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RESEARCH ARTICLE

MicroRNA-30e inhibits proliferation and invasion of non-small cell lung cancer via targeting SOX9

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Abstract

Previous studies have reported that microRNA-30e (miR-30e) is dysregulated in multiple human cancers. However, the expression, functions and molecular mechanism of miR-30e in NSCLC remain unknown. In this study, we found that miR-30e was expressed at a low level in NSCLC tissues and cell lines. In NSCLC cell lines, enforced expression of miR-30e could inhibit cell proliferation and invasion in vitro. In addition, miR-30e negatively regulated SOX9 expression through directly binding to the 3′UTR of SOX9, and an inverse correlation was found between miR-30e and SOX9 mRNA expression in NSCLC tissues. Moreover, knockdown of SOX9 led to decreased proliferation and invasion of NSCLC cells. Taken together, miR-30e acts as a tumor suppressor in NSCLC, and inhibits cell proliferation and invasion possibly by directly targeting SOX9. These findings might provide novel therapeutic targets for NSCLC.

Keywords MicroRNA-30e · Proliferation · Invasion · SOX9 · Non-small cell lung cancer

Introduction

Lung cancer is one of the most common types of malignancy in humans and a leading cause of cancer-related death among both men and women around the world [1, 2]. Non-small cell lung cancer(NSCLC), an aggressive type of lung cancer, has a higher tumor recurrence and metastasis [3, 4]. The 5-year survival rate of NSCLC patients still has shown no significant increase [2], Therefore, development of novel effective therapeutic strategies for patients with NSCLC is essential.

MicroRNAs (miRNAs) are a new series of endogenous, single-stranded and non-coding RNAs, whose length is about 21–25 nucleotides [5]. miRNAs can directly bind to the 3′-untranslated region (3′UTR) of their target genes to promote gene degradation or inhibit translation [6, 7]. miRNAs have been demonstrated to be involved in cell proliferation, differentiation, migration, invasion and other biological processes [8–10]. Especially, abnormally expressed miRNAs have been observed in a wide range of human cancer. Moreover, miRNAs may function either as oncogenes or tumor suppressor, depending on the regulated tumor types and their targeted genes [11, 12]. These findings suggested that miRNAs might be a therapeutic target for human cancers.

In this study, we found that miR-30e was decreased in NSCLC tissues and cell lines by qRT-PCR. Function analysis showed that miR-30e overexpression suppressed NSCLC cell proliferation and invasion in vitro. We further identified SOX9 as a target for miR-30e.Taken together, our data suggested that miR-30e could inhibit NSCLC progression via SOX9.

Yanwei Cui and Lei Zhao contributed equally to this work and should be considered co-first author.

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Materials and methods

Human tissues

NSCLC tissues and paired normal adjacent tissues (NATs) were obtained from 52 patients undergoing surgery resection at the Department of Thoracic surgery (The First Affiliated Hospital of Dalian Medical University, China). None of these patients received adjuvant therapy before surgery. Both NSCLC tissues and NATs were immediately frozen in liquid nitrogen and then stored at − 80 °C prior to RNA isolation. Written informed consent was provided by all patients and this study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Dalian Medical University.

Cell lines and cell culture

Four NSCLC cell lines (H1299, H460, SPC-A1, A549) and one normal human bronchial epithelial cell line (16HBE) were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). All cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) at 37 °C in a humidified atmosphere with 5% CO_2.

Cell transfection

miR-30e mimics, miRNA mimic negative control (miR-NC), small interfering RNA(siRNA) targeting SOX9 (si-SOX9) and its negative control (si-NC) were generated by GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was adopted to perform cell transfection following the manufacturer's protocol.

Quantitative real-time PCR

Total RNA was extracted from tissues or cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantified using an ND-2000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Inc., Wilmington, DE, USA), following the manufacturer's protocols. Reverse transcription was carried out with M-MLV (Promega, Madison, WI, USA). Relative expressions of miR-30e and SOX9 mRNA were detected by SYBR Premix Ex Taq™Kits (TaKaRa, Tokyo, Japan) using Applied Biosystems® 7900HT Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). U6 (for miRNAs) and GAPDH (for SOX9) were used as control. Relative expression levels were determined according to the 2-ΔΔCt method. Primer: miR-30e forward 5′-GTGCCTCACTGCGTCTC-3′, reverse 5′-GAAAGCCGG TGCGTAGCTG-3′; SOX9 forward 5′-AGGAAGTCGGTG AAGA ACGG-3′, reverse 5′-CGCCTTGAAGATGGCGTT G-3′; GAPDH forward 5′-TGACTTCAA CAGCGACAC CCA-3′, reverse 5′-CACCCTGTTGCTGTAGCCAAA-3′; U6 forward 5′-GACCGAGTGTAGCAAGG-3′, reverse 5′-GTTCTTCCGAGAACATATAC-3′.

MTT assay

Transfected cells were harvested 24 h after transfection and re-seeded in 96- well plates at a density of 3000 cells/well. Cells were then incubated at 37 °C for 24, 48, 72, and 96 h. At each time point, 20μL MTT solution (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) was added in each well and incubated for another 4 h at 37 °C. The culture medium was removed and 150μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added into each well. Finally, the optical density (OD) at 490 nm was detected using an enzyme-linked immunosorbent assay reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell invasion assay

Cell invasion ability was detected using transwell chambers (8 μm pore size; BD Biosciences, San Jose, CA, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA). Transfected cells were harvested 24 h after transfection, and 5×10^4 transfected cells were washed twice with PBS, resuspended in 200 μL serum-free DMEM and added into the upper chamber. 500 μL DMEM containing 20% fetal bovine serum (FBS) was filled into the lower chamber as a chemoattractant. After incubation at 37 °C for 48 h, non-invaded cells were wiped away carefully with cotton wool. The invaded cells on the lower surface were fixed in 4% paraformaldehyde, stained with 0.5% crystal violet, photographed and counted in five randomly selected areas under a 200× microscope field.

Bioinformatics prediction and luciferase reporter assay

TargetScan (http://www.targetscan.org/) and miRanda (http://www.Microrna.org/microrna/) were used to predict the potential targets of miR-30e.

For luciferase reporter assay, HEK293T cells (Shanghai Institute of Biochemistry and Cell Biology, China) were co-transfected with pmirGLO-SOX9-3′UTR wild-type (Wt) or pmirGLO-SOX9-3′UTR mutant (Mut), and miR-30e mimics or miR-NC using Lipofectamine 2000 according to the manufacturer's instructions. After incubation for 48 h at 37 °C, cells were harvested and luciferase activity was detected using Dual-Luciferase® Reporter Assay system (Promega

Corporation, Madison, WI, USA) following the manufacturer's protocol.

Western blot and antibody

The cells were harvested and lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (Roche, Mannheim, Germany). The protein concentration was measured using the BCA assay (KeyGen Biotech). Equal amounts of protein were separated by 10% SDS–polyacrylamide gels and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with the specific antibodies (appropriate concentration referred to specification) and the protein band was detected with enhanced chemiluminescence system.

Antibody: SOX9 antibody (1:1000 dilution; sc-166505; Santa Cruz Biotechnology, CA, USA), GADPH antibody (1:1000 dilution; sc-166574; Santa Cruz Biotechnology, CA, USA), goat anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (1:5000 dilution; Abcam, Cambridge, UK).

Statistical analysis

All data were presented as the mean ± standard deviation. SPSS 15.0 was used for statistical analyses. $P < 0.05$ was considered as statistically significant difference.

Results

MiR-30e is downregulated in NSCLC

The expressions of miR-30e in 52 pairs of NSCLC tissues and matched NATs were detected by using qRT-PCR. The results showed that miR-30e was significantly downregulated in 52 NSCLC tissues compared with NATs (Fig. 1a, $P < 0.05$). Then, the expression level of miR-30e was detected in four NSCLC cell lines and one normal human bronchial epithelial cell line (16HBE). As shown in Fig. 1b, there was lower expression of miR-30e in NSCLC cell lines than in HBE ($P < 0.05$). These results suggested that lower level miR-30e may be associated with NSCLC carcinogenesis and progression, and should be considered a potential biomarker for predicting poor prognosis in NSCLC.

MiR-30e overexpression suppresses NSCLC cell proliferation and invasion in vitro

To investigate the effect of miR-30e overexpression on cell proliferation and invasion of NSCLC, H1299 and A549 cells were transfected with miR-30e mimics or miR-NC. After 48 h transfection, miR-30e was significantly increased in H1299 and A549 cells transfected with miR-30e mimics compared with miR-NC (Fig. 2a, $P < 0.05$). It showed that miR-30e mimics were effective as endogenous miR-30e in vitro. In addition, following transfection, MTT assay and cell invasion assay were performed. As shown in Fig. 2b, c, upregulation of miR-30e obviously suppressed the proliferation ($P < 0.05$) and invasion ($P < 0.05$) of H1299 and A549 cells.

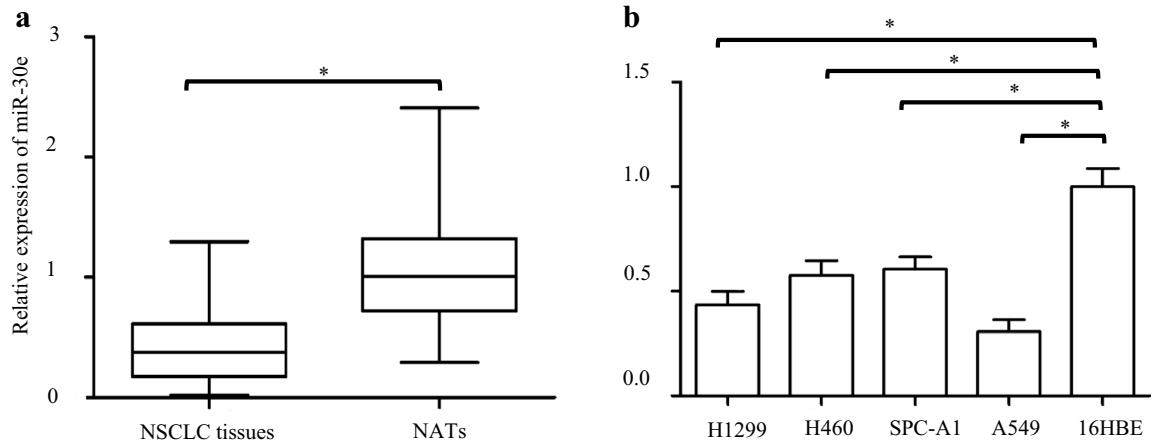

Fig. 1 MiR-30e is downregulated in NSCLC. **a** qRT-PCR was performed to measure miR-30e expressions in 52 pairs of NSCLC tissues and matched NATs. **b** The expression levels of miR-30e in NSCLC cell lines (H1299, H460, SPC-A1, A549) and normal human bronchial epithelial cell line (16HBE) were assessed by using qRT-PCR. The data were represented as the mean ± S.D. of three independent experiments, *$P < 0.05$

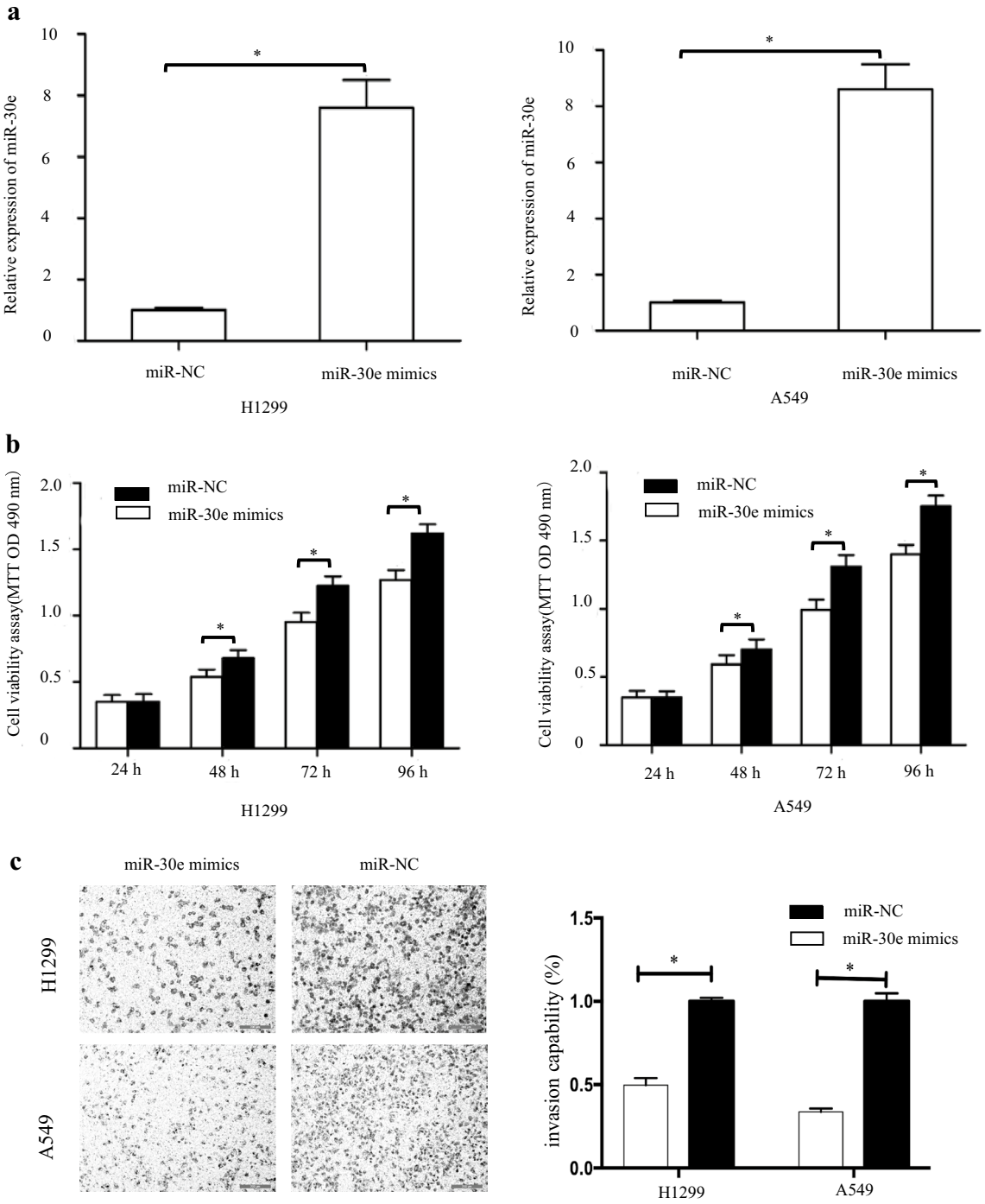

Fig. 2 MiR-30e overexpression suppresses NSCLC cell proliferation and invasion in vitro. **a** H1299 and A549 cells were transfected with miR-30e mimics or miR-NC. miR-30e expression was increased in H1299 and A549 cells by using qRT-PCR. **b** Proliferation of H1299 and A549 cells transfected with miR-30e mimics or miR-NC was evaluated by using MTT assay. **c** miR-30e overexpression decreased cell invasion of H1299 and A549 cells. The data were represented as the mean ± S.D. of three independent experiments, $^{*}P < 0.05$

SOX9 is the direct target gene of miR-30e

To explore the underlying mechanism of miR-30e in NSCLC, bioinformatics analysis was performed to predict the targets of miR-30e. SOX9 was identified as a candidate target of miR-30e (Fig. 3a,). qRT-PCR and western blot revealed that SOX9 expression was downregulated in miR-30e mimics-transfected H1299 and A549 cells at both mRNA and protein level as predicted (Fig. 3b, c, $P < 0.05$). To examine whether the 3′UTR of SOX9 could

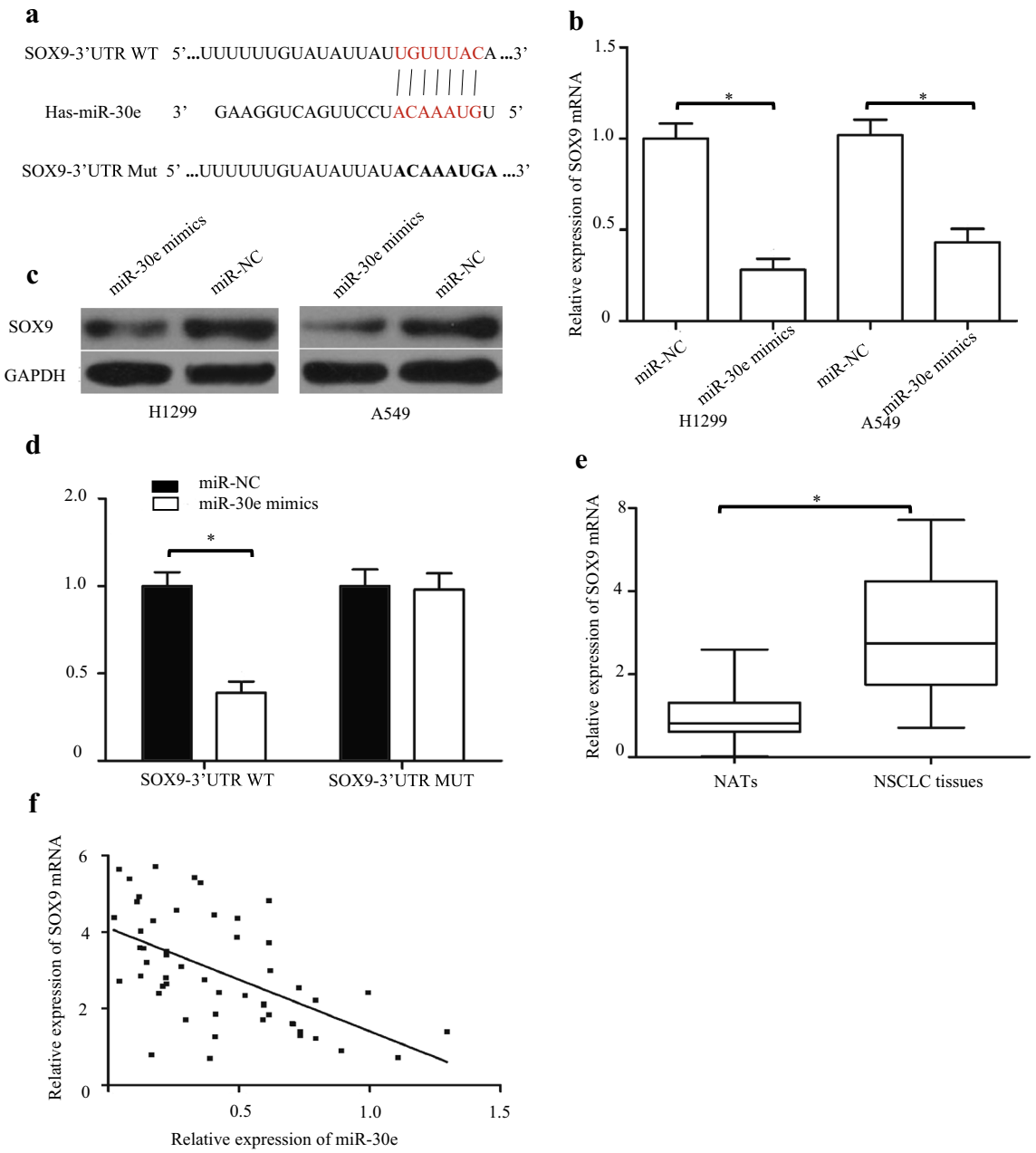

Fig. 3 SOX9 is the direct target gene of miR-30e in NSCLC. **a** Sequence alignment of miR-30e with the putative binding sites in the wild-type regions of SOX9. **b** qRT-PCR analysis of SOX9 mRNA expression in A549 and H1299 cells transfected with miR-30e or miR-NC. **c** Western blot of SOX9 protein expression in H1299 and A549 cells transfected with miR-30e mimics or miR-NC. **d** Dual-luciferase reporter assay showed that miR-30e mimics reduced the intensity of fluorescence in HEK293T cells transfected with SOX9-Wt, while it had no effect on the SOX9-mut vector. **e** qRT-PCR analysis of SOX9 mRNA expression in 52 pairs of NSCLC tissues and NATs. **f** Pearson's correlation analysis between the expression levels of the miR-30e and SOX9 mRNA in NSCLC tissues. $r = -0.5626$. The data were represented as the mean ± S.D. of three independent experiments, $^*P < 0.05$

be directly targeted by miR-30e, luciferase reporter assay was performed. The result showed (Fig. 3d) that restoring the expression of miR-30e decreased the luciferase activities of pmirGLO-SOX9-3′UTR Wt ($P < 0.05$), but had no effect on pmirGLO-SOX9-3′UTR Mut. In addition, SOX9 expression in 52 pairs of NSCLC tissues and NATs was measured using qRT-PCR. SOX9 mRNA expression levels were evaluated in NSCLC tissues compared with NATs as expected (Fig. 3e, $P < 0.05$). Moreover, a statistically significant inverse correlation was observed by Spearman's correlation analysis between miR-30e and SOX9 mRNA expression level in NSCLC tissues (Fig. 3f, $r = -0.5626$,

$P < 0.001$). Collectively, these results suggested that SOX9 is a direct target of miR-30e.

Downregulation of SOX9 inhibited H1299 and A549 cell proliferation and invasion in vitro

To confirm whether the tumor suppressor role of miR-30e in NSCLC is mediated by SOX9, we knocked down SOX9 in H1299 and A549 cells. Western blot analysis showed that SOX9 was downregulated in H1299 and A549 cells, following transfection with si-SOX9 (Fig. 4a, $P < 0.05$). Following transfection, MTT assay and cell invasion assay showed that si-SOX9 obviously inhibited cell proliferation and invasion of H1299 and A549 cells (Fig. 4b, c, $P < 0.05$). These results further suggested that SOX9 was the direct functional target of miR-30e in NSCLC.

Discussion

NSCLC is the most common type of lung cancer and has the highest mortality rate in China [13]. However, the mechanisms underlying NSCLC occurrence and development have largely remained elusive. Therefore, it is urgent to explore

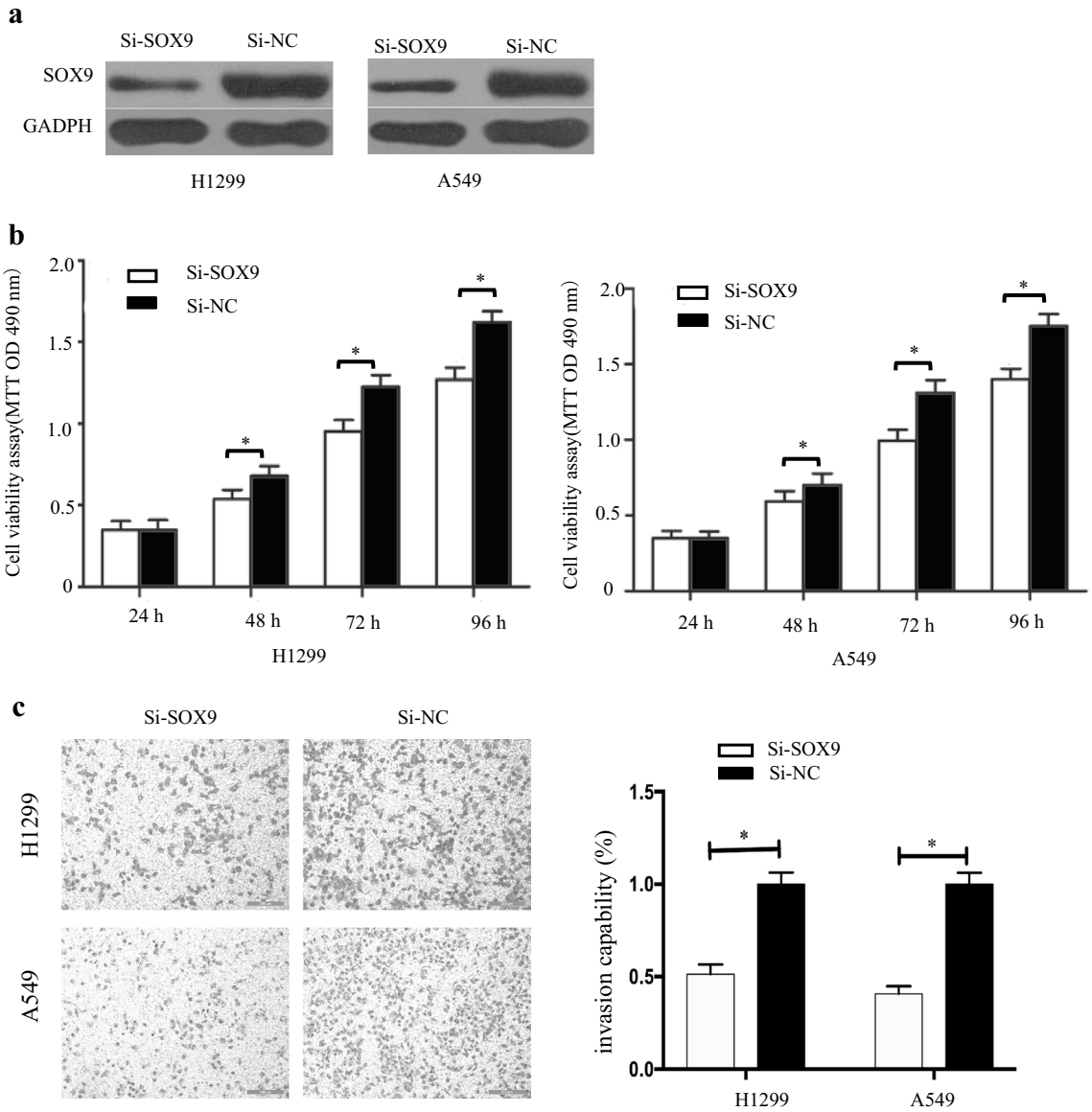

Fig. 4 Downregulation of SOX9 inhibited H1299 and A549 cell proliferation and invasion in vitro. **a** The expression of SOX9 protein in H1299 and A549 cells transfected with si-SOX9 or si-NC was detected by western blot. **b** Cell proliferation of H1299 and A549 cells transfected with si-SOX9 or si-NC was detected by MTT assay. **c** Cell invation of H1299 and A549 cells transfected with si-SOX9 or si-NC was detected by cell invasion assay. The data were represented as the mean ± S.D. of three independent experiments, $^*P < 0.05$

novel therapeutic targets for NSCLC patients. miRNA-based anti-tumor treatments are being developed to target multiple cancer-driven effector genes in tumor-suppressing and oncogenic signaling pathways involved in cell proliferation, apoptosis, invasion, metastasis, and survival [14].

Previous studies have reported that miR-30e was abnormal in multiple types of human cancer. miR-30e functions as a tumor suppressor in human malignancies by affecting different signaling pathways [15, 16, 17]. These findings indicated that miR-30e could be a diagnostic, prognostic biomarker and therapeutic target for human cancers. Here, we found that miR-30e is significantly downregulated in NSCLC tissues and NSCLC cell lines. In addition, overexpression of miR-30e suppressed cell proliferation and invasion of NSCLC. These results suggested that miR-30e played a crucial role in NSCLC growth and metastasis.

Reports have showed that miRNAs need to act on the 3′UTR of target genes. The identification of miR30e targets is important for understanding its roles in tumorigenesis and tumor development. To explore the molecular mechanism underlying the tumor-suppressive roles of miR-30e in NSCLC, bioinformatics analysis was performed to predict targets for miR-30e.In multiple potential target genes, SOX9 was selected for further validation since SOX9 has been demonstrated to be involved in NSCLC carcinogenesis and progression [18]. In addition, SOX9 was identified as a direct target of many diverse miRNAs in NSCLC, including miR-124 [19], miR-206 [20], and miR-32 [21]. Here, we further confirmed that SOX9 was a direct and functional target gene of miR-30e by using luciferase reporter assay, qRT-PCR and western blot analysis. Furthermore, SOX9 mRNA was increased and inversely correlated with miR-30e expression level in NSCLC tissues. Moreover, SOX9 downregulation had similar effects with miR-30e overexpression on proliferation and invasion in NSCLC cells. Taken together, our current study verified that the biological roles of miR-30e on NSCLC were possibly via negative regulation of the expression of its novel identified target, SOX9.

SOX9, a member of the sex-determining region Y (SRY) box family, has been demonstrated to be frequently upregulated in various human cancers such as prostate cancer [22], breast cancer [23] and colorectal cancer [24]. A study by Jiang et al. showed that SOX9 was expressed in high levels in lung adenocarcinoma. Lower expression of SOX9 reduced adhesive and anchorage-independent growth and decreased tumorigenicity in vivo [25]. Zhou et al. reported that SOX9 mRNA and protein were particularly overexpressed in NSCLC tissues and cell lines and obviously associated with the histological stage of NSCLC patients. Multivariate analysis illustrated that SOX9 high expression may be an independent prognostic indicator for the survival of NSCLC patients [26]. Functional experiments indicated that SOX9 knockdown suppressed NSCLC cells growth and invasion [3]. These findings suggested that miR-30e/SOX9-based targeted therapy could be investigated as a new therapeutic strategy for NSCLC.

In summary, this study showed that miR-30e was downregulated in both NSCLC tissues and cell lines. We found that the expression of miR-30e in H1299 and A549 cells could inhibit proliferation and invasion, potentially by directly targeting SOX9. These findings of this study have provided novel therapeutic targets for the future treatments of patients with NSCLC. In the future, we will further explore the differences in the expression of these two genes in different types of lung cancer, and also further study the clinical significance of these two genes that provide a better diagnosis and treatment basis in lung cancer.

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Compliance with ethical standards

Conflict of interest All the authors declared no conflicts of interest in this work.

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