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MiR-410 affects the proliferation and apoptosis of lung cancer A549 cells through regulation of SOCS3/JAK-STAT signaling pathway

M. LI, R. ZHENG, F.-L. YUAN

Department of Respiration, Yantai Yeda Hospital, Yantai, Shandong, China

Ming Li and *Rui Zheng* contributed equally to this work

Abstract. – OBJECTIVE: Janus kinase (JAK) – signal transducer and activator of transcription (STAT) signal pathway participates in regulating cell proliferation, differentiation, and apoptosis, and is correlated with non-small cell lung cancer (NSCLC) onset. Suppressors of cytokine signaling 3 (SOCS3) negatively regulates JAK-STAT pathway. SOCS3 is down-regulated in NSCLC tissues, with an elevation of miR-410 expression. This study thus intends to investigate if miR-410 plays a role in mediating NSCL set and underlying mechanism in this re ry process.

PATIENTS AND METHODS: NSCLC pati were collected for tumor and adjacent tissu among which, miR-410 and SO expressi were measured. Dual lucifer ter gen assay was employed to con the t ting re- lationship between miR- and SO 3. Their expression levels were c red b and BEAS-2B cells. ltu cells e treated with anti- 410 a SOCS3. Expression levels CS3, p-JA p-STAT3, and Bcl-2 were ed along w e apoptotic rate of c

RESULTS: Bioinform analysis revealed targeted ing site bet miR-410 and 3'-UTR o CS3 mRNA. Co ed to those in tumo ssues significant increase of miR-41 redu of SOCS3 were found in NSCLC < 0.05) al luciferase reporter gene a ndicat hat SOCS3 was target- gula y 410. Significantly higher m 0 and OCS3 levels were shown in A54 ells, co red to those in BEAS-2B cells. Tr tion of anti-miR-410 and/or SOCS3 in OCS3 expression and apoptosis re significantly induced, while JAK1, JAK2, STAT3 phosphorylation were statistically ased with the reduction of the Bcl-2 level (0.05).

CONCLUSIONS: miR-410 level was increased while SOCS3 expression was declined in NSCLS tissues. MiR-410 induces the apoptosis of A549 cells ou wnregulati JAK/STAT3/SOCS3 sig aling p ay, which provides new insights for the thera of pulmonary carcinoma.

Words:
iR-410, SOCS K-STAT3, DDP, Bcl-2, NSCLC.

Introduction

Pulmonary carcinoma is the most leading cause of death among all malignant tumors, and severely threatens public health[1]. Multiple cytokines and receptor-activated Janus kinase (JAK) – signal transducer and activator of transcription (STAT) signal pathway widely participate in cell proliferation/apoptosis, angiogenesis, and other biological processes. Abnormal transduction of JAK-STAT is closely correlated with tumor occurrence, invasion, metastasis, and prognosis[2]. STAT3 belongs to the tumor-facilitating member in STAT family. One of its important mechanisms involves up-regulating Bcl-2 protein expression to promote cell proliferation and tumor occurrence[3]. Suppressors of cytokine signaling (SOCS) can block tyrosine phosphorylation and JAK-STAT pathway transduction via inhibiting JAK kinase activity[4]. As an important member of SOCS family, SOCS3 exerts potent functions to directly inhibit JAK kinase activity and STAT phosphorylation, thus negatively regulating JAK-STAT3 signal pathway[5]. The decreased SOCS3 expression is correlated with occurrence and progression of multiple tumors including breast cancer[6], colorectal carcinoma[7], oral cancer[8], and prostate cancer[9]. Previous studies[10,11] on cancer revealed a significant reduction of

Corresponding Author: Fenglong Yuan, MD; e-mail: h4693103829@sina.com

SOCS3 expression, indicating a possible tumor suppressor role of SOCS3 in lung cancer occurrence.

MicroRNA (miR) is a type of endogenous small molecule non-coding single-stranded RNA with 21-24 nucleotide length. It can regulate target gene expression via degrading mRNA or inhibiting translation, and has become a focus in tumor-related research[12]. Previous studies showed a significantly increased level of miR-410 in pulmonary carcinoma tissues[13], indicating its possible role in the promotion of lung cancer pathogenesis[14].

Patients and Methods

Major Reagent and Materials

Lung cancer A549 cell line and normal human pulmonary epithelial cell line BEAS-2B were purchased from Yanyu Biotech (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) medium was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Bioind (C well, CT, USA). Streptomycin-penicill Annexin V-Fluorescein isothiocyanate (F)/ Propidium Iodide (PI) apoptotic reagent purchased from Solarbio (Beijing, China). R extraction reagent TriPure RN lation R agent was purchased from B napolis, IN, USA). Lipofectamine 0 was rchased from Invitrogen (Walth MA, I tiTect SYBR Green P PC purc d from Qiagen (Ven etherla iR-410 nucleotide fragme PCR pri ere synthesized by R Beijing, C . Rabbit anti-human p AK1 -JAK2 monoclonal antibody all purcha m Cell Signaling Techno (Danvers, MA, A). Rabbit anti-hu SOCS olyclonal antibody was provi Abc (Cambridge, MA, USA). Mouse anti-h TAT3 noclonal antibody and 2 po al a dy were purchased from Cruz nology (Santa Cruz, CA, US Goat a abbit and anti-mouse IgG-H L) were purchased from Shengxing n, Shan'xi, China). BCA protein antification kit was purchased from Baitaike ech (Beijing, China). Luciferase gene re- plasmid pLUC Luciferase vector was purchased from Ambion (Waltham, MA, USA). Dual-luciferase Reporter Assay System was purchased from Promega (Madison, WI, USA).

Clinical Information

A total of 38 NSCLC patients who received treatment in China-Japan Union Hospital of Jilin University from December 2015 to A st 2016 were recruited for this study. The males and 12 females in the patien up, with aging between 46 and 71 years age age = 52.9±12.5 years). All patients rec primary surgery in China-Japan Uni Hosp Jilin University. No patient ha eived any radio-, immune theran biol ical the Tumor tissues and ad t tiss ere remov d from NSCLC pati ts gery fo sting indexes.

This study been pre-a d the Ethical Commit ina-Japan Hospital of Jilin Uni ty. A bjects have signed the informed consent befo uitment in this study.

C Culture

549 and BE S-2B cells were cultured in EM contain 10% FBS and 1% penicill eptomycin fter re-suspension, cells were cul ber with 5% CO_2 at 37°C. Cells were p 1:3 ratio after full growth.

ruction of Luciferase Reporter lasmid and Dual Luciferase Reporter Gene Assay

Full-length fragment of 3'-UTR of SOCS3 gene or polymerase chain reaction (PCR) products containing mutant form were sub-cloned into the pLUC vector. After transformation, the plasmid was sequenced for determining the correct insertion, and were named as pLUC-SOCS3-3'-UTR-wt, pLUC-SOCS3-3'-UTR-mut. Lipofectamine 2000 was used to transfect pLUC-SOCS3-3'-UTR-wt (or pLUC-SOCS3-3'-UTR-mut) and miR-410 mimic (or its negative control sequence miR-NC) into HEK293T cells. After 48 h, dual luciferase activity assay kit (Promega, Madison, WI, USA) was used to test dual luciferase activity following the manual instruction.

Construction of SOCS3 Over-Expression Plasmid

CDS fragment of SOCS3 gene was amplified. The target fragment size was determined by gel electrophoresis. After digestion in Xho I and BamH I enzyme, fragments were ligated into the pIRES2 plasmid to transform JM109 competent cells. Ampicillin resistant plate was used to screen positive colony, which was amplified and extracted recombinant plasmid

containing target fragment. Correct insertion of SOCS3 gene fragment was determined by sequencing and was named as pIRES2-SOCS3. Empty vector pIRES2-Blank was used as the control group.

Transfection of A549 Cells

In vitro cultured A549 cells were divided into five groups: anti-miR-NC group; anti-miR-410 group; pIRES2-Blank group; pIRES2-SOCS3 group; and anti-miR-410 + pIRES2-SOCS3 group. 72 hours after transfection, cells were collected for mRNA, protein expression, and apoptosis assay.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) for Gene Expression

TriPure RNA Isolation Reagent was used to extract total RNA. QuantiTest SYBR Green RT-PCR Kit was used to test gene expression by qRT-PCR. In a 20 μL system, one added 10.0 μL 2X QuantiTest SYBR Green RT-PCR Master Mix, 1.0 μL of forward/reverse primers, 2 μg Template RNA, 0.5 μL QuantiTest RT [illegible] and ddH_2O. Primer sequences used were [illegible] 410P_F: 5'-CCGCC AATAT AACAC AC[illegible]G GCC-3'; miR-410P_R: 5'-TCAAG TACCC AC[illegible] GCGGT-3'; U6P_F: 5'-ATTGG AACGA TAC[illegible] AGAAG ATT-3'; U6P_R: 5'-GGA[illegible]TTC AC[illegible] GAA TTTG-3'; SOCS3P_F: 5[illegible]GCCTC AAGAC CTTC-3'; SOCS[illegible] 5'-G[illegible]C TG-CGC TCCAG TAGAA-[illegible]l-2P[illegible] GGTCA TGTGT GT[illegible]3'; [illegible]5'-CG[illegible] CAGGT ACTCA[illegible]CAT [illegible] β-actinP_F: 5'-GAACC CTA[illegible]GCCAA C[illegible]β-actinP_R: 5'-TGTCA CG[illegible]TTT CC-3'. [illegible]R conditions were: 9[illegible]C for [illegible]in pre-denature, followed by [illegible] cycles each [illegible]aining 94°C 15 s denatu[illegible]0°C 30 s annea[illegible]and 72°C 30 s elon[illegible]n. Ap[illegible] Biosystems 7500 (Waltham, M[illegible]A) r[illegible]me quantitative PCR was used for te[illegible] expre[illegible]n.

Western [illegible]

C[illegible]s were c[illegible]ected and extracted for proteins us[illegible] immunoprecipitation assay (RIPA) ly-[illegible]ter quantification, 40 μg samples [illegible]ere loaded and separated in sodium dodecyl [illegible]te-polyacrylamide gel electrophoresis (SDS-P[illegible]). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane, which was blocked in 5% defatted milk powder for 60 min room temperature incubation. Primary antibody (SOCS3 at 1:300, p-JAK1 at 1:200, p-JAK2 at 1:200, p-STAT3 at 1:200, Bcl-2 at 1:300, and β-actin at 1:600) was added for 4°C overnight incubation. After phosphate buffer [illegible] Tween-20 (PBST) rinsing, secondary [illegible] (1:5000 dilution) was added for 60[illegible] incubation. The membrane was rinsed [illegible]PBST and quantified for protein expression u[illegible]hanced chemiluminescence (ECL) m[illegible]od.

Flow Cytometry for [illegible] Apoptosis

Cells from all grou[illegible]ere col[illegible]ed and rinsed in PBS by centrifu[illegible]ati[illegible]ere re-[illegible]end-ed in Binding B[illegible], wit[illegible]ential[illegible]ion of 5 μL Annexin[illegible]ITC and P[illegible]r[illegible]in staining, Beckm[illegible]lter Gallios[illegible] cytometry was used[illegible]est c[illegible]optosis (Brea, CA, USA).

Statistical Analysis

[illegible]PSS 18.0 (SPSS Inc., Chicago, IL, USA) was [illegible] for data an[illegible]is. Continuous data are presented as means[illegible]standard deviation (SD), and were [illegible]using one-way ANOVA, with the Tu[illegible]st-hoc test. A statistical significance was defined when $p < 0.05$.

Results

Abnormal Expression of MiR-410 and SOCS3 in NSCLC Tissues

qRT-PCR results showed that miR-410 expression was significantly elevated in NSCLC tumor tissues compared to that in adjacent tissues ($p < 0.05$) (Figure 1A). Immunofluorescence test showed that SOCS3 level was significantly decreased in NSCLC tissue compared to that in adjacent tissues (Figure 1B). These data indicated that the reduction of SOCS3 expression might be associated with abnormal up-regulation of miR-410, which was involved in NSCLC pathogenesis.

Targeted Regulation Between MiR-410 and SOCS3

Bioinformatics analysis results showed the existence of targeted binding site between miR-410 and 3'-UTR of SOCS3 mRNA (Figure 2B). Transfection of miR-410 mimic significantly depressed relative luciferase activity in HEK293T cells transfected with pLUC-SOCS3-3'-UTR-wt ($p < 0.05$) (Figure 2B), indicating that miR-410 contributed to targeted regulation to SOCS3.

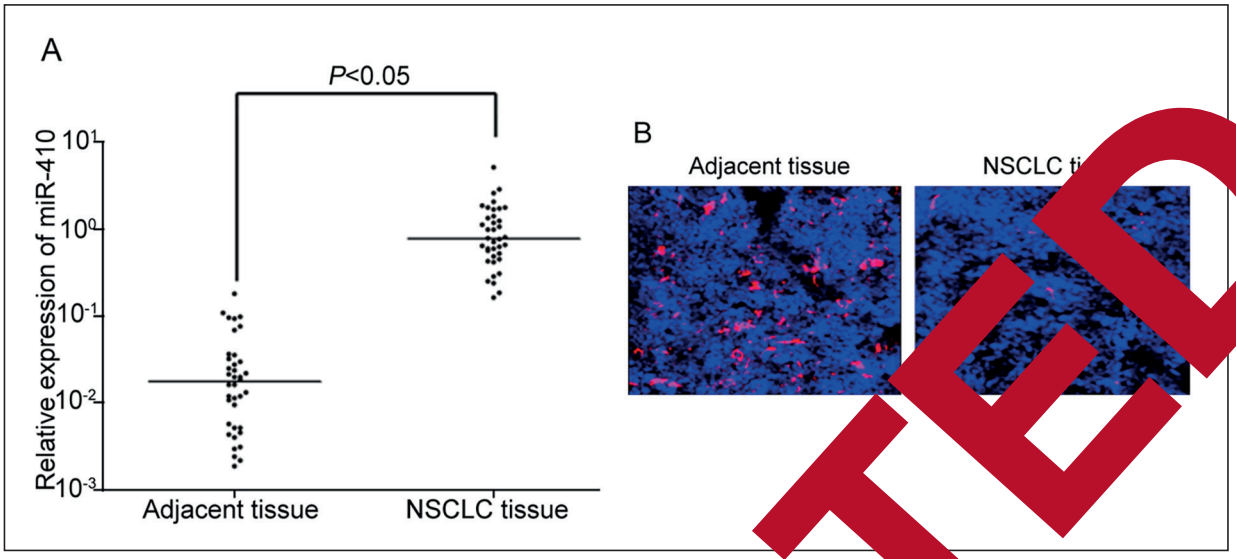
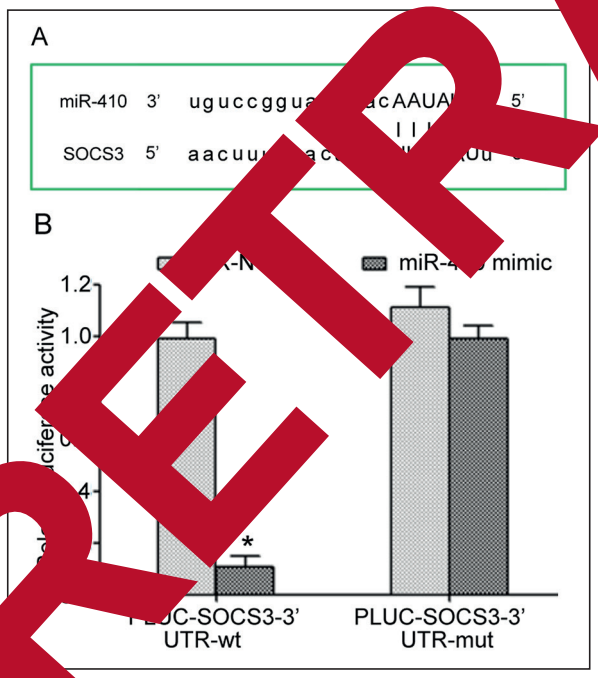


Figure 1. Abnormal expression of miR-410 and SOCS3 in NSCLC [illegible]. **A,** qRT-PC[illegible] miR-410 expression. **B,**

MiR-410 Inhibition Elevated SOCS3 Expression, Decreased Bcl-2, and Increased A549 Cell Apoptosis

Compared to those in BEAS-2B cells, the levels of miR-410 and bcl-2 were signi[illegible] elevated in A549 cells, whilst SOCS3 m[illegible]A [illegible]ession was s[illegible]tically decreased ($p < 0.05$) ([illegible]e 3A). Sim[illegible]ly, Western blotting detection sho[illegible]at S[illegible]3 protein level was apparently reduce[illegible]cl-2 expression was increased in A549 cells, compared to those in BEAS-2B cells [illegible]3B). We further used A549 cells as the *in* [illegible]del, on which detailed role of miR-410, SOCS3 in NSCLC pathogenesis mechanism were elucidated. The transfection of anti-miR-410 and/or SOCS3 over-expression plasmid remarkably up-regulated SOCS3 expression in A549 cells (Figure 3C and 3D), and impeded phosphorylation of JAK1, JAK2, and STAT3 (Figure 3D). Our data also indicated that the down-regulation of miR-410 and increase of SOCS3 level decreased expression of anti-apoptotic factor Bcl-2 (Figure 3C and 3D), and increased the apoptosis of cells (Figure 3E).



Fig[illegible] 2. Targeted regulation between miR-410 and SOCS3. **A,** Targeted binding sites between miR-410 and 3'-UTR of SOCS3 mRNA. **B,** Dual luciferase reporter gene assay. *, $p < 0.05$ comparing between miR-410 mimic and miR-NC.

Discussion

After binding with cell surface receptor, cytokines can transduce extracellular signals into the nucleus via signal transduction pathway cascade, in which JAK-STAT plays a critical role in responding extracellular factor, transducing signal cascade response, and regulating cell growth/apoptosis[15]. Cytokines, growth factors dimerize receptor and phosphorylate JAK via the binding with intracellular receptor as ligands, and induce the activation of STAT. Abnormal activation of STAT3 is closely correlated with irregulated cell

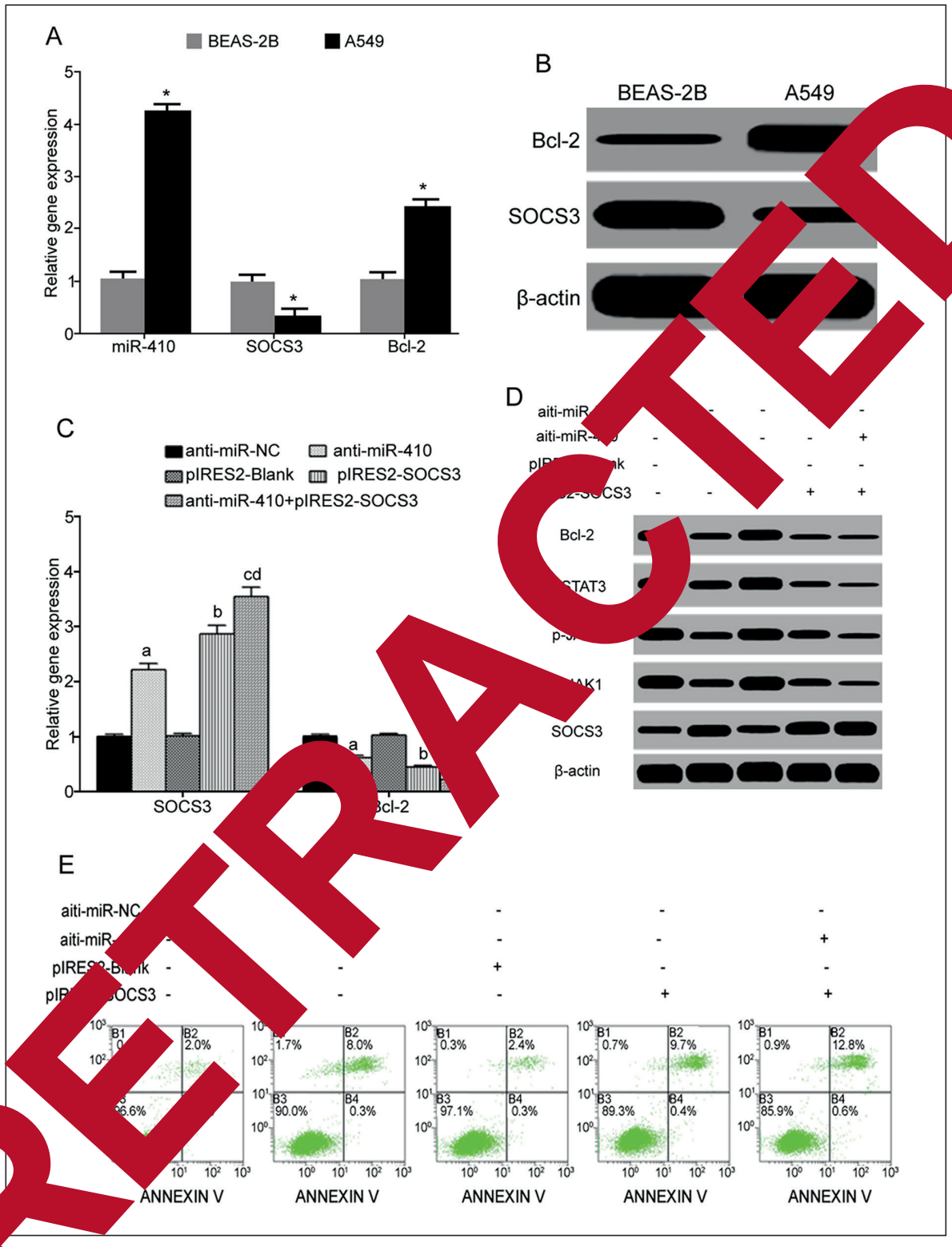


Figure 3. MiR-410 inhibition elevated SOCS3 expression, decreased Bcl-2 and increased A549 cell apoptosis. **A,** qRT-PCR for gene expression in BEAS-2B and A549 cells. **B,** Western blot for protein expression in BEAS-2B and A549 cells. **C,** qRT-PCR for gene expression in A549 cells with transfection. **D,** Western blot for protein expression in A549 cells after transfection. a, $p<0.05$ comparing between anti-miR-410 and anti-miR-NC group; b, $p<0.05$ comparing between pIRES2-SOCS3 and pIRES2-Blank group; c, $p < 0.05$ comparing between anti-miR-410 + pIERS2-SOCS3 and anti-miR-NC group; d, $p < 0.05$ comparing between anti-miR-410 + pIRES2-SOCS3 and pIRES2-Blank group.

proliferation and malignant transformation, and has become one major question in the study of JAK/STAT signal pathway and tumor pathogenesis[16]. SOCS represents the negative feedback regulatory factor in JAK-STAT signal pathway, and exerts a critical role in maintaining intracellular homeostasis. SOCS3 eventually blocks JAK-STAT signal pathway transduction through the inhibition of JAK phosphorylation and JAK kinase activity[17].

This study revealed that miR-410 level was significantly elevated in NSCLS tumor tissues compared to that in adjacent tissues, with a decrease of SCOS3 expression. Li et al[13] also reported higher miR-140 expression in NSCLC tissues compared to adjacent tissues while Zhang et al[14] found abnormally elevated miR-410 expression in NSCLC tissues. Our result agrees with previous finding. Evidence[10,18] revealed abnormally decreased SOCS3 expression in NSCLC tissues. Interestingly, in lung cancer tissues, SOCS3 expression was also decreased[19,20]. In this work NSCLC tissues were detected with lower SOCS3 expression, which was in line with previous studies.

It has been shown that miR-410 level wa... normally increased in lung cancer cells ... A540, SPC-A1, and H1299, compared to n... human bronchial epithelial cells HBE[13]. Z... et al[21] also showed lower SOCS3 expressio... A549 cells compared to HBE... Moreove... lower SOCS3 expression ... al... und in... lung cancer cell lines H... and ...9 compared to normal human ...ryoni... cell H1703[18]. We spe...late... R-410... d SOCS3 may be in...d in th... hogenesis of lung cancer. Du... ferase repo... ene assay further show... ...nsfection ...miR-410 mimic signifi...ntly st...sed relative luciferase activi... emonstrati... rgeted regulation correla... between miR-4... d SOCS3. We then ...essed t... effect of decreasing level of mi... or ... expression of SOCS3 on JAK-STA... g path... and cell apoptosis. Li ...[13] si... that ...r-expression of miR-410 ...cantly ...ed proliferation, migration, and ...vasion ...ulmonary cancer cells, probably related with miR-410 targeted inhibition ...pression and further enhancement ... phosphorylation activity of Akt. Zhang et ... showed that miR-410 could up-regulate S... A2 expression via activating Wnt/β-catenin signal pathway, and could facilitate proliferation, migration, and invasion of lung cancer cells. Over-expression of miR-410 also enhanced survival and viability of pulmonary cancer cells in nude mice. Consistently, our result unrevealed that the reduction of miR-410 induced the cell apoptosis and inhibited the cell ...lif... eration. The decrease of miR-410 also... up-regulation of SOCS3. In a sim... fashion, previous evidence[21,22] showed th... er-expression of SOCS3 significantly weak... motility and migration of A540 cells ...d dec... pro-liferation potency of lung ...cer cells. ... pression of SOCS3 sign...antly i...eased ... cell apoptosis, and ...ened ...feration/mi-gration potency[10]. ...hi... ...greem... with our finding. So... a va... of miR... s such as miR-520a-... miR-92a, ...27... rticipate in the reg... f lung can... . Notably, this work ...fied ... the decrease of miR-410 caused A549 cell ap...is and inactivation of the ...AT pathwa... over-expression of S...3, indicating the involvement of miR-410 ...e regulation ...lung cancer. However, the *in* ... test still req...s further evaluation of miR-4... clinical p...ice.

Conclusions

...emonstrated that NSCLC patients were detected with a high level of miR-410 and low SOCS3 expression compared to the normal group. MiR-410 can facilitate the activity of JAK-STAT3 signal pathway and cell proliferation via targeted inhibition on SOCS3 expression, which provides leads for the future therapy against lung cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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