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MiR-1269a acts as an onco-miRNA in non-small cell lung cancer via down-regulating *SOX6*

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Abstract. – OBJECTIVE: Lung cancer, especially non-small cell lung cancer (NSCLC), remains one of the leading death-causing malignant tumors worldwide. MicroRNAs (miRNAs) have been identified to participate in the development and progression of NSCLC. However, the role of miR-1269a in NSCLC still needs to be elucidated. The objective of this study was to investigate the function of miR-1269a in NSCLC and its underlying mechanism.

PATIENTS AND METHODS: Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) was utilized to measure the expression level of miR-1269a in NSCLC tissues and cell lines. After transfection with miR-1269a mimics or inhibitors, the expression level of miR-1269a in NSCL up- or down-regulated. Cell Counting Kit- 8) assay and colony formation assay we sed to measure cell proliferation ability. Flow cy try assay was applied to verify the cell cycl tributions of established cell lines. The pote target of miR-1269a was determi al-luciferase and Western blot

RESULTS: miR-1269a was nifica over-expressed in NSCLC tissue an that adjacent normal tissues. The expr of mi also up-regulated in NSC Up-regulation of mi 269a d the abilities of cell prolifer n, colony f n, and induced cell cycle ion. Meanwh n-regulation of miR eased the ca cities of cell prolifera n, colo mation, and arrested the cell cy It was furth licated that *SOX6* was veri as a target of 269a in NSCLC and ov expressed SOX6 coul rescue the effect of m 269a u egulation.

USI S: Our study demonstrated that could f tion as an onco-miRNA in and p ote NSCLC growth via -regu th xpression of *SOX6*.

Key rds:
1269a, NSCLC, Proliferation, *SOX6*.

Introduction

N -small cell lung cancer (NSCLC), which accounts for 85% of lung cancer, is one of the most common malig NSCLC is also the most comm easo mor m ity in the world[1, 2]. ough the lo of diagnosis and ent have imp apidly, the prognosis N is still very unoptimistic[3]. Therefore, it is i t to find more specific and co lusive eviden NSCLC progression, w serve as a nov rget for the biologi treatment of NSCLC.

MicroRNAs iRNAs) are a subtype of -coding RN with 20-22 nucleotides in len g iRNAs a involved in many biological pro cers, including NSCLC[4,5]. In a general iRNAs can bind to the 3'-UTR of ecific target genes and reduce protein expres tually influencing the proliferation, me sta or oncogenesis of cancers[6,7]. For example, miR-216a may suppress the growth of renal cell carcinoma via suppressing TLR4[8]. In colorectal cancer, miR-218 is indicated as a good prognostic ending and may enhance cell apoptosis through targeting BIRC5[9]. Moreover, in lung cancer, miR-182 inhibits EMT and the metastasis of cells by targeting the Met gene, while miR-106b-5p may promote cell growth and inhibit cell apoptosis via regulating BTG3[10,11].

miR-1269a is an onco-miRNA firstly identified in colorectal cancer, which may promote the development and metastasis of prostate cancer via a feedback loop related to TGF-β[12]. Meanwhile, miR-1269a can affect the occurrence and progression of hepatocellular cancer through targeting LRP6 and SPATS2L[13]. However, the expression of miR-1269a in NSCLC and the underlying mechanism has not been fully elucidated. In this study, we detected the expression of miR-1269a in 49 paired NSCLC and para-tumor tissues as well as in NSCLC cell lines. Subsequently, we constructed several functional experiments to identify the effect of miR-1269a on NSCLC proliferation and cell cycle. SOX6 was also verified as a potential target for miR-1269a in NSCLC. Our study demonstrated miR-1269a as an onco-miR-

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NA in NSCLC via regulating SOX6, which might provide a novel target for the biological therapy of NSCLC.

Patients and Methods

NSCLC and Adjacent Normal Tissues

All the 49 pairs of NSCLC tissues and adjacent normal tissues were obtained from surgical resection on NSCLC patients in the China-Japan Union Hospital of Jilin University from 2009 to 2011. All the samples were conserved in liquid nitrogen for subsequent experiments after removal. Informed consent was obtained from all patients and/or their families. Our study was approved by the Ethics Committee of the China-Japan Union Hospital of Jilin University.

Cell Lines and Cell Culture

All the 6 NSCLC cell lines (H1975, SPCA1, PC-9, H1299, H460, A549) and the human normal bronchial epithelium cell line (BEAS-2B) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). All the cell[illegible] cultured in DMEM (Dulbecco's modified [illegible] medium) (Invitrogen, Carlsbad, CA, US[illegible]taining 10% v/v fetal bovine serum (FBS) ([illegible], Rockville, MD, USA), 100 UI/mL penicillin [illegible] 100 μg/mL streptomycin (Gibco, [illegible]kville, M[illegible] USA), respectively, and incub[illegible]7°C, 5[illegible] CO_2 cell incubator.

Quantitative Real-Tim[illegible] Chain Reaction ([illegible]-PCR)

Total RNA of [illegible]LC tissue[illegible]d normal tissues, and NS[illegible]ell lines we[illegible]tracted using TRIzol [illegible]gen[illegible]itrogen, Car[illegible]bad, CA, USA). SYBR Green PC[illegible]ter Mix Kit (TaKaRa, Dalia[illegible]hina) was use[illegible]etect the expression le[illegible] of miR-1269a. Hou[illegible]keeping U6 was appl[illegible]s an int[illegible]rnal control. The relative expressio[illegible] of S[illegible]6 to glyceraldehyde 3-phosphate dehyd[illegible] (GAPD[illegible] which was used as an [illegible]ternal c[illegible] was [illegible]sured by using the SYBR [illegible] Kit ([illegible], Dalian, China). The relative [illegible]ression le[illegible] was calculated by the $2^{-\Delta\Delta CT}$ me[illegible]d. Each experiment was repeated at least for th[illegible]mers for miR-1269a, U6, SOX6, [illegible] GAPDH were synthesized by Genewiz Co. ([illegible]hou, China). Primer sequences used were as fo[illegible] microRNA-1269a, F: 5'-ACGTTGGATGA[illegible]TCTCATGATAGGCCATC-3', R: 5'-ATTGCGTGTCGTGGAGTCGGCAATGC-3'; SOX6, F: 5'-GCTGGAGGAGATGACTCGGAC-3', R: 5'-GTTCGGTCCACTATTGATGGGG-3'.

Cell Transfection

miR-1269a mimics, negative con[illegible] (NC), inhibitors, and inhibitors negative co[illegible] (INC) were bought from Genewiz Co. (Sucho[illegible]na). Cells were transfected with these [illegible]mposite[illegible]ing lipofectamine3000 (Invitro[illegible] Carlsbad, [illegible]) according to the manuf[illegible]rer's instruction[illegible] pcDNAs for SOX6 o[illegible]xpressi[illegible] were obtained from Ribobio Co. (G[illegible]hina) a[illegible] were transduced with [illegible]ofect[illegible]3000 ([illegible]rogen, Carlsbad, CA, [illegible]A). The eff[illegible] of [illegible]nsfection was confirm[illegible]RT-PCR.

Cell Cou[illegible]ting Ki[illegible] (CCK-8) Assay

Cel[illegible] were treated [illegible] miR-1269a mimics, N[illegible]itors, or INC, [illegible]pectively, and were [illegible]ted into 96-well plates. Subsequently, after [illegible]turing for 0 h, [illegible]4 h, 48 h, 72 h, a total of 10 μL [illegible]CCK8 reagen[illegible] (Dojindo, Kumamoto, Japan) w[illegible]dded to t[illegible] cells for incubation for 1 h at 37[illegible] [illegible]A 550 Microplate Reader (Bio-Rad, He[illegible], CA, USA) was utilized to measure [illegible] absorbance at 470 nm. The experiments were [illegible] for three times.

Colony Formation Assay

H1975 and A549 cells were seeded into 6-well plates at the density of 400 per well, and were cultured for 2 weeks. After dyeing with crystal violet, the numbers of colonies with a diameter greater than 50 μm were measured. The experiments were repeated at least for three times.

Cell Cycle Analysis

Flow cytometry was employed to detect cell cycle distributions. H1975 and A549 cells were washed with cooling phosphate-buffered saline (PBS) after miR-1269a mimics or inhibitors transfection. Then, the cells were re-suspended in 1000 μL binding buffer mixing with 10 μL Propidium Iodide (PI) (Thermo Fisher, Waltham, MA, USA). Cell cycle was detected by flow cytometry (FACS, Arlesheim, Switzerland). The percentage of cells on G0/G1, S, and G2/M phase was recorded and measured.

Dual-Luciferase Assay

Dual-Luciferase Reporter Assay System Kit (Promega, Madison, WI, USA) was used to detect the luciferase activity. Vectors of the wild-type group or mutant group were constructed by

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inserting a luciferase reporter vector with the region of the SOX6-3′-UTR containing the specific wild-type or mutant miR-1269a binding site, respectively. H1975 cells were co-transfected with the luciferase reporter vector and miR-1269a mimics according to the instruction of Lipofectamine3000 (Invitrogen, Carlsbad, CA, USA). Then, the luciferase activity was measured. The experiments were repeated for three times.

Western Blot Analysis

Total protein of the cells was extracted and quantified according to the Protein Extraction Kit Instructions (Beyotime, Nanjing, China). Loading Buffer Mix (Beyotime, Nanjing, China) was added, and the proteins were denatured at 100°C for 5 min. A total of 10 µL protein was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). 5% non-fat milk suspended in Tris-buffered saline-Tween (TBS-T) (Beyotime, Shanghai, China) was used to block non-specific protein loci for 2 h. The membranes were incubated with anti-SOX6 at 4°C over[illegible]. After washing with TBST for 3 times, th[illegible]branes were incubated with HRP-labeled [illegible]dary antibody for 2 h at room temperature. [illegible] washing with phosphate-buffered saline-tw[illegible] (PBS-T) for 3 times, the membran[illegible]ere imag[illegible] on a gel imager. Finally, gra[illegible]f relativ[illegible] bands were analyzed. The e[illegible]ession[illegible] GAPDH was used as a reference, [illegible]he ratio[illegible]SOX6 to GAPDH was calculated an[illegible]tic[illegible]

Statistical Anal[illegible]

All quantitati[illegible] were expres[illegible]s mean ± standard d[illegible]atio[illegible]e difference between groups was compared [illegible]ng One-way ANOVA, follo[illegible] by the Post-[illegible]est (Least Significant D[illegible]erence). Statistical P[illegible]uct and Service Solu[illegible]s (SPSS) 16.0 Version Software (SPSS Inc[illegible]icago[illegible]L, USA) and GraphPad Prism Versio[illegible]ftware ([illegible]Jolla, CA, USA) were employed[illegible]ta a[illegible]sis. $p<0.05$ was considered [illegible]tistica[illegible]cant.

Results

[illegible]-1269a Was Over-Expressed in [illegible]LC Tissues and Cell Lines

T[illegible]validate the expression level of miR-1269a in NSCLC, we detected miR-1269a expression in 49 pairs of NSCLC tissues and adjacent normal tissues. Results showed that the expression of miR-1269a in NSCLC tissues was [illegible] higher than that of adjacent normal[illegible]ues ([illegible]gure 1A). The relative expressi[illegible]was shown in Figure 1B. Meanwhile, w[illegible]easured the expression level of miR-1269a i[illegible]CLC-derived cell lines and 1 norm[illegible]l bronc[illegible]pithelial cell line BEAS-2B[illegible]sults dem[illegible]ted that the expression o[illegible]iR-1269a was [illegible]kably higher in NSC[illegible] cell li[illegible] (Figure 1[illegible]). All these data above[illegible]hat mi[illegible]1269a might act as an[illegible]ogen[illegible]SCLC.

Up-Regula[illegible]of miR-126[illegible]elerated the Cell[illegible]oli[illegible]ion of NSC[illegible] Cells

Next, t[illegible] explore[illegible]effect of miR-1269a on NSCLC cells, we ov[illegible]ressed miR-1269a in H[illegible]s by transfect[illegible]miR-1269a mimics. T[illegible]e expression of miR-1269a was up-regulated [illegible]out 8.03-fold i[illegible] the miR-1269a mimics group c[illegible]pared to the[illegible]gative control group (Figure 2[illegible]The CCK8[illegible]say demonstrated that transfec[illegible]with[illegible]R-1269a mimics significantly promote[illegible] proliferation of H1975 cells when compared with the NC group (Figure 2B). Me[illegible]we found that more colonies were for[illegible]ed [illegible]H1975 cells of the miR-1269a up-regulation group compared with the control group (Figure 2C, 2D). Furthermore, we detected the cell cycle of established H1975 cells and discovered that miR-1269 over-expression induced the transition of G0/G1 to S phase (Figure 2E). These data showed that up-regulation of miR-1269a accelerated cell growth and induced cell cycle transition of NSCLC cells.

Down-regulation of miR-1269a Inhibited the Cell Proliferation of NSCLC Cells

Similarly, we then knocked down the expression of miR-1269a in A549 cells. The expression level of miR-1269a in A549 cells was markedly decreased after miR-1269a inhibitors transfection (Figure 3A). The CCK8 experiment demonstrated that the knockdown of miR-1269a inhibited the cell proliferation of A549 cells (Figure 3B). Colony formation assay illustrated the same result with the CCK8 assay (Figure 3C, D). Moreover, miR-1269a inhibitors significantly blocked A549 cells in the G0/G1 phase when compared with the INC group (Figure 3E). These results indicated that down-regulation of miR-1269a inhibited cell proliferation and induced G0/G1 arrest of NSCLC cells.

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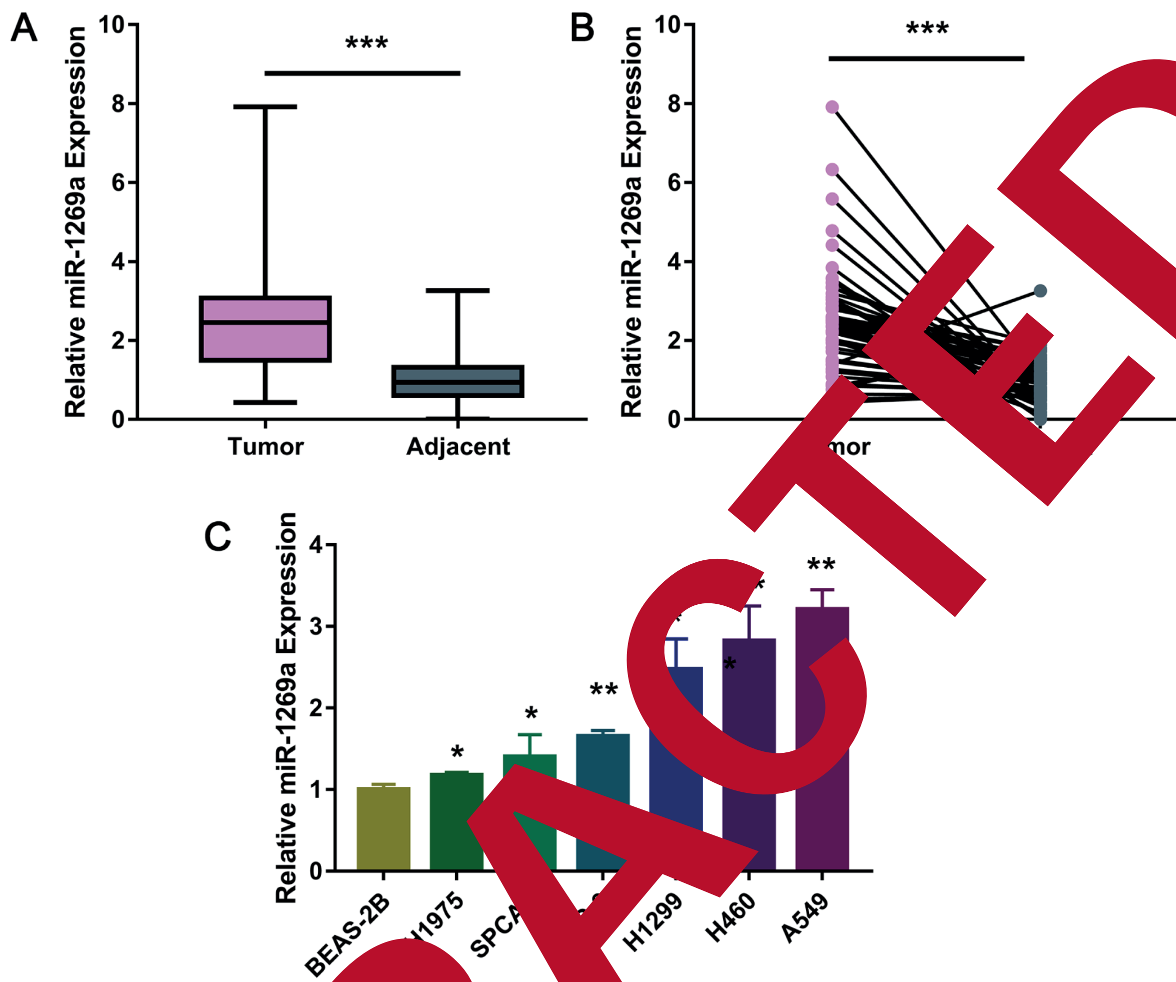

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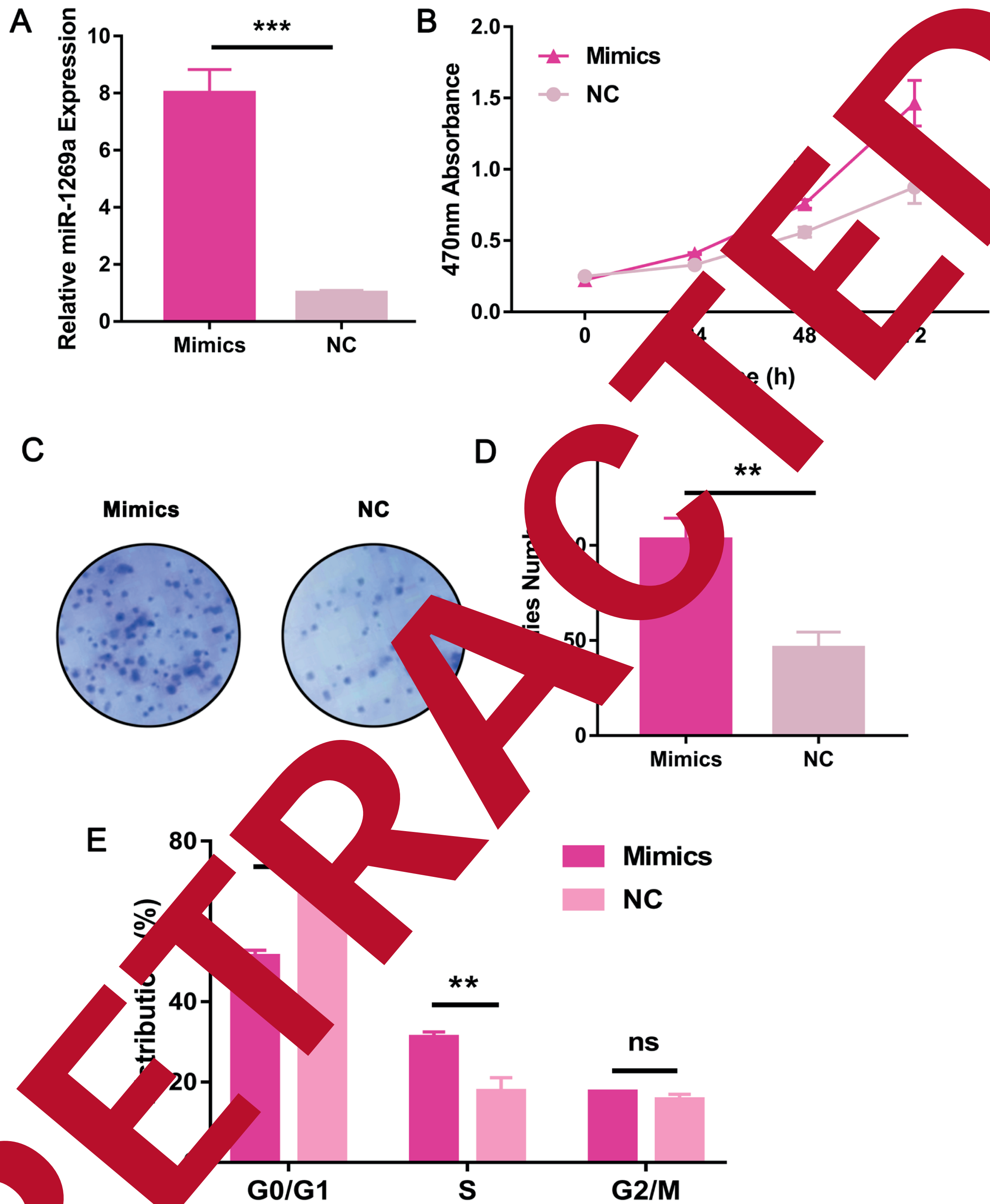
Figure 1. MiR-1269a expressi[illegible] NSCLC t[illegible]es and cell l[illegible]es. ***A-B***, The expression levels of miR-1269a in NSCLC tissues and paired adjacent lung tissu[illegible] analy[illegible]. ***C,*** MiR-144-5p expression in BEAS-2B cells and NSCLC cell lines (H1975, SPCA1, PC-9, H12[illegible]re shown as mean ± SD. The experiments were repeated for three times. $^*p<0.05$, $^{**}p<0.01$ [illegible]<0.001.

SOX6 Was a Direct [illegible]t of miR-1269a in NSCL[illegible]

Furt[illegible]more, to investiga[illegible] the underlying mech[illegible]ism of miR-1269a in NSCLC, we searc[illegible] sever[illegible]atabases, including miRBase, Targe[illegible]d miRW[illegible]. We found that SOX6 [illegible]ight be [illegible]ential [illegible]rget for miR-1269a. We [illegible]onfirm[illegible]ssumption by constructing the [illegible]IR-REP[illegible] vector carrying mutant or wil[illegible]ype miR-1269a binding SOX6-3'-UTR re[illegible]4A). Dual-luciferase assay indi[illegible]ed that the activity of luciferase was signi[illegible]tly decreased in the wild-type group, but n[illegible]rence was found in the mutant group (Fig[illegible]e 4B). We detected the mRNA expression of SOX6 in NSCLC tissues and adjacent normal tissues, respectively, and found an evident decrease of SOX6 mRNA expression in the NSCLC group (Figure 4C). Meanwhile, Western blot was used to measure the protein expression of SOX6 protein in NSCLC cells. Over-expression of miR-1269a inhibited the protein expression of SOX6 in H1975, whereas down-regulation of miR-1269a increased the protein expression of SOX6 protein in A549 (Figure 4D, 4E). In addition, we analyzed the relationship between miR-1269a and SOX6 in 49 pairs of NSCLC tissues and adjacent normal tissues, and found that there was a significant negative correlation ($R^2=0.5928$, $p<0.0001$) (Figure 4F). All the results illustrated that SOX6 was a target gene of miR-1269a in NSCLC.



Fi... xpression of miR-1269a promoted proliferation and induced cell cycle of H1975 cells. ***A,*** H1795 cells were ...sfected w... either miR-1269a mimics or negative control (NC). ***B,*** Cell viability was detected by the CCK8 assay. ***C,*** ...ny formation assay performed in H1975 cells. ***D,*** Quantitative analysis of the relative colony numbers to the negative ... ***E,*** After transfection for 48 h, H1975 cells were stained with propidium iodide, and the distribution of cell cycle was dete...ed by flow cytometric analysis. $*p<0.05$, $**p<0.01$, $***p<0.001$.

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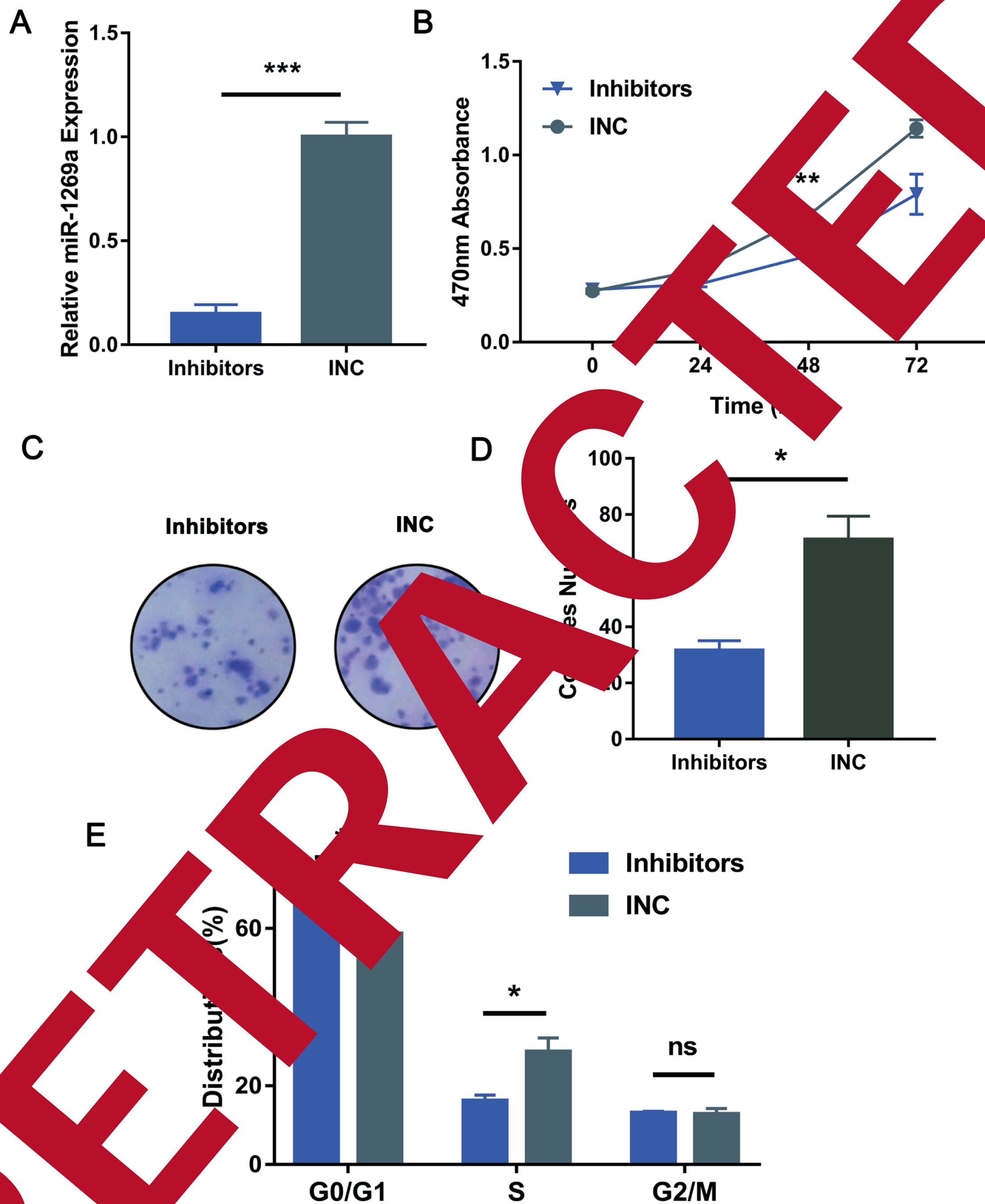

Fig 3. Down-regulation of miR-1269a inhibited proliferation and cell cycle of A549 cells. *A,* A549 cells were transfected wit 269a inhibitors or inhibitors negative control (INC). *B,* Cell viability was detected by the CCK8 assay. *C,* C assay performed in A549 cells. *D,* Quantitative analysis of the relative colony numbers to the INC group. E, er transfection for 48 h, A549 cells were stained with propidium iodide, and the distribution of cell cycle was determined cytometric analysis. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

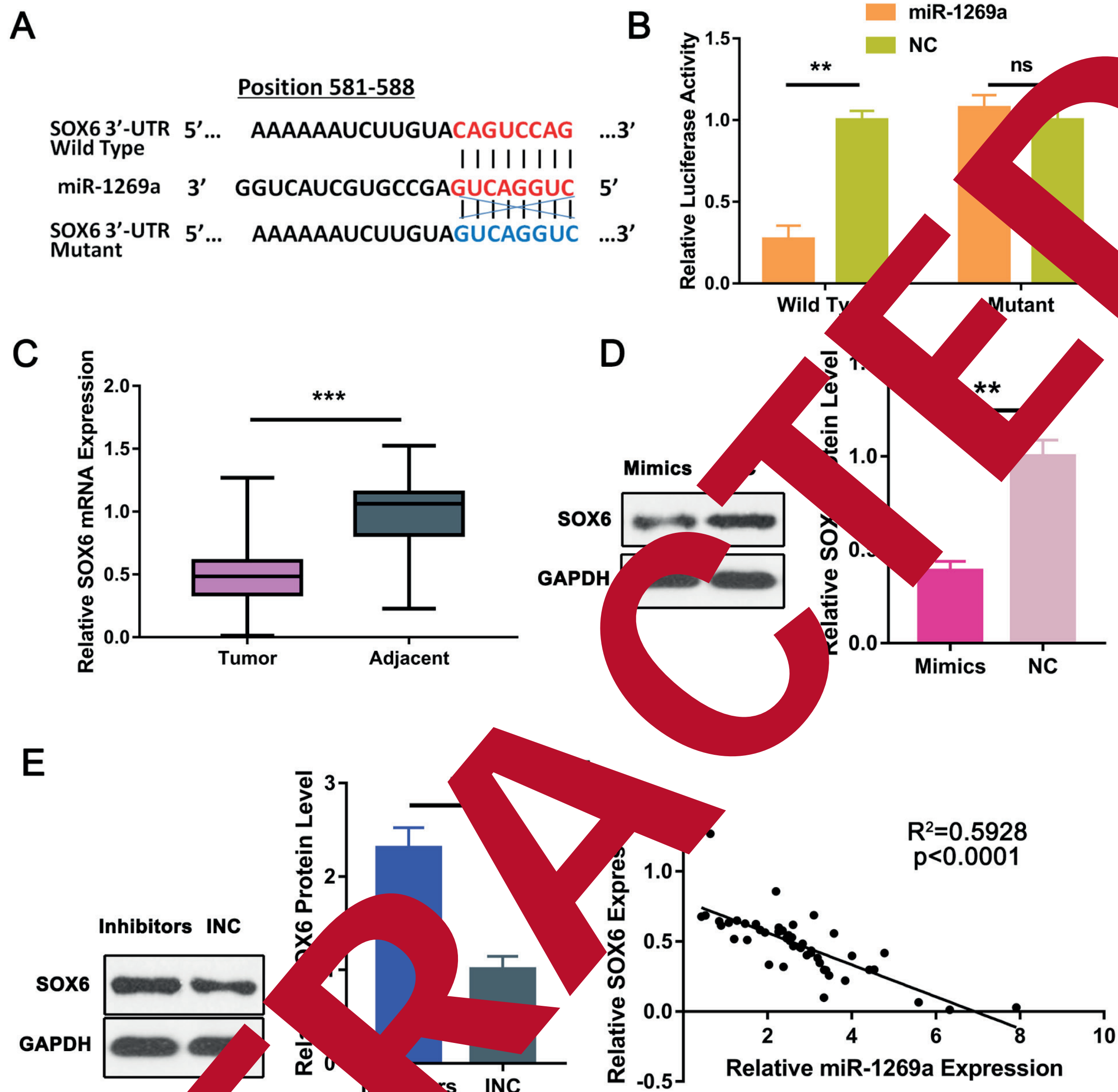

Figure 4. MiR-[illegible]a [illegible] to SOX6 in N[illegible]CLC. ***A,*** The predicted binding site of miR-11269a at the 3'-UTR of SOX6 mRNA. B, Luci[illegible]se repor[illegible]y using pMIR-REPORT (vector), pMIR-REPORT SOX6-3'UTR-wt, or SOX6-3'-UTR-mut plasmid in H1975 cells transfe[illegible]ith miR-1269a mimics or NC miRNA. ***C,*** The mRNA expression of SOX6 in NSCLC tissues an[illegible]red adjacent norma[illegible]s were analyzed by qRT-PCR. ***D-E,*** The protein expression of SOX6 in established H1975 a[illegible]549 cells was detected by Western blot analysis. F, Correlation between miR-1269a expression and SOX6 mRNA level i[illegible]SCLC tissues (R^2=0.5928, p<0.0001). $^{*}p$<0.05, $^{**}p$<0.01, $^{***}p$<0.001, ns=non-sense.

mi[illegible]269a P[illegible]oted NSCLC Growth Via re[illegible]ating SOX6

[illegible]nfirm our results, we over-expres-[illegible] SOX6 in H1975 cells transfected with miR-[illegible]9a mimics. The protein expression of SOX6 w[illegible]ificantly increased after SOX6 up-regulation (Figure 5A). Next, CCK8 and colony formation assays were used to explore the changes of cell proliferation. The enhancement of miR-1269a mimics on H1975 cell growth was significantly offset by SOX6 up-regulation (Figure 5B, 5C, 5D). Meanwhile, SOX6 over-expression restored the decreased distributions of G0/G1 phase caused by miR-1269a up-regulation (Figure 5E). These data demonstrated that miR-1269a could act as onco-miRNA via repressing SOX6 expression.

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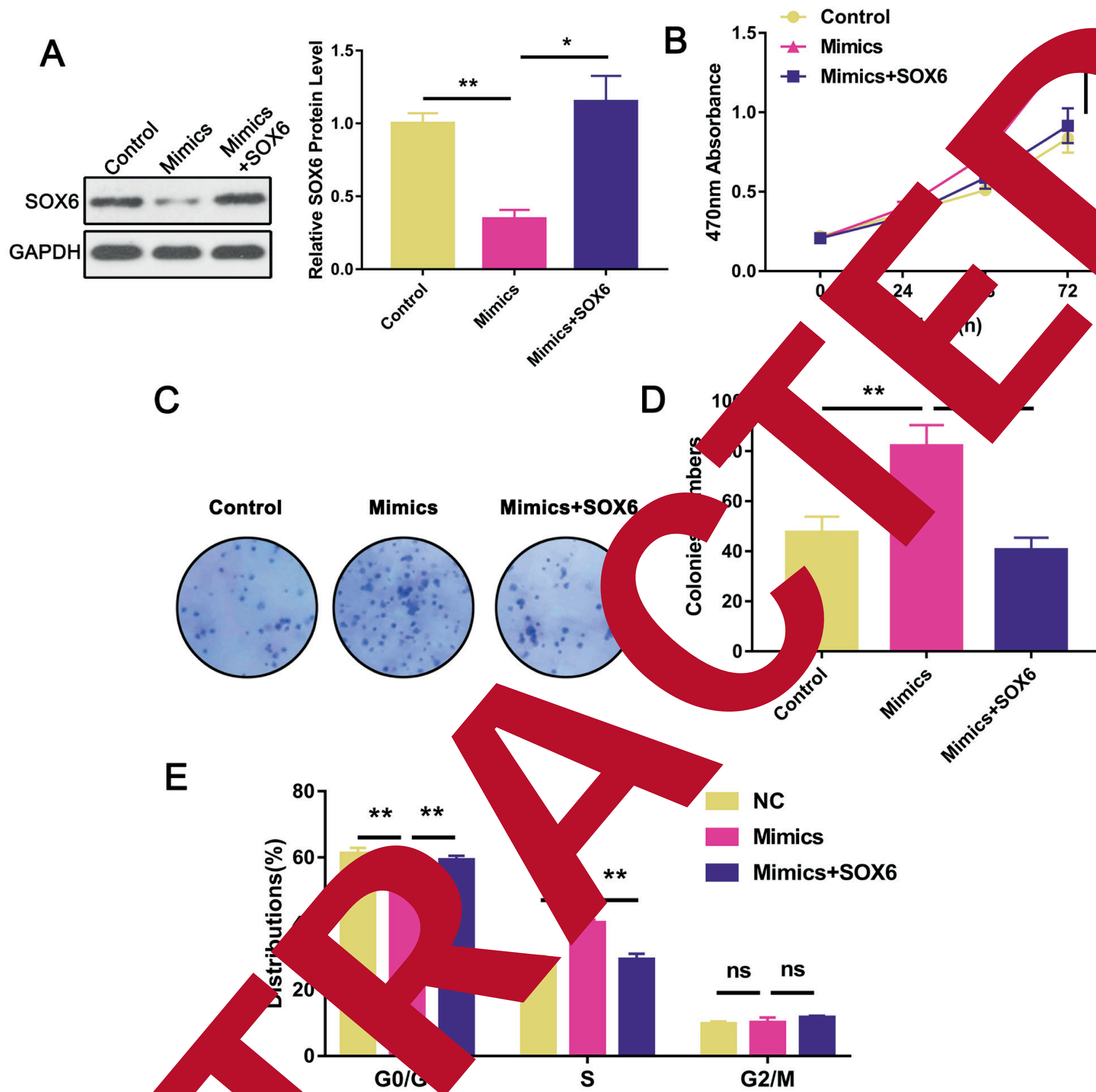

Figure 5. [illegible]X6 over-expressio[illegible]ished miR-1269a-mediated promotion of the growth of H1975 cells. ***A,*** The protein expressi[illegible] SOX6 was detected by [illegible]estern blot analysis. GAPDH was used as an internal control. B, Cell proliferation ability [illegible]A549 cells treated with negative control, mimics, or mimics+SOX6 were measured by the CCK8 assay. ***C-D***, Cell gro[illegible]ility wa[illegible]easured by colony formation assay. ***E,*** The distribution of cell cycle was determined by flow cytometric analys[illegible] represent[illegible] as mean ± SD. The experiments were repeated for three times. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

[illegible]iscussion

[illegible], especially NSCLC, contributes to [illegible] highest [illegible]cidence and mortality of malignan[illegible][14,15]. Despite of the continuous improvement a[illegible]elopment of various therapeutic technologie[illegible] in recent years, the prognosis of NSCLC remains poor[16,17]. MiRNAs, a classic class of regulatory factors involved in the biological behavior of many cancers, have been found to be a meaningful target for the diagnosis, prognosis and treatment of tumors[18,19].

miR-1269a has been found to be involved in the biological processes of many tumors. For example, it can participate in the occurrence and development of primary liver cancer, affect the re-

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sistance of prostate cancer in castration therapy, and involve in the oncogenesis of gastric cancer. These effects are often achieved by regulating the expression of specific target genes, including MYCBP2, ZNF70, SPATS2L, and LRP6[12,13,20,21]. However, the role of miR-1269a in NSCLC has not yet been confirmed. Here, we firstly identified that the expression of miR-1269a was increased in NSCLC tissues and cell lines compared to normal controls. Several functional experiments illustrated that over-expression of miR-1269a promoted cell proliferation and induced G0/G1 to S transition, while knockdown of miR-1269a inhibited cell growth and induced G0/G1 arrest of NSCLC cells. These results suggested that ectopic miR-1269a expression could affect the development and progression of NSCLC.

Furthermore, we found SOX6 as a potential target of miR-1269a in NSCLC. SOX6, a member of the Sox transcription-factor family, has been shown to be a tumor suppressor in many researches. The expression of SOX6 is low in multiple tumors. SOX6 usually affects cell proliferation and cell cycle distributions by affecting the expression of its downstream p21 and cyclin D1[22-24]. For example, in malignant tumor[illegible] as liver cancer, esophageal cancer, osteosarc[illegible] pancreatic tumor, SOX6 acts as a tumor su[illegible]sor gene. Moreover, its decreased expression ma[illegible]mote the occurrence and progression of tumors[illegible] In addition, SOX6 can be regulated [illegible] many mi[illegible]NAs. For example, in human e[illegible]quamou[illegible] cell carcinoma, miR-208 acc[illegible]ates ce[illegible]owth by down-regulating SOX6 [illegible] miR-76[illegible] promotes human colorectal cancer ce[illegible]ifera[illegible]ting SOX6. miR-96 i[illegible]eases [illegible]iferation and metastasis of hepat[illegible]llular carci[illegible]hrough regulating SOX6[29-[illegible] [illegible] we confirm[illegible] SOX6 could regulate [illegible]CL[illegible]wth via acting [illegible]s a direct target gene of miR-1269a, [illegible]h was familiar to mutual evide[illegible] of many previ[illegible]dies. Meanwhile, with ov[illegible]xpression of SOX6, t[illegible] promotion effect of mi[illegible]269a up-regulation was significantly offset. Ou[illegible] de[illegible]strated miR-1269a could act as an oncog[illegible]CLC vi[illegible]pressing the expression of SOX6. [illegible]ver, m[illegible] researches are needed for [illegible] illustr[illegible] mechanism of miR-1269a in NS[illegible].

Conclusions

[illegible]study demonstrated that miR-1269a was sign[illegible]cantly over-expressed in NSCLC tissues and cell lines. Moreover, miR-1269a might promote cell proliferation and cell cycle of NSCLC cells via down-regulating the tumor suppressor SOX6. These findings might provide a n[illegible] for NSCLC diagnosis and biological [illegible]apy.

Conflict of Interest

The Authors declare that they ha[illegible]o confl[illegible]erest.

Re[illegible]nces

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