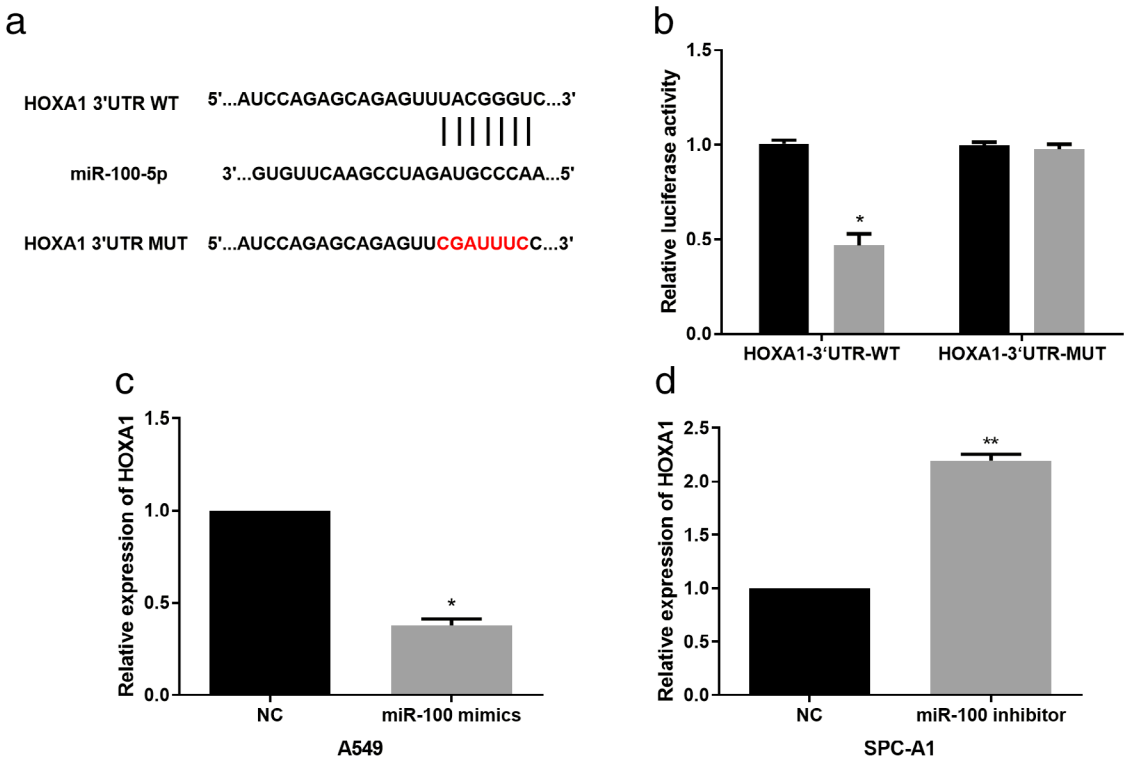

Figure 4 HOXA1 was a direct target of miR-100 in NSCLC cells. (**a**)The WT and MUT binding sites of miR-100 on HOXA1 3′-UTR. (**b**) Dual-luciferase reporter assay was used to confirm the association between miR-100 and HOXA1 in NSCLC cells (■ NC and ■ miR-100 mimics). (**c**, **d**) Regulatory effects of miR-100 on HOXA1 expressions in NSCLC cells. $*P < 0.05$, $**P < 0.01$.

with the malignant clinicopathologic characteristics. Statistical analysis of the clinicopathologic data indicated that miR-100 expression in NSCLC tissues was significantly related to lymph node metastasis ($P = 0.0026$) and tumor TNM stage ($P = 0.0030$). However, there was no association between its expression and patient age, gender, tumor size, histologic type, or smoking ($P > 0.05$; Table 2). Moreover, Kaplan-Meier analysis indicated that NSCLC patients in the low miR-100 group had a significantly poor overall survival (OS) compared with those in the high miR-100 group (Fig 1b)

miR-100 upregulation suppressed NSCLC proliferation

To confirm the above findings, we next detected the miR-100 expressions in NSCLC cells. As expected, we found that miR-100 expressions in all NSCLC cells were significantly downregulated when compared to the normal cells (Fig 2a). miR-100 gain-function and loss-function assays were performed to determine the biological functions of miR-100 in A549 or SPC-A1 cells, according to their endogenous low and high miR-100 expressions. The efficiency of transfections were confirmed using qRT-PCR (Fig 2b,c). Subsequently, MTT assays showed that miR-100 overexpression dramatically repressed NSCLC cell proliferation ability, whereas miR-100 inhibitor had the opposite effect (Fig 2d,e).

miR-100 overexpression suppressed NSCLC cell invasion and migration

Transwell assay was then performed to determine the impacts on NSCLC invasion and migration. Data revealed that miR-100 restoration significantly suppressed A549 cell invasion and migration capacities (Fig 3a,b). In contrast, we found that miR-100 knockdown promoted SPC-A1 cell invasion and migration in comparison with the control group (Fig 3c,d). All these findings implied that miR-100 exerted antitumor functions in NSCLC.

HOXA1 was an important target formiR-100

To further explore the downstream regulatory mechanism of miR-100 in NSCLC, we next explored potential targets of miR-100. According to Targetscan, HOXA1 was an important target of miR-100, and potential miR-100 target sequences in the HOXA1 3′UTRs were identified (Fig 4a). We then performed dual-luciferase reporter analysis to confirm the association between HOXA1 and miR-100. As shown in Fig 4b, miR-100 mimics prominently decreased the luciferase activity in the HOXA1-3′UTR-WT group, whereas the relative

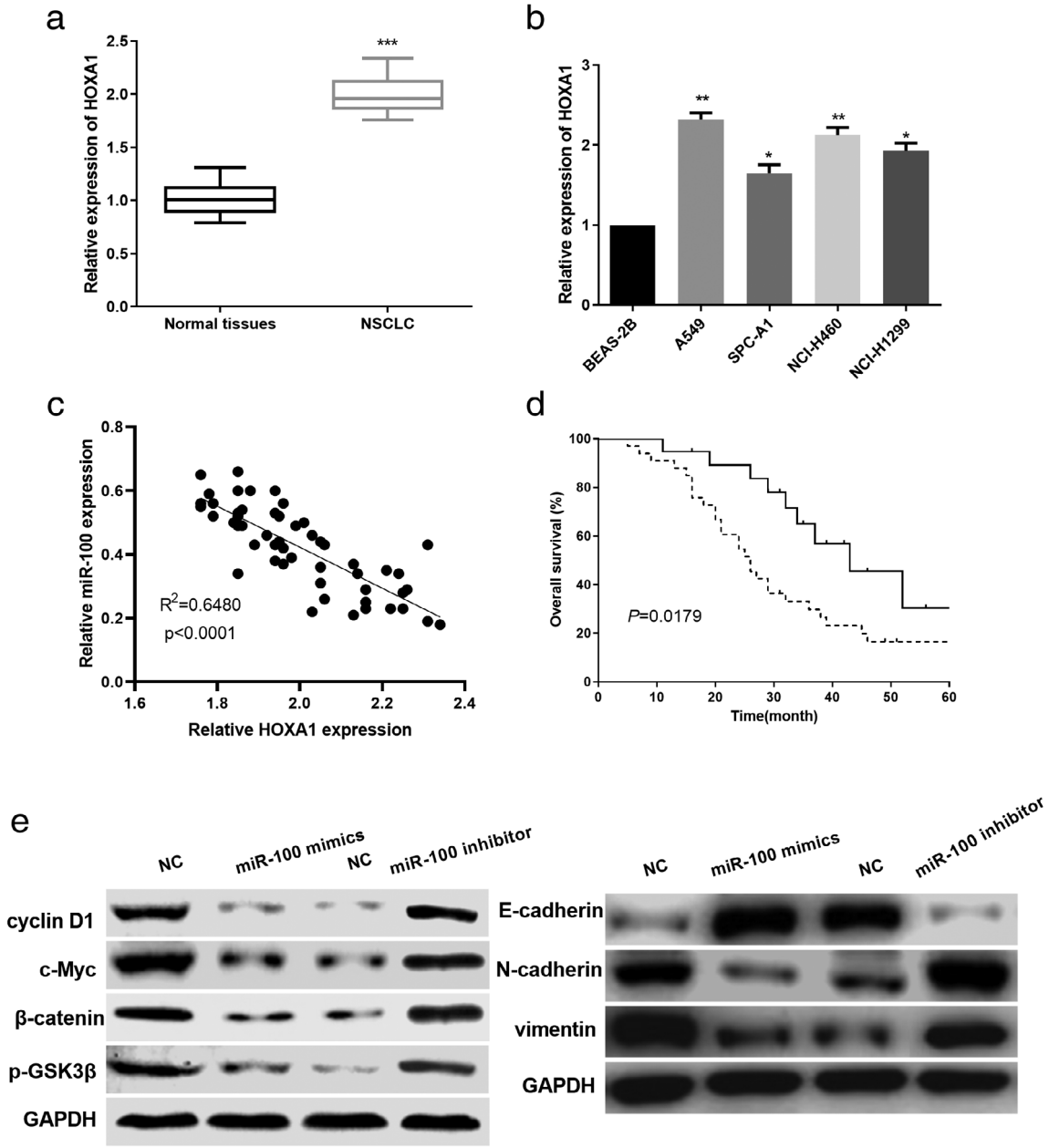

Figure 5 miR-100 regulated NSCLC EMT and Wnt/β-catenin. (**a**, **b**) qRT-PCR assays were performed to detect the expressions of HOXA1 in NSCLC tissues and cells. (**c**) A negative correlation between miR-100 and HOXA1 expressions in NSCLC tissues was identified. (**d**) Survival curves of NSCLC patients with low and high HOXA1 expressions (—— HOXA1(-) and – – – HOXA1(+)). (**e**) Western blots were applied to determine the functions of miR-100 in NSCLC EMT and Wnt/β-catenin pathway. $^{*}P < 0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$.

luciferase activity in the HOXA1-3′UTR-MUT group exhibited no notable changes in cells treated with miR-100 mimics. To further confirm the regulatory roles of miR-100 in HOXA1 expression, we measured HOXA1 expressions in A549 and SPC-A1 cells transfected with miR-100 mimics or inhibitor. We found that miR-100 overexpression was able to suppress HOXA1 expressions, whereas miR-100 inhibition had the opposite effects in NSCLC cells (Fig 4c,d).

miR-100 regulated EMT and Wnt/β-catenin in NSCLC cells

As HOXA1 was identified as a direct target for miR-100 in NSCLC cells, we then determined the clinical value of HOXA1 in NSCLC. First, the expressions of HOXA1 in NSCLC tissues cells were detected using qRT-PCR and the results demonstrated that HOXA1 expressions were significantly upregulated in NSCLC tissues and cells (Fig 5a,b).

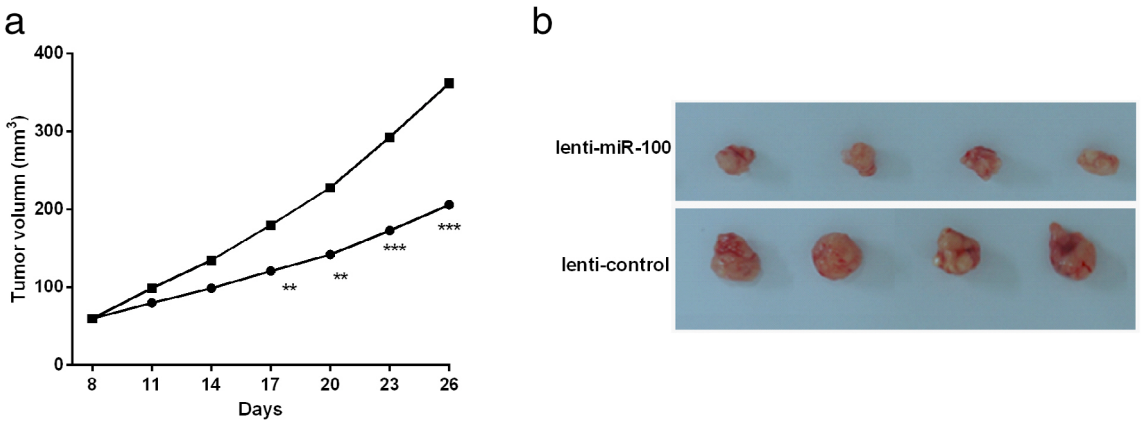

Figure 6 miR-100 inhibited NSCLC tumorigenesis in vivo. (**a**) Growth curve of tumor volumes in different groups (—●— lenti-miR-100 and —■— lenti-control). (**b**) Representative images of tumors in different groups. $^{**}P < 0.01$, $^{***}P < 0.001$.

Thereafter, a negative correlation between miR-100 and HOXA1 expressions in NSCLC tissues was identified (Fig 5c). Moreover, Kaplan-Meier analysis was applied to examine the prognostic values of HOXA1 in NSCLC patients. We found that NSCLC patients with high HOXA1 expressions had shorter overall survival (OS) than those with low HOXA1 expressions (Fig 5d). Western blot was then conducted to determine the potential mechanisms underlying the functions of miR-100 in NSCLC. Results on the impact of miR-100 on Wnt/β-catenin in NSCLC cells indicated that the expressions of cyclin D1, c-Myc, activated β-catenin and p-GSK3β were significantly reduced by miR-100 overexpression (Fig 5e). We subsequently detected the EMT-related markers E-cadherin, N-cadherin and vimentin for evaluating the effects of miR-100 on EMT cell process. It was found that miR-100 overexpression enhanced the E-cadherin expressions, whereas it decreased the N-cadherin and vimentin expressions (Fig 5e).

miR-100 overexpression suppressed NSCLC tumor growth in vivo

In vivo xenograft assays were performed to observe the effect of miR-100 on NSCLC growth. It was found that miR-100 overexpression remarkably restrained the growth of A549 xenograft tumors and the tumor growth rate in the lenti-miR-100 group was significantly lower than those in the control group (Fig 6a,b). These results indicated that overexpression of miR-100 could suppress the tumor sizes and growth rates of NSCLC.

Discussion

NSCLC is one of the most malignant tumors and is usually diagnosed at advanced stages, leading to poor survival.[25] During NSCLC tumorigenesis, a variety of epigenetic and genetic alternations typically occur. Moreover, despite advances in novel diagnostic and surgical approaches, the survival rates of NSCLC remain unsatisfactory, and new therapeutic biomarkers have yet to be explored.[26] Therefore, in order to seek more effective treatment strategies, contemporary studies pay attention to the molecular mechanism involved in the genetic or epigenetic process of NSCLC.[27] Recent tumor research has proved that miRNA is able to regulate various biological processes such as apoptosis, proliferation and metastases.[28–30] Previous studies have elucidated that the alterations of miRNA expressions could influence NSCLC tumorigenesis. Many upregulated miRNAs such as miR-1269a, miR-146, and miR-378 have been reported to promote NSCLC cells.[31–33] In contrast, some downregulated miRNAs such as miR-148a, miR-200c, and miR-204 have been reported to repress NSCLC development.[34–36] Thorough understanding of the association between abnormally expressed miRNAs and NSCLC development may therefore help to identify novel diagnostic and therapeutic biomarkers for the treatment of NSCLC.

miR-100 has been identified in various human cancers. In the study by Luan *et al.* overexpression of miR-100 was found to inhibit human glioblastoma cell chemosensitivity, proliferation and migration through FGFR3.[37] miR-100 inhibited nasopharyngeal carcinoma cell invasion and migration by regulating IGF1R in the report by Sun *et al.*[38] In NSCLC, previous studies showed that miR-100 overexpression inhibited NSCLC development, including growth, migration and chemosensitivity, via directly targeting FGFR3.[39] In addition, miR-100 functioned as a poor prognostic factor for NSCLC patients and miR-100 overexpression could lead to growth inhibition, G2/M cell cycle arrest and apoptosis enhancement in NSCLC cells by post-transcriptionally regulating PLK1 expression.[40] According to another study, low expression level of miR-

100 in NSCLC patients was found to be closely associated with poor prognosis of patients.[41] All these studies demonstrated the importance of miR-100 in NSCLC via regulation of different target genes. Based on the above research, we further investigated the functions and potential mechanisms of miR-100 in the regulation of the progression of NSCLC via regulating other targets. In this study, miR-100 was frequently downregulated in NSCLC tissues and relevant with the malignant phenotypes of NSCLC patients. Moreover, miR-100 overexpression was able to suppress NSCLC cell proliferation, invasion and migration capacities via modulating EMT and Wnt/β-catenin. Additionally, in vivo xenograft assays also demonstrated that miR-100 significantly suppressed NSCLC growth.

Increasing studies have shown that HOXA1 could influence different cellular processes including apoptosis, proliferation, and EMT, and overexpression of HOXA1 is sufficient to cause malignant transformations of nontumorigenic epithelial cells.[42] A study by Zhang *et al.* showed that HOXA1 upregulation enhanced NSCLC tumorigenesis and progression.[43] In particular, a previous study showed that downregulation of HOXA1 gene affects small cell lung cancer cell survival and chemoresistance under the regulation of miR-100.[44] However, the correlation between miR-100 and HOXA1 in NSCLC remain unclear. Herein, we found that HOXA1 was upregulated in NSCLC tissues and indicated a poor prognosis in NSCLC patients. Furthermore, we identified that HOXA1 was a candidate target of miR-100 in NSCLC cells. We demonstrated that the functional effects of miR-100 in NSCLC were regulated by HOXA1.

In conclusion, our findings indicated that the downregulated miR-100 in NSCLC was associated with poor prognosis and adverse phenotypes in NSCLC patients. The functional assays indicated that upregulation of miR-100 prominently suppressed NSCLC cell proliferation, invasion and migration via the repression of HOXA1, as well as the regulation of EMT and Wnt/β-catenin. These results may be helpful to better understand NSCLC pathogenesis and provide beneficial clues for the diagnosis and treatment of NSCLC.

Disclosure

The authors declare that there are no conflicts of interest.

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