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LINC01093 promotes proliferation and invasion of non-small cell lung cancer cells *via* targeting akt signaling pathway

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Abstract. – OBJECTIVE: To explore the expression of LINC01093, a long non-coding ribonucleic acid (lncRNA) in non-small cell lung cancer (NSCLC) tissues, and cells and its regulatory role in NSCLC cell proliferation and invasion.

PATIENTS AND MATERIALS: The expression of LINC01093 in NSCLC tissues and cells was detected via quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) experiment. The specific sequences interfering in LINC01093 were designed and transiently transfected into A549 and SPCA-1 cells using Lipofectamine™ 2000, and 48 later the transfection efficiency was detected, and the impacts of small interfering (si-LINC01093) on NSCLC cell proliferation were observed via methyl thiazolyl tetrazolium (MTT) and colony assays, the influence of LINC01093 on cell cycle distribution of NSCLC cells was determined through flow cytometry, and the changes in the invasion and migration abilities of NSCLC cells were evaluated via transwell assay after interfering in the expression of LINC01093. Finally, the expression changes of the molecular markers in the protein kinase B (Akt) signaling pathway in the downstream of LINC01093 were detected via Western blotting.

RESULTS: According to the results of qRT-PCR, the expression level of LINC01093 was up-regulated in NSCLC tissues and cells. After interfering in the expression of LINC01093, the results of MTT and colony forming assays revealed that the proliferation ability of NSCLC cells was weakened, according to the findings in flow cytometry, the cells were arrested in the G0 phase, the transwell assay results manifested that the cell migration and invasion abilities were weakened, and the results of the Western blotting suggested the changes in the expressions of molecular markers in the Akt signaling pathway.

CONCLUSIONS: The expression of LINC01093 is up-regulated in NSCLC tissues and cells, and facilitates the proliferation, invasion, and metastasis of NSCLC cells via the Akt signaling pathway.

Key words:
Non-small cell lung cancer, LINC01093, Proliferation, Invasion and migration, Akt signaling pathway.

Introduction

In recent years, the incidence and mortality rates of lung cancer have been increasing, and it is one of the cancers with the highest incidence and mortality rates worldwide[1]. Non-small cell lung cancer (NSCLC) represents 75-80% of the total lung cancer cases[2]. For the lack of typical clinical manifestations, it is relatively difficult to perform the early-stage screening for NSCLC, and most patients stayed at an advanced stage when diagnosed for the first time. Additionally, poor prognosis is also a grave issue facing in the current treatments[3]. Therefore, seeking effective markers for the early diagnosis and prognosis is of great scientific significance and clinical value.

Gene therapy for tumors is gradually becoming a research hotspot now, and the search for NSCLC-associated oncogenes and tumor suppressor genes provides new research directions for the diagnosis and treatment of NSCLC as well[4]. Long non-coding ribonucleic acids (lncRNAs) measuring more than 200 nt in length regulate the proliferation, apoptosis, metastasis, and other biological functions of cancer cells, playing a role

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as "tumor suppressor gene" or "oncogene"[5]. Latest researches have found that several lncRNAs, such as lncRNA MALAT1[6], lncRNA5 [growth arrest-specific gene 5 (GAS5)][7] and lncRNA HOX antisense intergenic RNA (HOTAIR)[8], are aberrantly expressed in NSCLC and participate in its pathological occurrence and development. New lncRNAs related to NSCLC should still be investigated through further studies.

LINC01093 is located on chromosome 4q35.1 and 1529 bp in full length. According to a literature report, the expression of LINC01093 is down-regulated in hepatocellular carcinoma, and lowly-expressed LINC01093 can be taken as an independent predictor for the prognosis of hepatocellular carcinoma patients[9]. He et al[10] found that LINC01093 is lowly expressed in hepatocellular carcinoma and related to the TNM stage in patients with this disease. In addition, they discovered that the molecular mechanism experiment results have established that LINC01093 binds to IGF2BP1 to degrade GLI1 messenger RNAs (mRNAs) and repress the proliferation and other biological features in hepatocellular carcinoma. However, the expression and biol[illegible] function of LINC01093 in NSCLC tissu[illegible] not yet been reported.

In this study, it was discovered for the first [illegible] that the expression of LINC01093 was upregu[illegible]ed in NSCLC tissues and cells[illegible] the high[illegible] expressed LINC01093 accele[illegible] proliferation, invasion, and metast[illegible] of N[illegible]C cells via the protein kinase B ([illegible] signali[illegible]way. The above findings offe[illegible] eti[illegible] molecular targets to [illegible]rse t[illegible]ation of the malignant phenoty[illegible]f NSCLC.

Patients and Methods

Tissue Specimens

A[illegible] of 56[illegible]CLC patients undergoing surge[illegible]he D[illegible]tment of Chest Surgery in China-Jap[illegible] Hospi[illegible]f Jilin University from [illegible] to 2[illegible]ere s[illegible]ted as subjects, and their [illegible]ous ti[illegible] para-cancerous tissues at 2 cm [illegible]y from [illegible] tumor were taken. Postoperati[illegible]hese patients were definitely diagnosed [illegible] examination. Before the opera[illegible], all the patients aged 31-75 years old did not [illegible]rgo radiotherapy, chemotherapy, or immunoti[illegible]or take targeted therapeutic drugs. This study was approved by the Ethics Committee of our hospital, and the enrolled patients signed the written informed consent. The cancerous tissues and para-cancerous normal tissues collected were cryopreserved at -180°C.

Cell Culture

Human bronchial epithelial [illegible] (16HBE) and NSCLC cell lines (adenocar[illegible]a: A549 and SPC-A1, squamous ca[illegible]noma: [illegible]ES-1) (Shanghai Institute of B[illegible]hemistry a[illegible] Biology, Chinese Aca[illegible]y of S[illegible]iences) cultured with the D[illegible]cco's [illegible]ified Eagle's Medium (DMEM)/R[illegible] P[illegible] Memor[illegible] Institute-1640 (RPMI[illegible]40) [illegible] (Hycl[illegible], South Logan, UT, U[illegible] containi[illegible]% [illegible] bovine serum (FBS[illegible]lone, South [illegible] UT, USA) in a therm[illegible]te[illegible]bator at 37°C with 5% CO_2.

Quantitative Real [illegible] Reverse Transcription-Polymer[illegible]e Chain Reaction (qRT-PCR)

[illegible]6HBE cells, [illegible]CLC cells, and cancerous or [illegible]-cancerous t[illegible]es were lysed with an appropr[illegible] amount [illegible]RIzol (Invitrogen, Carlsbad, CA, [illegible]dded with chloroform for extraction [illegible]ortexing. After high-speed centrifugation, the isopropanol was added to accelerate [illegible]A precipitation, and the precipitates were [illegible]ash[illegible] using anhydrous alcohol, centrifuged, collected, and dissolved with diethylpyrocarbonate-treated water. Then, the concentration of RNAs was determined. The total RNAs (1 μg) were reversely transcribed into complementary deoxyribonucleic acids (cDNAs). The qRT-PCR program was set as follows: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing and extension at 60°C for 31 s, for 40 cycles and performed, and then the data were analyzed using the 7300 system SDS software. Finally, the relative quantification (RQ) value was calculated based on cycle threshold (Ct) using the formula $RQ=2^{-\Delta\Delta Ct}$ and reflected the relative expression level of the genes detected.

Primers and Small Interfering (si)RNAs

The primer sequences were: linc 01093 Forward 5'-AGTTCTTCTAGATTGCGTT-3', linc 01093 Reverse 5'-ATTGACATGGGATGCCCTT-3', glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Forward 5'-CCCAC TTGAAGGGTGGAGCCAA-3', GAPDH Reverse 5'-TGGCATGGACTGTGGTCA TGA-3', si-linc 01093 #1, sense 5'-CGACAGUGCUIGUGAACUUTT-3', and antisense 5'-CCAUUCAGUACGAGAGUCCTT-3'; si-linc 01093 #2, sense 5'-ACUGACG CUGAC-

CAUGUGATT-3', and antisense 5'-ACUGGU-CAUCAGU AUCCCATT-3'. si-linc 01093 #3, sense 5'-CCAAGGCUGGAACUGAUATT-3', and antisense 5'-GC AACGAAUCCGGAUCG-CATT-3'. The primer and interfering sequences were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

Methyl Thiazolyl Tetrazolium (MTT) and Colony Forming Assays

MTT assay was performed according to the following specific steps: the NSCLC cells were transfected with si-LINC01093 and inoculated into a 96-well plate (3×10^3 cells/well), and each well of cells was incubated with 10 μL of MTT solution in the incubator for 1 h. Subsequently, they were added with 100 μL of formazan dissolving solution and continued to be incubated for 4 h. Finally, an ultraviolet spectrophotometer was used to determine the optical density (OD) at 570 nm. The colony forming assay was conducted as follows: after being transfected with si-LINC01093, the NSCLC cells were seeded into a 6-well plate (1.2×10^3 cells/well), and the medium was replaced every three days. After 14 d, with the m removed, the cells were covered and fixe methanol for 15 min, stained with crystal et for 15 min and washed using Phosphate-B Saline (PBS) twice.

Flow Cytometry

Cell cycle distribution w etected cording to the following procedur rstly, si 01093 and si-NC were transient sfe CLC cells, and 6 h la he c e inoculated into a 6-well plate cultured 8 h. Then, the cells were l d, re-suspe n 75% ethanol, and f at overnight. Finally, the content of DNA in cells determined via flow cytometr d PI staining, e percentage of cells in n time phase was c ulated using the softw

Trans say

The lo cham of transwell was added 00 μ medium containing 10% feta ovine se (a Millipore chamber pre-pa ith Matrigel and a Millipore chamber w ell invasion and migration assays, pectively; Millipore, Billerica, MA, USA), and t 2×10^5 cells (200 μL) were added to the up hamber and cultured in the incubator for 48 h. With the liquid in the upper chamber discarded, the chamber was taken out carefully, and the cells which failed to penetrate through the chamber membrane were removed using the wet cotton swab. Then, the resulting cells w d in methanol for 2 min, stained with c for 5 min, and washed using PB nally, the chamber membrane was cut and ted.

Western Blotting

The cells were collecte om the ex group and control grou d add with p lysis buffer, and the c entratio proteins was determined using the ch nic aci CA) protein assay ki ier ford, USA). Then, 10% se on and s were prepared, and a loading volu s calculated based on ab entioned pro in concentration deter ned, 20 proteins were loaded and transf onto polyvi ne difluoride (PVDF) m (Millipore, B rica, MA, USA) by t et transfer method at 80 V for 30 min and 20 V for 1 espectively. Subsequently, the branes were ubated with the primary antib of phosp lated Akt (p-Akt), T-Akt, p21, and gen, Carlsbad, CA, USA) at 4°C overnigh ed using Tris-Buffered Saline with Tween-20 (TBST), incubated with the secondary at at room temperature, rinsed with TBST d p ed on the ChemiDoc XRS+ imaging system for development.

Statistical Analysis

In statistical processing, Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. The *t*-test was used for analyzing the measurement data. The differences between the two groups were analyzed using the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ denoted that the difference was statistically significant.

Results

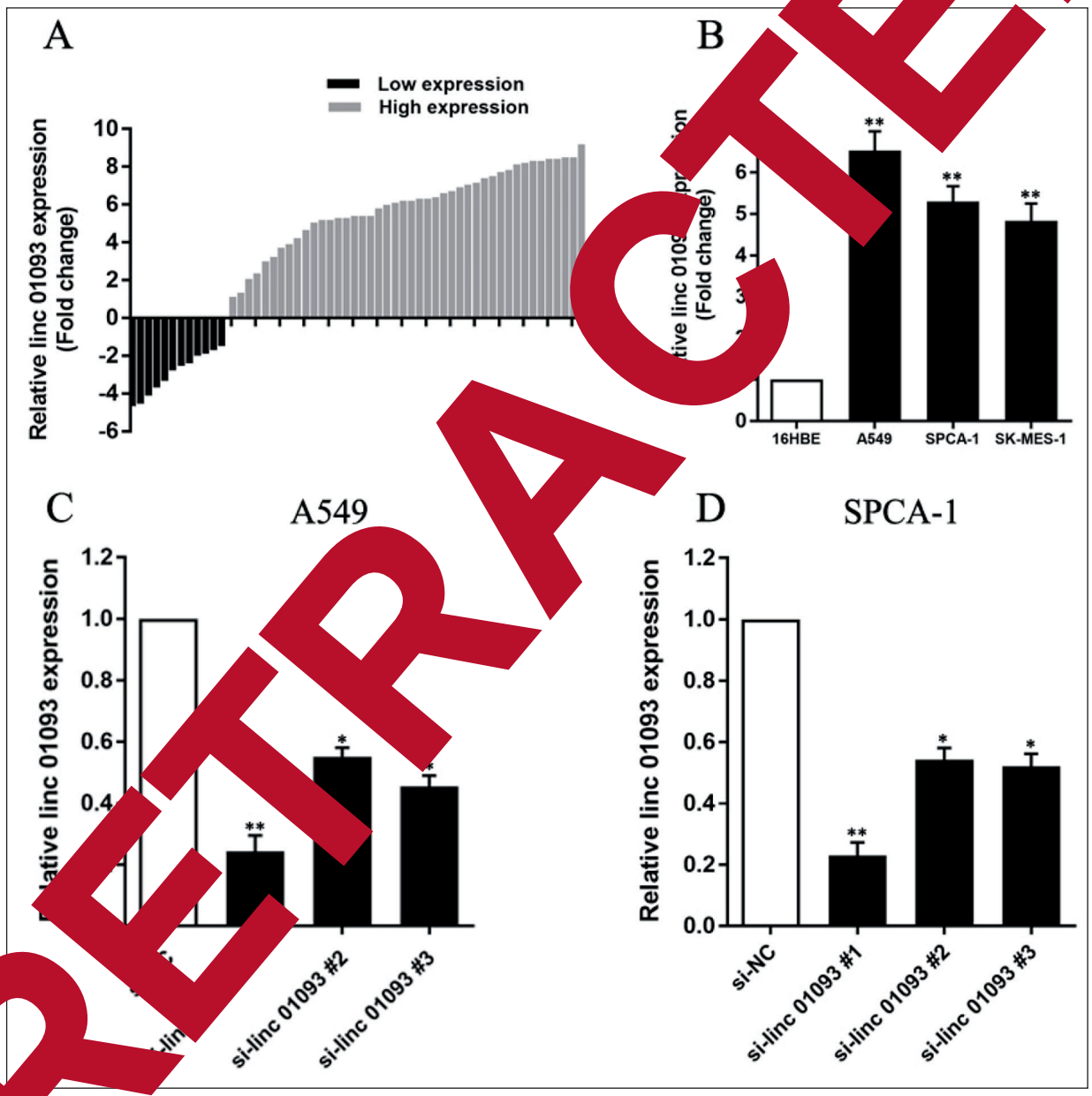
The Expression of LINC01093 Was Upregulated in NSCLC Tissues and Cells

The expression level of LINC01093 in the cancerous and para-cancerous tissues from 56 NSCLC patients was determined via qRT-PCR, and it was found that, compared with that in para-cancerous tissues, the expression level of LINC01093 was raised in NSCLC tissues (Figure 1A). Then,

the expression level of LINC01093 in NSCLC cells (A549, SPCA-1, and SK-MES-1) and human bronchial epithelial cells (16HBE) was measured via qRT-PCR, and the results revealed that the expression of LINC01093 was up-regulated in NSCLC cells (Figure 1B). Additionally, to explore the biological function of LINC01093 in NSCLC cells, A549 and SPCA-1 cells were taken as the model cells and transiently transfected with the specific interfering sequences of LINC01093 (si-LINC01093) designed by the research group. 48 h after transfection, the interference e[illegible] was measured (Figures 1C, 1D).

Influence of LINC01093 on [illegible]LC Cell Proliferation

Firstly, A549 and SPCA-1 [illegible]lls we[illegible]ient-ly transfected with si-LIN[illegible]093 and si-[illegible]



[illegible]re 1. Expression of LINC01093. **A,** Expression level of LINC01093 in 56 pairs of NSCLC tissues and para-cancerous [illegible]determined via qRT-PCR: compared with that in para-cancerous tissues, the expression of LINC01093 is up-regulated in [illegible]ses and down-regulated in 12 cases, with GAPHD as the internal reference. **B,** The relative expression level of LINC01093 in NSCLC cells and 16HBE cells measured via qRT-PCR. **C,** The transfection efficiency of the designed sequences interfering in LINC01093 detected via qRT-PCR. (**$p<0.01$, and *$p<0.05$).

then they were inoculated into a 96-well plate (3×10^3 cells/well). After inoculation, OD was measured at five observation time points 0, 24, 48, 72, and 96 h, and the growth curve was plotted. It was discovered that the interference in the expression of LINC010193 inhibited the proliferation of NSCLC cells (Figures 2A, 2B), which is consistent with the colony forming assay results (Figures 2C, 2D). Furthermore, the influence of LINC01093 on NSCLC cell cycle distribution was explored. Firstly, the cells in both the experiment group and control group were seeded into 6-well

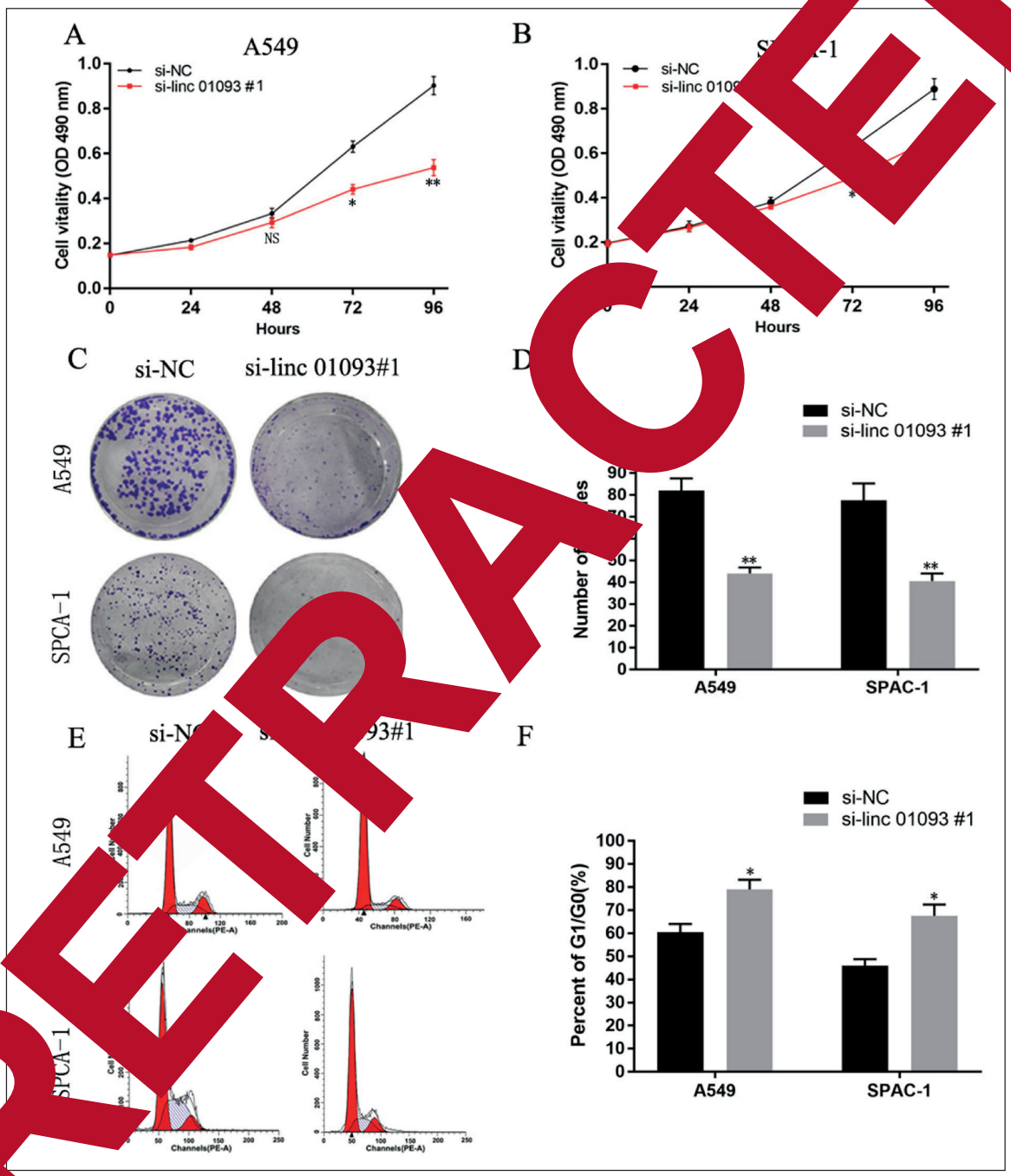
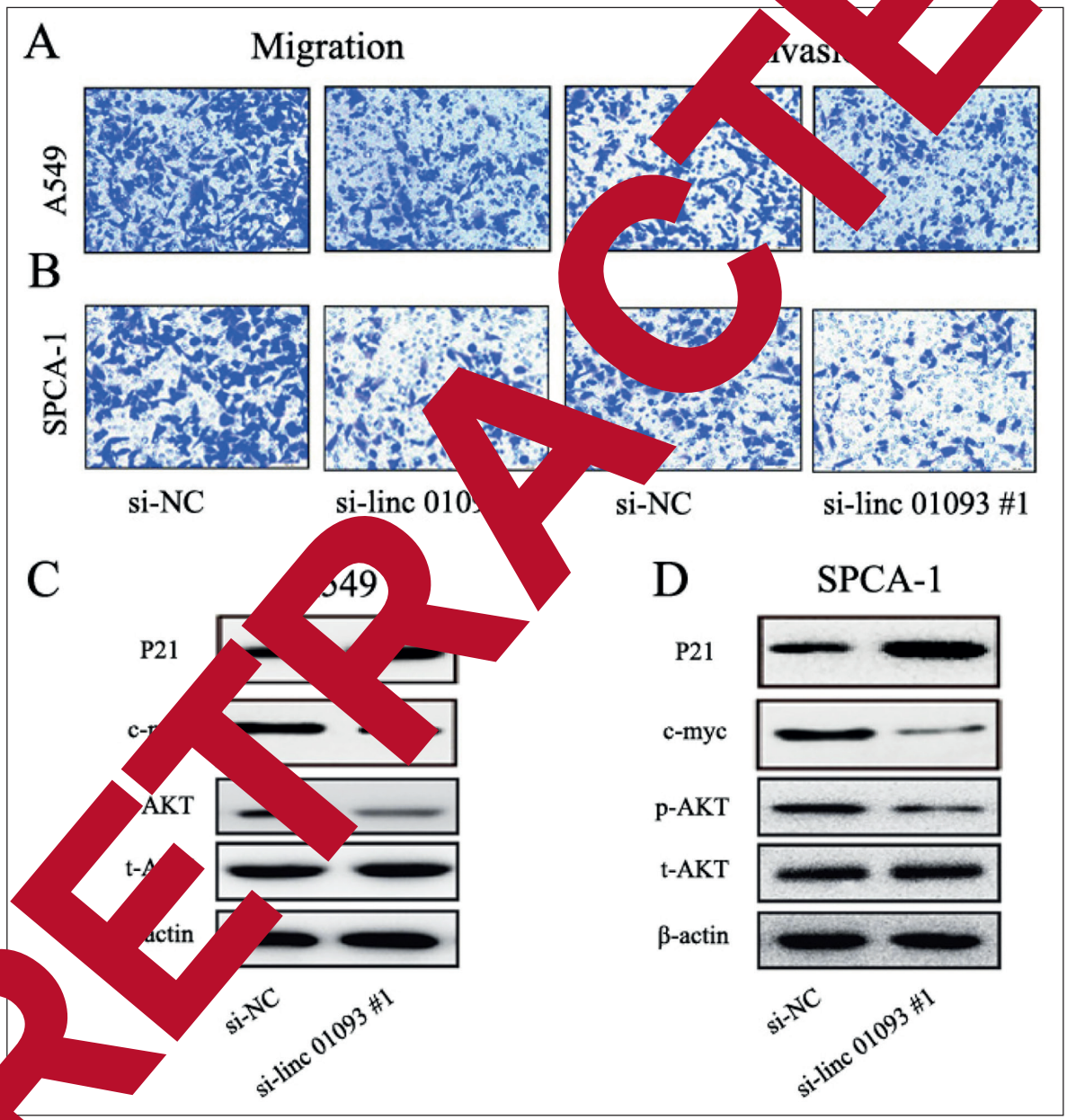


Figure 2. Influence of LINC01093 on NSCLC cell proliferation. **A-B,** The impacts of si-LINC01093 on the proliferation ability of A549 and SPCA-1 cells detected via MTT assay. **C-D,** Impacts of si-LINC01093 on the proliferation ability of A549 and SPCA-1 cells detected via colony forming assay (magnification: 10×). **E-F,** Cell cycle distribution detected using flow cytometry after intervening in the expression of LINC01093 in A549 and SPCA-1 cells. (**p<0.01, and *p<0.05).

plates, separately and 48 later, they were collected. Then, the cell cycle distribution was detected via flow cytometry, and the results showed that the knockdown of LINC01093 arrested NSCLC cells in G1/0 phase (Figures 2E, 2F).

Impact of LINC01093 on the Migration and Invasion of NSCLC Cells

Firstly, the cells in the experiment group and control group were inoculated into chambers, separately, and cultured for 48 h. Then, the liquid in the upper chamber was discarded, and the resulting cells were fixed using meth[illegible] stained with crystal violet. The re[illegible] fested that the intervention in the [illegible]ression of LINC01093 weakened the mig[illegible] and invasion abilities of NSCLC cells (Fi[illegible]3A, 3B). To verify the potential mol[illegible]lar me[illegible]m by which LINC01093 exert[illegible]e biologic[illegible] tions, the changes in [illegible]cular [illegible]arkers i[illegible]



[illegible]3. LINC01093 acts through the AKT signaling pathway. **A-B,** Changes in cell migration and invasion abilities detected via [illegible]well assay after interfering in the expression of LINC01093 in A549 and SPCA-1 cells (magnification: 40×). **C-D,** The changes in the expressions of the molecular markers in the AKT signaling pathway detected by Western blotting after interfering in the expression of LINC01093 in A549 and SPCA-1 cells.

Akt1 signaling pathway were detected via Western blotting in this study. It was discovered that, compared with those in the control group, the protein expressions of p-Akt, p21, and c-Myc in the cells were changed in the experiment group (Figures 3C, 3D).

Discussion

NcRNAs can fall into short strand ncRNAs (≤200 nt) and lncRNAs (>200 nt) according to the length. LncRNAs have previously been recognized as the "wastes" produced in the transcription, and we believed that they represented the low fidelity of RNA polymerases in such a process[11]. Over the past years, lncRNAs have attracted extensive attention as oncogenes or tumor suppressor genes, and their dysfunction is closely correlated with the occurrence of multiple tumors, including NSCLC[12].

Shen et al[13] followed 78 NSCLC patients for 5 years, and they found that the expression level of metastasis-associated to lung adenocarcinoma transcript 1 (MALA1), which is th st lncRNA researched in the lung cancer, is related to the survival time of patients. ey also discovered that the median survival of patients is 52 and 60 months in MALA high expression and low exp n group respectively. The above fin ate that MALAT1 is associated w the pi osis of NSCLC patients and tha serves inde-pendent factor reflecting p osi high expression of LA ests a poor prognosis in the ents. GA xl, a new lncRNA discov Han et al[14], ated on the down-stre of and acquire via antisense trans ip ion. Acc g to the findings of the resea compared wi mal tissues adjacent SCLC tissues, the ncerous tissues show ecreas xpression level of GAS6-Axl wh xpre is ne atively correlated with TNM AS6, host gene of GAS6-t can tivel omote the proliferation, ion, and filtration of NSCLC cell urther st es have found that GAS6-Axl is ely correlated with GAS6 in NSCLC t lls, suggesting that GAS6-Axl y regulate the expression of GAS6 to partic- in the occurrence and development of NS-C owever, there have not yet been reports on the expression and biological function of LINC01093 in NSCLC tissues and cells.

The present research discovered for the first time that the expression of LINC01093 was up-regulated in NSCLC tissues and cells t highly expressed LINC01093 could te proliferation, migration, and inva Since the relationships of LINC01093 w he clinico-pathological features of NSCLC ts were not further analyzed in thi tudy, e in-formation of such patient ll be comp probe the correlations INC01093 expre with the stage and p osis of e patients in subsequent researche the esent st the potential mecha n o n of L 01093 was prelimina explored e above experiment ts.

The fa o a serine/th onine protein kinase, ha 3 maj mbers, namely Akt1, 2 and 3 large amou f literature[15] have re-ve Akt is abnor y activated in such gnancies as liver cancer, breast cancer, and oid cancer. e Akt facilitates the growth proliferation umor cells, Akt has been re-g as an im ant target for researching and dev nor drugs, for instance, some broad-sp Akt inhibitors, like AZD5363, ave been subjected to clinical trials, as the drug breast and gastric cancers[16,17]. Similar-ly, in any studies[18,19], the Akt family has been found to bear close relationships with the proliferation and metastasis of NSCLC cells, while the interaction between LINC01093 and the Akt signaling pathway in NSCLC has not yet been reported. The present study discovered that after interfering in the expression of LINC01093, the expression of T-Akt was not changed, but on the other hand, that of p-Akt did.

Conclusions

Altogether, the expression of LINC01093 is up-regulated in NSCLC tissues and cells, and it facilitates the proliferation, invasion, and metastasis of NSCLC cells via the Akt signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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